The manufacturing of lactobacillus microcapsules by freezing with egg yolk: The analysis of microstructure and the preservation effect against freezing and acid treatments

AUTHOR(S):

Fang, Bowen; Watanabe, Hironoshin; Isobe, Kazuhiro; Handa, Akihiro; Nakagawa, Kyuya

CITATION:

Fang, Bowen ...[et al]. The manufacturing of lactobacillus microcapsules by freezing with egg yolk: The analysis of microstructure and the preservation effect against freezing and acid treatments. Journal of Agriculture and Food Research 2021, 6: 100221.

ISSUE DATE:

2021-12

URL:

http://hdl.handle.net/2433/279269

Journal of Agriculture and Food Research 6 (2021) 100221

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.sciencedirect.com/journal/journal-of-agriculture-and-food-research

The manufacturing of *lactobacillus* microcapsules by freezing with egg yolk: The analysis of microstructure and the preservation effect against freezing and acid treatments

Bowen Fang [a,**], Hironoshin Watanabe [b], Kazuhiro Isobe [c], Akihiro Handa [c], Kyuya Nakagawa [b,*]

[a] Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Japan
[b] Department of Chemical Engineering, Faculty of Engineering, Kyoto University, Japan
[c] R&D Division, Kewpie Corporation, 2-5-7 Sengawa-cho, Chofu-shi, Tokyo, 182-0002, Japan

ARTICLE INFO

Keywords:
Lactobacillus
Egg yolk
Freezing
Micro-encapsulation
Aggregation
Acid tolerance

ABSTRACT

Encapsulation is an effective strategy to prevent probiotics from environmental damages. The survivability of *Lactobacillus* is generally reduced under frozen or acid environments. In this study, an attempt was made to encapsulate *Lactobacillus* into the egg yolk aggregates formed upon freezing. *Lactobacillus* broth was mixed with liquid egg yolk and frozen at selected temperatures and time periods. After thawing, the number of surviving bacteria was determined. The results showed that freezing with the addition of egg yolk improved *Lactobacillus* survivability. It was confirmed that freezing increased the number of egg yolk aggregates, and those aggregates coated *Lactobacillus* after freezing. The encapsulated *Lactobacillus* was treated at pH 2.5 for 10 min, and the results showed that the encapsulation process increased the acid resistance of *Lactobacillus*. These results indicate that freezing-induced encapsulation with egg yolk could effectively protect *Lactobacillus* against freezing and acid treatment. This finding could be useful for the design and preservation of probiotics-based food products.

1. Introduction

Probiotics are defined as "Live microorganisms that when administered in adequate amounts confer a health benefit on the host" [1]. The benefits of consuming probiotics include, but are not limited to, improvement of gut flora, prevention of diarrhea, and curing of irritable bowel syndrome [2,3]. *Lactobacillus* is a group of gram-positive bacteria that is usually found in dairy products and pickled foods [4,5]. The health benefits of *Lactobacillus* spp. have been known for a long time. However, now there is a renewed interest in the therapeutic use of *Lactobacillus* for cholesterol reduction, cancer prevention, improvement of lactose tolerance, curing of Crohn's disease, etc. [6–9]. The viability of the probiotics is an important factor to consider during the development of *Lactobacillus*-based probiotic products. Viability mainly depends on two factors: the efficiency of the *Lactobacillus* strain and survivability.

An efficient *Lactobacillus* strain can be obtained via separation and purification [10]. For survivability enhancement, encapsulation is the mainstream approach. Encapsulation is coating the bacteria cells with a layer or layers of encapsulant, it could effectively protect the bacteria from environmental hazards. For example, Cook et al. reported that incorporating probiotics into microcapsules was an effective method to reduce cell death during gastric intestinal passage; it also offered the opportunity to control the release of probiotics [11]. Kim et al. encapsulated *Lactobacillus* and exposed them to low pH conditions. The results showed that the cells without encapsulation were completely destroyed, while the viable count of encapsulated samples only declined by 3 log [12]. Rather et al. encapsulated *Lactobacillus* using alginate. As a result, the coated bacteria showed higher survival in gastric juice as well as better heat tolerance [13]. Besides having the good preservation effect, encapsulation is also viable in food or pharmaceutical industry. Mixing the encapsulant and the bacteria together is the simplest encapsulation. Meanwhile, encapsulation can also be achieved using freeze-drying, spray-drying, crosslinking and freezing [14–16].

In previous studies by the authors, when protein (casein and egg white powder) suspensions were frozen for a long time, the structures of dairy and egg protein particles were modified by the freeze-concentration effect [16–19]. Since the characteristics of protein particles can be modified by freezing, the encapsulation effects of these

* Corresponding author.
** Corresponding author.
E-mail addresses: fangbowenzju@hotmail.com (B. Fang), kyuya@cheme.kyoto-u.ac.jp (K. Nakagawa).

https://doi.org/10.1016/j.jafr.2021.100221
Received 19 July 2021; Received in revised form 7 October 2021; Accepted 8 October 2021
Available online 14 October 2021

protein particles are supposed to be controllable by adjusting the freezing condition. In this research, we wanted to study how egg proteins encapsulate *Lactobacillus* and what how the encapsulation affects the survivability of the encapsulated bacteria. Since egg white contained bactericidal substances, it was removed and only the yolk was used for *Lactobacillus* encapsulation. This study was supposed to discuss about the possibility of developing novel yolk-based microencapsulated probiotics through freeze-thaw process, and the preserving *Lactobacillus* by microencapsulation.

