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An alternative vaccine target for bovine Anaplasmosis based on enolase, a moonlighting protein

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The discovery of new targets for preventing bovine anaplasmosis has moved away from focusing on proteins that have already been extensively studied in *Anaplasma marginale*, including the Major Surface Proteins, Outer Membrane Proteins, and Type IV Secretion System proteins. An alternative is moonlighting or multifunctional proteins, capable of performing various biological functions within various cellular compartments. There are several reports on the role of moonlighting proteins as virulence factors in various microorganisms. Moreover, it is known that about 25% of all moonlighting is involved in the virulence of pathogens. In this work, for the first time, we present the identification of three enolase proteins (AmEno01, AmEno15, and AmEno31) in the genome of Mexican strains of *A. marginale*. Using bioinformatics tools, we predicted the catalytic domains, enolase signature, and amino acids binding magnesium ion of the catalytic domain and performed a phylogenetic reconstruction. In addition, by molecular docking analysis, we found that AmEno01 would bind to erythrocyte proteins spectrin, ankyrin, and stomatin. This adhesion function has been reported for enolases from other pathogens. It is considered a promising target since blocking this function would impede the fundamental adhesion process that facilitates the infection of erythrocytes. Additionally, molecular docking predicts that AmEno01 could bind to extracellular matrix protein fibronectin, which would be significant if we consider that some proteins with fibronectin domains are localized in tick gut cells and used as an adhesion strategy to gather bacteria before traveling to salivary glands. Derived from the molecular docking analysis of AmEno01, we hypothesized that enolases could be proteins driven by the pathogen and redirected at the expense of the pathogen's needs.

KEYWORDS

multifunctional proteins, enolase, erythrocytes, ticks, extracellular matrix, veterinary
diseases

1. Introduction

The performance of more than one function (moonlighting) by a single protein has been recognized as a common phenomenon with significant implications in metabolic processes and other functions in bacteria, plants, yeasts, fungi, parasites, and vertebrates (1–8). Moonlighting proteins were described in the late 1980s as structural proteins in the lens of the eye (crystallins) with a second and even a third function (9–11). Most of the functions of moonlighting proteins are related to physiologically relevant biochemical or biophysical functions (12–14). The repertoire functions of moonlighting proteins reported in the last years include their

participation as enzymes of the TCA cycle (aconitase, *Homo sapiens, Saccharomyces cerevisiae, Mycobacterium tuberculosis*) (15, 16); glucose metabolism (aldolase, *Arabidopsis thaliana*, hexokinase; *Plasmodium vivax*) (17, 18); chaperones (GroEL, *S. cerevisiae*, HSP60; *Enterobacter aerogenes*) anti-oxidant proteins (thioredoxin, *Escherichia coli*) (19); virulence-associated functions (elongation factor Tu and enolase, *Mycoplasma pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Plasmodium* spp.) (20, 21); among other roles in different organisms that have been widely reported (6, 22–24) In the last years, due to sequencing technologies and metagenomics advances, more moonlighting proteins and their functions are being discovered in diverse organisms (25).

Currently, two large groups have been proposed to classify moonlighting proteins: 1) "trigger enzymes" and 2) intracellular/ secreted moonlighting proteins (4, 26). The first subset comprises enzymes that regulate transcription or translation by directly binding to DNA or RNA or by binding to other proteinaceous translation or transcription factors (27). The second subset includes the most extensive known moonlighting proteins, with activities as housekeeping enzymes, chaperones, translation factors, adhesion, DNA-binding proteins, and many others that are secreted and either reside attached to the cell surface, acting as receptors for soluble proteins or small molecules, or function in the fluid phase, often for intracellular signaling (4). In this regard, as moonlighting proteins perform their canonical and moonlighting functions in separate cell compartments (cytoplasm and the cell surface), this dual cellular localization of the protein strongly suggests a multifunctional activity (28).

The presence of moonlighting proteins is relevant in bacteria, and their study focuses on elucidating their alternative functions since they are present in both pathogenic and commensal bacteria (23).

In this regard, *Anaplasma marginale* is a Gram-negative intracellular pathogen known as the causal agent of bovine Anaplasmosis, an infectious, non-contagious disease characterized by progressive hemolytic anemia, abortions, loss of condition, milk production, and even death (6, 29, 30). Up to now, seven genomes (~1.2 Mbp) of Mexican strains of *A. marginale* have been reported and annotated (31–33). Due to their reduced genome size, moonlighting proteins in this pathogen could be a strategy to efficiently maximize their proteins' use (31, 34).

Currently, our interest focuses on moonlighting proteins of this vector-borne pathogen since they could participate in its pathogenicity or evasion of the host immune system, as it has been reported for many pathogens, which employ moonlighting/ multitasking proteins as virulence factors to interfere with multiple cellular processes, in different compartments at different times during infection, augmenting their virulence (6). Thus, we performed a deep and sharp genomic analysis that allowed us to identify potential moonlighting proteins in *A. marginale*, including enolase (AmEno), which have not been reported before in this pathogen. Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is an intensely studied moonlighting protein that converts 2-phosphoglycerate to phosphoenolpyruvate in glycolysis. Besides participating in this metabolic pathway, the enolase facilitates binding to host cells, as reported in *Anaplasma phagocytophilum*, in which enolase binds to the host plasminogen. In 2018, Gao et al. (35) demonstrated that recombinant enolase from *A. phagocytophilum* can bind and activate plasminogen and promote

conversion to plasmin, thus being crucial to pathogen infection. In addition, this enolase was considered a potential target to control anaplasmosis infection. In *Borrelia burgdorferi,* the surface-expressed enolase plays an essential role during pathogen invasion by binding mammalian plasminogen (36, 37). Recently, Xie et al., (38) confirmed that *Mycoplasma hyopneumoniae* enolase is localized on its surface and is capable of adhesion to swine tracheal epithelial cells.

In this work, we performed bioinformatic analysis, three-dimensional (3D) modeling, and docking of the *A. marginale* enolase, AmEno. This study aimed to identify *in silico* their potential to interact with different proteins from the extracellular matrix (ECM), erythrocyte membrane (EM), and the zymogen plasminogen that circulates in the mammals' blood. In addition, it could guide the development of a rational and sharp strategy to understand the interaction and functions of enolase and some ligands, which are essential for the success of the pathogen establishment.

