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

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ABSTRACT

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

1. Introduction

Milk has been considered as the most complete food for human being as it can provide a complex mixture of all macronutrients including proteins, carbohydrates and fat, as well as micronutrients such as minerals, vitamins, and other nutrients [1,2]. To extend the shelf-life of milk, thermal treatment is usually used to inactivate microorganisms in milk. However, thermal treatment is known to cause loss of nutrients and affects the flavors of milk [3,4], and induces protein denaturation [5,6]. As an alternative method, ultrasound technology can be used for microbial inactivation, while avoiding the undesirable effects caused by conventional heat treatments [7].

Ultrasound refers to a frequency of 20 kHz or above [8], and ultrasound instruments operate with frequencies ranging from 20 kHz to 10 MHz [9]. Power ultrasound refers to high intensity-low frequency (20–100 kHz) systems, whereas frequencies higher than 100 kHz are normally associated with high-frequency low power ultrasound [8,9]. Microbial inactivation by ultrasound treatment is mainly due to the acoustic cavitation [10,11]. Cavitation means "the formation and the subsequent dynamic life of bubbles in liquids" [12]. When ultrasound passes through a liquid medium, microbubbles grow due to the compression and decompression of the ultrasound waves, until they reach a critical size at which they collapse violently. The collapse of cavitation bubbles produces extreme temperatures and pressures [8]. There are two types of cavitation, transient cavitation which causes large scale variations in bubble size leading to a violent bubble collapse, whereas stable cavitation generates relatively small amplitude, weak and symmetrically oscillating bubbles [12–14]. The mechanical effects induced by acoustic cavitation include shear forces and micro-jettings. Shear forces caused by microbubble's collapse near a surface can generate shear stresses higher than 100 kPa [15,16]. When cavitation bubbles collapse near a surface (or within a cloud of bubbles), the collapse is no longer symmetrical and micro-jets are formed [17] – the speed of the liquid jet acting on a solid surface can exceed 100 m/s [18]. Shock waves can also be generated during cavitation collapse [19], and they can induce pressures of 40–60 kbar at 20 kHz [20]. Inactivation of bacteria by ultrasound treatment may be due to both the mechanical effects and the free radicals generated by cavitation. The shear forces cause the disruption of cell membranes of bacteria [21,22]. Particularly in the case of high-frequency ultrasound, in addition to the mechanical effects, free radicals are produced [23–25]. These free radicals are a result of the dissociation of the water molecules due to the extreme conditions resulting from the collapse of microbubbles [26]. The free radicals produced in aqueous solutions, which include hydroxyl

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radicals, attack the chemical structure of cell wall and can cause DNA damage, destroy enzymatic activity, and damage liposomes and bacteria membranes [27–31].

Bovine milk contains 3.0–3.5% (w/v) of proteins, which mainly consist of caseins and whey proteins [32]. Caseins, namely, α_{s1}-casein (α_{s1}-CN), αs_2-casein (α_{s2}-CN), β-casein (β-CN) and κ-casein (κ-CN) are in the proportion of 4:1:4:1, and exist in milk as small aggregates (60–400 nm diameter) termed casein micelles. Whey proteins include α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) with a ratio of 1:3 [32–34], and some minor proteins such as bovine serum albumin (BSA) and immunoglobulins (Igs) [35,36]. The whey proteins are prone to denaturation during heating. Although whey protein denaturation can be exploited to modify their functional properties and obtain desired modifications in final products (e.g. achieving high gel strength of yoghurt), it can cause some undesired effects including deposit formation, milk instability and gelling [37–39].

The main aim of this study is to determine the effects of both low-frequency (20 kHz) and high-frequency (850 kHz) ultrasound treatment on *Enterobacter aerogenes* suspended in skim milk. Further, the effect of ultrasound on the state of milk proteins is also considered. Ultrasound inactivation of microorganisms in milk was reported previously, on bacteria including *Salmonella* (35–40 kHz) [40]; *Escherichia coli, Saccharomyces cerevisiae* and *Lactobacillus acidophilus* (20 kHz, 750 W) [7]; and *Listeria innocua* ATCC 51742 (24 kHz, 120 μm amplitude) [41]. However, there are no reports on the effect of ultrasound treatment on *E. aerogenes* in milk. Moreover, there are only few published papers on the effect of ultrasound on milk proteins [42–46]. The findings of these studies will be discussed in the light of the results obtained in the present paper.