2. Materials and methods

2.1. Lactobacillus survivability after freeze-thaw process

Fresh shell eggs were purchased from a local chicken farm, and the yolk was manually separated from the egg white. A small amount of egg white on the vitellin membrane was carefully removed using a paper towel. Collected yolk was gently homogenized for experiments. Broth buffer was prepared by dissolving 30 g of tryptic soy broth powder (Becton Dickinson Corp.) in 500 mL of autoclaved water. Active lactic culture powder (0.1 g) (Cosme Co., Ltd) was inoculated in the broth buffer and incubated at 37 °C for 1 day. After incubation, the inoculated broth buffer was mixed 1:1 with egg yolk (for control groups, egg yolk was replaced with the same volume of 0.4% NaCl buffer). Then, the microtubes were frozen at −20 °C or −35 °C for 1 h, 1 day, 3 days, and 5 days. After freezing, the microtubes were moved to room temperature (20 ± 1 °C) and thawed for 1 h. After thawing, the buffer was diluted 50-fold with 0.4% NaCl. For unfrozen samples, the freeze-thaw process was skipped. After dilution, 1 ml of buffer was spread on a dry medium for microbial count (Dai Nippon Printing Co., Ltd), and incubated at 37 °C for 2 days. Then, the image of the incubated medium was analyzed using ImageJ software. An image of the frozen-thawed *Lactobacillus*-yolk mixture was obtained using a scanning electric microscope (SEM) device (JSM-6700F, JEOL Ltd., Japan) operated at 10 kV accelerating voltage.

2.2. Lactobacillus acid tolerance

Lactobacillus broth was obtained using the same method described in section 2.1. *Lactobacillus* broth was mixed with egg yolk (1:1) and frozen at −20 °C or −35 °C for 1 h (egg yolk was replaced with 0.4% NaCl buffer for control groups). After thawing, 0.1 mL of the samples was added to 1 mL of HCl buffer (pH 2.5) and mixed. The acid treatment was continued for 10 min at room temperature, until neutralization was achieved with the same amount of NaOH. The samples were then spread on agar plates and incubated at 37 °C for 2 days. Finally, the number of colonies was recorded and analyzed using ImageJ.

2.3. Tunable resistance pulse sensing (TRPS) analysis

Fresh liquid egg yolk (Kewpie Co. Ltd., Japan) was diluted with 0.4% NaCl buffer 1-fold, and frozen at −20 °C or −35 °C for 1 h, 1 day, 3 days, and 5 days. After being thawed at room temperature, the samples were diluted 10-fold by 0.4% NaCl, and subjected to particle analysis using a commercial TRPS device (qNano, IZON Co. Ltd, New Zealand). A micropore film (NP 4000, 2–10 μm) was stretched to 45 mm (nanopore size of 4 μm). A standard carboxylated polystyrene particle suspension (CPC4000E, IZON Co. Ltd, New Zealand) was used for system calibration. For each measurement, 35 μL of the samples were used. Pressure and voltage were set as 10 bar and 0.04 V, respectively. Each measurement was carried out 7 times. Data were analyzed using the Izon Control Suite 3.3. The particle diameter and particle number were analyzed based on the height and number of signal pulses. The particle surface characteristics were analyzed from the full width at half maximum (FWHM) duration of each signal pulse. Data obtained from the experiments were analyzed using one-way ANOVA ($n = 7$, $p = 0.05$).

3. Results and discussion

3.1. Egg yolk protected lactobacillus during freezing process

Lactobacillus broth was firstly processed by freezing. After being thawed, the average number obtained after 2 days of anaerobic incubation at 37 °C is shown in Fig. 1. It is presented in the figure that while the number of *Lactobacillus* colonies decreased during the freezing process at both −20 °C and −35 °C, egg yolk prevented a decrease in colony formation. For the *Lactobacillus* samples frozen at −20 °C, the average colony number decreased from 1540 to 1100 during the 5-day-freezing process. When the freezing temperature was −35 °C, the average colony number decreased to 250 on the fifth day. These results are consistent with common knowledge that *Lactobacillus* is less survivable at harsher temperatures. After egg yolk was added to the *Lactobacillus* broth, the average colony number of all samples increased. In the case of unfrozen samples, the average colony number of the yolk-added samples was 9.3% higher than that of the blank samples, but the increase was not significant ($p > 0.05$). This insignificant increase could be explained by the fact that egg yolk provided nutrients and was advantageous for the incubation of *Lactobacillus* on the plates.