2. Materials and methods

2.1. Identification of moonlighting enolases in *Anaplasma marginale* genomes

All seven *A. marginale* draft genomes reported (MEX-01-001-01, MEX-14-010-01, MEX-15-099-01, MEX-17-017-01, MEX-30-184-02, MEX-30-193-01, and MEX-31-096-01) had been previously annotated automatically using the RAST (version 2.0) server (39). Derived from this annotation, we identified one enolase gene in each of the seven genomes. Besides, sequences of enolases from different organisms were retrieved from a Blastp search at NCBI. Additionally, a search in AlphaFold Protein Structure Database (40) and MoonProt 2.0 (41) allowed a comparison of *A. marginale* enolase with those reported as pathogen virulence proteins. The selected sequences were used in the phylogenetic reconstruction.

2.2. Phylogenetic reconstruction

Enolase sequences were selected from the Domains Eukarya and Bacteria (Table S1). All multiple alignments were performed with Clustal Omega (42) and visualized with Jalview (43). A neighbor-joining method was used to reconstruct a phylogeny using Mega 11 software (44) with a Poisson substitution model and a bootstrap value of 1,000 replicates.

2.3. Bioinformatics analyses of *Anaplasma marginale* enolases

The magnesium (Mg^{2+}) binding sites, which are essential for the catalytic activity of the enolase and the conserved domains, were predicted in the Conserved Domains database (CDD-NCBI) (44) and ScanProsite (45). Transmembrane regions were predicted with DeepTMHMM (46). The subcellular localization of the proteins was predicted in PSORTb 3.0 (46), and the secondary structure and function were predicted in PSIPRED Workbench (47). The topology of the proteins was predicted in CATH (48).

2.4. Three-dimensional (3D) modeling

SwissModel is a protein structure homology-modeling server widely used to predict the 3D structure of proteins (49). We used this server to predict the 3D structures of the *A. marginale* enolases from strains MEX-01-001-01 (AmEno01), MEX-15-099-01 (AmEno15), and MEX-31-096-01 (AmEno31). The rest of the enolases from strains MEX-30-193-01, MEX-30-184-02, MEX-14-010-01, and MEX-17-017-01 are essentially identical to MEX-01-001-01; consequently, these structures were not modeled.

Homology modeling is currently an accurate method to generate reliable 3D protein structure models, using experimental protein structures from PDB ("templates") to build models for evolutionary-related proteins ("targets"). All generated models in SwissModel are based on the GMQE (Global Model Quality Estimate) and QMEAN model quality.

2.5. Molecular docking and interaction analysis

The docking of the modeled AmEno01 and five possible ligands was performed in ClusPro (50) to analyze their binding affinity. The PDB ID numbers of the ligands are plasminogen (4DUR), Fibronectin (3M7P), Spectrin (3LBX), Ankyrin (4RLV), and Stomatin (4FVF).

ClusPro is a server that uses a fast Fourier transform (FFT) method called Piper, where one of the proteins is placed at the origin of the coordinate system on a fixed grid, the second protein is placed on a movable grid, and the interaction energy is written as a sum of a few correlation functions. The algorithm rotates the ligand with 70,000 rotations. The 1,000 rotations/translation combinations out of the 70,000 rotations with the lowest score are chosen, and these 1,000 ligand positions are clustered with a 9 Å C-alpha RMSD radius (51). In ClusPro, we generated docking models using AmEno01 and the five potential ligands. For each molecular docking, ten models were generated that were downloaded in PDB format and visualized in UCSF ChimeraX (52). The model with the highest score of the ten docking models was selected to visualize the contact surface model in HDOCK (53).

The analysis of the interactions between amino acids of the five AmEno01-ligand complexes and the visualization were performed in PDBsum (54). For this, we first used the option PDBsum Generate to upload each docking model and generate a PDB code. Once we retrieved the PDB code of the five docking models, we used it as an entry in PDBsum to analyze the AmEno01-ligand interactions.

3. Results

3.1. Identification of moonlighting enolases in Mexican *Anaplasma marginale* genomes

We identified one enolase per genome in the seven *A. marginale* Mexican strains. The enolases of strains MEX-30-184-02 (GenBank KAB0450913.1), MEX-17-017-01 (KAB0451331.1), MEX-30-193-01 (KAB0450361.1), MEX-14-010-01 (TZF77690.1), and MEX-01-001-01 (RCL19410.1) had 425 amino acids of length. The enolase of strain MEX-15-099-01 (KAA8472002.1) had 450 amino acids,
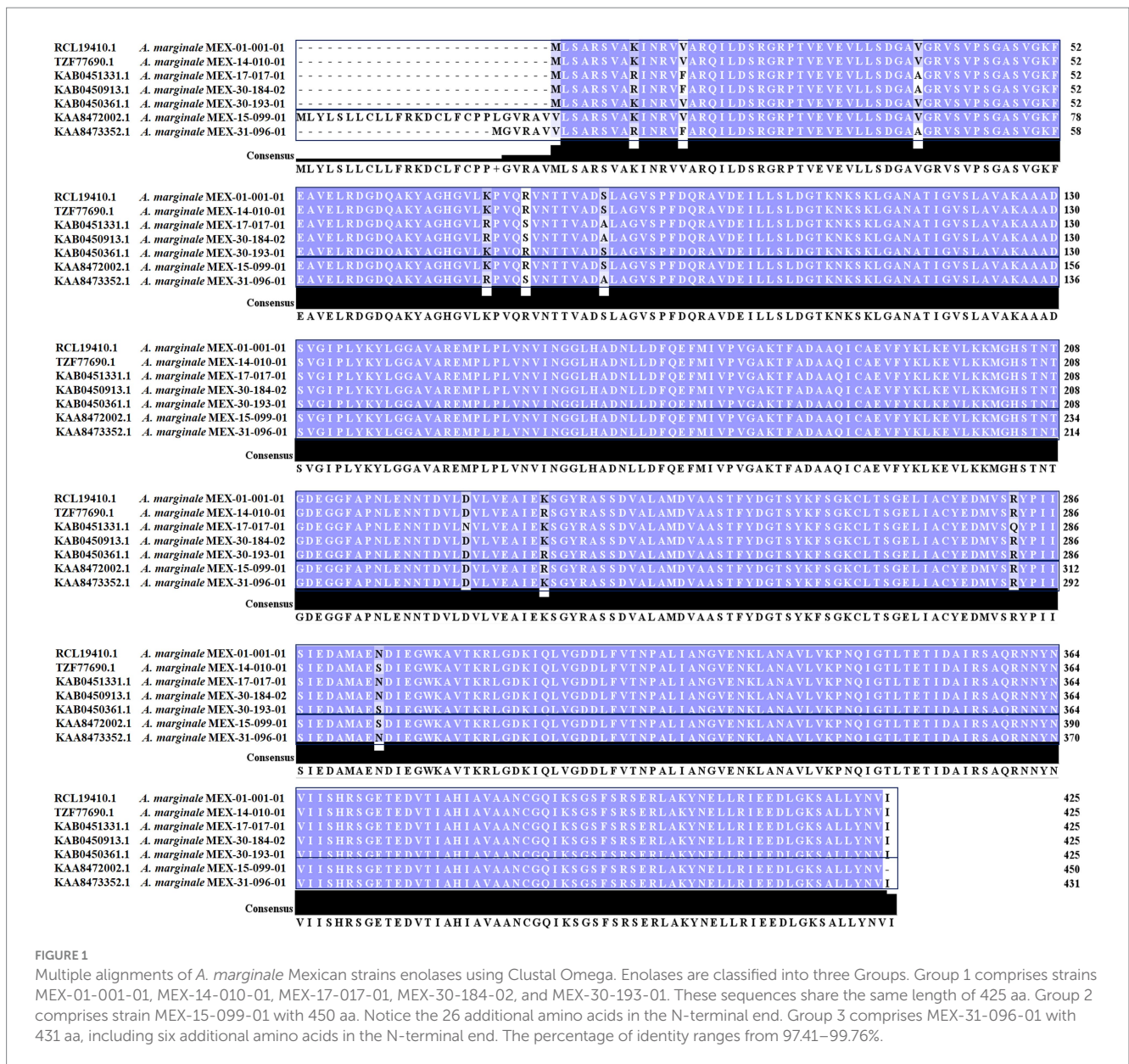
including 26 additional amino acids (MLYLSLLCLLFRKDCLF CPPLGVRAV) in the N-terminal end, and finally, the enolase of strain MEX-31-096-01 (KAA8473352.1) had 431 amino acids, considering six additional amino acids (MGVRAV) in the N-terminal end. Only eleven differences in amino acid sequences were observed in the seven enolase sequences (Figure 1). We performed bioinformatics predictions to all the enolases of the three Groups; however, the modeling and docking analyses we present here were performed only with the strain MEX-01-001-01, AmEno01 sequence. This model was representative of the three *A. marginale* enolases. Nevertheless, to confirm that 3D modeling and molecular docking of the enolases of Groups 2 and 3 did not vary substantially due to the differences at the sequence level, we also performed a docking of these proteins with the ligands. However, we found no significant variation at the structure or interaction level (data not shown).