2. Material and methods

2.1. Material, bacterial and milk samples preparation

The following chemicals were purchased form Sigma Aldrich (St. Louis, Missouri, USA): Calcium chloride ($CaCl_2$), ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA), dithiothreitol (DTT), BisTris and sodium citrate. The following chemicals were purchased form Merck KGaA (Darmstadt, Germany): Guanidine hydrochloride (GdnHCl) and acetonitrile (gradient grade). For Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC), α-casein (α-CN), β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin standards were purchased from Sigma Aldrich (USA).

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

E. aerogenes culture was obtained from a stock kept in a −80 °C freezer in the Environmental Microbiology Lab, School of Biology Sciences, The University of Auckland. The bacteria suspension was made by incubating the bacteria stock (100 μl stock into 100 ml Nutrient Broth) at 37 °C overnight under shaking (200 rev min^{-1}) to reach a stationary phase. The bacterial suspensions then were transferred into 50 ml tubes, and centrifuged (Biofuge Stratos, Heraeus, Germany) (10,000*g*, 4 °C, 10 min) to obtain a bacterial cell pellet. After quickly moving the supernatant, the pellet was washed twice with sterilized water. Then the washed bacteria suspensions were diluted to $\sim 10^9$ CFU/ml by measuring the optical density (OD) using a HeλIOS β UV Visible Spectrophotometer (Thermo Electron Corporation, UK) at an absorbance of 600 nm. 1 ml of the washed *E. aerogenes* suspension was added into 9 ml water or 9 ml skim milk to make bacteria in water or bacteria in skim milk samples. The final bacteria count, both in water or different skim milks, was $\sim 10^8$ CFU/ml. Bacteria counts were performed using a modified Miles–Misra method [47] as described in our previous work [21,22].

2.2. Ultrasonication

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

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

The calorimetric method was used for determination of ultrasound power [48,49]:.

$$P = mC_p(\Delta T/\Delta t) \quad (1)$$

where C_p is the specific heat capacity of the sample, m is its mass, ΔT is the increase in the temperature, and Δt is the applied ultrasound time. For water, C_p = 4.18 J/(g K) was used [50], and for skim milk a value of 3.98 J/(g K) for C_p was used [51]. Heat capacity is expressed as J/(g K) in SI units, which equates 1/4186 cal/g/°C in c.g.s. units [52].

2.3. RP-HPLC analysis

Milk proteins were separated by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) and the method was adapted with slight modification from previous publications [32,53–55]. Two solvents were used to carry out gradient elution. Acetonitrile (HPLC grade), Milli-Q water, and trifluoroacetic acid (TFA) in a ratio of 100:900:1 (v:v:v) were mixed to make up Solvent A, and in a ratio of 900:100:1 to make up Solvent B. Solvent A and Solvent B were ultrasonicated in Soniclean sonication bath (Soniclean Pty. Ltd., Thebarton, S.A., Australia) to get rid of micro(air)bubbles before operating HPLC instrument. A reversed-phase analytical column Jupiter 5 μm C18 300 Å (250 mm length × 4.6 mm i.d., Phenomenex) and a Jupiter C4 column (250 × 4.6 mm, Phenomenex) were used in an Agilent 1260 Infinity HPLC system to separate caseins and whey proteins, respectively. RP-HPLC was performed at room temperature under the following chromatographic conditions: injection volume of final solution, 50 μl; flow rate, 1.0 ml/min; detection wavelength: 220 nm for caseins and 205 nm

for whey proteins. The solvent gradient program for caseins was set to 0–40 min, 30–50% B; 40–42 min, 50–100% B; 42–43 min, 100–100% B; 43–46 min, 100–30% B; 46–51 min, 30–30% B; 51–55 min, re-equilibration. The gradient program for whey proteins was set as followings: 0–1 min, 35–35% B; 1–8 min, 35–38% B; 8–16 min, 38–42% B; 16–22 min, 42–46% B; 22–24 min, 46–90% B; 24–25 min, 90–90% B; 25–30 min, 90–35% B; 30–35 min, 35–35% B; 35–40 min, re-equilibration.

