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Application of Ultrasound Technology for Functional Meat Products

Thesis presented by
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MSc (Food Technology)

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To obtain the degree of
Doctor of Philosophy – PhD in Food Science and Technology

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Shikha Ojha

Declaration

I, Shikha Ojha, hereby declare that this thesis is my own work and effort, and that it has not been submitted for another degree, neither at the National University Ireland, Cork nor elsewhere. Where other sources of information have been used, they have been acknowledged.

Shikha Ojha

Abstract

With increasing consumer awareness of health and nutrition, the meat processing industry is eager to engage in a search for innovative ways of processing and developing novel meat products with potential health benefits. Novel processing technologies including ultrasound (US) technology can offer several benefits for versatile applications in food processing. In relation to meat processing, US technology has previously been investigated for many potential applications including accelerated brining, tenderisation, rapid cooking, faster thawing and decontamination of muscle-based food products. This thesis assesses the feasibility of employing US technology to assist in the development of functional meat products. To achieve this objective, a series of five studies were carried out. The first study investigated the efficacy of high intensity US on the fermentation profile of *Lactobacillus sakei* (a starter culture used for meat fermentation) in a meat model system. This study observed that both stimulation and retardation of *L. sakei* is possible, depending on the ultrasonic power and sonication time employed. Furthermore, to underline the fundamental mechanism influencing the behaviour of microorganisms subjected to ultrasonic frequencies of 20, 45, 130 and 950 kHz on growth kinetics, phenotypic behaviour and cell morphology were assessed in study 2. Results presented in this study showed that the physiological response of *L. sakei* to US is frequency-dependent and US can influence metabolic pathways. The influence of US frequency on the behaviour of *L. sakei* culture in a meat matrix was also studied. Additionally, this study investigated possible synergistic effects between US and *L. sakei* addition on drying kinetics, moisture mobility and key nutritional (fatty acid profile, protein, amino acids, and organic acids) in beef jerky samples. Given the complexity and number of parameters involved, a reliable multivariate statistical strategy was adopted. The results presented in this study showed a significant effect of US pre-treatment on various

physicochemical properties as a result of employing both *L. sakei* and US. The individual effects of *L. sakei* and US were prominent compared to interactive effects. Conversely, the effect of US frequency on *L. sakei* culture observed in model systems (Chapter 2 and 3) was not evident when assessed in a solid meat matrix. Two specific studies were carried out to investigate US-assisted diffusion of ingredients into meat matrices with the objective of improving the nutritional profile and healthy image of meat. Results from salt diffusion studies showed improved diffusion rates for sodium salt compared to a static brining system can be obtained. However, no significant differences were observed in the case of sodium salt replacers. A second aspect of US-assisted diffusion focused on the incorporation of essential fatty acids (FA) to improve the lipid profile of pork meat. This study has demonstrated the positive effect of US application in improving diffusion of encapsulated FA into meat, thereby resulting in higher levels of essential FA compared to the static diffusion system. It can be concluded from the thesis work conducted that the effectiveness of US technology is application dependent. The commercial utilisation of US technology remains challenging for solid food applications, especially for meat products due to the complex nature of meat. However, further research is needed prior to commercial uptake of US technology for a range of applications.

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Chapter 1: Literature review

Overview

This chapter outlines application of novel technologies for meat processing. Various novel thermal and non-thermal technologies are discussed with special focus on ultrasound technology for meat processing applications. This chapter has been published as a part of the following peer reviewed papers and book chapter.

Ojha, K. S., Kerry, J. P., Duffy, G., Beresford, T., & Tiwari, B. K. (2015). Technological advances for enhancing quality and safety of fermented meat products. Trends in Food Science & Technology, 44(1), 105-116.

Ojha, K. S., Mason, T. J., O'Donnell, C. P., Kerry, J. P., & Tiwari, B. K. (2017). Ultrasound technology for food fermentation applications. Ultrasonics Sonochemistry, 34, 410-417.

Troy, D. J., Ojha, K. S., Kerry, J. P., & Tiwari, B. K. (2016). Sustainable and consumer-friendly emerging technologies for application within the meat industry: An overview. Meat Science, 120, 2-9

Ojha, K. S., Kerry, J. P., & Tiwari, B. K. (2017). Emerging technologies for the meat processing industry, Advanced Technologies for Meat Processing (Eds. Fidel Toldrá, Leo M. L. Nollet), Second Edition. ISBN: 9781498754590, CRC Press.

1.1. Introduction

Meat and meat products are important sources for protein, fat, essential amino acids, minerals and vitamins and other functional nutrients (Biesalski, 2005). The growing concern of consumers regarding food health and safety issues has led to the development of functional meat products that promote health and well-being, beyond their nutritional benefits (Grasso, Brunton, Lyng, Lalor, & Monahan, 2014; Työppönen, Petäjä, & Mattila-Sandholm, 2003). This has driven researches in the meat processing sector to increase their efforts in producing such functional meat products, which have; incorporated functional ingredients, reduced salt, nitrates/nitrites, cholesterol and fat and modified product fatty acid profiles (Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010). The responsibility to produce high quality, sustainable and cost-effective meat products rests with producers, manufacturers, distributors and retailers to ensure consumer demands are met. Dealing with such a perishable product in a dynamic market place, it is not surprising that new and innovative technologies are constantly being developed and applied within the muscle-food processing sector in an attempt to enhance certain meat quality attributes and extend shelf-life and storage stability. Consistent and high quality meat production is one of the most important requirements of the meat industry in order to maintain and expand markets. The continually increasing demand to improve meat quality and safety has challenged meat processors, the scientific community and food process engineers to develop innovative techniques to produce sustainable meat products, whilst minimising environmental impact. Preservation and consumption of meat requires adequate processing techniques to ensure microbial safety and maintain quality with extended shelf-life. With increasing competition and tighter cost margins, the meat industry is eager to engage in a search for novel innovative ways of processing meat, while maintaining quality and safety attributes. Research on novel

processing and assessment technologies is ongoing around the world with numerous potential meat industry applications. Even though emerging technologies have demonstrated numerous advantages and potential applications for the food industry, there are limited commercial applications due to several factors. In some instances, industry uptake of new technologies is muffled by a lack of knowledge about these new technologies and their impact on product quality and safety.

Novel innovative meat processing techniques are aimed to achieve these key elements of sustainability by reducing environmental impact of meat processing by reducing wastage, minimising the use of natural resources (e.g. energy and water) and providing safe, nutritious and high quality product to consumers. Achieving sustainability in meat production system requires a holistic view spanning not just the end product and the production process but also the entire supply chain. Conventional methods of meat processing and preservation (e.g. heat processing, low temperature preservation or dehydration) have been used for centuries. However, the last century has seen a dramatic increase in the development of new technologies, which have, in many cases, been hyped, as replacements for conventional methods. However, in spite of much excitement relating to their discovery and potential, the anticipated uptake of novel processing techniques by the meat industry has not occurred owing to several factors associated with cost, scale up issues and potential consumer perception of novel technologies.

1.2. Novel technologies

With increasing consumer awareness and demand for fresh, safe, nutritious and healthy meat products, meat processors are continually investigating new and innovative food preservation technologies for potential commercial application. Novel thermal and non-thermal food

processing and preservation technologies, including radiofrequency (RF), microwave, infrared, ohmic heating, high pressure processing (HPP), pulsed-UV light, pulse electric field (PEF), power ultrasound, cold atmospheric plasma and ozone processing have gained much attention in recent years. These technologies can offer several benefits, including increased process efficiency, improved product safety, enhanced quality attributes and extended shelf-life stability of products.

1.2.1. Novel thermal technologies

Thermal processing of meat is considered to be the most common preservation technique used alone, or in combination with other novel food processing techniques. Various thermal processing techniques are employed for preserving meat and developing new products which can be classified as; conventional dry (e.g. roasting), moist (e.g. steaming) or novel thermal (e.g. microwave, RF, infrared or ohmic heating) applications. Novel thermal techniques used alone, or in combination with conventional thermal techniques have been employed to improve product safety and shelf-life, while minimising changes in meat quality. Conventional oven cooking employs high velocity, forced hot air convection which can cause surface deterioration, overheating and oxidation, thereby leading to a poor quality product. Some of the major disadvantages of conventional cooking of meat and meat products are longer cooking times and non-uniform heating of products (Chen, Ren, Seow, Liu, Bang, & Yuk, 2012; McKenna, Lyng, Brunton, & Shirsat, 2006). Conduction-based cooking generally results in longer cooking times and in the non-uniform heating of products, whereas in the case of novel thermal methods, food is heated mainly by radiation and/or convection due to the generation of heat within the product and by conduction to some extent. Generation of heat within the products consequently reduces cooking times and this can potentially lead to a more uniform heating without compromising meat safety and quality.

Dielectric heating (RF or microwave heating) is regarded as a volumetric form of heating which achieves quicker cooking times and can potentially lead to more uniform heating (Chen, Ren, Seow, Liu, Bang, & Yuk, 2012) due to dielectric energy. Dielectric energy induces molecular friction in water molecules to produce heat, which is partly dependent on the moisture content of food. Jeong, Lee, Choi, Lee, Kim, Min, et al. (2007) studied the effect of fat level, both with and without the addition of salt, on the cooking pattern and physicochemical properties of ground pork patties cooked by microwave energy. The authors observed that temperatures at the edge of the pork patties increased faster than those at the center or in the mid-way positions within the microwave cooker. Total cooking loss, drip loss, and reduction in diameter and thickness were higher in patties with 20% fat compared to those with 10% fat, thereby indicating the influence of fat content and salt on cooked meat using microwave energy. Yarmand and Homayouni (2009) investigated the effect of domestic microwave (700 W), industrial microwave (12000 W) and conventional oven heating/cooking of goat and lamb meat to reach an internal temperature of 70 °C. They observed higher cooking losses in microwave heating/cooking compared to conventional oven cooking, probably due to the separation of fat cells from the muscle matrix when in the presence of an electromagnetic field.

Radiofrequency (RF) heating employs electromagnetic energy for heating of food, thereby resulting in a shorter cooking time and a more uniformly heated product. In a study by Tang, Cronin, and Brunton (2005) it was shown that rapid RF cooking of turkey meat accelerated cooking time by a factor of 4 when compared to steam cooking, with no detectable sensory differences determined between samples. Unlike microwave and RF heating, infrared cooking involves heating of products by radiant energy which is generated externally and absorbed by food surfaces. Infrared heating has the distinct advantage of cooking food products in a

shorter processing time, while still maintaining product quality and safety. For example, Sheridan and Shilton (1999) studied the efficacy of cooking hamburger patties by mid infra-red (λ_{max} = 2.7 µm) and far-infrared (λ_{max} = 4.0 µm) sources. They observed that the change in core temperature follows closely the change in surface temperature which results in a short cooking time and is independent of the fat content of the samples when subjected to the higher energy source. However, with the lower energy source, the rate of core temperature rise is dependent on the fat content and a target core temperature can be achieved quickly for samples containing high fat. Gande and Muriana (2003) conducted a study that involved a radiant heat oven for pre-package pasteurisation of various meat products, including; turkey bologna, roast beef, corned beef and ham. The treatment time varied from 60 to 120 sec, and the air temperature ranged from 246 to 399°C. The authors reported a 1.25 to 3.5 log inactivation of *L. monocytogenes* after the products were passed through a radiant oven. Similarly, Huang (2004) obtained a 3.5 to 4.5 log reduction of *L. monocytogenes* in turkey frankfurters with an average initial inoculum of 106 to 107 CFU/cm^2.

Ohmic heating is another novel thermal process technique which employs electrical resistance to heat the food product. Ohmic heating can penetrate throughout the food instantly, compared to dielectric heating and radiant energy. Ohmic heating has shown several food industry applications with an aim of producing high quality, convenient and safe food products (Ramaswamy, Marcotte, Sastry, & Abdelrahim, 2014). Application of ohmic heating in meat was extensively reviewed by Yildiz-Turp, Sengun, Kendirci, and Icier (2013). Application of Ohmic heating in meat processing has shown its effectiveness in inhibiting microbial growth by providing uniform temperature distribution throughout the product and cooking faster and instantly inside the product. Studies have shown that meat products processed using ohmic heating resulted in shorter cooking times, higher cooking yields, more

consistent appearances and better eating quality properties compared to conventionally-processed products (Dai, Miao, Yuan, Liu, Li, & Dai, 2013; Engchuan, Jittanit, & Garnjanagoonchorn, 2014).

1.2.2. Novel non-thermal technologies

Several novel non-thermal technologies have been widely investigated for extending the shelf-life of food products while maintaining a fresh-like quality. Application of novel technologies has been reviewed extensively (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015; Kentish & Feng, 2014; Thirumdas, Sarangapani, & Annapure, 2015). Many of these technologies have been exploited by meat processors for niche applications. Scientific literature suggests that these technologies will assist meat processors in meeting both consumer demands for high quality, superior nutrition and safer products, in addition to conscientious demands for energy efficient processes (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015). Non-thermal techniques, including HPP, ultrasound, cold plasma, pulsed-UV light, pulsed electric field and ozone processing have some promising commercial applications for the meat industry.

In the meat industry, HPP involving pressures over 100 MPa is mainly employed to increase shelf-life, improve the food safety profile and to alter key quality parameters, including texture, colour and water holding capacity of meat and meat products (Bajovic, Bolumar, & Heinz, 2012). The effect of HPP on meat proteins is well documented. HPP induces changes in the secondary, tertiary and quaternary structure of meat proteins by influencing protein conformations and various molecular interactions (e.g. hydrogen bonds, hydrophobic interactions and electrostatic bonds) depending on the type of meat and processing conditions employed (Campus, 2010). Modification in meat proteins at a molecular level can

lead to changes in meat texture, water holding capacity and gelation capacities, thus adding a new dimension in meat-based product development (Sikes, Tobin, & Tume, 2009; Sun & Holley, 2010) In addition, HPP has successfully been used to reduce microbiological risks from vegetative pathogenic and spoilage microorganisms commonly encountered in meat, including *E. coli*, *S. typhimurium*, *L. monocytogenes*, *S. enteritica, S. aureus*, *Y. entreocollitica* and *Debaryomyces hansenii* (Jofré, Aymerich, Bover-Cid, & Garriga, 2010; Kruk, Yun, Rutley, Lee, Kim, & Jo, 2011).

The application of pulsed electric field processing has been demonstrated to inactivate pathogenic and spoilage microorganisms at ambient temperatures, thereby extending the overall shelf-life of different food products without inducing significant negative changes in sensorial and nutritional attributes (Bendicho, Barbosa-Cánovas, & Martín, 2002; Griffiths & Walkling-Ribeiro, 2014; Sampedro, Rodrigo, Martinez, Rodrigo, & Barbosa-Cánovas, 2005). Apart from microbial inactivation, pulsed electric fields can induce changes in the structure and texture of meat, potentially improving its functional properties or aiding in the development of new products (Faridnia, Ma, Bremer, Burritt, Hamid, & Oey, 2015; Topfl & Heinz, 2007). Studies have shown that pulsed electric field treatment can enhance meat tenderness and quality attributes of beef muscles (Bekhit, Suwandy, Carne, van de Ven, & Hopkins, 2016; Suwandy, Carne, van de Ven, Bekhit, & Hopkins, 2015a, 2015b). Despite several promising applications, significant challenges have been encountered for large-scale commercial applications. One of the limiting factors of the technology is the electric field strength (20 – 50 kV/cm) required to achieve microbial inactivation. While electric field strengths required to deliver antimicrobial properties are possible at a laboratory or pilot-scale, commercial scale units require higher voltages or special electrode arrangements in order to achieve desired conditions for microbial inactivation.

Pulsed-UV light has shown efficacy against a wide range of food borne pathogens and spoilage microorganisms. Studies have demonstrated the effectiveness of pulsed light to inactivate microorganisms on the surface of several muscle foods, including beef steaks (Dunn, Ott, & Clark, 1995), ready-to-eat sausages (Uesugi & Moraru, 2009), chicken breast (Keklik, Demirci, & Puri, 2010) and ready-to-eat cooked ham (Hierro, Barroso, la Hoz, Ordóñez, Manzano, & Fernández, 2011). Dunn, Ott, & Clark (1995) reported a 2-log reduction in microbial growth in beef steaks subjected to a pulsed light treatment at 5 J cm^{-2}. Pulsed light was shown to be effective in reducing *L. monocytogenes* and *S. Typhimurium* on the surface of ready-to-eat dry cured meat products (1.5–1.8 log cfu/cm^2 at a fluence of 11.9 J/cm^2), with no significant negative effect on organoleptic properties (Ganan, Hierro, Hospital, Barroso, & Fernández, 2013). Intense pulsed light decontamination treatment was successfully applied to reduce *L. monocytogenes* and *E. coli* O157:H7 on stainless steel meat contact surfaces (Rajkovic, Tomasevic, Smigic, Uyttendaele, Radovanovic, & Devlieghere, 2010).

Ozone (gas or aqueous phase) has been used for surface decontamination of meat and meat products. Lacumin, Manzano, and Comi (2012) investigated the use of ozonated air to limit mould growth on meat products. The gaseous ozone treatment prevented the growth of *Aspergillus ochraceus* and the presence of ochratoxin on the surface of sausages without adversely influencing ripening, physicochemical parameters, peroxide value or sensory characteristics of treated sausages. Cárdenas, Andrés, Giannuzzi, and Zaritzky (2011) analysed the effects of ozone treatment at refrigeration temperatures on microbial counts and key quality parameters (surface colour and rancidity) in beef samples. Gaseous ozone (154 x 10^{-6} kg m^{-3}) treatment after 3 or 24 hr of inoculation with *E. coli* in culture media resulted in complete inactivation at temperatures of 0° and 4°C. However, the treatment was less effective in beef tissue and quality characteristics, such as surface colour and lipid

peroxidation, were undesirable. In the US, ozone is being used commercially as an equipment sanitizer for pre-operational and in-process sanitation within ready-to-eat meat processing plants. The use of ozone-enriched cold water, instead of organic acid (sodium lactate) applications, has resulted in annual savings of $124,000 (Rice, Graham, Sopher, Zhang, Barbosa-Cánovas, Balasubramaniam, et al., 2011).

The application of cold atmospheric plasma or non-thermal plasma in the food industry has been reviewed extensively (Niemira, 2012). Non-thermal plasma is composed of highly reactive species, including gas molecules, primarily composed of photons, ions and free electrons which are effective against a wide range of microorganisms, including pathogens, biofilm-formers and bacterial spores commonly associated with meat (Noriega, Shama, Laca, Díaz, & Kong, 2011). The plasma technique has recently been tested on a range of meat and meat products, including pork (Fröhling, Durek, Schnabel, Ehlbeck, Bolling, & Schlüter, 2012; H.-J. Kim, Yong, Park, Choe, & Jo, 2013), chicken (Lee, Jung, Choe, Ham, Lee, & Jo, 2011), ham (Lee, Jung, Choe, Ham, Lee, & Jo, 2011; Song, Kim, Choe, Jung, Moon, Choe, et al., 2009), beef jerky (B. Kim, Yun, Jung, Jung, Jung, Choe, et al., 2011) and bacon (B. Kim, et al., 2011). These studies demonstrated that the application of cold atmospheric pressure plasma treatment to meat surfaces resulted in product decontamination, thus improving safety and the shelf-life stability of meat products.

1.3. Ultrasound technology

The history of scientific advances and discovery of ultrasound are rooted in the study of sound, with Sir Isaac Newton first proposing his theory of sound waves in 1687 (Mason, 1998). The fundamental difference between sound and ultrasound is the wave frequency. Generally, sound waves are divided into three categories that encompass different frequency ranges -

(i) *Audible waves* are waves that lie within the range of sensitivity of the human ear (10 Hz – 20 kHz); (ii) *Infrasonic waves* are waves having frequencies below the audible range (<16 Hz), (iii) *Ultrasonic waves* are waves having frequencies above the audible range (>20 kHz) and less than microwave frequencies (upto 10 MHz). Depending on the frequency, ultrasonic waves can be divided into three categories that encompass different frequency ranges: (i) power ultrasound (20 – 100 kHz); (ii) high frequency or extended range for sonochemistry (20 kHz – 2 MHz) and (iii) diagnostic ultrasound (>1 MHz) as shown in Figure 1.1. Generally, from an application perspective, ultrasonic can also be broadly classified as low intensity (<1 W/cm^2) and high intensity (10 – 1,000 W/cm^2) sonication. Ultrasound is employed in various sectors including chemical, bioprocessing, food processing, pharmaceutical, medical and defence (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Chemat & Khan, 2011). Within the food industry, high frequency ultrasound is typically used as a non-destructive, non-invasive analytical technique for quality assurance, process monitoring and control, whereas low frequency sonication is employed for process intensification. High frequency ultrasound employs low power levels which exert no or minimal physical and chemical alterations in the material through which the wave passes, hence can be employed for food analysis and quality control. In contrast, the low frequency ultrasound employs higher power levels for desirable physical and chemical modifications for various bioprocessing applications.

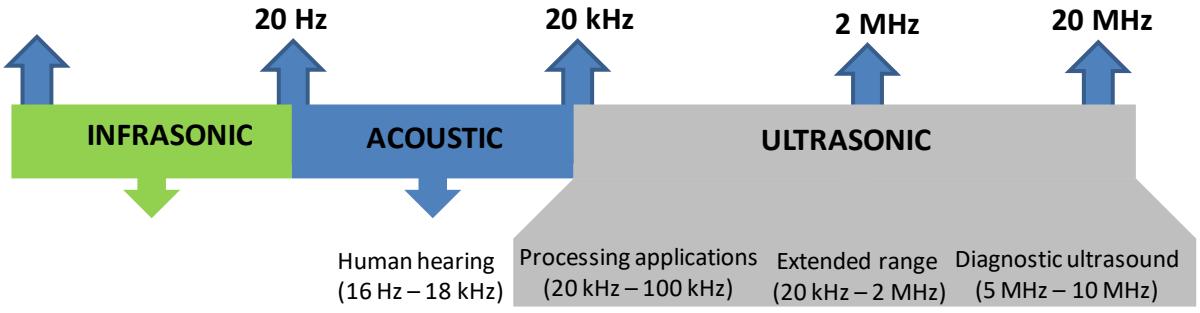


Figure 1.1. Ultrasound frequency range for food applications

1.3.1. Principles and mechanisms

Ultrasound is a form of vibrational energy in the frequency range of 20 kHz to ca. 10 MHz. Vibrational energy is produced by ultrasonic transducers which convert electrical energy to vibrational sound energy. Ultrasonic transducers are also capable of converting sound waves into electrical energy and are available in a range of size and frequencies depending on the application. Piezoelectric and magnetostrictive are the two most commonly used transducers (Mason & Dietmar Peters, 2002). Various transducer types and generation of low or high frequency ultrasonic waves for a wide range of food and non-food applications have been reviewed extensively (Mattiat, 2013; Nakamura, 2012). Low frequency ultrasound generated using a transducer is transmitted using either an ultrasonic bath or probe based system operating at various frequencies. Placement of the transducer is important for even distribution of ultrasonic energy for process intensification. The major advantage of a bath system arrangement is that transducers are not in direct contact with the sample, but significant losses of acoustic energy occur to the vessel and surroundings.

Physical and chemical phenomena of ultrasound associated with ultrasound frequencies include agitation, vibration, pressure, shock waves, shear forces, microjets, compression &

rarefaction, acoustic streaming, cavitation and formation of free radicals. The physical effects of ultrasound are dominant at the lower frequency range of 20 – 100 kHz with a higher level of transient cavitation whereas chemical effects are dominant in range of 200 – 500 kHz due to generation of large number of active bubbles (Feng, Barbosa-Cánovas, & Weiss, 2011). Acoustic streaming is dominant at higher frequencies (>1 MHz) with less physical and chemical effects associated with cavitation. Acoustic streaming is a physical force of the sound due to a pressure gradient which is capable of displacing ions and small molecules. Cavitation or Blake threshold is defined as the lowest acoustic pressure at which bubble formation is observed. The phenomenon of the creation, expansion, and implosive collapse of microbubbles in ultrasonically irradiated liquids is known as ‘‘acoustic cavitation’’ (Figure 1.2). At higher frequencies, acoustic pressures are lower and hence cavitation rarely occurs. Whereas at low frequencies, ultrasound waves of high acoustic pressures are possible, hence cavitation is observed.

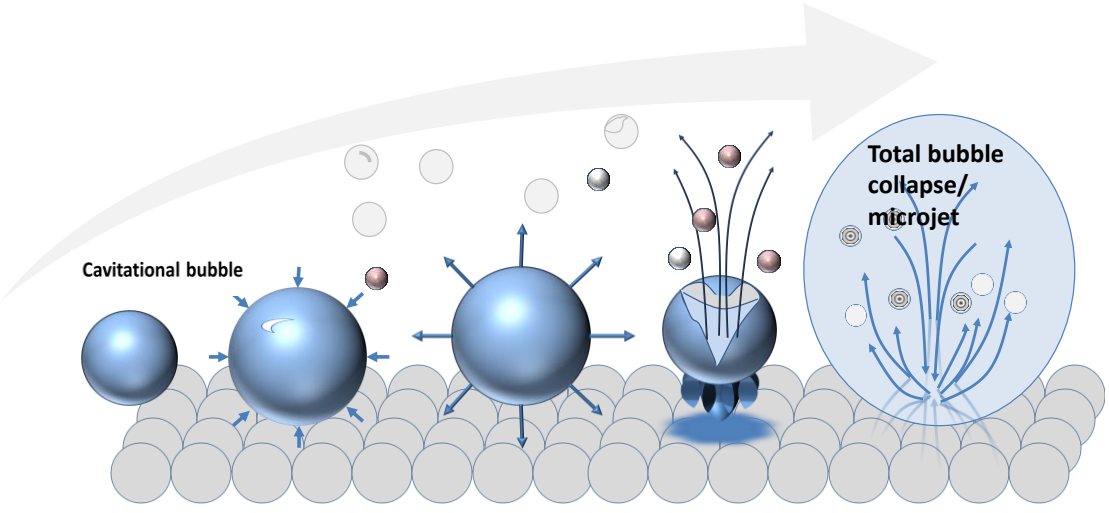


Figure 1.2. Formation and collapse of cavitation

The main driving force for the physico-chemical and biological effects of sonication is acoustic cavitation. When ultrasound propagates through any medium, it induces a series of

compression and rarefaction in the molecules of the medium. Such alternative pressure changes cause formation and ultimately collapse of bubbles in a liquid medium. This phenomenon of the creation, expansion, and implosive collapse of microbubbles in ultrasonically irradiated liquids is known as ‘‘acoustic cavitation’’. Cavitation itself is generally regarded as a problem in that it causes erosion where liquids move rapidly across metal surfaces, e.g., in pipes and impellors; however, when the energy of cavitation is harnessed it provides a remarkable energy source. This is essentially what sonication provides: controlled cavitation energy. The cavitation bubbles formed are roughly divided into two types namely transient cavitation (inertial) and stable cavitation (non- inertial). Stable cavitation bubbles are bubbles that oscillate, often non-linearly, around some equilibrium size during many cycles of acoustic pressure. They exist for long enough to gather together and form large bubble clouds. Stable cavities are relatively long-lived gas bubbles and exist for many compression and rarefaction cycles (Piyasena, Mohareb, & McKellar, 2003). Transient cavitation or inertial cavitation, are bubbles which exist for a very short period, sometimes less than one cycle, and collapse violently (Abramov, Abramov, Bulgakov, & Sommer, 1998; Laborde, Bouyer, Caltagirone, & Gérard, 1998). Stable bubbles are considered to be weakly and symmetrically oscillating bubbles, whereas transient bubbles are considered to be ‘‘active cavitation’’ bubbles. The collapse of cavitation bubbles is a near adiabatic process and results in the generation of high temperatures and localised high pressures within the bubble (Suslick, Hammerton, & Cline, 1986).

The cavitation bubbles are generated by the ultrasound wave as it passes through the liquid. Like any sound wave, it is transmitted as a series of compression and rarefaction cycles affecting the molecules of the liquid. When the negative pressure of the rarefaction cycle exceeds the attractive forces between the molecules of the liquid, a void is formed. This void

or cavity in the structure takes in a small amount of vapour from the solution so that on compression it does not totally collapse, but instead continues to grow in size in successive cycles to form an acoustic cavitation bubble. Most liquids contain nuclei about which cavitation bubbles originate. These nuclei may consist of dispersed particles, prominences on immersed surfaces, and minuscule gas bubbles. In fact, unless especially treated, liquids contain dissolved or entrained gas. Most liquids contain nuclei about which cavitation bubbles originate. Once nucleation has taken place, a gas bubble will grow by rectified diffusion over many periods of the driving pressure oscillations until it breaks up. It should be noted that the bubbles may originate by virtue of dissolved gases (gaseous cavitation) or form by vaporisation of the liquid itself (vaporous cavitation). Unlike in a single bubble system, the bubble growth in a multi-bubble system involves two processes, namely, rectified diffusion and bubble coalescence (Thakur & Nelson, 1997). Occurrence of intense cavitation depends upon the relationship amongst the dimensions of the nuclei, the wavelength and the intensity of the field. Gas bubbles in a liquid profoundly affect the acoustic behaviour of the liquid due to the dispersion and attenuation of the ultrasound that follows (Villamiel & de Jong, 2000). There are many thousands of such bubbles in a liquid, some of which are relatively stable but others expand further to an unstable size and undergo violent collapse to generate temperatures of about 5,000 K and pressures of the order of 50 MPa (Butz & Tauscher, 2002; Mason & Peters, 2002; Piyasena, Mohareb, & McKellar, 2003; Vollmer, Kwakye, Halpern, & Everbach, 1998) at a minuscule level. Theoretical calculations using hydrodynamic models of cavitational collapse have reported temperature and pressure estimates of 2000-10,000 K and 100-1000 MPa (Rayleigh, 1917). However, as the size of the bubbles is very small relative to the total liquid volume, the resulting heat produced is rapidly dissipated with little appreciable change in global conditions. Both stable and transient cavitation can be detected

by analysing the acoustic radiation originating from the bubbles (Richardson, Pitt, & Woodbury, 2007). Temperature and pressure changes that occur from these implosions cause shear disruption, thinning of cell membranes and cell disruption. The implosion of cavitating bubbles also generates turbulence at microscopic level, high-velocity inter-particle collisions and agitation in micro-porous particles of the matrix which accelerates the diffusion (Shirsath, Sonawane, & Gogate, 2012; Toma, Vinatoru, Paniwnyk, & Mason, 2001; Valachovic, Pechova, & Mason, 2001). Ultrasonic wave also facilitates hydration and swelling of the matrix with an enlargement of pores which increases the diffusion of solvent into the matrix and increase mass transfer.

The chemical effects of ultrasound have been studied extensively over the last century. Under the extreme temperature and pressure conditions, highly reactive radicals are generated. For example, if water is the medium, $H^{\bullet}$ and $OH^{\bullet}$ radicals are generated by the dissociation of water ($H_2O \rightarrow OH^- + H^+$). Both stable cavitation and an increase in the number of active bubbles can be expected to increase the amount of hydroxyl radicals generated with an increase in ultrasound frequency. Ashokkumar, Sunartio, Kentish, Mawson, Simons, Vilkhu, et al. (2008a) observed an increase in $OH^{\bullet}$ radicals in water with an increase in ultrasonic frequencies from 20 kHz to 358 kHz followed by a decrease in $OH^{\bullet}$ yield with an increase in frequency from 358 kHz to 1062 kHz. A plausible explanation can be the shortening of acoustic cycle at high frequencies thus restricting the amount of water vapour that can evaporate into the bubble during the expansion phase of the acoustic cycle. This decrease in the amount of water vapour within the collapsing bubble can lead to a decrease in the amount of $OH^{\bullet}$ radicals (Ashokkumar, et al., 2008a). These free radicals are chemically reactive and are potentially damaging to any cell they come in contact with (Henglein & Kormann, 1985). These primary radicals can induce a wide variety of chemical reactions in the bulk solution

including the degradation of target compounds. The majority of degradation studies in bath and probe based systems are reported for volatile and non-volatile organic compounds including aromatics, chlorinated alkenes, and dyes in model solutions (Goel, Hongqiang, Mujumdar, & Ray, 2004). The reported degradation of target compounds is mainly due to cavitation and sonochemical reactions occurring simultaneously or in isolation.

1.3.2. Ultrasonic energy measurement

One of the most important aspect for processing application of power ultrasound is the need to find the appropriate amount of acoustic energy to apply to the system. Almost all ultrasonic equipment has the capability to vary the power generated, i.e. the amplitude of vibration of the transducer which is a measure of the energy required to drive the transducer. However, it is important to know the absolute power entering in the system. Determination of energy used in the extraction process is difficult to measure because ultrasonic energy is absorbed, reflected or used in cavitation. Energy measurement can be determined either (i) chemical dosimetry or (ii) calorimetric method. Chemical dosimetry involves the measurement of H_2O_2 which is produced during sonication as a result of cavitation. Collapse of cavitational bubble produces sufficient energy to break H_2O to yield H+ and OH^- radicals. These short lived radicals are reactive and produce hydrogen peroxide which can be determined using iodine dosimetry method. H_2O_2 produced as a result of cavitation reacts with iodine solution to produce iodine molecules (Reactions 1.1- 1.4) which can be determined using UV/VIS spectrophotometer (Ashokkumar, Sunartio, Kentish, Mawson, Simons, Vilkhu, et al., 2008b). Measurement of H_2O_2 is complex in extraction system due to the presence of various components including ions and other colloidal components.

$$H_2O \rightarrow OH^- + H^+ \qquad \text{(Reaction 1.1)}$$

$$2OH^- \rightarrow H_2O_2 \qquad \text{(Reaction 1.2)}$$

$$H_2O_2 + 2I^- \rightarrow I_2 + 2OH^- \qquad \text{(Reaction 1.3)}$$

$$I_2 + I^- \rightarrow I_3^- \qquad \text{(Reaction 1.4)}$$

In a study, Tsukamoto, Yim, Stavarache, Furuta, Hashiba, and Maeda (2004) reported that the hydrogen peroxide formation rate is dependent on the amplitude of vibration of the transducer. As amplitude can be an indication of the ultrasonic cavitation this approach can serve as a reliable method for monitoring ultrasound power (O'Donnell, Tiwari, Bourke, & Cullen, 2010; Tiwari & Mason, 2012).

The calorimetric method is based on an assumption that ultrasound assisted system has no heat losses and can provide a rough estimate of energy introduced in the extraction system. Level of energy introduced in the system can be expressed as Ultrasonic Intensity (UI in W/cm^2) or Acoustic Energy Density (AED in W/cm^3 or W/mL). UI can be measured by determining the power (W) introduced into the system. Determination of UI or AED requires a measurement of power (P, Equation 1.1), and this can then be converted into UI by dividing by the area of the emitting face of the transducer (Equation 1.2), or density by taking into account the total volume (Equation 1.3). Ultrasonic power (P), ultrasonic intensity (UI) and acoustic energy density (AED) can be determined using Eq 1.1- 1.3. UI is the amount of energy emitted in the sample via radiating surface area of the transducer. AED is the amount of ultrasound energy per unit volume of sample and can be used as a guide for scale up of extraction system.

$$P = mC_p \left[\frac{dT}{dt}\right]_{t=0} \qquad [1.1]$$

$$UI = \frac{P}{A} \quad [1.2]$$

$$AED = \frac{P}{V} \quad [1.3]$$

where, (*dT/dt*) is the initial rate of change of temperature over time ($^oC\ s^{-1}$), dT/dt can be determined by fitting temperature change vs time data obtained for temperature rise using standard thermocouple against time. C_p is the specific heat of the medium, (kJ $kg^{-1}\ ^oC^{-1}$), *P* is ultrasonic power (W), *m* is sample mass (kg) and *V* is sample volume (mL).

1.4. Ultrasound applications in meat processing

Although commercial applications of power ultrasound have been demonstrated in some industries (e.g. chemical, cosmetic, textile, polymer, and petrochemical), it has recently gained interest in the food industry including meat processing. Power ultrasound has been known to influence technological properties and quality of fresh and processed meat. However, limited research studies are available demonstrating the application of ultrasonic for various unit operations in meat value chain. The application of ultrasound in meat processing to date includes surface decontamination, tenderisation, brining, freezing, thawing, cooking and cutting of meat (Alarcon-Rojo, Janacua, Rodriguez, Paniwnyk, & Mason, 2015). Table 1.1 demonstrates application of ultrasound for various meat processes.

Table 1.1. Application of ultrasound for meat processing

Meat	Condition	Effect of ultrasound	Reference
Decontamination of meat			
Chicken wing surface	2.5 $Wcm^{-2,}$ 40 kHz, 3 or 6 min	Microorganism reduction. Higher reduction with higher time. E. coli more sensible to ultrasound.	(Kordowska-Wiater & Stasiak, 2011)

fresh pork jowl	SonoSteam treatment, steam (130 °C, 3.5–5 atm) and ultrasound 30–40 kHz for 0.5 to 2 sec.	Less skin and surface bacteria	(Morild, Christiansen, Sørensen, Nonboe, & Aabo, 2011)
Chicken carcasses	SonoSteam treatment, a combination of steam at 90–94 °C and ultrasound at 30–40 kHz through specially designed nozzles	Campylobacter and total count viable reduction.	(Musavian, Krebs, Nonboe, Corry, & Purnell, 2014)
Bovine *Semitendinosus* muscle	Ultrasonic frequency of 40 kHz and an intensity of 11Wcm^{-2}	Reduced growth of mesophilic, psychrophilic bacteria and total coliforms in beef stored at 4 ◦C	(Caraveo, Alarcon-Rojo, Renteria, Santellano, & Paniwnyk, 2015)
Tenderisation of meat			
Beef (*semimembranosus)* 24 h post mortem and matured for 24, 48, 72 or 96 h at 2 °C	2 W cm^{-2}, 45 kHz, 2 min	No effect on pH or colour. Reduced hardness.	(Stadnik & Dolatowski, 2011)
Hen breast meat 0, 1, 3, or 7 days at 4 °C	24 kHz, 12 W cm^{-2}, 15 s period	Reduced shear force. No change in cooking loss.	(Xiong, Zhang, ZhanG, & Wu, 2012)
Chicken breast and soybean gels	20 kHz, 450 W for 0, 3, 6, 9 and 12 min (4 or 2 s pulses)	More viscoelastic gel, improved WFB and textural properties. Homogeneous fine network microstructures	(Zhao, Wang, Zou, Li, Kang, Xu, et al., 2014)
Beef *Longissimus lumborum* muscles	Ultrasonic probe (20 kHz) at power of 100 and 300W	Simultaneous application of both ultrasound and papain enzyme improved the tenderness of beef	(Barekat & Soltanizadeh, 2017)
Brining			
Pork *longissimus dorsi*	40 kHz; 37.5 W/dm^3	Higher salt and water diffusion	(Cárcel, Benedito, Bon, & Mulet, 2007)

Pork *longissimus dorsi*	2–4Wcm^{-2}, 20 kHz	Higher salt diffusion. Diffusion coefficient increases with ultrasound intensity.	(Siró, Vén, Balla, Jónás, Zeke, & Friedrich, 2009)
Pork *longissimus thoracis* and *lumborum*	0, 40, 56, 72 Wcm^{-2}, 34–40 kHz, 2, 4, 6 h	Reduction of salting time without changes in sensory attributes.	(McDonnell, Lyng, Arimi, & Allen, 2014)
Bovine *longissimus dorsi* muscles	20 kHz ultrasonic probe at different power levels (150, 200, 250 and 300W)	ultrasound intensity could significantly improve the D values of NaCl and water. Space between myofibrils increased with increased ultrasound intensity.	(Kang, Wang, Zhou, Zhang, Xu, & Guo, 2016)

1.4.1. Decontamination of meat

Power ultrasound has the ability to generate physical (micromechanical) and chemical effects resulting in the disruption of cellular structures. The bactericidal effect of ultrasound is largely attributed to intracellular cavitation which can cause thinning of cell membranes, temperature increase and production of free radicals (P Butz & B Tauscher, 2002; Chemat & Khan, 2011). It is proposed that in the compression and rarefaction cycles of ultrasound energy, fluctuating pressures induce formation and collapse of microscopic cavitation bubbles resulting in micro mechanical shockwaves. These shockwaves can disrupt cellular structural and functional components leading to cell lysis (Hoover, 2000). Free radicals, mainly hydroxyl radicals are produced during the collapse of cavitation bubbles as a result of sonolysis of water. These radicals (e.g. OH^-, H^+) through series of reactions produce hydrogen peroxide (H_2O_2) and other reactive species. Cavitation, production of radicals, along with microstreaming can cause thinning of the cell membrane and DNA damage leading to microbial inactivation (Alarcon-Rojo, Janacua, Rodriguez, Paniwnyk, & Mason, 2015; Earnshaw, Appleyard, & Hurst, 1995; Turantaş, Kılıç, & Kılıç, 2015). The inactivation of various

pathogenic and spoilage microorganisms including *E. coli*, *Listeria monocytogenes*, *Salmonella* sp., and *Bacillus* sp. pertinent to meat products is extensively reported in the literature (Piyasena, Mohareb, & McKellar, 2003). However, most studies indicate lower antimicrobial efficacy of ultrasound depending upon the conditions employed. Thus, a combination of ultrasound with pressure (manosonication), ultrasound and heat (thermosonication), ultrasound, heat and pressure (manothermosonication) or the combination of ultrasound with other non-thermal approaches has often been recommended for improving efficacy (Pagán, Manas, Alvarez, & Condón, 1999; Pagán, Manas, Raso, & Condón, 1999). Kordowska-Wiater and Stasiak (2011) investigated the removal of Gram-negative bacteria (*Salmonella anatum, E. coli, Proteus sp. and Pseudomonas fluorescens*) from the surface of chicken skin after treatment with ultrasound (40 kHz and 2.5 W cm^{-2} for 3 or 6 min) in water and in aqueous 1% lactic acid. Sonication in water alone or in lactic acid for 3 min resulted in a 1.0 CFU cm^{-2} reduction on the skin surface, but longer treatment (6 min) resulted in a reduction of over 1.0 CFU cm^{-2} in the water samples and 1.5 log CFU cm^{-2} in the lactic acid treated samples. Ultrasound treatment in combination with lactic acid may be a suitable method for decontamination of the poultry skin. Herceg, Markov, Sobota Šalamon, Režek Jambrak, Vukušić, and Kaliterna (2013) studied the effect of high-intensity ultrasound on the inactivation of suspensions containing *E. coli*, *Staphylococcus aureus*, *Salmonella sp.*, *Listeria monocytogenes* and *Bacillus cereus* treated with an ultrasound probe operating at 20 kHz and amplitudes of 60, 90 and 120 µm for 3, 6 and 9 min at 20, 40 and 60 °C. Increasing any of these three parameters improved the inactivation rates of bacteria in suspension. The results also showed increased inactivation after prolonged treatment time, especially in combination with high temperature and amplitude level. Ultrasound in combination with steam have shown potential for inactivation of pathogenic and spoilage microorganism. For instance, a

study investigated the decontaminating effect of SonoSteam treatment (ultrasound+steam) on broiler carcasses under commercial processing conditions with a rate of 8,500 carcasses per hour (Musavian, Krebs, Nonboe, Corry, & Purnell, 2014). SonoSteam treatment achieved reductions of approximately 1.0 log for *Campylobacter* and 0.7 log for Total Viable Counts with no significant changes in sensorial properties of chicken carcasses.

1.4.2. Meat tenderisation

As tenderness is one of the most important attributes of meat quality, researchers and meat processors have always been looking for efficient tenderisation processes. One method of increasing meat tenderness and sensory attributes is by employing ultrasound. Although ultrasonics for meat tenderisation was reported as early as the 1970s by Zayas and Orlova (1970), the actual mechanism is still unclear and results are inconsistent in some cases. Tenderisation effect of ultrasound is attributed to various mechanisms including physical disruption of muscle tissues through cavitation induced effects such as high shear, pressure and temperature; high proteolysis through the release of either cathepsins from lysosomes and/or Ca^{2+} from intracellular stores; Ca^{2+} activates calpains (S. Jayasooriya, Bhandari, Torley, & D'arcy, 2004; S. D. Jayasooriya, Torley, D'arcy, & Bhandari, 2007; Turantaş, Kılıç, & Kılıç, 2015).

Several studies have focused on the effect of ultrasound on meat tenderisation showing positive and no significant effects on meat tenderisation (Table 1.1). For example, Dickens, Lyon, and Wilson (1991) determined the effect of ultrasound (40 kHz, 15 min) on the texture and cooking loss of the cooked broiler breast meat and observed that the ultrasound treatment did not affect cooking loss, but improved texture significantly. Dolatowski and Twarda (2004) reported that ultrasound treatment (45 kHz, 2 W/cm^2 or 25 kHz, 2 W/cm^2)

during the rigor mortis period (up to 24 h post mortem) resulted in improved tenderness and higher water holding capacity in the *semimembranosus* muscles. On contrary, Got, Culioli, Berge, Vignon, Astruc, Quideau, et al. (1999) employed high-frequency (2.4 MHz) ultrasound to treat pre-rigour meat and observed an initial increase in firmness (compression force) of raw meat compared to untreated or post-rigour sonicated meat. After 14 days of ageing, this group observed no difference between untreated and pre-rigour or post-rigour ultrasound treated meat. Ultrasound in combination with other methods is also reported to be effective for improving marination, tenderness and modifying the functional properties of meat and poultry products (Turantaş, Kılıç, & Kılıç, 2015). For example, Xiong, Zhang, ZhanG, and Wu (2012) reported that contribution of mechanical disruption by ultrasound (24 kHz for 4 min) and endogenous proteolytic enzymes can be a suitable method for improving tenderness and decreasing the cooking loss of chicken meat. Barekat and Soltanizadeh (2017) evaluated the effects of ultrasonic alone, enzyme treatment, and simultaneous application of both ultrasound and papain on the tenderness and certain other physicochemical properties of *Longissimus lumborum* muscles. Results showed that the most proteolytic activity and the higher tenderness value were obtained when the combined treatment was applied at ultrasonic power of 100 W for 20 min. They also concluded that the combination of ultrasound radiation and papain treatment could be employed for the production of tender beef pieces.