3.2. Phylogenetic reconstruction

We reconstructed a phylogenetic tree to determine the phylogenetic relationship of AmEno01 and other enolases reported. It is known that enolases are well-defined in alpha, beta, and gamma groups in mammals. In the phylogenetic reconstruction, we found this classification in Chordata. The enolases of ticks *Rhipicephalus* spp. and *Ixodes scapularis* were clustered in a clade belonging to Arthropoda, and those from Protist organisms were separated from Animalia. In Bacteria, enolases MEX-01-001-01 (AmEno01), MEX-14-010-01 (AmEno14), MEX-17-017-01 (AmEno17), MEX-30-184-02 [(AmEno30-02), and MEX-30-193-01 (AmEno30-01)] were clustered in a unique clade that we named Group 1. The enolase MEX-15-099-01 (AmEno15) and Brazilian strains Jaboticabal and Palmeira clustered in Group 2. Finally, the enolase MEX-31-096-01 (AmEno31) was clustered with the reference strain *A. marginale* St. Maries in Group 3 (Figure 2). Additionally, the sequence identity of Mexican strains ranged from 97.65 to 99.53% in Group 1; the sequence identity between AmEno15 and Brazilian strains was 100%; and the identity between AmEno31 and the reference strain St. Maries was 98.38%.

3.3. Bioinformatics analyses of *Anaplasma marginale* enolases

The analysis in the CDD database showed four Mg^{2+} binding sites identified in AmEno01, AmEno14, AmEno17, AmEno30-02, AmEno30-01, AmEno15, and AmEno31, which were S, D, E, and D, varying in position along the sequences (Table 1). These residues are significant because of their role in the enolase catalytic activity. The enolase signature was identified in the analysis of ScanProsite. Additionally, DeepTMHMM predicted a localization inside the cell for all enolases since no transmembrane regions were identified, and no signal peptide was predicted with PSORT. Beta strands, alpha helixes, and coils were also identified (Supplementary Figure S1). To identify and compare the sequences of enolase signature, the amino acids of the catalytic site, and the loops of the active site, we contrasted enolases AmEno01, AmEno14, AmEno17, AmEno30-02, AmEno30-01, AmEno15, and AmEno31with information previously reported for *bona fide* enolases of *H. sapiens, T. cruzi, S. pneumoniae, A. phagocytophilum* and, *R. microplus* (Table 1). We found that the

FIGURE 1
Multiple alignments of *A. marginale* Mexican strains enolases using Clustal Omega. Enolases are classified into three Groups. Group 1 comprises strains MEX-01-001-01, MEX-14-010-01, MEX-17-017-01, MEX-30-184-02, and MEX-30-193-01. These sequences share the same length of 425 aa. Group 2 comprises strain MEX-15-099-01 with 450 aa. Notice the 26 additional amino acids in the N-terminal end. Group 3 comprises MEX-31-096-01 with 431 aa, including six additional amino acids in the N-terminal end. The percentage of identity ranges from 97.41–99.76%.

amino acids with a significant role in the catalytic site of the enolases were essentially H, E, E, E, D, K, R, S, and K, which varied in the sequence position. Additionally, we found variants of the enolase signature and plasminogen-binding site in Mexican strains (Table 1 and Figure 3).

