Molecular insight into binding behavior of caffeine with lactoferrin: Spectroscopic, molecular docking, and simulation study

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ABSTRACT

The majority of bioactive substances in the human diet come from polyphenols. Here, we use spectroscopy, molecular docking, molecular dynamics simulations, and in vitro digestion to look at the relationship between caffeine (CAF) and bovine lactoferrin (BLF). The correlation analysis of the CAF-BLF fluorescence quenching process revealed that the reaction was spontaneous and that the CAF-BLF fluorescence quenching process may have been static. The predominant intrinsic binding forces were hydrogen bonds and van der Waals forces, which were also supported by molecular docking and molecular dynamics simulations. Through Fourier infrared and circular dichroism spectroscopy experiments, it was found that CAF changed the secondary structure of BLF and might bind to the hydrophobic amino acids of BLF. Compared with BLF, CAF-BLF showed inhibitory effects on digestion in simulated in vitro digestion. It will be helpful to better understand the interaction between CAF and BLF and provide the basis for the development of innovative dairy products. Key words: caffeine, lactoferrin, spectroscopy, structure, digestibility

INTRODUCTION

An iron-binding glycoprotein found in milk called bovine lactoferrin (BLF) has a high capacity to bind iron (Jia et al., 2021). Its molecular weight is around 80 kDa (Krolitzki et al., 2022). Bovine lactoferrin is used extensively in the food and medical industries as a high-value dietary protein (Krolitzki et al., 2022). Studies have shown that BLF has antibacterial, anti-inflammatory, and antitumor properties in addition to its ability to regulate the immune system of mammals, lessen gastrointestinal discomfort, maintain iron balance in the body, and promote the absorption of calcium and magnesium and other nutrients (Ochoa et al., 2020; Cao et al., 2021; Jing et al., 2021). Bovine lactoferrin controls cell growth and eliminates damaging free radicals (Zhang et al., 2020). The BLF that enters the small intestine guards against oxygen-free radicals in the body (Wang et al., 2019). Yet, if the gastric fluids degrade the BLF, then BLF cannot fulfill its purpose. In this situation, BLF lost some of their biofunctional characteristics and became less bioavailable (van der Strate et al., 2001; Wang et al., 2017, 2021). The gastrointestinal system must therefore receive BLF in a structurally sound and biologically functional state.

Proteins and polyphenols can easily form complexes and, as is well known, have a very strong affinity for one another. The structure and functional characteristics of proteins may change as a result (Jiang et al., 2019). Covalent and noncovalent interactions are the 2 main forms of interactions between polyphenols and proteins. Nevertheless, quinone polymers and other unfavorable byproducts such as covalent protein-polyphenol interactions could develop (Yildirim-Elikoglu and Erdem, 2017). High temperatures and alkaline environments can significantly influence the stability of phenolic compounds, and covalent polyphenol-protein complexes can reduce the biological activity and bioavailability of dietary polyphenols (Trombley et al., 2011; Cao and Xiong, 2017). Noncovalent protein-polyphenol interactions are more common than covalent bonds, which include hydrogen bonding, hydrophobic interactions, and van der Waals gravity (Yildirim-Elikoglu and Erdem, 2017). Khan et al. studied the interaction between sunset yellow dye and β-LG using various spectral and molecular modeling techniques. The thermodynamic results showed that the interaction between sunset yellow dye and β-LG using various spectral and molecular modeling techniques. The thermodynamic results showed that the interaction between sunset yellow dye and β-LG was mainly hydrophobic (Khan et al., 2022). Al-Shabib et al. clarified the binding mechanism of catechin and β-LG and found through spectral tests that the interaction between β-LG and catechin occurred spontaneously mainly through hydrophobic interaction (Al-Shabib et al., 2020). The changes in enthalpy (ΔH) and the amount of entropy shift (ΔS) measured by Al-Shabib et al. by fluorescence indicate

Received April 20, 2023.
Accepted June 29, 2023.
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that the main interaction between the natural polyphenol compound rutin and β-LG is hydrophobic (Al-Shabib et al., 2018). Precupas et al. used isothermal titration calorimetry, differential scanning calorimetry, circular dichroism, and molecular docking methods to study the interaction between caffeic acid and BSA, and the results showed that the presence of caffeic acid enhanced the stability of BSA (Precupas et al., 2017).

