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Research Article

Molecular Simulation of Animal Milk Lactoferrins as Antiviral Agents against Rotavirus: Binding Mechanisms and Therapeutic Implications

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Abstract

Rotavirus infections impose a significant global health burden, particularly affecting infants and young children and causing severe gastroenteritis. To combat this viral pathogen, there is a growing interest in exploring the therapeutic potential of lactoferrins derived from different farm animal milk as antiviral agents. This study employed molecular simulation techniques to investigate the intricate binding mechanisms between different animal milk lactoferrins and rotavirus, providing insights into their molecular interactions. These animals included cow, sheep, camel, goat, horse, buffalo, in addition to humans. Molecular dynamics simulation techniques were employed using Gromacs software to simulate the interaction between animal milk lactoferrins and rotavirus. Precise computational models and simulations were conducted to investigate the binding mechanisms and identify critical amino acid residues involved. Our findings indicate that cow lactoferrin exhibits superior interaction with rotavirus compared to other lactoferrin sources. We identified specific binding sites and crucial amino acid residues responsible for these interactions. These results provide insights into the molecular determinants governing the strong binding affinity and specificity of lactoferrins towards rotavirus. This study provided valuable insights for the design of targeted antiviral strategies against rotavirus infections. Animal milk lactoferrins, particularly cow lactoferrin, demonstrated a promising potential as an antiviral agent against retroviruses. These findings enhanced our understanding of the molecular mechanisms underlying lactoferrin-mediated antiviral activity, paving the way for future experimental and clinical investigations in this field and supporting the development of efficient antiviral therapeutics against rotavirus.

***Keywords*:** Lactoferrin, docking, antiviral, molecular dynamics

Introduction

Rotavirus is the leading cause of paediatric diarrheal disease and mortality among children younger than 5 years (Tate et al., 2016). Rotavirus infections still cause over 200,000 deaths each year, primarily in developing nations, despite the widespread introduction of rotavirus immunizations over a decade ago (Omotade et al., 2023).

Rotaviruses, members of non-enveloped double-stranded RNA viruses of the Reoviridae family, with a naked icosahedral triple-layered capsid, possess a genome with 11 double-stranded RNA (dsRNA) segments encoding structural proteins (VP) and five to six non-structural proteins. Based on serological or genetic variability of the middle layer VP6 protein, ten groups (A-J) or species have been defined (Hu et al., 2012). Group A rotavirus is by far the most commonly found in humans, the leading viral agent of acute gastroenteritis (AGE) in human populations. It has been determined that there are more than 35 different glycoprotein (G) genotypes (VP7 gene) and 50 different protease (P) genotypes (VP4 gene) in the RVA strain, which is categorized using a binary genotyping system based on these proteins (Hoque et al., 2020). However, only a few of these are commonly observed in humans: G1–G4, G9, G12, and P[4], P[6], and P[8] (Liu et al., 2012; Isa et al., 2006). Two virion-associated components, VP8* and VP5*, are produced when the rotavirus infectivity is activated by protease cleavage of VP4 (Dormitzer et al, 2002; Fiore et al., 1991).

The long-term objective to reduce the burden worldwide must continue to be consistent scaling and implementation of successful therapies. Infection prevention through active immunization has been the focus of numerous efforts. During the past decades, in order to obtain a rotavirus vaccine, various strategies have been applied. However, even with ongoing efforts to obtain effective rotavirus infection, none of the currently available vaccinations are clinically beneficial against any

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type of diarrhoea, and RV continues to be the leading cause of gastroenteritis-related deaths globally (López et al, 2005; Kapikian et al., 1991). Moreover, there is a need for alternative means of protection besides vaccination, as rotaviruses also cause severe infections in patients undergoing bone marrow transplantation (Rennels et al., 1996). Parenteral or oral rehydration is a vital supportive treatment for rotavirus diarrhoea (Marrie et al., 1982), but finding particular antiviral agents is essential to halt the spread of the illness. Despite previous research demonstrating that various treatments have antiviral action in rotavirus-infected tissue cultures (Kitaoka et al., 1986), there is currently no effective antiviral treatment available.

The bioactive elements in milk have received a lot of attention over the last ten years. As an illustration, Lactoferrin exhibits antiviral, antifungal, antibacterial, antitumor, antiparasite, and immunoenhancing activities (Shahidi et al., 2020; Rashidian et al., 2023). Lactoferrin's antiviral effects are demonstrated against a wide range of viruses, both enveloped and naked, by preventing virus attachment to target cells by binding to viruses or their receptors, inhibiting viral adhesion and entry into host cells (Krzyzowska et al., 2022). Even though the levels of protection provided against rotavirus infection vary in different communities, breastfeeding has been shown to lessen the incidence of enteric illnesses in babies. However, there is no clear relationship between the level of antiviral antibodies in milk and the degree of protection (Javadmanesh & Azghandi., 2018; Tahmoorespur et al., 2020; Daneshmand et al., 2019).

