DHA-mediated milk protein treated by ultrasound-assisted pH-shifting for enhanced astaxanthin delivery and processed cheese application

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INTRODUCTION

Astaxanthin (ASTA) is a xanthophyll carotenoid found in various microorganisms and marine animals (Liu et al., 2019). ASTA in aqueous solution has a low oral absorption and poor water solubility, making it less bioavailable in a hydrophilic environment and limiting its application in functional foods and dietary supplements (Edelman et al., 2019). An effective solution to these problems is through the delivery of ASTA into the food delivery system. Milk proteins have been reported to show potential in the application of carrier due to their strong ability to bind hydrophobic, hydrophilic, and charged bioactive compounds and stabilize the biological activity of the encapsulated compounds. Several studies have applied milk proteins as carriers to improve the utilization efficiency of bioactive compounds by improving solubility and stability, enhancing bioavailability, and maintaining the flavor of active ingredients (Xie et al., 2022). However, most of the current studies on milk protein carriers employ Whey protein isolate (WPI) (Liu et al., 2016; Pan et al., 2020; Rodrigues et al., 2020), whereas the application of milk protein concentrate (MPC) and micellar casein (MCC) in such carriers has been less reported. Caseins and whey proteins constitute the major protein group present in milk. Caseins are rigid spheres that have a hydrophilic surface and hydrophobic interior (Tomadoni et al., 2020), and casein micelles exhibit an open, dynamic structure and a hydrophobic state in milk, allowing the incorporation of whey proteins into micelles (Sun et al., 2023). Previous research has demonstrated that WPI can effectively encapsulate many bioactive substances and improve their solubility, antioxidant capacity, stability, and bioaccessibility (Liu et al., 2016; Pan et al., 2020; Rodrigues et al., 2020). Therefore, it is essential to explore the potential of other milk proteins as carriers for ASTA delivery.
2020; Rodrigues et al., 2020). However, WPI is known to be unstable during heat treatments of above 70°C and irreversibly forms aggregates of different molecular sizes (Wijayanti et al., 2014). Compared with WPI, MCC is more thermally stable as a carrier, ensuring that the MCC-encapsulated bioactives are retained more efficiently in high-temperature–treated foods (Qin et al., 2023). In addition, the application of MCC as a carrier for bioactive substances can significantly improve encapsulation efficiency; water dispersibility; bioavailability; and storage, physical, and ionic stability (Barick et al., 2021).

In recent years, both the pH-shifting and ultrasound methods have proved to be effective for protein modification (Xu et al., 2021; Sun et al., 2022), due to their ability to improve the functional properties of proteins (Ryan and Foegeding, 2015; Sun et al., 2022; Zhao et al., 2022). In particular, ultrasound-assisted pH-shifting treatment can effectively enhance the efficiency of the protein encapsulation of bioactives and improve their water solubility and antioxidant properties (Xu et al., 2021). Furthermore, some studies have shown that lipid-based systems can effectively promote the bioavailability of lipid-soluble nutrients (Mercke Odeberg et al., 2003; Chen et al., 2020). More specifically, the oil phase in the system can solvate solid lipophilic bioactive compounds and increase the system molecular dispersion (Chen et al., 2020). Furthermore, the previous study has shown that fatty acid as ligands can change the spatial conformation of proteins and improve the hydrophobicity of proteins, thus improve the loading capacity and affinity of proteins to hydrophobic small molecules (Huang et al., 2022). Proteins could bind small amounts of fatty acids at their specific binding sites by self-assembly to form fatty acid–protein complexes. The structure of the complex varies depending on the protein and lipid ratio. However, the most common structural organization is the core-shell structure, in which a dynamic, partially expanded and extended layer of proteins surrounds the micelle-like fatty acid core (Pedersen et al., 2020). Research has shown that lipid-present protein complexes are more effective than their lipid-free equivalents in increasing the water dispersion and encapsulation of bioactives, as well as improving protein affinity for bioactives and complex stability (Bojko et al., 2008; Liu et al., 2020). Long-chain docosahexaenoic acid (DHA) is one of the ω-3 series of polyunsaturated fatty acids, and is essential for humans (Liu et al., 2021a).