To stimulate the central nervous system, caffeine (CAF) is used. It boosts brain activity and the body’s circulation of hormones similar to cortisol and adrenaline (Belščak-Cvitanović et al., 2015; Corrêa et al., 2021). As a result, caffeine is regarded as a superb nonvolatile alkaloid substance (Rashidinejad et al., 2022). Unfortunately, caffeine has a bad taste, and consuming too much of it can harm your nervous and cardiovascular systems. According to studies, adding protein can help solve solubility issues and lessen the unpleasant sensation (Santa Rosa et al., 2021). To comprehend the forces that bind protein-polyphenol complexes together, this study explored the molecular interactions between BLF and CAF. To aid in future research on the functional qualities of these complexes in food, we also detailed the structural characteristics of the complexes generated by BLF and CAF. Finally, an in vitro simulation of the CAF-BLF complex and BLF digestion alone was carried out to investigate the protein-polyphenol complex's protection and stability maintenance method of protein function.

MATERIALS AND METHODS

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

Materials

Yuanye Biotechnology Co. Ltd (Shanghai, China) was paid for bovine lactoferrin (BLF, 95%), and crude bovine lactoferrin was purchased from Beston Pure Dairies Pty LTD. (South Australia, Australia). Caffeine (CAF, 99.96%) came from Chengdu Biotechnology Co., Ltd., and Kangwei Century Biotechnology (Beijing) Co. Ltd. and Beijing Solaibao Technology Co. Ltd. both sold BCA protein quantification kits and SDS-PAGE gel preparation kits. Coomas Bright Blue G-250 was purchased from Tianjin Comeiou Chemical Reagent Co. Ltd. Rainbow 130 Broad-spectrum protein marker (15–130 kDa) was purchased from Beijing Soleibao Technology Co. Ltd. We purchased SDS from Tianjin Fuchen Chemical Reagent Factory. Protein was dissolved in PBS (0.1 mol L⁻¹, pH 7.4). Analytical grade reagents were also employed in the experiment, and ultra-pure water was the water used in the experiment. In further studies, none of the samples underwent more purification.

Reagent Configuration

With the aid of a PBS buffer, the BLF protein solution was made into a 10 µM working solution. Caffeine was produced with a PBS buffer to create a working solution with a 500 µM concentration. This solution was maintained in brown glass tubes and diluted as needed for further experiments. In a certain proportion, water, glacial acetic acid, and ethanol made up the decolorizing solution. A specific ratio of Tris, glycine, SDS, and water made up the electrophoresis buffer. Following preparation, all reagents were kept in a refrigerator at 4°C.

UV-Visible Spectrum

The UV absorption spectra of the samples were measured by Puxi T9CS double-beam UV-visible (UV-Vis) spectrophotometer. To produce 9 distinct BLF: dropwise additions of a 500 µM CAF solution were made to a pure sample of protein after 4 mL of a 10 µM BLF protein sample solution had initially been added to the quartz cuvette (1:0, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:10). The 240 to 400 nm spectral range was scanned.

Fluorescence Spectrum

The fluorescence spectrum of a 4-mL sample of the BLF protein at a concentration of 10 µM was scanned using an RF-6000 fluorescence spectrophotometer. The protein was then added dropwise to the CAF solution at a concentration of 500 µM to generate 9 distinct BLF: CAF molar ratios (1:0, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:10). At 280 nm, the excitation wavelength was set. The scanning range of 290 to 450 nm data were used to determine the emission wavelength. The excitation and emission light’s slit width were set to 5 nm. Three distinct temperatures—290 K, 300 K, and 310 K—were used in the trials.

Fourier-Transform Infrared Spectroscopy

To create Fourier-transform infrared spectroscopy (FTIR) samples, BLF (10 µM) and CAF (500 µM) solutions were combined in ratios of 1:0 and 1:8. The test sample was smeared onto spectral grade KBr tablets for analysis after being pulverized and compressed.
into tablets. The measurements, which had a resolution of 4 cm$^{-1}$ and covered the range from 4,000 to 400 cm$^{-1}$, were made using a Bruker Invennio S Fourier infrared spectrometer.

**Circular Dichroism Experiment**

A Characin CD spectrometer was used to gather CD spectral information from the protein solution. A drop-wise addition of CAF solution with an amount of 500 μM was made to the pure sample of protein after adding 1 mL of the 10 μM BLF sample of protein solution to the quartz cuvette to produce 3 distinct BLF: molar ratios of CAF (1:0, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:10). Far UV spectrum data were gathered by scanning the wavelength region between 190 and 260 nm. To create the circular chromatograms for each system and the contents of each protein secondary structure component, the data were processed using CDNN software (version 2.1).

