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Exploring the influence of ultrasound on the antibacterial emulsification stability of lysozyme-oregano essential oil

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ABSTRACT

A lysozyme-oregano essential oil (Lys-OEO) antibacterial emulsion was developed via ultrasonic treatment. Based on the general emulsion materials of ovalbumin (OVA) and inulin (IN), the addition of Lys and OEO successfully inhibited the growth of *E. coli* and *S. aureus*, two representatives of which were Gram-negative and Gram-positive bacteria respectively. The emulsion system in this study was designed to compensate for the limitation that Lys could only act on Gram-positive bacteria, and the stability of the emulsion was improved using ultrasonic treatment. The optimal amounts among OVA, Lys and OEO were found to be the mass ratio of 1:1 (Lys to OVA) and 20% (w/w) OEO. The ultrasonic treatment at the power of 200, 400, 600, and 800 W and time length of 10 min improved the stability of emulsion, in which the surface tension was below 6.04 mN/m and the Turbiscan stability index (TSI) did not exceed 10. The multiple light scattering showed that sonicated emulsions were less prone to delamination; salt stability and pH stability of emulsions were improved, CLSM image showed emulsion as oil-in-water type. In the meantime, the particles of the emulsions were found to become smaller and more uniform with ultrasonic treatment. The best dispersion and stability of the emulsion were both achieved at 600 W with a zeta potential of 7.7 mV, the smallest particle size and the most uniform particle distribution.

1. Introduction

Emulsion was a multiphase mixture consisting of two or more mutually immiscible liquids, one of which was mostly dispersed as small spherical droplets in the other phase [1]. It was not only widely used in the dissolution, encapsulation and controlled release of active ingredients in food, pharmaceutical and chemical industries [2], but also had great potential in the preparation of antibacterial materials [3–5]. The current industrialized antibacterial materials, which were mostly chemically synthesized, were facing great challenges of biosafety and environmental friendliness [3,6].

Natural antimicrobial materials derived from microorganisms, animals and plants (e.g., bacteriocins, lysozyme and plant essential oils) were considered alternatives to artificial antimicrobial materials due to their green processing and safety [7]. Lysozyme (Lys) was a natural alkaline protein found in organs, tissues, secretion, and especially chicken egg white, where Lys accounted for 3.5% of the egg white proteins [8,9]. Lys was composed of 129 amino acid residues and could inhibit the growth of bacteria by hydrolysing the peptidoglycan in bacterial cell walls [10,11]. The abundant source and the natural antibacterial properties of Lys could lead to its wide applications in food industry, medicine and agriculture [12]. However, the antibacterial activities of Lys mainly acted on Gram-positive bacteria but hardly on Gram-negative bacteria [13]. Broadening the antibacterial effects of Lys was of great importance. Oregano essential oil (OEO) was an extract from the stem and leaves of the whole herb of oregano. The carvacrol

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and thymol contained in OEO could alter the permeability of cell membrane and show good antibacterial activity against Gram-negative bacteria [14–16]. Combining Lys with OEO might be an efficient and convenient way to overcome the drawback of Lys, but the hydrophobic nature of OEO was a big challenge for its application [17].

Emulsion was thermodynamically unstable and its stability was usually reduced with different physicochemical environment [18,19]. Ultrasound was a green physical process that could affect the functional properties of emulsions by changing the structure and surface properties of proteins through mechanical shear and cavitation [20]. It was found that ultrasound treatment could reduce the β-LG allergenicity of milk by changing the structure of bovine milk proteins [21]. It could also improve the emulsifying properties of mussel myofibrillar protein by altering its fibrin structure [22]. The effects of ultrasonic treatment on Lys-OEO emulsion, including antibacterial properties, stability and structure remained unclear.

This experiment developed a well dispersed emulsion against both Gram-positive and Gram-negative bacteria by the addition of Lys and OEO as well as the introduction of ultrasound. Ovalbumin (OVA) which was commonly used in the preparation of base emulsions [23], and inulin (IN) which could improve the performance of ovalbumin emulsions [24,25], were selected as the materials for basic emulsions. The antibacterial effects of Lys and OEO were optimized. The effect of ultrasonic power on the emulsifying stability such as particle size, surface tension, salt stability and pH as well as rheological properties and microstructure of Lys-OEO emulsion was studied. The results could provide technical support for the application of Lys-based antibacterial emulsions and the development of new antibacterial emulsions.

2. Materials and methods

2.1. Materials and reagents

Lysozyme (Lys, purity > 98%) was purchased from Sigma-Aldrich (Missouri, USA); Oregano essential oil (OEO, purity > 98%) was purchased from Xi'an Tongze Biotechnology Co. (Xi'an, China); Ovalbumin (OVA, biotechnology grade) was purchased from Shanghai Maclean Biochemical Technology Co.(Shanghai, China); Inulin (IN, analytically pure) was purchased from FANINON, Qinghai, China (Xining, China); Nile Red (analytical pure) was purchased from Shanghai Maclean Biochemical Technology Co.(Shanghai, China); Fluorescein isothiocyanate (analytical pure) was purchased from Wuhan Ye Yuan Biotechnology Co. (Wuhan, China); Staphylococcus aureus and Escherichia coli were purchased from Shanghai Luwei Technology Co. (Shanghai, China). All the rest reagents used in the experiment were analytically pure and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of Lys-OEO emulsions

2.2.1. Preparation of Lys emulsions

The Lys emulsions were prepared following the method of Hu [25] with slight modifications. Lys was mixed with fixed mass of OVA in different mass ratios of 2:1, 3:2, 1:1, and 1:2, respectively. Then IN was added at an equal amount with OVA, stirred for 12 h (84-1A, Shanghai Siler Apparatus Co., Ltd., China), and then stored at 4 °C. The Lys emulsion was optimized by the antibacterial studies in section 2.3.

2.2.2. Addition of OEO

The emulsions were first prepared following the best results in section 2.2.1 and stirred for 12 h. Then OEO was added to the emulsion at 0%, 8%, 10%, 12% and 20% (w/w) and sheared at 10,000 rpm/min for 3 min using a shear (IKA18, Kinematica Co., Switzerland) to produce a fresh emulsion. The amount of OEO was optimized by the antibacterial studies in section 2.3. The emulsion containing optimized amounts of Lys and OEO was named Lys-OEO emulsion.

2.3. Antibacterial activities

Staphylococcus aureus (*S. aureus*, ATCC6538) and Escherichia coli (*E. coli*, CMCC(B)44102) were used as the target. *S. aureus* at a dilution of 5×10^8 CFU/mL and *E. coli* at a concentration of 5×10^7 CFU/mL were mixed in equal volumes with the emulsion and incubated in a shaker at 37 °C and 200 rpm for 48 h. The surviving *S. aureus* and *E. coli* in the mixture were inoculated onto plates after dilution and incubated in a biochemical incubator at 37℃ for 36 h. The growth of *S. aureus* and *E. coli* under different ultrasound treatment conditions were compared via colony counting.

2.4. Ultrasonic processing

The Lys-OEO emulsions were treated by different powers of ultrasound at 0, 200, 400, 600 and 800 W for 10 min. Effects of ultrasonic power on the surface tension, particle size, zeta potential, multiple light scattering, salt stability and pH stability were studied.

2.5. Stability of Lys-OEO emulsions

2.5.1. Surface tension

The interfacial properties of Lys-OEO emulsions at the oil/water interface were measured at 25 °C using a Tracker-type interfacial rheometer (TRACKER, TECLIS Interface Technologies Ltd., France). Poured 6.0 mL Lys-OEO emulsion into a container, submerged the sample needle into the emulsion and formed 10 μL oil droplets by motor control. The entire measurement time was set to 3,600 s to allow the adsorption of the Lys-OEO emulsion particles at the interface to reach balance.