1.4.3. Brining

Low frequency ultrasound in the range of 20-100 kHz is known to enhance several mass transfer processes, including brining and curing of meat. It is well recognised that ultrasound allows faster and uniform diffusion of brine solution into the meat tissues. Various physical and chemical phenomena including agitation, vibration, pressure, shock waves, shear forces, microjets, compression and rarefaction, acoustic streaming, cavitation and radical formation

are responsible for ultrasonic effect. The main driving force for enhancing salt diffusion is acoustic cavitation. It has been proved that power ultrasound treatment can directly increase the diffusion coefficient, which can shorten the curing time. Cárcel, Benedito, Mulet, and Riera (2003) immersed sliced pork in saturated brine solution at 2^{o}C for 45 min with different agitation. It was reported that sodium chloride and water content in the pork samples were higher with than without ultrasound treatment. Siró, Vén, Balla, Jónás, Zeke, and Friedrich (2009) also revealed that ultrasonic brining significantly improved salt diffusion in meat, in comparison with static brining. McDonnell, Lyng, Arimi, and Allen (2014) reported that it took 2 h to finish curing of pork ham, achieving 2.25% NaCl content, using ultrasound treatment while it needed about 4 h for the control treatment. Some authors pointed out that the mass transfer was not affected until intensity thresholds were reached. Cárcel, Benedito, Bon, and Mulet (2007) reported thresholds as 39 and 51 W cm^{-2}. But Siró, Vén, Balla, Jónás, Zeke, and Friedrich (2009) exhibited the mass transfer enhancement effect of ultrasound (20 kHz) at ultrasound intensity as low as 2-4 W cm^{-2}. They also reported an exponential increase of the diffusion coefficient with the ultrasound intensity. Recently Kang, Wang, Zhou, Zhang, Xu, and Guo (2016) evaluated the effects of ultrasound intensity and salt concentration of brining for accelerating brine transfer into beef. The results of diffusion coefficient study showed that ultrasound intensity could significantly improve the diffusion coefficient values of NaCl and water with best result at ultrasound intensity of 20.96 W/cm^2.

1.4.4. Other niche applications

Ultrasound has the ability to improve the characteristics associated with heat transfer, which is essential for cooking of food including meat. Several ultrasound cooking processes have also been developed for meat products. Ultrasound cooking (1000 W, 20 kHz) of beef samples

in a water bath reduced the cooking time (6.7 min) and energy consumption (0.08 W/g) and gave a higher yield (85.3%) than convection cooking in a broiler (12.3 min, 0.22 W/g, 76.1%) (Pohlman, Dikeman, Zayas, & Unruh, 1997). Ultrasound cooking provides a significantly faster cooking rate, lesser cooking losses and higher post-cooking moisture content with greater myofibrillar tenderness (Chemat, Zill e, & Khan, 2011; McClements, 1995; Skene, Martin, Delaney, Paluch, & Capodieci, 2004). Ultrasound has been used to improve the production of processed meat products. For instance, Manna, Russell, Voic, Nov al, Ng, and Pantano (2000) patented an ultrasonic device for the production of casing-less sausages. The patent describes an ultrasonic tool for treatment of a cylindrical shape to create the skin. The skin resulting from ultrasonic treatment has sufficient tensile properties to hold the cylindrical shape during final processing. Similarly, Kortschack and Heinz (2004) also describe a method to utilise ultrasound along with heat for the formation of a thin, sealed, shape-stabilizing independent coagulation skin on the surface of raw sausages

Application of acoustic waves for food thawing is an innovative technology in food processing. Acoustic thawing has potential application for commercial thawing of frozen food if the appropriate frequencies and sound power are chosen. For example, (Gambuteanu & Alexe, 2013) observed no significant differences in the chemical, microbiological or textural properties between the meats thawed by low intensities of ultrasound (25 kHz; $0.2Wcm^{-2}$ or $0.4Wcm^{-2}$) compared to thawing in water. Whereas, (Hong, Chun, Jo, & Choi, 2014) observed a rapid thawing of pork using ultrasound treatment. This study explored the effects of water or brine (2% NaCl, w/v) immersion thawing combined with ultrasound treatment (40 kHz, 150 W) on the quality characteristics of pork. They reported that the acoustic thawing shortens the defrost time, reducing cook loss and improving tenderness.

1.5. Thesis objectives

Recent researches, advances and developments have led to the introduction of more efficient and versatile ultrasound potential applications in food processing. In particular to meat processing, ultrasound technology has shown potential for accelerated brining, tenderisation, rapid cooking, faster thawing and decontamination of meat and meat products. This thesis assesses the feasibility of using ultrasound technology to assist in development of functional meat products. In order to advance the application of ultrasound in meat processing and to gain greater insight into ultrasound assisted mechanisms, three main objectives encompassing several objectives were set as listed below:

Objective 1. To investigate the effect of ultrasound and underlining mechanisms of action on starter culture used for meat fermentation.

1.1. To investigate the effect of ultrasound treatment on the growth behaviour of *L. sakei* in MRS broth and in beef extract.

1.2. To characterise the cell-free extract for molecular weight distribution after fermentation.

1.3. To test the efficacy of cell-free extract against selected pathogenic microorganisms (*Staphylococcus aureus*, *Listeria monocytogenes Escherichia coli* and *Salmonella typhimurium*)

1.4. To investigate the effect of different ultrasound treatements on growth, morphology, viability and metabolic activities of *L. sakei*.

Objective 2. To investigate efficacy of ultrasound for diffusion of novel/functional ingredients with specific health function into the meat matrix.

2.1. To investigate and compare the effect of ultrasound on brining kinetics for pork meat slices using a brine solution of NaCl or a salt replacer.

2.2. To assess the potential for ultrasound application to improve the diffusion of encapsulated oils into a pork meat matrix.

2.3. To investigate the effect of US frequency and *L. sakei* culture on drying kinetics of beef jerky samples and elucidate changes in water distribution during drying process.

Objective 3: To investigate the effect of ultrasound on physicochemical properties of meat products.

3.1. To access the effect of ultrasound and salt replacer on physicochemical properties of pork meat samples.

3.2. To assess the effect for ultrasound application and cooking in terms of fatty acid profiles of pork meat subjected to encapsulated oils.

3.3. To investigate the effect of US frequency, the addition of *L. sakei* culture and drying time on key nutritional and physicochemical properties.

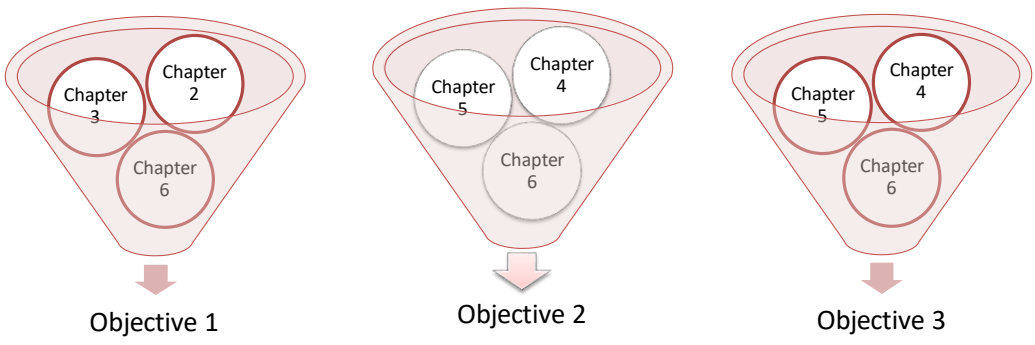


Figure 1.3. Association between thesis chapter and objectives.

Chapter 2: Effect of high intensity ultrasound on the fermentation profile of *Lactobacillus sakei* in a meat model system

Overview

This study investigated the efficacy of high intensity ultrasound on the fermentation profile of *Lactobacillus sakei* in a meat model system. The following paper has been published from this study.

- Ojha, K. S., Kerry, J. P., Alvarez, C., Walsh, D., & Tiwari, B. K. (2016). Effect of high intensity ultrasound on the fermentation profile of *Lactobacillus sakei* in a meat model system. Ultrasonics Sonochemistry, 31, 539-545.

2.1. Introduction

Ultrasound technology has been employed in various food and bioprocess engineering applications and its use has been directed at attempting to improve process efficiency (Barba, Grimi, & Vorobiev, 2015; Roselló-Soto, Galanakis, Brnčić, Orlien, Trujillo, Mawson, et al., 2015; Zinoviadou, Galanakis, Brnčić, Grimi, Boussetta, Mota, et al., 2015). Application of ultrasound alone, or in combination with other techniques, can be employed to inactivate enzymes and spoilage or pathogenic microorganisms. Inactivation of microorganisms during sonication is due to singular, or a multiple combination of several chemical and physical effects, including; thinning/disruption of cell membranes, localised heating, intracellular cavitation and sonolysis of water ($H_2O \rightarrow H^+ + OH^-$) leading to the production of free-radicals (O'donnell, Tiwari, Bourke, & Cullen, 2010). The effectiveness of ultrasound on microorganisms is strongly influenced by intrinsic and extrinsic factors, including; microbial ecology (e.g. type of microorganism, medium type and composition), ultrasound parameters (e.g. ultrasound power and frequency), sonication time, pH and temperature (Moncada, Aryana, & Boeneke, 2012). For example, studies have shown that Gram-positive bacteria are more resistant to ultrasound compared to Gram-negative bacteria, probably due to the presence of a thick peptidoglycan layer in the cell membrane of Gram-positive bacteria (Monsen, Lövgren, Widerström, & Wallinder, 2009). In recent years, application of ultrasound technology has expanded beyond simple inactivation of microorganisms. Ultrasound has demonstrated its capacity to enhance the growth of beneficial microorganisms and activity of enzymes, resulting in the production of biologically-active macromolecules. Beneficial effects of sonication is mainly attributed to the formation of pores on microbial cell membranes, thereby providing a channel for transport of essential nutrients and removal of toxic

substances across these membranes (F. Yang, Gu, Chen, Xi, Zhang, Li, et al., 2008; Yeo & Liong, 2012, 2013).

Probiotics are live microorganisms primarily derived from *Lactobacillus* and *Bifidobacterium* species which, when ingested in sufficient quantities, can exert beneficial health effects in the host (Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015). Lactic acid bacteria (LAB) are Gram-positive microorganisms which are widespread in nature and adapted to grow under different environmental conditions. Application of *Lactobacillus* sp. in food products has shown to inhibit the growth of pathogenic and spoilage microorganisms, improve organoleptic properties of food (Castellano, Holzapfel, & Vignolo, 2004; McLeod, Nyquist, Snipen, Naterstad, & Axelsson, 2008) and reduce the rate of food spoilage through competitive control of faster food spoilage microorganisms. Additionally, *Lactobacillus* sp. are reported to have beneficial effects on gut health and play a vital role in the alleviation of metabolic diseases (Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011; Tremaroli & Bäckhed, 2012). *L. sakei* is generally regarded as a safe strain in food, with its ability to grow at low temperatures and high salt concentrations. Consequently, *L. sakei* strain has been used as a starter culture in the development of various fermented food products. Bacteriocins and other metabolites obtained from probiotics, including *L. sakei,* have been demonstrated to inhibit the growth of various pathogenic microorganisms and thus broaden the food industry application range for *L. sakei* as a probiotic starter culture and bio-preservative.

It is evident that ultrasonic treatment can inactivate or activate microorganisms depending on the ultrasound control parameters of ultrasonic power, frequency and sonication time. For example, high intensity sonication treatment of milk containing *Bifidobacterium* sp. has been shown to enhance the production rates of organic acids and the growth of *Bifidobacterium* sp., while concurrently reducing fermentation time (Nguyen, Lee, & Zhou, 2009, 2012).

Similarly, Yang, Zhang, and Wang (2010) demonstrated that ultrasound pre-treatment, followed by incubation, enhances the growth of *Brevibacterium* sp. However, the effect of high intensity sonication on the growth behaviour of *L. sakei* has not been reported to date. The objective of this study was to (i) investigate the effect of ultrasound treatment on the growth behaviour of *L. sakei* in MRS broth and in beef extract; (ii) characterise the cell-free extract for molecular weight distribution after fermentation and (iii) test the efficacy of cell-free extract against selected pathogenic microorganisms.

2.2. Material and Methods

2.2.1. Culture and sample preparation

L. sakei DSM 15831 was obtained from DSM, Germany. The bacteria were cultured in Man Rogosa Sharpe (MRS; Oxoid Ltd, Cambridge, UK) medium at 30 ^{o}C in an microaerophilic chamber for 48 h. Microbial cells were harvested at 5000 g for 10 min and subsequently added to meat model system (MRS broth). MRS broth is considered as a best meat model system for fermentation studies (Leroy & De Vuyst, 2001). MRS broth (Oxoid Ltd, Cambridge, UK) was prepared as per the manufacturer's instruction. Additionally, fermentation studies were also carried out in the meat extract. Meat extract was prepared by cooking minced beef meat (500 g) in 2 L of water at 90 ^{o}C for 30 min, followed by centrifugation at 4000 × g. Fat collected from the upper surface of sample was removed manually and the supernatant autoclaved at 121 ^{o}C for 15 min, and subsequently cooled to room temperature.

2.2.2. Ultrasound treatment

MRS broth and meat extract solution (100 mL) containing *L. sakei* (1×10^6 cfu/mL) culture was sonicated in sterilised containers. The ultrasonic treatment of samples was carried out using a 550 W ultrasonic probe (XL2020 Heat Systems, Misonix Inc., Farmingdale, NY, USA) operating at 20 kHz with a 13 mm diameter ultrasonic probe operating in an amplitude range of 24 – 120 µm. The energy input was controlled by setting the amplitude of the ultrasound probe and ultrasonic power (P) power dissipated to the sample was calculated by using the method outlined by Tiwari, Muthukumarappan, O'Donnell, and Cullen (2008) using Eq. 2.1. Samples were subjected to sonication treatment at varying power level (0 - 68.5 W) and sonication time (0 – 9 min) operating at a frequency of 20 kHz. The temperature of the samples during sonication was monitored using thermocouples (Radionics, Ireland). Samples were sonicated at a temperature of ca. 20 ^{o}C and the final temperature of samples ranged from 30 – 40^{o}C depending on the applied ultrasonic power. These conditions were selected based on preliminary studies carried out prior to this study. Samples without ultrasound treatment were considered as the control. Samples were incubated at 30 ± 0.5^{o}C for 24 h in a temperature controlled incubation chamber (Model HCP 246, Memmert, Germany).

$$P = mC_p \left(\frac{dT}{dt}\right)_{t=0} \qquad [2.1]$$

Where (dT/dt) is the change in temperature over time (°Cmin^{-1}), C_p is the specific heat of water (4.18 kJkg^{-1}°C^{-1}), and m is the mass (kg).

2.2.3. *L. sakei* growth measurement

The growth of control and ultrasound treated *L. sakei* was monitored by measuring the optical density change at a regular interval of 30 min using 96 well plates in a temperature controlled microbial growth analyser (Multiskan^{TM} Microplate Spectrophotometer, Thermo Scientific) at a wavelength of 595 nm.

2.2.4. Cell-free extract preparation

Cell-free extract samples were obtained by centrifuging the samples at 4000 × g for 15 min at 20˚C. The supernatant obtained was filtered using a sterile 0.22 µm pore size filter unit (Millex GS Millipore) and subsequently, freeze-dried. Freeze-dried samples were stored at 4^{o}C for molecular weight analysis, SDS-PAGE and antimicrobial activity.

2.2.5. Lactic acid estimation

Lactic acid estimation was carried out using the method outlined byNguyen, Lee, and Zhou (2012) with slight modifications. HPLC (Waters, e2695 Seperation module) equipped with an auto-sampler and Rezex ROA-Organic acid H^{+} (8%) (350 mm × 7.8 mm) column (Phenomenex, UK) was utilised. Fermented MRS broth and meat extract solution (1 g) was mixed with distilled water (25 mL) and centrifued at 10000 × g for 10 min. samples were filtered using a 0.45 µm syringe filter.

2.2.6. Molecular weight analysis and SDS-PAGE

High Performance Size Exclusion Chromatography (HP-SEC) for each sample was carried out using a Waters Alliance 2795 Chromatography Separations Module (Waters Corp., Milford, USA) coupled to a Waters 2996 PDA detector at a wavelength of 254 nm. Separation was achieved using Tris-HCl 0.1 M as a mobile phase (0.85 mL/ min) and serial connected Zorbax GF-450 (9.4 x 250 mm, 6 µm) and Zorbax GF-250 (4.6 x 250 mm, 4 µm) columns. A calibration

curve was developed using albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), aprotinin (6.5 kDa), angiotensin II acetate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, 1.046 kDa) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, 0.555 kDa).

SDS–PAGE was carried out using a Compact PAGE AE-7350 (Atto, Tokyo, Japan) and ready-made 5–20% polyacrylamide gels (c-PAGEL compact gel C520L; Atto), as recommended by the manufacturer. Each extract solution (100 µg/mL) was mixed with an equal volume of 80 mM Tris–HCl buffer (pH 6.8) containing 140 mM SDS, 8 M urea, 20% glycerol, 0.01% bromophenol blue and 200 mM dithiothreitol (2 × sample buffer) and heated to 100°C for 5 min. Following denaturation, an 8 µL aliquot was subjected to electrophoresis. BLUE Star Pre-stained Protein-Ladder (Nippon Genetics, Tokyo, Japan) was used as a reference. On completion of the analysis, proteins were visualized by staining with Quick-CBB Plus (Wako, Osaka, Japan).

2.2.7. Antimicrobial activity

Various concentrations of cell-free extracts for selected samples were tested for antimicrobial activity against the following strains of bacteria: *Staphylococcus aureus* NCTC 8178, *Listeria monocytogenes*, *Escherichia coli* DSM 1103 and *Salmonella typhimurium* SARB 69. The strains were stored on ceramic beads in glycerol at 80^{o}C prior to use. A bead of each strain was streaked on to a nutrient agar plate and incubated for 18 h at 37 ^{o}C. A single colony was removed from each plate and inoculated into tubes containing 25 mL of sterile Mueller–Hinton Broth (MHB) and incubated for 22 h at 37^{o}C. Overnight cultures were vortexed and aliquots diluted appropriately in sterile MHB to produce solutions containing ca. 1 × 10^{6} cells/mL. Cell numbers were confirmed by plate counting.

2.2.8 Mathematical modelling and statistical analysis

To evaluate the sigmoidal shape of the *L. sakei* growth curve, the modified Gompertz was fitted to the optical density data. The Gompertz model (Eq. (2.2)) is commonly used for its flexibility and asymmetrical sigmoid shape.

$$OD_t = OD_0 + \frac{[OD_{max} - OD_0]}{[1 + e^{(-B(t-M)}]} \quad [2.2]$$

Where, OD_0 and OD_{max} are the initial and maximum optical density [-]; OD_t is the optical density at incubation time (h); *B* is the maximum relative growth (h^{-1}) at $t = M$ and *M* is time (h) at which the absolute growth rate was maximum. Maximum relative growth (*B*, h^{-1}) and *M* can be used to calculate the SGR and lag phase of the growth curve. The SGR and lag phase for the modified Gompertz model can be calculated by using Eqs. 2.3 and 2.4)

$$Specific\ growth\ rate\ (\mu_{max}) = \frac{[OD_{max} - OD_0]}{e} \times B \quad [2.3]$$

$$Lag\ phase\ (\lambda) = M - \frac{1}{B} \quad [2.4]$$

Where "*e*" is the base for natural logarithms

To investigate the effect of ultrasonic power (W) and sonication time (min) on SGR and lag phase, values were fitted to a second order polynomial equation. Eq. 2.5 is the general form of the quadratic polynomial model regression which was employed to describe the effects of the two independent variables, namely; Ultrasonic power (X_1, W) and sonication time (X_2, min) on SGR (μ, h^{-1}) and lag (λ, h). Using Eq. 2.5, linear (X_1, X_2), quadratic (X_1^2, X_2^2) and

interactive (X_1X_2) effects of independent variables on dependent variables (Y) were determined.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i,j=1}^{2} \beta_{ij} X_i X_j \quad [2.5]$$

Where Y is the predicted response; β_0 the constant (intercept); β_i the linear coefficient; β_{ii} the quadratic coefficient and β_{ij} is the cross-product coefficient, X_i and X_j are independent variables.

The response surface regression (PROC RSREG) of SAS (Vr. 9.3, SAS Institute, NC, USA) was used to analyse the experimental data. Three-dimensional plots were developed using Design Expert (V. 7.1.6). Goodness of the model fit was evaluated using the coefficient of multiple determinations (R^2). Analysis of variance (ANOVA) and separation of means was carried out using the PROC ANOVA procedure (SAS V.9.1, SAS Institute, NC, USA). Treatment means were separated using Tukeys' test and considered significantly different at $P< 0.05$.

2.3. Result and Discussion

2.3.1. Growth kinetics and model fitting

The optical density at 595 nm versus incubation time for various ultrasonic treatments at a constant frequency of 20 kHz in MRS broth was fitted to the Gompertz model. The Gompertz model has been widely applied to describe the growth of *Lactobacillus* sp. (Budinich, Perez-Díaz, Cai, Rankin, Broadbent, & Steele, 2011; Sharma & Mishra, 2014) which approximates cell growth over time and takes into account growth inhibition as cells move into the late growth phase (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990). The Gompertz model showed a good fit with the experimental data, with a low standard error (SE) of < 0.042 and high regression coefficients (R^2) of > 0.985. Figure 2.1 shows the effect of selected ultrasonic treatments, including control on the growth curve of *L. sakei* for an incubation

period of 24 h. SGR (μ, h^{-1}) and lag phase (λ, h) were obtained from the Gompertz model (Eq 3 & 4). SGR and lag phase for untreated samples were 3.43× 10^{-1} h^{-1} and 2.09 × 10^{-9} h, respectively. SGR and lag phase for samples subjected to ultrasound treatment were found to vary from 2.08 × 10^{-1} h^{-1} to 3.94 × 10^{-1} h^{-1} and 1.6 × 10^{-9} h to 1.52× 10^{1} h, respectively. The highest SGR value (3.94× 10^{-1} h^{-1}) and shortest lag time (1.62× 10^{-9} h) were obtained at lowest ultrasonic power of 2.99 W for 5 min. Conversely, the lowest SGR (2.08 × 10^{-1} h^{-1}) and longest lag (1.52× 10^{1} h) were observed for sample treated at 49.1 W for 7 min. In general, a significant decrease (*P <0.05*) in SGR and increase in lag phase was observed with an increase in ultrasonic power and sonication time (Table 2.1).

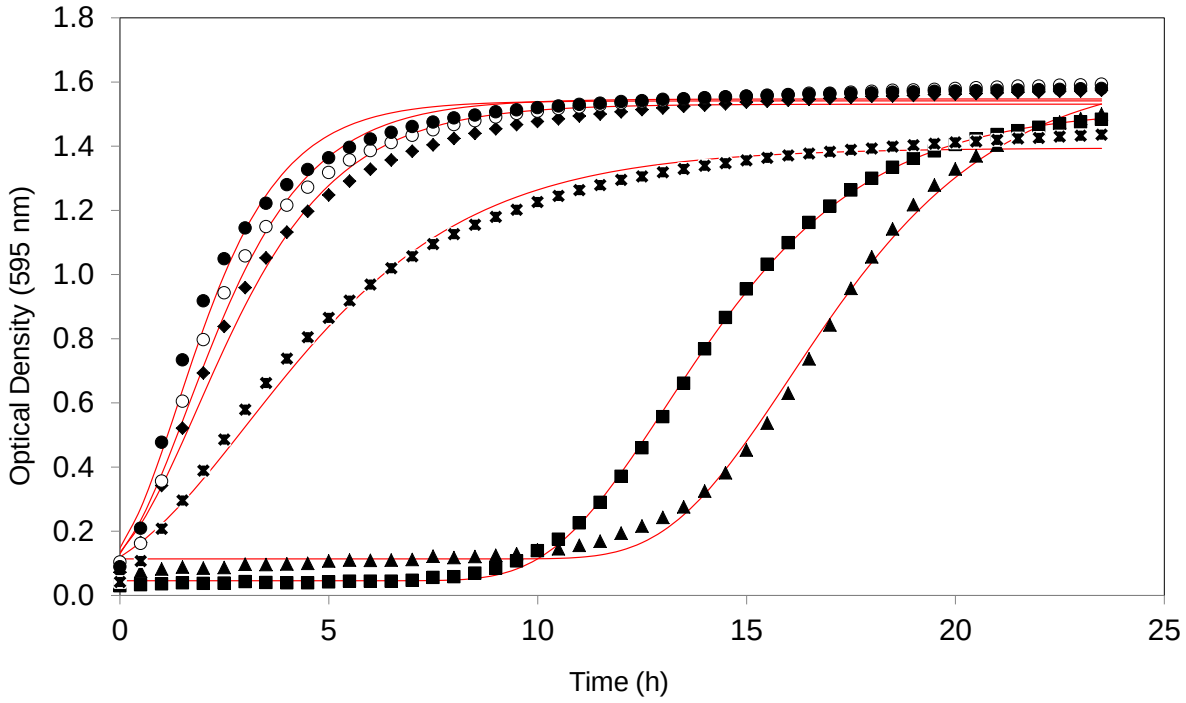


Figure 2. 1. Effect of ultrasonic treatment of 2.99 W for 5 min (●); 11.34W for 3min (◆); 11.34 W for 7 min (**x**); 49.1W for 7 min (▲); 68.5W for 5 min (■) and control (○) on the growth of *Lactobacillus sakei* at 30 °C in MRS broth fitted to the Gompertz model [—].

Table 2. 1. Experimental design matrix and responses

Power (W)	Sonication time (min)	SGR (μ_{max}, h^{-1})	Lag (λ, h)	SE	R^2
11.3 (-1)*	3.0 (-1)	2.99×10^{-1}	2.02×10^{-9}	0.036	0.991
68.5 (+α)	5.0 (0)	2.25×10^{-1}	11.26	0.020	0.999
29.7(0)	5.0(0)	2.21×10^{-1}	9.40×10^{-1}	0.015	0.999
11.3(-1)	7.0(+1)	2.97×10^{-1}	3.68×10^{-9}	0.026	0.996
29.7(0)	9.0(+α)	2.09×10^{-1}	11.65	0.026	0.998
49.1(+1)	7.0(+1)	2.08×10^{-1}	15.21	0.024	0.997
2.9(-α)	5.0(0)	3.95×10^{-1}	1.62×10^{-9}	0.042	0.985
29.7(0)	5.0(0)	2.46×10^{-1}	9.35×10^{-1}	0.018	0.999
29.7(0)	5.0(0)	2.30×10^{-1}	5.46×10^{-1}	0.016	0.999
29.7(0)	1.0(-α)	2.77×10^{-1}	2.96×10^{-9}	0.031	0.994
29.7(0)	5.0(0)	2.43×10^{-1}	1.05	0.014	0.999
11.3(0)	3.0(-1)	2.62×10^{-1}	2.94×10^{-9}	0.032	0.994
29.7(0)	5.0(0)	2.67×10^{-1}	9.35×10^{-1}	0.016	0.999

SE Standard error of fit

*() values in bracket indicate coded values equation for the rate and lag

Growth parameters obtained from the Gompertz model were fitted to a second order polynomial regression model. From the experimental data obtained, the second-order response functions (SGR and lag phase) were expressed as a function of the independent variables (Ultrasound power and sonication time) as shown in Eq. 2.6 and 2.7; while eliminating terms which were not significant (*P <0.05*).

$$\text{SGR } (\mu, h^{-1}) = 0.34 - 0.0043\,P + 6.39\times 10^{-5}\,P^2 \quad [2.6]$$

$$\text{Lag } (\lambda, h) = 1.87 - 1.70\,T + 0.186\,T^2 + 0.0499\,PT \quad [2.7]$$

Figures 2.2 (i) and (ii) illustrate the linear and quadratic effects of independent variables on SGR and lag phase. The significance of experimental factors which affect the treatment process may be quantified from the model coefficients, multiple determinations and probabilities generated from the RSREG procedure of SAS software. The predicted response models (Eq 2.6 and 2.7) for SGR and lag were found to be significant with *p* values of 0.0045

and 0.0004, respectively. The predicted model showed a good fit with the experimental data with high regression coefficients (R^2) of 0.84 and 0.91 for SGR and lag phase, respectively. Canonical analysis of response surface models revealed that both linear and quadratic parameters were significant ($P< 0.05$) for both SGR and lag phase. The cross product or interaction effect of ultrasonic power and sonication time for the models developed was not significant (p=0.188) for SGR, whereas it was significant for lag (p=0.042). Analysis of the independent factors (ultrasonic power and sonication time) influencing SGR and lag phase showed that only ultrasonic power significantly affected (p=0.0038) SGR, whereas lag phase was significantly affected by both ultrasonic power (p=0.001) and sonication time (p=0.0024).

Low ultrasonic intensity of < 2 W/cm^2 has been reported to enhance the growth rate of *Staphylococcus epidermidis, Pseudomonas aeruginosa and Escherichia coli* (Pitt & Ross, 2003). High intensity ultrasound is known to inactivate various microorganisms, both on food surfaces and in suspensions (Bilek & Turantaş, 2013; Tiwari & Mason, 2012). Results presented in this study demonstrate that the *L. sakei* growth was enhanced at low power and reduced at higher power compared to control. The underlying mechanism is probably due to changes in permeability and morphology of the bacterial cells. Pitt and Ross (2003) proposed that ultrasound increases the rate of transport of oxygen and nutrients required for microbial cell growth and increases the rate of transport of waste products away from the cells which allows faster microbial growth rate. However, increased lag phase and reduced SGR at higher power levels could have occurred due to intracellular cavitation that disrupts cellular structural and functional components up to cell lysis (Butz & Tauscher, 2002). Since temperature was not maintained during ultrasonic treatments an increase in temperature up to 40 ^{o}C was observed. Increase in temperature is inevitable during ultrasound treatment and rate of temperature increase is employed to measure ultrasound power (see section 2.2.2.).

This sub-lethal temperature increase may have some influence on various physical and chemical phenomena related to ultrasound.

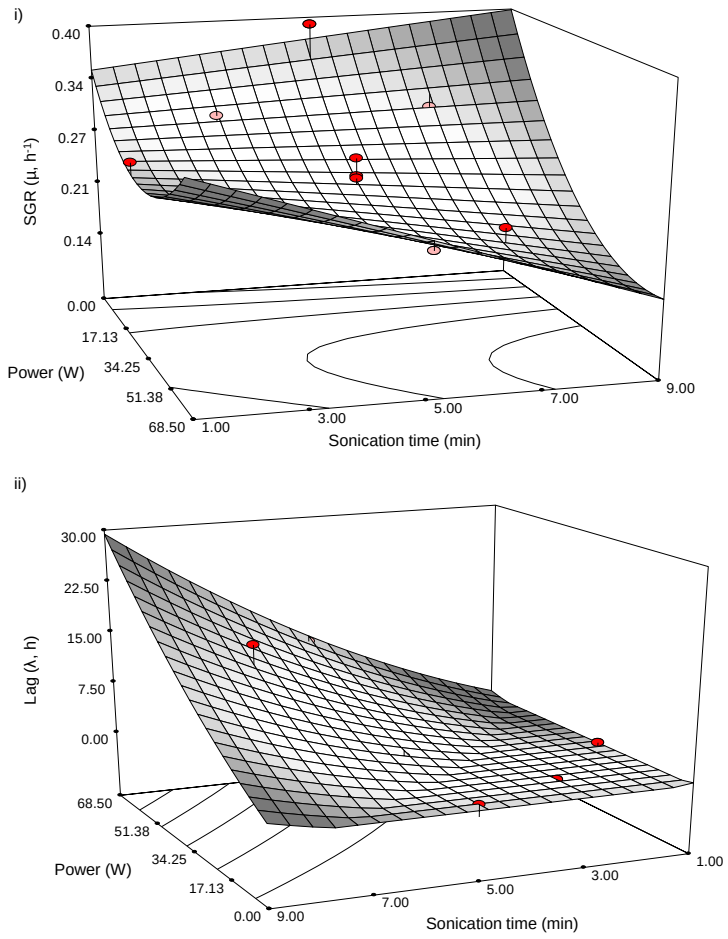


Figure 2. 2. Response surface plot illustrating the effect of ultrasound power (P, W) and sonication time (min) on (i) SGR and (ii) Lag values in MRS broth.

2.3.2. Cell-free extraction characterisation

2.3.2.1. Lactic acid content

Lactic acid content was found to be 181.42 µg/mL and 16.19 µg/mL for MRS broth and meat extract after 24 h fermentation period. No significant differences (*P<0.05*) were observed for lactic acid production between ultrasound treatments and control. Lactic acid production was

lower in the case of meat extract compared to MRS broth because no fermentable sugars were included in the meat extract. Lactic acid production during the fermentation process has been reported to induce self-inhibition along with depletion of nutrients (Leroy & De Vuyst, 2001). This indicates that *L. sakei* growth parameters were influenced by ultrasonic power and treatment time, and production of lactic acid did not result in reduced specific growth rate or increased lag phase.

2.3.2.2. HPSEC and SDS-PAGE analysis

Cell-free extracts obtained after 24 h fermentation were analysed for their molecular weight distribution using High Performance Size Exclusion Chromatography and SDS-PAGE. Figure 2.3i and 2.3ii show the HPSEC chromatogram and Figure 2.4i and 2.4ii show SDS-PAGE profiles of cell-free extracts after fermentation of MRS broth and the meat extract, respectively. A significant decrease in average molecular weight of protein from 3.6 kDa (control) to 3.2 kDa at low ultrasonic power 2.99 W for 5 min whereas, no significant difference was observed at a higher power level in the case of MRS broth. The profile of the peak obtained for MRS broth was very similar for control and ultrasound treated sample; but the peaks found in the control corresponded to slightly higher molecular-sized proteins. For example, the first peaks detected in the control corresponded to a molecular weight of 5.5 and 3.8 kDa; whereas for ultrasound-treated samples, a molecular weight of 5.0 and 3.5 kDa was obtained, resulting in different average molecular weights as shown in Table 2.2. In the case of meat extracts, a single peak of 0.9 kDa was observed. It has been postulated that ultrasound can promote protein hydrolysis, thereby leading to a decrease in molecular weight (Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014). Conversely, no appreciable change in the molecular weight of the protein was observed in the case of meat extract.

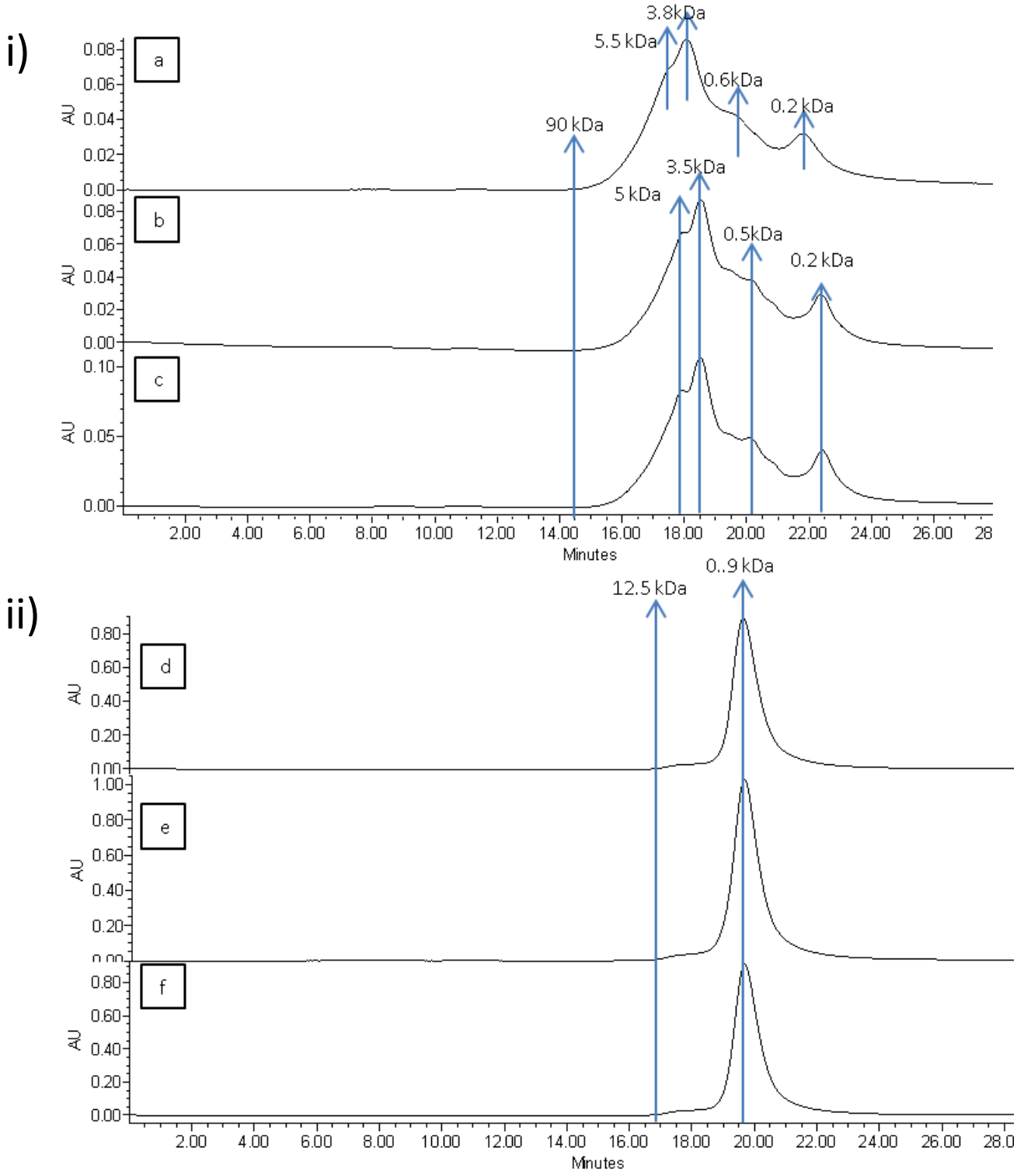


Figure 2. 3. Size exclusion chromatograms of supernatants obtained using meat model (i) and beef extract (ii) where a) Control, b) 2.99 W for 5 min and c) is 49.1W for 7 min.

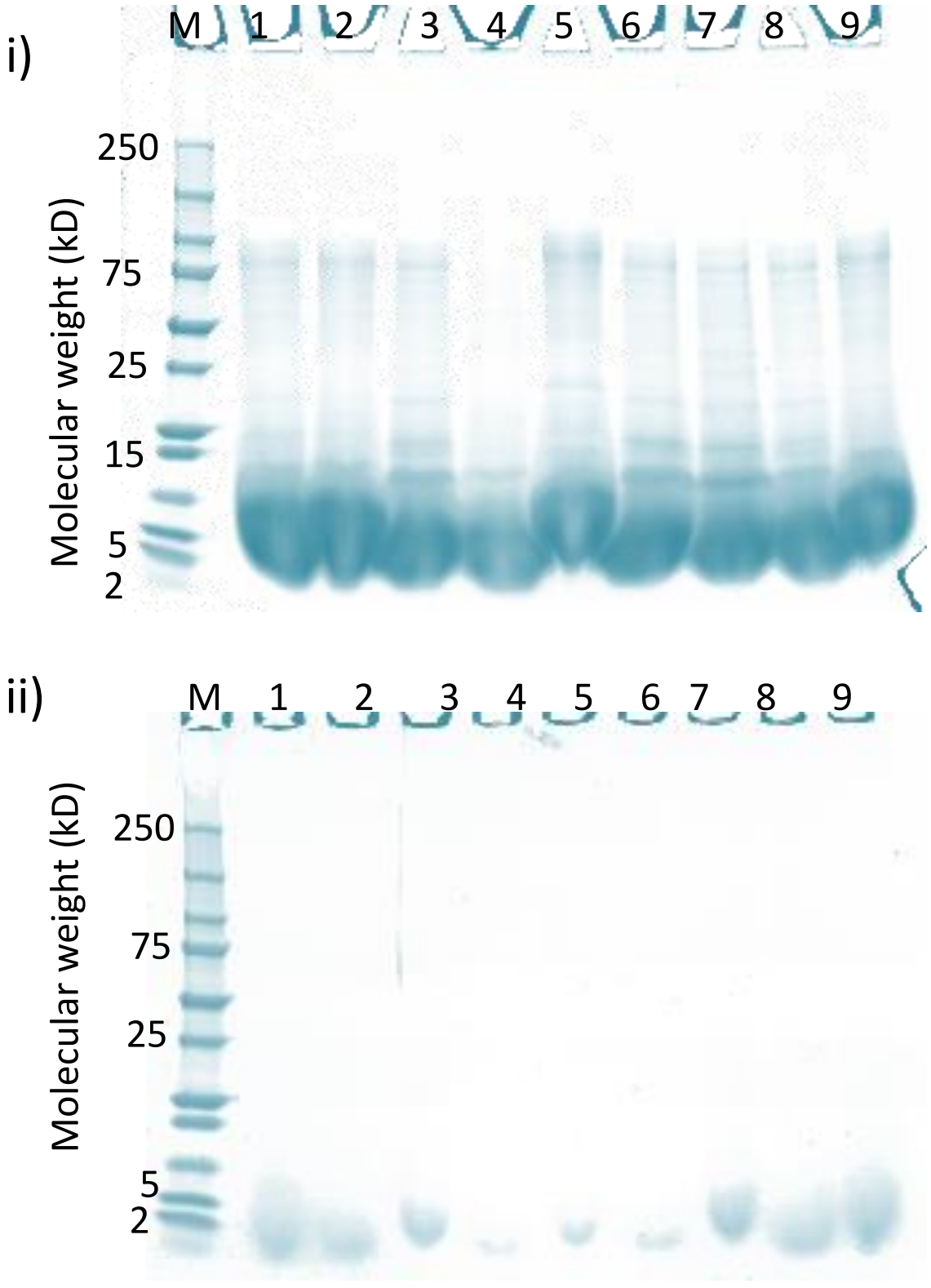


Figure 2. 4. SDS-PAGE profile of cell free extract obtained after fermentation of (i) MRS broth and (ii) meat extract. (M: Protein marker; 1: Control; 2: 11.3W for 3 min; 3: 68.5W for 5 min, 4: 29.7 W for 5 min; 5: 11.3 W for 7 min; 6: 29.7 W for 9 min, 7: 49.1 W for 7 min, 8: 2.99 W for 5 min, 9: 29.7 W for 1 min)

Table 2. 2. Characteristics of cell free extract

Treatment	Average molecular weight (kDa)	Lactic acid concentration (µg/mL)	Minimum Inhibitory Concentration (mg/mL)			
			E. coli	*L. monocytogenes*	*S. aureus*	*S. typhimurium*
Meat model system						
Control	3.601	181.42	25.0	25.0	25.0	25.0
2.99 W for 5 min	3.214	181.57	25.0	12.5	12.5	25.0
49.1W for 7 min	3.178	181.87	25.0	12.5	6.25	25.0
Meat extract						
Control	0.872	16.19	100.0	50.0	100.0	50.0
2.99 W for 5 min	0.868	15.89	50.0	50.0	50.0	50.0
49.1W for 7 min	0.858	16.12	50.0	25.0	50.0	50.0

Molecular weights of cell-free extracts obtained using HPSEC were confirmed by SDS-PAGE. The bands shown in Figure 2.4 are in agreement with the data obtained by HPSEC. In general, the SDS-PAGE patterns for cell-free extract did not show major changes as a consequence of applying ultrasonic treatment. However, large differences were observed between meat extracts and MRS broth. Protein bands, in a broad range of 2 kDa to 75 kDa were observed in the case of MRS broth, whereas, protein bands in a narrow range of 2 – 5 kDa were observed in the case of meat extracts. A smear of low molecular weight protein bands (<10 kDa) is visible in both meat extract and MRS broth, thereby indicating the generation of low molecular weight peptides and free amino acids (Candogan, Wardlaw, & Acton, 2009). However, it was not possible to confirm the molecular size reduction, since the bands obtained were broad and not well defined as shown in Figure 2.4.

2.3.2.3. Antimicrobial activity

The cell-free extracts were tested for inhibition against key pathogenic Gram-positive (*S. aureus* and *L. monocytogenes*) and Gram-negative (*E. coli* and *S. typhimurium*) bacterial strains. All ultrasonically-treated and control cell-free extracts of *L. sakei* in MRS broth and meat extract showed inhibition against the growth of all microorganisms investigated (Figure 2.5). In general, cell-free extracts obtained from ultrasound-treated *L. sakei* samples showed enhanced inhibitory activity compared to the control. MIC values for *E. coli* and *S. typhimurium* was 25 mg/mL with no significant difference ($P<0.05$) between control and ultrasound treated samples in the case of MRS broth. However, in the case of meat extract, ultrasound-treated samples (50 mg/mL) showed lower MIC values compared to control (100 mg/mL). MIC values for *L. monocytogenes* and *S. aureus* were found to be lower for 2.99 W for 5 min and 49.1 W for 5 min compared to control. Lowest MIC values for *S. aureus* and *L. monocytogenes* were 6.25 mg/mL and 12.5 mg/mL, respectively, at 49.1 W for 7 min. The antimicrobial activity of various probiotic strains has been reported extensively (Gálvez, López, Pulido, & Burgos, 2014; Gao, Jia, Gao, & Tan, 2010; Ghanbari, Jami, Kneifel, & Domig, 2013). The key mechanism seems to be due to number of factors, including; lowering of the pH, lactic acid production and the production of various antimicrobial compounds including bacteriocins, non-bacteriocins and non-lactic acid compounds (Fayol-Messaoudi, Berger, Coconnier-Polter, Lievin-Le Moal, & Servin, 2005). Antibacterial activity observed in this study can be mainly attributed to bacteriocins and non-lactic acid compounds present in the cell-free extracts, because no significant changes in the lactic acid content of samples tested against pathogenic microorganisms. Bacteriocins produced by *L. sakei* have been isolated from various fermented products including, sausages and sour dough and these exhibit a

broad range of antimicrobial activity against both Gram-negative and Gram-positive bacteria (Gao, Jia, Gao, & Tan, 2010).