**Molecular Docking Simulation**

Molecular docking simulation tests were carried out with the help of AutoDock 4.2. In this experiment, the crystal structure of BLF (PDB ID: 1BIY) was downloaded from the Protein Data Bank (PDB) website charge. After being loaded into pymol, it underwent a series of modifications to become an automatic docking-compatible system file, including the removal of water, the addition of hydrogen atoms, the correction of erroneous amino acids, and an adjustment of charge. PubChem (https://pubchem.ncbi.nlm.nih.gov/) provided the CAF structure, which was then translated to PDB format using Open Babel (PubChem CID: 2519; Santa Rosa et al., 2021). The Lamarckian Genetic Algorithm was used for semi-flexible molecular docking, and we selected the grid box size to cover the complete protein at 126 × 126 × 114 Å. To find the best conformation after docking and further examine the microenvironment of the CAF-BLF complex binding site, pymol was employed to visually investigate the predictions.

**Molecular Dynamics Simulation**

Gromacs2022.3 software was used for molecular dynamics simulation (Van Der Spoel et al., 2005). Gaussian 16W was applied to hydogenate small compounds and determine RESP potential, and AmberTools22 was used to apply the GAFF force field to tiny molecules. Potential information would be added to the molecular dynamics system’s topology file. Atmospheric pressure of 1 bar and a static temperature of 298 K were used for the simulation. Amber99sb-ildn was used as the force field, water molecules were used as the solvent (TIP3p water model), and an appropriate amount of Na$^+$ ions were added to balance out the system’s overall charge. With a coupling constant of 0.1 ps and a duration of 100 ps, the simulation system first used the steepest descent method to minimize energy before running through the isothermal isovolumic ensemble (NVT) equilibrium and isothermal isobaric ensemble (NPT) equilibrium for 100,000 steps in each case. The simulation of free molecular dynamics was then completed. The operation took 10 ns to complete and had 5,000,000 steps with a step length of 2 fs. The software’s built-in tool was applied to examine the trajectory when the simulation was complete. Each AA trajectory’s root-mean-square variance (RMSD), root-mean-square fluctuation (RMSF), and protein rotation radius were merged with the free energy (MMPBSA), free energy topography, and other data (Abraham et al., 2015).

**Simulated In Vitro Digestion Experiment**

An in vitro digestion system with oral, gastric, and intestinal phases was used to track the digestive patterns of BLF and the CAF-BLF complex. The activity of digestive enzymes and the concentration of bile salts at each stage of digestion were measured experimentally or by recommended standardized measurements (Brodkorb et al., 2019; Zhang et al., 2019; Wan et al., 2023). INFOGEST method was used to simulate the digestive process in vitro (Brodkorb et al., 2019).

Five milliliters of 300 µg/mL BLF and CAF-BLF samples were mixed with 4 mL of oral digestive fluid and 1 mL of 750 U/mL α-salivary amylase, respectively, and shaken for 2 min at a low speed (40–60 rpm) at 37°C and away from light. After the completion of oral digestion, a 1 mL reaction sample was immediately placed on ice to terminate digestion and was used as the oral digestion group (SSF). The rest of the solution continued gastric digestion. The remaining samples after oral digestion were mixed with 6.5 mL of gastric digestive fluid and 2.5 mL of 15,000 U/mL pepsin, and the pH was adjusted to 3.0. Shake at a low speed (40–60 rpm) for 120 min at 37°C and avoid light. After the completion of gastric cavity digestion, a 1-mL reaction sample was immediately placed on ice to terminate digestion and was used as the gastric cavity digestion group (SGF). The rest of the solution continued intestinal digestion. The remaining samples after oral digestion were mixed with 6.5 mL of gastric digestive fluid and 2.5 mL of 15,000 U/mL pepsin, and the pH was adjusted to 3.0. Shake at a low speed (40–60 rpm) for 120 min at 37°C and avoid light. After digestion, the reaction samples were placed on ice immediately...
to terminate digestion and were used as the intestinal cavity digestion group (SIF). The digestive liquids at different stages were centrifuged at 4°C, 3,000 × g, for 5 min, and the supernatant was taken and refrigerated at −80°C for subsequent analysis.

**Bicinchoninic Acid Assay**

Ten percent TCA was uniformly distributed throughout a portion of the digestive solutions from each system, and the combination was then submerged in ice water at 4°C for 30 min. In a frozen centrifuge, the solution was removed and centrifuged for 10 min at 888 × g at 4°C. After removing the supernatant, the digestibility of proteins in each system was evaluated with a BCA test. To get the average, the experiment was run 3 times.