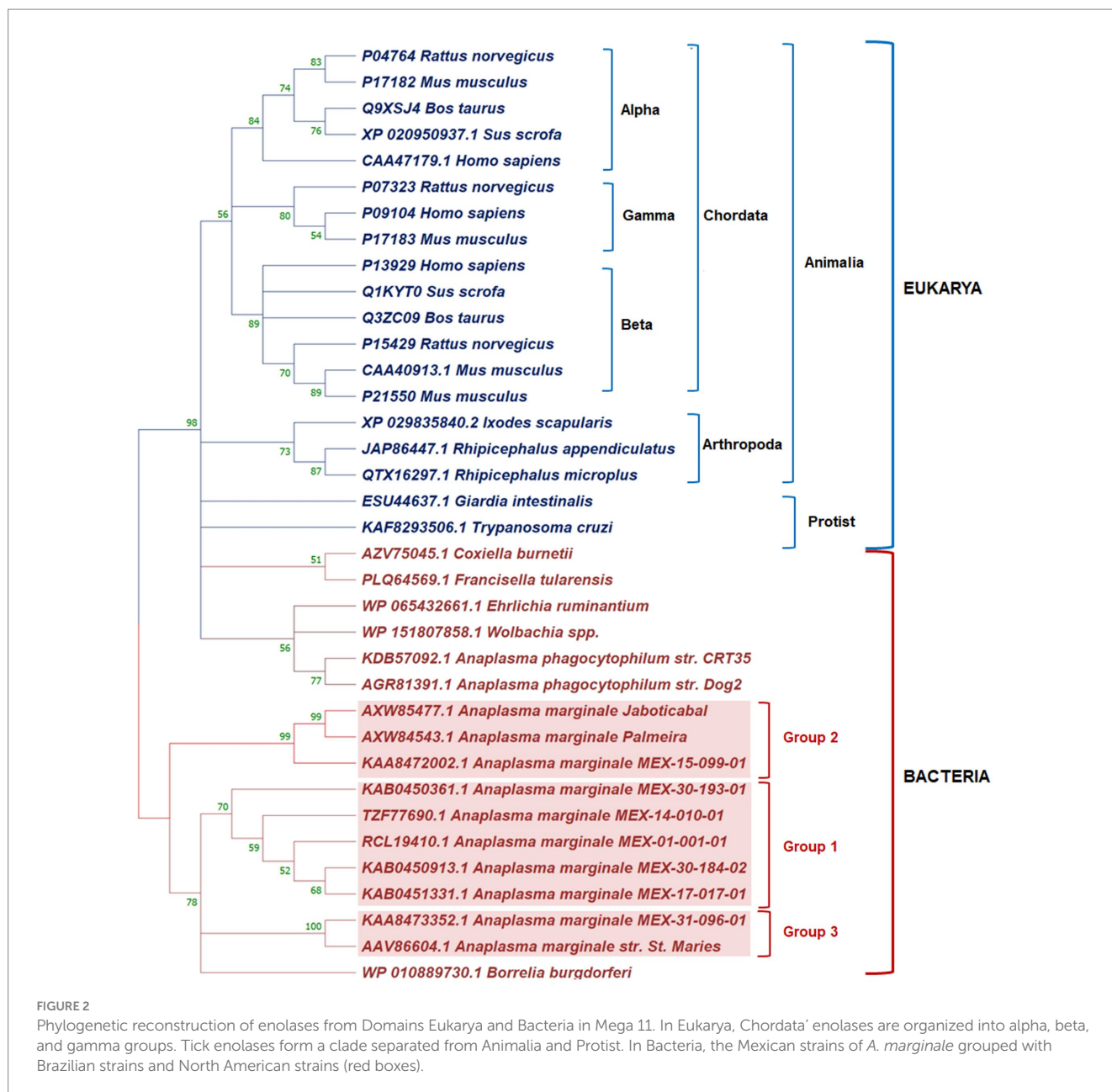## 3.4. Three-dimensional (3D) modeling and molecular docking

We selected the three representative enolases AmEno01, AmEno15, and AmEno31, as input target sequences in the SwissModel server. The results in SwissModel for templates matching with the target sequence were sorted by higher GMQE value, and the enolase from *Enterococcus hirae* (PDB 1IYX) with a value of GMQE of 0.88 was selected as a template for modeling the three enolases. The identity and coverage percentages between template 1IYX and the

modeled AmEno01 were 56.83 and 98%, respectively; for modeled AmEno15, were 56.49 and 92%, respectively; and for modeled AmEno31 were 56.83 and 97%, respectively (Figures 4A–C).

The GMQE value for the modeled enolases AmEno01, AmEno15, and AmEno31 were 0.82, 0.80, and 0.82, respectively, which is a significant accuracy value; hence, these were reliable models. QMEAN value for modeled AmEno01, AmEno15, and AmEno31 was 0.82 ± 0.05.

Since AmEno01, AmEno15, and AmEno31 presented a very similar 3D structure (Figure 4D), with a topology of an alpha-beta barrel (TIM barrel) in the C-terminal end and a two-layer sandwich in the N-terminal end (Figure 4E), we performed the docking with only AmEno01 as a representative model of Mexican strains enolases.

Therefore, the model of AmEno01 was downloaded in PDB format to be used in molecular docking in ClusPro. Five protein–protein dockings were performed in ClusPro, where AmEno01 was considered as a receptor, and the proteins plasminogen (4DUR),

FIGURE 2
Phylogenetic reconstruction of enolases from Domains Eukarya and Bacteria in Mega 11. In Eukarya, Chordata' enolases are organized into alpha, beta, and gamma groups. Tick enolases form a clade separated from Animalia and Protist. In Bacteria, the Mexican strains of *A. marginale* grouped with Brazilian strains and North American strains (red boxes).

fibronectin (3M7P), spectrin (3LBX), ankyrin (4RLV), and stomatin (4FVF), as ligands.

The ten generated docking models by ClusPro were retrieved as "models with balanced coefficients since we do not know what forces dominate the complex protein–protein," as recommended in the ClusPro manual. One of the ten docking models for each AmEno01-ligand was visualized by UCSF ChimeraX. The molecular dockings are shown in Figure 5, and contact surface models for the five interactions of AmEno01-ligands are shown in Supplementary Figure 2.

In addition, the five molecular dockings were evaluated using the Ramachandran plots to validate the quality of the docking by PDBsum. According to this, the obtained values for each docking were 86.6% (AmEno01-3M7P), 87.3% (AmEno01-3LBX), 86.6% (AmEno01-4FVF), 83.9% (AmEno01-4RLV), and 76.3% (AmEno01-4DUR).

Lastly, the analysis of protein–protein (AmEno01-ligand) performed in PDBsum for the five docking models allowed the identification of the residue interactions across the interface. Thus, the salt bridges, disulfide bonds, hydrogen bonds, and non-bonded contacts were identified for each docking model (Supplementary Figure 3).

4. Discussion

The functions reported for enolases as moonlighting proteins in the last years extend their potential applications in the little-explored area of veterinary diseases, such as bovine Anaplasmosis. *A. marginale* is the causal agent of this disease, and seven genomes from Mexican strains have been reported without any moonlighting protein reported in this pathogen up to now (5, 55, 57). However,

TABLE 1 Features of some enolases reported for animals, protists, and bacteria, including *A. marginale* enolases.

