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Ultrasonication and the quality of human milk: variation of power and time of exposure

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Donor human milk is pasteurized to prevent the potential risk of the transmission of pathogens to preterm infants. Currently, Holder pasteurization (human milk held at 62·5 °C for 30 min) is used in most human milk banks, but has the disadvantage that it results in excessive inactivation of important bioactive components. Power-ultrasound (20–100 kHz) is an emerging technology for the preservation of foods and could be an alternative method for the treatment of human milk. The aim of this study was to investigate the effect of different ultrasound settings on the elimination of *Escherichia coli* and the retention of bile salt stimulated lipase (BSSL) activity. Ultrasonication with a constant power decreased *Esch. coli* viability exponentially over time until the processing temperature increased to sub-pasteurization level to between 51·4 and 58·5 °C, then a $\log_{10}$ 1·3 decrease was observed ($P<0·05$). BSSL activity decreased to 91% until a temperature of 51·4 °C and then it decreased to 8% between 51·4 and 64·9 °C. Ultrasonication with a constant energy and various power and exposure times showed the highest temperature (53·7 °C) when treated with the longest exposure time and lowest ultrasound-power (276 s at 3·62 W) compared with 37·6 °C for 39 s at 25·64 W. The findings predict that the viability of *Esch. coli* could be reduced by $\log_{10}$ 5 with a minimal loss of activity of BSSL by applying 13·8 kJ of energy in 12 ml of human milk using high ultrasound power over a short exposure time to ensure that the temperature remains below the critical level for protein denaturation. Alternatively, the use of lower power settings such as the 26 W used in the present studies would require a cooling system to ensure the human milk BSSL was protected against temperature denaturation.

Keywords: Donor human milk, holder pasteurization, ultrasonication, *Esch. coli*, bile salt stimulated lipase.

Donor human milk is the best alternative when mothers' own milk is not available for the preterm infant because it contains nutritional, bioactive, protective and developmental components that cannot be found in infant formula (Wight, 2001). However, to prevent the potential risk for the transmission of pathogens from donor mothers to preterm infants, the donor milk is pasteurized.

Holder pasteurization is a low-temperature long-time (LTLT) heat preservation method that is widely used in milk banks. Human milk is heated in a water bath and held at 62·5 °C for 30 min (Arnold & Tully, 1994; Balmer & Williams, 1995; Hartmann et al. 2007). This treatment is capable of a $\log_{10}$ 5 reduction of bacteria including *Escherichia coli, Staphylococcus epidermidis, Enterobacter cloacae, Bacillus cereus* and *Staphylococcus aureus* (Czank et al. 2009). However, bioactive proteins are only partially preserved during this process. For example, 72% of sIgA, 22% of lactoferrin and 39% of lysozyme were retained after Holder pasteurization. Furthermore, the retention of bile salt stimulated lipase (BSSL) was reported to be less than 1% (Henderson et al. 1998; Tully et al. 2001; Czank et al. 2009). BSSL transforms triacylglycerol into monoglycerides and free fatty acids which aids in digestion of fat for the infant (Hernell & Bläckberg, 1994). BSSL is a very heat labile enzyme and depending on exposure time the inactivation starts at 45 °C (Wardell et al. 1984). It is likely that the inactivation of BSSL through the Holder pasteurization process is responsible for the lower growth rates of

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preterm infants fed pasteurized donor human milk compared with preterm infants fed mothers' own milk (Williamson et al. 1978; Andersson et al. 2007).

Treatments with power-ultrasound (20–100 kHz) are an emerging technology for the preservation of food alone (D'Amico et al. 2006; Chouliara et al. 2010; Wang et al. 2010) and in combination with other novel processing technologies (Walkling-Ribeiro et al. 2009a, b; Arroyo et al. 2011). Power-ultrasound creates cavitation, that is, the formation, growth and implosive collapse of bubbles in liquids. During the collapse of these bubbles localised hot spots occurs with temperatures of roughly 5000 °C, pressures of about 50 MPa and a lifetime of a few microseconds (Suslick, 1990). The pressure changes resulting from these implosions create shock waves that disrupt the cellular membranes of bacteria resulting in cell lysis (Allison et al. 1996; Cameron et al. 2008). The combined effect of ultrasound and heat on human milk has been found to inactivate *Esch. coli* and *Staph. epidermidis* with a greater retention of sIgA, lysozyme, lactoferrin and BSSL than with Holder pasteurization (Czank et al. 2010).