**SDS-PAGE**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to evaluate the digestion materials in 12% (wt/vol) acrylamide gels. Eight distinct sample types were examined: (A) oral digestion of BLF; (B) oral digestion of CAF-BLF; (C) oral and gastric digestion of BLF; (D) oral and gastric digestion of CAF-BLF; (E) oral, gastric, and intestinal digestion of BLF; and (F) oral, gastric, and intestinal digestion of CAF-BLF. The above samples were loaded into the centrifugal tubes (volume 1.5 mL) after being thoroughly mixed with the 4:1 volume-to-volume 5× SDS-PAGE loading buffer. They were then heated in a water bath at 100°C for 3 to 5 min. It was then chilled for subsequent use in an ice bath. Each lane was loaded with aliquots of the following samples: Marker, SSF (BLF), SSF (CAF-BLF), SGF (BLF), SGF (CAF-BLF), SIF (BLF), and SIF (CAF-BLF). Different settings were established at different voltages for separation and electrophoresis, respectively. The electrophoresis stopped when the indicator contacted the ground. The gel was scraped after electrophoresis, washed with deionized water, and then stained with Coomassie Brilliant Blue G-250 for 2 h on a shaker. The gel was taken out, washed with water that had been twice distilled, and then allowed to soak in a decolorizing solution all night. The decoloring solution should be swapped out periodically.

**RESULTS AND DISCUSSION**

**Experimental Analysis of UV-Vis Spectra**

An inexpensive, straightforward, adaptable, and nondestructive technique for analyzing organic materials and some inorganic stuff is UV-Vis absorption spectroscopy (Rocha et al., 2018). It is used to investigate how proteins change structurally and how their complexes form. In the UV-Vis spectroscopy, proteins and polyphenols can be absorbed. The structure of the protein skeleton is reflected at 200 nm. It is an aromatic AA at 280 nm. Typically, electron transitions between π-molecular orbitals are responsible for their UV-visible absorption spectra (Poklar Ulrih, 2017). Hence, UV-Vis spectroscopy investigations were used to investigate the interaction mechanism between CAF and BLF.

The UV-Vis spectra of BLF at various caffeine doses are depicted in Figure 1A. At first, it was largely possible to overlook the CAF absorption peak strength in the protein UV spectrum’s wavelength range. Second, the UV absorption intensity of the BLF amino residue at 280 nm dramatically increased with the rise in CAF content. The CAF-BLF complex system’s UV absorption spectrum in the enlarged image showed a little red shift. This redshift of the absorption peak might be explained by CAF interacting with the hydrophobic AA residues of BLF molecules to form a hydrophobic cavity (Poklar Ulrih, 2017).

Regular changes in the UV absorption spectra of CAF upon addition further suggested that CAF and BLF could interact to create complexes, while further research would be required to confirm the mechanism of the complex system’s interaction. Next, we will use a variable temperature fluorescence experiment to further calculate the thermodynamic values of the binding process.

**Fluorescence Quenching**

**Fluorescence Quenching Type Between Caffeine and Lactoferrin.** The fluorescence emission of a protein solution containing these fluorophores is typically decreased when ligands are added due to the fluorescence quenching of small molecules. However, the inner-filter effect, which occurs when the ligand appropriately absorbs light in the excitation and emission wavelength range, has a great effect on the UV-Vis absorption capabilities of the ligand and the fluorescence attenuation of the protein (Rubio et al., 1986). The fluorescence intensity was adjusted using the following equation to remove the inner-filter effects:

\[
F_{\text{cor}} = F_{\text{obs}} \times \frac{A_{\text{ex}}}{A_{\text{em}}} \times \frac{A_{\text{ex}}^{2} + A_{\text{em}}^{2}}{2},
\]

where \(A_{\text{ex}}\) and \(A_{\text{em}}\) are the quencher and lactoferrin’s absorption intensities at excitation and emission wavelengths, and \(F_{\text{cor}}\) and \(F_{\text{obs}}\) are the fluorescence intensities observed and corrected, respectively.
The behavior can be explained by a variety of quenching methods, but the 2 that are most frequently used are dynamic quenching and static quenching. Dynamic quenching is the reduction of fluorescence intensity without changing the structure of the protein caused by the interaction of quenching small molecules with fluorescent excited state proteins. The development of a nonluminescent ground state complex between fluorescent molecules and ground state quenching molecules results in static quenching of the fluorescence quenching process, which weakens fluorescence and alters the molecular structure of proteins (Zhu and Ryan, 2016). Because greater temperatures might lead to bigger diffusion coefficients, the bimolecular quenching constants will rise in the dynamic quenching process as the temperature rises. In contrast, because complexes become less stable as temperature rises, the static quenching constants are anticipated to decrease (Zhang et al., 2013). Dynamic quenching and static quenching can be identified based on these traits. To further explore, we used the Stern-Volmer Equation 2 to process experimental data:

$$\frac{F_0}{F} = 1 + K_{SV} [Q] = 1 + K_q \tau_0 [Q],$$  \hspace{1cm} [2]

where $F_0$ and $F$ are lactoferrin’s strengths with and without a quenching agent, respectively. The quencher concentration is $Q$. Stern-Volmer quenching constant is $K_{SV}$. The quenching rate constant is $K_q$. It is lactoferrin’s typical half-life, which is 10 ns when the quencher is not present. By doing a linear regression on the $F_0/F$ and $Q$ pictures, the Stern-Volmer equation may calculate the value of $K_{SV}$.