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

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

2.4. Viscosity measurement

Viscosity measurements were performed using an SI Analytics Ubbelohde capillary (diameter 1.50 ± 0.01 mm, viscometer constant $K = 0.3\ mm^2/s$) fitted into the viscosity Measuring Unit Visco-Clock (Schott, SI Analytics, Mainz, Germany). A temperature controller (SD07R-20-A12E, Ply Science, USA) was used to control a constant temperature of 25 °C. The viscosity (η) was calculated using [56]:

$$\eta = \eta_0 \frac{t}{t_0} \frac{\rho}{\rho_0} \quad (2)$$

where η_0(=0.890 mPa s) is the viscosity of water at 25 °C, ρ_0(=1.00 g/ml) is its density. t_0 and t are the flow-through times in the capillary of the water and the sample, respectively. All measurements were performed on duplicate samples and the flow-through time measured 6 times for each sample.

2.5. TEM and Cryo-TEM observation

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

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

2.6. Particle size measurement

Particle size measurements were performed using the Malvern Zetasizer Nano ZSP (Malvern Instruments Ltd., UK). A calcium-imidazole buffer (pH 6.7) made of 5 mM $CaCl_2$, 20 mM imidazole, and 30 mM NaCl, was used as dispersant [57]. All measurements were performed at least in duplicate at 25 °C, and each sample was measured 10 times.

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

3. Results and discussion

3.1. Bacterial inactivation in water and skim milk

Initial populations of *E. aerogenes* in water or milk samples were about $\sim 1 \times 10^8$ CFU/ml. Bacterial suspensions in water and skim milks (5, 10 and 15 wt%) were treated by both low-frequency (20 kHz) at different powers of ~8 to 9 W and high-frequency (850 kHz) at a power of 50 W for 0, 5, 10, 20, 40, and 60 min. Power values used in this study correspond to power densities of ~0.5 to 0.6 W/ml for low-frequency and 0.2 W/ml for the high-frequency treatment. The log reduction of *E. aerogenes* as a function of sonication time is shown in Fig. 1. For the low-frequency ultrasound treatment, the log reduction was found to decrease linearly with an increase in sonication time. The bacteria were more sensitive to ultrasonication when dispersed in water than in milk. The behavior of *E. aerogenes* in water when treated by low-frequency ultrasound treatment was previously reported [21,22], and the inactivation is mainly due to the mechanical effects induced by acoustic cavitation [10,11,22]. The log reductions were −3.64 (±0.04), −2.73(±0.23), −2.31(±0.41) and −2.21(±0.03) for the bacteria in water, 5% milk, 10% milk and 15% milk, respectively when ultrasonicated for 60 min. Clearly the milk concentration has an effect on the bacteria inactivation, with the higher the milk concentration the lower the number of bacteria inactivated. This is likely due to the increase in viscosity of the milk samples with the increase in concentration. Viscosity measurements showed that the

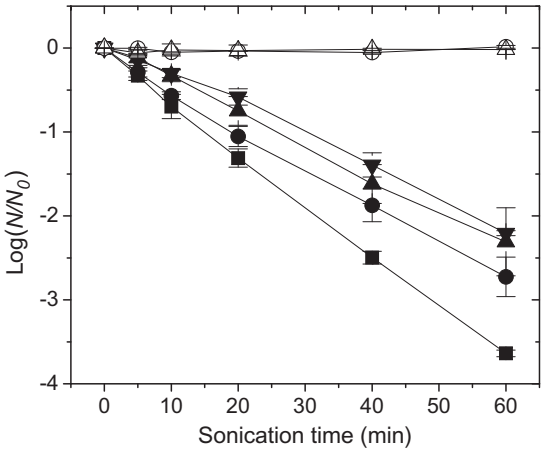

Fig. 1. Log of survival ratio (Log (N/N_0)) of *Enterobacter aerogenes* in skim milk and water as a function of sonication time. In water, 20 kHz, 8.0 W (■); In 5% skim milk, 20 kHz, 8.2 W (●); In 10% skim milk, 20 kHz, 8.5 W (▲); In 15% skim milk, 20 kHz, 9.2 W (▼); In 5% skim milk, 850 kHz, 50 W (○); In 10% skim milk, 850 kHz, 50 W (△). Error bars correspond to standard deviation.

viscosities were 1.072(±0.002), 1.293(±0.007), and 1.735(±0.009) mPa s, for 5, 10 and 15 wt% skim milks, respectively. The occurrence of cavitation decreases in high viscous media, due to acoustic impedance by high viscous liquids [58].