Cheese has become increasingly popular among consumers and its consumption is rapidly rising. Various bioactive substances have been added to food products to make them rich in nutritional and healthcare functions. Therefore, improving the functional properties of cheese by adding bioactive substances has a strong marketing potential. Based on this, we introduced DHA on the basis of ASTA delivery via 3 different components of milk protein under ultrasound-assisted pH-shifting treatment to investigate the functional properties of DHA-mediated milk proteins. The aim of the study was to further improve the encapsulation rate, antioxidant properties, bioaccessibility, and storage stability of milk proteins to ASTA. This work provides a basis for understanding the interaction of ASTA with DHA-mediated milk proteins and promotes the development of ASTA for applications in functional dairy products.

MATERIALS AND METHODS

Materials

MPC (approximately 80% protein content) and MCC (approximately 90% protein content) were acquired from the commercial supplier Leprino Foods Nutrition (Singapore). WPI (approximately 91% protein content) was donated by Agropur. DHA and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Macklin Biochemical Technology Co. (Shanghai, China). ASTA (≥98% purity) was acquired from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Cheddar cheese (food grade) and cream (food grade) were acquired from Irish Dairy Board China Co., Ltd. (Shenzhen, China). Sodium citrate (food grade) was obtained from Weifang Ensign Industry Co., Ltd. (Shandong, China). Xylitol (food grade) was purchased from Futaste Pharmaceuticals Co., Ltd. (Shandong, China). Gelatin (food grade) and Gellan gum (food grade) were acquired from Henan Gaobao Industrial Co., Ltd. (Henan, China). Ultrapure water was produced from a Direct-Q® 5 UV, Millipore SAS (Massachusetts, USA). All chemicals and reagents are analytical grade reagents unless otherwise noted.

Fabrication of the milk protein complexes

Three protein solutions (10 mg/L) containing MPC, MCC, and WPI proteins dissolved in ultrapure water were adjusted to pH 11 with NaOH (2 mol/L) and DHA-ethanol solution (30 mmol/L) was then added. The solutions were subjected to sonicate for 5 min at 20 kHz and 300 W power on an ultrasound device (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd.) equipped with a 12 mm horn (pulse-on and -off times of 2 and 3 s, respectively). Following this, the sample solutions were set for 30 min and adjusted to pH 7 (±0.02) with HCl (1 mol/L) to obtain the milk protein–DHA complex. Based on the aforementioned pH-shifting and sonication method, ASTA-milk protein complexes were prepared by adding 1 mL (0.6 mg/mL) of ASTA to
the 50mL milk protein solution (10 mg/L). The ASTA-milk protein complexes without DHA were denoted as A-MPC, A-MCC, and A-WPI, and the complexes with DHA were denoted as A-MPC-D, A-MCC-D, and A-WPI-D. All operations were conducted in triplicate and all samples were stored at 4°C for the subsequent observations.

CHARACTERIZATION OF COMPLEX PROPERTIES.

Determination of particle size, polydispersity index, zeta potential, and turbidity

Based on a previously reported method (Liu et al., 2012), the particle size and polydispersity index (PDI) were determined via the dynamic light scattering technique using a Zetasizer Nano-ZS90 (Malvern Instruments Limited, Worcestershire, UK). Before the measurements, the emulsion samples were diluted 5 fold with ultrapure water. Zeta potential measurements of the samples were performed using laser Doppler velocimetry (Malvern) based on a previously reported the method (Wu et al., 2020).

Turbidity detection was performed using a UV756 spectrophotometer (Yoke Instrument Co. Shanghai, China), with ultrapure water used as the blank. The turbidity was taken as the absorbance of each sample at 860 nm. All the measurements were carried out at 25°C (Liu et al., 2012).

ASTA embedding rate measurements

The ASTA embedding rate was determined following a previously reported method with some modifications (Huang et al., 2022). Briefly, 1 mL of protein complex solution and 1 mL n-hexane were mixed and centrifuged at 5000 rpm for 10 min using H1850R high speed refrigerated centrifuge (Xiangyi centrifuge instrument Co. Hunan, China). The supernatant was separated and dissolved in 9 mL of organic solvent (dichloromethane: methanol = 2:1, vol/vol). The absorbance of unembedded ASTA in solution was determined by UV (UV) spectrophotometer at 476 nm. The ASTA content was calculated using the ASTA standard curve under the same conditions and the ASTA embedding rate was calculated using equation (1):

\[
\text{Encapsulation rate of astaxanthin} = \frac{C_1 - C_2}{C_1} \times 100\%, \quad [1]
\]

where \(C_1\) is the total ASTA content in the solution; and \(C_2\) is the unwrapped ASTA amount.