Figure 1B shows the effect of caffeine on lactoferrin at temperatures of 300 K. As seen in the image, the interaction between caffeine and lactoferrin resulted in a considerable drop in lactoferrin fluorescence intensity with an increase in caffeine concentration.

The relationship between $F/F_0$ and caffeine concentration is plotted from the data in Figure 1B (see Figure 2). There was a good linear relationship between them. At 290 K, 300 K, and 310 K temperatures, the quenching process rate constant $K_q$ was computed (see Table 1). It could be seen from the experimental data that the value of $K_q$ was much higher than the dynamic quenching constant $2.0 \times 10^{10} \text{ L mol}^{-1} \text{s}^{-1}$ of the maximum diffusion collision. The technique by which caffeine
quenched the fluorescence of lactoferrin might therefore be static quenching rather than dynamic quenching.

Binding Constant and Binding Site Analysis Between Caffeine and Lactoferrin. Equation 3 can be used to explain static quenching. The slope and intercept of \( \log\left(\frac{F_0 - F}{F}\right) \) and \( \log\left[\frac{1}{(Q - P)(F_0 - F)/F_0}\right] \) in the double-logarithm equation can be used to calculate the binding constant \( K_A \) and the binding site \( n \) for static quenching:

\[
\log\frac{F_0 - F}{F} = n\log K_A - n\log \left(\frac{1}{(Q - P)(F_0 - F)/F_0}\right),
\]

where \([P]\) is the concentration of BLF, \( K_A \) is the binding constant, \( n \) is the number of binding sites, and \( F_0 \) and \( F \) are the strengths of lactoferrin with or without a quenching agent. The quencher concentration is \( Q \).

Figure 3 shows the \( \log\left(\frac{F_0 - F}{F}\right) \) and \( \log\left[\frac{1}{(Q - P)(F_0 - F)/F_0}\right] \) relationships during quenching at different temperatures, the \( K_A \) and \( n \) can be determined from the y-axis intercept and slope, which are shown in Table 1. It could observe that the \( K_A \) decreased with the rising temperature, which further suggested that the quenching between CAF and BLF was static quenching. Additionally, a medicine that is substantially protein-bound will normally have a \( K_A \) value between \( 10^5 \) and \( 10^7 \) L mol\(^{-1}\), while a drug that is moderately or weakly protein-bound will have a \( K_A \) value between \( 10^2 \) and \( 10^4 \) L mol\(^{-1}\) (Yamashita et al., 2013). The binding force between the CAF and BLF was therefore modest to moderate in vivo.

Thermodynamic Analysis and Interaction Force of Caffeine and Lactoferrin. Hydrogen bonds, electrostatic forces, van der Waals forces, and hydrophobic interactions are among the mechanisms through which tiny molecules and proteins interact. One way to assess this interaction is to examine the change in thermodynamic characteristics. The calculation equation was as follows:

\[
\log K_A = -\frac{\Delta H}{2.303RT} + \frac{\Delta S}{2.303R},
\]

\[
\Delta G = \Delta H - T\Delta S,
\]

where \( K_A \) is the connection constant in Equation 3, \( T \) is the thermodynamic temperature, \( R \) is the gas’s stability, \( \Delta H \) and \( \Delta S \) are the changes in enthalpy and the amount of entropy shift, respectively, and \( \Delta G \) is the free energy of Gibbs.