Molecular modelling and simulations are potent tools that are frequently used in the field of structural biology to predict the structure and function of native and recombinant proteins and peptides (Javadmanesh & Azghandi, 2017; Javadmanesh et al., 2021). Due to the antirotaviral properties of some milk derivatives, in this study, cow, sheep, camel, goat, horse, buffalo, and human lactoferrins were evaluated for their inhibitory effect on human rotavirus VP8*. The current study gives us comprehensive information on the application of modelling and simulations to determine key residues of milk lactoferrin responsible for its stability and interactions with human rotavirus.

Materials and Methods

Molecular modelling and in silico virtual screening

Structural data for protein-protein interaction inhibitors were retrieved from the Protein Data Bank (**www.pdb.org**) (Dutta et al., 2008). Crystal structure of the human rotavirus VP8* with the (PDB code:2DWR) (Blanchard et al., 2007) was selected as the receptor. The lactoferrin structures from different livestock species in addition to human are as follows: Human Lactoferricin (PDB code:1Z6V) (Hunter et al., 2005), Camel apo-lactoferrin (PDB code:1DTZ) (Khan et al., 2001), Equine apo lactoferrin (PDB code: 1I6B) (Kumar et al., 2002), goat lactoferrin (PDB: 1JW1) (Kumar et al., 2002), Bovine lactoferrin (PDB code:1BLF) (Moore et al., 1997), and Differric buffalo lactoferrin (PDB: 1BIY) (Karthikeyan et al., 2000) were used to investigate their inhibitory effect on human rotavirus VP8*. On the other hand, as the 3D x-ray crystallographic data of the protein Sheep lactoferrin were not available, the sequence of R9QXS6 was retrieved from UniProt (www.uniprot.org) (Apweiler et al., 2007), and loaded into the SWISS-MODEL server (Bordoli et al., 2009) to create three different 3D homology models of the protein. The top-ranked homology model built using human apolactoferrin as the template (PDB ID =1CB6) [40] was subjected to protein preparation. Finally, the verified homology model of sheep lactoferrin with good quality was used for the molecular docking investigations in this work.

Molecular docking of complexes

Molecular docking, as a powerful tool, can be applied to dock the binding of a peptide or ligand at the preferred location and orientation on a macromolecule. we carried out a reliable docking approach for docking lactoferrin proteins in Rotavirus VP8 employing a program in the HADDOCK web server (https://haddock.science.uu.nl/), which is freely available for observing and analysing protein-protein interactions (van Zundert et al., 2016). In order to control the docking process, HADDOCK distinguishes itself from ab initio docking approaches by encoding information from known or predicted protein interfaces in ambiguous interaction restraints (AIRs). These AIR files offer information about the active residues of the macromolecule (de Vries et al., 2010). The HADDOCK score and Z-score result that has the lowest value is thought to represent the best molecular interaction. The binding affinity at 25 °C is predicted using Prodigy (Jiménez-García et al., 2019), which calculates G and Kd. Ligplot+ (Laskowski et al., 2011) was used to visualize the interacting residues of proteins.

Molecular dynamics (MD) simulation of protein–protein complex

To gain deeper insights into the binding modes of proteins, MD simulation studies were carried out on the ligand-receptor complexes that have higher docking scores. THE GROMACS V 2020.1 package was used to run the MD simulation to elucidate the effectiveness of the screened compounds by molecular docking (Abraham et al., 2015). The force field considered in calculations was the CHARMM36 (Huang et al., 2013), and the topologies of the ligands were generated using the CGenFF (https://cgenff.umaryland.edu) with the SPC explicit model of solvation. To neutralize the system, an appropriate number of Na^+ and Cl^- ions were added. The complex species were subjected to energy minimization using the steepest descent algorithms, followed by 1 ns NVT and 1ns NPT. Subsequently, MD simulations were carried out utilizing the Berendsen thermostat (Berendsen et al., 1984). The V-rescale temperature coupling method and the Parrinello-Rahman coupling method were used to generate periodic boundary conditions at a constant temperature of 300 K and 1 atm pressure. The long-range electrostatic interactions were calculated using the Particle Mesh Ewald method (Darden et al., 1993). Finally, 100 nsMD simulations were subjected, and in all cases, snapshots of the trajectory were saved every 10ps. The trajectory files from MD simulations were analysed for root mean square fluctuation (RMSF), root-mean-square deviation (RMSD), radius of gyration (Rg), solvent-accessible surface area (SASA), and H bond occupancy, contributing to investigating the stability and structural dynamics during MD simulations.

MM/PBSA (binding free energy) calculations

The Molecular Mechanics Poisson-Boltzmann surface area (MM-PBSA) approach (Homeyer & Gohlke, 2012) was used to estimate the relative binding free energy of each protein-protein complex using the g mmpbsa tool of GROMACS. The MM-PBSA method has become one of the most widely used methods for computing interaction energies, and it is frequently used to study biomolecular complexes.

In general terms, the binding free energy ($\Delta G_{binding}$) of a protein-protein complex in solvent is computed as the difference in free energy between the complex (Gcomplex) and the sum of the free energies of the protein (Greceptor) and ligand (Gligand):

$$\Delta G_{binding} = G_{complex} - [G_{receptor} + G_{ligand}]$$

The last 20ns snapshots were extracted in order to assess binding free energy.

