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Milk Proteins as Molecular Gatekeepers: Comparative Modulation of Sulfonamides, Natural Phenolics, and Zinc–Polyphenol Complexes at the Food–Drug Interface

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Abstract

Milk is a complex biochemical mixture in which proteins significantly influence the behaviour of xenobiotics and bioactive compounds. Interactions between milk proteins and substances such as veterinary drugs or natural bioactives can modify molecular stability, binding dynamics, and exposure pathways, affecting food safety and the One Health concept. This study presents a comparative, matrix-focused investigation on how three chemically distinct ligand classes, sulfanilamide antibiotics, naturally occurring phenolic compounds and zinc–polyphenol complexes, interact with major milk proteins, β-lactoglobulin and casein. Protein–ligand interactions were examined using steady-state fluorescence spectroscopy to assess quenching behaviour and comparative interaction trends. Molecular docking was employed as a qualitative tool to provide structural context. Distinct interaction patterns were observed across ligand classes, reflecting differences in molecular structure, hydrophobicity, and coordination chemistry. Importantly, zinc coordination modified interaction profiles relative to the corresponding free ligands, indicating that metal coordination can affect ligand–protein interactions within the milk matrix. These findings support the concept that milk proteins may function as matrix-dependent modulators of ligand behaviour. The study is positioned as a hypothesis-generating framework highlighting the importance of food matrices as active biochemical environments. Herein, we provide a foundation for hypothesising how the milk matrix affects residue behaviour and bioactive interactions, with relevance to veterinary pharmacology and food safety risk assessment.

Keywords: milk proteins; β-lactoglobulin; protein–ligand interactions; zinc–polyphenol complexes; fluorescence spectroscopy; molecular docking; veterinary drug residues; One Health

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1. Introduction

Milk is more than just a basic nutritional fluid; it is a complex biochemical environment. Aside from its macronutrients, it contains a dynamic network of proteins, lipids, and micronutrients that can interact with various endogenous and exogenous compounds [1,2]. For example, milk proteins such as β-lactoglobulin and casein can effectively bind small molecules through hydrophobic interactions, hydrogen bonds, and electrostatic forces. These interactions influence how molecules are distributed, stabilised, and made available within the dairy matrix, making milk proteins active modulators of compound behaviour rather than mere carriers. From a food safety and One Health perspective, protein–ligand

interactions in milk are vital, particularly concerning veterinary medicines and naturally occurring bioactives [3–5]. Veterinary drugs, such as sulfonamide antibiotics, administered to food animals can leave residues in milk despite regulatory controls. Additionally, natural phenolic compounds from essential oils or plant-based feeds are present in dairy products. Although these compounds vary greatly in structure, origin, and regulatory status, they can all bind to milk proteins, potentially altering their physicochemical properties after secretion into milk [6–8].

An increasing number of studies show that β-lactoglobulin and casein can bind various xenobiotics, thereby influencing properties such as solubility, fluorescence, and photostability [9,10]. However, most research examines single ligand types under specific conditions, limiting the ability to compare how different chemicals interact with milk proteins within a consistent framework. This is particularly evident when synthetic veterinary drugs and natural bioactives coexist in the same food matrix [11–13]. Additionally, zinc-based metal–polyphenol coordination complexes are emerging as hybrid bioactive systems in food chemistry, toxicology, and translational research. Zinc, an essential trace element with important physiological functions, and polyphenols, known for their antioxidant and antimicrobial properties, can form complexes that alter their molecular shape, charge, and electronic structure, potentially affecting interactions with biological macromolecules [14–16]. Despite increasing interest in zinc–polyphenol systems, their interactions with milk proteins remain underexplored, particularly compared with those of free polyphenols and traditional veterinary drugs [17,18]. These interactions are more than simple academic exercises. When small molecules bind to milk proteins, they act as molecular 'gatekeepers,' affecting how compounds are retained, masked, or released within the dairy matrix. This regulation, influenced by the matrix, can affect residue behaviour, exposure assessment, and interpretation of food safety data [19,20]. Notably, these effects may not be predictable from the molecules' intrinsic properties alone and may depend on interactions between their chemical characteristics and the food matrix composition.

This study introduces a matrix-focused, comparison framework for examining interactions between three ligand types: sulfonamide antibiotics, phenolic compounds from essential oils, and zinc–polyphenol complexes, with key milk proteins, β-lactoglobulin and casein [21–23]. Protein–ligand interactions were analysed using fluorescence spectroscopy to evaluate quenching and interaction behaviour, complemented by in silico molecular docking to elucidate the interactions at the structural level [24–26]. The aim is not to demonstrate biological effects or toxicity, but to explore how these ligand groups interact with proteins. Viewing milk proteins as molecular gatekeepers that selectively modulate the activity of synthetic drugs, natural phenolics, and metal–polyphenol systems, this work provides a hypothesis-driven approach relevant to veterinary pharmacology, food safety, and One Health initiatives [27,28]. It also highlights the importance of food matrices as active biochemical interfaces in studying bioactive compounds in milk.

2. Materials and Methods

2.1. Milk Proteins

Bovine β-lactoglobulin (β-LG) and casein were used as representative milk proteins due to their abundance in bovine milk and their well-established ligand-binding properties. β-lactoglobulin (≥90% purity, Sigma-Aldrich, St. Louis, MO, USA) and casein (technical grade, Sigma-Aldrich) were used without further purification. Proteins were dissolved in 10 mM phosphate buffer (pH 7.4) prepared using analytical-grade reagents and equilibrated at room temperature before analysis. Protein concentrations were determined spectrophotometrically using established molar extinction coefficients, and working solutions were

freshly prepared for each experimental series. Casein systems were treated as simplified colloidal assemblies and do not fully represent native casein micelle structure in milk [29].