In Figure 4, the \( \log K_A \) versus \( 1/T \) curve is displayed. After linearly fitting the data, Table 1 lists the values of \( \Delta H \) and \( \Delta S \). Using Equation 5, the \( \Delta G \) value is determined and listed in Table 1. The hydrogen bonds and van der Waals force appeared to be the primary internal bonding forces between caffeine and lactoferrin as CAF and BLF’s \( \Delta H \) and \( \Delta S \) values were −40.54 kJ mol\(^{-1}\) and −63.3 kJ mol\(^{-1}\), respectively (Ali and

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**Table 1. Stern-Volmer quenching constants, binding constants, number of binding sites, and thermodynamic parameters of lactoferrin-caffeine system at different temperatures**

<table>
<thead>
<tr>
<th>T (K)</th>
<th>( K_{SV}^2 ) (× 10(^4) L mol(^{-1}))</th>
<th>( K_q^2 ) (× 10(^12) mol(^{-1}) s(^{-1}))</th>
<th>( K_A^2 ) (× 10(^3) L mol(^{-1}))</th>
<th>( n )</th>
<th>( \Delta H^3 ) (kJ mol(^{-1}))</th>
<th>( \Delta S^3 ) (J mol(^{-1}) K(^{-1}))</th>
<th>( \Delta G^3 ) (kJ mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>290</td>
<td>3.75</td>
<td>3.74</td>
<td>3.80</td>
<td>1.29</td>
<td>−40.54</td>
<td>−63.30</td>
<td>−21.766</td>
</tr>
<tr>
<td>310</td>
<td>3.46</td>
<td>3.46</td>
<td>3.24</td>
<td>1.06</td>
<td>−20.506</td>
<td>−20.506</td>
<td>−20.506</td>
</tr>
</tbody>
</table>

\( T \) = thermodynamic temperature.

\( K_{SV} \) = the quenching constant; \( K_q \) = the quenching rate constant; \( K_A \) = the binding constant.

\( \Delta H \) = the change in enthalpy; \( \Delta S \) = the change in the amount of entropy shift; \( \Delta G \) = the change in the free energy of Gibbs.
Al-Lohedan, 2017). $\Delta H < 0$ indicated that the reaction was exothermic, and $\Delta G < 0$ indicated that the reaction between caffeine and lactoferrin was spontaneous.

**FTIR Spectroscopy.** To determine the secondary structure of proteins, FTIR is performed. Fourier-transform infrared spectroscopy can provide comprehensive information regarding chemical composition (Yang et al., 2022). The advantages of FTIR over circular dichroism spectroscopy include the ability to analyze protein structure in solids, crystals, and aqueous solutions, as well as the effectiveness of each wave number’s absorbance. PeakFit 4.12 software was used to calculate the area of each peak of the amido I band in FTIR spectra, and the secondary structure of bovine lactoferrin after caffeine was added was calculated. The results are shown in Table 2. The secondary structure of lactoferrin was changed after the binding of lactoferrin with caffeine, in which the proportion of $\alpha$-helix and $\beta$-turns decreased, and the proportion of $\beta$-sheet and random coil increased. It was possible that CAF’s binding to hydrophobic areas of the BLF molecule, which disrupted some hydrogen bond networks, was what caused the observed reduction in $\alpha$-helix structure (Xiong et al., 2016). Three important characteristic absorption bands are included in the FTIR spectrum, 1,700 cm$^{-1}$ to 1,600 cm$^{-1}$ (amide I band) represents the tensile vibration of C=O, 1,600 cm$^{-1}$ to 1,500 cm$^{-1}$ (amide II band) represents the bending vibration of N–H and C–H, and 1,300 cm$^{-1}$ to 1,260 cm$^{-1}$ represents the vibration of C–O and C–O–C (Wang et al., 2022). As shown in Figure 5(A), the addition of caffeine increases the peak value of lactoferrin, and C=O is stretched.

**Circular Dichroism.** The interaction of caffeine with BLF may have an effect on the structure of BLF by a variety of spectral approaches. As a result, the BLF’s structure needs to be verified. The secondary structure of lactoferrin and caffeine was examined using a circular dichroic spectrometer. The alteration of secondary structure can be seen in the far UV band (200–250 nm; Khalil and Kashif, 2023). A common technique for determining the secondary structure of proteins is circular dichroism. The modifications to the $\alpha$-helix, $\beta$-sheet, $\beta$-turn, and random coil architectures are depicted in Figure 5(B). With a rise in CAF concentration, the contents of the $\beta$-sheet and random curling structures showed an upward trend ($P < 0.05$), while the $\alpha$-helix and $\beta$-turn structures showed a decreasing trend. It was consistent with earlier observations to show that the far UV circular dichroism spectrum had a minimum at 208 nm and was primarily $\alpha$-helix in structure. The $\alpha$-helix’s p-p* transition can be found in the 208 nm band (Jash et al., 2015; Liu et al., 2021). The intensity of the far UV CD spectrum of BLF decreased during titration as CAF concentration increased, but the peak value remained constant, indicating a reduction in helical structure (Figure 5B), indicating that caffeine binding caused secondary structural changes in the conformation of BLF. These findings were similar to those of Gong et al. (2021), who found that binding purple potato anthocyanins to casein and whey protein resulted in decreased helix and turn contents and increased sheet and random coil contents. Because CAF-BLF was more likely to result in a more compact and structured $\beta$-sheet than BLF, this suggested that CAF-BLF’s protein structure might be more stable (Jing et al., 2021).