Results

Docking Studies

In molecular biology, protein docking is a fundamental technique for identifying key residues involved in the interaction of two proteins (Ereifej et al., 2011; van der Kraan et al., 2005). HADDOCK web-server was used to perform protein-protein docking to investigate the potential binding mode of interaction between lactoferrin proteins and Rotavirus VP8. Van der Waals intermolecular energy, electrostatic intermolecular energy, desolvation energy, distance restraints energy, and buried surface area are weighted together to calculate the HADDOCK score.

Along with the HADDOCK score, the Z-score is expressed as the number of standard deviations from the average a particular cluster is located in terms of score. Negative Z-scores illustrate a strong HADDOCK cluster. As can be seen from Table 1, cow lactoferrin has the highest Z-score: HADDOCK score (-2.7: -96.0 +/- 9.9 kcal/mol). The contribution of van der Waals energy and electrostatic energy was observed to be -37.0+/- 10.3 kcal/mol and -300.3 +/- 16.5kcal/mol, respectively.

The residues involved in cow-Rotavirus lactoferrin interaction are Lys179, Gln200, Asp201, Gln3, Arg20, Asp186, Thr185, Arg172, Glu179 and Asp133.Fig., On the other hand, human-Rotavirus and camel-Rotavirus lactoferrin have the lower Z-scores: HADDOCK scores (-1.5: -49.4 +/- 6.9) and (-1.4: -77.4 +/- 6.6) respectively, while the Z-scores: HADDOCK scores for horse-Rotavirus and goat-Rotavirus are (-2.3: -83.3 +/- 3.1) and (-2.7: -96.0 +/- 9.9).

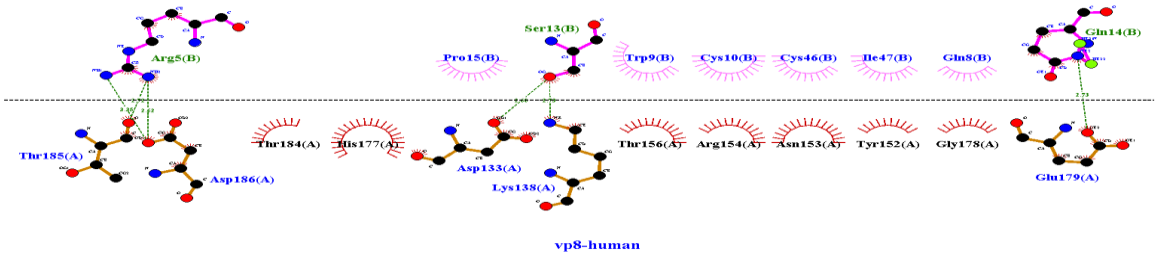
Molecular Dynamics Simulation

A 100 ns molecular dynamics simulation was performed on lactoferrin-Rotavirus VP8 complexes. RMSD of the protein backbone, RMSF, and Rg were calculated. Moreover, Hydrogen bands and SASA were investigated in this study, Figures 1 and 2-S.

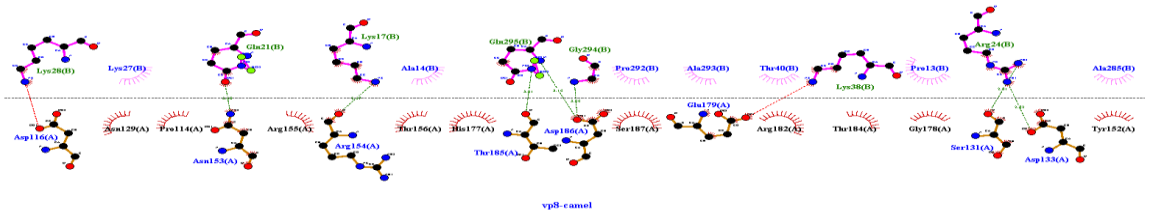
The high stability of the complex is indicated by the lower RMSD value of the protein backbone. As can be seen from Figure 2, the overall RMSD is satisfactory for all combinations. Here, the RMSD value for studied lactoferrins against VP8 is between 0.143 Å and 0.154 Å during the simulation period. The initial fluctuations were noted till 50ns, and then RMSD shows stability for all complexes. It means that the complexes are completely stable for the 100 ns simulation.

Table 1. Predicted interactions for the VP8 obtained with HADDOCK

PDB Code	HADDOCK score	Van der Waals energy	Electrostatic energy	Desolvation energy	Buried Surface Area	Z-Score
Human	-49.4 +/- 6.9	-34.5 +/- 4.7	-87.5 +/- 54.7	2.0 +/- 3.1	1020.5 +/- 95.9	-1.5
Camel	-77.4 +/- 6.6	-42.0 +/- 7.3	-224.7 +/- 14.3	9.2 +/- 1.1	1456.3 +/- 106.2	-1.4
Horse	-82.9 +/- 10.7	-21.3 +/- 9.6	-393.9 +/- 40.8	16.8 +/- 5.4	1508.6 +/- 39.3	-2.1
Goat	-83.3 +/- 3.1	-51.4 +/- 3.8	-245.4 +/- 39.2	17.2 +/- 1.4	1781.6 +/- 74.0	-2.3
Cow	-96.0 +/- 9.9	-37.0+/- 10.3	-300.3 +/- 16.5	0.8 +/- 1.0	1723.6 +/- 186.2	-2.7
Sheep	-90.2 +/- 5.4	-36.4 +/- 4.9	-323.6 +/- 13.8	9.1 +/- 1.9	1348.6 +/- 34.9	-2.2
Buffalo	-68.2 +/- 16.7	-33.7 +/- 15.7	-261.6 +/- 38.8	17.2 +/- 3.0	1700.5 +/- 248.1	-1.7