Species (accession number and length)	Enolase signature	Mg²⁺ binding sites	aa catalytic sites	Loops of active site	Plasminogen binding sequence	Ref.
H. sapiens (Alpha enolase, P06733; 434 bp)	^{340}LLLKVNQIGSVTES^{353}	S^{40} D^{245} E^{293} D^{318}	H^{158} E^{167} E^{210} E^{293} D^{318} K^{343} R^{372} S^{373} K^{394}	^{37}SGASTGIY^{44} ^{157}SHAGNKLA^{164} ^{248}ASEFFRSGKYDLD FKSPDDPSRYI^{271}	^{251}FFRSGKY^{257}	(55)
R. microplus (QTX16297.1; 434 bp)	^{341}LLLKVNQIGSITEA^{354}	S^{40} D^{245} E^{292} D^{319}	H^{158} E^{167} E^{210} E^{294} D^{319} K^{344} R^{373} S^{373} K^{395}	^{37}SGASTGIH^{44} ^{157}CHAGNKLA^{164} ^{248}ASEFCKDGKYDLDFKNQTSDPSKH^{272}	^{251}FCKDGKY^{257}	This work
Bos taurus (Alpha and beta enolase Q9XSJ4, Q3ZC09; 434 bp)	^{340}LLLKVNQIGSTES^{353}	S^{40} D^{243} E^{289} D^{318}	H^{158} E^{167} E^{210} E^{293} D^{318} K^{343} R^{372} S^{373} K^{394}	Alpha enolase ^{37}SGASTGIY^{44} ^{157}SHAGNKLA^{164} ^{248}ASEFYRSGKYDLDFKSPDDPSRYIT^{272} Beta enolase ^{248}ASEFYRSGKYDLDFKSPDDPSRYIT^{272}	^{251}FYRSGKY^{257}	This work
T. cruzi (KAF8293506.1; 429 bp)	^{340}LLLKINQIGTITEA^{353}	S^{40} D^{243} E^{289} D^{318}	H^{156} E^{165} E^{205} E^{291} D^{319} K^{343} R^{372} S^{373} K^{394}	^{37}SGASTGIH^{44} ^{155}KHAGNALP^{162} ^{245}ASETYDENKKQYNLTFKSPEATWVT^{270}	^{251}FFRSGKY^{257}	(56)
S. pneumoniae (Q97As2.1; 434 bp)	^{340}ILIKVNQIGTLTET^{353}	D^{242} E^{291} D^{318}	H^{155} E^{164} E^{205} E^{291} D^{318} K^{343} R^{372} S^{373} K^{394}	^{39}SGASTGEH^{46} ^{154}SHSDAPIA^{161} ^{245}SSEFYDKERKVYD YTKFEGEGAAVR^{269}	^{248}FYDKERKVY^{256}	(57)
A. phagocytophilum (KDB57092.1; 429 bp)	^{338}VLIKPNQIGTLSET^{351}	S^{48} D^{248} E^{289} D^{316}	H^{161} E^{170} E^{211} E^{289} D^{316} K^{341} R^{370} S^{371} K^{392}	^{45}SGASVGKN^{52} ^{160}LHADNGLD^{167} ^{250}ASTFYDGKIYKFSG^{264}	^{254}FYDGKIYK^{261}	This work

(Continued)

TABLE 1 (Continued)

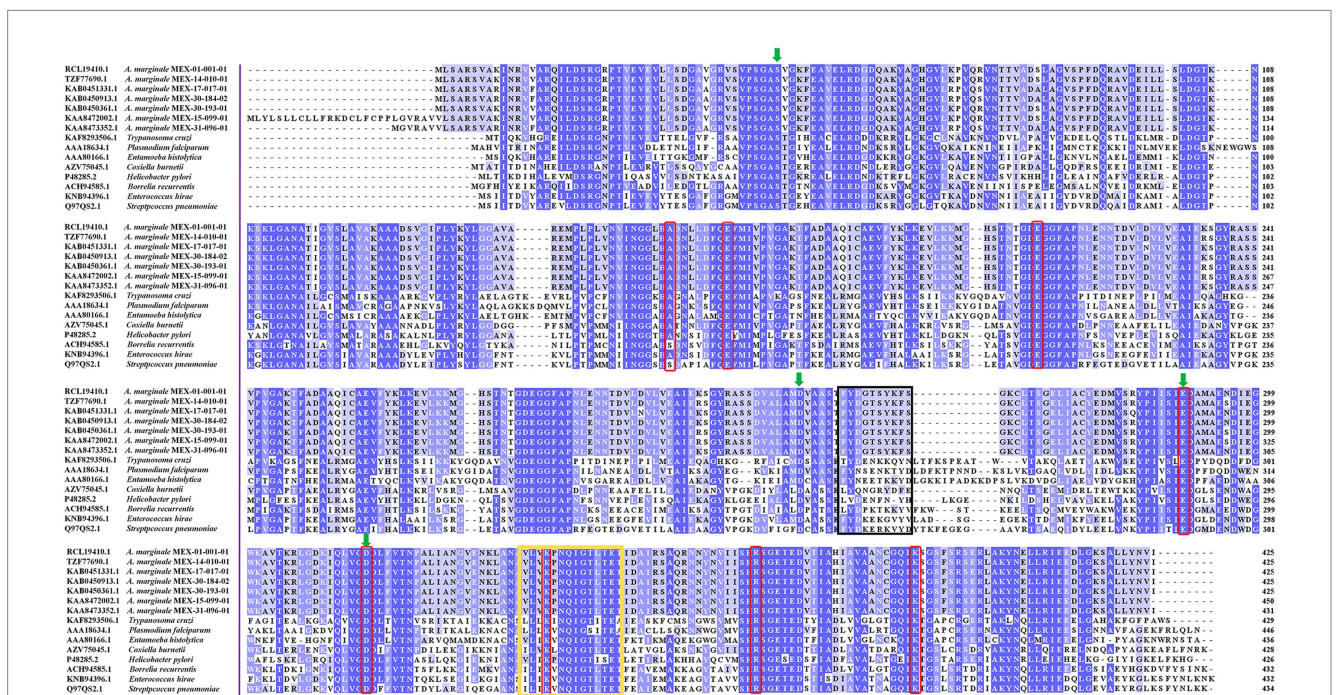| Species (accession number and length)	Enolase signature	Mg²⁺ binding sites	aa catalytic sites	Loops of active site	Plasminogen binding sequence	Ref.
A. marginale MEX-30-184-02 MEX-17-017-01 MEX-30-193-01 MEX-14-010-01 MEX-01-001-01 (RCL19410.1 TZF77690.1 KAB0451331.1 KAB0450913.1 KAB0450361.1; 425 bp)	³³⁸VLVKPNQIGTLTET³⁵¹	S⁴⁸ D²⁴⁸ E²⁸⁹ D³¹⁶	H¹⁶¹ E¹⁷⁰ E²¹¹ E²⁸⁹ D³¹⁶ K³⁴¹ R³⁷⁰ S³⁷¹ K³⁹²	³⁷SGASVGKF⁴⁴ ¹⁵⁹ LHADNLLD ¹⁶⁷ ²⁵⁰ASTFYDGKIYKFSG²⁶⁴ ²⁵⁰ ASTFYDGTSYKFSGK ²⁶⁴	²⁵⁴FYDGTSYK²⁶¹	This work
A. marginale MEX-15-099-01 (KAA8472002.1; 450 bp)	³⁶⁴VLVKPNQIGTLTET³⁷⁷	S⁷⁴ D²⁷⁴ E³¹⁵ D³⁴²	H¹⁸⁷ E¹⁹⁶ E²³⁷ E²⁹¹ D³¹⁵ K³⁶⁷ R³⁹⁶ S³⁹⁷ K⁴¹⁸	³⁷SGASVGKF⁴⁴ ¹⁸⁵LHADNLLD¹⁹³ ²⁷⁶ASTFYDGTSYKFSGK²⁹¹	³⁷SGASVGKF⁴⁴ ¹⁶⁵LHADNLLD¹⁷³ ²⁵⁶ASTFYDGTSYKFSGK²⁷¹	This work
A. marginale MEX-31-096-01 (KAA8473352.1; 431 bp)	³³⁸VLIKPNQIGTLSET³⁵¹	S⁵⁴ D²⁵⁴ E²⁹⁵ D³²²	H¹⁶⁷ E¹⁷⁶ E²¹⁷ E²⁹⁵ D³²² K³⁴⁷ R³⁷⁶ S³⁷⁷ K³⁹⁸	²⁷⁹FYDGTSYK²⁸⁷	²⁵⁹FYDGTSYK²⁶⁷	This work