2.5.2. Particle size and zeta potential

The Lys-OEO emulsions were diluted with ultrapure water to a mass fraction of 0.02%. The zeta potential was determined using a laser particle sizer (Nano ZS + MPT-2, Malvern, USA) at room temperature, three times in parallel for each sample. Similarly, the Lys-OEO emulsions were dispersed in ultrapure water to a mass fraction between 1% and 5% and the mean particle size was determined by laser particle sizer (Nano ZS + MPT-2, Malvern, USA) at room temperature, three times in parallel for each sample.

2.5.3. Multiple light scattering

The multiple light scattering of Lys-OEO emulsions was measured by Turbiscan Lab (Lundyson Technology Co., Ltd., Beijing). Three cycles were set in the measurements, in which the first cycle was scanned every 3 min for 3 h, the second cycle was scanned every 30 min for 4 h, and the third cycle was scanned every 60 min for 5 h.

2.5.4. Salt stability and pH stability

The stability of Lys-OEO emulsions at different pH and ionic strength was investigated using the particles in emulsion particle as an indicator. To determine the pH stability of the emulsions, 0.01, 0.02, 0.05 and 0.1 mol/L NaCl water solutions were added to Lys-OEO emulsions, stirred for 1 h, left to stand for 24 h and then the particle size distribution was measured. To determine the salt stability of the Lys-OEO complex emulsion, the pH of Lys-OEO emulsions was adjusted to 3, 5, 7, 9, 11, left to stand for 24 h, and then the sample size distribution was measured as mentioned in section 2.5.2.

2.6. Rheological properties

The effects of ultrasonic power on rheological properties of Lys-OEO emulsions were analysed referring to the method of Xiong et al. [26] with slight modifications. Lys-OEO emulsions were treated by 200, 400, 600 and 800 W ultrasound for 10 min, respectively. Then a rotational rheometer (Haake Rheostress 6000, Thermo Fisher Co., USA) was used

to determine the rheological properties of the emulsions. A 35 mm diameter parallel plate with a gap of 0.5 mm was used. Viscosity scan, amplitude scan, frequency scan, thixotropy scan and creep scan were performed, where in viscosity scan, the range of shear rate was set from 0.1 to 100 s^{-1}, in amplitude scan, the frequency was fixed at 1 Hz and the shear strain was set from 0.001% to 1000%; in frequency scan, the amplitude was fixed at 0.5% and the frequency was set from 0.01 to 10 Hz; in creep scan, the range of shear rate was set from 0.1 to 100 s^{-1} and the strain was selected as 1 Pa, in the thixotropic scan, the shear rates were set at 0.1 s^{-1} for 120 s, 10 s^{-1} for another 120 s, and then back to 0.1 s^{-1} for 120 s, recording changes in viscosity with time.

2.7. Microstructure

The surface morphology of Lys-OEO emulsions was observed by an optical microscope (CX31RYSF, OLYMPUS, Philippines). 10 μL emulsion was dropped on a clean slide, covered with a coverslip, placed under the microscope and observed using 20x objective lens.

The structure of Lys-OEO emulsions was observed using confocal laser scanning microscopy (CLSM, Leica TSC SP8, Leica, Germany) by the method of Du et al. [27] with slight modifications. 20 μL Nile Red (1 mg / mL) and 20 μL FITC (1 mg / mL) dyes were dropped into 1 mL Lys-OEO emulsions. After mixed thoroughly, 20 μL mixture was taken on a slide, covered with a coverslip, and observed under a 20 × lens with excitation wavelengths at 488 nm and 638 nm.

2.8. Effects of ultrasound on antibacterial properties

The emulsions were prepared using the best results in section 2.2.2. Then the emulsions were sonicated for 10 min at 0, 200, 400, 600 and 800 W using an ultrasonic cell crusher (SCIENTZ-IID, Ningbo Xinzhi Biotechnology Co., Ltd., China). The samples were placed in ice bath during ultrasound to control the temperature. The effects of ultrasound on antibacterial activities were studied following the procedures in section 2.3.

3. Results and discussion

3.1. Preparation of Lys-OEO emulsions

3.1.1. Optimization of Lys

Fig. 1A & 1B showed the effects of Lys on the inhibition of *E. coli* and *S. aureus*, respectively. *E. coli* was a common type of Gram-negative bacteria, and *S. aureus* was one of the representatives of Gram-positive bacteria. With the increased amounts of Lys, the growth of *S. aureus* was greatly inhibited, especially when the mass ratio of OVA to Lys reached 1:1, no active *S. aureus* was found. In the test, though *E. coli* grew slower with the increasing amount of Lys, the growth did not stop even when the mass ratio of OVA to Lys reached 1:2. Lys could effectively hydrolyse the β-1,4-gLycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamide (NAG) of peptidoglycan in bacterial cell walls and therefore showed strong antibacterial activities on Gram-positive bacteria [28]. However, the cell wall of Gram-negative bacteria usually contained less peptidoglycan and had an additional protective layer of lipopolyaccharides outside the peptidoglycan layer [8,28]. Therefore, the antibacterial activities of Lys on Gram-negative bacteria were limited. Fig. 1C demonstrated the particle size destruction in different mass ratios of Lys. The average particle sizes were found first increase and then decrease with the increasing amounts of Lys. Particles were evenly distributed in all samples, and>80% of the particles were found between 6 and 30 μm. The mass ratio of OVA and Lys was selected as 1:1 for subsequent studies.

3.1.2. Optimization of OEO

Fig. 2A & 2B showed the bacterial inhibition of OEO in *E. coli* and *S. aureus* on the basis of the addition of Lys, respectively. With the increasing amounts of OEO, the number of *E. coli* colonies on the plate gradually decreased, and the strongest inhibition was found in 20%(w/w) of OEO. However, due to the hydrophobic nature of OEO, 20% OEO had significantly affected the stability and dispersibility of the emulsion. As shown in Fig. 2C, although the particle size tended to increase with the addition of OEO, >80% of the particles were still in the range of 4 and 30 μm. Based on the study of OEO, 20% (w/w) addition of OEO was mixed with different proportions of protein to prepare the Lys-OEO complex emulsion.

3.2. Effect of ultrasonic power on the stability of the Lys-OEO emulsions

3.2.1. Effect of ultrasonic power on the surface tension

The interfacial behaviour of emulsions was closely related to the stability of emulsions. The lower the surface tension of emulsions, the better the stability [29]. As was shown in Fig. 3, the surface tension of the un-sonicated emulsion was much higher than that of the sonicated emulsions, the initial surface tension of the un-sonicated emulsion was found at 7.60 mN/m and that of the sonicated emulsions below 6.04 mN/m. With the increase of adsorption time, the surface tension was found decrease in all samples and reach an approximate equilibrium at 3000 s. The results suggested that the ultrasound treatment accelerated the adsorption rate at the oil/water interface and improved the stability of the emulsions. This was probably because that the ultrasonic