(A)



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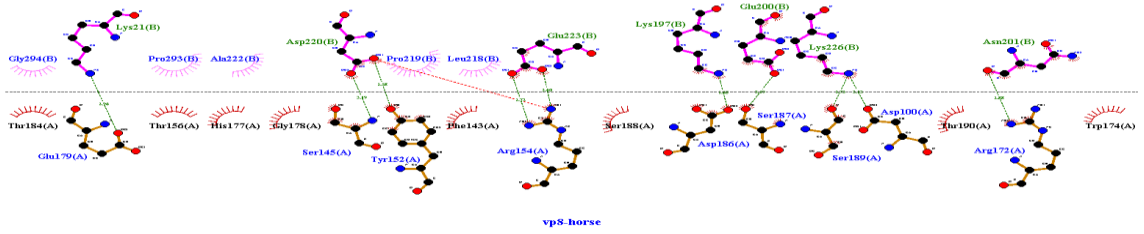

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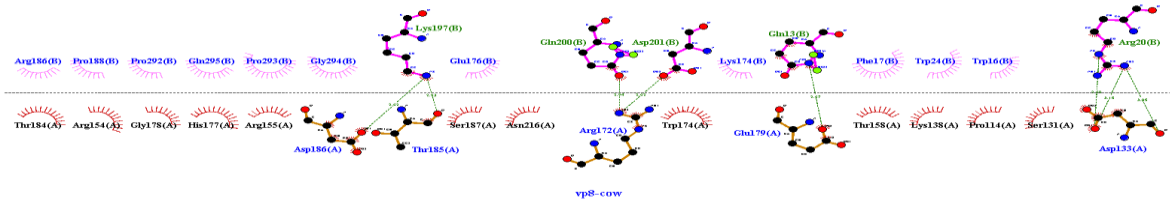

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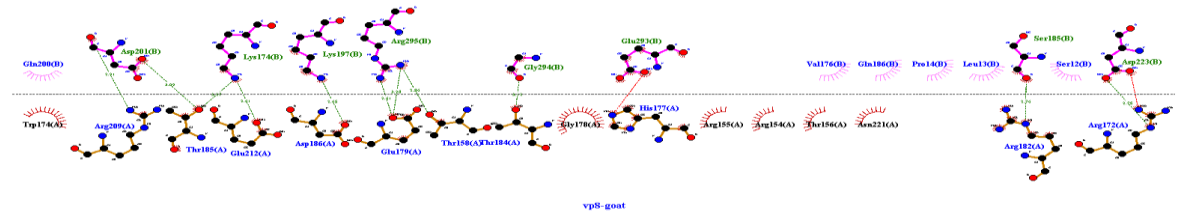

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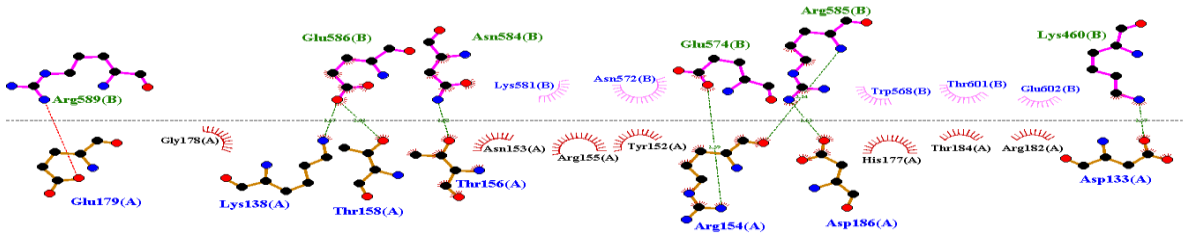
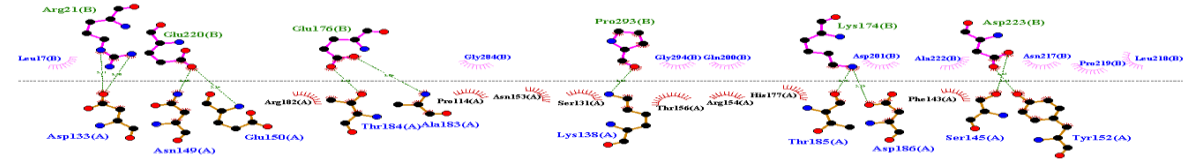


(F)



(G)

Figure 1. In silico analysis of the binding interactions between Rotavirus and (A) Human, (B) Camel, (C) Horse, (D) Cow, (E) Goat, (F) Sheep, and (G) Buffalo lactoferrins.