2.2. Ligands

Three chemically distinct ligand classes were investigated:

1. Sulfonamide antibiotics, selected as representative synthetic veterinary drugs commonly monitored as milk residues. (sulphanilamide)
2. Phenolic compounds derived from essential oils, representing naturally occurring bioactives present in animal feed or dairy matrices. (eugenol, thymol)
3. Zinc–polyphenol complexes, representing hybrid metal–organic systems of emerging interest in food chemistry and translational toxicology. (tocopherol and resveratrol)

Sulfanilamide, eugenol, and thymol were obtained from commercial suppliers and prepared as stock solutions in ethanol. Zinc–polyphenol complexes were prepared by coordination of Zn^{2+} with polyphenols at a 1:1 molar ratio under stirring conditions. Zinc–polyphenol complexes were assumed based on coordination chemistry principles; the structures were confirmed by UV and IR spectroscopy. The Table S1 can be found in the Supplementary Materials. Free phenolic compounds were used in their native form. Zinc–polyphenol complexes were prepared by coordination of Zn^{2+} ions with selected polyphenols at defined molar ratios under controlled conditions, following previously established protocols. Complexes predominantly involve oxygen-donor coordination sites on the polyphenols, resulting in altered electronic distribution and molecular geometry relative to the free ligands. All ligand solutions were prepared in appropriate solvents and diluted in buffer to minimise solvent effects in spectroscopic measurements [16].

2.3. Fluorescence Spectroscopy

Fluorescence measurements were performed using a JASCO fluorescence spectrophotometer (Tokyo). Excitation and emission slit widths were set at 5 nm and measurements were conducted at 25 °C. Potential contributions from ligand fluorescence overlap and inner filter effects were considered. Protein–ligand interactions were evaluated using steady-state fluorescence spectroscopy, exploiting the intrinsic fluorescence of aromatic amino acid residues in milk proteins. Protein solutions were excited at 280 nm, and emission spectra were recorded over the range of 300–450 nm. Incremental additions of each ligand were performed while maintaining constant protein concentration. Appropriate blank corrections were applied to account for ligand absorbance and background fluorescence. Fluorescence quenching data were analysed using the Stern–Volmer equation with interpretation focused on comparative trends rather than absolute quantitative values. Binding constants and apparent binding parameters were estimated using established fluorescence binding models. The analysis focused on comparative trends across ligand classes rather than on absolute thermodynamic values [17]. Fluorescence intensities were corrected for inner filter effects using the following equation:

$$F_corr = F_obs \times 10^{(A_ex + A_em)/2}$$

where F_corr is the corrected fluorescence intensity, F_obs is the observed fluorescence intensity, and A_ex and A_em are the absorbance values at the excitation and emission wavelengths, respectively. This correction was applied to minimise optical artefacts arising from ligand absorbance, particularly at higher concentrations.

2.4. In Silico Molecular Docking

Docking simulations were performed using iGEMDOCK v2.1. Molecular docking studies were conducted to provide structural interpretation of the experimentally observed protein–ligand interactions. Three-dimensional structures of β-lactoglobulin and representative casein models were obtained from publicly available protein databases or, where appropriate, from homology models (1BEB, 6RCG). Protein structures were prepared by removal of crystallographic water molecules, addition of polar hydrogens, and assignment of partial charges. Ligand structures were energy-minimised before docking [30]. Docking poses were ranked using scoring functions, and interpreted comparatively to indicate relative interaction favourability. Interaction profiles were analysed for hydrogen bonding, hydrophobic contacts, and electrostatic interactions. Molecular docking provides a static approximation of protein–ligand interactions and does not account for protein flexibility or solvent effects. To ensure methodological reliability, the docking protocol was evaluated through redocking of a reference ligand into the β-lactoglobulin structure (PDB: 1BEB). The reproduced binding pose showed spatial agreement within the known binding region, supporting the suitability of the docking parameters for comparative interaction analysis. Docking results are therefore interpreted comparatively to assess relative interaction tendencies rather than absolute binding energies. Although molecular dynamics simulations were not performed in the present study, the docking-derived interaction patterns provide a structurally consistent basis for future dynamic evaluation of ligand stability within the β-lactoglobulin binding cavity. For zinc–polyphenol complexes, coordination geometry and metal-mediated interactions were specifically examined to assess how metal complexation influenced binding modes relative to free polyphenols.

2.5. Data Analysis and Statistical Treatment

All fluorescence experiments were conducted in triplicate, and results are expressed as mean ± standard deviation. Data fitting and graphical analyses were performed using standard scientific data analysis software. Comparative interpretation focused on relative differences between ligand classes and protein systems, rather than absolute binding affinities. The integrated experimental–computational approach was employed to support a qualitative, hypothesis-generating framework for understanding milk protein–ligand interactions.

3. Results

3.1. Fluorescence Quenching Behaviour of Milk Proteins

Intrinsic fluorescence spectroscopy revealed that both β-lactoglobulin and casein exhibited measurable fluorescence quenching upon titration with all ligand classes examined. The extent and nature of quenching varied depending on the ligand type and protein system. In Figure 1 there is a representation of the crystal structures of β-lactoglobulin and its binding pocket that was used for this work.

For β-lactoglobulin, the addition of sulfonamide antibiotics resulted in a progressive decrease in fluorescence intensity, accompanied by minimal shifts in the emission maxima, suggesting predominant quenching of tryptophan residues without major conformational rearrangements in the protein structure. Stern–Volmer plots showed linear behaviour over the tested concentration ranges, consistent with a dominant static quenching mechanism. The interaction of thymol and eugenol with milk proteins was systematically investigated using steady-state fluorescence spectroscopy, focusing on β-lactoglobulin (LG) and casein nanoparticles of different sizes (15 nm and 60 nm). Changes in fluorescence intensity within the aromatic emission region provided insight into ligand-induced perturbations of the protein microenvironment and their dependence on protein identity, colloidal organization,

and ligand concentration. Free phenolic compounds induced a stronger quenching effect compared with sulfonamides at equivalent molar ratios. In several cases, modest red shifts in emission maxima were observed, indicating changes in the microenvironment around aromatic residues and suggesting increased exposure to a more polar environment upon ligand binding.