Intrinsic fluorescence spectroscopy analysis

The interaction between ASTA and milk protein and DHA was monitored using fluorescence spectroscopy according to the method described in another study (Liang and Subirade, 2012). The protein complex solution was diluted with phosphate buffer saline (PBS, 0.1 mol/L, pH 7.4) to 1 mg/mL. The excitation and emission slits were set at 5 nm and 10 nm, respectively, and the fluorescence emission spectra was recorded from 290 to 450 nm at an excitation wavelength of 280 nm.

Surface hydrophobicity

Surface hydrophobicity was determined using ANS as a fluorescence probe, referring to the method in a previous study (Han et al., 2020). The protein solution was diluted with PBS buffer (0.1 mol/L, pH 7.4) to 0.5 mg/mL, and 30 μL of ANS (8 mmol/L) solution was then added to each 6 mL of sample dilution. The samples were subsequently left standing in the dark for 15 min. The fluorescence intensity of the samples was measured with a F-2700 fluorescence spectrophotometer (Rili Co. Xianggang, China) at the excitation wavelength of 390 nm, emission wavelength of 470 nm, and a slit width of 5 nm.

Antioxidant activity

Antioxidant activity was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+), and hydroxyl radical (-OH) scavenging ability. DPPH radical scavenging assay was performed using a UV756 spectrophotometer (Yoke Instrument Co. Shanghai, China) at 517 nm according to the method with slight modifications (Xu et al., 2021). ABTS radical scavenging activity was determined at 734 nm by referring to the method (Wang et al., 2012). Hydroxyl radical scavenging activity was quantified at 517 nm by referring to the method of Yuan et al. (Yuan et al., 2013).

In vitro simulated digestion

With a few adjustments to the method followed by Wang et al. (Wang et al., 2022), in vitro simulated gastric fluid digestion was performed by mixing 5 mL samples with 5 mL simulated gastric fluid (SGF) (2 g/L NaCl, 7 mL/L HCl, 1.6 g/L pepsin, and pH 1.5) and adjusting the pH to 2. The mixture was then shaken at 37°C for 2 h at 150 rpm. The gastric phase’s final mixture (10 mL) was mixed with 10 mL simulated intestinal fluid (SIF) (8.8 g/L NaH₂PO₄, 8.76 g/L NaCl) and adjusted the pH to 7. This was followed by mixing with 80 mg of bile.
salts and 150 mg of trypsin, and digested at 37°C and 100 rpm for 4 h. The mixture was quickly removed and cooled to obtain the gastrointestinal digest. ASTA dispersed in dimethyl sulfoxide was subjected to the same process for comparison. The bioaccessibility of ASTA was subsequently measured at 476 nm on a UV-visible spectrophotometer according to the method of Boonlao et al. (2020) with some modifications.

**Retention rate of ASTA during storage and thermal treatment**

The retention of ASTA in the complex samples was determined every 7 d during 35 d of storage in brown glass vials at 4°C. The thermal stability of ASTA in the complex samples at different temperatures was determined following previously reported methods with slight modifications (Liu et al., 2019; Li et al., 2023). 1 mL sample solution was mixed with 9 mL organic solvent (dichloromethane: methanol = 2:1, vol/vol) to extract ASTA. ASTA was determined according to the approach described in ASTA embedding rate measurements, and the ASTA retention rate was calculated using Eq.(4):

\[
\text{ASTA retention rate} \% = \frac{B_0}{B_1} \times 100%,
\]

where \(B_0\) is the ASTA concentration in the sample under different treatment conditions; and \(B_1\) is the initial ASTA concentration in the sample.

**Preparation of the processed cheese**

The ASTA solvent was replaced with anhydrous ethanol due to the toxicity of dimethyl sulfoxide. The solutions of the A-MPC-D, A-MCC-D, and A-WPI-D complexes were freeze-dried to make powders as excipients for the processed cheeses. The corresponding processed cheeses were denoted as MPC-C, MCC-C, and WPI-C, respectively. Cheese with the direct incorporation of MPC and ASTA were used as the control (Control-C). Table 1 reports the formulation specifications of the processed cheese. The cheese was made according to the method followed by Li et al. (2023) with a few modifications.

### Characterization of Processed Cheese Properties.

**Textural characteristics** The texture of cheese at 4°C for 48 h was measured using a texture analyzer (TA-XT plus, Stable Micro Systems, UK) according to the method of Li et al. (2023) with some modifications. All samples were cut into 1.5-cm cubes. The following parameter setting were implemented for the measurements: pretest probe descent speed of 2.0 mm/s; test speed of 1.0 mm/s; post-test probe return speed of 5.0 mm/s; downward pressure deformation of 50%; trigger force of 20 g; and probe type of P/36.