The root mean square fluctuation (RMSF) analysis then provides the complex variations with temporal evolution for each atom (Figure 3). RMSF (root mean square fluctuations) calculation shows higher fluctuation for the horse, while the buffalo shows the lowest fluctuations over the whole time, which is presented in Figure 3. The residues of around 150 regions show significant fluctuation for goat, camel, horse, cow, human, and buffalo complexes. On the other hand, the greatest fluctuations were observed, almost 0.22 Å for sheep around residue 180, which indicates the conformational changes in protein structure under the physiological condition, which could be a barrier to ligand activity.

The compactness and folding mechanism of proteins are related to the radius of gyration (Rg). The compactness of Rotavirus lactoferin upon binding with the lactoferrins was assessed over the course of MD simulation. The lower Rg value is determined by an increase in the protein compactness. Figure 4 shows that the Rg values for complexes drop over time. This suggests that the protein in the protein-protein combination is stabilizing and exhibiting less conformational fluctuation than that seen in the protein alone. Based on the Rg analysis, the most compressed complex was that of sheep (1.514nm), while the camel is the least compressed one.

On the other hand, Molecular dynamics (MD) simulations coupled with free energy calculations have emerged as powerful tools for studying protein-protein interactions at the atomic level.

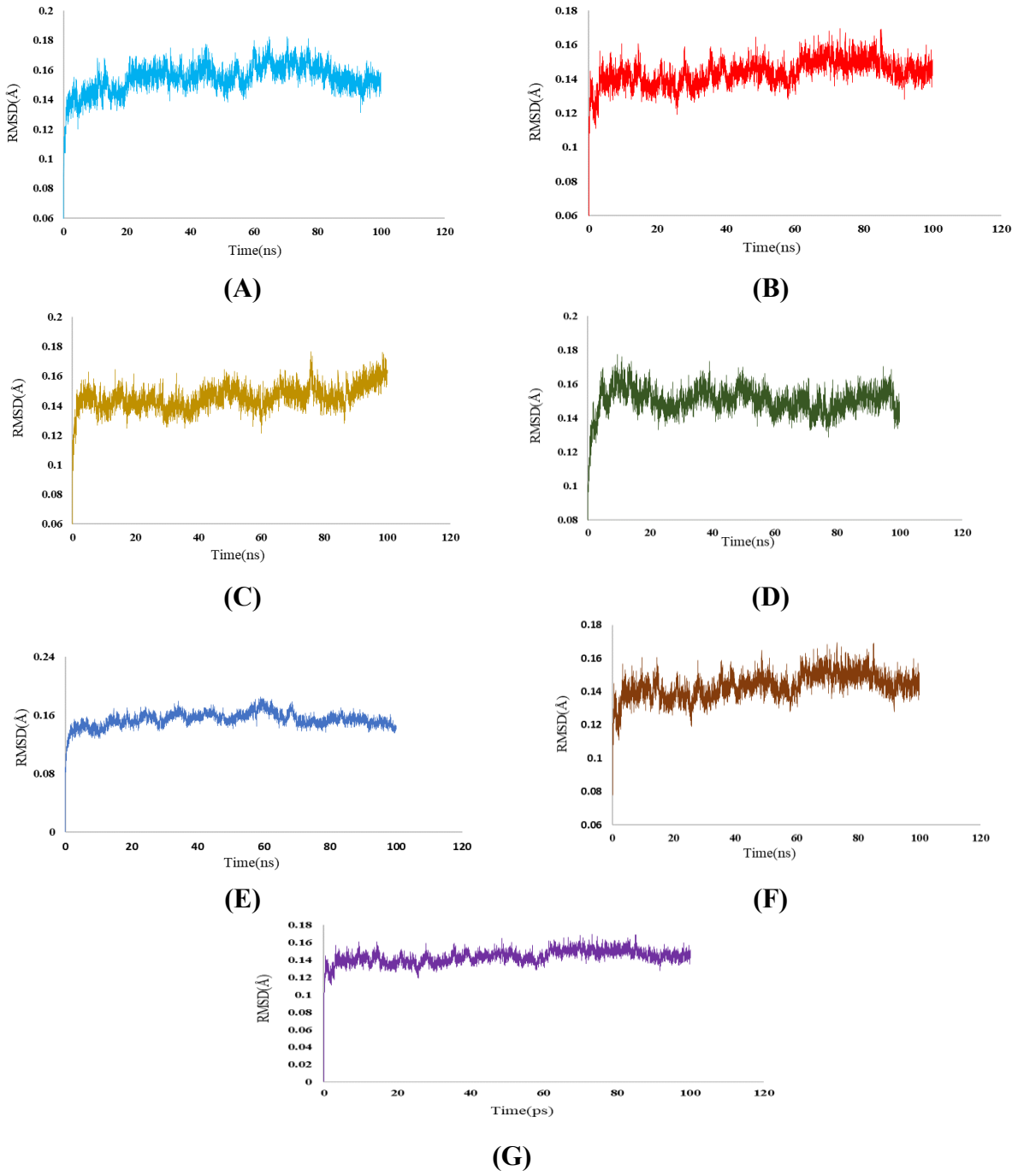


Figure 2. RMSD Analysis during 100ns simulation to study interactions between Rotavirus lactoferrin and (A) Human lactoferrin (B) Camel lactoferrin (C) Horse lactoferrin (D) Cow lactoferrin (E) Goat lactoferrin (F) Sheep lactoferrin (G) Buffalo lactoferin