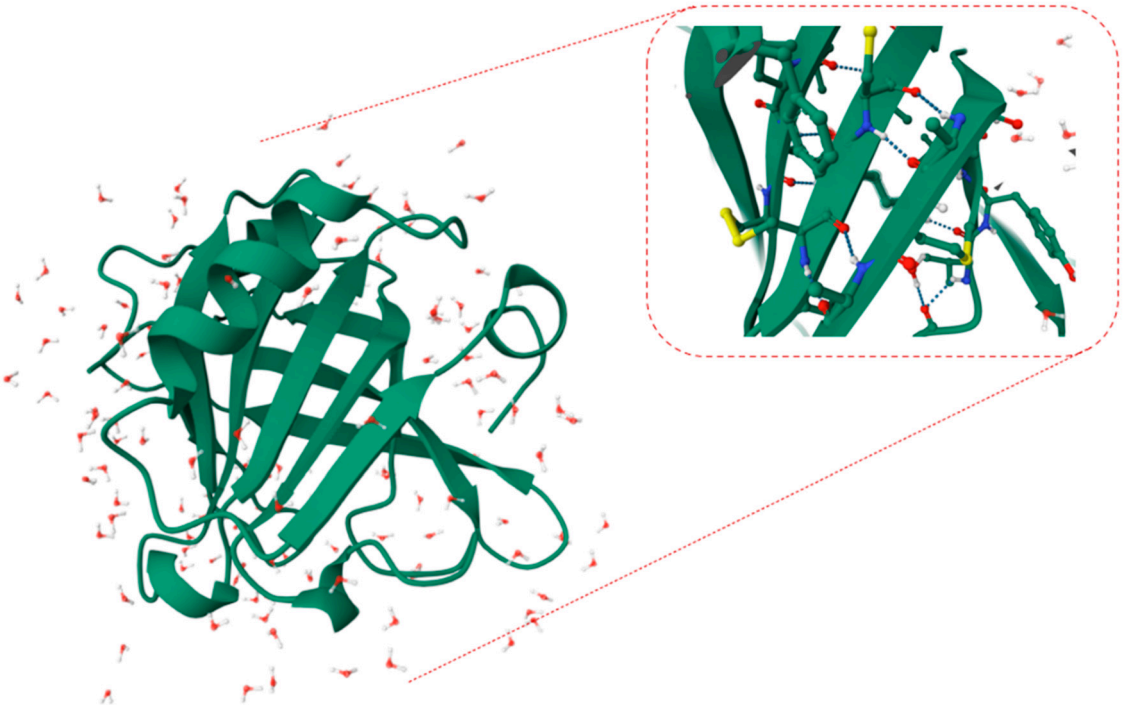

Figure 1. β-lactoglobulin protein structure and its binding pocket used for the molecular docking studies as downloaded from protein database (1BEB).

Both thymol and eugenol induced pronounced, concentration-dependent changes in the fluorescence emission of casein nanoparticles. For the 15 nm casein system, the response to both ligands were distinctly non-monotonic, characterized by fluorescence quenching at low-to-intermediate concentrations followed by marked intensity recovery and enhancement at higher concentrations. This behavior was consistently observed in normalized fluorescence analyses and indicates a transition between competing interaction regimes as ligand concentration increased. At lower concentrations, the observed quenching suggests proximity of thymol or eugenol to aromatic residues, leading to non-radiative energy dissipation. As ligand concentration increased, fluorescence enhancement became dominant, consistent with suggested possible ligand partitioning into hydrophobic region of the disordered casein matrix. Such partitioning is expected to reduce solvent exposure of aromatic residues, resulting in fluorescence intensification. The strong response of the 15 nm nanoparticles highlights the higher surface-area-to-volume ratio and greater conformational flexibility of smaller casein aggregates. In contrast, 60 nm casein nanoparticles displayed similar qualitative trends but with reduced amplitude. The attenuation of both quenching and enhancement phases indicates that larger aggregates are less susceptible to ligand-induced microenvironmental reorganization. This size-dependent dampening supports the interpretation that nanoscale organization plays a critical role in governing the accessibility and redistribution of hydrophobic ligands within casein assemblies.

Compared with casein, β-lactoglobulin exhibited more constrained and protein-specific fluorescence responses to both ligands. For 15 nm LG nanoparticles, thymol induced predominantly monotonic fluorescence quenching across the concentration range, with only limited recovery at higher concentrations. This behavior is consistent with ligand

binding in proximity to aromatic residues within structured binding regions, resulting in efficient quenching rather than extensive microenvironmental shielding.

Eugenol interaction with 15 nm lactoglobulin nanoparticles followed a more complex, non-monotonic pattern. An initial fluorescence enhancement at low concentration was followed by substantial quenching at intermediate levels and partial recovery at higher concentrations. This suggests that eugenol engages lactoglobulin through competing modes, potentially involving surface-associated interactions at low concentration and deeper penetration or redistribution at higher ligand levels. The overall magnitude of fluorescence modulation in lactoglobulin was lower than that observed for casein, reflecting the more compact and ordered structure of β-lactoglobulin. For 60 nm lactoglobulin nanoparticles, both ligands produced only modest fluorescence changes. Normalized intensities remained close to baseline values, indicating limited sensitivity of larger lactoglobulin aggregates to ligand-induced perturbations. This reduced responsiveness parallels the behavior observed for 60 nm casein but is more pronounced in lactoglobulin, underscoring the combined influence of protein structure and nanoparticle size.