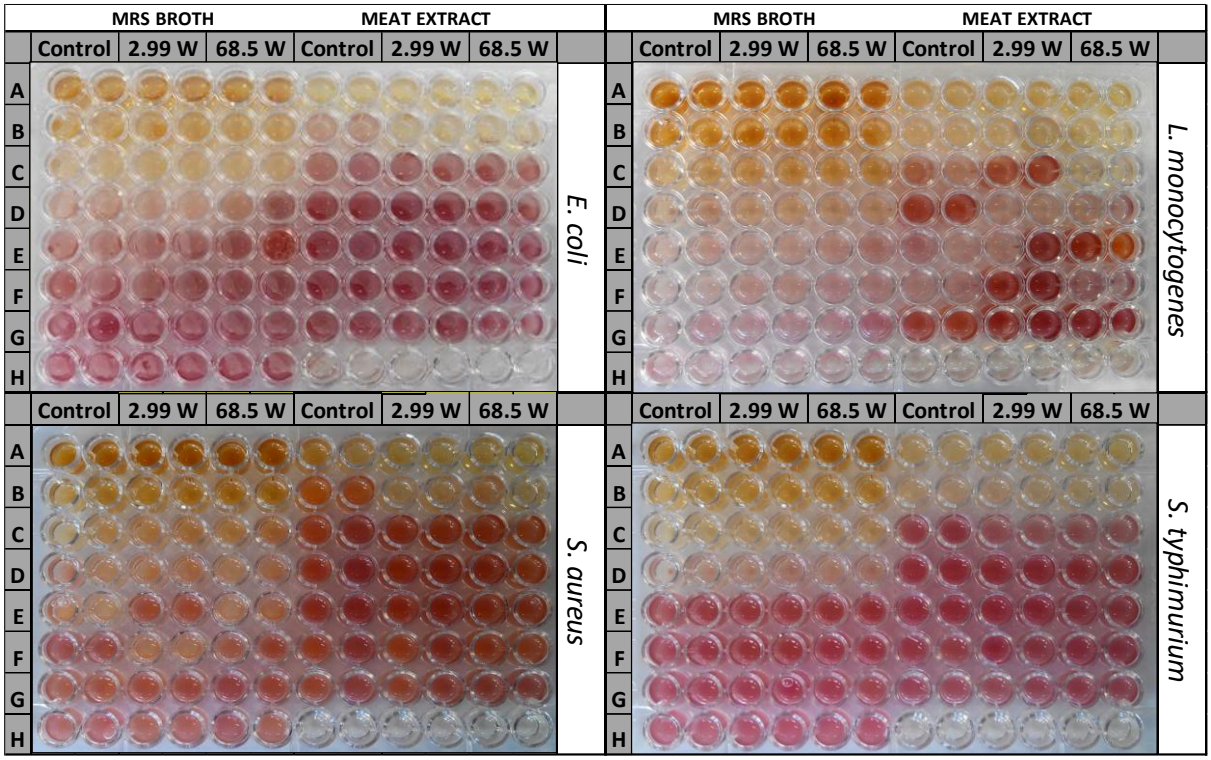


Figure 2. 5. Antimicrobial activity of cell free extract against *E. coli*, *L. monocytogenes, S. aureus* and *S. typhimurium*. (A, B, C, D, E, F, G and H corresponds to 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg of cell free extract/mL)

2.4. Conclusions

This study demonstrates that ultrasonic treatment can control the growth rate of *L. sakei* as evident from growth model parameters obtained by fitting the Gompertz model. Second order polynomial models for SGR and lag phase showed strong dependency of *L. sakei* growth on both the level of ultrasonic power and sonication time. Cell-free extracts obtained after 24 h fermentation period using MRS broth and meat extract showed inhibition against pathogenic microorganisms as evidenced by lower MIC values for ultrasonic-treated samples compared to untreated control. Overall the versatility of ultrasound processing on the stimulation and retardation of *L. sakei* and on the potential that exists to apply it to other

probiotics employed in food fermentation. Additionally, cell-free extracts obtained could be further exploited for food bio-preservation purposes.

Chapter 3: Integrated phenotypic-genotypic approach to understand the influence of ultrasound on metabolic response of *Lactobacillus sakei*

Overview

The objectives of this study were to investigate the influence of ultrasonic frequencies of 20, 45, 130 and 950 kHz on growth kinetics of *Lactobacillus sakei* and to establish molecular mechanism influencing the behaviour of microorganisms subjected to ultrasonic wave. This chapter has been submitted in the following peer reviewed research papers.

Ojha K. S., Burgess C. M., Duffy G., Kerry J. P. and Tiwari B. K. (2018). Integrated phenotypic-genotypic approach to understand the influence of ultrasound on metabolic response of *Lactobacillus sakei.* PlosOne (Accepted).

3.1. Introduction

A range of agri-food and bio-processing applications offered by ultrasound has been widely documented. In the past two decades, ultrasound has gained renewed interest for modifying the cell membrane permeability, thus delivering gene or macromolecules, chemotherapeutic drug and genetic materials into cell nuclei. However, the efficiency at which these molecules were delivered into the cell varies considerably between studies owing to various extrinsic and intrinsic control parameters (Karshafian, Samac, Bevan, & Burns, 2010). Ultrasound induced cell permeabilisation has been termed as "sonoporation'', which can be defined as the transient and reversible increase in the permeability of plasma cell membranes when exposed to ultrasonic waves (Lentacker, De Cock, Deckers, De Smedt, & Moonen, 2014). Sonoporation results in the formation of pores on microbial cell membranes, thereby providing a channel for transport of essential nutrients and removal of toxic substances across these membranes. Such an alteration of the membrane bilayer has been reported to affect cellular functions such as nutrient transport, enzymes activities, and cell growth and multiplication. Considering this, appropriate selections of ultrasound intensities are crucial for the viability of cells and enhancing the production of bioactive metabolites for specialised applications (F. Yang, Gu, Chen, Xi, Zhang, Li, et al., 2008; Yeo & Liong, 2013). However, an increase in ultrasonic power or exposure time can lead to inactivation or cell death due to thinning/alteration of cell membranes resulting in leakage of cellular content, localized heating, and production of free radicals. The effectiveness of ultrasound on microorganisms is strongly influenced by various factors, including but not limited to, microbial ecology (e.g. microorganism type, growth medium and composition), ultrasound parameters (e.g. ultrasonic power, intensity and frequency), exposure time, pH and temperature (Moncada, Aryana, & Boeneke, 2012). For example, studies have shown that Gram-positive bacteria are

more resistant to ultrasound compared to Gram-negative bacteria, possibly because Gram-positive bacterial cells possess a thick and more robust cell wall due to cross-linking of peptidoglycan and teichoic acid (Monsen, Lövgren, Widerström, & Wallinder, 2009).

Ultrasound can enhance the growth rate of microbial cells due to its ability to increase the rate of transport of oxygen and nutrients to the cells and increase the rate of transport of waste products away from the cells. Studies have shown the effect of ultrasound on viability, physiological characteristics and growth at various stages. For example, Lanchun, Bochu, Liancai, Jie, Yanhong, and Chuanren (2003), (Lanchun, Bochu, Zhiming, Chuanren, Chuanyun, & Sakanishi, 2003) reported that the appropriate ultrasonic frequency can accelerate the growth of *Saccharomyces cerevisiae* by nearly 33.3%, with the stationary phase reached four hour earlier compared to the control. Similarly, Jomdecha and Prateepasen (2011) studied the effect of pulse ultrasonic irradiation on the lag phase of *S. cerevisiae*. The lag durations were changed significantly due to ultrasonic energies and durations. In particular, application of sufficient amounts of ultrasonic energies were shown to reduce the lag time, resulting in accelerated yeast growth whereas, a higher level of ultrasonic energy delayed growth by increasing the lag phase. High intensity ultrasound treatment of milk containing *Bifidobacterium* sp. has been shown to enhance the production rates of organic acids and the growth of *Bifidobacterium* sp., while concurrently reducing fermentation time (Nguyen, Lee, & Zhou, 2009, 2012). Similarly, Yang, Zhang, and Wang (2010) demonstrated that ultrasound pre-treatment, followed by incubation, enhances the growth of *Brevibacterium* sp.

Lactic acid bacteria (LAB) are Gram-positive microorganisms which are widespread in nature and adapted to grow under different environmental conditions. Application of *Lactobacillus sp.* in food products have shown to inhibit the growth of pathogenic and spoilage

microorganisms and improve organoleptic properties of food (Castellano, Holzapfel, & Vignolo, 2004; McLeod, Nyquist, Snipen, Naterstad, & Axelsson, 2008). Additionally, *Lactobacillus* sp. are reported to have beneficial effects on gut health and play a vital role in the alleviation of metabolic diseases (Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011; Tremaroli & Bäckhed, 2012). *Lactobacillus sakei* is a lactic acid bacterium of commercial importance because of its ability to ferment various nutrients available in food. *L. sakei* is regarded as a safe strain of LAB, with an ability to grow at diverse environmental conditions, including low temperatures and high salt concentrations.

Genomics and/or proteomics approaches are being used to investigate the microbial response to environmental conditions, which provides valuable information at the point of cell harvesting. A high-throughput assessment of metabolic activities of cells can be achieved by employing a phenotype microarray (PM) technique. The PM technique has highlighted differences in growth requirements, nutrient utilisation, sensitivity to toxins, and genetic diversity in bacteria, fungi and mammalian cells. The PM technique is a convenient way of measuring live cell performance under different environmental conditions (Greetham, 2014). Hence this technique was employed in this study to understand the metabolic changes occurring at phenotypic level. The objective of this study was to investigate the effect of different ultrasound frequencies on growth, morphology, viability and metabolic activities of *L. sakei*.

3.2. Materials and methods

3.2.1. Culture and sample preparation

L. sakei DSM 15831 was obtained from DSM, Germany. The bacteria were cultured in de Man Rogosa Sharpe (MRS) broth (Oxoid Ltd, Cambridge, UK) medium at 30 ^{o}C in a microaerophilic chamber for 48 h. Microbial cells were harvested at 7000×g for 10 min and subsequently added to either to MRS broth or phosphate buffer saline (PBS) to achieve a target population of ca. 10^7 cfu/mL for subsequent ultrasound treatment and analysed further. MRS broth and PBS (Oxoid Ltd, Cambridge, UK) were prepared as per the manufacturer's instruction.

3.2.2. Ultrasound treatment

Different ultrasound frequency exposures were examined using a 20 kHz probe system and multiple bath systems operating at frequencies of 45, 130 and 950 kHz. For the 20 kHz ultrasound probe system, a *L. sakei suspension* (100 mL) was placed in a sterile container (250 mL) and sonicated (for 10 min) by submerging the probe tip (Ø 1.9 cm) (Model: 500 HD, Hielscher Ultrasonics GmbH, Germany) into the bacterial suspension. In the case of the bath systems, treatments were carried out by placing the sterile flat bottom glass tubes (30 mL) containing 20 mL of *L. sakei* suspension into the ultrasonic baths of 45 kHz (Elma Schmidbauer GmbH, Germany), 130 kHz (Elma Schmidbauer GmbH, Germany) and 950 kHz (Kaijo Shibuya America Inc.) for 1 h. Samples without ultrasound treatment were considered as the control. These conditions were selected based on prior studies carried out to establish exposure time (data not shown). For all treatments, the temperature was maintained at 4.0±1.0 °C by circulating cold water using a temperature-controlled refrigerated water circulation system. Three biological replicates were carried out for each of the treatment. The energy input (power, W) was measured using calorimetric method outlined previously by Tiwari,

Muthukumarappan, O'Donnell, and Cullen (2008). Acoustic cavitation pressures namely direct field pressure (Po, kPa), stable cavitation pressure (Ps, kPa) and transient cavitation pressure (Pt, kPa) were measured using a Hydrophone (HCT-3010) attached to a cavitation meter (Model: MCT-2000, Onda Co). Various ultrasonic parameters obtained are listed in Table 3.4.

3.2.3. *L. sakei* growth measurement

The growth of control and ultrasound treated *L. sakei* was monitored by measuring a change in optical density (OD) at a regular interval of 30 min intervals using 96 well plates in a temperature controlled microbial growth analyser (Multiskan™ Microplate Spectrophotometer, Thermo Scientific) at a wavelength of 595 nm and a temperature of 30°C over a 24 h incubation time. Population of control and ultrasound treated *L. sakei* cultures (immediately after treatment) were enumerated in duplicate on MRS pour-plates after 72 h incubation at 30 °C.

3.2.4. Phenotypic microarray assay

The control and ultrasonic treated *L. sakei* were examined for phenotypic divergence using an Omnilog™ phenotypic microarrays (using PM 1 to 4) (BioLog Inc., Hayward, California). Control and ultrasound treated *L. sakei cell* suspensions were prepared and PM plates were inoculated by following manufacturers' instructions. Control and ultrasound treated cell suspensions (ca. 105 cells/ml) were prepared and 50 µL of the cell suspension were inoculated into the wells of the PM panels. These plates were incubated at 30 °C for 48 h in an Omnilog™ micro plate reader (BioLog Inc., Hayward, California). The digital imagery of this instrument tracks changes in the respiration of *L. sakei cultures* growing in individual wells over the

incubation time. Incubation and recording of phenotypic data was performed automatically by the OmniLogTM instrument. The Omnilog™ output for a given plate consists of an OD reading for each well, recorded at 15 min intervals over the 48 h incubation period. The data output for the control and ultrasound treated *L. sakei* cells were analyzed using DuctApe software (Galardini, Mengoni, Biondi, Semeraro, Florio, Bazzicalupo, et al., 2014). Negative controls (wells containing the inoculated Omnilog™ growth medium, but without any substrate for each PM plate was used to normalise differences in inoculums and redox dye oxidation between samples) were subtracted from each reading for each plate.

3.2.5. Genotypic and Phenotypic analysis

DuctApe was employed for the analysis of genomic and phenomic data to obtain metabolic differences occurring due to ultrasound treatment. Briefly, three modules of DuctApe software suite include 1) dgenome for genomic data analysis of *L. sakei* subsp. *carnosus* DSM 15831 (Control); 2) dphenome for PM data analysis (PM 1 – 4) of the ultrasound treated and control; 3) dape for combined analysis of genomics and phenomics for metabolic reconstruction according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa & Goto, 2000), by combining outputs of the dgenome and dphenome modules. The genome sequence of *L. sakei* subsp. *carnosus* DSM 15831 was obtained from National Center for Biotechnology Information (NCBI) (NCBI, 2017). NCBI database resource organises information on various characterstics of range of organisms including genomic sequences. The genome of *L. sakei* subsp. *carnosus* DSM 15831 has a symmetrical identity of 88.005% with *L. sakei* subsp. *sakei* 23K. *L. sakei* subsp. *carnosus* used in this study is a type strain CCUG 31331 (R 14 b/a, DSM 15831) which has shown genomic variation within the subspecies *carnosus* (Nyquist, McLeod, Brede, Snipen, Aakra, & Nes, 2011). Intraspecies genomic diversity among *L. sakei* population has been extensively studied (Chaillou, Daty, Baraige,

Dudez, Anglade & Jones, 2009). Strain diversity among *L. sakei* populations and its linkages with evolutionary histories studied by Chaillou, Lucquin, Najjari, Zagorec & Champomier-Vergès (2013) has shown variations among genomic sequences and protein patterns. The protein pattern of *L. sakei* subsp*. carnosus* has been reported to be similar to that of the *L. curvatus* subsp*. Melibiosus*, with significantly different pattern obtained from *L. sakei* subsp *sakei* (Koort, Vandamme, Schillinger, Holzapfel, & Björkroth, 2004).

Experimental phenotypic microarray datasets obtained from the OmniLog™ Phenotype Microarray (PM) technology for the ultrasound treated and control samples were analysed with the DuctApe software (Ver 0.16.2) suite capable of combining genotypic and phenotypic data (Galardini, et al., 2014). DuctApe programme code written in Python was carried out on Ubuntu Version 15.05.

3.2.6. Imaging

Cryo−scanning electron microscopy (SEM) was carried out using the Zeiss Ultra Plus Field Emission Scanning Electron Microscopy with a Quorum Cryo Preparation chamber. Samples were plunged into liquid Nitrogen slush (-190°C) and transferred, under vacuum, into a sublimation/fracture/coating chamber. Here the samples were freeze fractured to expose the internal structure, sublimated at -100°C for 20 mins and coated in platinum. The samples were held under vacuum and transferred onto a cold stage in the SEM where it was maintained at a temperature of -170°C and imaged.

3.2.7. Mathematical modelling and statistical analysis

To evaluate the sigmoidal shape of the *L. sakei* growth curve, the primary growth parameters were obtained by fitting OD_{595nm} versus incubation time to three models namely Scale free, Biphasic and the modified Gompertz model using the DMFit excel based tool (József Baranyi

& Roberts, 1994). Readers are referred to József Baranyi and Roberts (1994) and Józeff Baranyi, Roberts, and Mcclure (1993) for theoretical derivation and to obtain information about the explicit form of the models employed to obtain the primary growth parameters. The primary growth kinetic parameters of *L. sakei* populations following US treatment i.e. specific growth rate (μ_{max}, OD unit/h) and lag phase (λ, h) were calculated. It must be noted that the μ_{max} obtained by fitting these models is only a potential rate because, theoretically this exact rate cannot be obtained due to the applied limiting functions which has effects even in the linear phase of the growth curve. However, the difference between real rate and rate obtained by the model is negligible if the m_{Curve} curvature parameter obtained from the model fit is sufficiently large (József Baranyi & Roberts, 1994).

Goodness of model fit was analysed based on coefficient of regression (R^2), Root Mean Squared Error (RMSE) and by analysing residuals, experimental vs predicted OD values. Analysis of Variance (ANOVA) and Tukey's test was carried out to separate means using SAS statistical software (SAS Ver 9.3). Mean values were considered significant at $P<0.05$. PROC CORR procedure of SAS was carried out to obtained correlation between various parameters.

3.3. Results

3.3.1. Effect of ultrasound on *L. sakei* population and growth curve

Figure 3.1 shows the effect of ultrasound on the inoculated *L. sakei* population (6.96 log cfu/mL) using a plate count method immediately after treatment at various US frequencies. The surviving *L. sakei* population ranged from 3.96 log cfu/ml (20 kHz) to 7.02 log cfu/mL (45 kHz). No significant changes ($P<0.05$) in *L. sakei* population were observed with respect to ultrasound frequency and control, with an exception of 20 kHz, where a significant ($P<0.05$) decrease of 3 log cfu/mL was observed.

The growth of the surviving *L. sakei* cells was monitored over a 24 h period using optical density measurements at 595nm. Figure 3.2 shows the plotted average optical density at each incubation time (h) for *L. sakei* subjected to ultrasound treatments at various frequencies of 20kHz, 45 kHz, 130 kHz, 950 kHz and control (no ultrasound treatment). The initial OD_{595nm} was in a range of 0.243 to 0.284 OD units irrespective of the treatment.

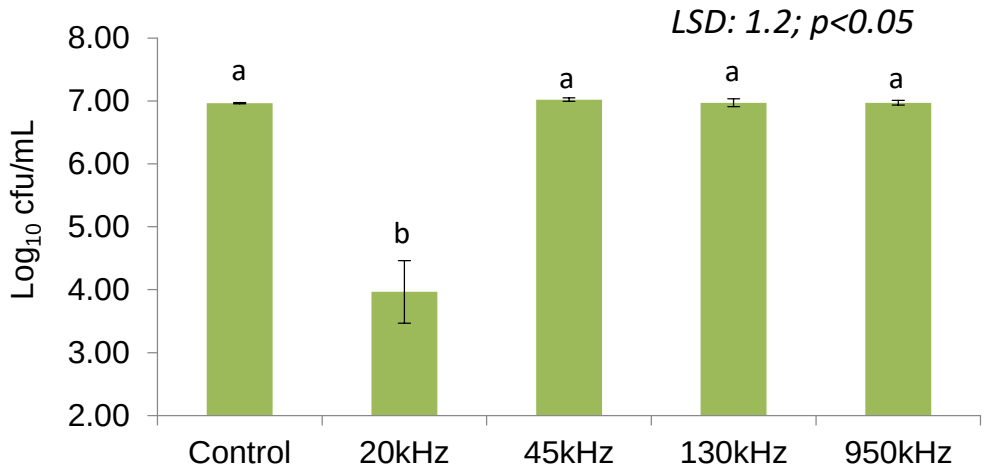


Figure 3. 1. *L. sakei* population of control (untreated) and surviving population after treatment at various ultrasound frequencies of 20, 45, 130 and 950 kHz

([ab]Columns with different letters are significantly different at $P<0.05$; LSD: Least significant difference)

As can be seen from Figure 3.1 the starting population of *L. sakei* following US treatment was in the range of 3.96 log cfu/mL (20 kHz) to 6.96 – 7.04 cfu/mL for all other treatments and control. The growth rate of the control remained lower at all times compared to ultrasound treated samples, irrespective of frequencies employed. It should be noted that different systems were employed for the ultrasound treatment which were operated at different frequencies. In the case of the 20 kHz treatment, *L. sakei* showed a longer lag time followed by a subsequent high growth rate. As can be seen from Figure 3.2, the slope of the exponential phase of treated and control *L. sakei* cells were parallel, indicating the growth behaviour was

relatively similar. However, a significant increase in lag time was observed for samples treated with 20 kHz frequency.

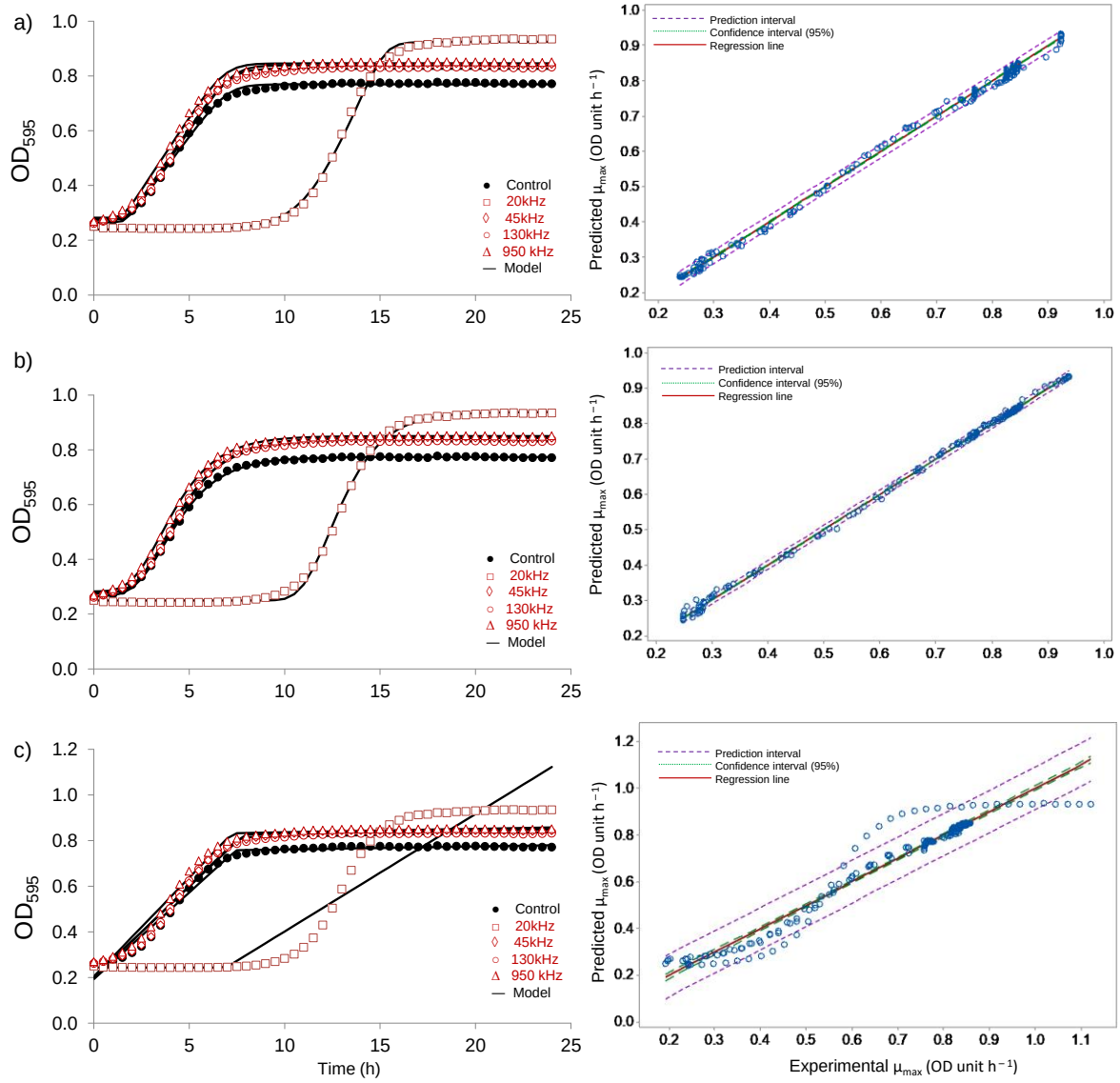


Figure 3. 2. *L. sakei* growth curve plot as measured by Optical Density over a 25 h incubation at 30°C following various ultrasound treatments plotted using Scale free (a), Gompertz (b) and Biphasic models (c) and corresponding model fitting.

Table 3. 1. *L. sakei* growth parameters along with 95% confidence interval, regression coefficient (R^2), root mean square error (RMSE) of model fit obtained for control and ultrasound treated *L. sakei* cells.

Treatment	Model	μ_{max} (OD unit h^{-1})	Lag time (λ, h)	Y_0 (OD unit)	Ymax (OD unit)	R^2	RMSE
Control	**Scale free**	0.099±0.003 [a] (0.094 – 0.104)	1.707±0.034 [b] (1.640 –1.773)	0.259	0.813	0.998	0.008
20 kHz		0.122±0.057 [a] (0.011 –0.233)	11.517±0.494 [a] (10.549 – 12.486)	0.251	0.947	0.999	0.011
45 kHz		0.103±0.001 [a] (0.102 –0.105)	1.790±0.025 [b] (1.741 –1.838)	0.270	0.840	0.997	0.009
130 kHz		0.105±0.001 [a] (0.103 –0.107)	1.925±0.024 [b] (1.877 –1.972)	0.269	0.849	0.998	0.009
950 kHz		0.104±0.001 [a] (0.101 –0.106)	1.560±0.022 [b] (1.517 –1.603)	0.273	0.850	0.997	0.010
	LSD	0.0441	0.3781				
Control	**Gompertz**	0.122±0.003 [c] (0.116 –0.129)	2.113±0.039 [b] (2.037 –2.188)	0.259	0.813	0.999	0.005
20 kHz		0.166±0.001 [a] (0.164 –0.168)	12.536±1.888 [a] (8.836 – 16.236)	0.251	0.947	0.999	0.010
45 kHz		0.128±0.001 [b] (0.126 –0.129)	2.187±0.022 [b] (2.145 –2.230)	0.270	0.840	0.999	0.005
130 kHz		0.129±0.001 [b] (0.127 –0.132)	2.326±0.023 [b] (2.281 –2.370)	0.269	0.849	0.999	0.005
950 kHz		0.128±0.002 [b] (0.124 –0.132)	1.955±0.026 [b] (1.904 –2.006)	0.273	0.850	0.999	0.004
	LSD	0.0033	1.4502				
Control	**Biphasic**	0.079±0.002[b] (0.075 –0.082)	7.617±0.124 [a] (7.373 –7.861)	0.259	0.813	0.989	0.017
20 kHz		$3.85\pm1.12\times10^{-10}$ [c] ($1.64 – 6.06\times10^{-10}$)	9.396±2.734 [a] (4.038–14.754)	0.251	0.947	0.902	0.095
45 kHz		0.080±0.001 [b] (0.078 –0.082)	7.710±0.032 [a] (7.647 –7.773)	0.270	0.840	0.988	0.020
130 kHz		0.079±0.001 [b] (0.078 –0.080)	7.966±0.082 [a] (7.804 –8.127)	0.269	0.849	0.986	0.022
950 kHz		0.084±0.001 [a] (0.082 –0.087)	7.334±0.049 [a] (7.238 –7.430)	0.273	0.850	0.990	0.019
	LSD	0.0019	2.0728				

([abc]Values with different letters are significantly different at $P<0.05$; LSD: Least significant difference)

Table 3.1 shows the growth parameters obtained by fitting OD_{595nm} versus incubation time (h) to three models, namely scale free, the Gompertz and biphasic model. Three models were employed to obtain key growth parameters of specific growth rate (μ_{max}, OD unit/h) and lag phase (λ, h). μ_{max} and λ were considered as key parameters required to investigate the effect of treatment on the growth behaviour of *L. sakei*. All three models gave the highest μ_{max} (OD unit/h) and λ (h) for *L. sakei* samples treated with 20 kHz and lowest for control. Initial (Y_0) and final (Y_{max}) absorbance ranged from 0.251 to 0.273 and 0.813 to 0.947 respectively. In general, all three models investigated to describe the growth behaviour of *L. sakei* predicted well with an exception for *L. sakei* subjected to 20 kHz. The biphasic model failed to predict growth behaviour for 20 kHz treated *L. sakei* samples (Figure 3.2c). μ_{max} obtained by three models ranged from 0.099 – 0.122 OD unit/h, 3.85×10^{-10} – 0.084 OD unit/h and 0.122 – 0.166 OD unit/h whereas, λ ranged from 1.56 – 11.51 h, 7.33 – 9.39 h and 1.95 – 12.536 h for scale free, biphasic and the Gompertz model respectively. Predicted growth models tested for the goodness of model fit by using R^2 and RMSE values, showed that the R^2 values for all models were >0.902 and RMSE values were <0.017 for all growth curves. However, the biphasic model showed higher RMSE values (0.017 – 0.095) and low R^2 values (0.90 – 0.99) compared to the scale free and the Gompertz model across all treatments including the control. A plot between model predicted and experimental OD values showed that values for both the Gompertz and scale free models were within the prediction and 95% confidence interval whereas, values obtained from the biphasic model did not show good model prediction (Figure 3.2). Further, the Biphasic model predicted low values for growth parameters compared to the other two models. Growth model parameters provided by both the Gompertz and scale free models were comparable. Considering goodness of fit, model predicted values and residual analysis, the Gompertz model was found to be best suited to describe the growth model for all

treatments. The Gompertz model showed a good fit with the experimental data with low standard error (SE) of < 0.042 and high regression coefficients (R^2) of > 0.985. Analysis of growth curve for control and ultrasound treated *L. sakei samples* using Gompertz will be discussed here after.

Correlation analysis (Table 3.2) shows that the stable cavitation pressure (*r=0.643; P<0.01*) and standing wave pressure (*r=0.489; P<0.05*) had a positive effect on μ_{max} (OD unit/h) whereas, both the stable (*r=0.580; P<0.05*) and transient (*r=0.509; P<0.05*) cavitation pressure showed positive correlation. The *L. sakei* population was significantly affected by ultrasonic power as evident by high correlation coefficient between power and log (cfu/mL) (*r =-0.977; P<0.0001*) and hence, it can be concluded that the higher power can inactivate bacterial cells. Surprisingly, low microbial count observed for 20 kHz samples showed longer lag phase followed by significantly higher growth rate.

Table 3. 2. Correlation analysis showing relationship between various ultrasonic parameters and *L. sakei* growth parameters

	Fo (kPa)	Po (kPa)	Ps (kPa)	Pt (kPa)	Power (W)	μ_{max} (OD unit/h)	λ (h)
Fo (kPa)	1.000						
Po (kPa)	0.389^{ns}	1.000					
Ps (kPa)	-0.476*	0.619**	1.000				
Pt (kPa)	-0.827****	-0.246^{ns}	0.410 ^{ns}	1.000			
Power (W)	-0.324 ^{ns}	-0.923****	-0.640**	0.408 ^{ns}	1.000		
µmax (OD unit/h)	-0.228 ^{ns}	0.489*	0.643**	0.420 ^{ns}	-0.376 ^{ns}	1.000	
λ (h)	-0.297 ^{ns}	0.368 ^{ns}	0.580*	0.509*	-0.238 ^{ns}	0.976****	1.000
Log (cfu/mL)	0.273 ^{ns}	-0.367^{ns}	-0.557**	0.229^{ns}	-0.977****	-0.998****	1.000

^{ns}Not significant (*P*<0.05); *significant at *P*<0.05; ****significant at *P*<0.0001;***significant at *P*<0.01.

The specific growth rate (μmax, OD units/h) and lag phase (λ, h) obtained from Gompertz model for the control sample was 0.122±0.003 OD units/h (CI: 0.116 –0.129) and 2.113±0.039 h (CI: 2.037 –2.188) respectively. Significantly (P<0.05) higher μ_{max} was observed for 20 kHz (0.166±0.001 OD units/h) compared to other frequencies investigated and the control. In general ultrasound treated samples showed significantly higher μ_{max} compared to the control. No significant differences were observed for μ_{max} with respect to 45, 130 and 950 kHz even though μ_{max} was significantly higher compared to the control. In the case of λ, no significant differences ($P<0.05$) were observed for control and ultrasound frequencies of 45, 130 and 950 kHz. Significantly higher λ (12.536±1.888 h (CI: 8.836 –16.236 h)) was observed for *L. sakei* samples subjected to 20 kHz frequency compared to the control and other frequencies investigated.

3.3.2. Genomic and phenomic characteristics

The genomic sequence of *L. sakei* subsp. *carnosus* DSM 15831 was used to study the metabolic response of *L. sakei* following ultrasound treatment. The control *L. sakei* strain showed proteome/genome size of 1904, out of which 1007 were mapped to KEGG with 877 KEGG orthology IDs, 95 pathways, 829 reactions and 586 unique reactions based on genomic sequence available for the *L. sakei* strain employed. As can be seen only 53.4% of genes were mapped to KEGG because most of genes are not involved in metabolism hence were not annotated through KEGG.

The metabolic abilities of control and ultrasound treated *L. sakei* were tested using the PM system (Biolog). Using PM plates 1 to 4, 571 different growth conditions were tested, including 190 different carbon sources, 95 nitrogen sources, 59 phosphorus sources and 35 sulphur sources. Metabolically active conditions were scored using a threshold calculation

based on growth curve data. The activity index (AV) was calculated using five clusters (k = 5) which were chosen through an elbow test.

Unique metabolic functions represent the number of wells for which a single treatment shows an exclusive phenotype, with respect to the others. Results showed that the highest number of unique “more active” metabolic features was detected in PM plates inoculated with 20 kHz treated culture and control (N=31 and N=23 respectively) suggesting a higher metabolic potential of these strains as compared to the others. Whereas, the 20 kHz treated culture and control culture strains exhibited “less active” phenotypic traits in only one and five conditions respectively, as shown in Table 3.3 In contrast, the 45 kHz treated culture seems to be the least metabolically active strain, as it showed a lower number of “more active” metabolisms (N=9) and the highest number of “less active” metabolisms (N=19). When looking at the single PM compound categories, the control culture was able to metabolise 19.27% of carbon sources, 25.00% of nitrogen sources and 31.25% of phosphorus and sulphur sources (Table 3.3). The *L. sakei* culture treated at 950 kHz frequency showed highest metabolic activity (24.48%) in carbon plates followed by 130 kHz (22.91%), 20 kHz (19.27%), Control (19.27%) and 45 kHz (7.81%). In the case of nitrogen plate, culture treated at 20 kHz had highest activity (31.25%) followed by control (25.00%), 950 kHz (21.87%), 130 (18.75%) and 45 kHz (8.33%). The control showed highest activity (31.25%) for phosphorus and sulphur sources followed by 20 kHz (26.04%), 45 kHz (18.75%), 130 kHz (14.58%) and 950 kHz (13.54%). Here, all the phenotypes were grouped based on the type of PM source molecules. Individual differences among each of the 571 PM metabolic conditions of the cultures treated at different US conditions were then assessed and represented in detail as circular plots (Activity Ring). Any difference in higher/lower metabolic reaction between the reference control culture and the culture treated at different ultrasonic conditions can be visualised using the ‘diff mode’

(Figure 3.3). Figure 3.3 shows concentric circles (from outer to inner ring) represent control *L. sakei* and delta activity of *L. sakei* treated at 950, 45, 20 and 130 kHz respectively, whereas each radial strip corresponds to a single tested phenotype. The higher colour intensity appeared in the carbon plates in the case of 950 and 130 kHz, confirming the lower metabolic activity of the control as compared to the culture treated at 950 and 130 kHz.

Table 3. 3. Unique and metabolic activity statistics obtained from DuctApe analysis

	Unique metabolic functions (% of wells with Δ ≥ 1)				
	Control	**20 kHz**	**45 kHz**	**130 kHz**	**950 kHz**
More active	23	31	9	8	5
Less active	5	1	19	3	6
Percentage (%)	7.29	8.33	7.29	2.86	2.86
Metabolic activity statistics (% of active wells with AV ≥2)					
Carbon sources	19.27	19.27	7.81	22.91	24.48
Nitrogen sources	25.00	31.25	8.33	18.75	21.87
Phosphorus and sulphur sources	31.25	26.04	18.75	14.58	13.54

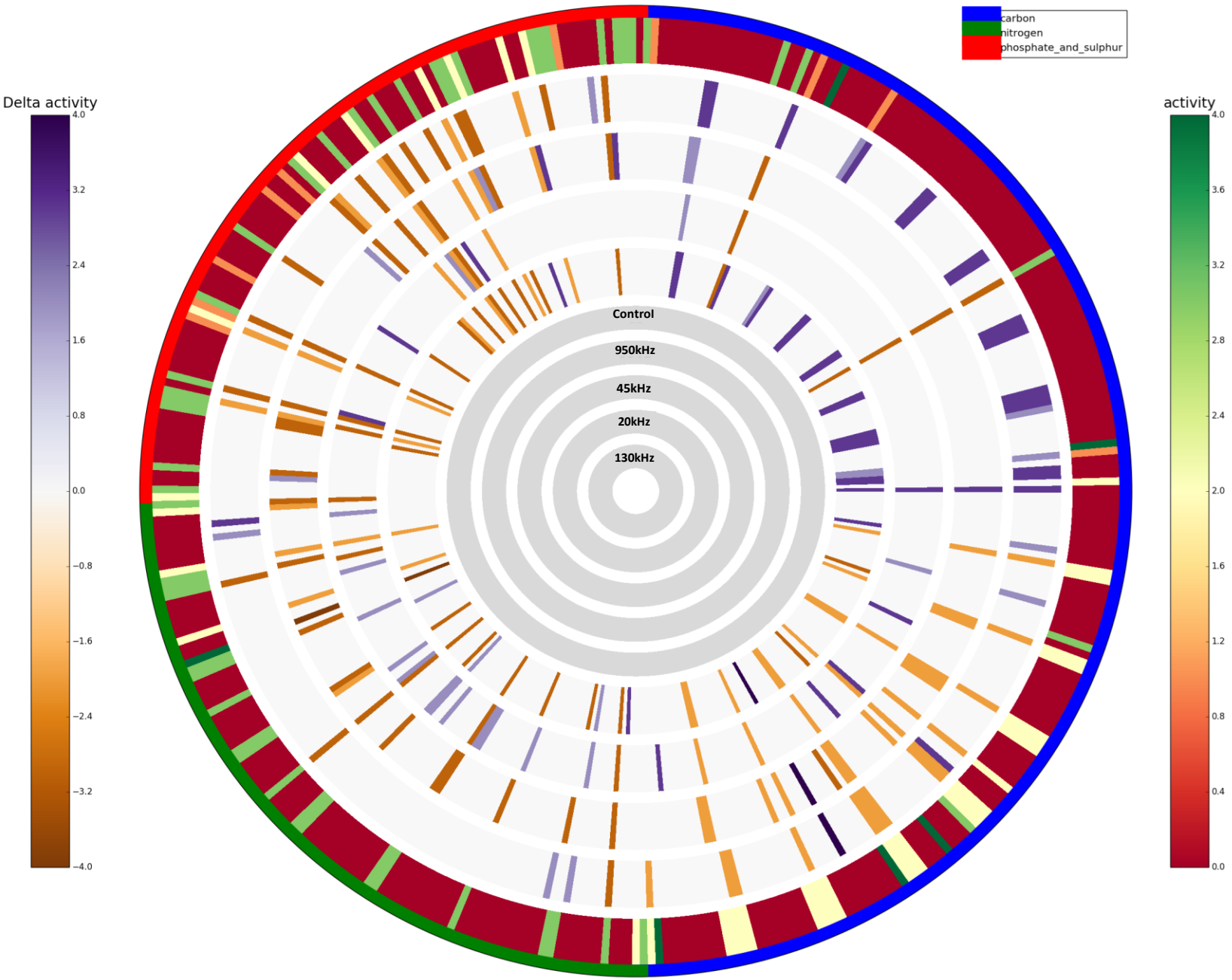


Figure 3.3. High-throughput metabolic activity in difference mode: the difference with the AV value of control culture is reported when AV≥2; it is grey otherwise; purple indicates a higher activity to; orange colour indicates a lower activity of ultrasound treated samples with respect control.

The utilisation of carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) by the *L. sakei* strain subjected to ultrasound treatment is shown in Figure 3.4. As can be seen from Figure 3.4, the utilisation of nutrients was significantly affected due to ultrasound treatment. Significant variations in the utilisation of carbon sources was observed for ultrasound treated and control samples. The control was able to ferment various sugars including ribose, lyxose, glucosamine, 2 deoxy-D ribose, arabinose and tagatose whereas low utilisation (AV<2) was observed for melibiose. Ultrasound treated *L. sakei* at 130 kHz and 950 kHz showed higher D-mannose, dulcitol, thymidine, sucrose, uridine, 2`-deoxyadenosine, adenosine, D-cellobiose and inosine as a carbon source compared to control or 20 kHz. In the case of carboxylic acids, relatively higher activities for butyric acid, itaconic acid, sorbic acid and tartaric acid was observed in the case of 20 kHz treated *L. sakei* samples compared to 45 kHz and control. The utilisation of malic acid and galacturonic acid was observed in the case of 130 and 950 kHz samples compared to other ultrasound treated and/or control samples. Among alcohols, higher activity value was observed in all cases for dihydroxyacetone and 2,3-butanedione however, 2-aminoethanol and 2,3-butanedione was utilised only in the case of ultrasound treated samples.

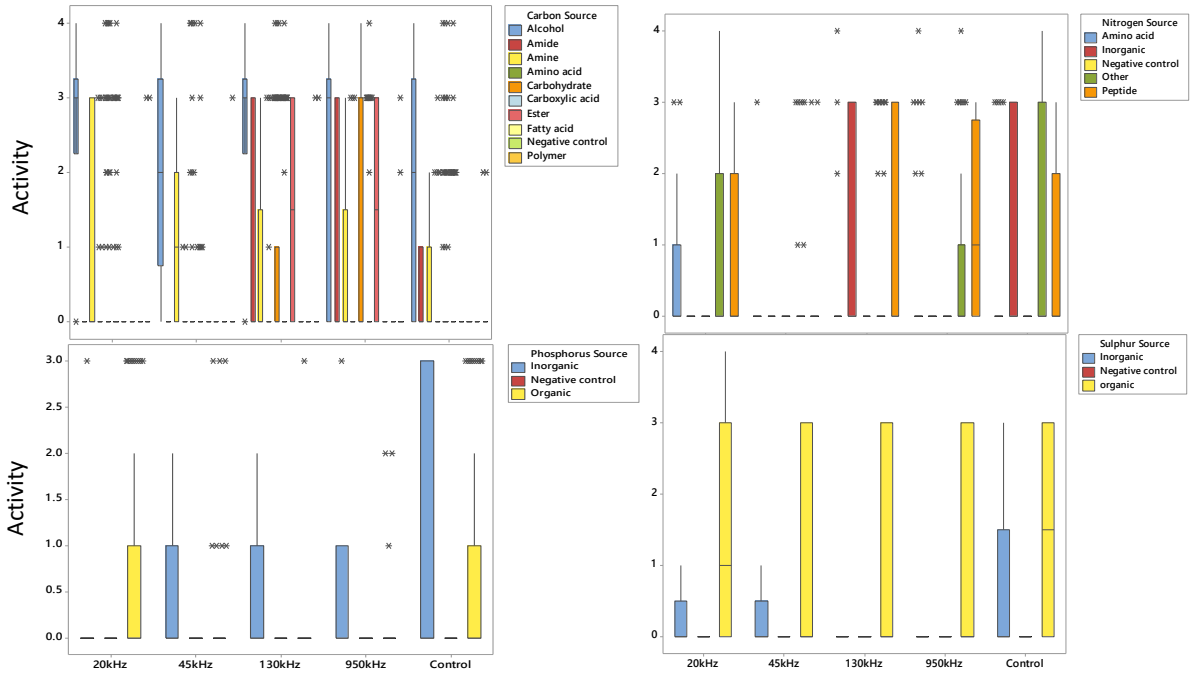


Figure 3. 3. The utilisation of various carbon, nitrogen, phosphorus and sulphur sources by *L. sakei* subjected to ultrasonic frequencies of 20 kHz, 45 kHz, 130 kHz, 950 kHz and control, (*indicates outliners).

Amino acids as a source of carbon was not utilised for control or ultrasound treatment whereas, alcohols as a source of carbon was utilised by all treated and control samples. Among nitrogen sources, ultrasound treated *L. sakei* did not show any utilisation for nitrate, with exception to the control. No utilisation of amino acids as a nitrogen source was also observed for all samples, with the exception of cysteine, tryptophan and ornithine. Some activity (AV=1) was observed for 20 kHz samples for threonine, valine and citrulline. Amino acids as a nitrogen source was utilised by 20 kHz samples compared to other treatments whereas, peptides as a nitrogen source seems not to be utilised by 45 kHz and inorganic form of nitrogen was not utilised by both 20 and 45 kHz. No significant changes in utilisation of organic sulphur was observed among various treatments with some variations observed for 20 kHz samples whereas, in the case of inorganic phosphorus higher activity was observed for

the control compared to ultrasound treatment. In the case of phosphorus, organic source of phosphorus was utilised in the case of 20 kHz and control whereas, inorganic source of phosphorus was exclusively utilised by the control compared with ultrasound treated with no utilisation observed for 20 kHz.