**In Vitro digestion simulation of ASTA in processed cheese** The bioavailability of ASTA in processed cheese was determined by in vitro digestion simulations according to the method with some modifications (Guo et al., 2022). In vitro gastrointestinal digestion assay was performed using 5 g of chopped cheese. The supernatant (micellar fraction) was collected and ASTA content in the micellar fraction of each sample was considered bioaccessible. Refer to the above in vitro simulated digestion for details of the experimental procedure.

**Storage stability of ASTA in processed cheese**

We followed the method described in Li et al. (2023), with some modifications. Samples were stored at 4°C for 28 d. The protective effect of milk protein carriers against ASTA was assessed by measuring the amount of ASTA preserved in the processed cheese every 7 d.

### Table 1 Formulation of processed cheese made from different milk protein carriers

<table>
<thead>
<tr>
<th>Samples</th>
<th>Control-C (g)</th>
<th>MPC-C (g)</th>
<th>MCC-C (g)</th>
<th>WPI-C (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw cheese</td>
<td>34.6</td>
<td>34.6</td>
<td>34.6</td>
<td>34.6</td>
</tr>
<tr>
<td>Milk protein carrier</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ASTA</td>
<td>0.0024</td>
<td>0.0024</td>
<td>0.0024</td>
<td>0.0024</td>
</tr>
<tr>
<td>Xylitol</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Milk powder</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Gel</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Butter</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Emulsified salt</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Total amount</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: the addition of ASTA in processed cheese is 24 μg/g cheese, the milk protein carrier used in the Control-C was only MPC.
Statistical analysis

All operations were conducted in triplicate and the data were expressed as the average ± standard deviation of the values of 3 sample replicates. Statistical analysis was performed in SPSS v. Twenty-two (IBM Corp., New York, USA) with a one-way ANOVA at a significance level of $P = 0.05$. OriginPro 8.5 (Origin-Lab, Massachusetts, USA) was used to plot the results.

RESULTS AND DISCUSSION

Particle size, Polydispersity index, and Zeta potential of milk protein–DHA complex

The DHA was dispersed in milk protein solution to obtain the final concentrations of 0.3 μmol/L, 0.36 μmol/L, and 0.42 μmol/L. Figure 1 presents the changes in the average particle size, PDI, and zeta potential of milk protein with DHA of varying concentrations. The average particle size was observed to increase significantly with the addition of DHA ($P < 0.05$), rising from 100.67 nm to 137.67 nm in MPC and from 103.49 nm to 128.47 nm in MCC. There was a particularly sharp increase in the average particle size from 4.41 nm up to 153 nm after the addition of 0.30 μmol/mL DHA in WPI (Figure 1A). Thus, the particle size of the treated milk proteins increased with the addition of DHA, especially for WPI. Fatty acids might make interfacial protein film in emulsion softened, which promoted the aggregation of particles to increase the size of particles. This is consistent with previous research that reports an increase in the particle size of bovine serum albumin induced by DHA (Huang et al., 2022). PDI represents the degree of particle size uniformity, and the smaller the PDI, the more uniform the particle size. Previous studies have proven that PDI values between 0.5 and 0.6 indicate that the nanoparticles belong to monodisperse systems (Sponton et al., 2015). The PDI values for all DHA-milk protein complexes were less than 0.6, indicating that the particle size of the milk protein and DHA compounds were distributed uniformly (Figure 1B). Figure 1C reveals that the addition of DHA slightly reduced the zeta potential compared with the milk protein without DHA. At the DHA addition of 0.36 μmol/mL, the MPC, MCC, and WPI proteins exhibited the lowest zeta potential of $-19.05$, $-16.9$, and $-19.65$ mV, respectively. Previous studies have shown that larger absolute zeta potential values increased the stability of the dispersive systems, that is, the compound stability was enhanced (Huang et al., 2022). Therefore, our results and previous research indicated that the addition of 0.36 μmol/mL DHA was optimal for the delivery of milk protein to ASTA in the subsequent fabrication.