In contrast, zinc–polyphenol complexes produced distinct quenching profiles. Although overall quenching efficiency was comparable to or greater than that of free phenolics, deviations from linearity were observed in Stern–Volmer plots at higher ligand concentrations. This behaviour suggests more complex interaction dynamics, potentially involving combined static and dynamic quenching components or heterogeneous binding environments. In Figure 2 there is a representation of the small molecules that the interactions with those milk proteins investigated.

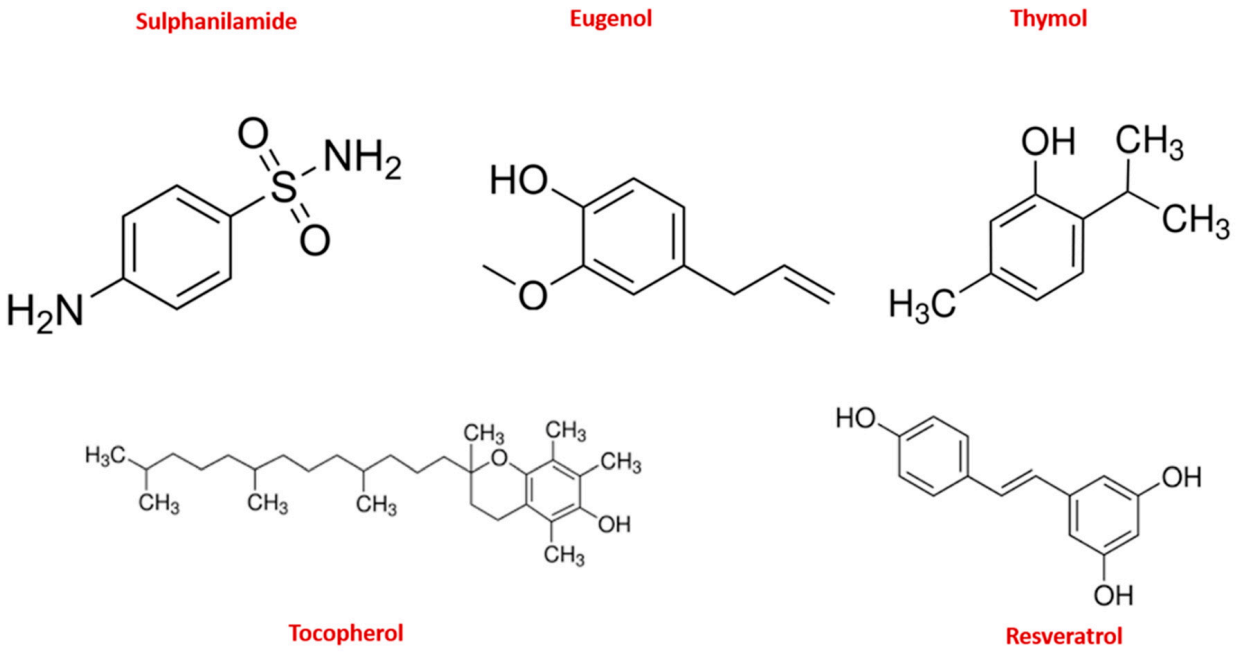

Figure 2. The small molecules that their interactions investigated with lactoglobulin and casein proteins.

For casein, fluorescence quenching trends were generally less pronounced than for β-lactoglobulin but followed similar qualitative patterns. Sulfonamides produced moderate quenching effects, whereas free phenolics and zinc–polyphenol complexes induced stronger fluorescence attenuation. The broader emission profiles of casein reflected its heterogeneous protein composition, yet clear ligand-dependent differences in quenching behaviour were consistently observed.

3.2. Comparative Interaction Behaviour Trends

Apparent binding parameters derived from fluorescence data revealed clear comparative trends across ligand classes and protein systems. For both β-lactoglobulin and casein, free phenolic compounds displayed higher apparent binding affinities than sulfonamide antibiotics under identical experimental conditions. This trend was consistent across multiple phenolic structures and reflects the strong contribution of hydrophobic interactions and hydrogen bonding to protein–phenolic association (Figure 3).

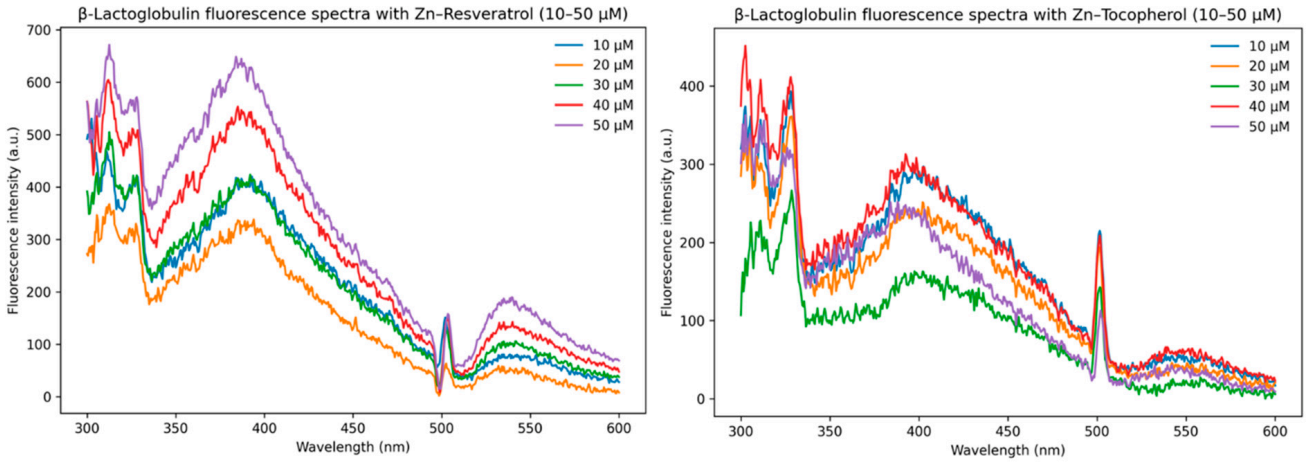

Figure 3. Fluorescence emission spectra of β-lactoglobulin following titration with the Zn-resveratrol and Zn–tocopherol complex (10–50 μM). Changes in fluorescence intensity indicate interaction between the complex and the protein, with less pronounced ligand-associated emission compared with Zn–resveratrol. Regarding Zn–tocopherol, changes in fluorescence intensity indicate interaction between the complex and the protein, with less pronounced ligand-associated emission compared with Zn–resveratrol.