The phenotypic variability identified from PM1-4 were combined with genome of the *L. sakei* subsp. *carnosus* DSM 15831. Figure 3.5 shows that the ultrasound treatment influenced the utilisation of various nutrients resulting in variations in various metabolic pathways. Metabolic reconstruction Citrate cycle (KEGG map20), Glycolysis/ Gluconeogenesis (KEGG map10) and pentose phosphate pathway (KEGG map 30) were influenced by ultrasound treatment compared to control (Figure 3.6). Various other metabolic pathways seems influenced by ultrasound treatments were degradation of lysine (KEGG map310), metabolism of biotin (KEGG map780), glutathione (KEGG map480), carbon (KEGG map1200), purine (KEGG map230), biosynthesis of valine, leucine and isoleucine (KEGG map290). Specifically, ultrasound treatment showed highest number of genome related sustrate used differently by ultrasound treated samples compared to control (Figure 3.5). This difference was noted mainly for biosynthesis of amino acids (KEGG map1230). Further, a higher utilisation of ribose for the ultrasound treated samples compared to the control was observed, ribose sugar is linked to pentose phosphate pathway. A considerable number of nutrients metabolised by ultrasound treated *L. sakei* culture namely associated with Pentose phosphate pathway (KEGG map30) were pyruvate (C00022), D-ribose (C00121), D-gluconate (C00257) and deoxyribose (C01801) with varied level of activities. Highest being noted for ribose and deoxyribose in the case of 20 kHz and 45 kHz (AV>5) compared to control.

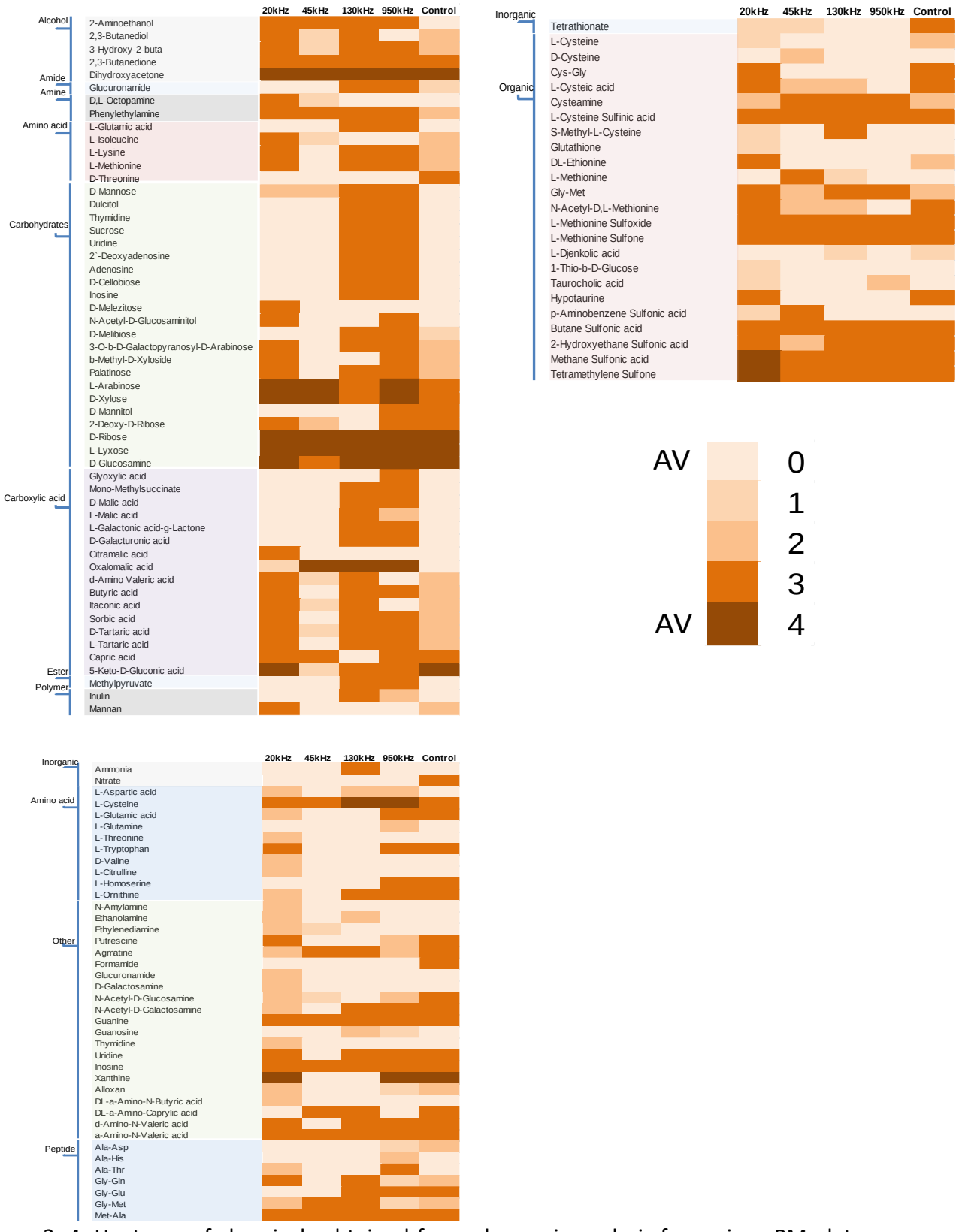


Figure 3. 4. Heatmap of chemicals obtained from phenomic analysis for various PM plates (PM1-2: Carbon source; PM3: Nitrogen source; PM4: Phosphorus and Sulphur source for control and ultrasound treated *L. sakei* culture.

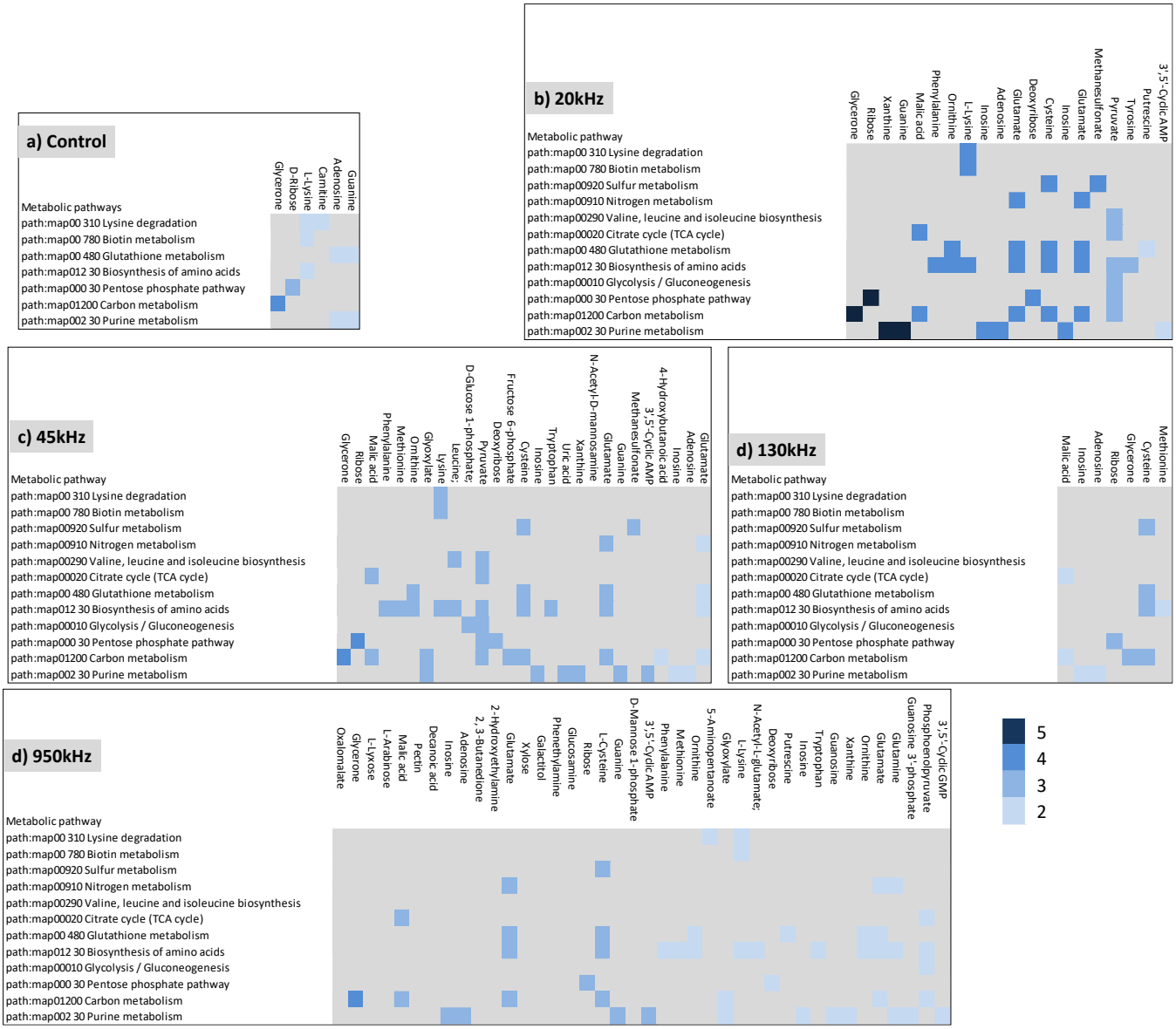


Figure 3. 5. Combined genotypic and phenotypic variability of *L. sakei* culture as influenced by various treatments.

3.3.3. Morphological changes

Figure 3.7 shows SEM images of control and 20 kHz treated *L. sakei* cells. It shows that the control (A) bacterial cells are intact with uniform surface whereas 20 kHz treated *L. sakei* cells show formation of pitting (B – D). Some bacterial cells visualised showed physical damage, leading to a formation of pores on the cell surface (highlighted by arrow) which may be reversible, while certain cells within the treated suspension showed rupturing of *L. sakei* cells

leading to leakage of cellular component (highlighted with a circle, D), causing irreversible damage to the cells.

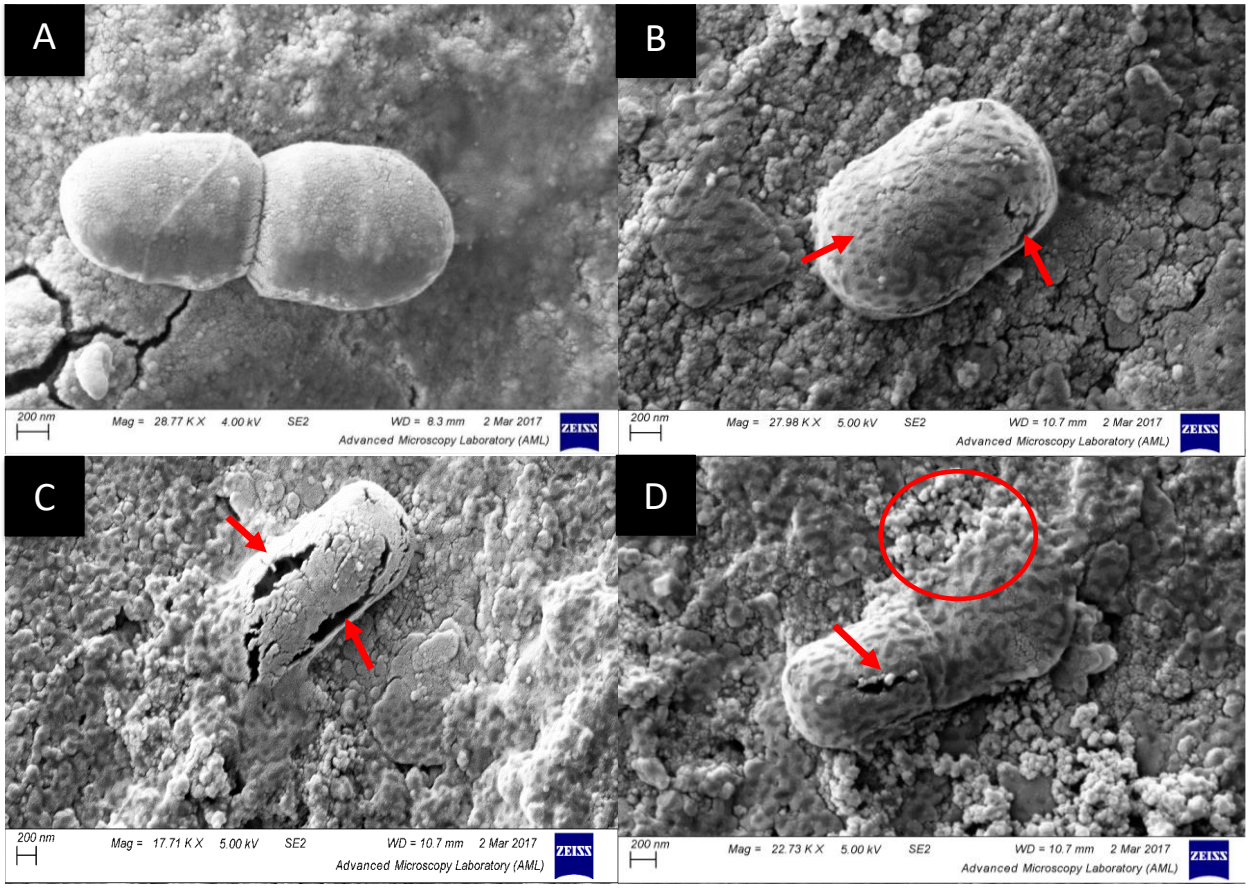


Figure 3. 6. Cryo SEM images of *L. sakei* strain (A: Control; B – D: 20 kHz samples). Arrow indicates formation of pores on cellular surface and circle shows the rupture of *L. sakei* cells.

3.4. Discussion

Previous studies have shown that ultrasound treatment can significantly inactivate bacteria depending on the ultrasound conditions. High power ultrasound treatment has been shown to inactivate various pathogenic and spoilage microorganisms including *Enterobacter aerogenes*, *Bacillus subtilis* and *Staphylococcus epidermidis*, *Escherichia coli* and *Listeria monocytogenes* in various media (Gao, Hemar, Ashokkumar, Paturel, & Lewis, 2014; Gera & Doores, 2011). Inactivation is mainly due to various physical and chemical phenomena

occurring due to inertial and non-inertial cavitation occurring alone or in combination. A previous study by these authors investigated the effect of ultrasound power at a frequency of 20 kHz and showed a significantly higher specific growth rate with a short lag phase at low US power (2.99 W), while a significant decrease ($P<0.05$) in specific growth rate and increase in lag phase was observed following treatment with increased ultrasound power and treatment time (Ojha, Kerry, Alvarez, Walsh, & Tiwari, 2016). In this study, since different ultrasonic systems i.e. probe (20 kHz) and bath system (25 – 950 kHz) were employed to investigate the ultrasonic effects. The influence of ultrasonic acoustic distribution owing to ultrasonic systems employed on *L. sakei* culture may have occurred. Moreover, variations in the growth rate and lag phase obtained at 20 kHz frequency (see Chapter 2) and results obtained in this study can be partly due to the fact that temperature was maintained at 4.0±1.0 °C in this study while temperature was not maintained in Chapter 2.

Use of OD data for the estimation of bacterial growth model parameters has been reported extensively (Dalgaard, Ross, Kamperman, Neumeyer, & McMeekin, 1994). The growth models investigated in this study are often argued to be of limited biological significance for being empirical in nature compared to mechanistic models. However, empirical models can help in elucidating the effect of a single environmental factor such as temperature, pH, treatment etc. on relevant biological growth parameters i.e. growth rate and lag phase (Leroy & De Vuyst, 2001). The Gompertz model has been previously reported to describe the growth behaviour of *Lactobacillus* species in range of media (Aryani, Zwietering, & den Besten, 2016; Mechmeche, Kachouri, Yaghlane, Ksontini, Setti, & Hamdi, 2017; Kumari Shikha Ojha, Kerry, Alvarez, Walsh, & Tiwari, 2016; Siragusa, De Angelis, Calasso, Campanella, Minervini, Di Cagno, et al., 2014). The empirical modelling approach using the Gompertz model approximates cell growth over incubation time and considers the growth inhibition as

microbial cells move into the late growth phase (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990).

Previous studies have shown the application of various growth models including scale free, Gompertz, logistic, and Richards models to describe the growth behaviour of a range of pathogenic, spoilage and probiotic strain (Sant'Ana, Franco, & Schaffner, 2012; Sharma & Mishra, 2014; Tomás, Labanda, de Ruiz Holgado, & Nader-Macías, 2002; Wijtzes, Rombouts, Kant-Muermans, Van't Riet, & Zwietering, 2001). Hence, these models were employed to describe the effect of ultrasound treatment on the growth behaviour of *L. sakei*. Previously, a study has shown that a decrease in lag phase is proportional to an increase in initial population because the culture requires less time to reach a concentration which can increase detectable optical density (Pla, Oltra, Esteban, Andreu, & Palop, 2015). The longer lag phase for 20 kHz samples can be attributed to the fact it took certain amount of time for injured cells to recover but which then grew at a faster rate compared to other treatments and control. It may be hypothesised that bacteria grew at faster rate due to enhanced movement of nutrients into the cell via pores formed as a result of ultrasound treatment. Studies have shown that the sublethally stressed populations, i.e., the bacteria population which is slightly damaged by environmental conditions are believed to be more susceptible to change and exhibit greater phenotypic variations and have different response to nutrients (Flowers & Ordal, 1979; Mossel & Van Netten, 1984; Roszak & Colwell, 1987) as can be deduced from the variations in metabolic pathways and *L. sakei* response to various nutrients investigated in this study. Further, microscopic images (Figure 3.7) show that ultrasound can induce pits or pores leading to a sublethal or lethal injury to the microbial cells. This injury can be attributed to sonoporation which would have led to the formation of reversible pores which can improve permeability of the cell membrane (Lentacker, De Cock, Deckers, De Smedt, & Moonen,

2014). However, formation of large size pores may cause permanent cell injury as can be seen from Figure 3.7D. in a study Wordon, Mortimer, and McMaster (2012) observed a extensive boundary damage and disintegration of *S. cerevisiae* cells as a result of ultrasound treatment at 20 kHz when exposed to ultrasound.

Studies have shown that low frequency high intensity ultrasound causes inactivation compared to high frequency low intensity ultrasound (Radel, McLoughlin, Gherardini, Doblhoff-Dier, & Benes, 2000). Inactivation of various microorganisms in model solution and food matrices has been reported at 20 kHz (Barba, Koubaa, do Prado-Silva, Orlien, & de Souza Sant'Ana, 2017; Shamila-Syuhada, Chuah, Wan-Nadiah, Cheng, Alkarkhi, Effarizah, et al., 2016). The effect of ultrasound on *L. sakei* suspensions can be attributed to dispersion effect on *L. sakei* clumps, disruption of bacterial cells, modification of cellular activity, either due to introduction of morphological changes on the cell membrane, or cavitation effects within the cell leading to changes in microbial growth parameters and inactivation. Physical and chemical phenomena of ultrasound associated with ultrasound frequencies include agitation, vibration, pressure, shock waves, shear forces, microjets, compression & rarefaction, acoustic streaming, cavitation and formation of free radicals (Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2017). These effects on the microbial cell are mainly due to physical and chemical mechanisms and/or combination of these occurring due to various types of cavitation namely direct field pressure (Po, kPa), stable cavitation pressure (Ps, kPa) and transient cavitation pressure (Pt, kPa). Po causes oscillation of microbial cells at the fundamental frequency whereas, Ps (non-inertial) cavitation causes oscillation of cavitating bubbles and is responsible for microstreaming. Pt (inertial cavitation) is mainly responsible for microstreaming, formation of shockwaves and jetting due to collapse of cavitating bubble. Collapse of cavitating bubble

causes formation of free radicals (e.g. OH^-) due to sonolysis of water ($H_2O \rightarrow OH^- + H^+$) and formation of hydrogen peroxide.

L. sakei has an ability to produce all amino acids with exception to aspartic and glutamic acids for its growth and survival (Champomier-Vergès, Chaillou, Cornet, & Zagorec, 2001). *L. sakei* being facultative heterofermentative in nature, hence can utilises glucose for glycolysis and ribose using phosphoketolase pathway. Irrespective of treatments, utilisation of ribose, arabinose, lyxose, xylose was unaffected whereas, no utilisation of glucose was observed. Arabinose utilisation was comparatively higher for 20 kHz, 45 kHz and 950 kHz with AV=4 compared to 130 kHz and control with AV=3. *L. sakei* are able to ferment many pentoses including ribose, arabinose, ribulose and xylulose by using the phosphoketolase pathway (McLeod, Zagorec, Champomier-Vergès, Naterstad, & Axelsson, 2010). Phenotypic characteristics of various *L. sakei* strains including subsp. *carnosus* studied by Koort, Vandamme, Schillinger, Holzapfel, and Björkroth (2004) showed arabinose, galactose, maltose, melibiose sucrose, trehalose can be utilised with weakly positive results for β-gentiobiose and gluconate. The genome of *L. sakei* strain was mapped with phenotypic variations for ultrasound treated and control *L. sakei* culture. Linking genome with high throughput phenotypic characteristics revealed significant differences in metabolic responses to various ultrasonic frequencies.

The genetic diversity of *L. sakei* strains has been studied and reported extensively (Claesson, Van Sinderen, & O'Toole, 2007; Jensen, Grimmer, Naterstad, & Axelsson, 2012; McLeod, Nyquist, Snipen, Naterstad, & Axelsson, 2008). Previous studies have shown that phenotypic variations exist and the ability of strains to utilise carbohydrates varies significantly depending on the environment and growing conditions (Chiaramonte,

Anglade, Baraige, Gratadoux, Langella, Champomier-Vergès, et al., 2010). Various metabolic pathways were influenced as a result of the ultrasound treatment and concomitant nutrient utilisation. The focus of the following discussion is restricted to the utilisation of ribose sugar and the effect of ultrasound treatment on the phosphate pentose pathway. The *L. sakei* strain employed in this study has been reported to have limited utilisation of ribose (McLeod, Nyquist, Snipen, Naterstad, & Axelsson, 2008). The major heterolactic fermentation pathway of ribose is via the phosphoketolase pathway. In accordance with the literature, low activity was observed for ribose utilisation in the case of the control whereas, higher activity was observed in the case of ultrasound treated *L. sakei* culture, indicating possible changes in protein expression for ribose utilisation via phosphate pentose pathway. The utilisation of ribose sugar leading to an impaired growth on ribose is reported due to the inactivation of *rbsK* which encodes ribokinase. Moreover, both *rbsK* and *rbsR*, encoding a ribokinase are shown to be divergent in various strains of *L. sakei* (Nyquist, McLeod, Brede, Snipen, Aakra, & Nes, 2011) which may have been expressed differently owing to ultrasound treatment. Catabolic pathways for utilisation of various sugars exist in almost all strains identified to date for *L. sakei* species. Release of ribose via ATP hydrolysis with intermediates such as inosine and IMP are the most abundant intermediates of ATP breakdown. Metabolism of ribose for energy production in *L. sakei* include conversion of Ribose-1P→Ribose-5P (enzyme:phosphopentomutase); Ribose→Ribose-5P (Enzyme: Ribokinases) and conversion of Ribose-5P →Ribulose-5P (enzyme: ribose-5-phosphate isomerase) (Chaillou, Champomier-Vergès, Cornet, Crutz-Le Coq, Dudez, Martin, et al., 2005). Metabolism of pyruvate is very important for the phosphoketolase pathaway which is converted to lactate by a NAD-dependent lactate

dehydrogenase. The phosphoketolase pathway along with glycolysis is responsible for production of lactic acid depending on the metabolism of glucose or ribose.

The comparative PM analysis of the growth or no-growth response of ultrasound treated or control strains provided significant differences as shown in Figure 3.5 and 3.6 Higher metabolic activity of ultrasound treated culture may be due to a change in gene expression leading to the utilisation of various nutrients.

Phenotypic alterations and application of genomic-phenomic approach of a culture subjected to environmental stress e.g. adaption to cold environment has been successfully demonstrated (Mocali, Chiellini, Fabiani, Decuzzi, de Pascale, Parrilli, et al., 2017). Phenotypic variations identified as a result of ultrasound treatment and links associated with the various metabolic pathways demonstrates the relevance of PM technology in providing in-depth analysis of *L. sakei* growth which may be useful in understanding the influence of processing technologies on microorganisms, which to now has been restricted mainly for inactivation and growth studies. This study signifies that the genomic-phenomic approach for understanding the key underlying mechanism and adaptive response to emerging technologies. Understanding phenotypic variations and differences in nutrient utilisation will also enable tailoring the food processes for specialised applications of probiotic strains.

Chapter 4: Ultrasound assisted diffusion of sodium salt replacer and effect on physicochemical properties of pork meat

Overview

The objective of this study was to investigate the effect of employing sonication on the diffusion of sodium salt (NaCl) and a salt replacer (SR) into pork meat. The following paper has been published from this study.

- Ojha, K. S., Keenan, D. F., Bright, A., Kerry, J. P., & Tiwari, B. K. (2016). Ultrasound assisted diffusion of sodium salt replacer and effect on physicochemical properties of pork meat. International Journal of Food Science & Technology, 51(1), 37-45.

4.1. Introduction

Meat curing is one of the most ancient meat preservation methods. It involves addition of salt(s) mainly NaCl and nitrates, which also contributes positively to the technological and sensory characteristics of the meat. NaCl has been traditionally used in curing processes and serves a number of key functions. NaCl is an essential ingredient in processed meat products, contributing to the water-holding capacity, colour, fat-holding properties, flavour and texture. Moreover, salt decreases water activity (a_w) and has significant effect on the shelf-life of products (Desmond, 2006).

Commercial salting techniques involve dry curing or brine curing or a combination of both. In such cases, movement of salt into and water out of the meat is governed by several factors, including meat type, salt concentration and curing time. Beyond the negative effects of slow salt uptake on meat quality, long duration of brine salting of meat can also prove expensive in terms of reduced productivity and yields, impact negatively in terms of space utilisation and have maintenance costs due to the corrosiveness of the brine. Furthermore, besides an interest among processed meat manufacturers in speeding up the uptake of salt in meat, achieving uniform salt profiles in meat is also desirable for a processed meat product (Turhan, Saricaoglu, & Oz, 2013). Therefore, processed meat manufacturers are in constant search for alternative salting processes that are faster and more efficient, which allow homogeneous salt distribution in meat, would reduce processing time and improve the quality of the product.

Moreover, with the increased knowledge and convincing evidence linking excess dietary intake of sodium chloride with coronary heart disease risks, consumer demand for reduced salt meat products has increased (Ruusunen & Puolanne, 2005). This demand coupled with

health recommendations and guidelines from regulatory agencies has led the meat industry in search of processes and salted products which are low in sodium content. Meat processors are continuously trying to develop reduced salt processed meat products because such products represent a significant part of the dietary sodium intake (Armenteros, Aristoy, Barat, & Toldrá, 2009). Sodium reduction in salted meat products is relatively difficult to achieve compared to other food products, due to the numerous sensorial and technological properties associated with NaCl (Desmond, 2006).

Numerous strategies have been proposed to reduce NaCl without much alteration in the curing process. Studies have shown that the partial substitution of NaCl by other chloride salts (e.g. KCl, $CaCl_2$ and $MgCl_2$) could be one of the best alternatives to reduce sodium content in meat products. Use of mixtures of salts with low sodium content may negatively influence the textural attributes and other sensory attributes of processed meat products (Lorenzo, Cittadini, Bermúdez, Munekata, & Domínguez, 2015). Several techniques include the use of salt replacers, application of novel technologies including high pressure processing and ultrasound. Ultrasound has been proposed to improve salt diffusion in meat matrix with an aim to reduce sodium salt content in food.

Ultrasound is known to enhance several mass transfer processes, including, brining of meat. It is now well recognised that ultrasound allows faster and uniform diffusion of brining solution into the meat tissues (Carcel, Benedito, Bon, & Mulet, 2007; Jayasooriya, Torley, D'Arcy, & Bhandari, 2007; Ozuna, Puig, García-Pérez, Mulet, & Cárcel, 2013). Studies have shown the effect of ultrasound on NaCl brining and various physicochemical properties including texture, water activity and cook loss. However, from extensive review of the literature, the effect of applying ultrasound on a salt replacer in brined meat has not been

reported. Hence, the objective of this study was to investigate and compare the effect of ultrasound on brining kinetics for pork meat slices using a 5% brine solution of NaCl or alternatively, using a salt replacer. The effect of ultrasound on physicochemical properties of pork meat samples was subsequently carried out.

4.2. Materials and methods

4.2.1. Raw material and sample preparation

The meat used for all experiments was porcine *M. semitendinosus* and was obtained from a local supplier (Dublin Meat Company, Blanchardstown, Co. Dublin, Ireland) at 6 days post mortem and vacuum packed. Muscles were stored at 4 °C for 24 h prior to brining. The pH of the meat was in the range of 5.6 – 5.8. All visible fat was removed from each muscle manually prior to brining. Samples were cut into slices of similar weight (approximately 100 g) and size (60 x 100 × 20 mm length x width x height). Sample location within the muscle was randomised with respect to treatment and a new muscle was used for each experimental replication. The brine solutions of NaCl (British Salt, UK) and a commercial salt replacer (GRINDSTED® SaltPro™, Danisco) (SR) were used at a concentration of 50 g L^{-1}. The ratio of meat weight relative to brine weight was set at 1:10 for all treatments (Siró, Vén, Balla, Jónás, Zeke, & Friedrich, 2009).

4.2.2. Brining treatments

Brining experiments were carried out in 1000 mL of 5% NaCl or SR brine solution in a 2000 mL glass beaker (Figure 4.1). During the experiments, brine temperature was kept constant at 4 ± 1 °C by placing the glass beaker into a temperature-controlled bath. For each treatment (replicated thrice) 6 meat samples were placed in the brined solution (SR or NaCl) and samples were withdrawn at a regular interval of 30 min. Samples were rinsed in deionised water,

blotted dry and stored at 4 °C for subsequent analysis. In all experiments, the final brining time was 120 min. This length of time was chosen based on results reported by Siró, Vén, Balla, Jónás, Zeke, and Friedrich (2009) and based on preliminary experimental work carried out prior to this study.

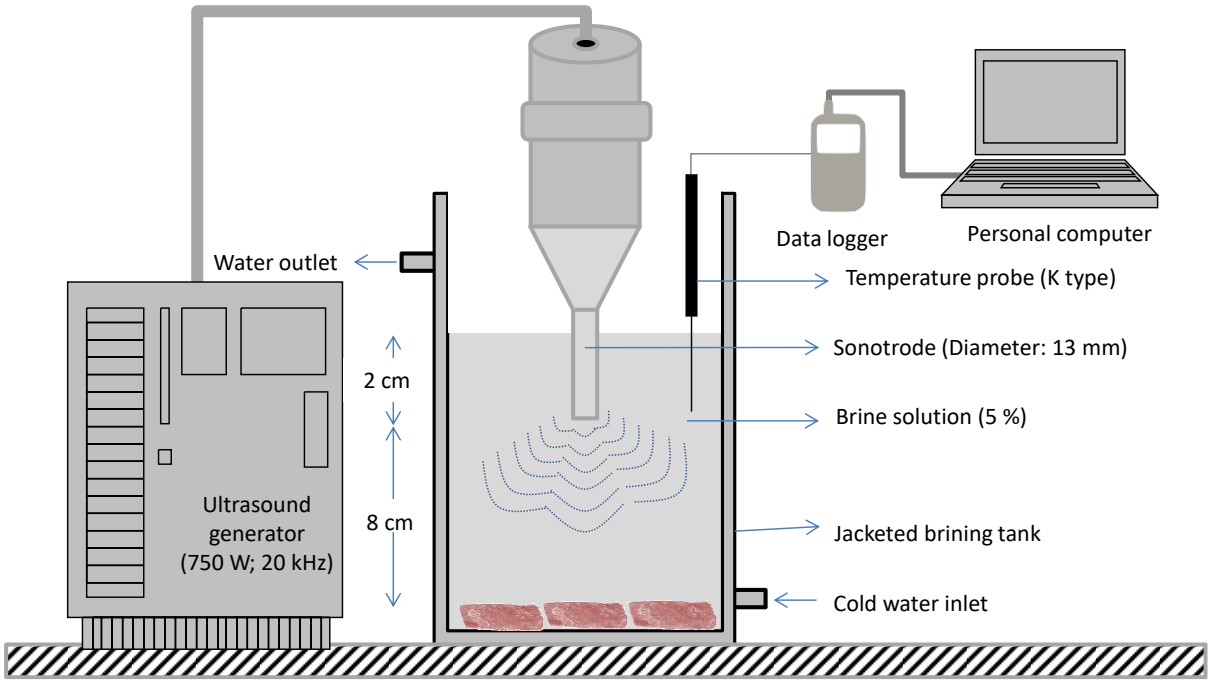


Figure 4.1. Schematic representation of experimental set up.

Ultrasound-assisted brining treatments were carried out using a 750 W ultrasonic processor (VC 750, Sonics and Materials Inc., Newtown, USA) operating at 20 kHz with a 13 mm diameter ultrasonic probe. The energy input was controlled by setting the amplitude of the sonicator probe. Extrinsic parameters of amplitude were 20% and 80%, which corresponds to the ultrasonic power (P) of 9.29 and 54.9 W cm^{-2} calculated using Eq (4.1 & 4.2). Samples were withdrawn as in the case of static brining. Ultrasounic power dissipated to the sample was calculated by using method outlined by Tiwari, Muthukumarappan, O'Donnell, and Cullen

(2008). P was determined by using Eq. 4.2 and the ultrasound intensity (UI) was calculated using Eq. 4.2

$$P = mC_p\left(\frac{dT}{dt}\right)_{t=0} \quad [4.1]$$

$$UI = \frac{4P}{\pi D^2} \quad [4.2]$$

Where (dT/dt) is the change in temperature over time (°Cmin^{-1}), C_p is the specific heat of water (4.18 kJkg^{-1}°C^{-1}), m is the mass (kg) and D is the diameter of the sonotrode (13 mm).

4.2.3. Physicochemical properties

Cook loss was estimated as per method outlined by Ciara K. McDonnell, James G. Lyng, Joshua M. Arimi, and Paul Allen (2014). Briefly samples were vacuum packed in a bag and cooked at 77 ^{o}C to a core temperature of 72°C. Cook loss was calculated as the difference between the initial and final (after cooking) weights, divided by the initial weight and expressed as % cook loss. NaCl content of pork samples was measured using the standard titrimetric Volhard method (Kirk & Sawyer, 1991).

Warner-Bratzler shear force (WBSF) was measured using the method outlined by Maher, Mullen, Moloney, Buckley, and Kerry (2004). Cooked samples were tempered at room temperature and left to cool at 4 ^{o}C overnight. Cored samples were sheared using the Instron model 5543 and Merlin series IX software, Instron Ltd., Buckinghamshire, UK. Average values of 8 replications for each sample were reported.

Nuclear magnetic resonance (NMR) relaxation measurements were carried out as described by (McDonnell, Allen, Duggan, Arimi, Casey, Duane, et al., 2013) on a Maran Ultra instrument

(Oxford Instruments, Abington, Oxfordshire, UK) with a resonance frequency of 23.2 MHz. Transverse relaxation (T_2) times were measured using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with the resultant relaxation decays analysed by tri – exponential unsupervised fitting in the RI Win – DXP software (Version 1.2.3 Oxford Instrument, Abington, Oxfordshire, UK).

Thermal transition properties of pork were measured using a TA Instruments DSC (Model No. DSC 2010, TA Instruments INC. New Castle, DE, USA). Pork samples were analysed as per method outlined by Keenan, Resconi, Kerry, and Hamill (2014). Onset temperature, protein denaturation (T_0), peak temperature (T_p) and the denaturation enthalpy (ΔH) were recorded.

4.2.4 Mathematical modelling and statistical analysis

The mass transfer coefficients (K_m, $min^{-1}m^{-2}$) for salt diffusion into pork samples were estimated using the experimental data. The parameter was adjusted using Eq 4.3 and 4.4 with a parameter estimation procedure based on the minimization of the error sum of squares. The model equation was solved by numerical integration using the Runge-Kutta method (Jackson, Enright, & Hull, 1978).

$$\frac{dM_M^S}{dt} = -K_m^S \cdot A_M \cdot \left(C_M^S - C_{OS}^S\right) \cdot V_M \qquad [4.3]$$

$$\frac{dM_{OS}^S}{dt} = -\frac{dM_M^S}{dt} \qquad [4.4]$$

Where, A_M is the total superficial area of the meat samples (m^2), C^S_M is the concentration of salt in the meat samples (g/m^3), C^S_{OS} is the concentration of salt in the osmotic solution (g/m^3),

K_m^S is the mass transfer coefficient for the brining process ($min^{-1}m^{-2}$), M_M^S is the mass of salt in the meat samples (g), t is the time (min), V_M is the total volume of the meat samples (m^3) and M_{OS}^S is the mass of salt in the osmotic solution (g).

Analysis of variance (ANOVA) was performed using the SAS procedure (SAS Version 9.1.3, statistical Analysis Systems). The LSD (least significance difference) intervals ($p < 0.05$) were calculated to distinguish significantly different treatments. Tukey's multiple comparison was used to compare treatment means. Pearson's correlation coefficients were analysed to determine a relationship between various physicochemical properties. Correlation coefficients and significance values were determined using PROC CORR (SAS Ver 9.1.3).

4.3. Result and discussion

4.3.1. Effect of ultrasound on salt diffusion into pork meat

Salt content was found to increase from 0.88±0.03 g/kg to 8.32±0.02 g/kg after 120 min of brining time for control (static brining) samples, whereas in the case of SR, NaCl content was found to increase from 0.70±0.01 to 2.38±0.02 g/kg as shown in Table 4.1. A significant increase ($P< 0.05$) in salt content within the first 30 min was observed for pork samples at an ultrasound intensity (UI) level of 54.9 W/cm^2 compared to the control brining treatment. NaCl content of pork samples subjected to 5% salt replacer, for both static brining and ultrasound-assisted brining, showed an increase in salt content; however, ultrasound did not show an improvement in salt content of pork samples at the UI levels of 9.0 and 54.9 W cm^{-2}.

Mass transfer coefficients for the brining process of pork for both NaCl and SR showed that ultrasonic treatment had a low influence on the mass transfer of salt into the pork samples at UI of 9.0 W cm^{-2} whereas, at high UI of 54.9 W cm^{-2}, the mass transfer coefficient was higher

than the control experiment (without ultrasonic treatment). K_m was found to be higher for ultrasonic-assisted brining process for 5% NaCl. However; in the case of SR utilisation, improvement in K_m was not evident at UI levels investigated. This is probably due to low level of sodium present in the SR. The actual composition of the commercial SR used in this study is not known and it can be assumed that the composition has other sodium salt substitutes and salt enhancers which could have significant impact on NaCl diffusion. In a study, Aliño, Grau, Toldrá, and Barat (2010) observed a variation in salt content of dry cured hams with a mixture of various chloride salts of sodium, potassium, magnesium and calcium. These authors observed higher penetrations rates for sodium and potassium salts, as opposed to calcium and magnesium salts.

Table 4.1. Effect of brining time and ultrasound on sodium salt content (g/kg) and diffusion coefficient (K_m, min^{-1} m^{-2}) of pork samples

Brining Time (min)	5 % Salt			5 % Salt Replacer		
	Control	9.0 W cm^{-2}	54.9 W cm^{-2}	Control	9.0 W cm^{-2}	54.9 W cm^{-2}
0	0.88*^{e}	0.91^{e}	0.74^{e}	0.70^{d}	0.72^{d}	0.680^{e}
30	4.32^{d}	3.49^{d}	7.99^{d}	0.75^{d}	0.76^{d}	0.803^{d}
60	5.37^{c}	4.07^{c}	8.96^{c}	1.10^{c}	1.06^{c}	1.212^{c}
90	5.99^{b}	7.50^{b}	10.09^{b}	1.17^{b}	1.11^{b}	1.286^{b}
120	8.32^{a}	11.16^{a}	10.88^{a}	2.38^{a}	1.60^{a}	1.710^{a}
K_m ($min^{-1}m^{-2}$)	1.07×10^{-2}	1.09×10^{-2}	1.79×10^{-2}	1.15×10^{-3}	7.91×10^{-4}	1.06×10^{-3}
R^2	0.935	0.942	0.783	0.756	0.892	0.949

*Average of three replications.

^{abcde}Values followed by same letter within a column are not significantly different (P< 0.05) for each parameter.

Improvement in salt content of pork, with an increase in UI, has been reported previously (Cárcel, Benedito, Bon, & Mulet, 2007; Mulet, Cárcel, Sanjuán, & Bon, 2003; Ozuna, Puig, García-Pérez, Mulet, & Cárcel, 2013). Siró, Vén, Balla, Jónás, Zeke, and Friedrich (2009) observed an increase in salt diffusion due to ultrasound application compared to brining

under static conditions and the diffusion coefficient exponentially increased with an increase in ultrasonic intensity (2–4 W cm^{-2}) at a frequency of 20 kHz. Cárcel, Benedito, Bon, and Mulet (2007) investigated the effect of ultrasonic intensities ranging from 5.2 – 75.8 W cm^{-2} on NaCl transfer rates. They observed a significant improvement in NaCl transfer rates at UI above 51 W cm^{-2}. In another study, Ciara K. McDonnell, James G. Lyng, Joshua M. Arimi, and Paul Allen (2014) did not observe a linear increase in salt migration, but an increase in UI intensities ranged from 10.7 to 25.4 W cm^{-2}. In this study, a significant increase in salt uptake was observed at a UI of 54.9 W cm^{-2} for NaCl compared to static brining or brining at a UI of 9.0 W cm^{-2}; which corroborates the findings of Cárcel, Benedito, Bon, and Mulet (2007) and Siró, Vén, Balla, Jónás, Zeke, and Friedrich (2009). The variations observed in previous studies are perhaps due to variations in the geometry of the brining tanks used, position of ultrasonic probes, temperature control and other processing variables which have an effect on ultrasonic energy distribution (Berlan & Mason, 1992). However, salt diffusion in meat is a complex phenomenon and the role of ultrasound in improving diffusion of salt is not clearly understood (Siró, Vén, Balla, Jónás, Zeke, & Friedrich, 2009).

Ultrasound improves salt diffusion in the meat matrix by microinjection of brine into the meat by the formation of microjets as a result of asymmetric cavitation near the solid surface (Mason & Lorimer, 2002). Changes in microstructure, as a result of sonication, may also improve the diffusion of NaCl into the meat matrix. Siró, Vén, Balla, Jónás, Zeke, and Friedrich (2009) observed a microstructural change, in particular, an increased distance between muscle fibres. Moreover, protein denaturation and diffusion of other components may also influence NaCl diffusion into the meat matrix.

4.3.2. Effect of ultrasound on physicochemical properties of brined pork

4.3.2.1. Water mobility by TD-NMR relaxometry

NMR relaxometry provides useful information about interactions between water and myofibrillar meat proteins as it is governed by exchange of water protons and exchangeable protons in proteins (Bertram, Engelsen, Busk, Karlsson, & Andersen, 2004). Figure 4.2(a) shows a typical representation of T_2 transverse measurements for fresh pork sample. After multi-exponential fitting of the curves, three peaks were observed. These peaks can be attributed to water closely associated ('bound') with macromolecules or protons located on macromolecular structures plasticized by water (T_{2b}), water located within the dense myofibrillar protein matrix (T_{21}), and extramyofibrillar water (T_{22}) (Bertram, Andersen, & Karlsson, 2001; Li, Liu, Zhou, Xu, Qi, Shi, et al., 2012).

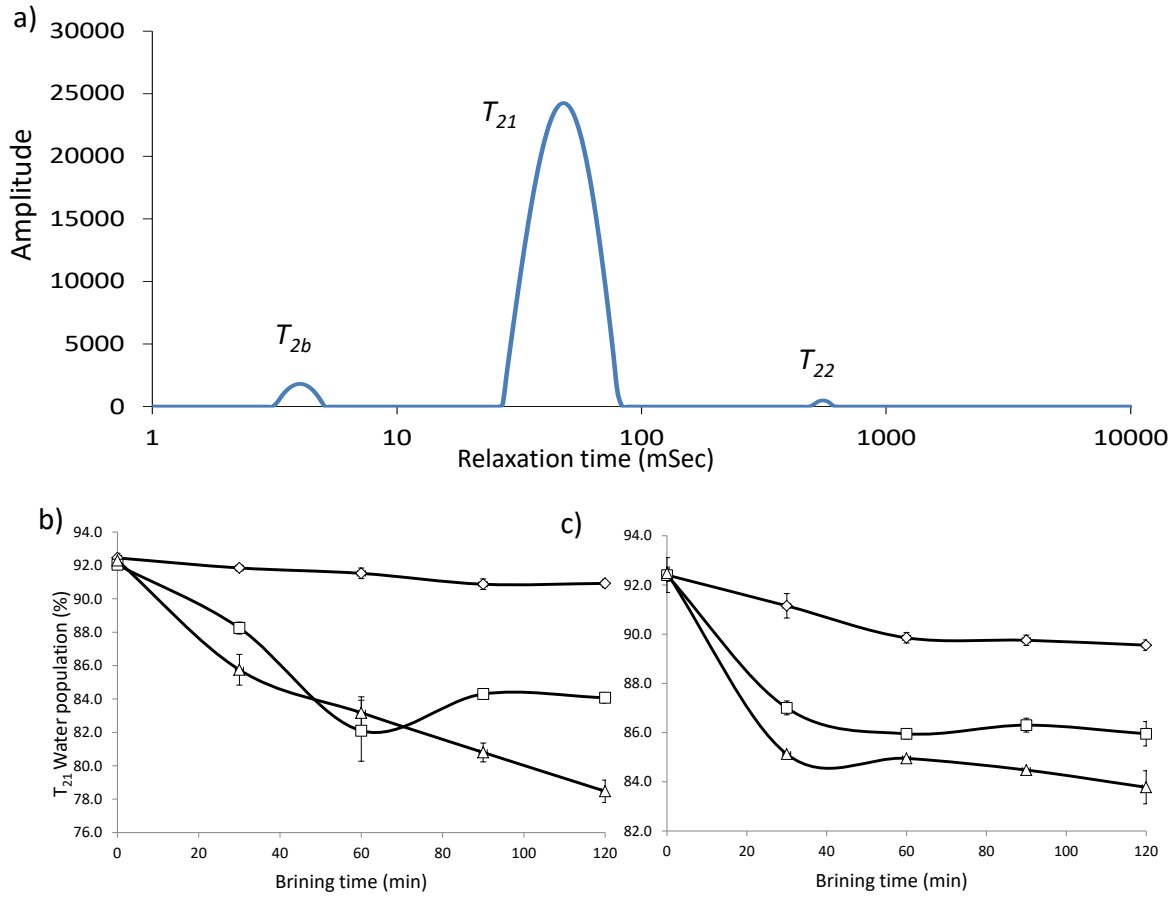


Figure 4.2. Distribution of multi exponentially fitted transverse relaxation (T_2) data of pork control sample (a); T_{21} water population for NaCl (b) and Salt replacer samples (c) [(◊) Control; (□) 9.0 W cm^{-2};(Δ): 54.9 W cm^{-2}].