The aim of this study was to investigate the effect of different ultrasound settings on the elimination of *Esch. coli* and preservation of BSSL activity in human milk. In a first experiment different ultrasound-energies were created by holding ultrasound-power constant and varying the exposure times. In a second experiment ultrasound-energy was held constant with varying ultrasound-power and exposure times.

Methods and Materials

Sample collection

Frozen human milk samples were donated by 3 mothers with excess milk supply. The collection of the milk was approved by the Human Research Ethics Committee of the University of Western Australia (RA/4/1/2369). All donors gave written consent for their donations to be used in research and all samples were de-identified.

Sample preparation

Escherichia coli K12 (ATCC1498, Southern Biological, Nunawading, Vic, Australia) was cultured in nutrient broth (Nutrient Broth No. 2, Oxoid Australia Pty Ltd, Adelaide, SA, Australia) over night at 37 °C and enumerated using the optical density method with previously constructed standard curves (Monod, 1949) measured with a spectrophotometer at 600 nm (PowerWave, Biotek, Winooski, VT, USA). The culture was then diluted and inoculated into previously thawed and pooled (3 donors) human milk to target a maximum accepted concentration in milk banks of 10^5 CFU/ml (colony forming units per millilitre) (Hartmann et al. 2007) and portioned into 12 ml samples and brought to 20 °C using a water bath (CC-1, Huber GmbH, Offenburg, Germany).

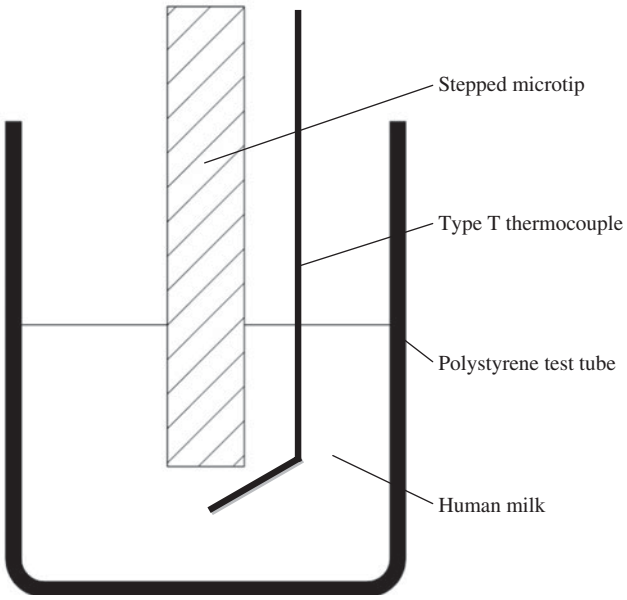

Fig. 1. Ultrasonication set-up.

Ultrasonication

A 20 kHz ultrasonic processor fitted with a 6 mm stepped microtip (VCX 130, Sonics & Materials Inc., Newtown, CT, USA) for a batch volume between 10 and 50 ml with a maximum amplitude of 123 µm was used for all experiments. Human milk (12 ml) was treated in batch mode (10 samples for each treatment condition) using 25 ml polystyrene test tubes (inner-diameter 25 mm) with 12 mm gap between the bottom of the tube and the microtip surface (approximately half the height of human milk). Temperature was recorded using a type T thermocouple (MT-29/5, Physitemp Instruments Inc., Clifton, NJ, USA) and USB data-logger (THERMES USB, Physitemp Instruments Inc., Clifton, NJ, USA) with data acquisition software (DASYlab10·0, Measurement Computing Corp., Norton, MA, USA) at a sampling rate of 16 Hz (Fig. 1). After treatment, the tube with human milk was transferred to an ice bath. Aliquots where taken and kept frozen at − 80 °C until the BSSL assay was performed. Cooled samples were transferred for immediate microbiological analyses.