Stern–Volmer analysis revealed consistent comparative trends across ligand classes. Apparent quenching constants (Ksv) followed the order: free phenolic compounds > putative zinc–polyphenol systems ≥ sulfonamide antibiotics, indicating stronger interaction propensity of phenolic structures with milk proteins. While absolute Ksv values were not used for quantitative thermodynamic interpretation, the observed hierarchy supports a structure-dependent interaction mechanism dominated by hydrophobic and aromatic contributions.

Ligand chemistry further modulated these effects. Thymol generally produced stronger quenching responses in β-lactoglobulin, whereas eugenol was more prone to induce fluorescence enhancement and non-monotonic behavior, particularly in casein systems. These differences likely reflect variations in ligand polarity and aromatic substitution patterns, which influence their distribution within protein microenvironments. Notably, zinc–polyphenol complexes exhibited altered binding behaviour relative to their corresponding free phenolics. In some cases, zinc complexation increased the apparent affinity for β-lactoglobulin, whereas in others it reduced or redistributed binding interactions. These findings indicate that metal coordination modifies ligand–protein interfaces rather than uniformly enhancing or suppressing binding. β-lactoglobulin exhibited a characteristic intrinsic fluorescence emission in the 320–360 nm region, consistent with tryptophan-dominated emission from a globular whey protein. Upon addition of Zn–tocopherol, concentration-dependent changes in fluorescence intensity were observed. A decrease in emission intensity was evident at lower to intermediate ligand concentrations, followed by partial recovery at higher concentrations. This non-linear behaviour suggests that multiple effects may contribute to the observed fluorescence response, including protein–ligand association and optical effects at elevated ligand levels.

In contrast, the addition of the Zn–resveratrol complex resulted in more pronounced spectral changes. Alongside alterations in the protein emission region, a distinct emission band emerged and intensified in the 380–410 nm range as ligand concentration increased. This band is attributed to ligand-associated fluorescence and partially overlaps with the tail of the protein emission spectrum. Consequently, apparent increases in overall fluorescence intensity at higher Zn–resveratrol concentrations are interpreted primarily as a result of ligand emission rather than enhancement of protein fluorescence. These observations indicate that both Zn–polyphenol complexes interact with β-lactoglobulin, with Zn–resveratrol producing stronger fluorescence perturbations due to its intrinsic emissive properties.

Casein displayed broader and weaker intrinsic fluorescence compared with β-lactoglobulin, consistent with its flexible and heterogeneous protein structure. Upon titration with Zn–tocopherol, modest changes in fluorescence intensity were observed, without sharp quenching or well-defined peak shifts. This suggests a more distributed or less specific interaction, which is expected given the absence of a rigid tertiary structure in casein. The Zn–resveratrol complex produced more noticeable fluorescence changes, particularly at higher concentrations, where an increase in long-wavelength emission was observed. As with β-lactoglobulin, this behaviour is attributed largely to ligand-associated fluorescence rather than direct changes in protein emission.

Overall, the fluorescence response of casein to both Zn–polyphenol complexes was less structured than that of globular proteins, reflecting the intrinsically disordered nature of casein and its different interaction environment. Differences between the two proteins were also evident. β-lactoglobulin generally exhibited higher apparent affinity across all ligand classes than casein, consistent with its well-defined ligand-binding cavity. Casein interactions appeared more diffuse, reflecting its ensemble nature and multiple low-affinity binding regions. Importantly, the results highlight that binding behaviour could not be predicted solely from ligand origin (synthetic vs. natural) but depended strongly on molecular structure and coordination chemistry.

3.3. Structural Interpretation of Protein–Ligand Interactions

Molecular docking simulations provided a structural context for the experimental observations. Docking of sulfonamide antibiotics to β-lactoglobulin consistently located the ligands within or near the central hydrophobic cavity, stabilised primarily by hydrophobic contacts and limited hydrogen bonding with polar residues lining the binding pocket.

Free phenolic compounds displayed more diverse binding orientations, engaging both hydrophobic regions and polar surface residues. Multiple docking poses were energetically comparable, supporting the experimentally observed flexibility in phenolic binding and the associated changes in fluorescence emission characteristics. Zinc–polyphenol complexes exhibited distinct docking behaviour compared with free polyphenols. The presence of the metal centre altered ligand geometry and charge distribution, leading to alternative binding orientations and, in some cases, engagement of residues not involved in binding of the free phenolic ligands. Metal-mediated interactions and coordination-driven steric constraints contributed to these altered binding modes. For casein models, docking results suggested multiple shallow interaction regions rather than a single dominant binding site. Ligand binding was distributed across accessible hydrophobic and charged regions, consistent with the broader and less site-specific fluorescence responses observed experimentally.

3.4. Molecular Docking Reveals Ligand-Class Discrimination Within the β-Lactoglobulin Cavity

To structurally rationalize the experimentally observed fluorescence trends, molecular docking simulations were performed using the crystal structure of bovine β-lactoglobulin (PDB: 1BEB). Docking scores were interpreted comparatively to assess relative binding

tendencies among ligand classes. Clear affinity discrimination was observed. Resveratrol exhibited the lowest docking score (−86.6), followed by tocopherol (−79.4), sulfanilamide (−72.4), eugenol (−65.1), and thymol (−64.7). This ranking closely mirrors the fluorescence quenching hierarchy, supporting concordance between spectroscopic and structural findings (Figure 4).