Significant changes were observed for water population at T_{21} and T_{22} relaxation times (Table 4.2). The majority of water present in samples was T_{21} which represent water trapped by the dense myofibrillar network. A significant (P<0.05) decrease in T_{21} was observed for both NaCl and SR after a brining time of 120 min. Correlation analysis showed a negative relationship between NaCl content and T_{21} values for both NaCl (*r=0.73; P=0.001*) and SR (*r=0.37; P=0.001*). This is probably due to the diffusion of salt into the sample through osmosis, thereby resulting in increased sodium content in the meat matrix. A higher salt content in the meat matrix will dissolve/solubilise some of the meat proteins, which will weaken the matrix that is trapping the T_{21} water population.

Table 4.2. NMR T_2 relaxation data and cook loss (%) for NaCl and salt replacer brined samples with static brine and UI of 9 W cm^{-2} and 54.9 W cm^{-2}.

Brining Time (min)	**5 % Salt**			**5 % Salt Replacer**		
	Control	**9 W cm^{-2}**	**54.9 W cm^{-2}**	**Control**	**9 W cm^{-2}**	**54.9 W cm^{-2}**
T_{2b}*						
0	3.52^{a}	3.60^{a}	3.67^{a}	3.60^{b}	3.55^{b}	3.57^{b}
30	3.68^{a}	3.52^{a}	4.70^{a}	4.90^{ab}	5.45^{ab}	5.95^{a}
60	3.77^{a}	3.95^{a}	4.42^{a}	5.65^{a}	6.10^{a}	5.47^{a}
90	4.17^{a}	3.27^{ab}	4.60^{a}	5.27^{a}	5.30^{ab}	5.05^{a}
120	3.62^{a}	2.00^{b}	4.50^{a}	5.05^{a}	5.15^{ab}	5.10^{a}
T_{21}						
0	92.45^{a}	92.04^{a}	92.30^{a}	92.40^{a}	92.40^{a}	92.47^{a}
30	91.85^{ab}	88.25^{b}	85.75^{b}	91.15^{b}	87.00^{b}	85.12^{b}
60	91.52^{ab}	82.10^{d}	83.17^{bc}	89.85^{c}	85.95^{b}	84.95^{bc}
90	90.87^{b}	84.30^{c}	80.80^{cb}	89.75^{c}	86.30^{b}	84.47^{bc}
120	90.92^{b}	84.07^{c}	78.47^{b}	89.55^{c}	85.95^{b}	83.77^{c}
T_{22}						
0	4.02^{b}	4.36^{d}	4.02^{e}	4.00^{c}	4.05^{b}	3.95d
30	4.47^{ab}	8.22^{c}	9.55^{d}	3.95^{c}	7.55^{a}	8.92^{c}
60	4.70^{ab}	9.45^{c}	12.40^{c}	4.50^{bc}	7.95^{a}	9.57^{bc}
90	4.95^{ab}	12.42^{b}	14.60^{b}	4.97^{ab}	8.40^{a}	10.47^{ab}
120	5.45^{a}	13.92^{a}	17.02^{a}	5.40^{a}	8.90^{a}	11.12^{a}
Cook loss (%)						
0	24.98^{a}	21.34^{a}	21.59^{a}	18.13^{a}	23.13^{ab}	25.32^{a}
30	21.56^{b}	19.67^{ab}	17.71^{b}	20.99^{a}	27.48^{a}	27.24^{a}
60	16.41^{c}	20.64^{a}	19.58^{ab}	20.15^{a}	25.49^{ab}	27.46^{a}
90	24.44^{a}	18.37^{ab}	21.99^{a}	22.06^{a}	16.88^{b}	26.89^{a}
120	14.10^{d}	15.54^{b}	18.31^{b}	22.25^{a}	17.43^{b}	24.22^{a}

*T_{2b}, T_{21}, T_{22}, relaxation times for water closely associated with macromolecules, water located within the myofibrillar protein matrix, and extramyofibrillar water, respectively

^{abcd}Values followed by same letter within a column are not significantly different (P< 0.05) for each parameter.

Ultrasound-assisted brining demonstrated higher decrease in T_{21} water population compared to static brine treatment. Moreover, this decrease was higher for samples processed at a UI of 54.9 W cm^{-2} compared to 9.0 W cm^{-2} samples, for both NaCl and SR. This can be explained by the fact that the ultrasound showed an improvement in salt migration into pork samples. However, NaCl content of pork samples subjected to SR did not show a significant

improvement in salt diffusion rates compared to the static SR brining, but showed a significant decrease in water population at the T_{21} relaxation time, thereby demonstrating the effect of ultrasonic waves. Ultrasound technology employs both physical and chemical effects to alter meat structure, with promising results reported for meat tenderisation (Ozuna, Puig, García-Pérez, Mulet, & Cárcel, 2013) and modification of meat structure (which is derived primarily from hot spots formed during acoustic cavitation). Decrease in the T_{21} population at higher UI indicates the release of trapped water and a simultaneous increase in the T_{22} population represents free water within the pork meat matrix i.e. extramyofibrillar water. T_{22} population was found to increase, with an increase in brining time for all samples. T_{22} water population was higher at a UI level of 54.9 W cm^{-2} compared to a UI level of 9.0 W cm^{-2} or static brining. Reduction of the T_{21} population and an increase in the T_{22} population may be due to increased salt intake and a change in physical properties of meat during curing process. Increased salt content leads to an enlarged electrostatic repulsion within myofibrils, which swell laterally, thereby resulting in osmotic dehydration (Vestergaard, Andersen, & Adler-Nissen, 2007).

4.3.2.2. Differential scanning calorimetry (DSC)

A typical DSC heat curve for the control sample and the control brine sample after 120 min is shown in Figure 4.3. Three thermal transitions were observed in the heat flow curve of pork samples with endothermic peak values in range of 48.8 – 53.3 ^{o}C, 70.3 – 72.9 ^{o}C and 79.6 – 80.1 ^{o}C, respectively (Table 4.3). These values are in agreement with those reported by Graiver, Pinotti, Califano, and Zaritzky (2006). They observed three major peaks for untreated pork samples which were linked to myosin (57.6 ^{o}C), sarcoplasmic protein and collagen (66.2 ^{o}C) and actin (80.3 ^{o}C).

A significant decrease in peak temperature for endothermic peak 1 representing myosin was observed with an increase in brining time for NaCl treated samples and this decrease was higher at UI level of 54.9 W cm^{-2} compared to control or UI level of 9.0 W cm^{-2}. However, in the case of the SR, no significant (*P<0.05*) change for all three endothermic peaks was observed for staticly-brined samples; with an exception to endothermic peak 1 for samples brined at UI of 54.9 W cm^{-2}. The effects observed are due to the higher sodium content, attributed to the use of normal processing NaCl in the meat after 120 min brining time and UI in the case of the NaCl brine. However, in the case of the SR, the effects observed were mainly attributable to the employment of ultrasound. Correlation analysis of the data showed a negative correlation (*r=–0.73*; *P<0.05*) between NaCl content and T_{max} (^{o}C, myosin) for NaCl-brined samples, whereas no correlation was found in the case of the SR (*r=0.05*).

Peak temperature data is also an indicative of myosin becoming destabilised by the presence of salt, as its concentration increased as a function of time due to ultrasound treatment. Increased salt content and ultrasound intensity resulted in destabilisation of the myosin and enhanced extraction of proteins due to ultrasonic cavitation. Implosion of cavitation bubbles, along with micromechanical shock waves and micro jetting, could have weakened and caused the dissociation of actomyosin, thereby encouraging the myosin structure to unfold (Ciara K McDonnell, James G Lyng, Joshua M Arimi, & Paul Allen, 2014). Similarly, Ciara K McDonnell, James G Lyng, Joshua M Arimi, and Paul Allen (2014) observed increased protein extraction due to the use of ultrasound treatment for meat products. It has been reported that an increase in salt content may decrease T_{max} for myosin, sarcoplasmic proteins, collagen and actin. In this study, a decrease in myosin, sarcoplasmic proteins and collagen was observed, with no significant changes observed in actin content. In a study, Kijowski and Mast (1988)

observed a shift in peak temperature, with an increase in salt concentration most likely due to a decrease in sarcoplasmic proteins and partial breakdown of myosin during brining.

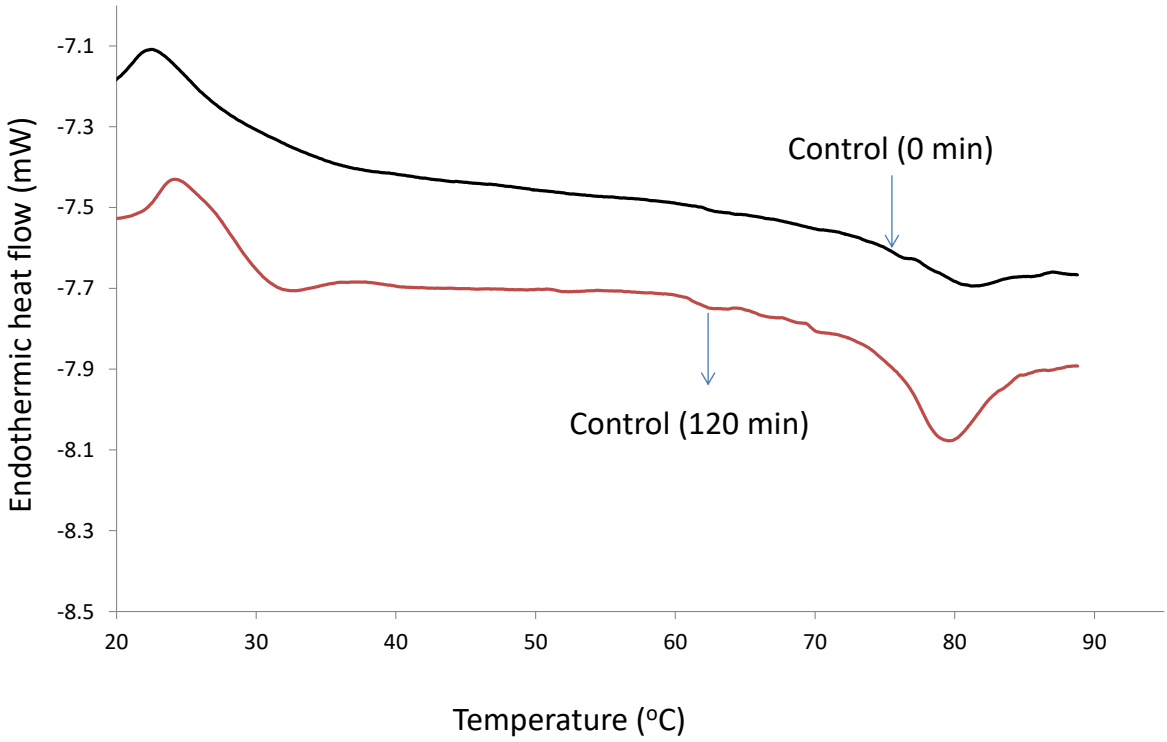


Figure 4. 3. Differential Scanning Calorimetric curve for control brined samples before and after brine treatment (120 min).

Table 4. 3. Peak temperature (°C) and enthalpy (ΔH, J/g) for pork samples

Brine type	Peak temperature (°C)						
		Endothermic peak 1		Endothermic peak 2		Endothermic peak 3	
	Brining time	0 min	120 min	0 min	120 min	0 min	120 min
NaCl (5%)	Control	53.0^{a}	42.8^{b}	72.8^{a}	71.7^{a}	80.1^{a}	78.7^{a}
	9 W cm^{-2}	48.6^{a}	46.4^{a}	69.8^{b}	72.8^{a}	78.8^{a}	78.8^{a}
	54.9 W cm^{-2}	49.9^{a}	38.3^{c}	70.3^{a}	67.2^{b}	80.0^{c}	82.1^{b}
Salt Replacer (5%)	Control	50.6^{ab}	56.9^{a}	72.4^{a}	72.5^{a}	79.6^{a}	78.2^{a}
	9 W cm^{-2}	49.4^{a}	36.8^{b}	72.5^{b}	76.3^{a}	79.2^{a}	81.9^{a}
	54.9 W cm^{-2}	48.9^{a}	45.2^{b}	71.4^{b}	74.7^{a}	80.0^{a}	79.3^{a}
	Delta H (x10^{-3}, J/g)						
NaCl (5%)	Control	10.2^{b}	28.7^{b}	24.0^{b}	28.0^{a}	18.8^{a}	22.3^{a}
	9 W cm^{-2}	21.4^{a}	27.7^{a}	19.8^{a}	11.9^{c}	20.0^{a}	16.5^{a}
	54.9 W cm^{-2}	21.4^{a}	22.2^{a}	19.4^{a}	15.6^{b}	18.5^{a}	18.4^{a}
Salt Replacer (5%)	Control	14.8^{a}	12.6^{a}	16.7^{b}	21.8^{b}	18.7^{a}	17.7^{a}
	9 W cm^{-2}	16.5^{b}	12.6^{c}	17.0^{b}	26.5^{a}	18.4^{a}	8.1^{b}
	54.9 W cm^{-2}	18.1^{a}	18.7^{a}	17.7^{a}	5.7^{b}	18.7^{b}	24.6^{a}

^{a,b,c}Values followed by same letter for each of the endothermic peak is not significantly different ($P< 0.05$).

4.3.2.3. Cook loss and WBSF

Significant changes in cooking losses were observed with an increase in brining time (Table 4.2). SR resulted in higher cooking losses compared to NaCl-brined samples. Reduction in cooking losses with increase in brining time was due to an increase in the sodium content in pork samples. Cook loss was negatively correlated with NaCl content (*r=-0.55, P< 0.05*) for sodium salt treated samples whereas, in the case of SR, the correlation coefficient was insignificant (*r=-0.12*). One of the main functions of NaCl in meat products is the extraction and solublisaiton of myofibrillar proteins which helps in binding of meat particles and reducing cooking losses. Correlation analysis did not show any significant relationship with water population associated with macromolecules. Similarly, Li et al., (2012) did not observe

any significant corrleation of cook loss with water population parameters, even though some changes in water distribution and mobility could have taken place. During cooking several structural changes occurs, namely; denaturation of myosin at around 40 ^{o}C, followed by shrinkage of collagen due to denaturation at around 55 – 60 ^{o}C and finally, shrinkage of muscles and connective tissues at around 60^{o}C (Bertram et al., 2004).

WBSF values were significantly reduced for both NaCl and SR samples at a UI of 54.9 W cm^{-2} compared to control or samples at a UI of 9.0 W cm^{-2} (Figure 4.4). Jayasooriya, Torley, D'Arcy, and Bhandari (2007) observed a significant decrease in WBSF values for bovine *M. Semitendinosus* and *M. Longissimus*. Some studies have shown that the sonication had no effect on meat tenderisation (Lyng, Allen, & McKenna, 1997) whereas, some studies reported a significant effect on meat tenderisation (Alarcon-Rojo, Janacua, Rodriguez, Paniwnyk, & Mason, 2015; Jayasooriya, Torley, D'Arcy, & Bhandari, 2007). Based on previously reported studies and results obtained in this one, it can be concluded that the tenderisation effect of sonication can be achieved only at higher ultrasonic intensities for longer processing times.

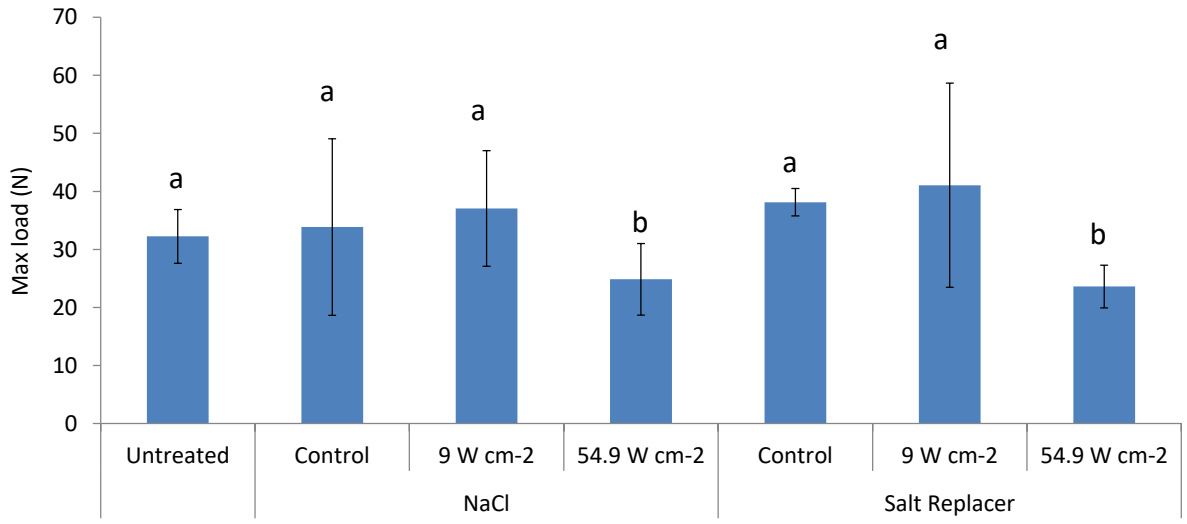


Figure 4. 4. WBSF values for brined samples before and after brine treatment (120 min).

4.4. Conclusions

The applicability of ultrasound-assisted brining was investigated for both NaCl brined and/or commercial SR and compared with a static brining system. The results presented in this study demonstrate that ultrasound can enhance salt diffusion into the meat matrix compared to a static brining system. However, improvement in salt diffusion was not evident in the case of SR. Reduction in cook loss for NaCl brined samples compared to SR brined samples clearly demonstrated the role that the use of NaCl has in terms of meat processing. Ultrasound-assisted brined samples showed improvement in WBSF values for pork samples at a higher UI for both NaCl and SR, indicating that the texture of brined samples can be improved by employing a higher UI for a longer duration.

Chapter 5: Ultrasonic-assisted incorporation of nano-encapsulated omega-3 fatty acids to enhance the fatty acid profile of pork meat

Overview

This study investigated ultrasound treatment to enhance the diffusion of nanovesicles of fish oil composed of 42% EPA (eicosapentanoic acid) and 16% DHA (docosahexanoic acid) into pork meat. The following paper has been published from this study.

- Ojha, K.S., Perussello, C. A., Garcia, C. A., Kerry, J. P., Pando, D., & Tiwari, B. K (2017). Ultrasonic-assisted incorporation of nano-encapsulated omega-3 fatty acids to enhance the fatty acid profile of pork meat. Meat Science 132, 99–106.

5.1. Introduction

Pork is a rich source of proteins, possessing high biological value and a number of bioactive molecules, including taurine, B vitamins and minerals. However, pork also contains high levels of lipids, which have been a topic of discussion for meat consumers due to their associated health implications. Relationships between dietary fat intake and incidence of various lifestyle disorders, including cardiovascular diseases, is well established and several health agencies have specific guidelines in this regard (Dugan, Vahmani, Turner, Mapiye, Juárez, Prieto, et al., 2015; Troy, Tiwari, & Joo, 2016). For instance, the World Health Organisation, recommends that various dietary fat fractions should contribute <15–30%, <10%, <5–8%, <1–2% and <1% of the total energy intake from total fat, saturated fatty acids, n(6)-polyunsaturated fatty acids, n(3)-polyunsaturated fatty acids and trans fatty acids, respectively (WHO, 2003). Lipid content of pork generally varies from 4-15% on a fresh basis, depending on several factors, including; geographical origin, genotype, feeding regime and meat cut (Park, Kim, Lee, Jang, Kim, Lee, et al., 2012; Wood, Enser, Fisher, Nute, Sheard, Richardson, et al., 2008).

According to Jiménez-Colmenero, Cofrades, Herrero, Fernández-Martín, Rodríguez-Salas, and Ruiz-Capillas (2012) and Olmedilla-Alonso, Jiménez-Colmenero, and Sánchez-Muniz (2013), the nutritional quality of meat products can be improved towards the reduction of caloric value and fat content, enhancement of fatty acid profile (e.g. reduction of saturated fatty acids and increase in MUFAs and PUFAs), reduction of cholesterol content, increase of amino acid quality, enrichment with minerals, vitamins and antioxidants, and reduction of sodium, nitrite and phosphate contents.

Genetic and environmental factors largely affect meat quality. Animal tissues vary in composition according to species, breed, age, sex, feeding, climate, rearing and slaughtering

conditions (Bonneau & Lebret, 2010). According to Żak and Pieszka (2009), genomic manipulation may result in improved carcass and meat quality (lean and fat content). Carcass and meat quality are influenced simultaneously by genes known as quantitative trait loci (QTLs) and environment. Additionally, animal feeding composition and portion affects growth rate, hormone metabolism and quantity and quality of fat within porcine muscles. The fatty acid profile of pork meat can also be modified by changing the dietary composition of fatty acids, either by adding vegetable oils or supplementing conjugated linoleic acid (CLA) to the pigs' diet, which improves feed conversion (Bonneau & Lebret, 2010; Rosenvold & Andersen, 2003). Studies have shown that the alteration of feeding regime e.g. grass-fed vs grain fed can improve meat fatty acid profile (Daley, Abbott, Doyle, Nader, & Larson, 2010).

In the last decade, specific strategies for increasing the level of fatty acids with beneficial health effects, while reducing the content of saturated fatty acids, have been a subject of active research (Horcada, Polvillo, Juárez, Avilés, Martínez, & Peña, 2016; Mapiye, Vahmani, Mlambo, Muchenje, Dzama, Hoffman, et al., 2015; Pouzo, Fanego, Santini, Descalzo, & Pavan, 2015; Xu, He, Liang, McAllister, & Yang, 2014). Different strategies have also been adopted to particularly focus on intramuscular fat (Scollan, Hocquette, Nuernberg, Dannenberger, Richardson, & Moloney, 2006). One such approach is the incorporation of encapsulated polyunsaturated omega-3 fatty acids into meat to enhance its fatty acid profile, while oils are preserved from oxidation and further degradation. Studies have shown that microencapsulated omega-3 fatty acids from a range of sources, such as fish, can be achieved successfully. Several techniques are available in order to encapsulate targeted fatty acids; the most popularly employed approaches being; coacervation, spray drying, spray chilling, extrusion coating and liposome entrapment by thin film hydration (TFH) (Kaushik, Dowling, Barrow, & Adhikari, 2015; Martín, Varona, Navarrete, & Cocero, 2010). Incorporation of

microencapsulated oils have been carried out in a range of food matrices for various potential health benefits (Bilia, Guccione, Isacchi, Righeschi, Firenzuoli, & Bergonzi, 2014; Gallardo, Guida, Martinez, López, Bernhardt, Blasco, et al., 2013; Jiménez-Colmenero, 2013). However, incorporation of encapsulated ingredients in solid foods such as meat is challenging compared to liquid foods, since diffusion rates are low and the oil is not effectively dispersed into a solidfood matrix. Among the techniques investigated to date, ultrasound has shown its potential for use in the assisted diffusion of a number of ingredients within food matrices. For example, the application of ultrasound can enhance NaCl diffusion rates, thereby allowing for faster and more uniform diffusion of NaCl into meat tissues (Carcel, Benedito, Bon, & Mulet, 2007; Jayasooriya, Torley, D'Arcy, & Bhandari, 2007; Ojha, Keenan, Bright, Kerry, & Tiwari, 2016; Ozuna, Puig, García-Pérez, Mulet, & Cárcel, 2013). However, the application of ultrasound for enhancing diffusion of microencapsulated ingredients has not been investigated to date. The objectives of this study were (a) to assess the potential for ultrasound application to improve the diffusion of encapsulated oils into a pork meat matrix, in terms of fatty acid profile of cooked and uncooked pork meat using a chemometric approach and (b) characterise both cationic (Lipo-CAT) and non-cationic (Lipo-N) nanoencapsulated fatty acids.

5.2. Material and methods

5.2.1. Preparation of nanovesicles

Fish oil for encapsulation was purchased from Neoalgae (Oviedo, Spain). EPA (eicosapentanoic acid) and DHA (docosahexanoic acid) contents were 42% and 16%, respectively. Two different nanovesicles were prepared using the Pronanosome Lipo-N and Pronanosome Lipo-cat blend techniques. The first one was employed to generate non-

cationic nanovesicles, while the second one yielded cationic nanovesicles. Although both formulations are confidential, all ingredients employed were of food grade. The nanovesicles were generated by means of the TFH method (Martín, Varona, Navarrete, & Cocero, 2010). Briefly, specific amounts of fish oil and the selected Pronanosome (Lipo-N or Lipo-cat) formulation were solubilized in chloroform; then the solvent was evaporated firstly using a rotatory evaporator and finally under nitrogen stream. The dried films obtained were rehydrated for 20 min using ultrapure water at 55ºC. Subsequently, the samples were homogenised at 15000 rpm for 15 min using a SilentCrusher M homogenizer (Heidolph). Finally, in order to obtain a homogenous size distribution, a further extrusion step (Avesting, LiposoFast LF50) was employed using a 500 nm pore size cellulose filter (Millipore). Non-encapsulated fish oil was removed using two purification steps. Firstly, samples were centrifuged at 5000 rpm for 1h and the supernatant, which contained the non-encapsulated oil, was carefully decanted. Following this step, the pellets were dialysed as follows: samples were filled into 10k MWCO SnakeSkin dialysis tubing (Thermo Fischer) and submerged in ultrapure water for 24h. The final composition of the nanovesicles was 100 mg/mL of Pronanosome formulation and 10 mg/mL of fish oil, respectively, for both cationic and non-cationic nanovesicles.

5.2.2. Nanovesicles characterisation

Vesicle characterisation was carried out according to the protocol described by Pando, Beltran, Gerone, Matos, and Pazos (2015). Measurement of size and determination of Polidispersity Index (PDI) of the samples were carried out using Dynamic Light Scattering (DLS) (Zetasizer NanoZS90, Malvern Instruments). Ten microliters of the sample was diluted into 990 µL of ultrapure water and the measurements were carried out in triplicate. Zeta potential was determined using the Mixed Measurement Mode-Phase Analysis Light Scattering

technology (M3-PALS) (ZetaSizer NanoZS90, Malvern Instruments). The particle concentration of the sample was determined using Nanoparticle Track Analysis (NTA) in a Nanosight LM10 device (Malvern Instruments). The samples were diluted at 1:200 using ultrapure water and three video captures were recorded to calculate the particle concentration of samples.

5.2.3. Sample preparation and ultrasound treatment

Meat used for all treatments was porcine *M. semitendinosus* which was obtained from a local supplier (Dublin Meat Company, Blanchardstown, Co. Dublin, Ireland) at 6 days post-mortem. The muscles were derived from Large White crosses, slaughtered at approx. 96 kg live weight. All visible fat was manually removed from each muscle, prior to slicing and subsequent treatments. Samples were cut into slices of approximately similar weight and size (4 x 4 x4 mm, length x width x height). Meat cubes were subjected to treatments immediately after slicing.

Pork meat pieces were submerged in the corresponding nanovesicles suspension in a glass beaker and were placed in an ultrasonic bath operating at a frequency of 25 kHz (Elma Schmidbauer GmbH, Germany) for either 30 or 60 min. Control (no US treatment) samples were placed in a beaker containing nanovesicles suspension. During the experiments, temperature was kept constant at 4.0±1.0°C by circulation of cold water using a temperature-controlled refrigerated water bath. After treatment, samples were removed, blot-dried and vacuum packed in high gas barrier laminated pouches. Control, ultrasound treated and raw pork samples were cooked in hot water bath (90 ^{o}C) to a core temperature of 70ºC for 10 min. The cooked and uncooked samples were cooled, vacuum packed and stored at -80 ^{o}C for fatty acid analysis.

5.2.5. Microwave- assisted preparation of fatty acid methyl esters (FAMEs)

Microwave-assisted FAME preparation was conducted using a MARS 6 Express 40 position Microwave Reaction System (CEM Corporation, Matthews, NC, USA). Reactions took place in PFA 55 mL reaction vessels. For FAME preparation, 1.0 g of pork samples was added to the reaction vessel containing a 10 mm stir bar. To this, 10 mL of potassium hydroxide (2.5%, w/v) in methanol was added along with 100 µL of internal standard (ISTD) (C23:0 methyl ester; final concentration following extraction is 0.1 mg/mL in pentane), and the reaction vessel was heated in the MARS 6 Express system to 130°C within 4 min and held at this temperature for 4 min. The reaction vessels were then removed from the carousel and cooled on ice for 5 min, or until they had reached room temperature before being opened. Derivatisation was then carried out by adding 15 mL of 5% (v/v) acetyl chloride in MeOH solution and heated to 120°C within 4 min and holding at this temperature for 2 min. The reaction tubes were removed again and cooled on ice to room temperature. To the cooled tubes, 10 mL of pentane was added, and the reaction tubes were shaken to extract the FAMEs into the upper pentane layer. Subsequently, 15 mL of a saturated salt solution was added, and the solution was mixed again. Following separation of the layers, the top pentane layer was removed and aliquoted into amber GC vials (1.5 mL).

5.2.6. Gas chromatography-flame ionisation detector (GC-FID) analysis

Gas chromatography was carried out using a Clarus 580 Gas Chromatograph fitted with a flame ionisation detector. A CP-Sil 88 capillary column (Agilent, Santa Clara, California, USA) with a length of 100 m x 0.25 mm ID and 0.2 µm film was used for the separation. The injection volume was 0.5 µL, and the injection port was set to 250°C. The oven was set to 80°C with an initial temperature ramp of 6.2°C/min to 220°C which was held for 3.2 min. A second temperature ramp of 6.3°C/min to 240°C followed and was held for 6.5 min (total runtime

was 35 min for each sample). The carrier gas was hydrogen at a flow of 1.25 mL/min, and the split ratio was set at 10:1. The FID was set at 270°C. Compounds were identified by comparing their retention times with those obtained from the Supelco 37 FAME standard (Sigma Aldrich, Wicklow, Ireland). The content of each fatty acid was calculated using the following equation (Eq. 5.1).

$$FA\ content = \frac{Peak\ Area\ (FAME)}{Peak\ Area\ (ISTD)}\ x\ \frac{ISTD\ weight}{Sample\ weight}\ x\ ISTD\ purity\ x\ 10\ x\ 0.96 \quad [5.1]$$

where, FA content is the content of fatty acid in the sample (mg/g), 10 is the dilution factor and 0.96 is the conversion factor for the internal standard.

5.2.7. Lipid quality factors

Atherogenic index (AI) and thrombogenic index (TI) were calculated as per Ghaeni, Ghahfarokhi, and Zaheri (2013):

$$AI = [(4 \times C14{:}0) + C16{:}0 + C18{:}0]/\ [\Sigma MUFA + \Sigma PUFA\ n6 + \Sigma PUFAn3] \quad [5.2]$$

$$TI = (C14{:}0+C16{:}0+C18{:}0)/(0.5MUFA+0.5PUFAn6+3PUFAn3+\ PUFAn3/PUFAn6) \quad [5.3]$$

5.2.8. Statistical analysis

Experiments were carried out as per experimental design shown in Table 5.1. Four parameters (cooking, ultrasound treatment, nanovesicles' formulation and sonication time) were investigated at two different levels. Principal Component Analysis (PCA) and Analysis of Variance (ANOVA) were performed using the software Statistica (StatSoft, ver 7.0). Means were separated using Tukey's test. Samples were considered significant at 95% confidence level ($p<0.05$). Factor loadings analysis was performed and graphs containing the experimental treatments were constructed using the first three principal components (PC1, PC2 and PC3).

Table 5.1. Experimental design (factorial 2^4).

Variable	Code	Level	
		-1	**+1**
Cooking	X1	No	Yes
Ultrasound	X2	No	Yes
Emulsion	X3	N	CAT
Immersion time (min)	X4	60	120

5.3. Results and discussion

5.3.1. Nanovesicles characterisation

The properties of the nanovesicles suspensions are shown in Table 5.2. The appearance of the suspension was white homogeneous liquid. There was no statistical difference ($p>0.05$) regarding the average size of the vesicles. Similarly, the PDI values were not statistically different as a result of the formulation employed.

Table 5.2. Characteristics of nanovesicles employed for fish oil encapsulation based on their formulation.

Emulsion	Average size (nm)	Polidispersity index	Zeta potential (mV)	Particle concentration (vesicles/mL)
Lipo-N	352.8±8.5[a]	0.266±0.037[a]	14.4±0.1[b]	5.8 x 10^{12a}
Lipo-Cat	345.9±5.1[a]	0.319±0.010[a]	51.3±1.0[a]	7.2 x 10^{12a}

[a,b] Values followed by same superscript are not significantly different ($P< 0.05$).

On the other hand, zeta potential was significantly affected by nanovesicle formulation ($P< 0.05$). This value is an indication of suspension stability: absolute values closer to 0 are found in low-stable suspensions and consequently, prone to aggregation and precipitation, while higher absolute values are found in highly-stable suspensions. In this case, Lipo-N can be

considered as having incipient stability, while Lipo-Cat suspension possessed a good stability (American Society for Testing and Materials, 1985). Finally, particle concentration is placed in the same order of magnitude, supporting the fact that minor differences can be found based on formulation pertaining to fatty acid content. Thus, it can be assumed that size and size distribution did not impact upon the results obtained in this study. On the contrary, zeta potential is the one factor that might have an impact on the performance of the vesicles in terms of lipid contents in treated meats.

5.3.2. Principal component analysis

Similarities and differences between samples were analysed by Principal Component Analysis, as illustrated in Figures 5.1 and 5.2. According to the scatterplot of the loading factors, while most of the fatty acids remained unchanged after treatment, PUFAn3, PUFAn6 and AI, TI and n6/n3 were affected by the process variables (Figure 5.1a). In fact, several omega-3 and omega-6 fatty acids (e.g. C18:3n3, C20:5n3, C22:6n3 and C20:4n6) are located in the same quarter as total PUFAn3 and PUFAn6, confirming the consistency of this PCA analysis. Since most of the samples were located in the second and third quarters of the PC1 X PC2 plot, the samples could be further differentiated based on spectral details described by the second (PC2) and third (PC3) principal components. The PC1 × PC2 scatterplot of the principal component scores (Figure 5.1b) allowed identification of a slight separation between cooked and uncooked samples by P2, and between Lipo-N and Lipo-CAT nanovesicles also by P2.

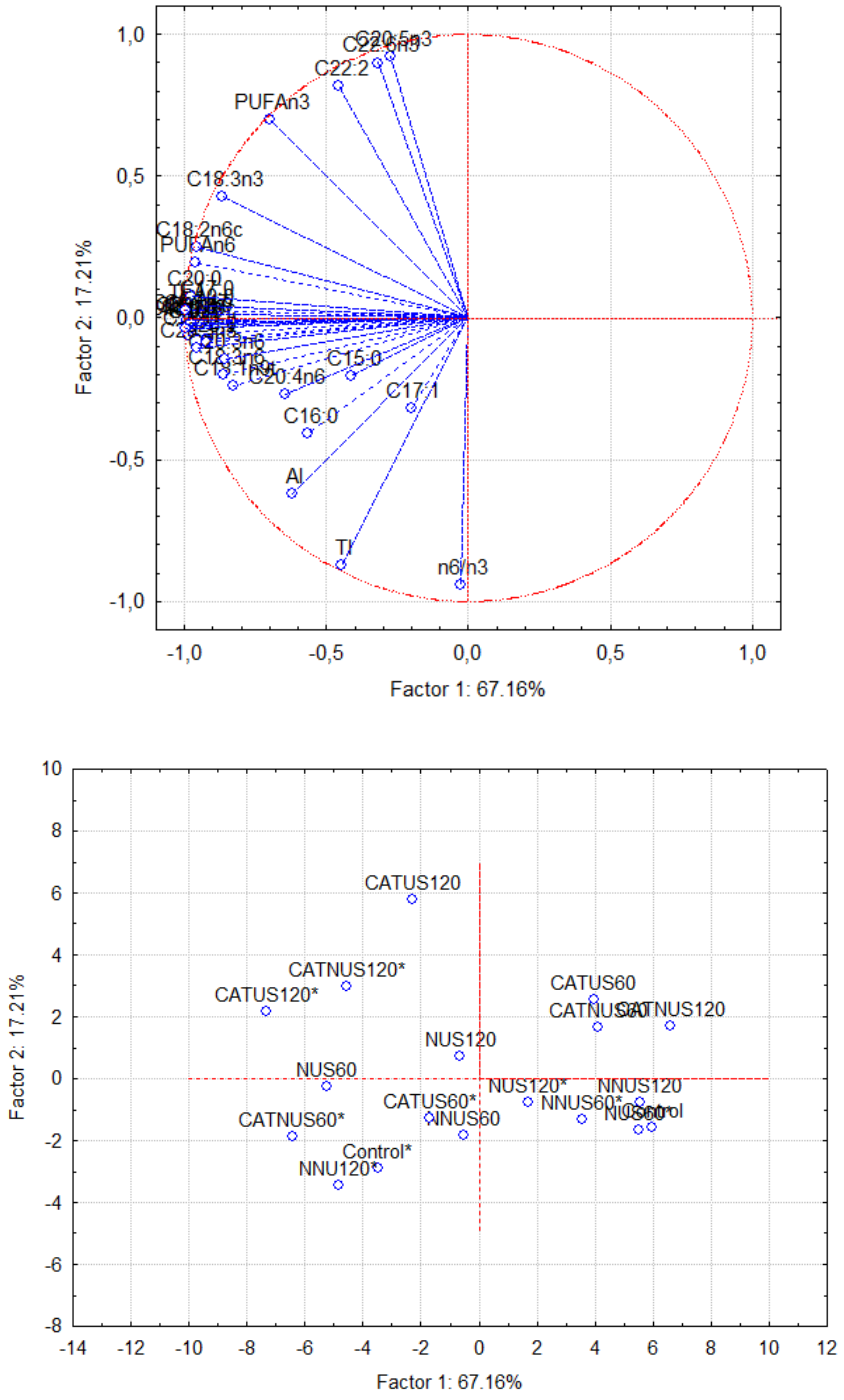


Figure 5. 1. PCA (PC1 X PC2) showing the differences between samples of pork meat treated according to the experimental design outlined in Table 5.1: (a) Loadings and (b) Scores. (Cooked samples are marked with*)

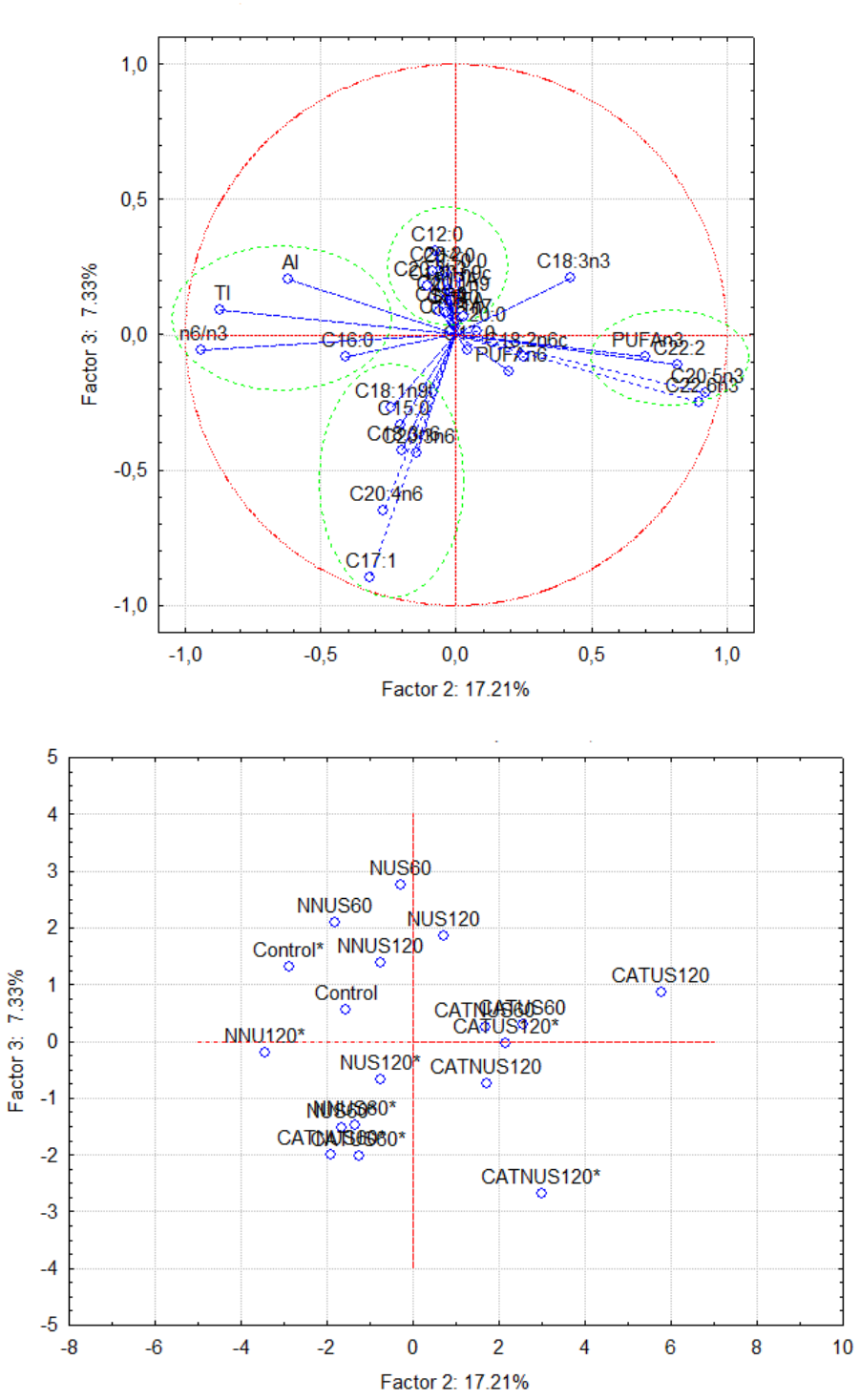


Figure 5.2. PCA (PC2 X PC3) showing the differences between samples of pork meat treated according to the experimental design outlined in Table 5.1: (a) Loadings and (b) Scores. (Cooked samples are marked with*)

Although PC1 and PC2 represented around 85% of the data variance, the similarities and differences between samples were better evidenced by PC2 and PC3 (Figure 5.2), indicating

that the data are explained only in the details. The scatterplot of the loading factors (Figure 5.2a) showed four clear distinct groups, as indicated in green. Samples subjected to CAT emulsion are clustered in the region correspondent to higher levels of PUFAn3 and PUFAn6 (1st and 4th quarters), PUFAn3 prevailing over PUFAn6. These samples showed, in particular, high levels of C18:3n3/α-linolenic acid, C22:6n3 (DHA), C22:2 (cis-13,16- docosadienoic acid methyl ester) and C20:5n3 (EPA). On the other hand, samples subjected to N emulsion have higher atherogenic and thrombogenic indices (AI and TI, respectively), meaning that they have greater amounts of saturated fatty acids. These samples also had higher ratios between omega-6 and omega-3 fatty acids.

PC3 clearly separated cooked from non-cooked samples (Figure5.2b), as previously represented by the PC1 × PC2 scatterplot (Figure 5.1b). The slight differentiation between samples which were and were not subjected to ultrasound by PC2 in Figure 5.2b indicates that ultrasound enhanced the content of unsaturated over saturated fatty acids. Differences between samples could be clearly observed after the introduction of a third PC (PC3), which accounted for 7.33% of the data variance. This means that the differences ascribed to the process variables are only subtle, which is in agreement with the results found by the Pareto/ANOVA analysis presented in the following section.

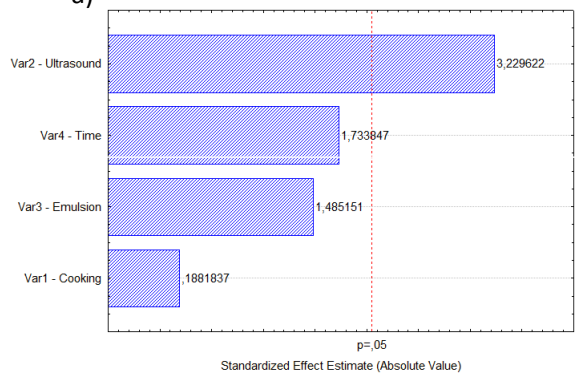
5.3.3 Effects of process variables on the fatty acid profile of pork meat

The effect of process variables was evaluated in 33 responses related to the fatty acid composition (Table 5.3), including individual fatty acids, AI and TI indices, and the ratio between n6/n3, following a Pareto analysis at a 95% confidence interval. The detailed results presented in Figure 5.3 are summarized in Table 5.3.

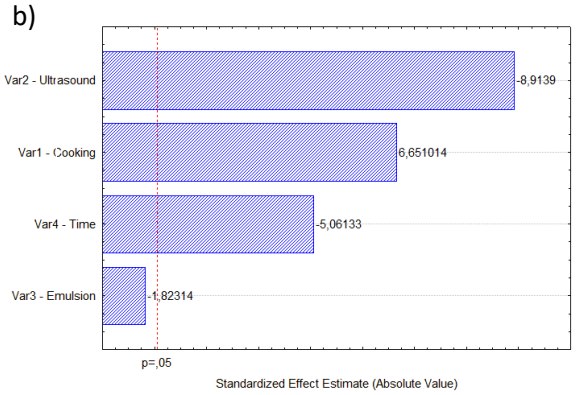
Table 5.3. Effects of process variables on the fatty acid profile of pork meat.