The beneficial effect of egg yolk was also observed in the frozen samples. After being frozen, the average colony number of all samples decreased. But the yolk-added samples had comparatively more colonies. After being frozen at −20 °C for 1 day, 3 days and 5 days, samples with egg yolk had higher average colony number for 16%, 9%, and 11%, respectively ($p < 0.05$). Moreover, after being frozen with egg yolk at −35 °C for 1 day, 3 days, and 5 days, the average colony number was higher than blank samples for 51%, 202%, and 245%, respectively ($p < 0.05$). These results indicate that the preservative effect of egg yolk was higher at −35 °C than at −20 °C. The difference in the preservative effect of egg yolk at the two temperatures could be attributed to the different death rates of *Lactobacillus*. At −20 °C, after being frozen for 5 days, the decrease in average colony number of blank and yolk-added samples was 28% and 27%, respectively. At −35 °C, however, the

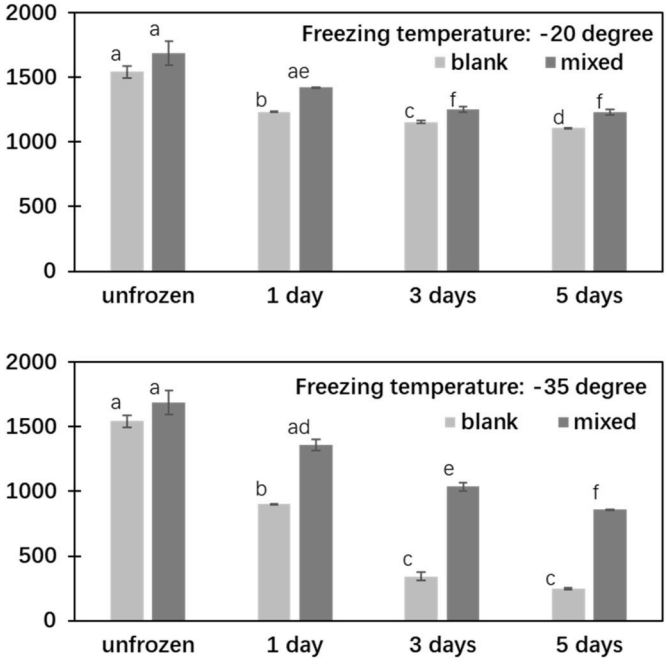

Fig. 1. Average colony number of plates incubated with *Lactobacillus* broth (blank) and egg yolk-mixed *Lactobacillus* broth (mixed). Samples were frozen at −20 °C and −35 °C for 1 day, 3 days and 5 days. Error bar is the standard deviation of 3 replicates. Significant difference between samples is represented by various English letters.

reduction in the average colony number of blank and yolk-added samples was 84% and 49%, respectively. In conclusion, the addition of egg yolk did not strongly affect the reduction of *Lactobacillus* during the freezing process at −20 °C for 5 days. However, during the freezing process at −35 °C for 5 days, adding egg yolk clearly protected *Lactobacillus* from freezing.

The analysis of colony numbers shows that the addition of egg yolk improved the survivability of *Lactobacillus* at −35 °C. To understand the mechanism behind this preservative effect, the 1:1 *Lactobacillus*-yolk mixture was frozen at −35 °C for 3 days.

After thawing, an image of the mixture was obtained using SEM (Fig. 2).

In Fig. 2, *Lactobacillus* cells can be clearly seen as particles with diameters less than 10 μm. The mass around the *Lactobacillus* cells was identified as egg yolk aggregate. The image shows that the egg yolk covered the *Lactobacillus* cells to form a microcapsule structure. Many researchers have reported the formation of microcapsule structure could be an effective way to protect *Lactobacillus* from environmental hazards [20–22]. In this research, the microcapsule structure gained from freezing process was supposed to protect *Lactobacillus* cells. The experiment results in Fig. 1 also support the protective effect of the freezing-induced microcapsule structure: after being frozen at −35 °C for 3 days, the average colony number of yolk-added *Lactobacillus* broth was approximately 3 times higher than that of the no-yolk sample.

Based on the survivability results and the SEM image, it could be concluded that egg yolk was an effective encapsulant to preserve *Lactobacillus*. The mechanism of the protective effect could be roughly attributed to the physical coating. However, we could not reject the possibility that some egg yolk substances (lecithin, thiamine, riboflavin, different lipids and proteins, etc.) contributed to increase *Lactobacillus* survivability. Hence, in the following steps of this research, we prepare to fractionate egg yolk to study the effect of different yolk substances [23,24]. The further research will be separating the egg yolk substances and mix the different substances with *Lactobacillus*. Then, the deeper discussion about the protection effect of egg yolk could be attained.

3.2. Egg yolk protected lactobacillus from acid treatment

The results described above indicate that the liquid egg yolk encapsulates *Lactobacillus* under freezing conditions. Subsequently, the protective effect of the microencapsulated structure against acidic environment was studied. The *lactobacillus*-yolk mixture was frozen at −20 °C or −35 °C for 1 h. After thawing, the samples were treated with acid solution. The results, as shown in Fig. 3, indicate that the addition of egg yolk, the freezing process, and the acid treatment, all affected the average colony number of samples.