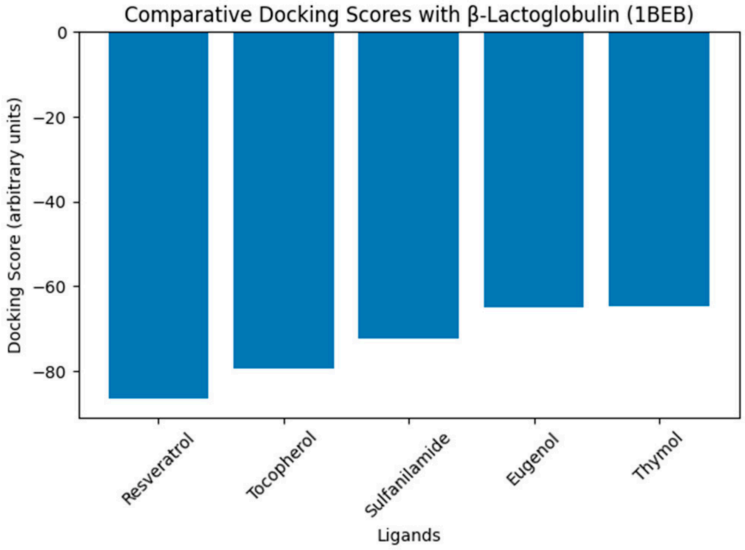

Figure 4. Comparative docking scores for resveratrol, tocopherol, sulfanilamide, eugenol, and thymol docked to bovine β-lactoglobulin (PDB: 1BEB) using iGEMDOCK. Bars represent total docking scores generated by the scoring function (arbitrary units). More negative values indicate stronger predicted interaction within the modeled binding region. Docking scores are interpreted comparatively and do not represent absolute binding free energies.

3.4.1. Deep Cavity Engagement by Resveratrol

Resveratrol occupied the canonical hydrophobic calyx of β-lactoglobulin, forming stabilizing interactions with aromatic residues TYR20 and TYR42, alongside polar stabilization through GLU44 and GLN59. The planar aromatic scaffold enabled π–π stacking within the cavity, providing strong van der Waals stabilization and electrostatic complementarity. This deep anchoring explains: Strong fluorescence perturbation, non-linear emission behavior and greater quenching efficiency.

3.4.2. Hydrophobic Surface Anchoring of Tocopherol

Tocopherol displayed a different binding topology. While still strongly stabilized, it engaged residues LEU140, MET145, LYS141, and ARG148, indicating interaction within a hydrophobic surface-accessible cavity region rather than deep aromatic stacking. The bulky chromanol ring and long aliphatic chain promote hydrophobic stabilization but limit cavity penetration. This structural pattern explains moderate quenching, limited emission wavelength shifts and less pronounced aromatic microenvironment disruption (Table 1).

Table 1. Docking scores and dominant interacting residues for ligands docked to β-lactoglobulin (PDB: 1BEB).

Ligand	Total Docking Score	Dominant Interacting Residues	Binding Region Character
Resveratrol	−86.6	TYR20, TYR42, GLU44, GLN59, LEU156	Deep hydrophobic cavity, π–π stacking
Tocopherol	−79.4	ASP137, LEU140, LYS141, MET145, ARG148	Hydrophobic surface cavity

Table 1. *Cont.*

Ligand	Total Docking Score	Dominant Interacting Residues	Binding Region Character
Sulfanilamide	−72.4	SER21, GLN59, TYR42, PHE151, LEU156	Cavity-associated polar contacts
Eugenol	−65.1	ARG124, THR125, LYS101, GLU127	Surface–cavity interface
Thymol	−64.7	TYR42, GLN59, PHE151, LEU156	Partial cavity occupation

3.4.3. Moderate Cavity Occupation by Sulfanilamide

Sulfanilamide occupied overlapping regions with phenolics, particularly involving TYR42, GLN59, PHE151, and LEU156. However, its smaller size and reduced hydrophobic surface area resulted in weaker stabilization compared with polyphenols. Interaction was dominated by hydrogen bonding and limited hydrophobic contacts.

3.4.4. Surface-Distributed Interactions of Thymol and Eugenol

Thymol and eugenol demonstrated weaker docking scores and more surface-oriented binding modes. Interactions involved ARG124, THR125, GLU127, and adjacent residues, suggesting partial cavity engagement or transient surface association (Figure 5).

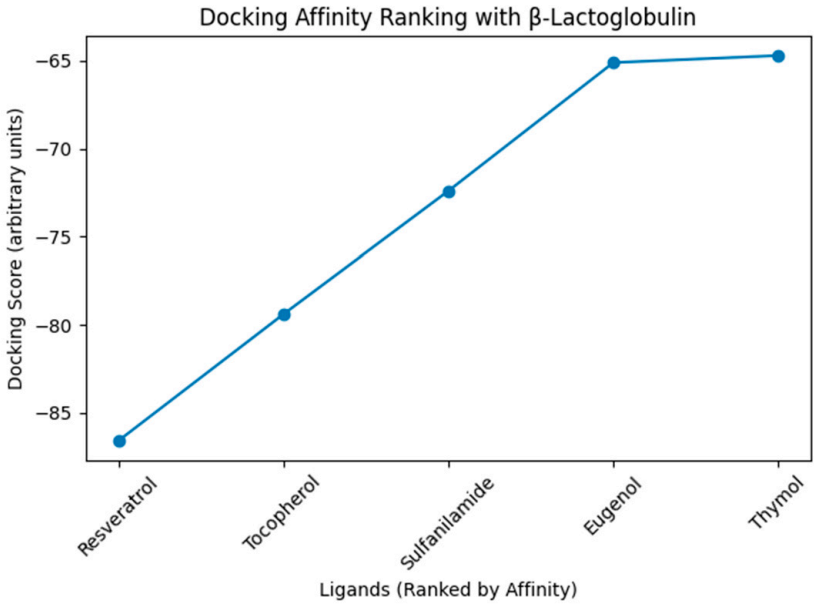

Figure 5. Ranking of investigated ligands according to predicted interaction behaviour toward bovine β-lactoglobulin (PDB: 1BEB), based on total docking scores. The monotonic separation highlights ligand-class discrimination within the hydrophobic calyx, with polyphenolic scaffolds exhibiting stronger predicted stabilization than essential-oil phenolics and sulfonamide under identical docking conditions.