Constant ultrasound-power and varying exposure times

The ultrasound processor was set on maximal amplitude and generated an ultrasound-power of 26 W. Human milk was treated for different times (19·2, 38·4, 57·6, 76·8, 96 and 115·2 s) to achieve an applied ultrasound-energy (500,1000, 1500, 2000, 2500 and 3000 J), respectively.

Constant ultrasound-energy and varying ultrasound-power and exposure time

The same ultrasound-energy (1000 J) was applied with different time-power ratios (276 s at 3·62 W; 114·5 s at

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8·73 W; 70·5 s at 14·18 W; 50·5 s at 19·8 W and 39 s at 25·64 W).

Bacteria analysis

Cooled untreated and ultrasonicated samples (5 µl) were plated in duplicates onto nutrient agar (Plain agar, Southern Biological, Nunawading, Vic, Australia/Nutrient Broth No. 2, Oxoid Australia Pty Ltd, Adelaide, SA, Australia) and incubated at 37 °C for 18 h. The numbers of colonies were then enumerated to determine the reduction in bacterial number following the ultrasound processing based on CFU/ml. Detection limit was at 200 CFU/ml.

BSSL assay

The BSSL activity in human milk was determined using a lipase assay kit (QuantiChrom Lipase Assay Kit, BioAssay Systems, Hayward, CA, USA). The frozen (− 80 °C) untreated and ultrasonicated samples were thawed and brought to 37 °C, diluted (1/100, v/v) with double deionized water and the assay was performed in duplicate according to kit instructions. The absorbance was determined with a spectrophotometer at 412 nm (PowerWave, Biotek, Winooski, VT, USA) and transformed into BSSL activity per litre (U/l) based on the formula in the kit instruction. The kit linear detection range is between 40 and 1600 U/l lipase activity in the 96-well plate assay. Unit definition: one unit of enzyme catalyses the cleavage of 1·0 µmole of substrate per min under the assay conditions (pH 8·5). The coefficient of variation (CV) within groups was <6% unless otherwise stated.

Statistical analysis

Results are presented as treatment means ± SD values unless otherwise stated. All analyses were carried out using R 2.9.2 for Mac OS X (R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, 2009). ANOVA models with Tukey's HSD Post Hoc test were used to compare the values between groups (statistical significant when $P<0{\cdot}05$, confidence level 95%). Graphical modelling was performed with OpenOffice 3.2.0 Spreadsheet (Sun Microsystems Inc. based on OpenOffice.org)

Results

Effect of holding ultrasound-power constant and varying the exposure times

A reduction of *Esch. coli* viability (CFU/ml) over time was observed when human milk was treated using ultrasound-power of 26 W. Untreated samples (0·0 s) inoculated with *Esch. coli* gave a total count of 221 600 ± 48 157 CFU/ml ($n=10$). The temperature before treatment was 20·0 ± 0·4 °C. After 76·8, 96·0, and 115·2 s of treatment the *Esch. coli* viability was reduced to 42 160 ± 13 598, 1611 ± 1334 and undetectable CFU/ml ($P<0{\cdot}05$) ($n=10$) with the temperature of the human milk increasing up to 51·4 ± 0·3, 58·5 ± 0·5, and 64·9 ± 1·2 °C, respectively ($P<0{\cdot}05$) ($n=10$). There was a significant exponential regression (R^2 of 0·976) for bacterial count from untreated samples up to an exposure time of 76·8 s (Fig. 2).