FIGURE 3

Enolases of parasites and bacteria aligned with Clustal Omega. The plasminogen binding sequence is shown in the black box. The sequence FYDGTSYKFS in *A. marginale* strains varies by 70% from the reported in *S. pneumoniae*, which binds to plasminogen by the sequence FYDKERKVYD. The enolase signature in *A. marginale* strains has seven amino acid changes compared to the sequences shown in the yellow box. The amino acids binding magnesium atoms are shown in green arrows and the amino acids of the catalytic site are shown in red boxes.
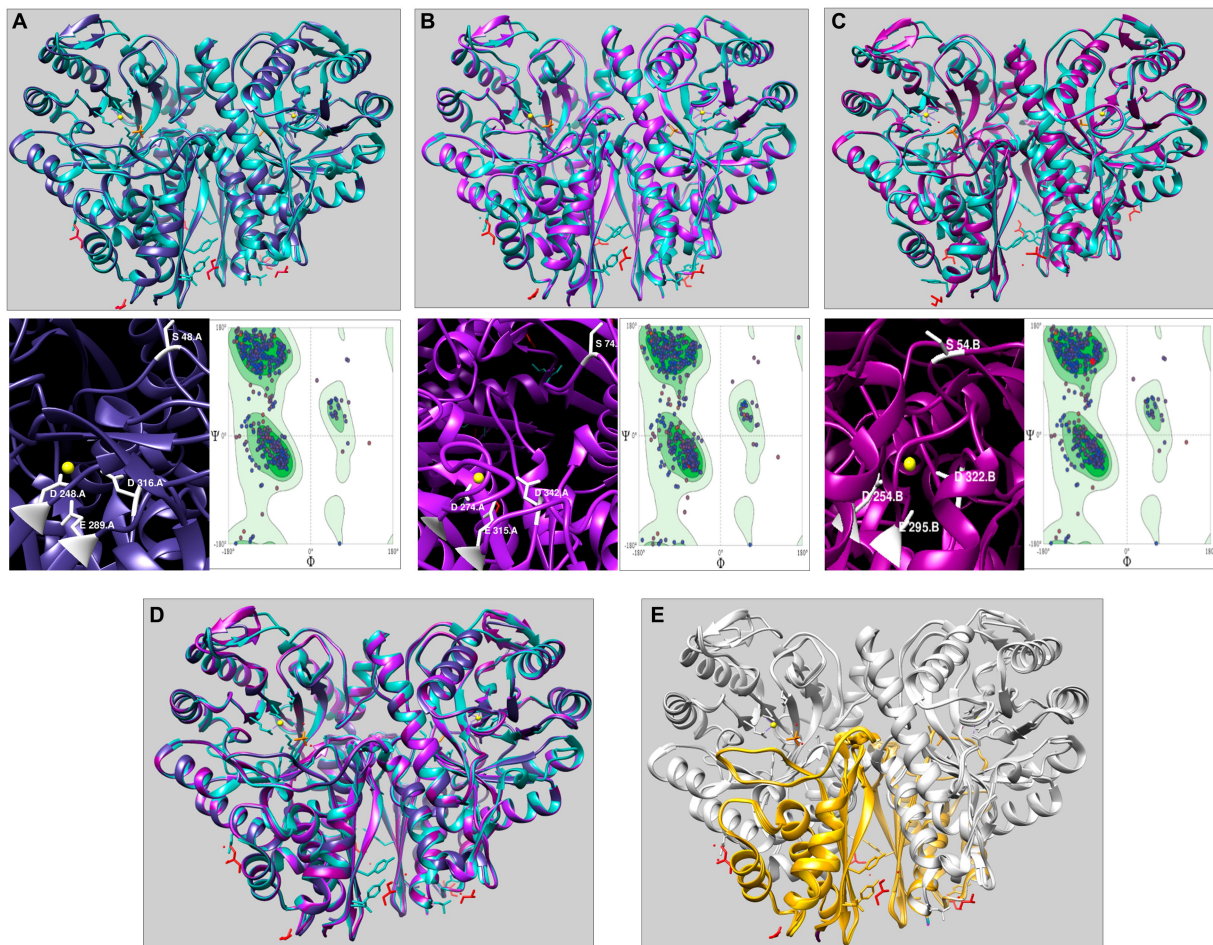
FIGURE 4
Three-dimensional modeling of enolases from Mexican strains of *A. marginale* using *Enterococcus hirae* enolase as the template (PDB 1IYX, cyan). In the three modeled enolases obtained in Swiss Model **(A)** AmEno01 (purple), **(B)** AmEno15 (magenta), and **(C)** AmEno31, the magnesium binding atoms S, D, E, and D (which vary in position) are shown. Models were considered feasible according to the Ramachandran plots. **(D)** Superposition of AmEno01, AmEno15, AmEno31, and 1IYX. **(E)** Enolases have the topology of a two-layer sandwich in the N-terminal end (golden) and an alpha-beta barrel (TIM barrel) in the C-terminal end (silver). For all models, magnesium atoms are shown in yellow circles; the structure of glycerol molecules is in red, and sulfates in orange. All 3D models were visualized and colored in ChimeraX.