**Molecular Modeling of CAF and BLF Interactions.** The development of CAF-BLF complexes can be understood at the molecular level using thermodynamic information from thermodynamic experiments. Yet, a clever and effective technique for learning more about binding locations is molecular modeling tech-

<table>
<thead>
<tr>
<th>Item</th>
<th>$\alpha$-Helix (%)</th>
<th>$\beta$-Sheet (%)</th>
<th>$\beta$-Turn (%)</th>
<th>Random coil (%)</th>
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<tbody>
<tr>
<td>Bovine lactoferrin (%)</td>
<td>26.26</td>
<td>27.28</td>
<td>17.15</td>
<td>29.31</td>
</tr>
<tr>
<td>Caffeine-bovine lactoferrin (%)</td>
<td>17.18</td>
<td>42.53</td>
<td>7.98</td>
<td>32.31</td>
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**Figure 4.** Van’t Hoff diagram of caffeine binding to lactoferrin.
The thermodynamics of interactions between relevant molecules can be precisely predicted using molecular simulations (Nunes et al., 2020). To evaluate the binding energy and contact force of protein-ligand complexes, identify the ligand’s presumed binding site, and investigate the microenvironment of the binding site, molecular docking was used (Shanmugaraj et al., 2015). In contrast, by replicating experimental parameters similar to solvents, pH, and the presence of ions, molecular dynamics could forecast interactions (Rocklin et al., 2013).

In Figure 6, the mimicked docking model that used the smallest binding energies for CAF and BLF was shown (the combination had the greatest stability when in this manner). It was obvious that the CAF and BLF active sites were intimately connected and integrated with it. Figure 6 displays the molecular docking simulation’s binding mode results. The results of the docking study showed that the residues of lactoferrin, including Arg 603, Leu600, Gly387, Arg323, Thr326, and Arg342, were involved in the interaction with CAF, which bonded to Arg603 by hydrogen bonds, and the remaining 5, Leu600, Gly387, Arg323, Thr326, and Arg342, by hydrophobic forces.

**Molecular Dynamics Simulation of CAF and BLF Interaction.** To evaluate the stability of CAF molecules binding to BLF and the interaction mechanism, 100,000 ps molecular dynamics simulations were performed. The RMSD value between the main atom of the protein and the initial structure was analyzed...
to test the trajectory stability of CAF molecules after binding to the protein. As shown in Figure 7A, after 30,000 to 50,000 ps simulation, RMSD values of CAF molecule and protein tended to be stable and low, which was close to the stability of protein molecule, indicating that CAF molecule had high binding stability. It was consistent with the experimental results of the circular dichroism experiment (Van Der Spoel et al., 2005; Abraham et al., 2015).

Solvent-accessible surface area (SASA) is the surface area of biomolecules that are exposed to the solvent. During the simulation, the scale of the probe radius is less than 1.4 Å (Van Der Spoel et al., 2005; Abraham et al., 2015). As shown in Figure 7C, the changes in SASA indicated that the microenvironment and surface hydrophobicity of the CAF-BLF complex changed and finally reached stability during the molecular simulation.

Cyclotron radius can be used to describe how tightly packed a protein structure is as well as how the protein’s peptide chain looseness changes over the simulation process. In the molecular dynamic simulation, the tightness and rigidity of the protein skeleton were evaluated by Rg, which could also reflect the strength of the protein stability at different temperatures (Van Der Spoel et al., 2005; Abraham et al., 2015). As can be seen from Figure 7D, the structure of the CAF-BLF complex was relatively stable in the time range of 30,000 to 54,000 ps.

Binding energy values and interactions between ligands and proteins are calculated by MM/PBSA, and the results are shown in Table 3. The binding energy between lactoferrin-caffeine was −66.26 kJ mol−1, which indicated that lactoferrin was automatically bound to CAF. The electrostatic force between lactoferrin-caffeine was −12.31 kJ mol−1 and the van der Waals force was −101.88 kJ mol−1. Also, there was one hydrogen bond between lactoferrin and caffeine. It showed that the electrostatic effect of lactoferrin and caffeine was much smaller than van der Waals forces and negligible. To bind CAF to lactoferrin, hydrogen bonds, and van der Waals contributions were crucial. Thus, the results confirm the findings of quenching fluorescence: both molecular dynamics simulations and thermodynamic experiments showed that van der Waals forces and hydrogen bonds were the main binding forces between caffeine and lactoferrin.