ASTA–Milk protein–DHA complex particle size and distribution, Polydispersity index, and Zeta potential

The average particle sizes of A-MPC, A-MCC, and A-WPI were 114.00 nm, 120.80 nm, and 12.89 nm, respectively, and increased by varying degrees with the addition of DHA ($P < 0.05$) (Figure 2A). For example, in the A-WPI-D complex, the particle size increased from 12.89 nm to 106.00 nm. This is in agreement with the results of Huang et al. (2022), who reported that DHA significantly increased the complex particle size of bovine serum albumin-embedded ASTA (Huang et al., 2022). The PDI values of A-MPC-D and A-MCC-D exhibited slightly increases compared with the A-MPC and A-MCC complexes; however, all PDI values remained below 0.6 (Figure 2B). It is noteworthy that the particle size of the ASTA-WPI complex with DHA increased significantly compared with the complex with no DHA, whereas a significant drop in PDI was observed ($P < 0.05$). According to the size distribution (Figure 2D-F) and PDI results, the complex of ASTA-milk proteins formed with DHA displayed a uniform monodisperse system (Sponton et al., 2015). Figure 2C reveals a downward trend of the zeta potential in the ASTA-milk protein with DHA compared with the samples with no addition of DHA. The reduced charge can be attributed to the DHA binding to the protein surface. In particular, DHA is known to contain anionic impurities that bind to proteins and reduce their charge. Moreover, DHA adsorption increases the shear surface on the protein surface, resulting in a decrease in the measured potential (Hu and McClements, 2014). This indicates that DHA can remove the charge on the protein surface from the shear plane.

Encapsulation rate and turbidity of ASTA in milk protein complexes

Figure 3A presents the effect of DHA addition on the ASTA encapsulation rate. Compared with the complex without DHA, the ASTA and milk protein complex with the addition of DHA exhibited a better encapsulation. This is because fatty acid ligands alter the spatial conformation of proteins, thereby increasing the delivery of active substances to proteins (Guo and Zhou, 2019). A-MPC-D exhibited the highest encapsulation rate of 98.81%, higher than the sample with no DHA addition. Similarly, A-MCC-D also presented a significant increase in ASTA encapsulation from 89.6% to 94.98%, and that of A-WPI-D rose by 3.07%. This is similar to the results of a study on dietary fatty acids.
and the protein-based encapsulation of fucoxanthin by Li et al. (2022), whereby the encapsulation efficiency improved by > 98%. In addition, fatty acids as protein ligands can also increase the hydrophobicity of proteins and their affinity for hydrophobic molecules (Maciázk-Jurczyk et al., 2012).

Turbidity reflects the solubility of ASTA in the milk protein complex. The addition of DHA resulted in a significant increase in the turbidity of A-MPC-D and A-MCC-D (Figure 3B). This may be due to the improvement of the solubility of ASTA in the presence of DHA, thus leading to an increase in the amount of ASTA delivered by the protein with the addition of DHA. Previous studies reported that the turbidity of bovine serum albumin-ASTA complex increased with the unsaturation of unsaturated fatty acids (Huang et al., 2022). This is consistent with the experimental results in this study. However, WPI itself has a high

Figure 1. Effect of DHA addition on the average particle size (A), PDI (B), and zeta potential (C) of milk protein. Note: Values with different letters in the same column are significantly different (P < 0.05).

Figure 2. Particle size (A), PDI (B), zeta potential (C), and particle size distributions (D, E, F) of the 3 milk protein complexes. Note: Values with different letters in the same column are significantly different (P < 0.05).
solubility and low turbidity, and the addition of DHA had little influence on the turbidity. Thus, DHA played different roles in the milk proteins. For MPC and MCC, DHA can act as a protein ligand to improve the binding of milk proteins to ASTA, contribute to the solubility of ASTA in the solution, and have a beneficial effect on the formation of complexes.

**Intrinsic fluorescence and surface hydrophobicity analysis**

The emission spectra of the intrinsic fluorescence of proteins can provide reliable information on the micro-environment of aromatic amino acids derived mainly from tryptophan, tyrosine, and phenylalanine, thus increasing our understanding on protein conformation, kinetic properties, and intermolecular interactions (Liu et al., 2021b). The intrinsic fluorescence intensity of individual proteins was stronger than that of the ASTA-protein and ASTA-protein–DHA complexes (Figure 4A-C), indicating that the addition of ASTA led to the endogenous fluorescence burst phenomenon. It also revealed the combination of ASTA small molecules and milk proteins. Moreover, the complexes of A-MCC-D and A-MPC-D exhibited a weaker fluorescence than the complexes without DHA. This demonstrates that the addition of DHA led to the endogenous fluorescence subtract phenomenon of MCC and MPC, further proving that hydrophobic interactions occurred between MCC, MPC, and DHA.