their presence in genomes of Mexican strains of *A. marginale* suggests they could have similar functions reported elsewhere for this type of protein. The *A. marginale* enolase (AmEno), is of great interest since this protein could have a significant role during the erythrocyte invasion of the pathogen, as it has been reported for *Mycoplasma suis* where the enolase would participate in the adhesion of the pathogen to porcine erythrocytes at early stages of the invasion process (58).

Using bioinformatics approaches allowed us to identify only one enolase sequence per genome in Mexican strains of *A. marginale*, seven enolase sequences. As the phylogenetic reconstruction showed, these sequences grouped with other enolases into three different subclades: Group 1 (five Mexican strains enolases of 425 aa), Group 2 (one Mexican strain enolase of 450 aa and Brazilian strains enolases Palmeira and Jaboticabal), and Group 3 (one Mexican strain enolase of 431 aa and North American strain St. Maries). This suggested that some enolases of Mexican *A. marginale* strains could be related to Brazilian and North American strains, while others could be exclusive to Mexico.

Although the main difference between enolases of Groups 1 to 3 are additional amino acids at the N-terminal end, they have similar secondary structures of beta strands, alpha helix, and coil structures. Additionally, these differences at the sequence level and length observed in Mexican *A. marginale* strains' enolases did not cause significant modifications in the 3D structural arrangements, as we elucidated when we performed 3D modeling of representative members of each group of enolases: AmEno01, AmEno15, and AmEno31. After predicting 3D structures for these three enolases and realizing their structure was practically identical, we selected AmEno01 for further analysis.

Concerning the AmEno01 3D modeling, our results revealed a dimeric structure of the protein as it has been observed in other enolase structures, including *Helicobacter pylori* (59), *Aeromonas hydrophyla* (60), *Candida albicans* (61), and *Mycoplasma pneumoniae* (62). Additionally, an enolase octameric structure has been reported in *Bacillus subtilis*, *Streptococcus* spp., and *Thermotoga maritima* (63–65), and a monomeric structure has also been identified *in vitro* conditions (66).
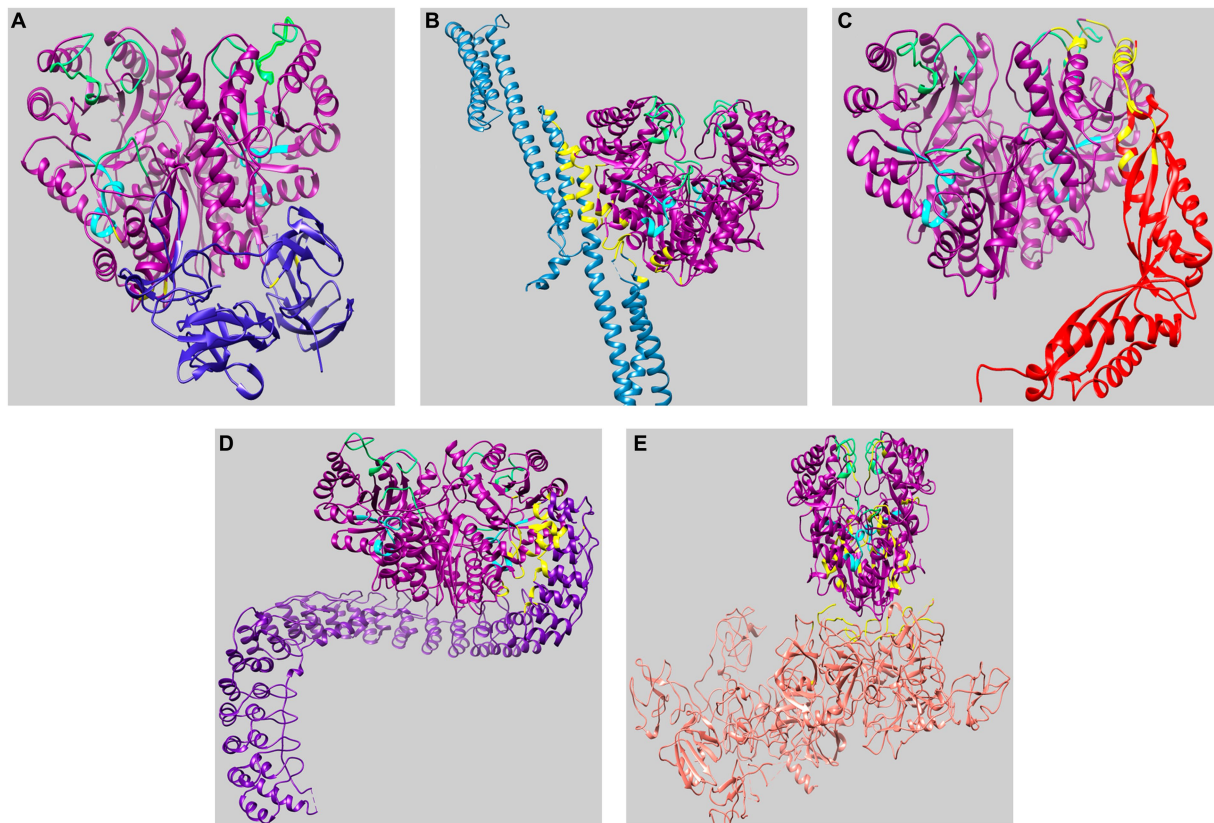
FIGURE 5
Visualization of molecular docking of AmEno01 with ligands retrieved in ClusPro. **(A)** fibronectin (3M7P); **(B)** spectrin (3LBX); **(C)** stomatin (4FVF); **(D)** ankyrin (4RLV); and **(E)** plasminogen (4DUR). The predicted amino acids participating in the interaction between AmEno01 and each ligand are shown in yellow, the enolase signature is shown in cyan, and the loops of the active site are shown in green. The docked structures were visualized and colored in ChimeraX.