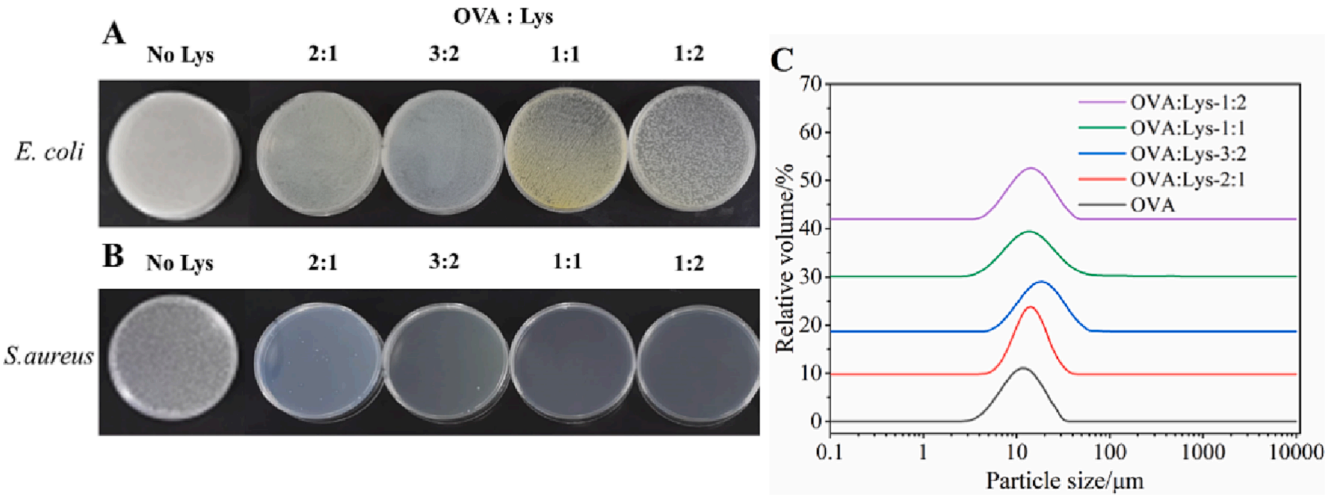

Fig. 1. Effects of the amounts of Lys on antibacterial activity and particle size distribution. (A) *E. coli*, (B) *S. aureus* and (C) Particle size distribution.

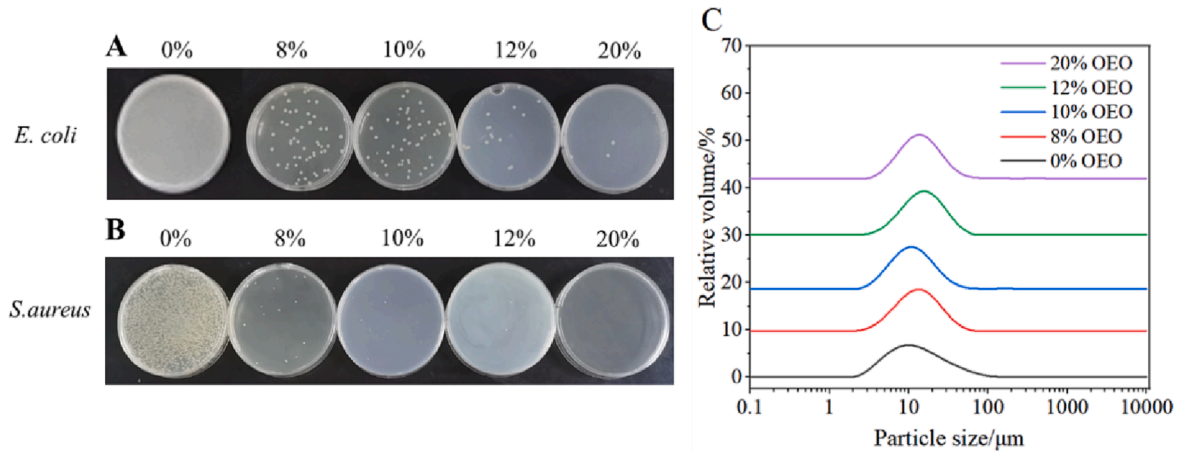

Fig. 2. Effect of OEO on bacteriostasis and particle size. (A) *E. coli*, (B) *S. aureus* and (C) Particle size distribution.

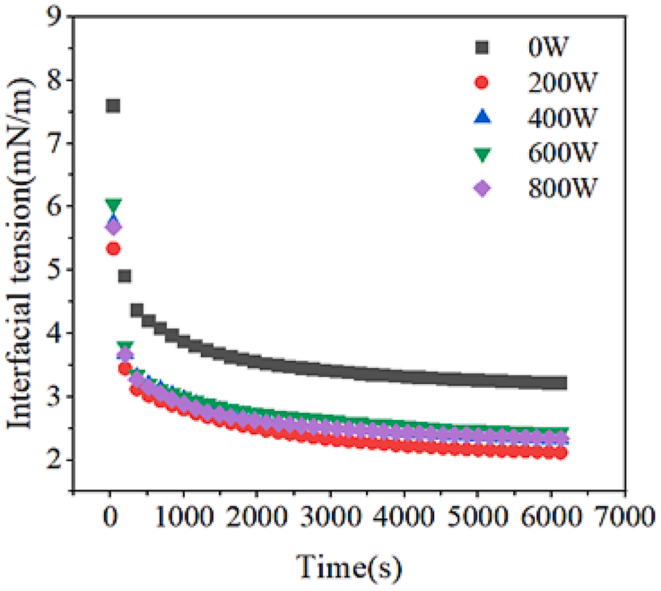

Fig. 3. Effect of ultrasonic treatment on the surface tension of Lys-OEO emulsions.

treatment exposed the hydrophilic groups inside the protein structure and in turn enhanced the adsorption capacity of the protein at the oil–water interface, leading to a decrease in surface tension, which was consistent with the findings of Ai et al. [30].

3.2.2. *Effect of ultrasonic power on particle size distribution and Zeta potential*

The effect of sonication on the dispersion and size of the emulsion droplets was evaluated in Fig. 4. The particle size of emulsion reduced after ultrasonic treatment. The probable reason was that the ultrasound treatment enhanced the interaction between the protein and IN, forming a complex that facilitated the stability of the emulsion, whereas in the un-sonicated emulsion the protein structure was not fully developed, so the interaction between the protein and IN was weak, reducing the interfacial binding ability of the protein and IN [25]. It was also shown that the interactions among droplets of ultrasound treated emulsion was inhibited due to spatial site resistance, which was more conducive to improving the stability of emulsions [30].

Zeta potential shown in Fig. 4 B was an important parameter to describe the surface charge of proteins [31]. The Zeta potential of the un-sonicated emulsion was 5.5 mV, and showed a trend of first increasing and then decreasing as the sonication power increased from 0 to 800 W. The largest Zeta potential of the emulsion was found 7.7 mV at 600 W, while the lowest potential was 4.41 mV at 800 W. The decrease Zeta potential at 800 W was probably because that excessive ultrasound power promoted the re-agglomeration of protein, reducing the electrostatic interactions between droplets and oil droplets [32].

3.2.3. *Effect of ultrasonic treatment on TSI and BS intensity*

The stability of the emulsion was evaluated using the Turbiscan stability index (TSI). The TSI represented the dynamic process of sample

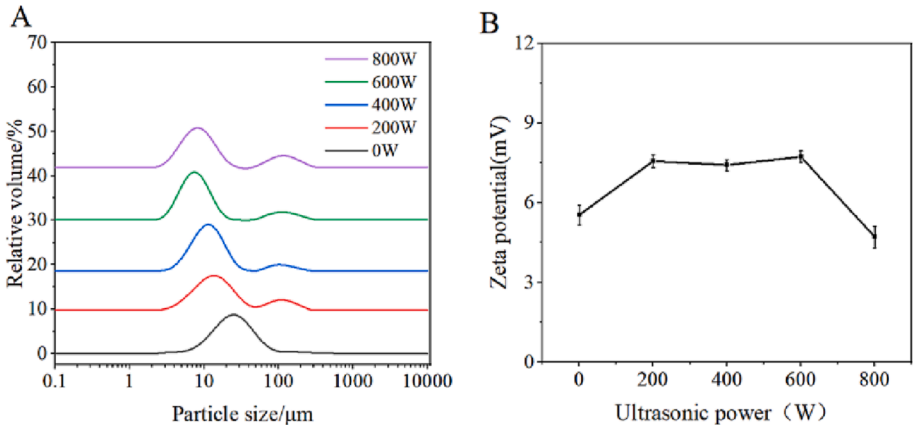

Fig. 4. Effect of ultrasonic treatment on (A) Particle size and (B) Zeta potential of Lys-OEO emulsions.

agglomerating and settling at different times, the smaller the TSI, the more stable the system [33]. As was shown in Fig. 5A, the TSI values of emulsions first increased rapidly, then increased slowly, and finally reached the equilibrium state. The TSI value of the non-sonicated emulsions rose rapidly and finally stagnated at about 18. Much slower increase of TSI values was observed in ultrasonicated emulsions and did not exceed 10 at the final equilibrium status. It indicated that the stability of emulsions was significantly improved after ultrasonication.