Other studies on the inactivation of bacteria in milk systems also reported a decrease in the log reduction when compared to the inactivation of bacteria in aqueous solutions. For instance, *S. cerevisiae* was found to be more resistant in UHT milk than in Saline solution, where there was a 2.10 and 3.62 log-reduction, respectively, when ultrasonicated for 10 min at 20 kHz with power of 750 W [7]. *L. innocua* ATCC 51742 was also found to be more resistant when sonicated (24 kHz, 120 μm amplitude) for 30 min in milks with different fat content [41]. The rate of inactivation increased with a decrease in fat content, and 2.5, 3.2, 4.5 and 4.9 log-reductions were obtained for whole milk (3.47% fat content), 2% and 1% butter fat content milks, and fat free milk, respectively. However, it was reported that the inactivation of *E. coli* ultrasonicated at 20 kHz for a power of 750 W for 10 min was similar when the bacteria were presented in saline solution (3.88-log reduction) and UHT milk (4.42-log reduction) at 20 kHz and 750 W for 10 min [7]. It is worth pointing out that the ultrasound conditions, especially power and time used, and the bacteria suspensions were different in all these studies.

In the case of high-frequency ultrasound treatment of *E. aerogenes* in milk (5 and 10 wt%) there was no change for the viable cells after ultrasonication up to 60 min at 50 W (Fig. 1, open symbols). This is not surprising, since the inactivation of bacteria by high-frequency ultrasound is mainly due to the generation of free radicals