3.5. Integration of Spectroscopic and Docking Findings

The strong agreement between docking score hierarchy and fluorescence quenching trends indicates that β-lactoglobulin discriminates ligands based on:

- Aromatic planarity;
- Hydrophobic surface area;
- Electrostatic complementarity;
- Steric accessibility;
- Coordination chemistry (for Zn complexes).

Importantly, binding selectivity was not determined by ligand origin (synthetic vs. natural), but by structural compatibility with the protein cavity. These findings reinforce the concept that milk proteins function as structure-dependent molecular gatekeepers rather than passive binding matrices (Figure 6).

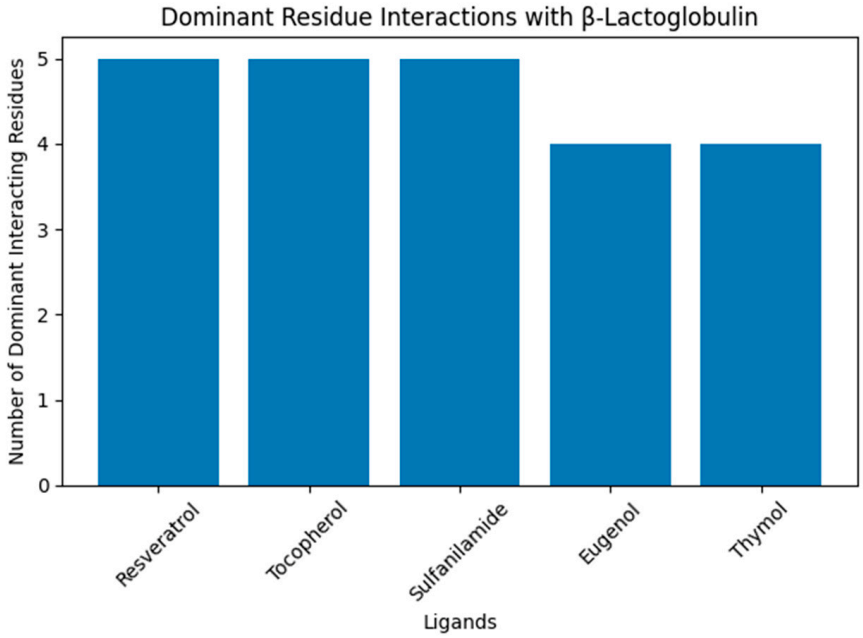

Figure 6. Number of dominant interacting residues identified for each ligand docked to bovine β-lactoglobulin (PDB: 1BEB), derived from residue-level interaction profiles in iGEMDOCK output. Counts reflect residues contributing most strongly to hydrogen bonding and van der Waals interactions within the binding region. This simplified interaction footprint complements the docking score hierarchy and illustrates differences in cavity engagement among ligand classes.

4. Discussion

4.1. Milk Proteins as Molecular Gatekeepers in the Dairy Matrix

The present study supports the concept that milk proteins may act as matrix-dependent modulators of ligand behaviour, selectively modulating the behaviour of chemically diverse compounds within the dairy matrix. Rather than acting as passive carriers, β-lactoglobulin and casein exhibited ligand-dependent interaction patterns that influenced fluorescence responses, apparent binding behaviour, and predicted binding modes. These findings reinforce the view that the milk matrix is an active biochemical interface that shapes the fate of both synthetic and naturally occurring compounds after their secretion into milk. It should be noted that the casein systems used represent simplified colloidal models and do not fully replicate native micelle structure. The gatekeeping role observed here can be operationally understood as a combination of compound retention, masking of physicochemical properties, and controlled molecular exposure. Importantly, these effects were not uniform across ligand classes, indicating that matrix-mediated modulation depends on the interplay between protein architecture and ligand chemical features rather than ligand origin alone.

4.2. Matrix-Dependent Modulation of Veterinary Drug Residues

Sulfonamide antibiotics showed measurable but relatively moderate interactions with both milk proteins, particularly compared with natural phenolics and zinc–polyphenol complexes. The predominantly static quenching behaviour and limited spectral shifts observed suggest binding that stabilises the ligand within the protein environment without

inducing major conformational changes. This pattern is consistent with the presence of defined binding regions in β-lactoglobulin and more diffuse interaction sites in casein.

From a food safety perspective, these observations underscore that residue behaviour in milk cannot be inferred solely from administered dose or intrinsic molecular properties. Protein binding within the milk matrix may influence residue persistence, detectability, and apparent availability, with implications for exposure assessment. While the present study does not address regulatory thresholds or depletion kinetics, it highlights that protein–ligand interactions constitute an additional layer of complexity in interpreting residue data.

4.3. Natural Phenolics and Protein Interactions in Milk

Free phenolic compounds exhibited stronger and more dynamic interactions with milk proteins than sulfonamide antibiotics. The enhanced quenching efficiency and modest emission shifts observed experimentally, together with docking-derived interaction diversity, suggest that phenolics engage milk proteins through a combination of hydrophobic contacts and hydrogen bonding across multiple surface regions. These findings align with previous reports describing the affinity of polyphenols for milk proteins and their ability to alter protein microenvironments [31,32]. In the context of the present comparative framework, the results indicate that naturally occurring bioactives may be preferentially retained or modulated by the milk matrix, potentially influencing their stability, bioaccessibility, and functional behaviour within dairy products.