The ASTA complex and the 3 milk proteins exhibited a lower hydrophobicity compared with the single milk protein (Figure 4D-F). This is because milk protein binds to ASTA through hydrophobic interaction, resulting in a decrease in hydrophobicity, which also proves that ASTA binds to milk protein. Furthermore, the addition of DHA significantly reduced the surface hydrophobicity of MPC and MCC ($P < 0.05$). This may be due to the binding of the hydrophobic molecule DHA to the hydrophobic sites on the protein surface, competing for the binding of the hydrophobic probe ANS to the hydrophobic sites of the protein. This decrease in surface hydrophobicity is consistent with the results of the fluorescence attenuation and the interaction between soy lecithin and whey protein in Sun et al. (2018), which exhibited a similar trend. However, the increase in surface hydrophobicity is due to protein defolding, exposing hydrophobic amino acid residues (Zhang et al., 2021). Our results suggest that DHA contributed to the de-folding degree of milk protein, thus exposing the hydrophobic component previously buried on the inside and increasing the surface hydrophobicity.

**Antioxidation analysis and bioaccessibility of ASTA in milk protein complexes**

The antioxidant activity of the complexes was evaluated based on the DPPH, ABTS, and ·OH radical scavenging activity, which indicate the ability of the antioxidant to transfer free radicals. The antioxidant abilities exhibited great improvements after astaxanthin was encapsulated with 3 types of milk proteins treated with ultrasound-assisted pH-shifting as carriers (Figure 5A-C). Compared with WPI, astaxanthin loaded by MPC and MCC presented a stronger antioxidant capacity, increasing by 8.43% for DPPH and 10.12% for ·OH. DHA further enhanced the radical scavenging activity of DPPH, ABTS$\textsuperscript{+}$, and ·OH compared with the ASTA-milk protein complex with no DHA. This is because the addition of DHA induced a stronger interaction between

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Encapsulation rate of ASTA in milk protein complex (A) and turbidity (B) and visual appearance (C) of the milk protein complex. Note: Values with different letters in the same column are significantly different ($P < 0.05$).
the protein and ASTA, resulting in more ASTA binding to the protein (Huang et al., 2022). However, the antioxidant capacity of unsaturated fatty acids may exert a positive effect, and thus, the antioxidant activity of the complex does not drain the corresponding contribution of DHA. This demonstrates that the delivery of ASTA into DHA-mediated milk proteins does not alter the internal structure and biological activity of ASTA itself (Du et al., 2022; Huang et al., 2022).

ASTA is reportedly prone to protonation and isomerization in acidic conditions, causing degradation that reduces its bioavailability (Du et al., 2022; Huang et al., 2022). ASTA showed an improved bioaccessibility after being encapsulated by milk proteins, particularly with the encapsulation loaded by MPC and MCC, and the great improvement for the samples with the addition of DHA (Figure 5D). For MPC and MCC with the addition of DHA, bioaccessibility increased by 10.60% and 11.48%, respectively. In contrast, the bioaccessibility of A-WPI-D exhibited a considerable reduction. This may be due to the aggregation of WPI by the addition of DHA, as evidenced by an increase in particle size. Larger particle size carriers have a poorly digestive stability and therefore do not effectively protect ASTA. Similar results were also observed in the study by Huang et al. (2022), in which a reduction in ASTA bioaccessibility compared with ASTA-BSA was induced by the introduction of DHA (Huang et al., 2022). Dietary lipids play a central role in promoting the bioavailability of ASTA by enhancing micellization during digestion and intestinal transport, and lipids in the food matrix are important for the intestinal absorption of ASTA in vivo. The addition of DHA ligands during the delivery of ASTA by MPC and MCC is more than enough to promote the absorption of ASTA. Previous research has reported that the bioaccessibility of micellarized carotenoids is inversely proportional to droplet size (Salvia-Trujillo et al., 2013; Sotomayor-Gerding et al., 2016). Furthermore, a DHA-BSA carrier with a large particle size displayed poor digestion stability (Huang et al., 2022), which is also demonstrated by the DHA-WPI carrier in this study. Thus, the mediating effects of DHA on protein carriers with ASTA varies with the carrier type. However, the underlying mechanisms require further research.