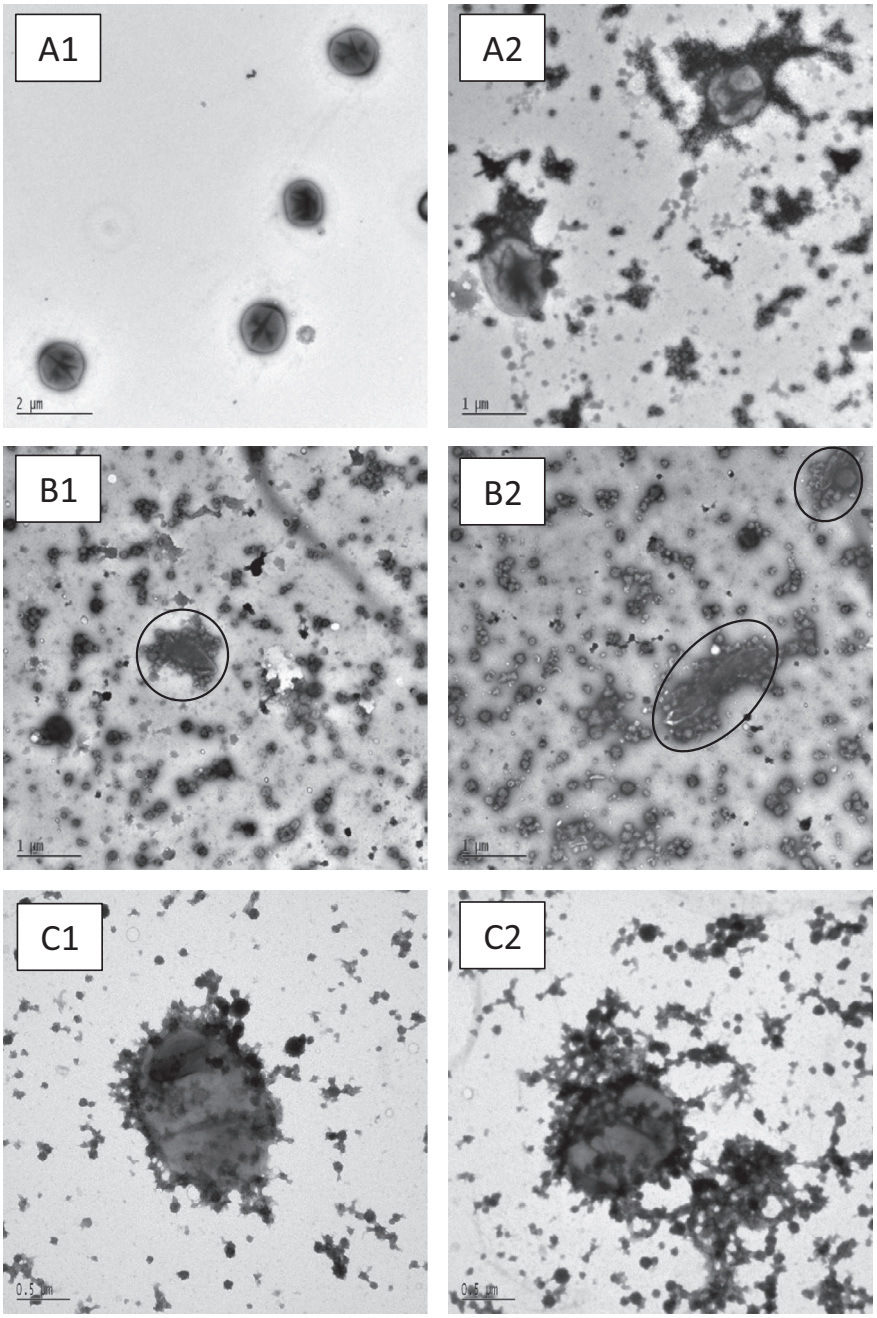

Fig. 2. TEM micrographs of *Enterobacter aerogenes* in water and 5% skim milk before and after ultrasonication. (A1) Before ultrasonication in water, (A2) before ultrasonication in milk, the cells remained stable and intact; (B1) and (B2) after ultrasonication at 20 kHz for 20 min (8.5 W), the bacteria had misshapen structures which were highlighted by circles; (C1) and (C2) after ultrasonication at 850 kHz for 60 min (50 W), the cells remained stable and intact.

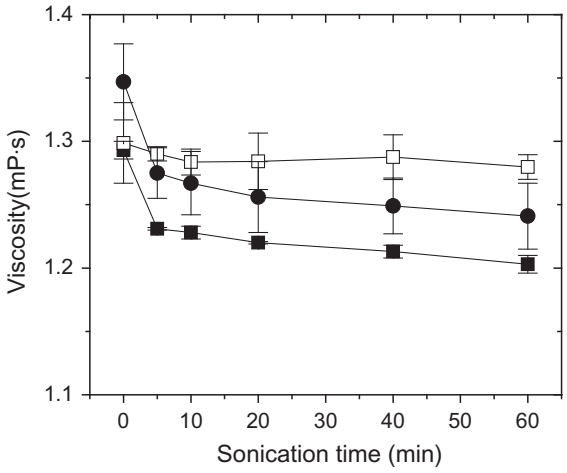

Fig. 3. Viscosity change of milk and bacteria (*Enterobacter aerogenes*) suspensions. Water in 10% milk, 20 kHz, 8.5 W (■); Bacteria suspension in 10% milk, 20 kHz, 8.5 W (●); Water in 10% milk, 850 kHz, 50 W (□). Error bars correspond to standard deviation.

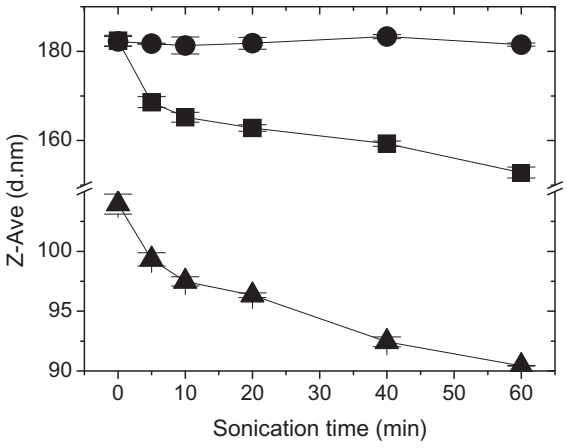

Fig. 4. The particle size diameter of milk (10% skim milk) and fat globules samples as a function of sonication time: milk, 20 kHz, 8.5 W (■); milk, 850 kHz, 50 W (●); Fat globules, milk: EDTA = 1:100 (v/v), 20 kHz, 8.5 W (▲). Error bars correspond to standard deviation.