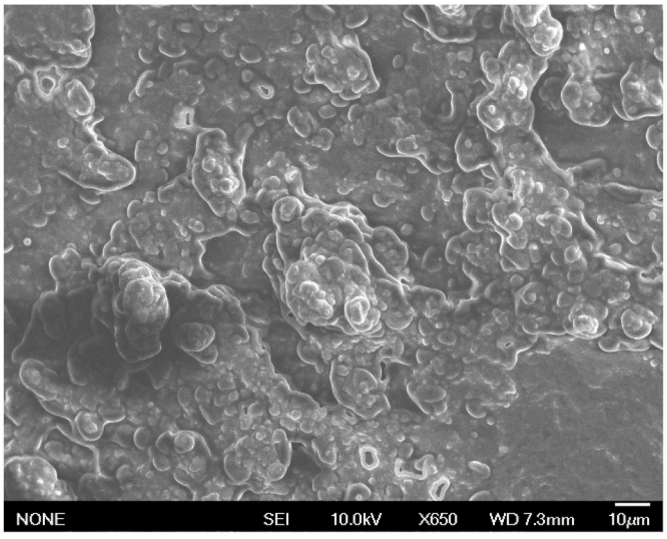

Fig. 2. SEM image of 3-day-frozen *Lactobacillus*-yolk mixture.

The addition of egg yolk improved the survivability of *Lactobacillus*. The 12 samples could be divided into 6 groups based on different freezing conditions and acid treatment (S1+S2, S3+S4, S5+S6, S7+S8, S9+S10, and S11+S12). In each group, more average colony numbers were counted from the egg yolk-added samples than the NaCl samples ($p < 0.05$). These results indicate that the encapsulation by egg yolk increases the survivability of *Lactobacillus*. In the case of unfrozen samples, the increase in the average colony number caused by egg yolk for acid-treated and acid-untreated samples was 35% and 21%, respectively. For the samples frozen at −20 °C, the increases were 97% and 74% for the acid-treated and acid-untreated samples, respectively. In the case of samples frozen at −35 °C, the increases for the acid-treated/untreated samples were 62% and 51%, respectively. These results indicate that the protective effect of egg yolk was more obvious in frozen samples than in unfrozen samples. It therefore seems that the freezing process strengthens the preservative effect of egg yolk. Moreover, −20 °C was found to be a better temperature than −35 °C for the preservation of *Lactobacillus*. In conclusion, egg yolk increased the survivability of *Lactobacillus*, and being frozen with egg yolk at −20 °C for 1 h had better protective effect than being frozen at −35 °C for 1 h or unfrozen.

In order to study the effect of acid treatment, the 12 samples were divided into 6 groups, based on whether egg yolk was added, and whether the freezing processes were carried out (S1+S3, S5+S7, S9+S11, S2+S4, S6+S8, and S10+S12). Then, the rate of decrease in the average colony number after acid treatment was calculated for each group. The results indicated that in the case of the egg yolk-lacking samples, the decrease in average colony number caused by acid treatment for unfrozen, −20 °C-frozen and −35 °C-frozen samples was 16%, 16.4%, and 19.2%, respectively ($p < 0.05$). However, for egg yolk-containing samples, the acid-caused reductions for the unfrozen, −20 °C-frozen and −35 °C-frozen samples were 6.6% ($p > 0.05$), 5.3% ($p > 0.05$), and 13.5% ($p < 0.05$), respectively. These results indicate that when samples were not mixed with egg yolk, the 10-min acid treatment significantly reduced the survivability of *Lactobacillus*, for which the decrease was higher than 16% in all conditions. However,

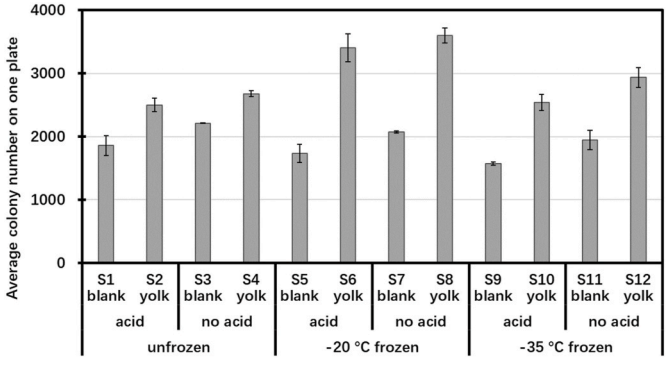

Fig. 3. The average colony number of one plate incubated with different *Lactobacillus* samples. Error bar is the standard deviation of 3 replicates. S1 represents unfrozen blank (no egg yolk) samples treated by acid; S2 represents unfrozen yolk-added samples and treated by acid; S3 represents unfrozen blank samples untreated by acid; S4 represents unfrozen yolk-added samples untreated by acid; S5 represents −20-degree-frozen blank samples treated by acid; S6 represents −20-degree-frozen yolk-added samples and treated by acid; S7 represents −20-degree-frozen blank samples untreated by acid; S8 represents −20-degree-frozen yolk-added samples untreated by acid; S9 represents −35-degree-frozen blank samples treated by acid; S10 represents −35-degree-frozen yolk-added samples and treated by acid; S11 represents −35-degree-frozen blank samples untreated by acid; S12 represents −35-degree-frozen yolk-added samples untreated by acid.

acid-induced damage was prevented by the addition of egg yolk. The addition of egg yolk also alleviated the loss of living bacteria. Especially for the yolk-added unfrozen and −20 °C-frozen samples, the acid-induced damages on bacteria survivability were slight and insignificant ($p > 0.05$). In summary, egg yolk protected bacteria from acid-induced damage. The preservative function of egg yolk was higher at −20 °C than at −35 °C or in unfrozen conditions.