Simulated In Vitro Digestion. It was crucial to understanding variations in the gastrointestinal stability of BLF because BLF was easily digested in the stomach and broken down into tiny particles by the human body, ensuring that nutrients were taken in the intestine and transported through the blood. The retention rate of CAF-BLF in the stomach and intestine was higher than that of BLF, although the retention rate of CAF-BLF

Figure 6. Molecular docking of caffeine-bovine lactoferrin (CAF-BLF).
in the oral was lower. This is clear from Figure 8(A). This demonstrated that BLF’s physiological activity in the intestine benefited from CAF’s ability to reduce the amount of digestion that took place in the stomach. The molecular weight of digestive fluid proteins, may be seen following the completion of various phases of digestion, as shown in Figure 8(B). The BLF protein and the CAF-BLF complex were broken down into minute peptide or AA fragments, the majority of which had a molecular weight of 50 KDa, during digestion in the mouth and stomach. However, when the BLF protein and CAF-BLF complex entered the intestinal digestion stage, they were broken down into smaller peptide and AA fragments, and there were hardly any noticeable bands on the electrophoretic map.

It was suggested that CAF might bind to pepsin and inhibit pepsin because hydrophobic interactions between polyphenols and digestive enzymes might decrease the digestion of proteins (Jing et al., 2021). When combined with the secondary structure test results, it was clear that CAF stabilized and compacted the structure of BLF, which might make pepsin’s activity on the active site of BLF difficult (Jing et al., 2021). The CAF-BLF complex was less digestible as a result. These findings conclusively showed that the CAF-BLF complex, which was created by combining CAF and BLF, could prevent the body from absorbing BLF. The findings served as a theoretical foundation for the preparation and manufacture of beneficial dairy products.

In conclusion, it has been shown through thermodynamic experiments, Fourier transforms infrared spectroscopy, circular dichroism spectra experiments, molecular docking, molecular dynamics simulation, and in vitro digestion experiments that CAF can spontaneously bind to BLF through hydrogen bonding and van der Waals forces. It will alleviate the problem where BLF is predigested by the stomach and unable to perform its function in the intestine. More research will be required to determine how CAF affects BLF’s functional qualities other than digestibility. Additionally, because infant formula milk powder and middle-aged

<table>
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<tr>
<th>Binding energy (kJ mol⁻¹)</th>
<th>Van der Waals force (kJ mol⁻¹)</th>
<th>Electrostatic force (kJ mol⁻¹)</th>
<th>Hydrogen bond number</th>
<th>Dissociation constant (×10⁻¹² M)</th>
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<td>−101.88</td>
<td>−12.31</td>
<td>1</td>
<td>2.42</td>
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</tbody>
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Figure 7. (A) Root means square deviation (RMSD) of caffeine molecule interaction with lactoferrin. (B) Root-mean-square fluctuation (RMSF) of caffeine molecule interaction with lactoferrin. (C) Solvent-accessible surface area (SASA) of caffeine molecule interaction with lactoferrin. (D) Cyclotron radius of caffeine molecule interaction with lactoferrin.
CONCLUSIONS

The effects of caffeine on the structure and digestive properties of lactoferrin were studied by multispectral techniques, computational molecular docking, and molecular dynamics simulations. The reaction of caffeine with lactoferrin was exothermic, a spontaneous binding process of static quenching. The formation of CAF-BLF and the conformational change of BLF were further confirmed by fluorescence, UV, FTIR, and circular dichroism spectra. The interaction between CAF and several hydrophobic AA residues in the pocket of the active site of BLF was predicted using molecular docking and molecular dynamics simulations. Van der Waals and hydrogen bonds accounted for many of the forces that interacted between CAF and BLF. In vitro digestion results showed that CAF could inhibit BLF digestion and make it function better. In consequence, these studies will add to the potential of lactoferrin and caffeine as nutritional molecules in functional foods.

ACKNOWLEDGMENTS

Funding was provided by Shaanxi Science and Technology Plan projects of China (2022KXJ-010, 2022ZDLNY04-09), Xi’an City Science and Technology Plan projects of China (22NYGG0012 and 2022JH-RYFW-0117), Science and Technology Plan projects in Xianyang city of Shaanxi Province (2021ZDZX-NY-0014), Forestry Science and Technology Innovation Project of Shaanxi Province (SXLK2021-0221), and the Fundamental Research Funds for the Central Universities in China (GK202306005). The authors have not stated any conflicts of interest.

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