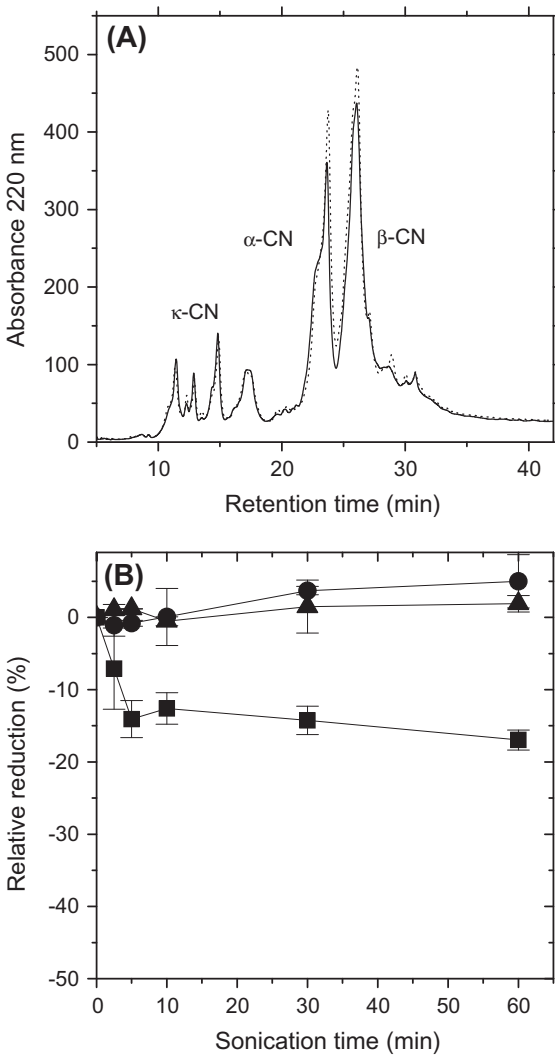

Fig. 5. (A) Chromatograms of caseins from skim milk by RP-HPLC: Control skim milk (solid line); Ultrasonicated skim milk, 30 min. 20 kHz, 8.5 W (Dotted line). (B) The relative reductions of concentration of caseins from RP-HPLC: κ-casein (■) α-casein (●), and β-casein (▲); Error bars correspond to standard deviation.

including hydroxyl radicals (OH.) and hydrogen peroxide [59,60]. In fact, a ~3-log reduction was achieved when the bacteria were sonicated in water at 850 kHz and 50 W for 60 min (Results not shown). Because milk is an excellent free radicals scavenger; as a result, the free radicals produced during high-frequency ultrasonication were consumed by milk. This is due to the presence of several antioxidants in milk including vitamins and enzymes which scavenge radicals or hydrogen peroxide [61]. In addition, milk proteins and hydrolysates are also reported to have antioxidative activities [62] and both caseins and whey proteins in skim milk showed antioxidant activities [63]. It should be noted that high-frequency ultrasonication also results in mechanical effects, which could inactivate bacteria in water, however at a much less extent to that generated by low-frequency ultrasonication. To the best of our knowledge, there is only one report on the inactivation of bacteria in milk by high-frequency ultrasound. Munkacsi and Elhami [64] applied ultrasound treatment (800 kHz, 8.4 W/cm^2 for 1 min) followed by UV treatment. While the combination of the two treatments resulted in the inactivation of total bacteria and coliform, ultrasound treatment alone resulted in a 100% survival.

To confirm the effect of ultrasonication on *E. aerogenes* suspensions TEM observations were carried out (Fig. 2). Before sonication, the bacterial cells remained stable and intact although it can be clearly seen that they are surrounded by the casein micelles of milk (Fig. 2A2). After low-frequency ultrasonication, the bacterial cells were disrupted into misshapen structures made of cell debris (indicated by circles in Fig. 2B1 and B2). However, treatment by high-frequency ultrasound did not affect the bacteria cells, which remained intact and whole (Fig. 2C1 and C2).