3.3. Changes in particle size and concentration of liquid egg yolk by freezing and aging

Liquid egg yolk samples were diluted one-fold and frozen at −20 °C or −35 °C for 1 h, 1 day, 3 days and 5 days. After thawing, the average particle diameter and average particle concentration were analyzed using TRPS, as shown in Fig. 4. As shown in the figure, during the 5-day aging process, a change in the diameter of aggregates was observed. However, the diameter was between 3.2 and 3.7 μm. While the change in particle diameter was no more than 15.2%, the change in particle number was much more significant. After being frozen for 5 days, the average particle number increased by 573% (frozen at −20 °C) and 394% (frozen at −35 °C). In summary, during the freezing process, the diameter of egg yolk aggregates changed slightly, while the average number of egg yolk aggregates changed significantly.

Assuming that the density of aggregates was unchanged during the freezing process, the increase in material (protein and lipid) content of the aggregates can be calculated from the average particle diameter and average particle number concentration. The results indicate that the material content in aggregates increased by 711% (when frozen at −20 °C) and 534% (when frozen at −35 °C) after the 5-day freezing period. Combined with the fact that the aggregate density increased during freezing, the increase would be higher than the calculation results [25,26].

The calculation showed that the material content of the aggregate increased after freezing. The increase in material content of aggregate after freezing could be explained by surface-induced denaturation. Protein molecules are composed of both hydrophilic and hydrophobic residues. When protein is dissolved in a solution, hydrophilic residues are exposed on the protein surface, and are therefore in contact with water molecules, while hydrophobic residues are hidden inside. When the dissolved protein molecule approaches a hydrophobic ice surface, the protein structure tends to unfold and expose the hydrophobic residues to the hydrophobic ice surface. Thus, the structure of the protein molecule is modified and the solubility is decreased. After thawing, therefore, instead of being dissolved in solution, the protein molecules tend to aggregate [25]. Hence, the amount of protein in the aggregates increases after freezing.

The increase in particles after freeze-thaw process could also be explained by another mechanism. In egg yolk, approximately 78% (in dry matter) of proteins are in the plasma, while the remaining 22% are in the granules [27]. Plasma is a protein/lipid-containing aqueous phase, which is comprised of small lipoprotein aggregates (17–60 nm). Granules are large, microscale lipoprotein particles. The removal of water during the freezing process causes an increase in salt concentration. The high salt concentration dissolves the small lipoprotein aggregates and liberates the protein molecules. The released protein molecules tended to self-aggregate and form the large particles. After thawing, even if the salt concentration returns to a low level, egg yolk proteins are already rearranged from small plasma particles to large granules [27,28]. In this experiment, the TRPS device detected only micron-level particles. Therefore, the number of aggregates increased during freezing. In addition, the change in average particle diameter was slight, whereas the change in average particle number concentration was almost linear. These findings suggest that freezing and thawing rearranged egg yolk protein but did not affect the average size of the protein granules.

3.4. Modification of particle surface characteristics by freezing

The duration of particles passing through the nanopore indicates the speed of particles in the electric field, which in turn depends on the surface electric characteristics of the particles [29]. The data on duration

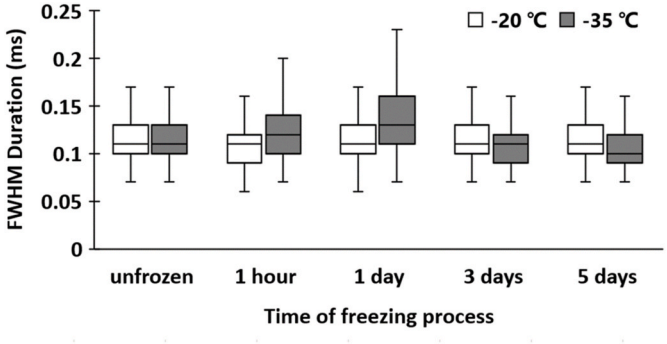

Fig. 5. Full width half maximum (FWHM) duration of egg yolk samples before and after being frozen at −20 °C and −35 °C for 1 h, 1 day, 3 days and 5 days.