	Response	Effect(s)
	MUFA	Not affected by any variables
	PUFAn3	Positively affected by US treatment
	PUFAn6	Not affected by any variables
	SFA	Not affected by any variables
	TFA	Not affected by any variables
	AI	Not affected by any variables
	n6/n3	Affected by US treatment and time (-), and cooking (+)
	TI	Affected by US treatment (-) and cooking (+)
SFA	C10:0	Not affected by any variables
	C12:0	Not affected by any variables
	C14:0	Not affected by any variables
	C15:0	Not affected by any variables
	C16:0	Not affected by any variables
	C16:1	Not affected by any variables
	C17:0	Not affected by any variables
	C17:1	Affected by cooking (+) and emulsion (-)
	C18:0	Not affected by any variables
	C20:0	Not affected by any variables
PUFAs and MUFAs	C18:1n9t	Positively affected by cooking
	C18:1n9c	Not affected by any variables
	C18:1n7	Not affected by any variables
	C18:2n6c	Not affected by any variables
	C18:3n6	Positively affected by cooking
	C20:1n9	Not affected by any variables
	C18:3n3	Not affected by any variables
	C20:2	Not affected by any variables
	C20:3n6	Positively affected by cooking
	C20:3n3	Not affected by any variables
	C20:4n6	Positively affected by cooking and US treatment
	C22:2	Positively affected by US treatment and time
	C20:5n3	Positively affected by US treatment and time
	C22:5n3	Positively affected by US treatment and cooking
	C22:6n3	Positively affected by US treatment and time

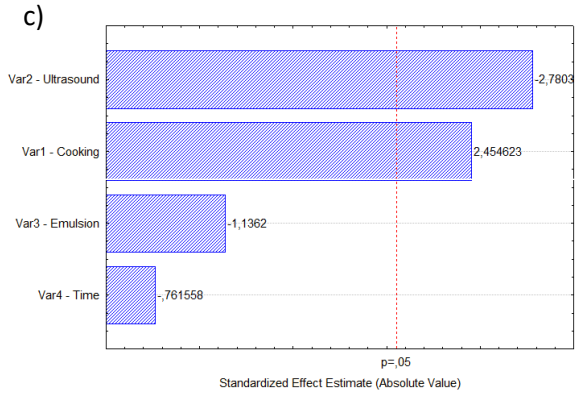
a)


Var2 - Ultrasound
3,229622
Var4 - Time
1,733847
Var3 - Emulsion
1,485151
Var1 - Cooking
,1881837
p=,05
Standardized Effect Estimate (Absolute Value)

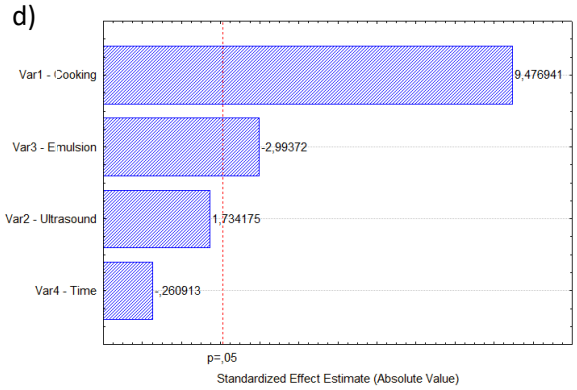
b)


Var2 - Ultrasound
-8,9139
Var1 - Cooking
6,651014
Var4 - Time
-5,06133
Var3 - Emulsion
-1,82314
p=,05
Standardized Effect Estimate (Absolute Value)

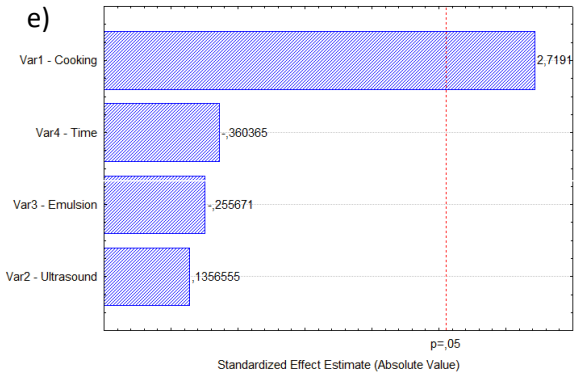
c)


Var2 - Ultrasound
-2,7803
Var1 - Cooking
2,454623
Var3 - Emulsion
-1,1362
Var4 - Time
-,761558
p=,05
Standardized Effect Estimate (Absolute Value)

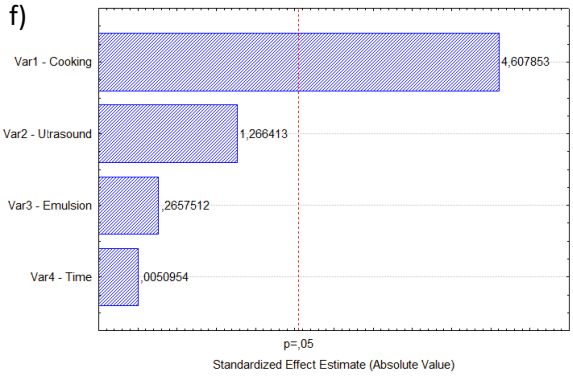
d)


Var1 - Cooking
9,476941
Var3 - Emulsion
-2,99372
Var2 - Ultrasound
1,734175
Var4 - Time
-,260913
p=,05
Standardized Effect Estimate (Absolute Value)

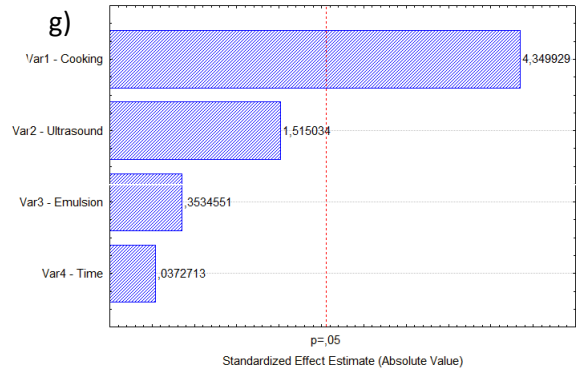
e)


Var1 - Cooking
2,7191
Var4 - Time
-,360365
Var3 - Emulsion
-,255671
Var2 - Ultrasound
,1356555
p=,05
Standardized Effect Estimate (Absolute Value)

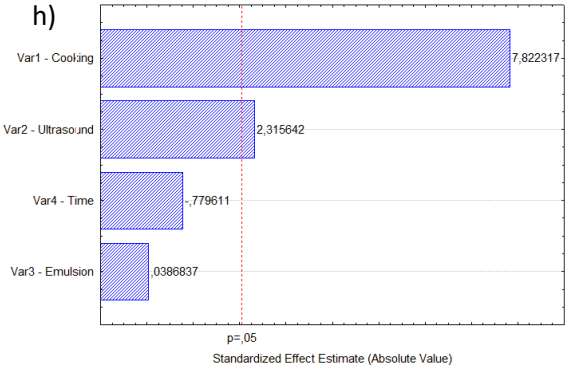
f)


Var1 - Cooking
4,607853
Var2 - Ultrasound
1,266413
Var3 - Emulsion
,2657512
Var4 - Time
,0050954
p=,05
Standardized Effect Estimate (Absolute Value)

g)


Var1 - Cooking
4,349929
Var2 - Ultrasound
1,515034
Var3 - Emulsion
,3534551
Var4 - Time
,0372713
p=,05
Standardized Effect Estimate (Absolute Value)

h)


Var1 - Cooking
7,822317
Var2 - Ultrasound
2,315642
Var4 - Time
-,779611
Var3 - Emulsion
,0386837
p=,05
Standardized Effect Estimate (Absolute Value)

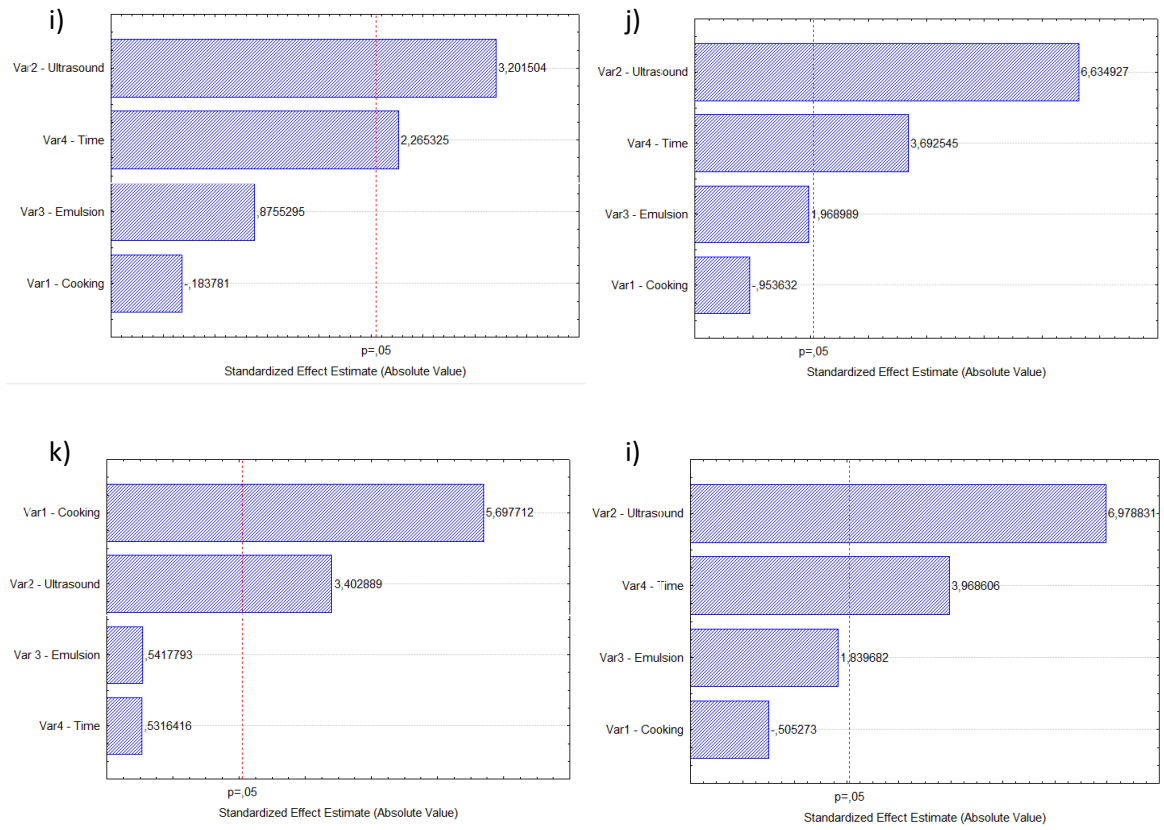


Figure 5.3. Pareto charts of the main effects of treatments. All fatty acids and indices analysed that were significantly affected (p> 0.05) by one or more of the four parameters studied are represented. Only 12 out of 33 responses analysed were affected: (a) PUFAn3; (b) n6/n3; (c) TI; (d) C17:1; (e) C18:1n9; (f) C18:3n6; (g) C20:3n6; (h) C20:4n6; (i) C22:2; (j) C20:5n3; (k) C22:5n3; (l) C22:6n3. Those remained unaffected are not represented.

It was found that the total fatty acids (TFA) were not affected by any of the treatments at the confidence interval aforementioned. This means that the overall fat content of the meat samples remained constant following the ultrasound treatment. However, it was found that the level of PUFA3 was positively affected by the treatment, regardless of the nanovesicle formulation. This effect is mainly ascribed to the increased amount of EPA (C20:5n3), which was the main component of the fish oil employed for encapsulation, and DHA (C22:6n3), which was the second fatty acid of importance in the fish oil studied. These two fatty acids were primarily affected by ultrasound treatment and time. Longer treatments significantly increased (P< 0.05) the amount of n3 fatty acids present in the final product. In general terms,

all long chain PUFAs (which are present in the encapsulated fish oil), experienced a positive effect following ultrasound treatment. These results indicate that ultrasound has a positive effect on the fatty acid profile of pork meat, enhancing the mass transfer of encapsulated oil into the meat samples. However, it has been reported that ultrasound had a negative effect on the fatty acid composition of edible oils (Chemat, Grondin, Sing, & Smadja, 2004). In addition, cooking resulted in higher contents of certain fatty acids (Figure 5.3), probably owing to the water loss incurred during cooking.

No significant differences ($p>0.05$) were found regarding the type of formulation employed for oil encapsulation. It means that both, cationic and non-cationic nanovesicles, can be successfully employed to increase the levels of MUFAs and PUFAs in pork meat.

5.3.4. Lipid quality indices

To further characterise the fatty acid profile, two lipid quality factors were studied. The atherogenic index (AI) indicates the relationship between the sum of the main saturated fatty acids and that of the main classes of unsaturated, the former being considered pro-atherogenic (favouring the adhesion of lipids to cells of the immunological and circulatory system), and the latter anti-atherogenic (inhibiting the aggregation of plaque and diminishing the levels of esterified fatty acid, cholesterol, and phospholipids, thereby preventing the appearance of micro- and macro-coronary diseases). The second factor analysed was the thrombogenic index (TI), which represents the tendency to form clots in the blood vessels. This is defined as the relationship between the pro-thrombogenetic (saturated) and the anti-thrombogenetic fatty acids (MUFAs, PUFAs-n6 and PUFAs-n3).

Table 5.4. Lipid quality factors in pork meat submerged in nanovesicles after different treatments.

Treat	Emulsion	US	Time (min)	n6/n3	AI	TI
Cooked	Control	Control	Control	10.75±0.00	0.49±0.00	1.06±0.00
	N	NOUS	60	10.59±0.02	0.43±0.01	0.95±0.02
	N	NOUS	120	10.72±0.11	0.49±0.5	1.07±0.11
	cat	NOUS	60	9.41±0.04	0.47±0.02	1.01±0.04
	cat	NOUS	120	7.24±0.10	0.45±0.03	0.90±0.10
	N	US	60	10.89±0.02	0.43±0.01	0.93±0.02
	N	US	120	9.42±0.01	0.45±0.01	0.96±0.01
	cat	US	60	9.53±0.05	0.45±0.01	0.95±0.05
	cat	US	120	7.74±0.03	0.46±0.01	0.93±0.03
Uncooked	Control	Control	Control	9.41±0.04	0.45±0.01	0.95±0.04
	N	NOUS	60	9.69±0.01	0.47±0.01	1.00±0.01
	N	NOUS	120	8.93±0.05	0.45±0.02	0.93±0.05
	cat	NOUS	60	7.39±0.02	0.44±0.01	0.87±0.02
	cat	NOUS	120	7.21±0.05	0.41±0.01	0.82±0.05
	N	US	60	9.55±0.16	0.45±0.07	0.97±0.16
	N	US	120	7.80±0.07	0.46±0.02	0.92±0.07
	cat	US	60	7.05±0.10	0.42±0.03	0.83±0.10
	cat	US	120	5.87±0.16	0.42±0.06	0.81±0.16

No statistical differences were determined when the AI was analysed (Table 5.3). The values of AI ranged from 0.41±0.01 to 0.49±0.00 (Table 5.4). These values are similar to those found in rabbit meat (Dal Bosco, Castellini, Bianchi, & Mugnai, 2004) and fermented pork sausages (Stajić, Živković, Perunović, Šobajić, & Vranić, 2011). On the other hand, the reduction of TI ascribed to the ultrasound treatment and the effect of time (Table 5.4) were statistically significant (Table 5.3), which means that the pork obtained showed a better relationship between pro-thrombogenic and anti-thrombogenetic fatty acids. The values for this index ranged from 1.07±0.05 (when ultrasound was not applied) to 0.81±0.16 (after 60 minutes of ultrasound treatment using cationic nanocapsules). These results suggest that healthier pork meat results from the novel technique applied here. In the same way, the ratio of n6/n3was

found to decrease with ultrasound treatment, as shown in Table 5.3 and Figure 5.3. This ratio is of key importance for well-balanced and healthy diets. In western diets this ratio is around 15-20/1, when it should be closer to 1/1 (Simopoulos, 2008). As the ratio n6/n3 in pork meat was reduced from 9.1±1.4 to 8.5±1.6 after the US treatment (Table 5.4), it resulted in a more balanced and healthier product. Compared to other studies (Kasprzyk, Tyra, & Babicz, 2015; Peiretti, Gai, Brugiapaglia, Mussa, & Meineri, 2015), the lipid quality indices of treated samples were found to have similar values of atherogenic index (0.42-0.47), higher (thus unwanted) values of thrombogenic index (1.01-1.15) and considerably higher (thus undesirable) n6/n3 ratios (12.61-35.32).

5.4. Conclusions

A combination of novel technologies e.g. nanoencapsulation and ultrasound, has been applied in order to improve the lipid profile of pork meat. This study has demonstrated the positive effect that ultrasound application had in increasing the amount of healthy fatty acids in pork meat. It was found that, regardless of the type of formulation employed, there was an increase in the content of DHA and EPA fatty acids. This increase was more noticeable as the length of ultrasound treatment was extended. The higher content of these fatty acids improved the lipid quality indices. Although the AI remained constant after treatment, TI and n3/n6 ratio values were beneficially modified.

Chapter 6: Application of ultrasound processing for the production of cultured and uncultured beef jerky

Overview

The objective of this study was to investigate the effect of extrinsic control factors namely ultrasonic frequencies, drying time and the addition of *Lactobacillus sakei* culture on various physicochemical and nutritional properties of beef jerky. This chapter has been published in the following three peer reviewed research papers.

- Ojha, K. S., Harrison, S. M., Brunton, N. P., Kerry, J. P., & Tiwari, B. K. (2017). Statistical approaches to assess the effect of *Lactobacillus sakei* culture and ultrasound frequency on fatty acid profile of beef jerky. Journal of Food Composition and Analysis, 57, 1-7.
- Ojha, K. S., Kerry, J. P., & Tiwari, B. K. (2017). Investigating the influence of ultrasound pre-treatment on drying kinetics and moisture migration measurement in *Lactobacillus sakei* cultured and uncultured beef jerky. LWT - Food Science and Technology, 81, 42-49.
- Ojha, K.S., Granato, D., Rajuria, G., Barba, F. J., Kerry, J. P., & Tiwari, B. K. (2018). Application of chemometrics to assess the influence of ultrasound frequency, *Lactobacillus sakei* culture and drying on beef jerky manufacture: Impact on amino acid profile, organic acids, texture and colour. Food Chemistry, 239, 544-550.



Beef slices with L. sakei ***Drying of beef slices*** ***Beef jerky***

6.1. Introduction

Jerky is one of the oldest forms of cured and dried meat products. Jerky products possess a high protein content and a unique flavour and texture profile. Jerky is traditionally prepared from thinly sliced whole muscles marinated and subsequently dried to an a_w value ranging from 0.70–0.85 (Yang, Hwang, Joo, & Park, 2009). Commercially this intermediate moisture foodstuff is often preserved using a hurdle concept involving interventions such as reducing a_w through the addition of preservatives such as organic acids, spices and nitrate/nitrite salts. The development of whole-muscle and/or restructured jerky from a range of meats by employing various curing ingredients, curing methods and drying conditions have been widely reported (Choi, Jeong, Han, Choi, Kim, Lee, et al., 2008; Jang, Kim, Hwang, Song, Kim, Ham, et al., 2015; Kucerova, Hubackova, Rohlik, & Banout, 2015).

Traditionally, jerky products are perceived as unhealthy. However, in recent years, consumption of jerky has increased significantly in Western meat-consuming countries. In a recent report of the Euromonitor International on sweet and savoury snacks in Ireland for 2015 it has been suggested that the air-dried protein product snack (beef jerky) was the “big breakout product”. Additionally, IBIS World report highlighted that the beef jerky accounted for 79% jerky sales within USA in 2014. The growth of sweet and savoury snacks has been heavily influenced by the changing health attitudes. Moreover, jerky, as a protein source, is the main driver for its popularity. Typically chopped and formed beef jerky contains approximately 23.4% moisture, 33.2% proteins and 25.6% lipids depending on the formulation. Protein content as high as 81% has been reported for pork jerky (Ruiz-Ramírez, Arnau, Serra, & Gou, 2006).

With growing consumer demand for high quality foods with good flavour, texture, nutrition and safety profiles, various strategies are being investigated and applied to jerky production. The focus has been to improve nutritional quality of traditionally produced jerky samples. Numerous applications of novel ingredients including antioxidants (Kołożyn-Krajewska & Dolatowski, 2012; Udabage, Augustin, Versteeg, Puvanenthiran, Yoo, Allen, et al., 2010), stabilisers (Dobson, Sanozky-Dawes, & Klaenhammer, 2007), probiotics (Ruiz-Ramírez, Arnau, Serra, & Gou, 2006) and non-conventional technologies, such as irradiation (McLeod, Zagorec, Champomier-Vergès, Naterstad, & Axelsson, 2010), plasma (Hammes, Bantleon, & Min, 1990) have been reported to improve the physicochemical properties, nutritional and safety profiles of beef jerky. Amongst these strategies the application of a starter culture (e.g. lactic acid bacteria) capable of preventing the growth of spoilage bacteria by producing bacteriocins has recently been reported (Biscola, Todorov, Capuano, Abriouel, Gálvez, & Franco, 2013; O'Connor, Ross, Hill, & Cotter, 2015). For example Pinto, Ponsano, Franco, and Shimokomaki (2002) reported that the inoculation of starter cultures (e.g. *Staphylococcus carnosus* and *S. xylosus*) can substantially improve sensorial quality of beef jerky.

Whilst consumers are most interested in the organoleptic quality, safety and healthiness of food products, manufacturers must also consider the cost of manufacture and are increasingly examining the use of novel processing strategies capable of reducing energy requirements and accelerating processing times. In recent years, several studies have reported the effects of power ultrasound on fresh and processed meat to assist curing, brining, drying and tenderisation of meat (Chapter 1). For example, ultrasound in combination with the application of a vacuum has been shown to increase drying rate of beef and chicken meat (Başlar, Kılıçlı, Toker, Sağdıç, & Arici, 2014). Application of ultrasound can also enhance mass transfer rates during brining/curing of meat mainly by disrupting the continuity of

cellular membranes due to various physical and chemical effects of the technology (C Ozuna, Cárcel, García-Pérez, Peña, & Mulet, 2015). However, whilst the processing benefits of ultrasound are clear some authors have reported that it can cause undesirable compositional changes in some foods leading to a reduction in nutritional and eating quality (Pingret, Fabiano-Tixier, & Chemat, 2013).

Additionally, over the last years, the combination of ultrasound with traditional preservation techniques, such as fermentation has attracted much interest from both researchers and the food industry. In this line, *Lactobacillus* species have been established as important food-associated lactic acid bacteria, which are widely used as starter culture for industrial meat fermentation, and with great potential as a bio-preservative in meat products (Hammes, Bantleon, & Min, 1990; McLeod, Zagorec, Champomier-Vergès, Naterstad, & Axelsson, 2010). Therefore, taking on the technological trend to use non-conventional processing techniques in the meat industry, the objective of this study was 1) to investigate the effect of US frequency and *L. sakei* culture on drying kinetics of beef jerky samples and elucidate changes in water distribution using Low Frequency-Nuclear Magnetic Resonance (LF-NMR); 2) to investigate the effect of US frequency, the addition of *L. sakei* culture and drying time on key nutritional (fatty acid profile, protein, amino acids, and organic acids) and physicochemical properties (texture and colour) employing a reliable multivariate statistical strategy.

6.2. Materials and methods

6.2.1. Sample preparation and ultrasonic pre-treatment

Eye of the round (*Semitendinosus*) obtained from a local supplier (Dublin Meat Company, Blanchardstown, Co. Dublin, Ireland) was used in this study. Muscle was stored at 4 °C and then cut into slices of similar size with a meat slicer (10 x 4 x 0.2 cm, L x W x H). The beef slices

were cured in two different curing solutions: (I) Cultured, containing 70% water, *L. sakei* DSM 15831 culture (10^4 cfu/mL), 1.5% salt, 1.0% sugar, 0.05% sodium nitrite and (II) Uncultured, containing 70% water, 1.5% salt, 1.0% sugar, 0.05% sodium nitrite (based on raw meat weight; v/w). The curing solution was calculated based on weight of beef slices in each sub-groups cultured (4 sub-groups: Control, 25 kHz, 33 kHz and 45 kHz) and uncultured (4 sub-groups: Control, 25 kHz, 33 kHz and 45 kHz). Curing solution along with beef slices from each sub-group were subjected to ultrasonic (US) treatments at frequencies of 25 kHz (Elma Schmidbauer GmbH, Germany), 33 kHz (Jencons, Leighton Buzzard, UK) and 45 kHz (Elma Schmidbauer GmbH, Germany) for 30 min along with controls (no US treatment). US treatments were carried out in ultrasonic bath systems maintained at a temperature of 30 ^{o}C. All samples were subsequently cured for 18 h at 4 ^{o}C. All cured beef jerky slices were dried using a hot air dryer (Gallendkamp Plus II, Weiss Technik, UK) at a temperature of 60 ^{o}C for up to 4 h. Two beef slices were withdrawn at drying times of 0 (after 18 h curing), 1, 2, 3 and 4 h and freeze dried prior to fatty acid analysis.

6.2.2. Drying of Beef Jerky

Cultured and uncultured cured beef jerky slices were dried using a hot air drying oven (Gallendkamp Plus II, Weiss Technik, UK) at a temperature of 60^{o}C for 4 h and using an air velocity which was maintained at 0.3 m/s. Beef jerky samples were placed in trays and were transferred to the hot air drying oven. Two slices from each treatment were withdrawn after every 30 min for 4 h and subsequently weight using precise weighing balance (Sartorius, Germany), after weight determination slices were placed back to the oven.

6.2.3. Physicochemical properties

6.2.3.1. LF-NMR transverse relaxation measurements

LF-NMR transverse relaxation measurements were carried out using a method described by McDonnell, Allen, Duggan, Arimi, Casey, Duane, et al. (2013) using a Maran Ultra instrument (Oxford Instruments, Abington, Oxfordshire, UK) resonating at a frequency of 23.2 MHz. Transverse relaxation (T_2) times were measured using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with the resultant relaxation decays analysed by tri–exponential unsupervised fitting using RI Win–DXP software (Version 1.2.3 Oxford Instrument, Abington, Oxfordshire, UK).

6.2.3.2. Texture analysis

Textural properties of jerky samples from both cultured and uncultured group was measured using a Texture Analyzer (Model: TA-XT2i; Stable Microsystems, UK), with a 25 kg load celland HDP/M3PB rig. Firmness (g force) and toughness of (g.sec) was measured by placing samples on top of the two adjustable supports of the base plate with the upper blade at a speed of 5 mm/s. Eight samples from each treatment were measured and average values recorded.

6.2.3.3. Instrumental colour analysis

Colour parameters were measured using a Hunter Lab colorimeter (Model: UltraScan XE, Reston, USA) dried beef jerky samples. The CIE L^* (lightness [0=black, 100=white]), a^* (–a^*=greenness, +a^*=redness) and b^* (–b^*=blueness, +b^*=yellowness) values were used to calculate the total colour difference (TCD) ($TCD=[(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$) , where ΔL^*, Δa^*, and Δb^* are differences between the untreated sample and the US-treated beef jerky. Five samples were measured for colour parameters and average was recorded.

6.2.4. Nutritional properties

6.2.4.1. Protein content and proteolysis index

Protein content of all the samples was determined using a LECO FP628 (LECO Corp., MI, USA) protein analyser based on the Dumas method according to the AOAC method 992.15 (1990). A sample extract of 0.25 g was used for protein estimation. Proteolysis index was determined as a percentage of the ratio between non-protein nitrogen obtained by precipitation of proteins with trichloroacetic acid and total nitrogen obtained using the Dumas method (Ruiz-Ramírez, Arnau, Serra, & Gou, 2006).

6.2.4.2. Amino acid analysis

The amino acid analysis (free and total content) of beef jerky was carried out according to the procedures outlined by Gambuteanu and Alexe (2015) with the aid of JEOL JLC-500/V AminoTac™ amino acid analyser (JEOL Ltd., Herts, UK). Beef jerky samples were deproteinised to determine the free amino acids content using a trichloroacetic acid solution at 240 g/L for 10 min. Samples were centrifuged (14,400×*g* for 10 min) and the supernatant was diluted with a sodium citrate buffer (0.2 mol/L; pH 2.2) and the content was diluted (1:2 v/v) with an internal standard (Norleucine) prior to injection. A λ=440 nm was used to detect proline, while λ=570 nm was used to detect the other amino acids. Aiming to assess the total amino acids content, beef jerky samples were hydrolysed using a 6 mol/L HCl solution at 110 °C for 23 h and all analyses were performed in triplicate.

6.2.4.3. Organic acid analysis

Organic acid analysis was performed according to the method outlined by Gupta, Jaiswal, and Abu-Ghannam (2013). Briefly, 1 g of jerky sample was mixed with 25 mL of distilled water, vortexed and centrifuged at 8720×*g* for 10 min at 4 °C, whereas the fermented broth samples

were directly centrifuged following the same conditions. The collected supernatant was filtered using 0.45 µm syringe filter and used for the determination of organic acids by HPLC. The HPLC analyses were carried out using the Waters Alliance HPLC (e2695 separation module) system equipped with W717 plus auto sampler, W486 UV detector and W410 differential refractometer detector connected in series. Chromatographic analysis was performed using an analytical Rezex ROA-Organic acid H^+ (8%) column (350 mm × 7.8 mm ID), fitted with a suitable guard cartridge (50 mm × 7.8 mm) (Phenomenex, UK). The analyses were carried out isocratically at a flow rate of 0.6 mL/min, employing 0.005 mol/L H_2SO_4 as mobile phase. A 20 µL aliquot was injected into a thermostatically controlled compartment set at 65 °C and the detection was carried out at 210 nm wavelength. The data acquisition and integration were performed using the Empower software package. The organic acids in the samples were identified by comparing the retention time and spectral data with that of standards, such as lactic acid and acetic acid (Sigma-Aldrich, Ireland).

6.2.3.4. Fatty acid profile

Microwave assisted FAME preparation was carried out using a MARS 6 Express 40 position Microwave Reaction System (CEM Corporation, Matthews, NC, USA) according to the method by Brunton, Mason, and Collins Jr (2015). Briefly, 0.5 g of freeze dried beef jerky samples were added to the reaction vessel containing a 10 mm stir bar. To this 10 ml of potassium hydroxide (2.5%, w/v) in methanol was added along with 100 µL of internal standard (C23:0 methyl ester; final concentration following extraction is 0.1 mg/mL in pentane). The reaction vessel was heated in the MARS 6 Express system to 130°C over 4 min and held at this temperature for 4 min. The reaction vessels were then removed from the carousel and cooled on ice for 5 min or until they had reached room temperature before they were opened. The derivatisation was then carried out in Microwave Reaction System by adding 15 mL of 5%

(v/v) acetyl chloride in MeOH solution and heating to 120°C over 4 min and holding at this temperature for 2 min. The reaction tubes were removed again and cooled on ice to room temperature. To the cooled tubes 10 mL of pentane was added and the reaction tubes were shaken to extract the FAMEs into the upper pentane layer. Following this, 15 mL of a saturated salt solution was added, and the solution was mixed again. Following separation of the layers, the top pentane layer was removed and aliquoted into amber GC vials (1.5 mL) containing sodium sulphate and analysed using gas chromatography.

Gas chromatography was carried out using a Clarus 580 Gas Chromatograph fitted with a flame ionisation detector. A CP-Sil 88 capillary column (Agilent, Santa Clara, California, USA) with a length of 100 m x 0.25 mm ID and 0.2 µm film was used for the separation. The injection volume was 0.5 µL, and the injection port was set to 250 °C. The oven was set to 80 °C with an initial temperature ramp of 6.2 °C/min to 220 °C which was held for 3.2 min. A second temperature ramp of 6.3 °C to 240°C followed and was held for 6.5 min (runtime 35 min). The carrier gas was hydrogen at a constant flow of 1.25 mL/min, and the split ratio was set at 10:1. The FID was set at 270°C. Compounds were identified by comparing their retention times with those of authentic FAMEs from the Supelco 37 FAME mix. The content off each fatty acid was calculated using following equation (Eq. 6.1).

$$FA\ content = \frac{Peak\ Area\ (FAME)}{Peak\ Area\ (ISTD)} \times \frac{ISTD\ Weight}{Sample\ Weight} \times ISTD\ purity \times 10 \times 0.96 \qquad [6.1]$$

Where, FA content is the amount of a given fatty acid in the sample (mg/g), 10 is dilution factor and 0.96 is the conversion factor for the internal standard which is already a FAME.

6.2.4.5. Nutritional quality indexes

Nutritional quality indices of beef jerky samples were analyzed from fatty acids composition data. The indexes of atherogenicity (AI) and thrombogenicity (TI) were calculated as proposed by Ulbricht and Southgate (Ulbricht & Southgate, 1991) and hypocholesterolemic/hypercholesterolemic (HH) index was calculated according to Santos-Silva et al. (Santos-Silva, Bessa, & Santos-Silva, 2002). AI, TI and HH indices were calculated using Equation 6.2 – 6.4, respectively. Other nutritional quality indices namely ratio of *n*–6/ *n*–3 PUFA and PUFA/SFA were also determined.

$$AI = \frac{[C12{:}0+4\times(C14{:}0)+C16{:}0]}{[\sum MUFA+\sum PUFA]} \quad [6.2]$$

$$TI = \frac{[C14{:}0+C16{:}0+C18{:}0]}{[0.5\times(\sum MUFA+\sum n6)+3\times\sum n3+\frac{\sum n3}{\sum n6}]} \quad [6.3]$$

$$HH = \frac{[C18{:}1cis9+C18{:}2n6+C20{:}4n6+C18{:}3n3+C20{:}5n3+C22{:}5n3+C22{:}6n3]}{[C14{:}0+C16{:}0]} \quad [6.4]$$

6.2.5. Mathematical modelling and statistical analysis

6.2.5.1. Drying kinetics

Moisture content, on a dry basis, is the weight of moisture present in the product per unit weight of dry matter in the product. For drying experiments, where weight losses were recorded, the instantaneous moisture contents at any given time can be obtained from Eq.6.5:

$$M = \frac{(M_o+1)W_o}{W_t} - 1 \quad [6.5]$$

Where W_o is the initial weight (g) of jerky sample after a curing period of 18 h, W_t is the weight (g) of sample at time t (min) and M_o is the initial moisture content (g water/g dry solids),

respectively. The initial moisture content was determined using the hot air oven method as per AOAC. The data obtained experimentally for control and ultrasound pre-treated beef jerky slices from both uncultured and cultured groups were plotted as a dimensionless variable moisture ratio (MR) *versus* time as calculated from Eq. 6.6:

$$Moisture\ ratio\ (MR) = \frac{(M_t - M_e)}{(M_o - M_e)} \quad [6.6]$$

Where M_t is the moisture content at any time t, M_e the equilibrium moisture content and M_o is the initial moisture content and all expressed as g water/g dry solids. The value of the equilibrium moisture content (Me) is relatively small compared to M_t or M_0. Thus, Eq. (6.1) can be simplified as $MR = Mt/Mo$ (Ju, El-Mashad, Fang, Pan, Xiao, Liu, et al., 2016; Xie, Mujumdar, Fang, Wang, Dai, Du, et al., 2017). Moisture diffusivity (D_f) for beef jerky samples were calculated by using Eq. 6.7 by analogy to the analytical solution to the Fick's second law of diffusion assuming negligible shrinkage, constant temperature, and constant moisture diffusivity (Zielinska & Michalska, 2016).

$$MR = \frac{8}{\pi^2} exp\left[-\frac{\pi^2 D_f\, t}{4L^2}\right] \quad [6.7]$$

Where, D_f is the effective moisture diffusivity (m^2/min), L is the thickness of the sliced beef (m).

Six empirical models were employed to describe drying kinetics were Henderson and Pabis, Wang and Singh, Page, Lewis (Newton), Weibull and Peleg (Table 6.1). The regression coefficient (R^2), Root mean square error (RMSE) and AICc (Akaike information criterion) values were calculated using Eq. 6.8 –6.10, respectively. R^2, RMSE and AICc values were used as the primary criteria for measuring best model fit.

$$R^2 = \frac{\sum_{i=1}^{N}(MR_i - MR_{pred,i}) \times \sum_{i=1}^{N}(MR_i - MR_{exp,i})}{\sqrt{\left[\sum_{i=1}^{N}(MR_i - MR_{pred,i})^2\right] \times \left[\sum_{i=1}^{N}(MR_i - MR_{exp,i})^2\right]}} \quad [6.8]$$

$$RMSE = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(MR_{exp,i} - MR_{pred,i}\right)^2} \quad [6.9]$$

$$AICc = 2n - 2log_e(\mathcal{L}(\hat{\theta}|y) + \frac{2n(n+1)}{N-n-1} \quad [6.10]$$

Where, $MR_{exp,i}$ is moisture content observed experimentally and $MR_{pre,i}$ is predicted moisture content; *SSE* is the sum of squared error, $2log_e(\mathcal{L}(\hat{\theta}|y)$ is the log-likelihood at its maximum point of the model estimated, *N* and *n* represent the number of observations and parameters assessed, respectively.

6.2.5.2. Statistical data analysis

Analysis of variance (ANOVA) was performed using SAS procedure (SAS Version 9.1.3, statistical Analysis Systems). Tukey's multiple comparison was used to compare treatment means. PROC CORR procedure of SAS was employed to determine Pearson's correlation coefficients (*r*) which were analysed to determine a relationship between various parameters.

In order to gain a broader view of the experimental data (excluding fatty acid profile data), principal component analysis (PCA) was applied to the whole dataset. The (I×J) data matrix was pre-treated using the unit variance standardization prior to PCA. Factor loadings analysis was also performed and graphs containing the experimental treatments were constructed using the first two principal components (PC1 vs PC2).

Multivariate analysis of fatty acid profiles for all treatments except for various nutritional indices was carried out using Factor analysis method. Factor analysis method is more

elaborate compared to principal component analysis which can be employed to describe variability among observed, correlated variables in terms of unobserved variables called factors. The observed variables can be projected as linear functions of the “factors.” Factor analysis with VARIMAX rotation was performed on the mean data matrix to classify samples without any presumption. The VARIMAX rotation is carried out to obtain a clear pattern of factor loading and is aimed at maximizing the variances of the squared normalized factor loadings across variables for each factor. Factor analysis was carried out using MINITAB (v17.0) software package.

6.3. Result and discussion

6.3.1. Drying kinetics

The effects of ultrasound frequencies on drying kinetics of marinated (uncultured and cultured) beef jerky slices are shown in Figure 6.1(a) & 1(b), respectively. In general, the moisture ratio (MR) decreased exponentially with time for control and ultrasound pre-treated samples from both cultured and uncultured groups. A variable effect was observed on the drying curves, depending upon culture treatment and ultrasonic frequency, as can be deduced from Figure 6.1.

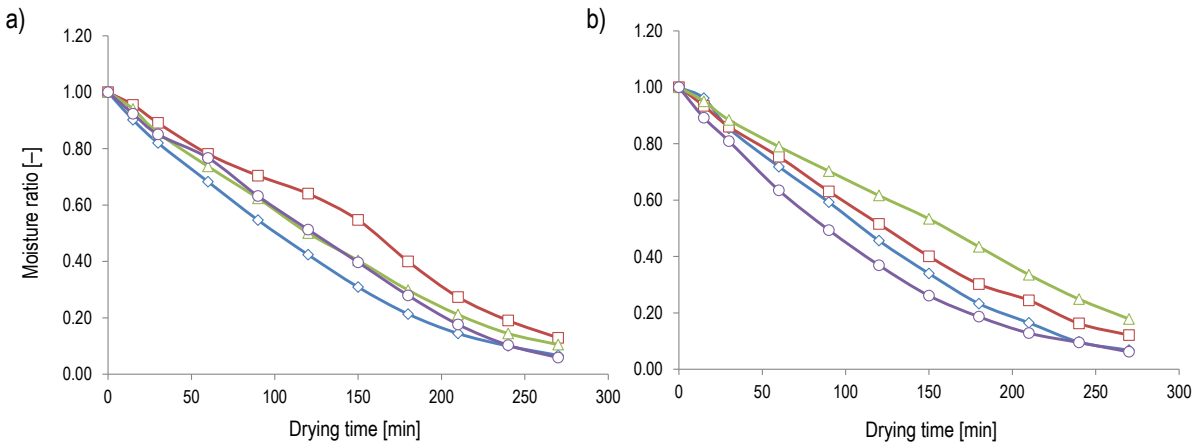


Figure 6.1. Moisture ratio [MR] vs. drying time [min] for a) uncultured and (b) cultured beef jerky slices pre-treated at various ultrasonic frequencies [Control (◊), 25 kHz (□), 33 kHz (Δ) and 45 kHz (o) respectively].

In general, a fast decrease in the MR [–] was observed for all treatments at initial stages followed by a slow decrease with drying time [min] at a drying temperature of 60°C. The moisture content decreased gradually for all samples, while a fast decrease in moisture content was observed at a frequency of 45 kHz, followed by the control, 25 kHz and 33 kHz, respectively, for cultured samples. In the case of uncultured samples, control samples showed the fastest decrease in moisture content, followed by 45 kHz, 33 kHz and 25 kHz. Previous studies have shown that ultrasound pre-treatment can enhance drying rate for various food matrices (Fernandes, Rodrigues, García-Pérez, & Cárcel, 2015; García-Pérez, Cárcel, Benedito, & Mulet, 2007). However, the effect of ultrasound assisted drying depends largely on food matrix being dried, ultrasonic processing parameters and drying temperature. For example, ultrasound pre-treatment of various food matrices showed a significant decrease in drying time, whereas in some cases, minor improvements were reported (F. A. N. Fernandes, M. I. Gallão, & S. Rodrigues, 2008; A Mulet, Carcel, Sanjuan, & Bon, 2003). Generally, during the drying process, migration of moisture is fast due to the evaporation of

surface moisture and decreases exponentially with an increase in drying time due to resistance offered by the matrix to moisture movement. In a study conducted by Başlar, Kılıçlı, Toker, Sağdıç, and Arici (2014), a significant decrease in drying time for ultrasound-assisted, vacuum-drying of chicken and beef meat samples was observed. There are several supporting studies which show that ultrasound enhances drying rate, owing to various mechanisms, thus modifying the diffusion boundary due to acoustic pressure waves, oscillating viscosities, compressions and expansions of materials leading to the formation of micro channels on surfaces which is required for fluid movement (Cárcel, García-Pérez, Benedito, & Mulet, 2012; A Mulet, Cárcel, Benedito, Rosselló, & Simal, 2003; Yao, 2016). Variation in drying rate in this study may be due to the diffusion of marination solution into the meat matrix due to the formation of micro channels on surfaces. Studies have shown that ultrasound application can increase brine diffusion rate into a range of meat matrices (J. A. Cárcel, J. Benedito, J. Bon, & A. Mulet, 2007; A. Mulet, Cárcel, Sanjuán, & Bon, 2003; César Ozuna, Puig, García-Pérez, Mulet, & Cárcel, 2013). This may occur due to ultrasound assisted microinjection of brine into meat through the formation of microjets as a result of asymmetric cavitation near the solid surface of the product (Mason & Lorimer, 2002). However, it has been reported that no linear increase in diffusion of brine solution into meat matrices was observed with respect to ultrasonic intensity (McDonnell, Lyng, Arimi, & Allen, 2014).

The successful application of ultrasound on meat drying rates has been reported, however, the mechanism of action is not yet clear. In this study, the effect of ultrasound frequency on drying rate for both uncultured and cultured samples was probably due to the effect of ultrasound on lactobacillus culture and diffusion of marination solution into the beef jerky samples. A significant moisture change was observed in marinated beef jerky samples after 18 h marination for ultrasonic pre-treated samples compared to fresh beef (72.0%). For

uncultured samples treated, at the lowest ultrasound frequency (25 kHz), a gain of 6.04% was observed whereas for 33 kHz and 45 kHz pre-treatments moisture gains of 5.60 % and 6.15%, respectively, were observed. In the case of cultured samples, no significant moisture gain was observed for the control group, whereas moisture gains of 5.12%, 4.11% and 3.58% were observed for ultrasound pre-treatments 33 kHz, 25 kHz and 45 kHz, respectively.

The observed changes were mainly due to uptake of marination solution. Similar gains in moisture have been reported for ultrasound pre-treatment prior to drying of fruit (F. A. Fernandes, Gallão, & Rodrigues, 2008; Oliveira, Gallão, Rodrigues, & Fernandes, 2011). However, in some cases, solid losses during ultrasound pre-treatments were also reported (Kadam, Tiwari, & O'Donnell, 2015; Oliveira, Gallão, Rodrigues, & Fernandes, 2011). A concentration gradient of soluble solids between beef slices and the marination solution resulted in water gain after pre-treatment and subsequent incubation. Increase in moisture uptake has been reported for marinated beef products, including; pork, poultry and beef, depending on composition of marination solution. Aktaş and Kaya (2001) observed an increase in moisture uptake for beef *Longissimus dorsi* muscle after marination at 4^{o}C for 24 h. In this study, moisture uptake was observed for ultrasound pre-treated samples, whereas no significant change in moisture uptake was observed for control samples. Research carried out by Cárcel, Benedito, Bon, and Mulet (2007) on ultrasound-assisted brine diffusion of pork muscle showed no significant change in moisture uptake in samples subjected to static brining and found that moisture uptake was dependent on ultrasonic intensity at a constant frequency of 20 kHz. Limited studies with muscle-based foods have, like this present study, also highlighted moisture uptake as a result of ultrasound pre-treatment in the case of Halal and non-Halal chicken breast (Leal-Ramos, Alarcon-Rojo, Mason, Paniwnyk, & Alarjah, 2011).