Retention of ASTA during storage and thermal stability

A higher retention of ASTA was observed in samples embedded by milk protein with DHA compared with those without the addition of the DHA ligand, and there were large differences in the degree of protection of ASTA during storage for the different compositions of milk proteins as carriers (Figure 6A). After 35 d of storage, the highest retention rate of ASTA was 94.26%
for A-MCC-D, suggesting that MCC exhibited the best protection capability for ASTA compared with MPC and WPI. In addition, the delivery of WPI with DHA resulted in a significant reduction in the retention of ASTA compared with the WPI carrier without DHA during storage. Similar results were also reported by Huang et al. (2022), whereby the introduction of unsaturated fatty acids was observed to reduce the storage stability of ASTA to some extent. In this study, obvious differences were found in the effect of DHA on the 3 milk proteins as carriers. This may be attributed to the characteristics of milk proteins and the interaction occurring in the milk protein complexes and DHA. The storage stability results were in close agreement with the particle size, zeta potential, and hydrophobicity behavior. Slight changes in the particle size of MPC and MCC were observed after the addition of DHA; however, that of WPI sharply increased, indicating that the WPI molecules happened to form aggregates with DHA.

The ASTA retention in all samples exhibited a decline with the increasing temperature (Figure 6B), which is similar to the lutein in gels after heat treatment reported by Li et al. (2023). The ASTA retention was greatly improved after loading in the milk protein complexes with and without DHA. This indicates that the milk proteins had a better protective effect on astaxanthin, yet this protective effect weakened with the increasing temperature. This is due to the disruption of the network structure of the milk protein complex, resulting in the release and degradation of the ASTA encapsulated therein in the presence of heat. The thermal stability of different milk protein complexes varied, with the highest observed for the MCC complex, in which astaxanthin retention rate was able to reach up to 90.19% after heating at 85°C for 30 min, followed by the MPC and WPI complexes. As the unstructured nature of casein is thermally stable, both casein micelles and caseinate (Daniloski et al., 2022). Moreover, previous research has demonstrated that pH-shifts can also improve the thermal stability of rice starch/casein based on high internal phase emulsions (Liu et al., 2023), and thus, the MCC complex can effectively protect astaxanthin. In contrast, whey proteins are thermally unstable and

![Figure 5. DPPH (A), ABTS (B), ·OH (C) radical scavenging activity, and bioaccessibility (D) of milk protein complexes and appearance of supernatant after centrifugation of sample digest (E). Note: Values with different letters in the same column are significantly different (P < 0.05).](image-url)
easily denatured at temperatures of approximately 70°C, in which β-lactoglobulin contains a free thiol group, which is readily exposed by thermal unfolding (Daniloski et al., 2022). Thus, MPC has a small portion of whey protein in addition to casein, resulting in a thermal stability that is less than that of MCC but higher than that of WPI.

Textural characteristics of cheese

Protein and moisture content are closely related to the texture of cheese (e.g., hardness, gumminess, and chewiness). Table 2 reveals that MCC-C exhibited high hardness, gumminess, and chewiness values, with significant differences between these values and those of the other samples. In the study by Xia et al. (Xia et al., 2022), MCC-C, with a higher casein content than standard milk, exhibited a lower moisture content, and the cheeses were subjected to more frequent collisions during mixing, which resulted in greater pressure being exerted on the curd particles. Cheese with a higher casein content is harder and more chewy, which is attributed to its higher dry matter and protein content, lower level of water-soluble nitrogen, and/or thicker strands of the protein network (Soodam and Guinee, 2018). In this study, WPI-C prepared from more whey protein exhibited the lowest hardness, gumminess, and chewiness values. Furthermore, MPC contained a smaller portion of whey protein in addition to casein, and thus, the textural properties of Control-C and MPC-C were also influenced by the presence of a small amount of whey protein. Previous research found that the uniform distribution of whey protein microgels through the protein matrix not only increased the moisture content of low-fat cheeses, but also prevented the formation of a denser casein network structure, resulting in a looser casein network (Wen et al., 2021). Note that Control-C was harder, gummier, and chewier than MPC-C, indicating that ASTA-protein carriers with different binding modes produce different structures during cheese production. This may be related to the binding and cross-linking of ASTA to proteins, reducing casein interactions, and decreasing the network structure in the cheese.