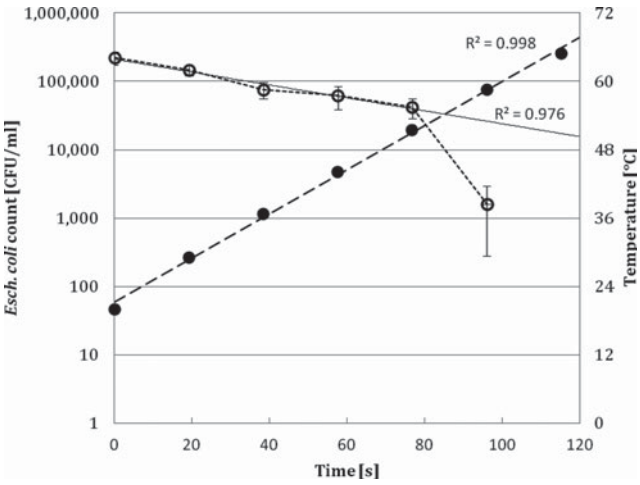

Fig. 2. Effect of holding ultrasound-power constant (26 W) and varying the exposure times on *Esch. coli* count (o, fine dashed line) and exponential regression for *Esch. coli* count between 0·0 and 76·8 s (solid line), and processing temperature (●) and its linear regression trend (long dashed line). All values are mean ± SD ($n=10$).

Untreated human milk had a BSSL activity of 46 253 ± 1969 U/l ($n=6$). The BSSL activity was significantly reduced to 93% between 0·0 and 19·2 s ($P<0{\cdot}05$) but then remained relatively constant between 19·2 and 76·8 s ($P>0{\cdot}9$) before decreasing significantly from 91 to 76% ($P<0{\cdot}05$) between 76·8 and 96·0 s and to 8% at 115·2 s ($P<0{\cdot}05$). Over this latter period the BSSL activity decreased from 42 052 ± 1365 U/l at 76·8 s and 34 962 ± 1268 U/l at 96·0 s (CV <6%) to only 3858 ± 1921 U/l (CV <50%) at 115·2 s ($n=6$) (Fig. 3).

Effect of holding ultrasound-energy constant and varying the ultrasound-power and exposure time

Untreated human milk inoculated with *Esch. coli* gave a total bacteria concentration of 204 760 ± 38 770 CFU/ml ($n=10$). Human milk treated with constant ultrasound-energy (1000 J) but varying ultrasound-power and exposure time showed a significant reduction of *Esch. coli* cell concentration compared with untreated human milk ($P<0{\cdot}05$). Furthermore, a significant reduction in the *Esch. coli* load was found between the human milk treated with the longest exposure time and lowest power (276 s at 3·62 W; 7700 ± 3731 CFU/ml) and all the other shorter but more powerful ultrasound settings ($P<0{\cdot}05$) (Fig. 4). However, there was no significant difference in *Esch. coli* elimination between the treatments 114·5 s at 8·73 W, 70·5 s at 14·18 W, 50·5 s at 19·8 W and 39 s at 25·64 W ($P>0{\cdot}1$).

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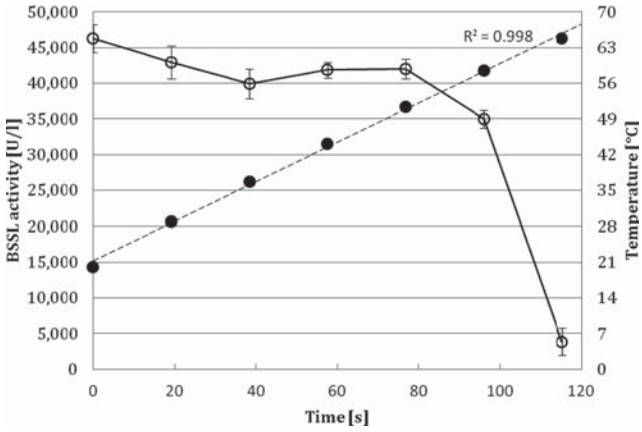

Fig. 3. Effect of holding ultrasound-power constant (26 W) and varying the exposure times on BSSL activity (o, solid line) and processing temperature (●) and its linear regression trend (long dashed line). All values are mean ± SD ($n = 6$).