6.3.2. Drying models

Non-linear regression analysis was carried out for six drying models as a function of drying time and moisture ratio and various statistical parameters (R^2, RMSE and AICc) were determined to measure the goodness of model fit. Model and statistical parameters (of drying models are listed in Table 6.1. For all models R^2 ranged from 0.941 to 0.998, RMSE ranged from 0006 to 0.075 and AICc values ranged from -105.40 to -50.43. For beef jerky samples investigated, the Wang and Singh model had the closest fit to the drying experimental data, as evident from the high R^2 values (≥ 0.994) and the low RMSE (≤ 0.023) and low AICc (<-74.535) values for both cultured and uncultured jerky samples. Model parameters (*a* and *b*) obtained by fitting the Wang and Singh model indicated that the relative magnitude of the parameter accurately reflects drying behaviour. Drying constant values (*a)* were in the range of -5.98×10^{-3} min^{-1} to -3.2×10^{-3} min^{-1} for uncultured and -6.73×10^{-3} min^{-1} to -3.39×10^{-3} min^{-1} for cultured jerky samples, whereas, drying constant values (b) varied from -4.22×10^{-7} min^{-2} to 9.28×10^{-6} min^{-2} for uncultured and 1.23×10^{-6} min^{-2} to 1.22×10^{-5} min^{-2} cultured samples. Model parameter (a) was lowest in the case of 45 kHz and highest for 33 kHz for cultured samples, whereas, in the case of uncultured samples it was lowest for control samples and highest for 25 kHz samples. The lower (*a)* values reflect the higher moisture removal rates. A similar trend was also observed for drying kinetics when fitted to other models. Various models have been proposed to model drying kinetics of various food products, including; beef and chicken (Başlar, Kılıçlı, Toker, Sağdıç, & Arici, 2014). Drying behaviour can be predicted using a range of models, however, in this study the Wang and Singh model was found to be the best fit. Best model fit can be judged based on various statistical parameters, however; AICc and RMSE values were the criteria used for model section, because R^2 alone cannot be

judged for model fitting. AICc tends to have performance advantages over other criteria for model fitting (Burnham, Anderson, & Huyvaert, 2011). AICc value rise with an increase in the number of model parameters and the lower the AICc value, the better is the model performance. AICc criteria has been adopted by several researchers to test the performance of drying kinetics models (Buttchereit, Stamer, Junge, & Thaller, 2010; Gowen, Abu-Ghannam, Frias, & Oliveira, 2008; Kadam, Tiwari, & O'Donnell, 2015). The D_f value of the of cultured and uncultured beef samples ranged between 0.90 to 1.33 × 10^{-8} $m^2.min^{-1}$ and 0.83 to 1.45 × 10^{-8} $m^2.min^{-1}$, respectively, as shown in Figure 6.2. The highest Df value was observed for control uncultured samples, and cultured samples pre-treated at 45 kHz. Df value was found to increase with an increase in ultrasonic frequency in the case uncultured samples, however, values remained significantly lower for control jerky samples in all cases. Calculated Df values were within the range (10^{-8} to 10^{-10} m^2/s) of those previously reported for drying of biological materials (Başlar, Kılıçlı, Toker, Sağdıç, & Arici, 2014; Zogzas, Maroulis, & Marinos-Kouris, 1996).

Table 6.1. Model parameters obtained from fitting drying models to beef jerky samples along with key statistical parameters

Model	Parameter	Uncultured				Cultured			
		Control	**25 kHz**	**33 kHz**	**45 kHz**	**Control**	**25 kHz**	**33 kHz**	**45 kHz**
Henderson and Pabis	*a*	1.036	1.057	1.048	1.056	1.068	1.045	1.040	1.031
$\mathrm{MR} = \mathrm{a}\exp(-\mathrm{kt})$	*k*	8.18×10^{-3}	5.53×10^{-3}	6.83×10^{-3}	7.12×10^{-3}	7.82×10^{-3}	6.56×10^{-3}	5.09×10^{-3}	8.94×10^{-3}
	R^2	0.987	0.950	0.982	0.963	0.980	0.984	0.974	0.994
	RMSE	0.038	0.069	0.043	0.065	0.049	0.040	0.046	0.026
	AICc	-64.230	-50.435	-60.915	-51.810	-58.080	-62.630	-59.260	-71.965
Wang and Singh	*a*	-5.98×10^{-3}	-3.2×10^{-3}	-4.8×10^{-3}	-4.59×10^{-3}	-5.34×10^{-3}	-4.71×10^{-3}	-3.39×10^{-3}	-6.73×10^{-3}
$\mathrm{MR} = 1 + \mathrm{at} + \mathrm{bt}^2$	*b*	9.28×10^{-6}	-4.2×10^{-7}	5.28×10^{-6}	3.74×10^{-6}	6.71×10^{-6}	5.22×10^{-6}	1.23×10^{-6}	1.22×10^{-5}
	R^2	0.999	0.994	0.999	0.996	0.997	0.999	0.998	1.000
	RMSE	0.010	0.023	0.011	0.020	0.019	0.011	0.012	0.006
	AICc	-95.105	-74.535	-90.375	-78.980	-79.380	-90.780	-89.385	-105.400
Page	*k*	2.49×10^{-3}	3.17×10^{-4}	1.38×10^{-3}	6.56×10^{-4}	1.05×10^{-3}	1.38×10^{-3}	8.35×10^{-4}	3.76×10^{-3}
$\mathrm{MR} = \exp(-\mathrm{kt}^{\mathrm{n}})$	*n*	1.250	1.545	1.319	1.4785	1.392	1.3	1.3465	1.1725
	R^2	0.997	0.984	0.997	0.991	0.998	0.998	0.991	0.999
	RMSE	0.019	0.039	0.018	0.032	0.016	0.016	0.027	0.010
	AICc	-79.925	-62.775	-79.875	-67.365	-82.830	-83.160	-71.390	-92.330
Lewis (Newton)	*k*	7.86×10^{-3}	5.13×10^{-3}	6.45×10^{-3}	6.68×10^{-3}	7.27×10^{-3}	6.22×10^{-3}	4.82×10^{-3}	8.64×10^{-3}
$\mathrm{MR} = \exp(-\mathrm{kt})$	R^2	0.984	0.941	0.977	0.957	0.972	0.980	0.969	0.993
	RMSE	0.042	0.075	0.049	0.071	0.058	0.045	0.051	0.029
	AICc	-66.115	-52.575	-62.025	-53.965	-58.060	-63.680	-61.235	-73.125
Weibull	*a*	0.9737	0.95545	0.9788	0.955	0.9886	0.9792	0.97	0.9864
$\mathrm{MR} = \mathrm{a}\exp(-\mathrm{kt}^{n})$	*k*	1.81×10^{-3}	1.02×10^{-4}	9.56×10^{-4}	2.64×10^{-4}	8.83×10^{-4}	9.80×10^{-4}	4.47×10^{-4}	3.20×10^{-3}
	n	1.323	1.7515	1.382	1.6445	1.425	1.363	1.463	1.2025
	R^2	0.998	0.988	0.997	0.994	0.998	0.998	0.993	0.999
	RMSE	0.016	0.034	0.016	0.026	0.015	0.013	0.023	0.009
	AICc	-78.310	-60.655	-77.165	-66.835	-78.525	-81.595	-68.960	-89.970
Peleg	*q*	149.2	312.1	199.65	214.1	177.75	203.05	293.45	122.55
$\mathrm{MR} = 1 - \mathrm{t}/(\mathrm{a} + \mathrm{bt}))$	*b*	0.48645	-0.03865	0.34795	0.23575	0.37325	0.3582	0.12383	0.58215
	R^2	0.997	0.994	0.998	0.996	0.995	0.998	0.998	0.997
	RMSE	0.017	0.023	0.014	0.021	0.024	0.014	0.012	0.017
	AICc	-81.455	-74.535	-85.955	-77.730	-73.750	-84.980	-89.590	-80.710

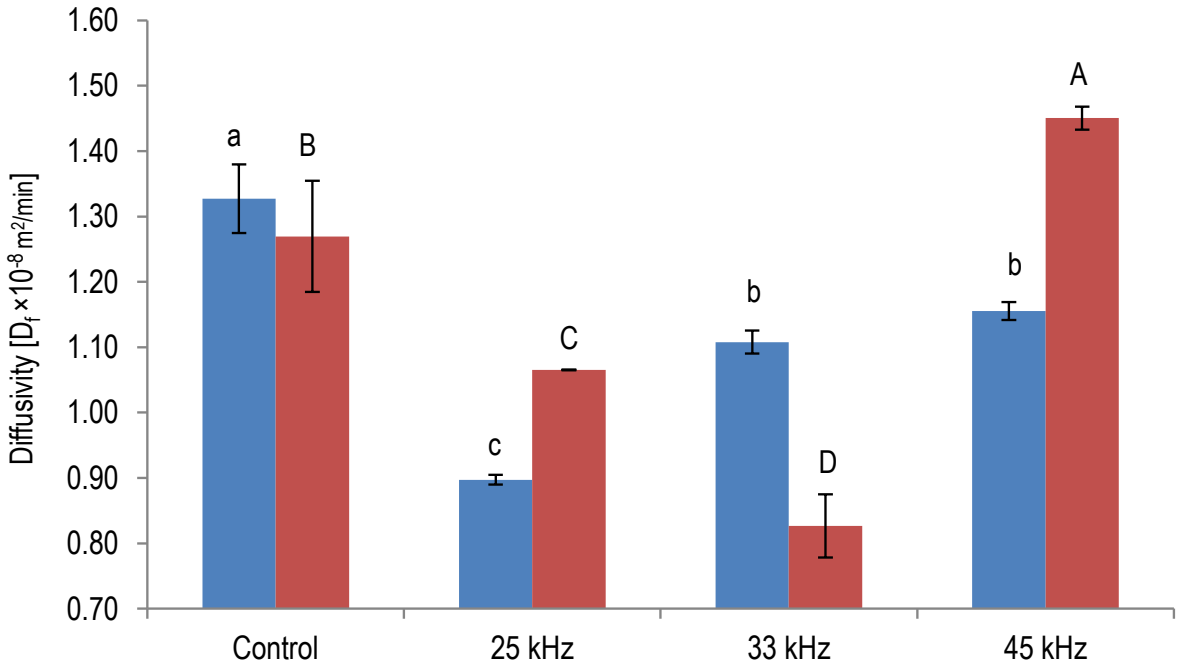


Figure 6.2. Diffusivity for uncultured [ ] and cultured [ ] beef jerky samples pre-treated at various ultrasonic frequencies.

6.3.3. Water mobility by TD-NMR relaxometry

A representative LF-NMR T_2 transverse measurement for uncultured and cultured samples after 18 h marination (i.e. before drying) and after the 4 h drying period is shown in Figure 6.3. Distributed exponential analysis of curve obtained for various samples revealed the presence of three distinct peaks obtained at relaxation time ranges of 0–10 ms (T_{2b}), 10–100 ms (T_{21}) and >100 ms (T_{22}) respectively. These peaks can be attributed to various fractions of water present in beef jerky samples. The first peak obtained at the shortest relaxation time (T_{2b}) represents bound water which is closely associated with macromolecules (mainly proteins). The second peak at T_{21} represents water present within the dense myofibrillar protein matrix, whereas, the third peak at T_{22} can be attributed to free-water present outside the myofibrillar protein matrix.

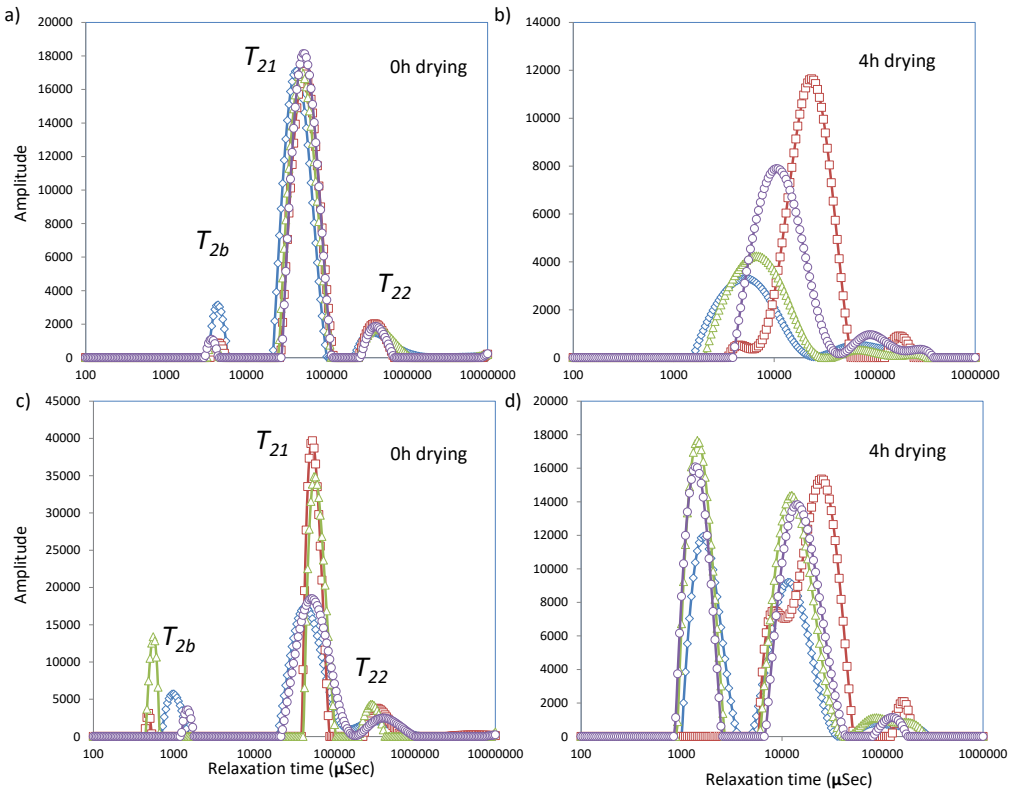


Figure 6.3. Distribution of multi exponentially fitted transverse relaxation (T_2) data for uncultured (a – b) and cultured (c – d) beef jerky slices pre-treated at various ultrasonic frequencies [Control (◊), 25 kHz (□), 33 kHz (Δ) and 45 kHz (o) respectively].

Presence of three water fractions at relaxation times and their association with muscle proteins has been previously reported (Huff-Lonergan & Lonergan, 2005; Pearce, Rosenvold, Andersen, & Hopkins, 2011). Ultrasound pre-treatment showed a shift in peaks for uncultured samples compared to cultured samples after 18 h of marination or 0 h drying (Figure 6.3a&b). In the case of cultured control samples, a higher level of bound water fraction was observed with a decrease in ultrasound pre-treated (Figure 6.3a), whereas, a shift in peaks were observed in the case of uncultured samples (Figure 6.3b). In this study, the largest fraction of water present in beef jerky samples was observed at T_{21} for cultured (in the range of 84.74–78.87%) and uncultured (90.51 to 66.47%) samples after 18 h of marination, whereas, during drying at 60^{o}C, the proportion of water obtained at T_{21} was found to decrease with an increase in water proportion at T_{2b}. An increase in water fraction at T_{21} indicates an increase in the

number of protons in the intra-myofibrillar space. Whereas, an increased water fraction at T_{22} population indicates a similar rise in number of protons, thereby representing an increase in the extra myofibrillar water population (Pearce, Rosenvold, Andersen, & Hopkins, 2011). An increase in the proportion of water at T_{2b} suggests a reduction in myofibrillar moisture and an increase in the bound water fraction obtained at T_{2b} due to the removal of myofibril and free-moisture during drying. Similar increases in the bound water fraction, indicating moisture mobility, was reported for beef granules during drying within a temperature range of 40–60^{o}C (X. Li, Ma, Tao, Kong, & Li, 2012). Analysis of variance showed that culture and drying time were the significant factors for all three relaxation times, whereas, ultrasound frequency was a significant factor for T_{21} ($p=0.0001$), T_{22} ($p=0.0010$) and an insignificant factor for T_{2b}. Interaction effects of drying time with culture and ultrasound frequency were significant for relaxation time and water proportion. Similar, changes for water population at T_{21} and T_{22} relaxation times were also reported for ultrasound-assisted brining of pork samples in a study which concluded that a reduction in the T_{21} population and an increase in the T_{22} population may be due to increased salt intake and a change in physical properties of meat during the curing process (Ojha, Keenan, Bright, Kerry, & Tiwari, 2016). The increased intake of curing solution owing to ultrasound pre-treatment can cause an enlarged electrostatic repulsion within myofibrils, thereby resulting in water mobility and osmotic dehydration (Vestergaard, Andersen, & Adler-Nissen, 2007).

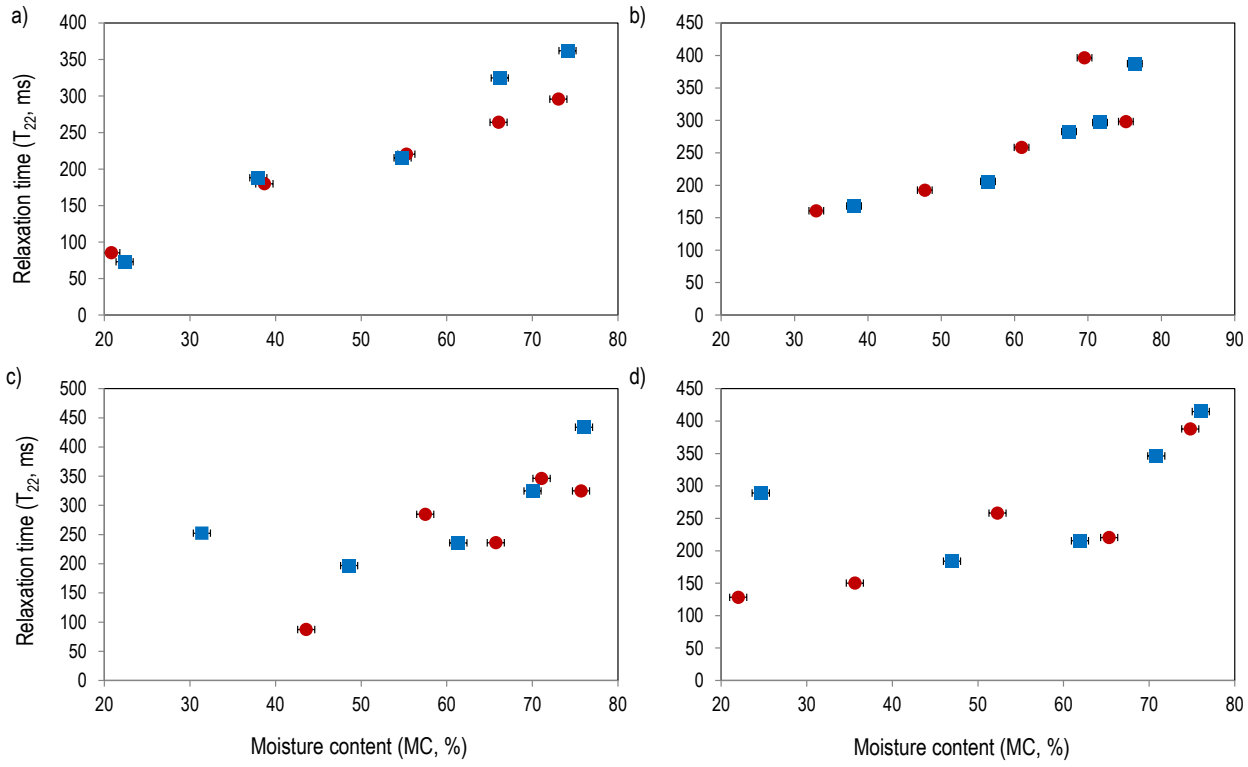


Figure 6.4. Relationship between relaxation time (T_{22}) and moisture content of beef samples during drying of cultured (●) and uncultured (●) control (a) and ultrasound pre-treated beef jerky samples at 25 kHz (b), 33 kHz (c) and 45 kHz (d).

A plot of moisture content (MC, %) and T_{22} relaxation time (free-water) indicated that a change in relaxation time is related to the MC of cultured and uncultured beef jerky samples (Figure 6.4). Similarly, (2014) showed a relationship between T_{21} and T_{22} with water holding capacity of tofu. Hence, moisture population data obtained from NMR can be used for indirect prediction of key moisture related measurements. In this study, a strong positive correlation was observed between MC and T_{22} ($r=0.790$, $P<0.0001$) and proportion of water at T_{22} (P_2) ($r=0.709$, $P<0.0001$) indicating that the MC of beef jerky samples is mainly associated with free-water. Correlation analysis also showed a strong positive relationship between drying time (h) and various water fractions and relaxation times (Table 6.2), with the exception of

T_{2b}, whereas, a significant negative relationship was observed between water fraction associated with T_{2b}. This is probably due to a shift in relaxation time during the drying process.

Table 6.2. Correlation analysis showing a relationship between various parameters

	Time (h)	P_0	P_1	P_2	T_{2b}	T_{21}	T_{22}	MC (%)
Time (h)	1.000	0.507^{***}	-0.437^{**}	-0.762^{****}	0.206^{ns}	-0.400^{*}	-0.822^{****}	-0.929^{****}
P_0		1.000	-0.994^{****}	-0.468^{**}	0.144^{ns}	0.282^{ns}	-0.305^{ns}	-0.615^{****}
P_1			1.000	0.366^{*}	-0.136^{ns}	-0.323^{*}	0.249^{ns}	0.557^{***}
P_2				1.000	-0.123	0.205^{ns}	0.565^{**}	0.709^{****}
T_{2b}					1.000	0.386^{ns}	0.214^{ns}	-0.205^{ns}
T_{21}						1.000	0.702^{****}	0.340^{*}
T_{22}							1.000	0.790^{****}
MC (%)								1.000

ns:Not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.001

6.3.4. Changes in protein, proteolysis index and amino acid profile

The true protein contents for uncultured samples ranged from 76.07±0.15 g/100 g of dry matter to 79.07±0.96 g/100 g of dry matter and 75.49±0.35 g/100 g to 76.03±0.12 g/100 g of dry matter for cultured beef jerky samples, respectively (Figure 6.5). From comparison, it can be seen that the cultured jerky has significantly less true protein than the uncultured jerky samples. No significant difference (P>0.05) was observed between true protein content of control cultured and uncultured samples whereas significant differences (P<0.05) were observed for US pre-treated samples. The highest true protein content was observed for samples processed at 25 kHz for uncultured samples (79.89±0.95 g/100 g of dry matter) and the lowest value was observed for beef jerky manufactured with 45 kHz (74.19±0.53 g/100 g of dry matter).

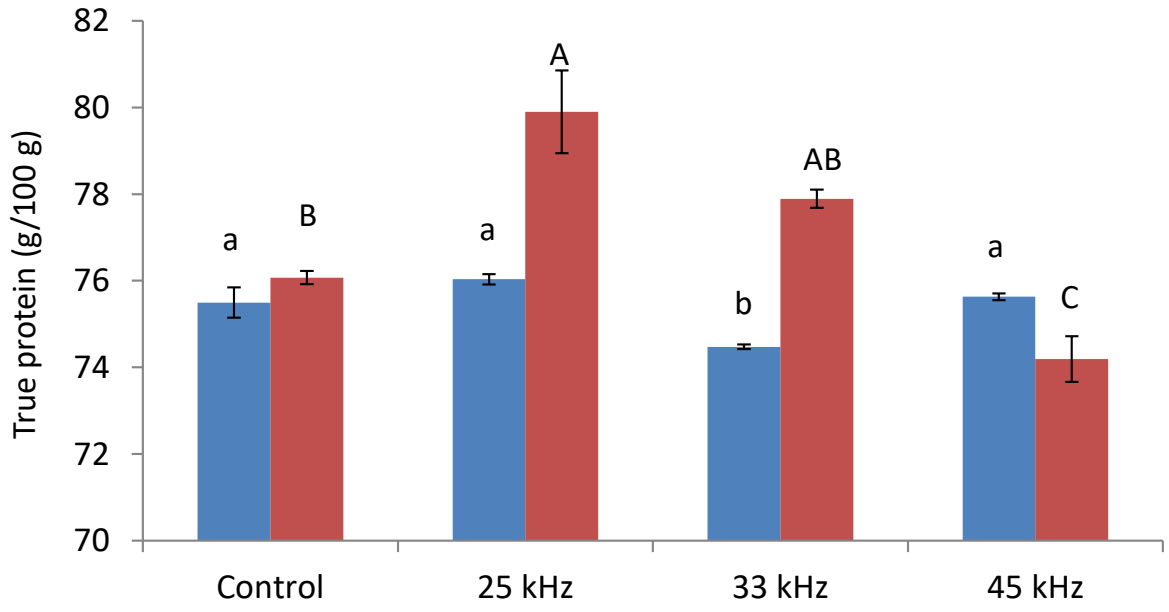


Figure 6.5. Changes in protein content (g/100g) for cultured ( ) and uncultured ( ) beef jerky samples.

Beef jerky samples pre-treated at US frequencies of 25 kHz, 33 kHz and 45 kHz showed low level of proteolysis after 18 h of marination compared to dried beef jerky samples. The addition of *L. sakei* coupled with US would have hydrolysed beef proteins, thus yielding short chain polypeptides or free amino acids. Total amino acid content of control samples for cultured and uncultured samples was 64.25 g/100 g and 63.75 g/100 g, respectively with highest observed for cultured samples pre-treated with 25 kHz (67.96 g/100 g) and lowest for cultured samples pre-treated at 45 kHz.

In the case of free amino acids, no significant differences were observed for control cultured (27.89 mg/ g) and uncultured (28.98 mg/ g) samples. US pre-treated samples showed higher level of free amino acid at 25 kHz for uncultured (41.48 mg/ g) samples and decreased with an increase in US frequencies. This indicates that US pre-treatment had significant effect during the marination and subsequent drying process on protein hydrolysis. It appears that

the breakdown of beef proteins occurred due to ultrasound as well as the culture treatment. Effect of US on proteolysis has already been reported. For instance, Abadía-García, Castaño-Tostado, Ozimek, Romero-Gómez, Ozuna, and Amaya-Llano (2016) observed that the degree of whey protein hydrolysis was dependent on ultrasonic pre-treatment and enzymes. Similarly, Uluko, Li, Cui, Zhang, Liu, Chen, et al. (2013) observed US pre-treatment can enhance hydrolysis of milk protein concentrates to produce short chain peptides with various biological activities.

US pre-treatment may have caused loss of protein structure for various natural enzymes present in beef slices required for hydrolysis. Studies have shown that the protein structure can be altered after US processing due to partial cleavage of intermolecular hydrophobic interactions, rather than peptide or disulphide bonds (Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014).

On the other hand, for cultured samples, it was not possible to establish a trend and the behaviour differed according the amino acid data and the US frequency. In fact, PCA was able to explain up to 71% of data variability using only two factors but no clear distinction between US frequencies was observed (Figure 6.6). The amounts of cystic acid, methionine sulfone, aspartic acid, threonine, serine, histidine, lysine, and arginine were higher in control (untreated) sample, while US frequency of 25 kHz gave the highest values for cysteine, 33 kHz for glycine and proline and 45 kHz for glutamic acid, alanine, valine, and phenylalanine. Using PCA, a separation of uncultured and cultured samples was basically based on the content of threonine, γ-aminobutyric acid (GABA), and aspartate.

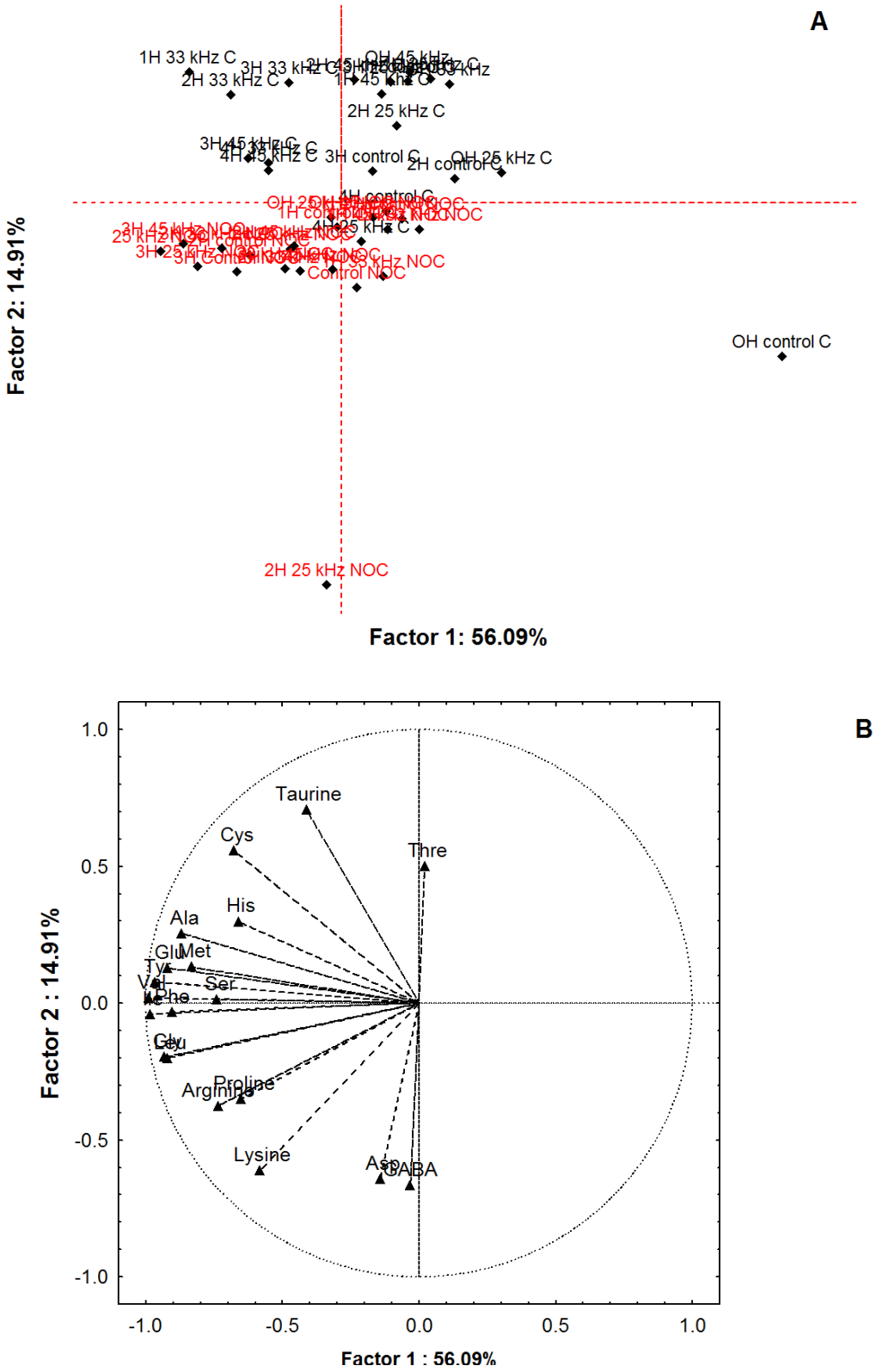


Figure 6.6. Principal component analysis (PCA) based on the amino acid profile of beef jerky subjected to ultrasound frequency (25, 33 and 45 kHz). NOC: Uncultured. 1 h, 2 h, and 3 h drying after 18 h marination.

Interestingly, taurine (2-aminoethanesulfonic acid) contents of control beef jerky samples for cultured and uncultured samples were 1.09±0.14 and 1.05±0.16 mg/ g, respectively. However, taurine content was found to be higher for US pre-treated samples compared to control for both cultured and uncultured samples (Figure 3a). Taurine is a sulphur-containing β amino acid which is reported to be abundantly present in free form in foods of animal origin. Higher level of taurine in US pre-treated samples indicates the US pre-treatment would have released bound taurine in jerky samples (Figure 6.7). Various biological and health benefits (eg. antioxidant, protection against ischemia-reperfusion injury, modulation of intracellular calcium concentration, antiatherogenic and blood pressure-lowering effects) of taurine have been reported in literature (Purchas, Rutherfurd, Pearce, Vather, & Wilkinson, 2004; Xu, Arneja, Tappia, & Dhalla, 2008; Zulli, 2011). Taurine is also reported to have a protective role in exercise induced muscle and is one of the key ingredients in sports nutrition (McPherson & Hardy, 2011).

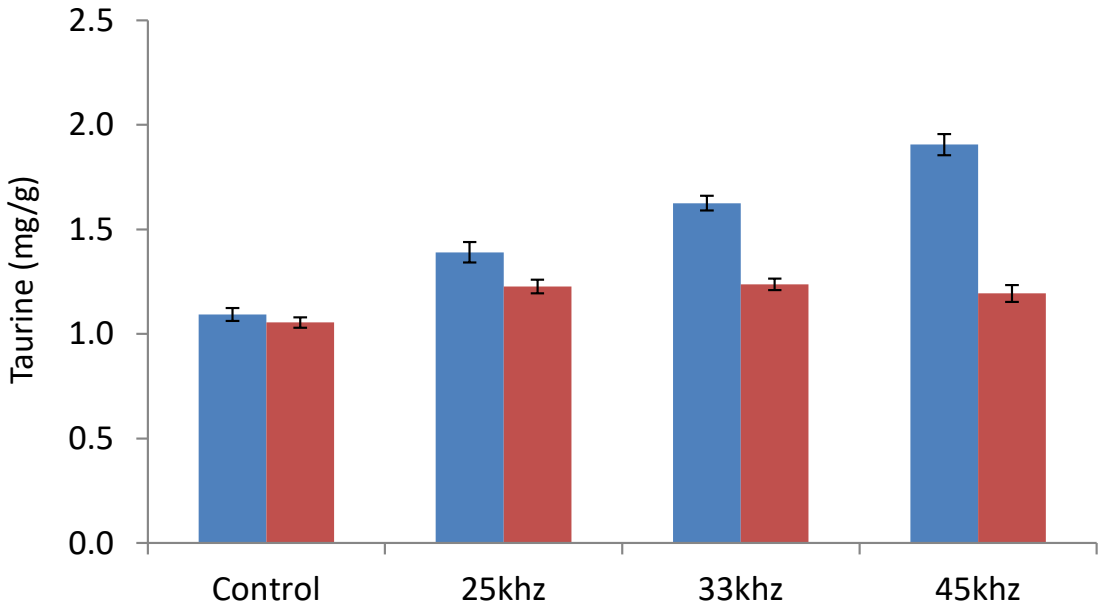


Figure 6.7. Taurine content (mg/g) for cultured ( ) and uncultured ( ) beef jerky samples.

Changes in true protein content, proteolysis index and amino acid profile can be attributed to proteolysis induced when US pre-treatment is applied (Gambuteanu & Alexe, 2013; Jayasooriya, Torley, D'arcy, & Bhandari, 2007; Stadnik & Dolatowski, 2011). It is also reported that the US treatments can lead to a significant decrease in molecular weight and protein fractionation (Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014). A higher taurine level in US pre-treated samples could be attributed to US-assisted extraction of taurine due to various sonochemical mechanisms. For instance, US have shown improved extraction yields of taurine from *Porphyra yezoensis* (Wang, Guo, Zhang, Wu, Wu, & Chen, 2015). Studies have shown that the US pre-treatment on hydrolysis of proteins using enzymes are mostly inconsistent. For example, US pre-treatment is shown to enhance the release of peptides from defatted wheat germ protein with no changes in degree of hydrolysis at various ultrasound power intensities and US frequencies range of 24–68 kHz (Stefanović, Jovanović, Grbavčić, Šekuljica, Manojlović, Bugarski, et al., 2014; Zhou, Ma, Yu, Liu, Yagoub, & Pan, 2013).

6.3.5. Influence of US frequency and drying time on organic acid composition of cultured and uncultured beef jerky samples

Changes in lactic acid and acetic acid content of jerky samples are shown in Figure 6.8. ANOVA analysis revealed a significant influence (P<0.05) of US frequency, drying time and *L. sakei* on organic acid composition. As can be seen in Figure 6.8, US frequency had a significant remarkable influence independent of *L. sakei* culture inclusion after 18 h of marination (0 h drying), observing a significant decrease when higher US frequencies of 33 and 45 kHz were applied. After 4 h drying, a decrease in lactic acid of US pre-treated cultured samples compared to control samples was observed. However, the trend was not clear for uncultured samples, obtaining a decrease of 25.6% after US application at 33 kHz while an increase of 9.2% was found at 45 kHz compared to control samples.

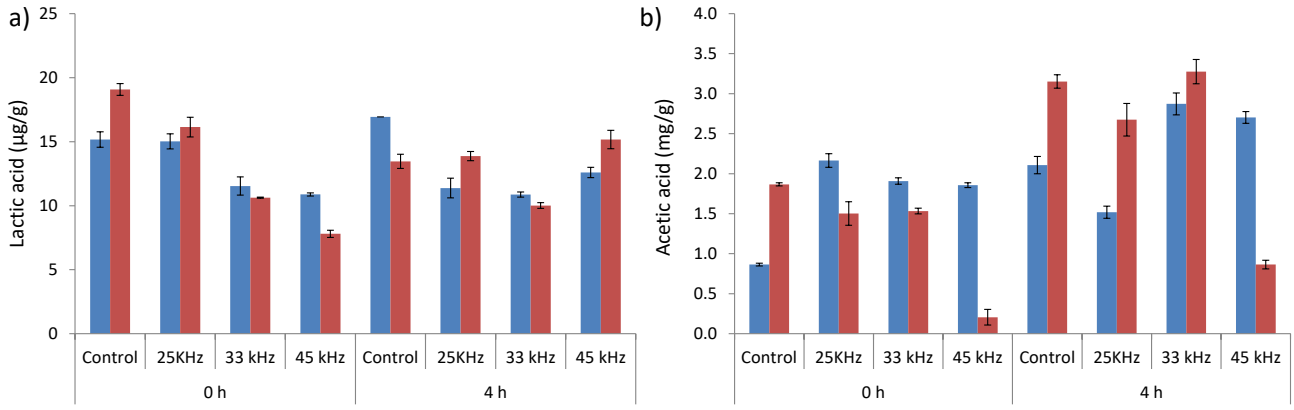


Figure 6.8. a) Lactic acid (µg/g) and (b) acetic acid (mg/g for cultured ( ) and uncultured ( ) beef jerky samples.

An increase in acetic acid content was observed after 18 h of marination for cultured samples subjected to US frequencies of 25, 33 and 45 kHz whereas, a decrease in acetic acid content was observed with an increase in US frequencies for uncultured samples. After 4 h of drying a significant increase of 36.3 and 28.2% in acetic acid content was observed for cultured samples pre-treated at 33 kHz and 45 kHz respectively. However, for uncultured samples a significant decrease of 72.5% was observed for samples pre-treated at 45 kHz. PCA showed no clear differences in acidity for samples inoculated with or without *L. sakei*. This result can be observed as samples were mixed in all quadrants in the projection presented in Figure 6.9. However, a trend was observed, cultured samples submitted to US frequency (33 and 45 kHz) showed higher acetic acid content, while uncultured samples showed a higher lactic acid content for samples pre-treated at 25 and 45 kHz.

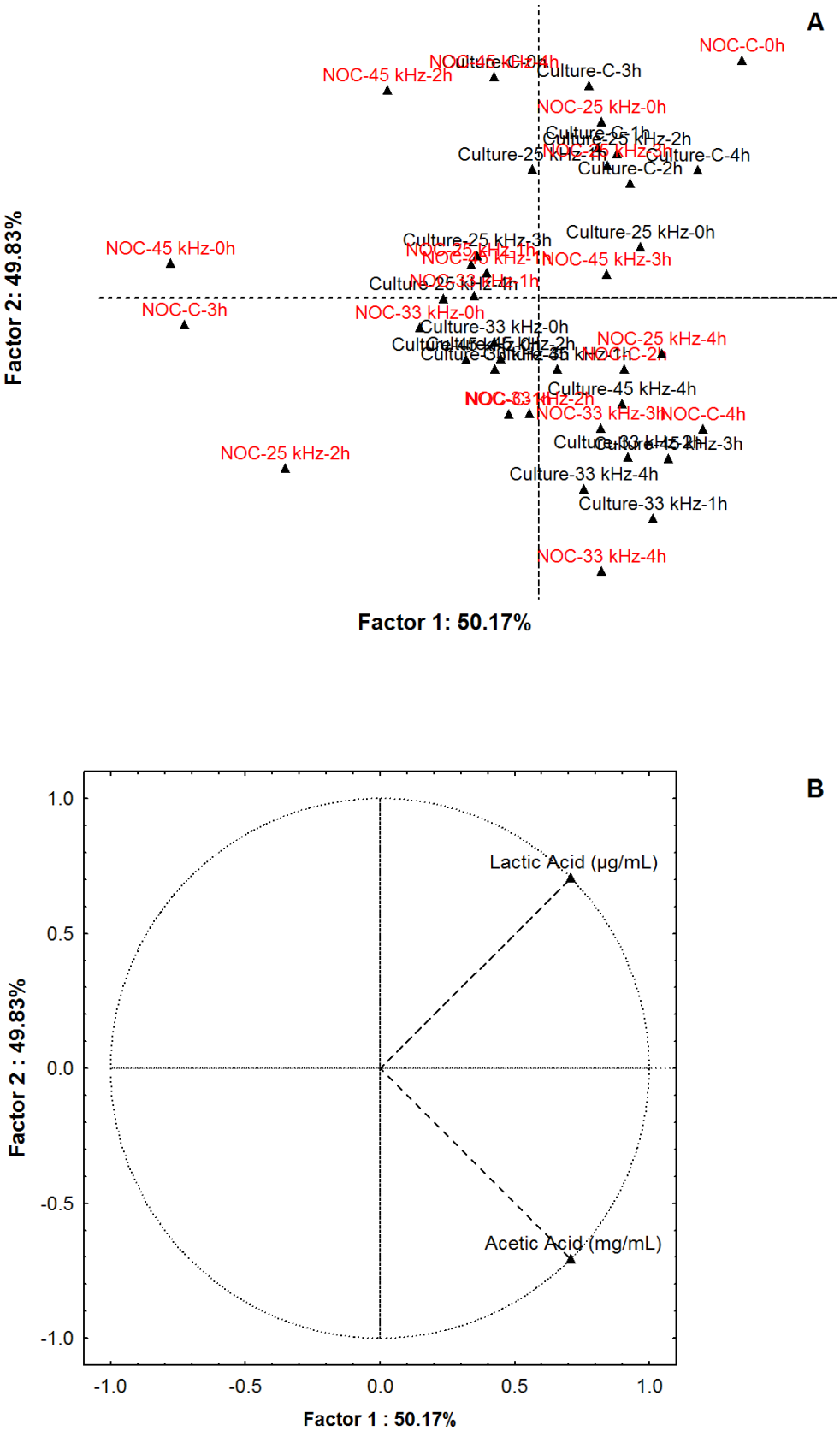


Figure 6.9. PCA based on the acetic and lactic acid profile of jerky beef subjected to ultrasound frequency.

6.3.6. Fatty acid profiling

Thirty-one fatty acids were identified and quantified in beef jerky samples. A representative chromatogram of beef jerky sample is shown in Figure 6.10. Medium and long chain fatty acids were identified and quantified at various drying times (0, 1, 2, 3 and 4 h) for cultured and uncultured beef jerky samples with or without ultrasound pre-treatment are not presented. In general, fatty acids in beef vary depending on the genotype, muscle type and feeding regime (Mapiye, Aalhus, Turner, Rolland, Basarab, Baron, et al., 2013; Scollan, Dannenberger, Nuernberg, Richardson, MacKintosh, Hocquette, et al., 2014). In the present study, beef slices had a high level of MUFAs, accounting for 45.6 – 53.8%, followed by the SFAs (36.3 – 47.8%) and PUFAs (4.8 – 13.7%). Similar fatty acid profiles of beef jerky samples were reported by Yang, Hwang, Joo, and Park (2009); these contained 42.9% SFAs, 53.2% MUFAs and 3.8% PUFAs. Among PUFAs, linoleic acid (C18:2*n*–6), α-linolenic acid (C18:3*n*–3), Arachidonic acid (C20:4*n*–6), Eicosapentaenoic acid (C20:5*n*–3) and Docosapentaenoic acid (C22:5*n*–3) were the main fatty acids identified and quantified in beef jerky samples. Presence of these long chain *n*–3 and *n*–6 PUFAs has been reported to confer various health benefits including improvement of maternal and offspring health, growth and development, cognitive function and psychological status (Mapiye, Vahmani, Mlambo, Muchenje, Dzama, Hoffman, et al., 2015; Pelliccia, Marazzi, Greco, Franzoni, Speziale, & Gaudio, 2013).