3.2. Effects of ultrasonication on viscosity and particle size of skim milk

To investigate the effect of ultrasound treatment on skim milk (10 wt%) without bacteria, viscosity measurements and particle size determination were carried out under different sonication times. High-frequency ultrasound treatment of milk for up to 60 min did not result in any change in the viscosity of the milk (Fig. 3). However, low-frequency ultrasound treatment resulted in a slight decrease in viscosity after 5 min treatment. The initial viscosity of skim milk was 1.293 (±0.007) mPa s at 25 °C, then decreased to 1.231 (±0.001) mPa s after 5 min ultrasound treatment. Longer time treatment by low-frequency did not result in a marked

decrease in the viscosity. The values of the viscosity measured were 1.228 (±0.005), 1.220 (±0.001), 1.213 (±0.005) and 1.203 (±0.007) mPa s individually for 10, 20, 40 and 60 min ultrasonication, respectively (Fig. 3).

To investigate the observed changes in viscosity, particle size measurements were performed on the same milk samples treated by low and high frequency ultrasound. The intensity mean-average diameter as a function of sonication time is reported in Fig. 4. In the case of high-frequency ultrasound treatment, similar to viscosity, there are no changes in the mean diameter as a function of sonication time. While in the case of low-frequency ultrasound treatment, the particle size did decrease from 182.4 (±1.2) nm before ultrasound treatment to 168.6 (±1.2) nm after 5 min ultrasonication. The mean-size diameter also further decreased slightly when the low-frequency sonication time was increased, to reach to 152.8 (±1.2) nm after 60 min sonication. Milk is a complex colloidal dispersion, and the dispersed phase is made of casein micelles and fat globules, while the dispersion medium is an aqueous solution that includes whey proteins and lactose [65]. Although the milk used in this study is reconstituted from skim milk, it still contains some fat droplets. Thus it was important to investigate the effect of ultrasonication on their size, particularly that ultrasound is well-known to reduce substantially the size of fat droplets [42,43,66,67]. To do so, non-sonicated and sonicated milk samples were diluted in 0.1 M EDTA solution to dissociate casein micelles, prior to particle size measurement. The particle size measurement showed clearly that the average size did decrease from 104.0 (±0.8) nm to 90.4 (±0.0) nm after low-frequency ultrasonication for 60 min.

The decrease in viscosity and in particle size of skim milk after treatment with low-frequency ultrasound was previously reported. It was reported that the size of particles in skim milk decreased [66,68] and the decrease was related to the break-up of fat globules [66]. The decrease in viscosity was also previously reported for skim milk [66] and concentrated milks [69]. However, the reduction in particle size of the fat droplets without a change in the volume fraction should not affect the viscosity. In fact, for dilute suspension of non-interacting spherical particles, the viscosity η_{dis} is given by Einstein formula $\eta_{dis} = \eta_{sol}\ (1 + 2.5\phi)$ [70]. This equation involves only the viscosity of the continuous phase η_{sol} and the volume fraction ϕ occupied the particles. Further investigations of the state of the individual proteins in milk after ultrasound treatment were performed using RP-HPLC.