Among these methods, the Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) approach has gained significant popularity for its ability to estimate binding free energies from the MD studies. The binding of free energy for the last 20ns of simulation was determined using the g_mmpbsa package. As can be seen from Table 2, the average of free binding energies obtained using g_mmpbsa for cow lactoferrin in complex with rotavirus lactoferrin is higher than others with (-1256.920 ±120.160 kcal/mol), which is consistent with docking results. Moreover, human lactoferrin in complex with rotavirus lactoferrin has a lower binding energy with (-183.586± 66.847 kcal/mol). It can also be observed in Table 2, the binding free energy for camel lactoferrin, horse lactoferrin, and goat lactoferrin in complex with rotavirus lactoferrin are (-1231.783 ± 116.969 kcal/mol), (-433.504 ± 70.893 kcal/mol), and (-925.341±129.740 kcal/mol), respectively. While this value for sheep and buffalo

lactoferrin in complex with Rotavirus lactoferrin is (-61.982±32.005 kcal/mol) and (-748.638 ± 103.000 kcal/mol).

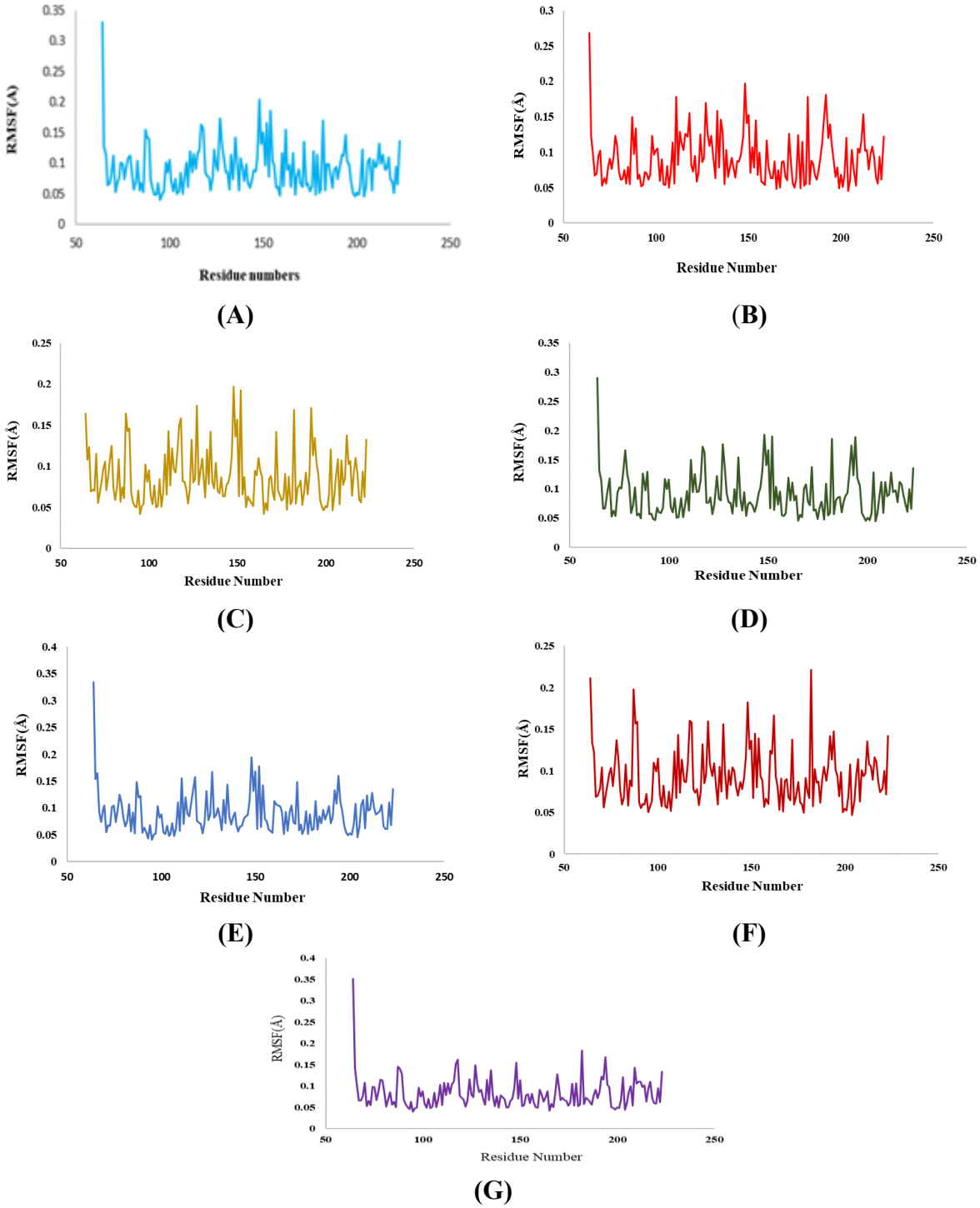


Figure 3. RMSF Analysis during 100ns simulation to study interactions between Rotavirus lactoferrin and (A) Human lactoferrin (B) Camel lactoferrin (C) Horse lactoferrin (D) Cow lactoferrin (E) Goat lactoferrin (F) Sheep lactoferrin (G) Buffalo lactoferrin