Functionally, in the AmEno01 3D structure, we identified the sequences of the loops of the active site (^{37}SGASVGKF^{44}, ^{159}LHADNLLD^{167}, ^{250}ASTFYDGKIYKFSG^{264}, and ^{250}ASTFYDGTSY KFSGK 264) and the enolase signature (^{338}VLVKPNQIGTLTET^{351}), which were similar to those sequences of the rest of the Mexican enolases´ strains.

On the other hand, it has been reported that two Mg^{2+} ions bind to the amino acids S, D, E, and D in the classical enolase active site, facilitating its catalytic reaction (67). In AmEno01, we found that amino acid S^{48} has a different spatial location than the one observed in other enolases; however we cannot discard its possible binding to the Mg^{2+} ion. In addition, in the predicted 3D models of AmEno01, AmEno15, and AmEno31, the Ramachandran plots, which predict the possible conformation of a protein, revealed that these models with a different spatial location of the S^{48} were feasible.

Subsequently, to analyze the potential of AmEno01 to bind ligands, we explored its interactions with proteins from the extracellular matrix or ECM (fibronectin), erythrocyte membrane (spectrin, ankyrin, and stomatin), and plasminogen by *in silico* approaches.

In this regard, our analysis of the AmEno01-fibronectin interaction showed that the Ramachandran plot contains 86.6% of residues in the most favored regions [A, B, L], suggesting the occurrence possibility of this interaction. The significance of this interaction relies on the ability to bind to fibronectin as a characteristic reported for many pathogens in the early steps of infection of host tissues (68). Interestingly, as *A. marginale* is a pathogen that does not infect tissues, we proposed that the potential of AmEno01 to bind fibronectin is highly relevant to infect ticks if we consider the possibility to adhere to tick gut cells. Like in spirochaete *B. burgdorferi,* whose membrane protein extract interacts with a protein with fibronectin III domains (Ixofin3D) identified in the gut of *Ixodes* spp., facilitating spirochaete congregation to the gut, and providing a molecular exit to the salivary glands before transmission to the human host (69, 70).

On the other hand, we decided to perform a docking analysis to elucidate if AmEno01 could adhere to erythrocyte proteins spectrin and ankyrin as part of the initial invasion process. According to the Ramachandran plot with 87.3 and 83.9% residues, respectively, in the most favored regions, our docking results suggested the binding of AmEno01 with spectrin and ankyrin. Therefore, we hypothesized that AmEno01 adhere to these proteins and probably mediate the initial erythrocyte invasion process. We must highlight that in *A. marginale*, the entry process to the erythrocyte is not well known; however, the ability to attach to host cells is essential for infection (71).

Some examples of enolase as an adhesion molecule to erythrocytes have been reported. Like *M. suis* enolase, that could act as an adhesion factor to porcine erythrocytes. Schreiner et al. (58)

found that *M. suis* recombinant enolase bound to erythrocytes lysates in a dose-dependent manner, and even transformants *E. coli* acquired the ability to bind to erythrocytes due to the expression of the enolase on their surface.

In addition to spectrin and ankyrin, another erythrocyte protein is stomatin, an integral protein that plays a role as a membrane-bound scaffolding protein modulating transport protein (72). According to this, the docking results of AmEno01 with stomatin showed 86.6% residues in the most favored regions in the Ramachandran plot. Therefore, we proposed that AmEno01 could recognize stomatin in the erythrocyte membrane as an essential step to adhesion and further internalization. In summary, spectrin, ankyrin, and stomatin could be potential targets to be experimentally assessed to avoid the invasion of *A. marginale* to erythrocytes.

As it is known, *A. marginale* infects erythrocytes but not tissues. Therefore, its binding to plasminogen, a zymogen that facilitates migration and invasion of pathogens to host tissues when converted to plasmin by its proteolytic activity, must be analyzed. Interestingly, we found that 76.3% of residues in the docking AmEno01-plasminogen are in favored regions of the Ramachandran plot, a percentage below the expected values to be considered a good quality model. Additionally, the plasminogen binding sequence identified in Mexican strains of *A. marginale* is different to the sequence of well-known enolases that bind plasminogen (73, 74).

In this regard, we theorized that AmEno01 could be binding to erythrocyte membrane proteins instead of binding to plasminogen; this last one is a strategy that parasites use to invade tissues such as *Leishmania mexicana* (cysticercus), *Taenia solium* and bacteria *S. pneumoniae, Bacillus antracis*, and *B. burgdorferi*, among others (37, 73, 75–77).

Finally, the role of enolases in many organisms is still under study; however, many advances in their functions as moonlighting proteins have been achieved (78). Thus, the study of moonlighting activities attributed to enolases in pathogenic bacteria such as *A. marginale* is relevant since it could be a candidate to control bovine Anaplasmosis. This proposal is based on the approaches performed with moonlighting proteins as potential vaccine candidates against several animal diseases. For example, a robust immune response in mice and piglets was observed when an enolase subunit of *M. suis* was used as antigen (20), or the protective immune response obtained when recombinant enolase of *P. vivax* was expressed in *E. coli* and used as an antigen against malaria (79).

In this regard, elucidating the functions of the enolases from Mexican strains of *A. marginale* could be the basis for developing strategies such as an anti-enolase antibody that interferes, avoids the invasion of erythrocytes, or blocks some other vital processes for the pathogenesis. In addition, we neither exclude that enolases could be proteins driven by the pathogen according to its needs nor discard their possible interaction with tick proteins that contribute to the pathogen's survival inside the vector.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

HA-D and RQ-C conducted the experiments. HA-D, RQ-C, and IA-E analyzed the data. RQ-C and HA-D envisioned and designed the study, and RQ-C and HA-D wrote the manuscript. RQ-C, IA-E, and HA-D edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1225873/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) fibronectin (3M7P); **(B)** spectrin (3LBX); **(C)** stomatin (4FVF) **(D)** ankyrin (4RLV), and **(E)** plasminogen (4DUR).

SUPPLEMENTARY FIGURE 2
(1) fibonectin (3M7P); **(2)** spectrin (3LBX); **(3)** stomatin (4FVF) **(4)** ankyrin (4RLV), and **(5)** plasminogen (4DUR).

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