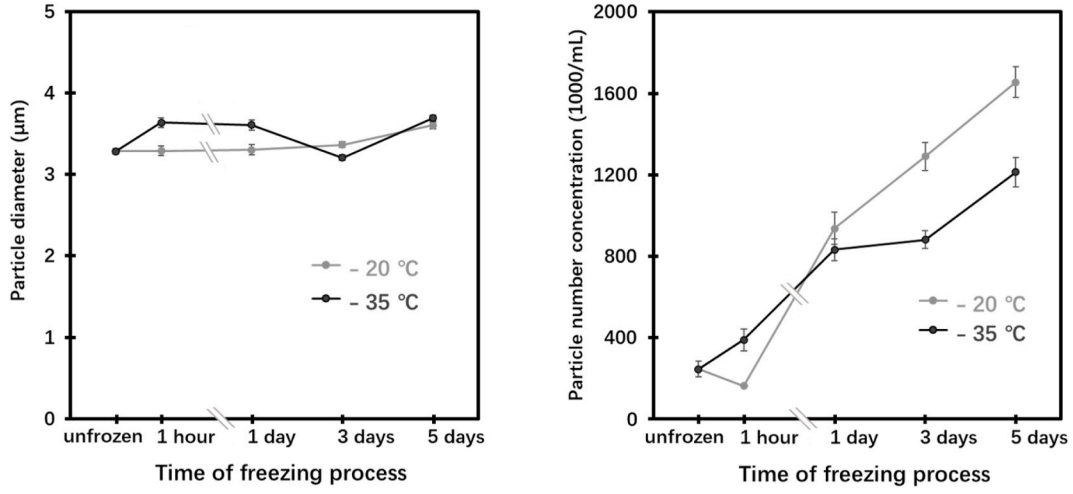

Fig. 4. Particle diameter and particle number concentration of egg yolk samples before and after freezing treatment at −20 °C and −35 °C for 1 h, 1 day, 3 days and 5 days. Error bar is the standard deviation of 7 replicates.

obtained by the TRPS device were processed as full width at half maximum (FWHM) values and are summarized in Fig. 5. In the figure, the variation in the FWHM duration of the samples corresponded with the size of the whisker box. The average value of the FWHM duration is shown as a line inside the whisker box. As can be seen in the figure, the box size of samples frozen at −20 °C was more stable than that of the samples frozen at −35 °C. In the case of samples frozen at −20 °C, the lower edges of the whisker box were not lower than 0.09, while the upper edges were not higher than 0.13. However, in the case of samples frozen at −35 °C, the lower edges of the whisker box were not lower than 0.09, while the upper edges were not higher than 0.17. These data indicate that the surface characteristics of egg yolk aggregates were more stable at −20 °C than at −35 °C. A similar conclusion can also be drawn from the average FWHM duration values. The average FHWM values for samples frozen at −20 °C were unchanged (0.11) during the 5-day freezing process. While the average FWHM value for samples frozen at −35 °C increased from 0.11 to 0.13 during the first day of freezing, it then decreased to 0.1 on the fifth day. As can be seen in the figure, the surface characteristics of egg yolk aggregates were stable when the freezing temperature was −20 °C, but changed when the freezing temperature was −35 °C.

3.5. Changes in egg yolk aggregate volume distribution during freezing

In addition to the information on average particle diameter, average particle number, and surface characteristics, the TRPS device also provided information about the volume distribution of aggregates. The volume distribution data are summarized in Fig. 6. As can be seen in the figure, the average total volume, regardless of the volume range, increased during the freezing process. The continuous increase in particle volume was caused by the increased number of free protein molecules, as indicated in Section 3.3. Fig. 6 also shows the dominant size range of the aggregates. It indicates that during the freezing process, the peak of the curves moved from 10×10^{-15} L to 15×10^{-15}. Besides the peak, the percentage of particle volume higher than 20×10^{-15} L also increased during the freezing process. These results indicate that the size of the aggregates was enhanced by the freezing process. The increase in particle size was caused by the increase in free protein molecules, as well as the aggregation of protein granules (Section 3.3).

Fig. 6 also shows the difference between the two freezing temperatures. During the first hour of the freezing process, the curves of both samples (−20 °C and −35 °C) increased. The freezing-induced increase at −35 °C was more obvious than at −20 °C. However, when the curves on the first and fifth days were compared, freezing at −20 °C caused a higher volume increase than that at −35 °C. At the beginning of freezing, the speed of ice formation was higher at −35 °C than at −20 °C. Therefore, the freeze-concentration effect at −35 °C was stronger, which caused more protein modification. In this way, the volume of aggregates increased faster at −35 °C than at −20 °C. However, after the samples were frozen for several days, the freeze-concentrated system was already stable. Under such stable conditions, the change in protein content was influenced by the amount of activated water. The samples frozen at −20 °C retained more activated water than samples frozen at −35 °C, so the liberation and rearrangement of protein molecules at −20 °C were more active than at −35 °C. These mechanisms could explain the phenomenon that in the first hour of freezing, −35 °C made more changes to the samples than −20 °C, but −20 °C made more changes on samples than −35 °C at the fifth day of freezing.

As explained in the previous sections, mixing with the same volume of liquid egg yolk increased the survivability of *Lactobacillus* after the freeze-thaw process. In the SEM image, *Lactobacillus* cells were covered by egg yolk aggregates and formed a microencapsulated structure, which could protect *Lactobacillus* from environmental damage. The *Lactobacillus*-yolk mixture was then treated with an acid solution. The results showed that the microencapsulated structure could enhance the survivability of *Lactobacillus* after acid treatment. In addition, the microcapsules produced at −20 °C had better preventive function than the microcapsules produced at −35 °C. Afterwards, the frozen-thawed egg yolk aggregates were analyzed using TRPS, which showed that characteristics such as average particle diameter, particle number concentration, and surface characteristics changed during freezing. These changes, especially the increase in aggregate number, influenced the formation and function of the microcapsules. Thus, the proper control of freezing conditions is vital to ensure the optimal quality and function of microcapsules produced by freezing.