Fig. 5B to 5F showed the relationship between the backscattered light (BS) intensity of the emulsion system and the height of the emulsions treated by different ultrasonic powers from 0 to 800 W. These images demonstrated that the height of the un-sonicated emulsion was corresponding to the horizontal section increased significantly (Fig. 5B), indicating that there was a large amount of stratification in the lower layer of the emulsion. The changes of height were much smaller in the sonicated emulsions, indicating that the emulsions were almost free of delamination. The best stability was found at 600 W, where the heights of emulsions all maintained at around 1500 μm (Fig. 5E). In addition, the intensity of backscattered light in the horizontal section of the sonicated emulsions was basically unchanged, so no oil phase was precipitated from the upper layer of the emulsions, and the original fluid state of emulsion was maintained [33].

3.2.4. Effect of ultrasonic treatment on the ionic strength and pH

The stability of the emulsions tested in different ion concentrations and pH conditions was shown in Fig. 6A and 6B, respectively. In the un-sonicated emulsion, the particle size decreased first and then increased with the increase of ionic strength, while the influence of pH showed an opposite trend. The effects of ionic strength and pH on the particle size of the ultrasound treated emulsions were much smaller, and at the power of 600 W, the most stable particle size was found.

3.3. Effect of ultrasonic treatment on the rheological properties

3.3.1. Viscosity

During ultrasound treatment, cavitation and the unfolding of protein chains usually led to rapid molecular movement, affecting the flow of the emulsion [34]. The apparent viscosity was often used to characterize the change in the flow [26]. As was shown in Fig. 7, all the emulsions exhibited shear thinning behaviour, and the viscosity of the emulsions first increased and then decreased with the increasing of ultrasonic power with the highest viscosity at 400 W. The decrease of viscosity with ultrasonic power from 400 to 800 W was probably because that the excessive power destroyed the ordered structure of protein to an irregular structure, and the irregular shape of the protein arrangement impeded the flow of the emulsion [27].

3.3.2. Amplitude sweeps and frequency sweeps

Energy storage modulus/loss modulus was the amount of energy stored/loss due to elastic/viscous deformation of the material and reflected the elastic/viscous size of the material [35]. Fig. 8A showed the effects of ultrasonic power on G' and G''. G' and G'' were found to decrease with the increase of strain in all samples. At lower strain between 0.001% and 0.1%, G' and G'' were basically unchanged, and the structural destruction by stress was not obvious at this time. When the strain gradually increased from 0.1% to 1%, the deformation became more serious, especially at 800 W, where G' decreased from 55.03 Pa to 0.07 Pa and G'' decreased from 15.19 Pa to 0.67 Pa, causing an intersection near 0.323%. At larger strain > 1%, G'' was gradually greater than G', and the emulsion was changed from elastic structure-dominated to viscous-dominated. Furthermore, Fig. 8B indicated that G' was always higher than G'' in the linear viscoelastic region between 0.01 Hz and 10 Hz. Therefore, the elastic properties of the emulsions were dominant and G' was always greater than G'' (G' ranged from 26.14 to

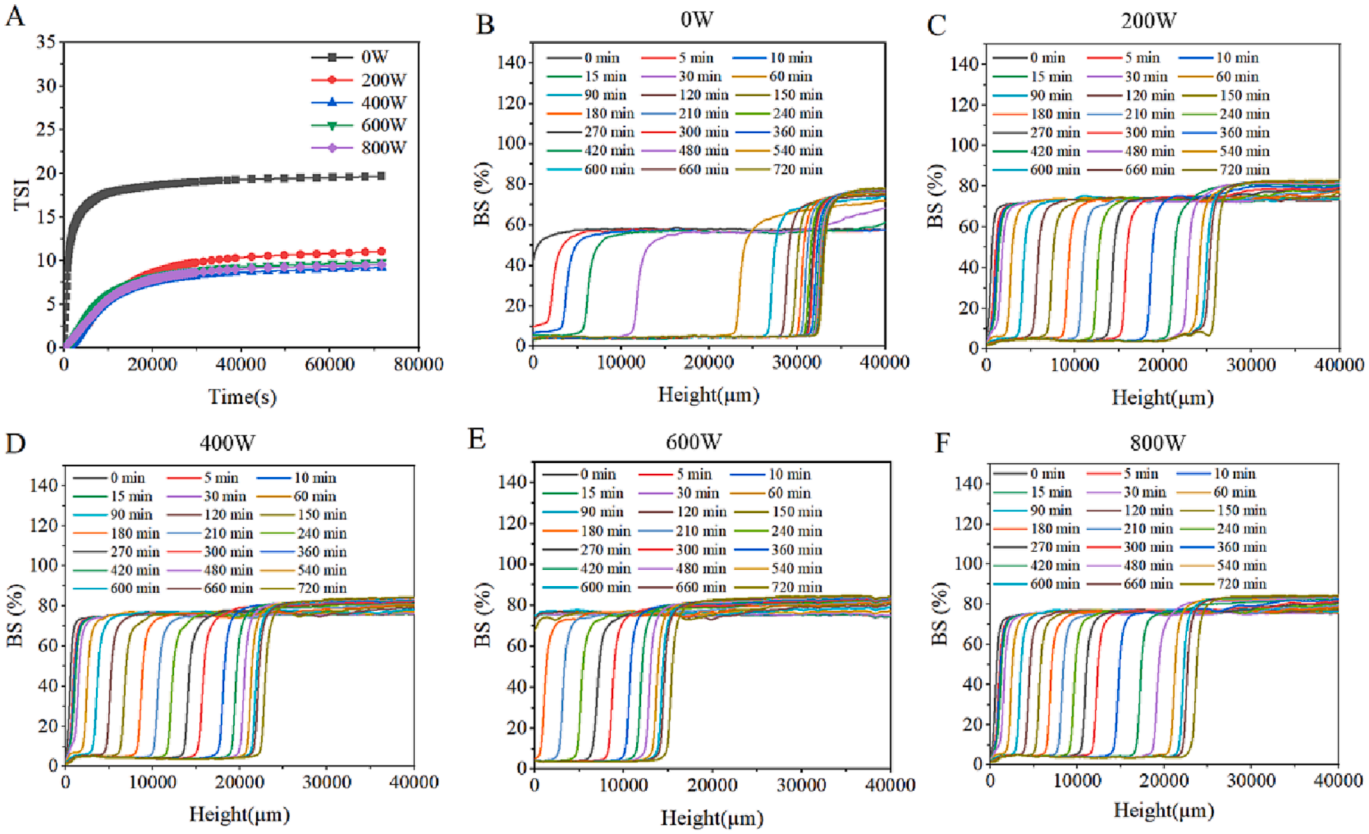

Fig. 5. Effect of ultrasonic treatment on TSI and BS intensity of emulsion: (A) TSI index vs. time; (B) BS intensity of un-sonicated emulsion; (C) BS intensity of emulsion treated at 200 W; (D) BS intensity of emulsion treated at 400 W; (E) BS intensity of emulsion treated at 600 W; (F) BS intensity of emulsion treated at 800 W

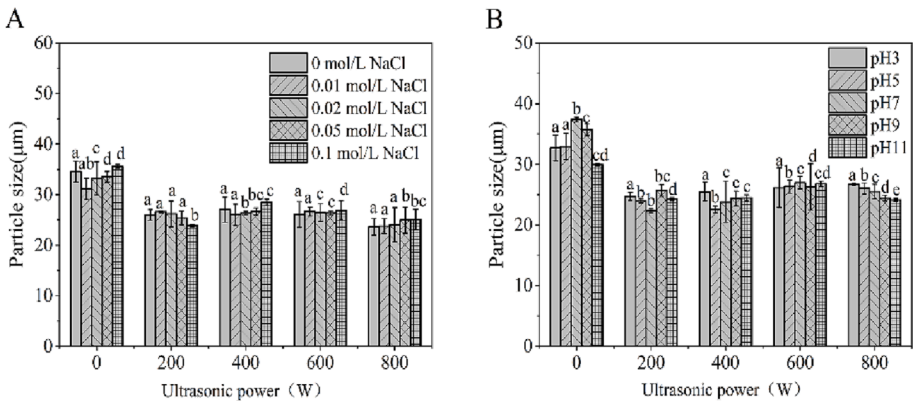

Fig. 6. Effect of (A) ionic strength and (B) pH on the particle size of Lys-OEO emulsions.