4.4. Impact of Zinc Coordination on Protein–Ligand Interfaces

A key finding of this study is that zinc coordination appears to modify interaction behaviour. However, as direct experimental validation of complex formation rather than UV and IR was not performed, these interpretations should be considered cautiously. Zinc–polyphenol complexes exhibited quenching patterns and docking profiles distinct from those of their free phenolic counterparts, reflecting changes in molecular geometry, charge distribution, and steric constraints introduced by metal coordination. Rather than uniformly enhancing binding, zinc complexation led to reconfigured interaction modes, sometimes increasing apparent affinity and in other cases redistributing binding across alternative protein regions. This highlights coordination chemistry as a critical determinant of ligand–protein interfaces within the milk matrix. These results are particularly relevant given the growing interest in metal–polyphenol systems in food chemistry, toxicology, and functional ingredient research.

4.5. Structural Basis of Matrix-Selective Modulation

The present data provide mechanistic evidence that β-lactoglobulin selectively discriminates among chemically diverse ligands through cavity architecture and residue-specific stabilization. The calyx of β-lactoglobulin is known to accommodate hydrophobic molecules; however, the present comparative analysis suggests that ligand geometry and π-electron density strongly influence cavity penetration depth and stabilization.

The superior docking performance of resveratrol reflects optimal aromatic stacking within TYR20/TYR42 regions, whereas sulfanilamide, despite partial overlap, lacks sufficient hydrophobic and π-interaction capacity for equivalent stabilization. Tocopherol, while highly hydrophobic, adopts a surface-accessible anchoring mode rather than deep cavity insertion, highlighting steric constraints as determinants of binding topology.

This structural discrimination supports the hypothesis that milk proteins regulate molecular retention and exposure based on physicochemical compatibility rather than regulatory classification. Metal coordination introduces geometric rigidity and altered charge distribution, reshaping ligand–protein interfaces. Zinc complexation may enhance

cavity complementarity in planar phenolic systems or redistribute interactions across alternative protein regions.

4.6. Implications for Food Safety and One Health

Milk represents a convergence matrix linking veterinary pharmacology and human exposure. Protein-mediated modulation may influence the residue detectability, bioaccessibility, molecular masking and release kinetics. Thus, matrix-level structural interactions should be integrated into exposure assessment models.

The interaction patterns observed in this study are relevant beyond dairy chemistry, extending to One Health and translational toxicology. Milk represents a convergence point between animal health, food safety, and human exposure, and protein-mediated modulation of compounds within this matrix may influence how synthetic drugs, natural bioactives, and hybrid systems are transferred across the food–animal–human interface. This work suggests the value of combined in vitro–in silico approaches for interrogating matrix-dependent effects at the molecular level. Such strategies can complement conventional residue monitoring by providing mechanistic insights into how compounds behave within complex biological fluids.

4.7. Study Limitations and Future Perspectives

This study was designed as a hypothesis-generating investigation, and several limitations should be acknowledged. Fluorescence measurements provide indirect evidence of protein–ligand interactions and may be influenced by ligand emission overlap and inner filter effects. Docking results should be interpreted as qualitative structural models rather than quantitative predictors of binding affinity. No direct structural or morphological characterisation (e.g., TEM, SEM, AFM) was performed; therefore, nanoscale structural interpretations remain hypothetical. Future studies incorporating time-resolved spectroscopy, calorimetric techniques, or in vivo validation could further elucidate the functional consequences of milk protein binding. Additionally, extending the comparative framework to other veterinary drug classes and metal complexes would strengthen understanding of matrix-mediated modulation across a broader chemical space. Overall, this study should be considered a hypothesis-generating framework highlighting matrix-dependent interaction behaviour.

4.8. Concluding Perspective

Taken together, the findings support a conceptual shift in how milk protein interactions are viewed from incidental binding events to selective, structure-dependent gatekeeping mechanisms. Demonstrating that synthetic drugs, natural phenolics, and zinc–polyphenol complexes interact with milk proteins in fundamentally different ways, this study provides a framework for rethinking residue behaviour, bioactive modulation, and risk interpretation within the dairy matrix.

5. Conclusions

This study presents a comparative, matrix-centred examination of how chemically distinct ligand classes: synthetic sulfonamide antibiotics, natural phenolic compounds, and zinc–polyphenol complexes, interact with the major milk proteins β-lactoglobulin and casein. Integrating fluorescence spectroscopy with molecular docking, this work suggests that milk proteins do not function as passive carriers but instead act as selective molecular gatekeepers, modulating ligand behaviour in a structure-dependent manner.

Distinct interaction patterns were observed across ligand classes, with free phenolics and zinc-coordinated systems exhibiting interaction behaviours markedly different from those of conventional veterinary drugs. Importantly, zinc coordination was shown to reshape polyphenol–protein interfaces, altering binding orientation and modify interaction profiles. However, these findings are based only on few spectroscopic (IR, UV) and computational approaches and require further experimental validation. These findings indicate that coordination chemistry is a critical determinant of protein–ligand interactions within the milk matrix. The results emphasise that compound behaviour in milk cannot be inferred solely from intrinsic molecular properties or regulatory classification. Instead, protein-mediated modulation within the dairy matrix represents an additional layer influencing residue behaviour, molecular availability, and interpretative risk assessment. The study provides a conceptual framework for understanding how synthetic drugs, natural bioactives, and hybrid metal–organic systems are differentially regulated at the food–drug interface. This work highlights the importance of considering food matrices as active biochemical environments and supports the use of integrated in vitro–in silico approaches in food safety, veterinary pharmacology, and One Health-oriented research and is positioned as a hypothesis-generating framework for future investigation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dairy7030030/s1, Table S1. Spectroscopic evaluation of the Zn-polyphenol complex formation.

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