In vitro digestion simulation of ASTA in processed cheese

The cheese samples were diluted and exposed to solutions containing pepsin, trypsin, and bile salts during simulated digestion. The proteins were hydrolyzed and astaxanthin was released and centrifuged into the micellar fraction (Li et al., 2023). The micellar phase typically includes phospholipids, bile salts, undigested soluble proteins, and peptides, contributing to the bioaccessibility of ASTA (Yuan et al., 2019). The bioaccessibility of ASTA in MPC-C, MCC-C, and WPI-C reached 43.64%, 39.47%, and 45.67%, respectively, all of which are significantly higher than Control-C (Table 3). The increased bioaccessibility of ASTA indicates that the milk protein carrier was an effective delivery vehicle for ASTA and its impact depended on the protein varieties. A tighter network and smaller pores could increase the diffusion resistance of the actives.
in the gel structure, thus reducing their release rate (Li et al., 2023). The results showed that the release of ASTA was highly correlated with the structure of milk proteins and their changes at the different digestion stages. The gel network was not easily hydrolyzed during the simulated digestion because of the high stability and stiffness values of MCC-C. In contrast, WPI-C is easily digested and decomposed. Our results are in agreement with those of previous studies. For example, in the study of Li et al. (2023), the bioavailability of embedded lutein in processed cheese increased to 34.86% via induction method for the preparation of whey isolate protein-milk fat emulsion gels. DHA-mediated bovine serum albumin carriers also improved ASTA bioavailability, and dietary lipids play a central role in promoting ASTA bioavailability by enhancing micellization during digestion and intestinal transport (Huang et al., 2022).

**Determination of storage stability of ASTA**

Figure 7 depicts the retention of ASTA in cheeses made from different milk protein carriers under storage conditions at 4°C. The retention of ASTA in Control-C was significantly lower than that of the other 3 experimental groups during the 28-d storage period. This indicates that ASTA with no embedding in its carriers was more susceptible to thermal decomposition during cheese processing and to the degradation or aggregation during storage, resulting in the release of solubilized ASTA and a reduction in the protective effect. The degradation effect of ASTA in the 3 cheeses from milk protein carriers was significantly inhibited during storage. The highest retention of ASTA was found in MCC-C, followed by MPC-C and WPI-C. This is consistent with the results of the milk protein mixture stability. Moreover, the airtight structure of the cheeses could also effectively encapsulate and protect the stability of the milk protein carriers. However, the retention rates of the 4 groups of samples converged from the 27th day onwards, indicating that protective effect of milk protein carriers on ASTA is limited and decreases with time.

**CONCLUSIONS**

This work investigates DHA in terms of 3 types of milk protein ligands for the embedding of ASTA under ultrasound-assisted pH-shifting treatment and its application to processed cheeses. The milk protein–DHA complex exhibited a strong embedding effect and excellent antioxidant properties of ASTA compared with the complex without DHA. For MPC and MCC, the addition of DHA significantly improved the hydrophobicity site, thermal stability, retention and bioaccessibility of ASTA, and was conducive to its application to cheese production. The results indicate the positive effect of all 3 milk protein carriers on cheese texture and the bioavailability and storage stability of ASTA, with MCC exhibiting the best protection of ASTA, followed by MPC and WPI. This study further confirms that the ultrasound-assisted-pH-shifting treatment of DHA-mediated ASTA delivered milk protein provides an effective strategy to improve the characteristics of ASTA in water, providing a strong basis for research aimed to solve the application challenges of ASTA in hydrophilic food systems.

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| Table 2 Results of cheese texture characteristics  |
|---------------------------------------------|-------------|
| Samples         | Control-C | MPC-C | MCC-C | WPI-C |
| Hardness (g)    | 713.2 ± 11.5b | 621.4 ± 76.7bc | 864.6 ± 76.2a | 558.0 ± 62.8c |
| Springiness     | 0.77 ± 0.04c | 0.78 ± 0.01c | 0.70 ± 0.02b | 0.78 ± 0.02c |
| Cohesiveness    | 0.58 ± 0.01bc | 0.56 ± 0.02bc | 0.50 ± 0.01c | 0.60 ± 0.01c |
| Gumminess (g)   | 415.8 ± 15.2bc | 350.8 ± 55.1c | 474.2 ± 45.6c | 332.1 ± 34.4c |
| Chewiness (g)   | 321.6 ± 30.1bc | 273.8 ± 47.2bc | 342.0 ± 31.8b | 260.2 ± 31.0b |
| Resilience      | 0.23 ± 0.01b  | 0.25 ± 0.01c  | 0.22 ± 0.02bc | 0.26 ± 0.01a  |

| a–c Values in the same column with different superscript letters are significantly different (P < 0.05). |

| Table 3 Bioavailability of ASTA in cheese |
|------------------------------------------|-------------|
| Samples        | Bioavailability (%) |
| Control-C      | 21.14 ± 0.40d   |
| MPC-C          | 43.64 ± 0.81b   |
| MCC-C          | 39.47 ± 0.56c   |
| WPI-C          | 45.67 ± 0.52a   |

| a–d values in the same column with different superscript letters are significantly different (P < 0.05). |


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