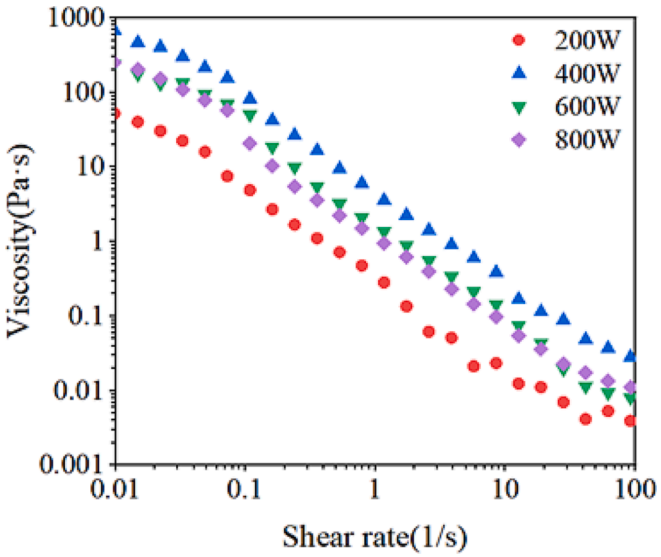

Fig. 7. Effect of ultrasonic treatment on the viscosity of Lys-OEO emulsions.

1096.90 Pa, while G'' ranged from 9.95 to 363.28 Pa), indicating that all samples had gel-like nature with excellent network structure [27].

3.3.3. Thixotropy and creep

The effect of ultrasound treatment on the viscoelastic relaxation behaviour of emulsions was further discussed by thixotropy and creep behaviour [36] in Fig. 9. As was shown in Fig. 9 A, when the ultrasonic power was higher, the emulsion system exhibited lower creep strain levels (e.g., 9.00% and 9.59% strains for ultrasonic power of 600 W and 800 W, respectively); after the emulsion was deformed under stress conditions and the stress effect was withdrawn instantaneously after a duration of 60 s, the deformation of the emulsion decreased rapidly (e.g., from 9% to 4.93% strain for ultrasonic power of 600 W), indicating that the structure of the emulsion gradually recovered to its original state. Based on the recovery of sample, it was clear that a larger ultrasonic power was beneficial to improve the viscoelasticity and stability of the emulsions. The thixotropic behaviour of the emulsion was shown in Fig. 9B, the emulsions treated by different ultrasound power reacted similarly, and the emulsion samples were equilibrated at a lower shear rate of 0.1 s^{-1} for 120 s. Sample viscosities within 120 s were in the range from 8.89 to 25.78 Pa·s. Then the shear rate was increased to 10 s^{-1} and the emulsions were equilibrated for 120 s, the viscosity decreased significantly to the range from 0.27 to 0.50 Pa·s. When the shear rate was adjusted to the initial value of 0.1 s^{-1} again, the viscosity increased from 9.41 to 18.62 Pa·s, which indicated a certain recovery of the emulsions, and the recovery of the emulsion treated by different sonication powers did not differ significantly.

3.4. Effect of ultrasonic treatment on the structure of Lys-OEO emulsions

3.4.1. Optical microscope

Fig. 10A displayed the situation of Lys-OEO emulsions treated by different ultrasonic powers. Obvious delamination was observed in the un-ultrasonic emulsion, while the ultrasonic-treated emulsions were more homogeneous. Differences in morphology of the emulsions treated at different ultrasonic powers were compared in Fig. 10B to 10F using an optical microscope. The droplets became smaller with the increase of

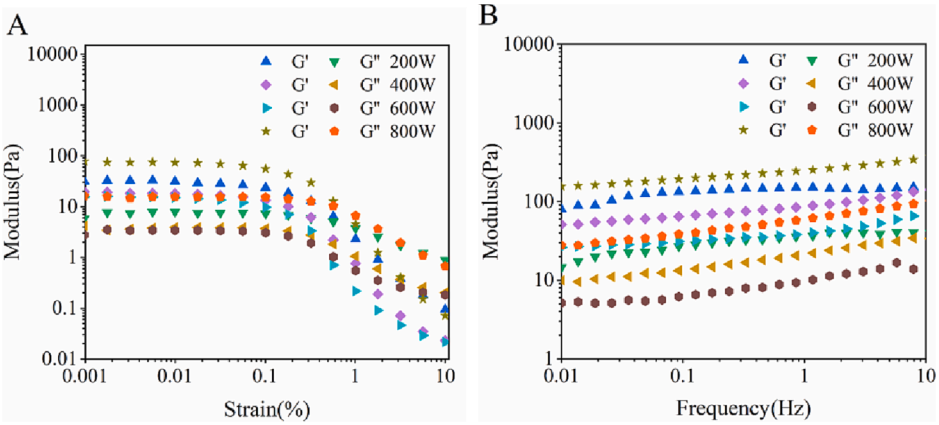

Fig. 8. (A) Amplitude sweeps and (B) Frequency sweeps of Lys-OEO emulsions treated by different ultrasonic powers.

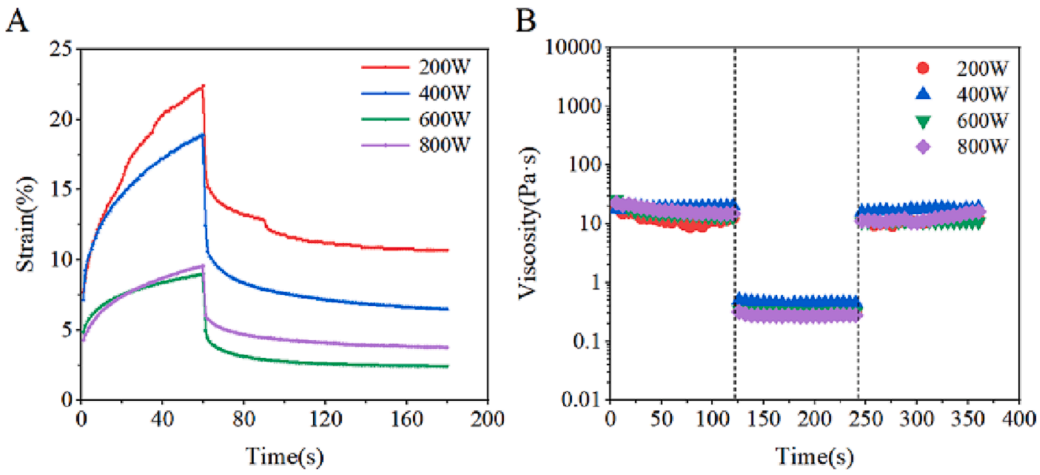

Fig. 9. (A) Strain responsive and (B) Thixotropic behavior of Lys-OEO emulsions treated by different ultrasonic powers.