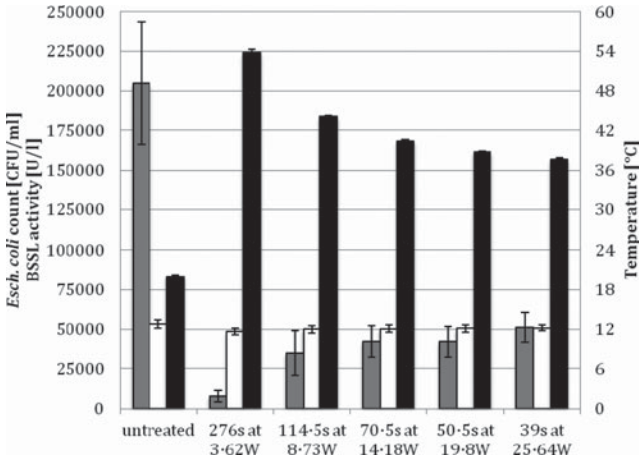

Fig. 4. Effect of holding ultrasound-energy constant (1000 J) and varying the ultrasound-power and exposure time. *Esch. coli* count (grey) ($n = 10$), BSSL activity (white) ($n = 7$) and final-temperature (black) ($n = 10$). All values are mean ± SD.

The temperature before ultrasonication was at 20·0 ± 0·2 °C. The temperature after treatment was highest for the treatment with the longest time and lowest power. The temperature then progressively and significantly decreased at each time by power change as exposure time decreased from 276 to 39 s ($P < 0·05$). Thus, the highest temperature of 53·7 ± 0·5 °C was observed at 276 s at 3·62 W and the lowest temperature of 37·6 ± 0·3 °C was observed at 39 s at 25·64 W ($n = 10$) (Fig. 4).

Untreated human milk had a BSSL activity of 53 408 ± 2590 U/l ($n = 7$) and was only significantly reduced by the longest treatment time at the lowest power (276 s at 3·62 W) with the activity decreasing to 48502 ± 2094 U/l ($n = 7$), that is, to 91% of the untreated human milk BSSL activity ($P < 0·05$) (Fig. 4).

Discussion

Effect of holding ultrasound-power constant and varying the exposure times

A previous study reported a synergistic effect when thermal and ultrasound treatments were used in combination (Czank et al. 2010). The time to reduce the bacterial content by $\log_{10}$ 5 was significantly reduced when applied to human milk that had a starting temperature of either 45 or 50 °C. However, ultrasound treatment of 60 ml human milk resulted in a consistent increase of 10·3 °C from the starting temperature which stabilised within three minutes of the application of ultrasound. In our study the application of ultrasound to 12 ml human milk with a starting temperature of 20 °C resulted in a linear increase in temperature over a period of 115·2 s (Fig. 2). We found an exponential decrease in the *Esch. coli* cell concentration up to 51·4 °C (76·8 s exposure to ultrasound) and followed by a rapid decrease ($\log_{10}$ 1·3) in viability between 51·4 and 58·5 °C (96 s). It is of interest that the *Esch. coli* cells, when exposed to heat only, decreased when the temperature was increased to 56 °C (Wills et al. 1982; Czank et al. 2009). A possible explanation for this trend could be that the rapid decrease in *Esch. coli* occurring as the temperature increased from 51·4 to 58·5 °C was related to a synergistic effect of ultrasound and temperature (Fig. 2), because the application of temperature alone using Holder pasteurization at 57 °C required a holding time of 16·4 min (Czank et al. 2009) to obtain a similar $\log_{10}$ decrease in *Esch. coli* to that observed as the temperature increase from 51·4 to 58·5 °C (Fig. 2).

In contrast to the inactivation of the coliform bacterium, the loss in activity of BSSL occurred in two stages; from 100 to 93% BSSL activity between 20 and 28 °C and then from 91 to 8% between 51·4 and 64·9 °C (Fig. 3). Since it has been reported that BSSL is heat stable up to 45 °C (Wardell et al. 1984), the initial significant but small decrease in BSSL activity may have been due to the exposure to ultrasound. However, the BSSL activity remained constant from 28 and 51·4 °C, and this suggests that if the temperature of the human milk is maintained below 51·4 °C the length of exposure to ultrasound would have a small effect on BSSL activity (Fig. 3). The high CV of 50% for BSSL activity at 115·2 s can be explained by the proximity to the linear detection range limit for this enzyme activity assay kit.