Discussion

The interaction between lactoferrins and rotavirus lactoferrin is an intriguing subject of research due to lactoferrin's potential role as an antiviral drug and its capacity to reduce rotavirus infection. Molecular dynamics (MD) simulation was used to explore some different lactoferrin-rotavirus interactions at the atomic level, shedding light on the binding mechanism and the dynamics of complex formation. The results of the molecular simulation study revealed that cow lactoferrin exhibited the highest affinity and interaction strength with rotavirus among the studied lactoferrin variants. Simulation results revealed that cow lactoferrin has the highest affinity and interaction strength with rotavirus, suggesting that it may have unique structural and functional properties that contribute to its higher antiviral effectiveness. The simulation demonstrated a strong and stable binding between specific regions of the lactoferrin molecule and the viral surface proteins, crucial for the virus's infection process. The majority of cow lactoferrin anti-RV activity has been found to occur during the virus's pre-attachment and entry phase, primarily through binding to viral particles rather than inhibiting cell receptors (Superti et al., 1997), but it additionally interferes with later steps during and after virus infection (Superti et al., 2001).

Table 2. Results of the binding free energy calculation for Rotavirus VP8 in complex with the (a) Human, (b) Camel, (c) Horse, (d) Goat, (e) Cow, (f) Sheep, (g) Buffalo Lactoferrins.

Targets	ΔG binding energy Kcal/mol	ΔG Vdw Kcal/mol	ΔG elec Kcal/mol	ΔG polar Kcal/mol	SASA energy Kcal/mol
(a)	-183.586± 66.847	-0.002 ± 0.001	-186.476 ± 5.700	3.044 ± 65.704	-0.152±1.468
(b)	-1231.783 ± 116.969	-137.696 ±14.300	-1591.220±88.516	516.803± 112.512	-19.670 ± 3.479
(c)	-433.504 ± 70.893	-119.855 ± 9.280	-1035.449 ± 92.531	740.381 ± 105.473	-18.581 ± 3.261
(d)	-925.341±129.740	-244.478± 21.990	-1375.256±82.401	730.588±159.458	-36.195 ± 4.165
(e)	-1256.920 ±120.160	-168.000 ±20.005	-1582.111 ±9.036	514.262 ± 117.289	-21.070± 3.689
(f)	-61.982±32.005	-131.641±7.455	-593.108±9.598	682.210± 44.689	-19.443±3.246
(g)	-748.638 ± 103.000	-223.053±21.357	-1276.927±58.901	784.331±111.120	-32.990±3.788

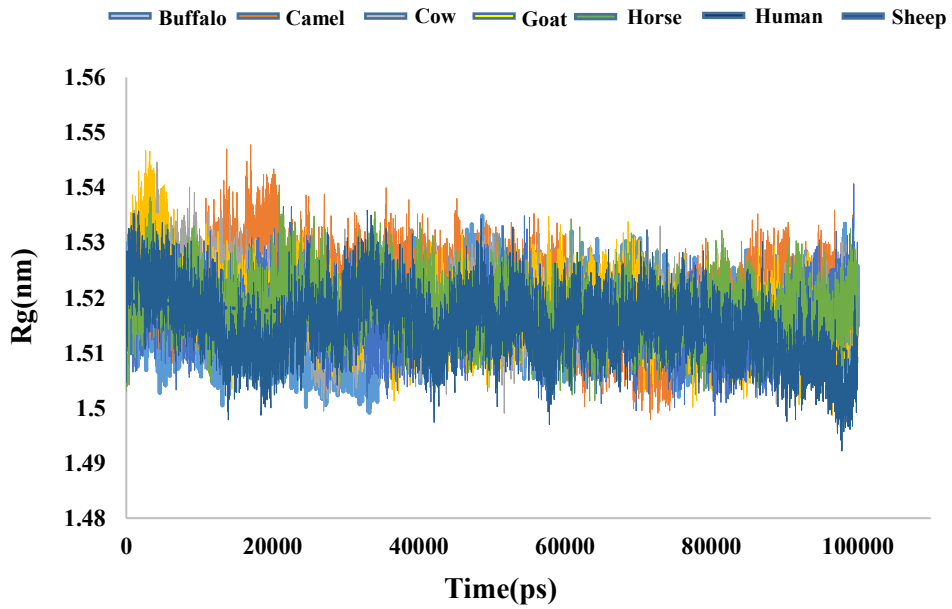


Figure 4. Comparative change in radius of gyration during 100 ns simulation of Rotavirus lactoferrin-studied protein lactoferrins

Crucial amino acid residues participating in the interaction are revealed by the structural insights obtained from molecular docking and dynamics simulations. For example, residues like Lys179 and Asp201 were found to be important in maintaining the complex in the case of cow lactoferrin.