4. Conclusion

We investigated the effect of mixing *Lactobacillus* with liquid egg yolk on the survivability of *Lactobacillus* after freezing. The results showed that egg yolk protected *Lactobacillus* under frozen conditions. This protective effect was attributed to the formation of a microencapsulated structure composed of a mixture of *Lactobacillus* cells and egg yolk aggregates. Then, the mixture of *Lactobacillus* and egg yolk was treated with an acidic solution. The results showed that mixing with egg yolk greatly enhanced the survivability of *Lactobacillus* in acidic environment. Moreover, the temperature used for producing the microcapsule mattered, as the microcapsules produced at −20 °C had a better preservative function than the microcapsules produced at −35 °C. Particle analysis revealed that freezing temperature influenced the formation of egg yolk aggregates, which were the encapsulants. Therefore, the control of the freezing temperature is important for the proper functioning of the microcapsules produced by the freezing process.

This study indicates that egg yolk, as an encapsulant, is beneficial for

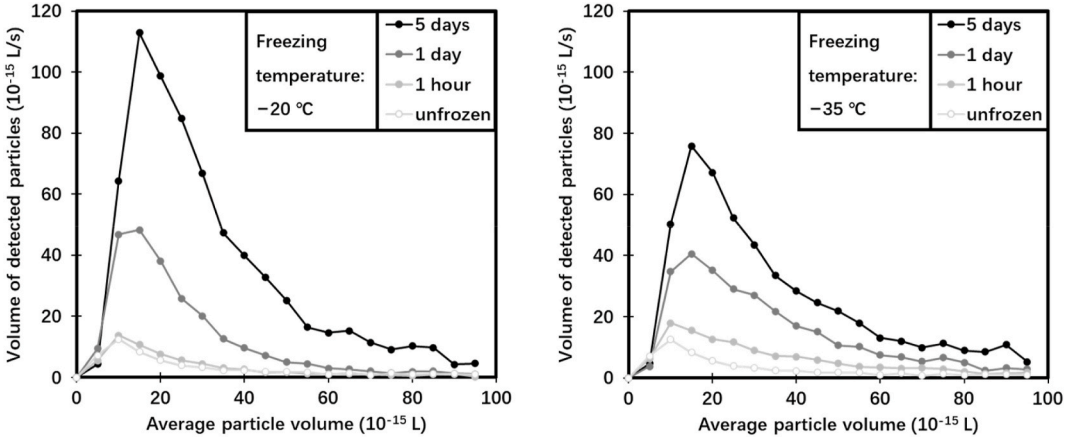

Fig. 6. Volume distribution (range: 0 - 10^{-13} L) of samples before and after being frozen at −20 °C and −35 °C for 1 h, 1 day and 5 days.

the preservation of *Lactobacillus* against acid treatment and freezing. It might therefore be a useful technology for improving the survivability and accessibility of bio-products.

Declaration of competing interest

No Conflict of Interest.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (C) [Grant Number 17K07813]; the PhD candidate was supported by China Scholarship Council [Grant Number 201808050207].