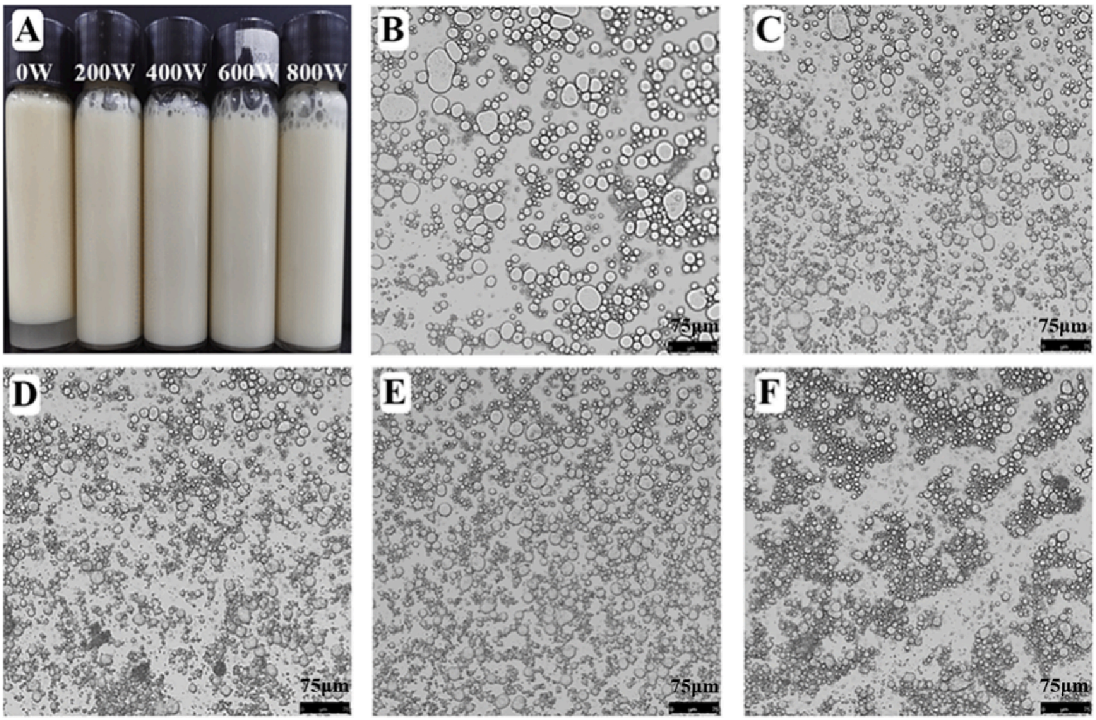

Fig. 10. (A) Pictures of Lys-OEO emulsion, (B-F) Optical microscope images of Lys-OEO emulsions treated by different ultrasonic powers: 0 W, 200 W, 400 W, 600 W and 800 W, respectively.

ultrasonic power, which was consistent with the results of particle size distributional. In particular, the droplets of the emulsion were uniformly distributed at 600 W, while at 800 W a clear tendency of oil droplet aggregation was observed, indicating that an excessive ultrasonic energy could cause protein aggregation and result in reduced stability [34].

3.4.2. CLSM

The interfacial composition of the emulsion and the adsorption of the emulsifier at the oil–water interface were observed via CLSM. It was shown in Fig. 11 that the FITC-stained proteins were represented by green fluorescent signals and the oil droplets stained using Nile Red were represented by red fluorescent signals. The particles were mainly distributed on the surface of the oil droplets, indicating that the emulsion type was oil-in-water. Compared with the emulsions without ultrasonic treatment, the distribution of which was uneven and prone to aggregation, the emulsions treated by ultrasound had more homogeneous distributed particles and significant smaller particle size, which was consistent with the change of particle size in the above studies. With the increase of ultrasonic power, particle distribution on the surface of the oil droplets became denser, and the formation of interfacial film were more obvious. When the ultrasonic power was higher than 400 W, an interfacial film was formed on the surface of the oil droplets, which hindered the aggregation of oil droplets and improved the physical stability of the emulsion [37].

3.5. Effect of ultrasound treatment on the antibacterial properties

The effect of different ultrasound powers on the antibacterial activities of the Lys-OEO emulsions was evaluated by *E. coli* (Fig. 12A) and *S. aureus* (Fig. 12B). Only *E. coli* showed a few colonies on the plate without ultrasound treatment, while no colony was observed on the other plate. The results indicated that the ultrasound treatment

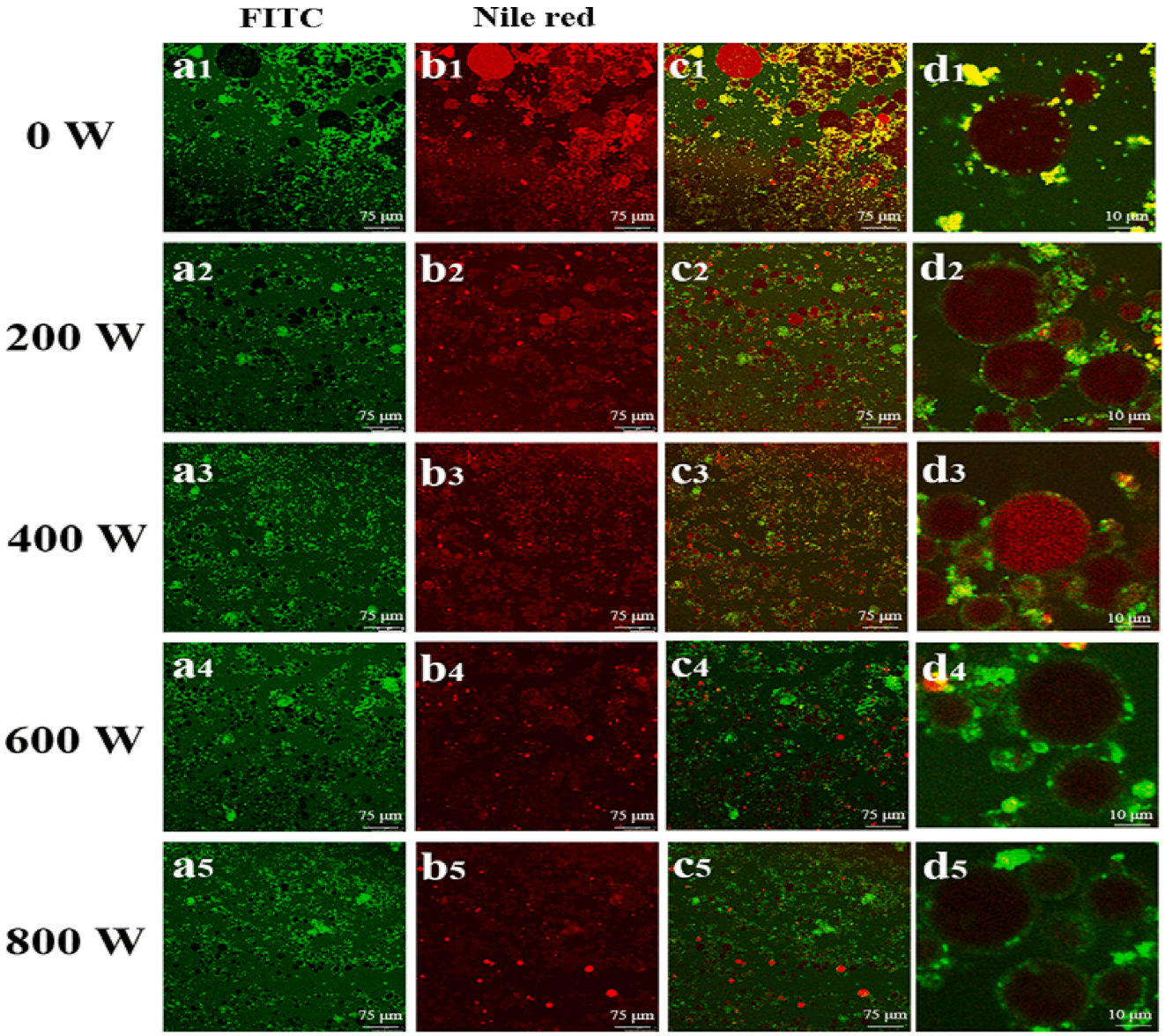

Fig. 11. CLSM of Lys-OEO emulsions treated by different ultrasonic powers (a: single FITC fluorescence image; b: single Nile red fluorescence image; c: Nile red and FITC dual channel superposition diagram; d: amplified image of the superposition diagram).