The creation of modified lactoferrin molecules with improved antiviral capabilities may benefit from this knowledge, which is essential for discovering the specificity of lactoferrin interactions. Furthermore, the study identified key residues and molecular motifs within cow lactoferrin that played a vital role in mediating this interaction. Cow lactoferrin's unique amino acid composition and conformational flexibility may allow for stronger and more prolonged binding to rotavirus particles, preventing viral attachment and reproduction more efficiently. This interaction may potentially inhibit the virus's ability to infect host cells, disrupt its replication process, or neutralize its pathogenic effects. By understanding the molecular basis of this interaction, researchers can explore the development of novel antiviral strategies or therapeutic interventions targeting rotavirus infections.

The high affinity of cow lactoferrin for rotavirus is demonstrated by the HADDOCK scores and Z-scores obtained from our docking investigations. These findings are consistent with earlier research that indicated the significance of van der Waals and electrostatic forces in protein-protein interactions (Song & Zhao, 2004; Roth et al., 1996). The idea that charge interactions have a major impact on binding kinetics is further supported by the considerable contribution of electrostatic energy found in our study. On the other hand, lactoferrins derived from camels and humans showed reduced binding affinities, which could be explained by the proteins' structural variations and conformational flexibility.

The stability and adaptability of the lactoferrin-rotavirus complexes are further clarified by the molecular dynamics simulations. A stable interaction, with cow lactoferrin retaining its structural integrity throughout time, is suggested by the constant RMSD values throughout the simulation. The RMSF analysis also clarifies the dynamics of individual residues, showing that some areas exhibit flexibility while others stay constant, suggesting possible hotspots for additional research. Notably, horse lactoferrin exhibits more fluctuations, indicating a higher degree of structural variability that may impact its capacity to bind.

Moreover, the measurement of the radius of gyration (Rg) suggests that the lactoferrin-rotavirus complexes are stabilized by conformational changes that occur during binding. Effective protein interactions require a more compact structure, which is demonstrated by the lowered Rg values, especially in the case of the cow and sheep complexes. These results indicate that binding of lactoferrin to rotavirus not only facilitates a stable interaction but may also enhance the functional properties of lactoferrin as a potential therapeutic agent. The predicted binding free energy calculations using the MMPBSA approach corroborate our docking results, reinforcing the conclusion that cow lactoferrin possesses a significantly higher binding affinity for rotavirus than the other lactoferrins studied. This high affinity is likely a result of the unique structural features of cow lactoferrin, which could be the focus of future therapeutic development efforts.

Our findings were intriguing and contradict the data given by Ereifej et al about the antibacterial properties of camel milk. According to Ereifej et al.'s study, camel milk has great antibacterial potential due to its reportedly enhanced lactoferrin concentration, which is 10 times more than that of cow's milk. However, our research suggests a different conclusion, encouraging us to investigate the underlying elements that contribute to these dissimilarities (Ereifej et al., 2011). Our findings are in accordance with previous studies by Graikini et al., which used a human intestinal model to demonstrate the in vitro antirotaviral activity of lactoferrin from various species. This study highlights the potential of lactoferrins, particularly those found in cow's milk, as effective treatment agents for rotavirus infections in young children and babies (Graikini et al., 2024). However, our study findings align closely with the research conducted by van der Kraan et al. (van der Kraan et al., 2005). The research methodology of the current research closely followed the approach outlined by Kraan et al., ensuring a comparable framework for our investigations.

In the study conducted by Graikini et al., the authors demonstrated that lactoferrins exhibit antiviral properties, likely due to their ability to bind to rotavirus and inhibit its infectivity (Graikini et al., 2024). Our molecular docking and dynamics simulations also demonstrate that cow lactoferrin has a higher binding affinity to rotavirus than lactoferrins from other species, suggesting that lactoferrin's structural characteristics may be essential to its antiviral activity. The discovery of particular binding residues in cow lactoferrin, including Lys179, Gln200, and Asp201, is consistent with the knowledge that some amino acids' hydrophilia and positive charge can improve

binding interactions with the negatively charged rotavirus surface.

The findings from this study provide a solid foundation for future research and may contribute to the development of effective antiviral strategies, improved vaccines, and innovative functional food products. Harnessing the power of molecular simulation techniques, scientists continue to uncover novel insights into the molecular world, driving advancements in medical research and therapeutic development.

Conclusion

In conclusion, our simulation analysis indicates that cow lactoferrin interacts with rotavirus better than lactoferrins from humans, goats, sheep, camels, horses, and buffalo. These variables may explain the reported differences in antiviral activity between lactoferrins from various species against rotavirus. These data pointed out the cow's lactoferrin as a possible candidate for further research as an antiviral agent against rotavirus. Experiments should be conducted in the future to corroborate these simulation-based findings and to investigate the underlying molecular pathways causing the improved interaction between cow lactoferrin and rotavirus.

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Conflict of interests
None.

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