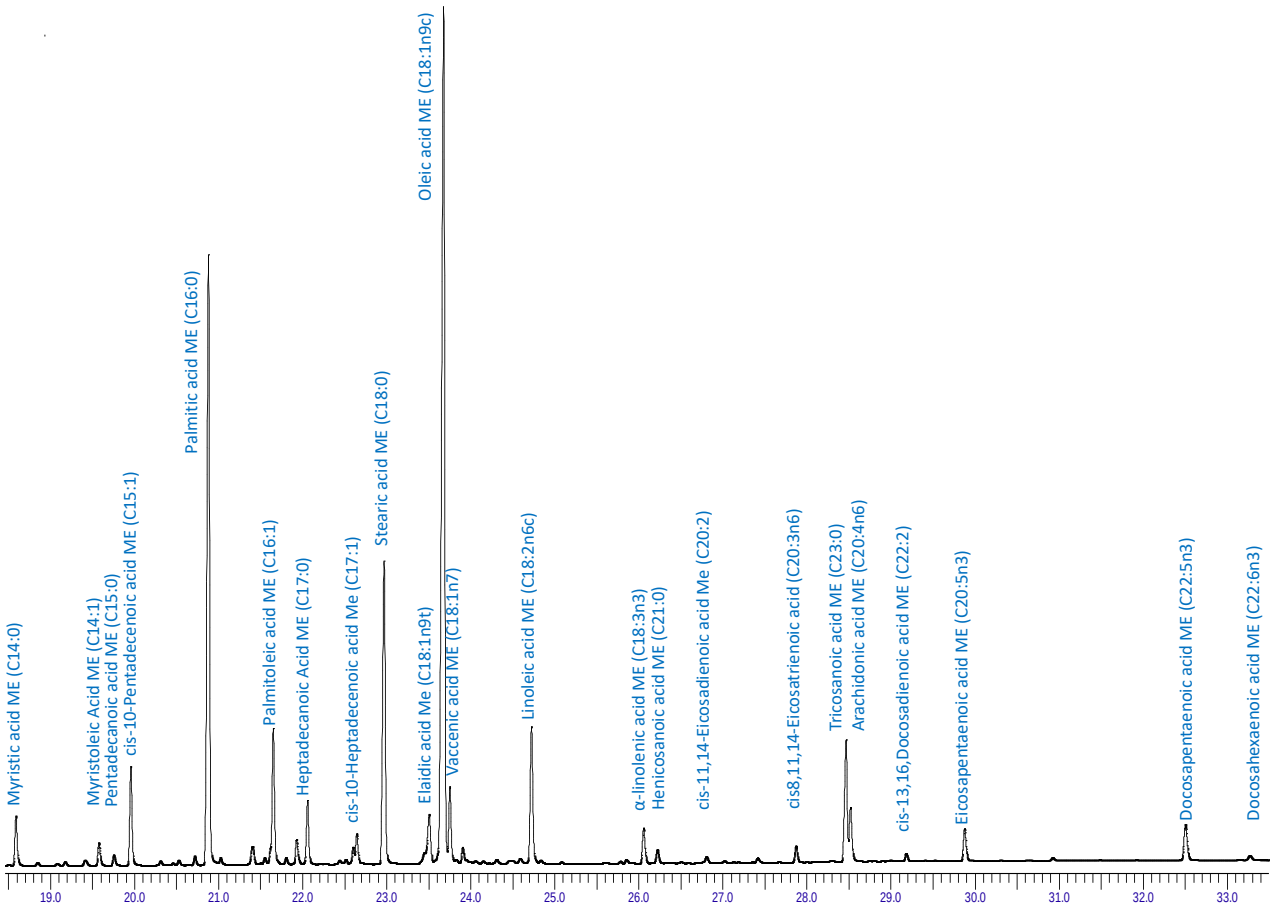


Figure 6.10. A representative chromatogram of fatty acid profile of beef jerky sample

6.3.7. Effect of culture of fatty acid profile

In this section, controls from both groups (cultured and uncultured) are compared to investigate the effect of culture on fatty acid profile of marinated beef slices. The proportion ∑SFA and ∑MUFA were significantly higher for cultured samples (42.98 g/100g and 50.41 g/100g of FA) compared to uncultured samples (40.81 g/100g and 46.85 g/ 100 g of FA) respectively. Whereas, ∑PUFA was higher for uncultured (12.23 g/100 g FA) compared to cultured samples (6.57 g/100g FA). Among the 31 fatty acids identified and quantified Oleic acid (C18:1), Palmitic acid (C16:0), Stearic acid (C18:0), Palmitoleic acid (C16:1) were the most abundant for both cultured and uncultured beef jerky slices. Other authors have also reported that palmitic acid, stearic acid and oleic acid are the predominant fatty acids in beef jerky

samples (Kim, Lee, Choi, & Kim, 2014). Culturing of the samples would have caused a higher extent of oxidation resulting in higher losses of PUFA's as these are more susceptible to oxidation. Studies show that the bacteria accelerate the oxidation of UFA's (Ansorena & Astiasarán, 2004; Chizzolini, Novelli, & Zanardi, 1998). Higher degradation of ∑PUFA for cultured samples can be due to lipolysis of long chain fatty acids owing to the inclusion of *Lactobacillus* culture along with the action of endogenous enzymes. For example, Hierro et al (1997) investigated the role of the starter culture (*Lactobacillus*, *Staphylococcus* and *Micrococcus*) on lipolysis of dry fermented sausages and concluded that the lipolysis is not only due to microbial lipases and also due to endogenous lipases present in meat. Contrary to this, Hu, Xia, and Ge (2007) observed higher PUFA content in sausages with mixed cultures of *Staphylococcus xylosus*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactobacillus casei* compare to the control silver carp (*Hypophthalmichthys molitrix*) sausages. The effect of starter culture on fatty acid composition in a range of fermented meat products, including sausages, during fermentation, ripening and storage has been reported (Karsloğlu, Çiçek, Kolsarici, & Candoğan, 2014; Qiu, Zhao, Sun, Zhou, & Cui, 2013; Visessanguan, Benjakul, Riebroy, Yarchai, & Tapingkae, 2006). Studies show that there is a tendency to hydrolyse certain fatty acids (e.g. linoleic, oleic, and stearic acid) probably because of the action of microbial lipases, which is dependent on the position and structural conformation of the fatty acids in the glycerides (Alford, Smith, & Lilly, 1971; Gambacorta, Sinigaglia, Schena, Baiano, Lamacchia, Pati, et al., 2009).In the present study among the main fatty acid identified in samples, culture treatment had a significant effect (*P<0.0001*) on C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2*n*–6, & C18:3*n*–3 levels. Correlation analysis (Table 6.3) showed a negative relationship between culture inoculation and ∑SFA (*r=- 0.465*, *P<0.01*), ∑MUFA (*r= - 0.430,*

P<0.01) whereas, a positive correlation were observed for PUFA (*r=0.379, P<0.05*) and *n*–6 PUFA (*r=0.497, P<0.001*) indicating a positive effect of culture treatment on PUFA.

Table 6.3. Correlation matrix for fatty acid derived nutrition indices of control and ultrasound pre-treated (25 kHz, 33 kHz and 45 kHz for 30 min) beef jerky samples from uncultured and cultured groups followed by drying for 4 hours at 60^{o}C

	Culture	Drying time	US treatment	∑SFA	∑MUFA	∑PUFA	∑PUFA(*n*–3)	∑PUFA (*n*–6)	*n*–6/ *n*–3	∑MUFA/ ∑SFA	∑PUFA/ ∑SFA
Culture	1.000	0.000	0.000	-0.465**	-0.430**	0.379*	0.102^{ns}	0.497***	0.549***	0.389**	0.710****
Drying time		1.000	0.000	-0.212^{ns}	-0.155^{ns}	-0.664****	-0.620****	-0.634***	-0.161^{ns}	0.260^{ns}	-0.124^{ns}
US treatment			1.000	0.038^{ns}	-0.011^{ns}	-0.140^{ns}	-0.130^{ns}	-0.133^{ns}	-0.062^{ns}	-0.189^{ns}	-0.183^{ns}
∑SFA				1.000	0.974****	0.407**	0.548***	0.299^{ns}	-0.232^{ns}	-0.776****	-0.776****
∑MUFA					1.000	0.380*	0.536***	0.266^{ns}	-0.260^{ns}	-0.762****	-0.762****
∑PUFA						1.000	0.898****	0.972****	0.270^{ns}	0.180^{ns}	0.180^{ns}
∑PUFA(*n*–3)							1.000	0.771****	-0.169^{ns}	0.027^{ns}	0.025^{ns}
∑PUFA(*n*–6)								1.000	0.481**	0.247^{ns}	0.248^{ns}
n–6/*n*–3									1.000	0.283^{ns}	0.284^{ns}
∑MUFA/∑SFA										1.000	-0.463***
∑PUFA/∑SFA											1.000

*Significant at p≤0.05; **Significant at p≤0.01; ***Significant at p≤0.001; ****Significant at p≤0.0001; ^{ns}Not significant

6.3.8. Effect of ultrasound frequency and drying on fatty acid profile

Ultrasonic frequency (kHz) and drying time (h) had a significant impact on fatty acid profiles. In the case of cultured beef jerky samples after 18 h of marination, a significant increase in SFA (%) was observed for samples sonicated at 33 and 45 kHz compared to control whereas, uncultured samples sonicated at 33 kHz had a higher proportion of SFA compared to control (Figure 6.11).

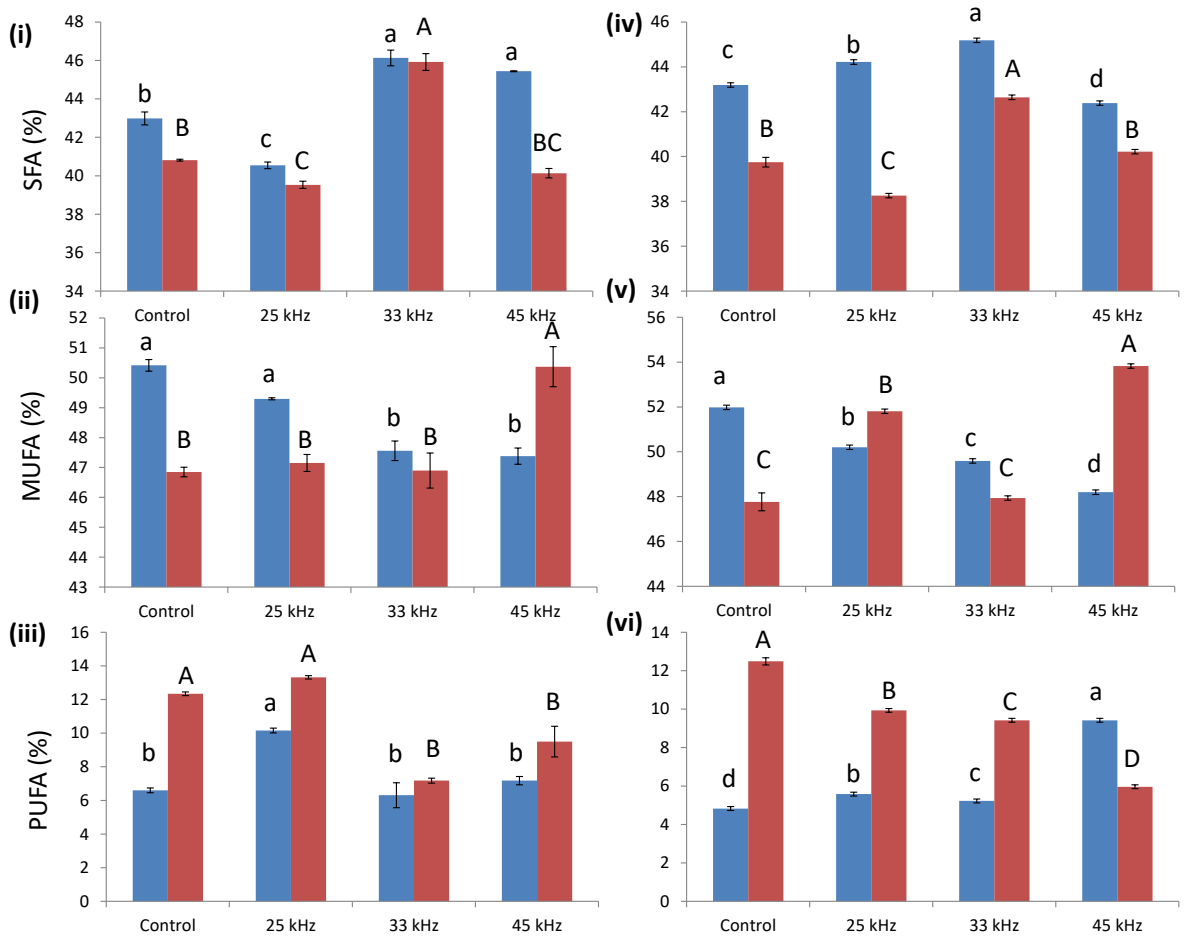


Figure 6.11. Proportion of SFA (%FA), MUFA (%FA) and PUFA (%FA) after marination (i – iii) and 4 h drying period (iv – vi) for cultured (■) and uncultured (■) beef jerky samples. [a-d, A-D; columns followed by same letters are not significantly different at $P<0.05$].

The proportion of MUFA was found to decrease significantly (P<0.05) from 52.0% (control) to 48.2% (45 kHz) with increase in ultrasonic frequency for cultured samples while the reverse was observed in the case of uncultured dried beef jerky samples. In the case of cultured beef jerky samples, a significant increase in PUFAs was observed with an increase in ultrasonic frequency with highest being observed for dried beef jerky samples pre-treated at 45 kHz (9.4%) compared to control (4.8%); a reverse trend was observed for uncultured dried beef jerky samples. ANOVA revealed that drying time was found to have a significant effect on C18:0 (*P<0.01*), C18:2*n*–6 (*P<0.0001*) and C18:3*n*–3 (*P<0.0001*) while drying time did not significantly affect other major fatty acids. A significant Interaction between drying time and culture was observed for C18:0, C18:2*n*–6 and C18:3*n*–3 only. Drying time and ultrasonic frequency interacted significantly for all major fatty acids with the exception of C16:1. Ultrasonic frequency did not affect most of the major fatty acids with the exception of C16:1 (*P<0.05)* and C18:2*n*–6 (*P<0.01*) whereas, interaction effects of ultrasonic frequency and culture was found to be significant for all major fatty acids. Correlation analysis showed a negative relationship between drying time and ∑PUFA (*r = –0.667, P<0.001*) and no significant correlations were observed between drying time and ∑SFA (*r=–0.222*) or ∑MUFA (*r=–0.155*) (Table 6.3). A significant decrease in ∑PUFA during drying of beef jerky samples could be due to oxidation of PUFAs to secondary oxidation products such as aldehydes, alcohols, ketones and furans. For example, formation of various compounds including n-alkanals, 2-alkenals, 1-alkanols, and alkylfurans are reported due to oxidation of abundant MUFA or PUFA present in beef during cooking (Elmore, Mottram, Enser, & Wood, 1999). Ultrasound has been reported to have minimal or no effect on fatty acid profiles of various foods when ultrasound is employed for extraction of oils from food matrices (Zhang, Wang, Li, Jiao, Chen, & Mao, 2008). However, some studies highlight the degradation of some fatty acids due to ultrasound

treatment to form volatile fatty acids leading to changes in flavour (Chemat, Grondin, Shum Cheong Sing, & Smadja, 2004). Moreover, cavitation induced by ultrasound will cause damage to cell membranes, which are rich in unsaturated fatty acids and therefore can contribute to fatty acid oxidation.

6.3.9. Effect of ultrasound frequency, culture and drying time on nutritional indices

Table 6.4. ANOVA of independent factors on major fatty acids of beef jerky samples

	Cultured					**Uncultured**				
Frequency	AI	TI	HH	*n*–6/ *n*–3	PUFA/SFA	AI	TI	HH	*n*–6/ *n*–3	PUFA/SFA
Control (no ultrasound)	0.642^{b}	1.187^{c}	1.615^{b}	1.398^{d}	0.112^{c}	0.536^{c}	0.913^{c}	1.808^{a}	1.882^{b}	0.314^{a}
25 kHz	0.641^{b}	1.231^{b}	1.631^{q}	1.534^{b}	0.125^{b}	0.523^{c}	0.891^{d}	1.787^{a}	1.681^{c}	0.260^{b}
33 kHz	0.681^{a}	1.296^{a}	1.546^{c}	1.614^{a}	0.116^{c}	0.604^{a}	1.121^{a}	1.654^{b}	2.255^{a}	0.221^{c}
45 kHz	0.632^{c}	1.036^{d}	1.608^{b}	1.450^{c}	0.222^{a}	0.564^{b}	1.043^{b}	1.766^{a}	1.684^{c}	0.148^{d}

The nutritional indices (AI, TI, HH, *n*–6/*n*–3 and PUFA/SFA) for dried beef jerky samples are shown in Table 6.4. Certain fatty acids have proatherogenic and antiatherogenic effects on cholesterol. Saturated fatty acids mainly C12:0, C14:0 and C16:0 are proatherogenic whereas, MUFAs and PUFAs are antiatherogenic fatty acids related by the index of atherogenicity (AI). AI relates to the risk of atherosclerosis and is considered as an indicator of the impact of fat on the cholesterol concentration. AI is based on those fatty acids which can increase (C12:0, C14:0 and C16:0) or decrease (∑MUFA, ∑PUFA) the level of cholesterol (Stajić, Živković, Perunović, Šobajić, & Vranić, 2011). The AI was found to vary from 0.63 to 0.68 in the case of cultured samples and from 0.52 to 0.60 in the case of uncultured samples. López-López, Cofrades, Cañeque, Díaz, López, and Jiménez-Colmenero (2011) reported similar AI values of 0.71 and 0.68 for raw and cooked formulated beef patties, respectively. In general, a lower AI value corroborates to the lower atherogenic potential. TI is an index of thrombogenicity

(TI) i.e. formation of clots within blood vessels. It is a ratio of prothrombogenic (C14:0, C16:0 and C18:0) and antithrombogenic fatty acids (MUFAs and PUFAs). Interestingly, C18:0 is believed to be thrombogenic but not atherogenic (Laudadio & Tufarelli, 2011). TI ranged from 1.04 to 1.30 and 0.89 to 1.12 for cultured and uncultured samples, respectively. Low values of AI and TI are preferred and indicate positive health benefits derived from the product. HH index, a ratio of hypocholesterolemic fatty acids and hypercholesterolemic fatty acids is related to the metabolism of cholesterol. Most PUFAs cause the strong hypocholesterolemic effects whereas, C12:0 and C14:0 have hypercholesterolemic effects. Higher value of HH index demonstrates low presence of cholesterol. HH index was found to vary from 1.546 to 1.615 and 1.654 to 1.808 for cultured and uncultured dried beef jerky samples, respectively. The PUFA:SFA ratio in beef jerky samples varied from 0.112 to 0.222 and 0.148 to 0.314 for cultured and uncultured samples, respectively. It has been reported that the ratio of PUFA:SFA in human diet should be >0.45(Sobczuk-Szul, Wroński, Wielgosz-Groth, Mochol, Rzemieniewski, Nogalski, et al., 2013). The ratio of *n*–6/ *n*–3 for cultured samples was generally lower (1.398 to 1. 614) compared to uncultured samples (1.681 to 2.255). These values are less than <5.0 as recommended by WHO/FAO (Migdał, Živković, & Migdał, 2009).Diets containing higher amount of *n*–6 PUFA or high *n*–6/ *n*–3 ratio have been reported to promote the pathogenesis of cancer, inflammatory and cardiovascular diseases (Simopoulos, 2002, 2008). Both PUFA:SFA and *n*–6/ *n*–3 ratio are considered as good indicators of nutritional value of dietary fat.

In order to classify the samples according to their fatty acid profile, multivariate analysis was performed on levels of individual fatty acids in beef jerky samples subjected to the various treatments under investigation. Three factors were retained based on the amount of variance explained and eigenvalues. According to factor analysis, 84.5% of total variance was explained

by three factors as shown in Table 6.5. Factor 1 explained 53.9% variation and was marked by high positive loadings for C12:0 –C21:0, C16:1, C17:1, C18:1, C20:1, C22:2. Factor 2 which explained 21.5% variation was marked by high negative loadings for C15:1, C18:2*n*–6, C20:3*n*–6, C20:4*n*–6, C20:5*n*–3, C22:5*n*–3 and C22:6*n*–3. Finally, factor 3 explained 8.0% variation, was marked with high positive loadings for C20:2 and C22:5*n*–3 and high negative loadings for C21:0 and C22:0 respectively. Factor 1 can be interpreted as the presence of higher level of saturated fats in beef jerky samples. The score plot of Factor 1 and 2 explaining total variability of 76.3% is shown in Figure 6.12. Figure 6.12 showed the position of cultured and uncultured beef jerky samples. The clear separation between samples is visible with cultured samples appearing in the upper part of the graph while samples without culture appearing in the bottom quadrant with negative factor values.

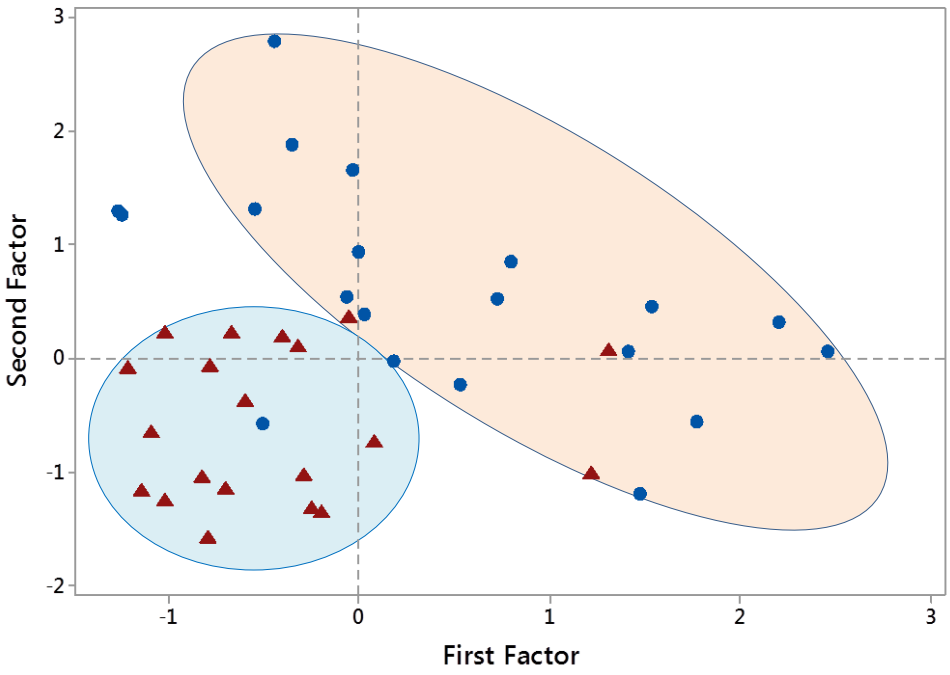


Figure 6.12**.** Score plot for Factor 1 and 2 for beef jerky cultured samples (●) and uncultured (▲) samples.

Table 6.5. Eigenvalues and varimax rotated factor loadings (Eigenvectors) for the fatty acid profile of beef jerky samples

	Factor 1	Factor 2	Factor 3
Eigenvalue	13.873	5.583	2.085
Variance explained (%)	53.4	21.5	8.0
Eigenvectors (↓)			
Lauric Acid (C12:0)	0.812	0.094	-0.389
Myristic acid (C14:0)	0.985	0.106	-0.026
Myristoleic Acid (C14:1)	0.855	0.198	0.242
Pentadecanoic acid (C15:0)	0.984	0.075	-0.058
cis-10-Pentadecenoic acid (C15:1)	-0.093	-0.725	0.475
Palmitic acid (C16:0)	0.995	0.025	0.011
Palmitoleic acid (C16:1)	0.869	0.034	0.384
Heptadecanoic Acid (C17:0)	0.972	-0.038	-0.07
cis-10-Heptadecenoic acid (C17:1)	0.941	-0.103	0.205
Stearic acid (C18:0)	0.953	-0.066	-0.153
Elaidic acid (C18:1*n*–9t)	0.831	-0.098	-0.031
Oleic acid (C18:1 *n*–9c)	0.991	-0.004	0.095
Vaccenic acid (C18:1*n*–7t)	0.820	-0.108	0.33
Linoleic acid (C18:2 *n*–6c)	0.330	-0.787	-0.082
Arachidic Acid (C20:0)	0.919	-0.069	-0.193
cis-11-Eicosenoic acid (C20:1 *n*–9)	0.960	-0.03	0.136
alpha-linolenic acid (C18:3 *n*–3)	0.912	-0.154	-0.222
Henicosanoic acid (C21:0)	0.744	-0.087	0.548
cis-11,14-Eicosadienoic acid (C20:2)	-0.001	-0.367	-0.56
Behenic acid (C22:0)	0.093	-0.356	0.427
cis8,11,14-Eicosatrienoic acid (C20:3 *n*–6)	0.033	-0.952	-0.185
Arachidonic acid (C20:4 *n*–6)	-0.034	-0.960	-0.026
cis-13,16,Docosadienoic acid (C22:2)	0.637	-0.355	-0.514
Eicosapentaenoic acid (C20:5 *n*–3)	-0.018	-0.905	-0.268
Docosapentaenoic acid (C22:5 *n*–3)	0.035	-0.653	0.172
Docosahexaenoic acid (C22:6 *n*–3)	-0.163	-0.915	-0.012

6.3.10. Effect of ultrasound frequency on colour and texture of beef jerky

Changes in firmness and toughness values of cultured and uncultured beef jerky samples are shown in Figure 6.13. Overall, cultured samples showed higher firmness and toughness values compared to uncultured samples. However, in contrast to the results found in this study, Meng, Long-Hao, Hong-Sheng, Yan-Qing, & Xin (2013) found an increase in tenderness after beef jerky fermentation compared with uncultured samples.

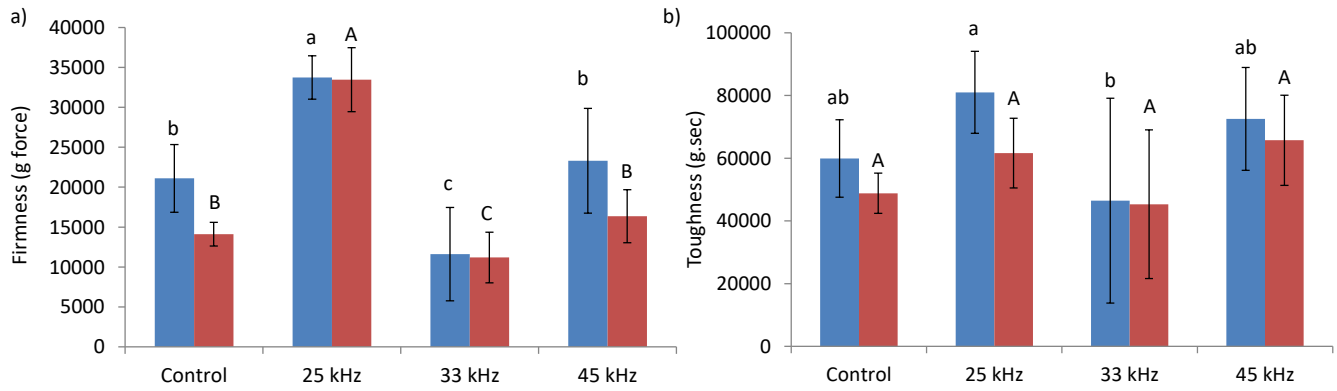


Figure 6.13. Changes in firmness (a) and toughness values for cultured ( ) and uncultured ( ) beef jerky samples.

Moreover, in the present study, significantly lower firmness and toughness values were observed for samples pre-treated at 33 kHz for both cultured and uncultured samples. Higher values of firmness values for cultured beef jerky samples pre-treated at 25 kHz can be attributed to decreased migration of marination solution into the beef jerky samples due to sonication, thus reducing hydration and swelling of myofibrils and increasing water holding capacity. This fact has a negative effect on tenderisation and juiciness of meat, thus resulting in firm jerky samples (Gault, 1985; Goli, Bohuon, Ricci, Trystram, & Collignan, 2011; Offer et al., 1989; Offer & Knight, 1988). Low power US frequency has shown enhanced diffusion of marination solution into the meat matrix. For example, Leal-Ramos, Alarcon-Rojo, Mason, Paniwnyk, and Alarjah (2011) reported improved marination rates for US-assisted marination of chicken breasts. Enhanced marination is reported to induce tenderisation due to several mechanisms including weakening of structures, due to swelling of meat, increased proteolysis by cathepsins (Berge, Ertbjerg, Larsen, Astruc, Vignon, & Møller, 2001). Higher firmness and

toughness may be due to uptake of higher level of marination solution resulting in firm texture in dried jerky samples.

Table 6.6. Changes in colour values for cultured and uncultured beef jerky samples.

	Treatment	*l*	*a*	*b*	TCD
Cultured	Control	24.5±0.7^{ab}	5.9±1.0^{a}	3.5±0.5^{ab}	0.0±0.0^{c}
	25 kHz	24.4±1.7^{ab}	4.7±0.9^{a}	3.9±0.8^{ab}	2.2±1.2^{bc}
	33 kHz	25.7±1.7^{ab}	6.4±1.8^{a}	4.7±2.4^{ab}	2.4±2.4^{bc}
	45 kHz	23.1±0.8^{b}	4.4±0.9^{a}	2.7±0.8^{b}	2.9±1.2^{bc}
Uncultured	Control	26.5±3.1^{ab}	5.6±0.8^{a}	5.1±1.6^{ab}	0.0±0.0^{c}
	25 kHz	27.7±3.1^{a}	6.1±1.3^{a}	6.4±1.9^{a}	3.3±1.8^{abc}
	33 kHz	24.3±1.9^{ab}	4.0±0.8^{a}	2.9±1.6^{b}	6.7±2.1^{a}
	45 kHz	25.9±2.9^{ab}	5.0±1.8^{a}	4.5±2.1^{ab}	4.9±2.7^{ab}

Hunter colour values of cultured and uncultured beef jerky samples subjected to US pre-treatments are shown in Table 6.6. As can be seen from the Table, there was not a clear trend in *L**, *a** and *b** values after applying US treatments. Overall, non-significant changes in *L**, a* and *b** values were found for cultured and uncultured samples, regardless of the US treatments. The results contrast with those obtained by Meng, Long-Hao, Hong-Sheng, Yan-Qing, & Xin (2013) who observed a significant increase in *a** and *b** values of fermented beef jerky samples compared to uncultured. However, it should be noted that fermentation conditions (i.e., fermentation time) were different from those reported in the current study. In addition, higher total colour differences were found for uncultured samples after applying US treatments compared to the US-treated cultured samples.

6.4. Conclusions

This study demonstrates that ultrasound pre-treatment has significant effect on drying behaviour and moisture mobility of cultured and uncultured beef jerky samples. However, improvement in drying rates for both cultured and uncultured samples was not evident from

the drying models generated. Significant increases in moisture gain after ultrasonic pre-treatment promoted brine uptake due to the combined effect of cavitation and concentration gradient phenomena. Among several drying models tested to predict the drying behaviour of beef jerky samples, the Wang and Singh drying model was found to be the best model as demonstrated by high R^2, low RMSE and AICc values. LF-NMR results showed moisture mobility during drying process with strong correlation with MC of jerky samples. LF-NMR can be employed to elucidate changes in water distribution and moisture content of beef jerky samples.

Significant effects on true protein content, amino acid profile, organic acid contents and fatty acid profiles were observed with no significant changes in colour values. The result presented in this study showed that beef jerky samples with improved amino acid profile including taurine content can be obtained by employing US pre-treatment. Interactive effect of US frequencies and culture treatment was evident. However, the individual effects of *L. sakei* and US pre-treatment at various frequencies cannot be established based on this study. In addition, principal component analysis was shown to be a promising statistical approach to monitor the quality traits of beef jerky.

Chapter 7. Conclusions and Recommendations

The responsibility to produce high quality, sustainable and cost-effective meat products rests with producers, manufacturers, distributors and retailers to ensure consumer demands are met. Dealing with such a perishable product in a dynamic market place, it is not surprising that new and innovative technologies and ingredients are constantly being developed and applied within the muscle-food processing sector in an attempt to improve health image, enhance certain meat quality attributes and extend shelf-life. With increasing competition, negative health image of meat and tighter cost margins, the meat industry has shown willingness to engage in seeking novel innovative ways of processing meat, while maintaining quality and safety attributes.

On the other hand, the healthy image of meat, more specifically processed meat products, gradually became eroded during the 1980's, when the lipid hypothesis focused attention on the fat contributed from meat (Kerry, Kerry, & Ledward, 2002) and through campaigns driven by various national and international agencies for reducing sodium salt consumption. For example, the WHO are driving measures to reduce worldwide populations daily intake of salt and saturated fat by raising consumers' awareness (WHO, 2003, 2012). Moreover, in Ireland, daily salt intakes are estimated to be 11.1 g for men and 8.5 g for women, and a reduction in salt intakes to 6 g/day was recommended by the Irish Food Safety Authority of Ireland (FSAI) in 2011 (Trieu, Neal, Hawkes, Dunford, Campbell, Rodriguez-Fernandez, et al., 2015). Considering these multiple challenges faced by the meat industry, an attempt was made in this thesis to investigate the application of novel technologies and novel functional ingredients to develop functional meat products.

Research on the application of novel processing technologies is ongoing around the world with numerous potential meat industry applications. Even though emerging technologies have demonstrated numerous advantages and potential applications for the food industry, there are limited commercial applications due to several factors. In some instances, industry uptake of new technologies is stifled by a lack of knowledge about these new technologies and their impact on product quality. Among several novel technologies, power ultrasound is an emerging technology which can assist in development of functional meat product because ultrasonics can be applied to induce gentle but targeted processes to improve the quality of processed foods and offer the potential for improving existing processes as well as for developing new processing techniques. Research on ultrasound applications in meat was restricted to certain meat processing applications as shown in comprehensive review presented in Chapter 1 with no application focusing on ultrasound technology for meat products manufactured using starter cultures. Further, limited efforts have been made to investigate potential application of ultrasound technology for developing functional meat products.

A two-pronged strategies were adopted, first, to advance the application of ultrasound and secondly, to investigate the ultrasound interaction with new ingredients for development of functional meat products. In first study, the efficacy of high intensity ultrasound technology on fermentation profile of starter culture was investigated. The study observed that both stimulation and retardation of *L. sakei* is possible, depending on the ultrasonic power and sonication time employed (Chapter 2). Study showed a higher SGR and a shorter lag phase at low power (2.99 W for 5 min) compared to control. Conversely, a decrease in SGR with an increase in lag phase was observed with an increase in ultrasonic power level. Cell-free extracts obtained after 24 h fermentation period using meat model system and meat extract

showed inhibition against pathogenic microorganisms (*Staphylcoccus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*) as evidenced by lower MIC values for ultrasonic-treated samples compared to untreated control. Conclusions drawn from this study on growth modulation of *L. sakei* can be extended to other probiotics and starter cultures employed in food fermentation. Additionally, cell-free extracts obtained could be further exploited for food bio-preservation purposes. It was hypothesised that ultrasound may have some physiological or morphological effect on *L. sakei's* response to ultrasound processing conditions. The majority of ultrasound applications and the mechanism of ultrasound have been investigated from a microbial inactivation point of view, limited efforts have focussed on improving microbiological activity, especially for beneficial microorganisms. Therefore, a further development in this area was required to comprehend a deeper understanding on the fundamental molecular mechanism influencing the behaviour of microorganisms subjected to ultrasonic waves. A study was carried out at a range of ultrasonic frequencies of 20, 45, 130 and 950 kHz on growth behaviour, morphological changes and metabolic responses of *L. sakei* (Chapter 3). Growth rates of *L. sakei* were frequency dependent, while scanning electron microscopy showed that ultrasound caused significant changes on the cell surface of *L. sakei* culture with the formation of pores. Phenotypic microarrays showed that all ultrasound treated *L. sakei* after exposure to various carbon, nitrogen, phosphorus and sulphur sources had significant variations in nutrient utilisation. Integration of this phenotypic data with the genome of *L. sakei* revealed that various metabolic pathways were being influenced by the ultrasound treatments. Phenotypic variations identified as a result of ultrasound treatment and links associated with the various metabolic pathways demonstrates the relevance of PM technology in providing in-depth analysis of *L. sakei* growth which may be useful in understanding the influence of processing

technologies on microorganisms. Chapters 2 & 3 provided a greater insight to understand effect of ultrasound on bacteria and demonstrated that the combined genomic-phenomic approach can be adopted to understand the key underlying mechanism and adaptive response of bacteria to emerging technologies. Understanding phenotypic variations and differences in nutrient utilisation via PM technique will also enable tailoring the food processes for specialised applications of probiotic strains.

It was anticipated that the effect observed on *L. sakei* in model system (Chapters 2 & 3) could be replicated in a realistic meat system. Based on this hypothesis, a comprehensive study (Chapter 6) was designed to investigate the effect of ultrasound frequency and *L. sakei* culture on various physico-chemical and nutritional properties of beef jerky. This study also investigated if there was any synergistic effect of using ultrasound in combination with *L. sakei* on drying kinetics; changes in water distribution using Low Frequency-Nuclear Magnetic Resonance (LF-NMR) and on key nutritional (fatty acid profile, protein, amino acids, and organic acids) and physicochemical properties (texture and colour). Given the complexity and number of parameters involved, a reliable multivariate statistical strategy was adopted. A significant effect of ultrasound pre-treatment on drying behaviour and moisture mobility of cultured and uncultured beef jerky samples was observed. However, improvement in drying rates for both cultured and uncultured samples was not evident from the drying models generated. Significant effects on true protein content, amino acid profile, organic acid contents and fatty acid profiles were observed with no significant changes in colour values. Interactive effects of US frequencies and culture treatment were evident. However, the effect of ultrasound frequency on *L. sakei* observed in model system (Chapter 2 and 3) was not evident when applied to a solid meat matrix.

Research studies presented in Chapters 4 and 5 revisited ultrasound-assisted diffusion of ingredients in meat matrices with an objective to improve the nutritional profile of meat products. Application of ultrasound was reaffirmed as an efficient technique for meat brining application as evidenced with improved diffusion rates for sodium salt compared to the previously studied static brining system. However, in the case of the salt replacer, benefits from ultrasound-assisted diffusion was not observed (Chapter 4). Although, diffusion conclusions were based on sodium estimation for both sodium salt and sodium salt replacer, no significant differences in diffusion coefficients (min^{-1} m^{-2}) were observed between ultrasound-assisted and static brining systems in the case of employing salt replacers. Therefore, application of ultrasound for enhanced diffusion of salt replacers cannot be established based on this study. Use of salt replacers with reduced Na content may be beneficial from a health perspective, but the use of salt replacers resulted in higher cooking losses compared to sodium-based salt. These findings highlight the challenge associated with replacing sodium salts in meat products. Hence, it would require a comprehensive assessment of various salt replacers available in the marketplace for meat application potential. Previous attempts on the complete replacement of sodium salts with other chloride salts of calcium, lithium, magnesium and potassium showed significant and negative effects on certain aspects of product texture, flavour, appearance, moisture and shelf-life.

Second aspect of ultrasound-assisted diffusion focused on the incorporation of essential fatty acids namely eicosapentanoic acid and docosahexanoic acid. Unlike salt, incorporation of other ingredients to improve nutritional profile of solid foods such as meat is challenging compared to liquid foods, since diffusion rates are low and uniform dispersion into a solid food matrix is difficult. Furthermore, the incorporation of essential fatty acids is more challenging due to a risk of altering meat taste/flavour and promoting lipid oxidation. To

overcome these challenges, a combination of encapsulation using thin film hydration methodology and ultrasound technology, to improve the lipid profile of pork meat was adopted (Chapter 5). This study has demonstrated the positive effect of ultrasound application in improving fatty acid profile of pork meat by improving diffusion of encapsulated fatty acids into the meat resulting in higher levels of DHA and EPA fatty acids compared to a static diffusion system. The higher content of these fatty acids in pork meat resulted in improved lipid quality indices leading to a production of healthier product.

From the experimental work carried out in this thesis, it can be concluded that ultrasound technology at best, can improve quality parameters with minimal or no negative impact on quality parameters for muscle-based food products. Application of ultrasound technology remains challenging for meat product application due to the complex nature presented by most meat systems. However, further research is needed before proposing a recommendation to the food industry on the potential of ultrasound for a range of applications.

Recommendations for future research

It is quite evident that a significant advancement was made in understanding the mechanisms of ultrasound-induced modulation of microbial activity. However, similar application of ultrasound should be investigated for yeast using PM technique. Ultrasound treatment showed the presence of pores on *L. sakei* cells, which was hypothesised as one of the factor to improve its performance apart from influencing it metabolic responses. It will be interesting if a thorough investigation on the application of ultrasound for controlling pore size on cells is carried out. If this can be achieved it will be a significant breakthrough to introduce target chemical into bacterial cells for specialised applications. Ultrasound-

mediated intracellular drug and gene delivery have been proven for clinical applications. However, this could be replicated for microbial cells to improve their functionality for food applications. One of the significant findings of Chapter 2, where cell free extract showed significant effects of various pathogenic bacteria should be carried forward. These cell free extracts obtained from ultrasound treated *L. sakei* can be employed for biopreservation of various food products. Similar, cell free extracts should also be investigated for other probiotics and starter cultures.

In relation to the development of novel products with an application of ultrasound for diffusion of various other beneficial ingredients (e.g. bioactives, peptides) with a range of biological activities such as antimicrobial, antioxidant, anticancerous properties could be investigated to improve healthy image of meat products. Although enhanced fatty acid profile of pork meat was achieved by incorporating encapsulated essential oils, however, the stability of such oil in meat system during storage requires further investigations including sensorial aspects of such meat. Application of ultrasound in combination with *L. sakei* culture on solid meat should significant effects. However, similar investigation on reformulated jerky samples i.e. jerky prepared using minced meat may be advantageous from new product development perspective.

One of the key challenges witnessed while carrying out the literature review for commercial applications of ultrasound for meat applications *per se* was the non-standardised reporting of extrinsic and intrinsic control parameters including ultrasound power level, amplitude, processing time and raw material characteristics. Ultrasonic technology is still in its early stages and requires a great deal of future research in order to develop the technology on an

industrial scale, and to more fully elucidate the effect of ultrasound on the properties of foods.

Chapter 8: Cited Literature

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Appendices

Appendix I: List of Publications

1. **Ojha K. S**., Burgess C. M., Duffy G., Kerry J. P. and Tiwari B. K. (2018). Integrated phenotypic-genotypic approach to understand the influence of ultrasound on metabolic response of *Lactobacillus sakei.* PlosOne (Accepted).
2. **Ojha, K. S.,** Granto, D., Rajuria, G., Barba, F.J., Kerry, J. P., & Tiwari, B. K. (2018). Application of chemometrics to assess the influence of ultrasound frequency, *Lactobacillus sakei* culture and drying on beef jerky manufacture. Food Chemistry 239, 544-550.
3. **Ojha, K.S.,** Perussello, C. A., Garcia, C. A., Kerry, J. P., Pando, D., & Tiwari, B. K (2017). Ultrasonic-assisted incorporation of nano-encapsulated omega-3 fatty acids to enhance the fatty acid profile of pork meat. Meat Science Meat Science 132 99–106.
4. Charoux, C. M. G., **Ojha, K. S.**, O'Donnell, C. P., Cardoni, A., & Tiwari, B. K. (2017). Applications of airborne ultrasonic technology in the food industry. Journal of Food Engineering, 208, 28-36.
5. **Ojha, K. S.,** Kerry, J. P., & Tiwari, B. K. (2017). Investigating the influence of ultrasound pre-treatment on drying kinetics and moisture migration measurement in *Lactobacillus sakei* cultured and uncultured beef jerky. LWT - Food Science and Technology, 81, 42-49.
6. **Ojha, K. S.**, Harrison, S. M., Brunton N. P., Kerry, J. P., & Tiwari, B. K. (2017). Statistical approaches to assess the effect of Lactobacillus sakei culture and ultrasound frequency on fatty acid profile of beef jerky. Journal of Food Composition and Analysis, 57, 1–7.
7. **Ojha, K. S.**, Mason, T. J., O'Donnell, C. P., Kerry, J. P., & Tiwari, B. K. (2017). Ultrasound technology for food fermentation applications. Ultrasonics Sonochemistry 34, 410-417.
8. Troy, D. J., **Ojha, K. S.**, Kerry, J. P., & Tiwari, B. K. (2016). Sustainable and consumer-friendly emerging technologies for application within the meat industry: An overview. Meat Science, 120, 2–9.
9. **Ojha, K. S.**, Keenan, D. F., Bright, A., Kerry, J. P., & Tiwari, B. K. (2016). Ultrasound assisted diffusion of sodium salt replacer and effect on physicochemical properties of pork meat. International Journal of Food Science & Technology, 51(1), 37-45.
10. **Ojha, K. S.**, Kerry, J. P., Alvarez, C., Walsh, D., & Tiwari, B. K. (2016). Effect of high intensity ultrasound on the fermentation profile of *Lactobacillus sakei* in a meat model system. Ultrasonics Sonochemistry, 31, 539-545.
11. **Ojha, K. S.**, Kerry, J. P., Duffy, G., Beresford, T., & Tiwari, B. K. (2015). Technological advances for enhancing quality and safety of fermented meat products. Trends in Food Science & Technology, 44(1), 105-116

Appendix II: Conferences

1. **Ojha, K.S.,** Perussello, C. A., Garcia, C. A., Kerry, J. P., Pando, D., & Tiwari, B. K (2017). Ultrasonic-assisted incorporation of nano-encapsulated omega-3 fatty acids to enhance the fatty acid profile of pork meat. 63rd International Congress of Meat Science and Technology, Cork, Ireland, August 13- 18.
2. **Ojha, K. S**., Kerry, J. and Tiwari, B.K. (2016). Investigating the influence of *Lactobacillus sakei* culture and ultrasound processing on quality of beef jerky. IUFoST, Dublin, Ireland, August 21-25.
3. **Ojha, K. S.**, Kerry, J. P. & Tiwari, B. K., (2016). The Influence of Ultrasonics on Viability and Physiological Characteristics of *Lactobacillus sakei*, 2nd international conference on ultrasonic-based applications Caparica, Portugal, June 6 – 8.
4. Charoux C.M.G., **Ojha K.S.**, Tiwari B.K., O'Donnell C.P. (2016). Investigation of the effects of airborne acoustic ultrasound on vegetative cells and spores. 2nd international conference on ultrasonic-based applications: from analysis to synthesis, Caparica, Portugal, June 6 – 8.
5. **Ojha, K. S.**, Kerry, J. P. & Tiwari, B. K., (2015). Effect of ultrasound on fermentation profile of *Lactobacillus sakei* in meat model system, 29th EFFoST International Conference, Athens, Greece.
6. **Ojha, K. S.**, Keenan, D. F., Kerry, J. P., & Tiwari, B. K. (2015). Ultrasound assisted diffusion of sodium salt replacer and effect on physicochemical properties of pork meat. IFT Annual meeting and Expo, Chicago, USA.

Appendix III: Book chapters

1. **Ojha, K. S.**, Kerry, J. P., & Tiwari, B. K. (2017). Emerging technologies for the meat processing industry, Advanced Technologies for Meat Processing, (Eds. Fidel Toldrá, Leo M. L. Nollet), Second Edition. ISBN: 9781498754590, CRC Press.
2. **Ojha, K. S.** & Tiwari, B. K. (2016). Novel Food Fermentation Technologies – Ojha K.S. & Tiwari, B.K. Novel Food Fermentation Technologies: Springer.
3. **Ojha, K. S.** & Tiwari, B. K. (2016). Ultrasound and food fermentation – Ojha K.S. & Tiwari, B.K. Novel Food Fermentation Technologies: Springer.
4. **Ojha, K. S.**, Tiwari, B. K., Kerry, J. P., & Cullen, P.J. (2016). Online meat quality and compositional assessment techniques, Emerging technologies for meat Processing: Wiley Publishing Co.
5. **Ojha, K. S.**, Kerry, J. P., Tiwari, B. K., & O'Donnell, C. (2016). Freezing for Food Preservation Reference Module in Food Science: Elsevier.
6. **Ojha, K. S.**, Tiwari, B. K., Kerry, J. P., & Troy, D. (2016). Beef Encyclopedia of Food and Health (pp. 332-338). Oxford: Academic Press.
7. **Ojha, K. S.**, Tiwari, B. K., O'Donnell, C., & Kerry, J. P. (2016). Emerging Nonthermal Food Preservation Technologies. Leadley, C.E Innovation and Future Trends in Food Manufacturing and Supply Chain Technologies (pp. 257-274): Woodhead Publishing.