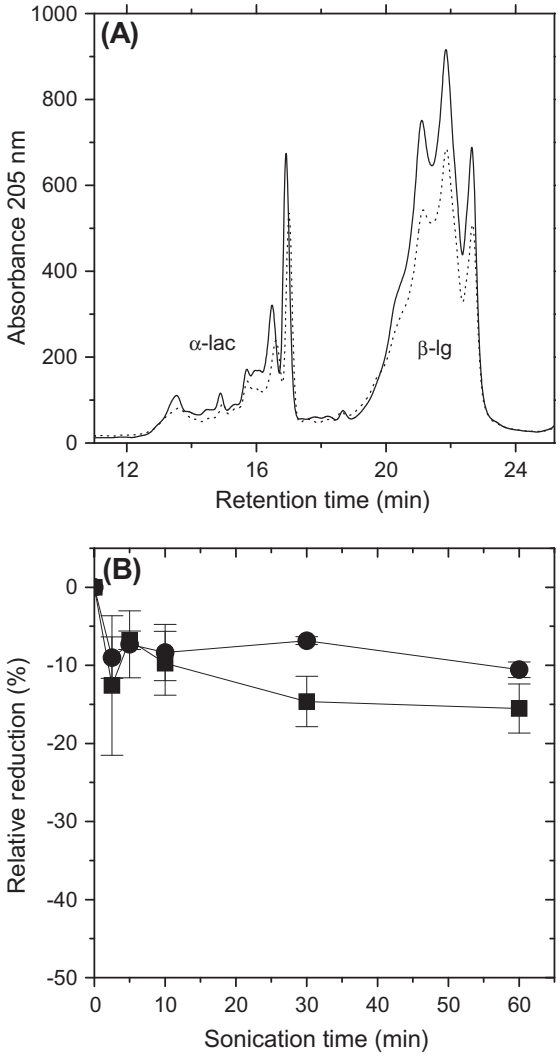

Fig. 6. (A) Chromatograms of whey proteins from skim milk by RP-HPLC: Control skim milk (solid line); Ultrasonicated skim milk, 30 min. 20 kHz, 8.5 W (Dotted line).(B) The relative reductions of concentration of whey proteins from RP-HPLC: α-lactalbumin (■) and β-lactoglobulin (●); Error bars correspond to standard deviation.

3.3. Effect of ultrasound treatment on milk proteins

Reversed-phase high-performance liquid chromatography (RP-HPLC), which separates proteins based on their molecular hydrophobicity [71], is considered as an accurate, rapid, and repeatable method to identify and quantify the concentration of milk proteins [32,33]. In this study, the caseins, namely κ-CN, α-CN (both α_{s1}-CN and α_{s2}-CN) and β-CN, and the whey proteins, α-Lac and β-Lg were considered. Typical RP-HPLC chromatograms of caseins and whey proteins are shown in Fig. 5A and Fig. 6A, respectively. The amount of each individual protein is obtained by integrating the corresponding RP-HPLC peak. The results are reported as the difference in area between the ultrasonicated milk and the non- ultrasonicated skim milk. The results of this exercise are reported in Fig. 5B and Fig. 6B for the caseins and whey proteins, respectively. In the case of caseins, the amounts of α-CN and β-CN remained nearly constant within experimental errors. However, the amount of κ-CN decreased by ~13% when the milk was sonicated for 10 min with a power of 50 W. Increasing further the treatment time did not result in a further decrease markedly the amount of κ-CN. In the case of whey proteins, the amount of both α-Lac and β-Lg decreased after 5 min sonication, and increasing the sonication time did not result in a further decrease in the amount of α-Lac and β-Lg. Amongst the whey proteins, approximately 8–15% and approximately 7–11% reductions were observed for α-Lac and β-Lg, respectively (Fig. 6B).

The results obtained in this study on the effect of ultrasound treatment on the individual milk proteins is in very good agreement with Shanmugam et al. [42], who also reported a decrease in whey proteins and κ-CN. In the case of decrease of whey proteins, these authors suggested that transient cavitation can easily damage the proteins causing denaturation of soluble whey proteins followed by their self-aggregation. Note however that aggregation of the whey proteins will lead to an increase in viscosity, while a reduction in viscosity is observed in this work. A decrease in the amount of κ-CN would lead to a decrease in viscosity. κ-CN is believed to be present mainly at the surface of the casein micelle [72]. If the cavitation generated by ultrasound is high enough to cleave the κ-CN, then the radius of the casein micelle would decrease slightly. The small decrease in the casein micelle size, and thus in their overall volume fraction, will lead to a reduction in viscosity. To confirm the decrease in casein micelle particle size, TEM micrographs obtained from both non-sonicated and sonicated milk (20 min, 8.5 W) were analysed using GIMP 2.8 software. After

analyzing 200 casein micelles under each condition, the results showed that the average diameter was changed from 111 ± 53 nm for the non-sonicated milk compared to 104 ± 57 nm for the sonicated sample. While these results should be taken with caution since particle size determination by microscopy is extremely difficult, they are nonetheless very encouraging since the decrease in the casein micelle reported by Shanmugam et al. [42] is in the order of magnitude (approximately 5 nm at the highest power they used). While this value seems small, it is certainly non-negligible and would explain the slight decrease in viscosity.

4. Conclusions

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

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

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