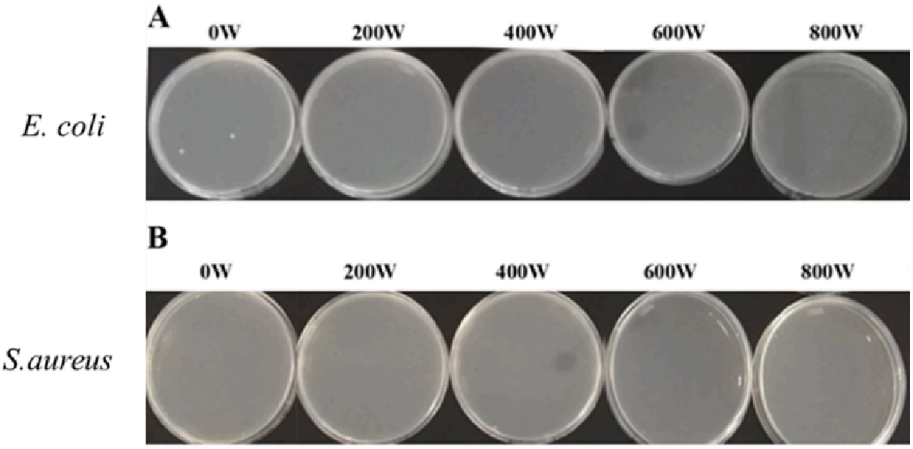

Fig. 12. Effect of ultrasonic treatment on antibacterial activity of the Lys-OEO emulsions: (A) *E. coli*, (B) *S. aureus*.

improved the antibacterial properties of the emulsion.

4. Conclusion

In this study, an emulsion with effective antibacterial activity and improved stability was developed using Lys and OEO with ultrasonic treatments. On the basis of common basic emulsion composed of OVA and IN, the addition of OEO together with Lys overcame the drawback of Lys that acted only on Gram-positive. The optimized Lys-OEO emulsion, which contained the amounts of Lys at a mass ratio of 1:1 to OVA and 20% (W/W) OEO successfully inhibited the growth of both Gram-positive and Gram-negative bacteria. Ultrasonic treatment not only contributed to the antibacterial activities of the Lys-OEO emulsion, but also improved the dispersion and stability. The emulsions ultrasonicated at 200, 400, 600 and 800 W for 10 min showed small interfacial tension and particle size, as well as high salt stability and pH stability. The results of stability measurement showed that the stability of emulsions tended to increase and then decrease with the increase of ultrasonic power. The ultrasonic power also changed the rheological properties of emulsions, and a higher ultrasonic power was beneficial to improve the viscoelasticity and stability of emulsions. In the optical microscope images, the particle size of emulsions decreased with the increase of ultrasonic power, but excessive ultrasonic power caused aggregation of droplets. CLSM showed that an interfacial protein layer formed on the surface of oil droplets to prevent droplets from aggregation. The emulsions treated at 600 W simultaneously achieved uniform particle size distribution, good stability, reduced apparent viscosity and a stable emulsion system. The research provided a technical support for the development of Lys-based antibacterial emulsions as well as an efficient ultrasonic method for the dispersion and stability of emulsions, and ir provided a promising future for emulsion in food preservation industry.

CRediT authorship contribution statement

Mengzhen Zhong: Methodology, Data curation, Writing – original draft. **Lulu Ma:** Formal analysis, Writing – review & editing. **Xin Liu:** Investigation, Formal analysis. **Ying Liu:** Investigation, Data curation. **Shuaishuai Wei:** Investigation, Formal analysis. **Ying Gao:** Formal analysis, Writing – review & editing. **Zhan Wang:** Methodology, Data curation. **Shang Chu:** Methodology. **Shijian Dong:** Resources, Data curation. **Yuping Yang:** Data curation. **Sihai Gao:** Supervision, Methodology, Formal analysis. **Shugang Li:** Project administration, Supervision, Conceptualization, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] J.V.M. Benetti, J.T.D. Silva, V.R. Nicoletti, SPI microgels applied to Pickering stabilization of O/W emulsions by ultrasound and high-pressure homogenization: rheology and spray drying, Food Res. Int. 122 (2019) 383–391, https://doi.org/10.1016/j.foodres.2019.04.020.

[2] N. Nikmaram, S. Roohinejad, S. Hashemi, M. Koubaa, F.J. Barba, A. Abbaspourrad, R. Greiner, Emulsion-based systems for fabrication of electrospun nanofibers: food, pharmaceutical and biomedical applications, RSC Adv. 7 (2017) 28951–28964, https://doi.org/10.1039/c7ra00179g.

[3] J. Su, Q. Guo, L. Mao, Y. Gao, F. Yuan, Effect of gum arabic on the storage stability and antibacterial ability of β-lactoglobulin stabilized d-limonene emulsion, Food Hydrocoll. 84 (2018) 75–83, https://doi.org/10.1016/j.foodhyd.2018.05.041.

[4] E. Mauriello, G. Ferrari, F. Donsi, Effect of formulation on properties, stability, carvacrol release and antimicrobial activity of carvacrol emulsions, Colloid Surf. B-Biointerfaces 197 (2021) 10, https://doi.org/10.1016/j.colsurfb.2020.111424.

[5] H. Cheng, M.A. Khan, Z.F. Xie, S.N. Tao, Y.X. Li, L. Liang, A peppermint oil emulsion stabilized by resveratrol-zein-pectin complex particles: Enhancing the chemical stability and antimicrobial activity in combination with the synergistic effect, Food Hydrocoll. 103 (2020) 13, https://doi.org/10.1016/j.foodhyd.2020.105675.

[6] F.P. Yi, K.W. Wu, G.F. Yu, C. Su, Preparation of Pickering emulsion based on soy protein isolate-gallic acid with outstanding antioxidation and antimicrobial, Colloid Surf. B-Biointerfaces 206 (2021) 8, https://doi.org/10.1016/j.colsurfb.2021.111954.

[7] M. Carocho, P. Morales, I.C.F.R. Ferreira, Natural food additives: Quo vadis? Trends Food Sci. Technol. 45 (2015) 284–295, https://doi.org/10.1016/j.tifs.2015.06.007.

[8] T. Silvetti, Use of Hen Egg White Lysozyme in the Food Industry 233–242 (2017), https://doi.org/10.1016/B978-0-12-800879-9.00022-6.

[9] P. Ferraboschi, S. Ciceri, P. Grisenti, Applications of Lysozyme, an Innate Immune Defense Factor, as an Alternative Antibiotic, Antibiotics 10 (2021) 1534.

[10] Y. Zhao, Z. Chen, J. Li, M. Xu, Y. Shao, Y. Tu, Changes of microstructure characteristics and intermolecular interactions of preserved egg white gel during pickling, Food Chem. 203 (2016) 323–330, https://doi.org/10.1016/j.foodchem.2016.02.044.

[11] K. Behera, F.A. Wani, A. Bhat, S. Juneja, M.K. Banjare, S. Pandey, R. Patel, Behavior of lysozyme within ionic liquid-in-water microemulsions, J. Mol. Liq. 326 (2021) 10, https://doi.org/10.1016/j.molliq.2021.115350.