References

[1] C. Hill, F. Guarner, G. Reid, G.R. Gibson, D.J. Merenstein, B. Pot, S. Salminen, Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic, Nat. Rev. Gastroenterol. Hepatol. 11 (8) (2014) 506.
[2] M. Bermudez-Brito, J. Plaza-Díaz, S. Muñoz-Quezada, C. Gómez-Llorente, A. Gil, Probiotic mechanisms of action, Ann. Nutr. Metabol. 61 (2) (2012) 160–174.
[3] O. Simon, W. Vahjen, L. Scharek, Micro-organisms as feed additives-probiotics, Adv. Pork Prod. 16 (2) (2005) 161–167.
[4] K. Makarova, A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, N. Polouchine, Comparative genomics of the lactic acid bacteria, Proc. Natl. Acad. Sci. Unit. States Am. 103 (42) (2006) 15611–15616.
[5] J. Zheng, S. Wittouck, E. Salvetti, C.M. Franz, H.M. Harris, P. Mattarelli, J. Walter, A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*, Int. J. Syst. Evol. Microbiol. 70 (4) (2020) 2782–2858.
[6] C. Gaspar, G. Donders, R. Palmeira-de-Oliveira, J. Queiroz, C. Tomaz, J. Martinez-de-Oliveira, A. Palmeira-de-Oliveira, Bacteriocin production of the probiotic *Lactobacillus* acidophilus KS400, Amb. Express 8 (1) (2018) 1–8.
[7] L. Rocha-Ramírez, R. Pérez-Solano, S. Castañón-Alonso, S. Moreno Guerrero, A. Ramírez Pacheco, M. García Garibay, C. Eslava, Probiotic *Lactobacillus* strains stimulate the inflammatory response and activate human macrophages, J. Immunol. Res. (2017), 2017.
[8] G. Tachedjian, M. Aldunate, C.S. Bradshaw, R.A. Cone, The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health, Res. Microbiol. 168 (9–10) (2017) 782–792.
[9] Z. Zhang, J. Lv, L. Pan, Y. Zhang, Roles and applications of probiotic *Lactobacillus* strains, Appl. Microbiol. Biotechnol. 102 (19) (2018) 8135–8143.
[10] X. You, L. Yang, X. Zhao, K. Ma, X. Chen, C. Zhang, Q. Zhang, Isolation, purification, characterization and immunostimulatory activity of an exopolysaccharide produced by *Lactobacillus* pentosus LZ-R-17 isolated from Tibetan kefir, Int. J. Biol. Macromol. 158 (2020) 408–419.
[11] M.T. Cook, G. Tzortzis, D. Charalampopoulos, V.V. Khutoryanskiy, Microencapsulation of probiotics for gastrointestinal delivery, J. Contr. Release 162 (1) (2012) 56–67.
[12] S.-J. Kim, S.Y. Cho, S.H. Kim, O.-J. Song, I.-S. Shin, D.S. Cha, H.J. Park, Effect of microencapsulation on viability and other characteristics in *Lactobacillus* acidophilus ATCC 43121, LWT-Food Science and Technology 41 (3) (2008) 493–500.
[13] S.A. Rather, R. Akhter, F. Masoodi, A. Gani, S. Wani, Effect of double alginate microencapsulation on in vitro digestibility and thermal tolerance of *Lactobacillus* plantarum NCDC201 and L. casei NCDC297, LWT-Food Science and Technology 83 (2017) 50–58.
[14] H.W. Tang, S. Abbasiliasi, P. Murugan, Y.J. Tam, H.S. Ng, J.S. Tan, Influence of freeze-drying and spray-drying preservation methods on survivability rate of different types of protectants encapsulated *Lactobacillus* acidophilus FTDC 3081, Biosc. Biotech. Biochem. 84 (9) (2020) 1913–1920.
[15] J.P. Farnsworth, J. Li, G.M. Hendricks, M.R. Guo, Effects of transglutaminase treatment on functional properties and probiotic culture survivability of goat milk yogurt, Small Rumin. Res. 65 (1–2) (2006) 113–121.
[16] B. Fang, K. Nakagawa, Modification of casein aggregate microstructures under frozen conditions: a study using tunable resistive pulse sensing, Food Hydrocolloids 98 (2020) 105259.
[17] K. Nakagawa, Y. Fujii, Protein-based microencapsulation with freeze pretreatment: spray-dried oil in water emulsion stabilized by the soy protein isolate–gum Acacia complex, Dry. Technol. 33 (13) (2015) 1541–1549.
[18] K. Nakagawa, M. Kagemoto, Characterization of casein-based nanoparticles formed upon freezing by in situ SAXS measurement, Colloids Surf. B Biointerfaces 103 (2013) 366–374.
[19] K. Nakagawa, S. Maebashi, K. Maeda, Freeze-thawing as a path to concentrate aqueous solution, Separ. Purif. Technol. 73 (3) (2010) 403–408.
[20] V. Chandramouli, K. Kailasapathy, P. Peiris, M. Jones, An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions, J. Microbiol. Methods 56 (1) (2004) 27–35.
[21] P.K. Okuro, M. Thomazini, J.C. Balieiro, R.D. Liberal, C.S. Fávaro-Trindade, Co-encapsulation of *Lactobacillus* acidophilus with inulin or polydextrose in solid lipid microparticles provides protection and improves stability, Food Res. Int. 53 (1) (2013) 96–103.
[22] M.P. Silva, F.L. Tulini, E. Martins, M. Penning, C.S. Favaro-Trindade, D. Poncelet, Comparison of extrusion and co-extrusion encapsulation techniques to protect *Lactobacillus* acidophilus LA3 in simulated gastrointestinal fluids, Lebensm. Wiss. Technol. 89 (2018) 392–399.
[23] N. Naderi, J.D. House, Y. Pouliot, Scaling-up a process for the preparation of folate-enriched protein extracts from hen egg yolks, J. Food Eng. 141 (2014) 85–92.
[24] N. Naderi, Y. Pouliot, J.D. House, A. Doyen, Effect of freezing, thermal pasteurization, and hydrostatic pressure on fractionation and folate recovery in egg yolk, J. Agric. Food Chem. 65 (35) (2017) 7774–7780.
[25] B. Fang, K. Isobe, A. Handa, K. Nakagawa, Microstructure change in whole egg protein aggregates upon freezing: effects of freezing time and sucrose addition, J. Food Eng. 296 (2021) 110452.
[26] B. Fang, K. Nakagawa, Effect of pH, ionic strength, and freezing treatment on a colloidal suspension of egg white aggregates, Food Struct. 27 (2021) 100181.
[27] M. Anton, Composition and structure of hen egg yolk, in: Bioactive Egg Compounds, Springer, 2007, pp. 1–6.
[28] T. Strixner, J. Sterr, U. Kulozik, R. Gebhardt, Structural study on hen-egg yolk high density lipoprotein (HDL) granules, Food Biophys. 9 (4) (2014) 314–321.
[29] E. Weatherall, G.R. Willmott, Applications of tunable resistive pulse sensing, Analyst 140 (10) (2015) 3318–3334.