The large discrepancy between the exponential loss of *Esch. coli* and the relative stability of BSSL activity between 20 and 51·4 °C suggests that initially ultrasound exposure caused cell death by disrupting the cell membranes of *Esch. coli* resulting in cell lysis (Allison et al. 1996; Cameron et al. 2008). Subsequently, between 51·4 and 58·5 °C there was a significant decrease in both BSSL activity and *Esch. coli* count suggesting that this resulted from the combined effect of ultrasound and temperature causing the denaturation of both BSSL and functional proteins present in *Esch. coli*.

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Effect of holding ultrasound-energy constant and varying the ultrasound-power and exposure time

At constant ultrasound-energy (1000 J), the increase in temperature was highest when the human milk was treated for the longest time at the lowest ultrasound-power (276 s at 3·62 W) and lowest when treated for the shortest time at the highest power (39 s at 25·64 W) (Fig. 4). This finding demonstrates that the longer exposure time, rather than high ultrasound power, was responsible for the increase in temperature. Although, treatment at the longest time at the lowest power was the most effective in reducing the viability of *Esch. coli*, this longer exposure time resulted in a temperature that was approaching those associated with protein denaturation and compromised retention of the BSSL activity (Figs. 3 & 4). Furthermore, the BSSL activity was reduced at this setting (276 s at 3·62 W) but only to 91% of the untreated activity (Fig. 4). These findings again focus on a critical temperature being important in relation to decreasing both *Esch. coli* cell numbers and BSSL activity.

Extrapolation of the exponential regression for the reduction in *Esch. coli* from 0·0 s to 76·8 s, that is, during the time that ultrasound alone appeared to reduce bacteria (Fig. 2), predicts that there would be a $\log_{10}$ 5 reduction in *Esch. coli* viability at 530 s. By 530 s with ultrasound power set at 26W, it can be calculated that the human milk would be subjected to 13·8 kJ of energy. With constant ultrasound-power it was found that the increase in temperature was linear up to 115·2 s (64·9 °C) (Fig. 2). Furthermore when the ultrasound-energy was held constant the heating effect on human milk was the lowest when it was treated with high ultrasound-power and short exposure times (Fig. 4). Therefore, these findings predict that the loss of activity of BSSL could be minimised and the viability of *Esch. coli* could be reduced by $\log_{10}$ 5 by applying 13·8 kJ of energy using high ultrasound power over a short exposure time to ensure that the temperature remains below the critical level for protein denaturation. Alternatively, the use of lower power settings such as the 26 W used in the present studies would require a cooling system to ensure the human milk BSSL activity was protected against temperature denaturation.

Although this study has only investigated the effects of ultrasonication on BSSL and *Esch. coli,* previous studies using either the LTLT Holder pasteurization or ultrasound processing methods have shown that other important proteins in human milk (sIgA, lactoferrin and lysozyme) were more resistant than BSSL to both forms of treatment types (Tully et al. 2001; Czank et al. 2010).

On the other hand, rod-shaped bacteria such as *Esch. coli* were reported to have less resistance to ultrasound cavitation than coccoid bacteria (Zenker et al. 2003). Therefore, it could be expected that a longer exposure time will be required for coccoid bacteria, such as *Staph. epidermidis* and *Staph. aureus*, that are also commonly found in human milk. In this connection, Czank et al. (2010) found that *Esch. coli* in human milk required 5·94 min exposure to ultrasound to achieve a $\log_{10}$ 1 reduction in bacteria load, whereas, *Staph. epidermidis* required 16·01 min for a $\log_{10}$ 1 reduction under similar conditions (Czank et al. 2010). Although, ultrasonication could be an alternative method to the thermal pasteurization of human milk, it would be necessary to ensure that the temperature of the human milk during ultrasonication remains below the level that causes a precipitous fall in the activity of BSSL (a marker enzyme for temperature denaturation of bioactive proteins). However a longer exposure time to ultrasound would be required to enhance possible synergistic treatment effects between temperature and ultrasound.

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Ethic approval

Human Research Ethics Committee of the University of Western Australia (RA/4/1/2369).

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