[12] G. Leśnierowski, T. Yang, Lysozyme and its modified forms: A critical appraisal of selected properties and potential, Trends Food Sci. Technol. 107 (2021) 333–342, https://doi.org/10.1016/j.tifs.2020.11.004.

[13] A.M. Abdou, S. Higashiguchi, A.M. Aboueleinin, M. Kim, H.R. Ibrahim, Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against Bacillus species, Food Control 18 (2007) 173–178, https://doi.org/10.1016/j.foodcont.2005.09.010.

[14] H. Lu, J.A. Butler, N.S. Britten, P.D. Venkatraman, S.S. Rahatekar, Natural Antimicrobial Nano Composite Fibres Manufactured from a Combination of Alginate and Oregano Essential Oil, Nanomaterials 11 (8) (2021) 2062.

[15] F.G. Niu, W.C. Pan, Y.J. Su, Y.J. Yang, Physical and antimicrobial properties of thyme oil emulsions stabilized by ovalbumin and gum arabic, Food Chem. 212 (2016) 138–145, https://doi.org/10.1016/j.foodchem.2016.05.172.

[16] M. Wu, Z.L. Zhou, J. Yang, M. Zhang, F. Cai, P. Lu, ZnO nanoparticles stabilized oregano essential oil Pickering emulsion for functional cellulose nanofibrils packaging films with antimicrobial and antioxidant activity, Int. J. Biol. Macromol. 190 (2021) 433–440, https://doi.org/10.1016/j.ijbiomac.2021.08.210.

[17] F. Plati, A. Paraskevopoulou, Micro- and Nano-encapsulation as Tools for Essential Oils Advantages' Exploitation in Food Applications: the Case of Oregano Essential Oil, Food Bioproc. Tech. 15 (5) (2022) 949–977.

[18] W.N. Wang, R.Y. Wang, J. Yao, S.N. Luo, X. Wang, N. Zhang, L.Q. Wang, X.Q. Zhu, Effect of ultrasonic power on the emulsion stability of rice bran protein-chlorogenic acid emulsion, Ultrason. Sonochem. 84 (2022) 10, https://doi.org/10.1016/j.ultsonch.2022.105959.

[19] D. Rousseau, Fat crystals and emulsion stability — a review, Food Res. Int. 33 (2000) 3–14, https://doi.org/10.1016/S0963-9969(00)00017-X.

[20] G. Schaafsma, J.L. Slavin, Significance of Inulin Fructans in the Human Diet, Compr. Rev. Food. Sci. Food Saf. 14 (2015) 37–47.

[21] F. Yang, L. Zou, Y. Wu, Z. Wu, A. Yang, H. Chen, X. Li, Structure and allergenicity assessments of bovine beta-lactoglobulin treated by sonication-assisted irradiation, J. Dairy Sci. 103 (2020) 4109–4120, https://doi.org/10.3168/jds.2019-17070.

[22] C.P. Yu, S.H. Li, S. Sun, H.J. Yan, H.N. Zou, Modification of emulsifying properties of mussel myofibrillar proteins by high-intensity ultrasonication treatment and the stability of O/W emulsion, Colloid Surf. A-Physicochem. Eng. Asp. 641 (2022) 14, https://doi.org/10.1016/j.colsurfa.2022.128511.

[23] X.J. Ma, J.Y. Gao, H.B. Chen, Combined effect of glycation and sodium carbonate-bicarbonate buffer concentration on IgG binding, IgE binding and conformation of ovalbumin, J. Sci. Food Agric. 93 (2013) 3209–3215.

[24] B. Ozturk, D.J. McClements, Progress in natural emulsifiers for utilization in food emulsions, Curr. Opin. Food Sci. 7 (2016) 1–6, https://doi.org/10.1016/j.cofs.2015.07.008.

[25] B. Hu, K.P. Wang, L.Y. Han, B. Zhou, J.X. Yang, S.G. Li, Pomegranate seed oil stabilized with ovalbumin glycated by inulin: Physicochemical stability and oxidative stability, Food Hydrocoll. 102 (2020) 10, https://doi.org/10.1016/j.foodhyd.2019.105602.

[26] Y. Xiong, Q.R. Li, S. Miao, Y. Zhang, B.D. Zheng, L.T. Zhang, Effect of ultrasound on physicochemical properties of emulsion stabilized by fish myofibrillar protein and xanthan gum, Innov. Food Sci. Emerg. Technol. 54 (2019) 225–234, https://doi.org/10.1016/j.ifset.2019.04.013.

[27] Q.W. Du, X.H. Ji, F. Lyu, J.H. Liu, Y.T. Ding, Heat stability and rheology of high-calorie whey protein emulsion: Effects of calcium ions, Food Hydrocoll. 114 (2021) 9, https://doi.org/10.1016/j.foodhyd.2020.106583.

[28] T. Wu, Q. Jiang, D. Wu, Y. Hu, S. Chen, T. Ding, X. Ye, D. Liu, J. Chen, What is new in lysozyme research and its application in food industry? A review, Food Chem. 274 (2019) 698–709.
[29] W. Xu, W.P. Jin, K.L. Huang, L. Huang, Y.C. Lou, J. Li, X.F. Liu, B. Li, Interfacial and emulsion stabilized behavior of lysozyme/xanthan gum nanoparticles, Int. J. Biol. Macromol. 117 (2018) 280–286, https://doi.org/10.1016/j.ijbiomac.2018.05.187.
[30] M.M. Ai, Z. Zhang, H. Fan, Y.Y. Cao, A.M. Jiang, High-intensity ultrasound together with heat treatment improves the oil-in-water emulsion stability of egg white protein peptides, Food Hydrocoll. 111 (2021) 13, https://doi.org/10.1016/j.foodhyd.2020.106256.
[31] S.G. Li, Y. Huang, F.P. An, Q. Huang, F. Geng, M.H. Ma, Hydroxyl radical-induced early stage oxidation improves the foaming and emulsifying properties of ovalbumin, Poult. Sci. 98 (2019) 1047–1054, https://doi.org/10.3382/ps/pey370.
[32] T.H. Zhang, Y.L. Zhao, X.E. Tian, J. Liu, H.Q. Ye, X. Shen, Effect of ultrasound pretreatment on structural, physicochemical, rheological and gelation properties of transglutaminase cross-linked whey protein soluble aggregates, Ultrason. Sonochem. 74 (2021) 9, https://doi.org/10.1016/j.ultsonch.2021.105553.
[33] K. Wang, G. Li, B. Zhang, Opposite results of emulsion stability evaluated by the TSI and the phase separation proportion, Colloids Surf. A Physicochem. Eng. Asp. 558 (2018) 402–409, https://doi.org/10.1016/j.colsurfa.2018.08.084.
[34] X.N. Sui, S. Bi, B.K. Qi, Z.J. Wang, M. Zhang, Y. Li, L.Z. Jiang, Impact of ultrasonic treatment on an emulsion system stabilized with soybean protein isolate and lecithin: Its emulsifying property and emulsion stability, Food Hydrocoll. 63 (2017) 727–734, https://doi.org/10.1016/j.foodhyd.2016.10.024.
[35] S. Belgheisi, A. Motamedzadegan, J.M. Milani, L. Rashidi, A. Rafe, Impact of ultrasound processing parameters on physical characteristics of lycopene emulsion, J. Food Sci. Technol.-Mysore 58 (2021) 484–493.
[36] J. Mewis, N.J. Wagner, Thixotropy, Adv. Colloid Interface Sci. 147–148 (2009) 214–227.
[37] K.M. Albano, V.R. Nicoletti, Ultrasound impact on whey protein concentrate-pectin complexes and in the O/W emulsions with low oil soybean content stabilization, Ultrason. Sonochem. 41 (2018) 562–571, https://doi.org/10.1016/j.ultsonch.2017.10.018.