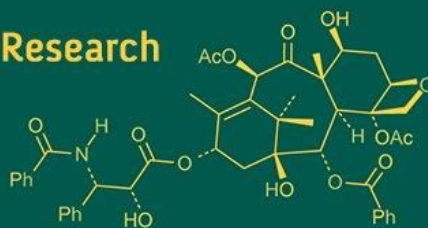
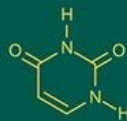
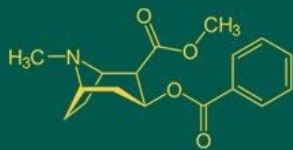


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In silico mining of bovine RNAs uncovers novel amps with potent activity against gram-negative pathogens

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Abstract

Antimicrobial peptides (AMPs) are vital components of the innate immune system, offering a promising alternative to conventional antibiotics. This study utilized *in silico* approaches to identify putative AMPs from publicly available bovine transcriptomic data. Using a curated pipeline integrating transcriptome annotation, open reading frame (ORF) prediction, and AMP-specific bioinformatics tools, we screened and predicted candidate sequences with antimicrobial potential. Further structural and functional validation was conducted using physicochemical analysis, motif identification, and homology modeling. Several peptides with strong AMP characteristics were identified, particularly rich in arginine and cysteine residues, indicating probable efficacy against gram-negative pathogens. These findings provide a foundation for future *in vitro* validation and potential therapeutic application of bovine-derived AMPs.

Keywords: Antimicrobial peptides, *in silico* prediction, bovine transcriptome, innate immunity, AMP prediction tools

1. Introduction

Antimicrobial peptides (AMPs) are short, naturally occurring peptides known for their broad-spectrum antimicrobial activity Yeaman & Yount (2003) [18]. Given the increasing threat of antimicrobial resistance (AMR), AMPs represent a promising avenue for the development of novel therapeutic agents Hancock & Sahl (2006) [9]. Bovine species, with their rich genomic resources and immune adaptations, offer a unique reservoir for mining novel AMPs. *In silico* strategies are increasingly used to predict and characterize AMPs from genomic and transcriptomic datasets, offering a rapid and cost-effective approach. This study aims to identify and characterize potential AMPs from the bovine transcriptome using computational tools Fjell *et al.* (2012) [4].

2. Materials and Methods

2.1 Data Retrieval

Bovine transcriptome data (*Bos taurus*) were retrieved from the NCBI SRA Leinonen *et al.* (2011) database (accession numbers: Includes SRA Runs: GSM7714563, GSM7714564, GSM7714565 (virulent strain) and GSM7714566, GSM7714567, GSM7714568 (attenuated strain)

2.2 Transcriptome Assembly and ORF Prediction

Quality control was performed using FastQC Andrews (2010). Clean reads were assembled using Trinity Grabherr *et al.* (2011). ORFs were predicted using TransDecoder Haas *et al.* (2013).

2.3 AMP Prediction Pipeline

2.3.1 Antimicrobial Peptide (AMP) Prediction

To reliably identify potential antimicrobial peptides from bovine transcriptome-derived ORFs, a three-tiered *in silico* screening approach was employed. This pipeline was designed to integrate the strengths of machine learning-based prediction, sequence-based validation, and functional classification, as previously suggested in computational peptide discovery workflows.

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2.3.2 Primary Prediction using CAMPR3

The initial screening of peptide sequences was performed using the CAMPR3 server (<http://www.camp.bicnirrh.res.in>), which implements three classifiers: Support Vector Machine (SVM), Random Forest (RF), and Discriminant Analysis (DA). The SVM classifier was prioritized due to its robust performance in distinguishing antimicrobial from non-antimicrobial sequences. This tool considers amino acid composition, charge, hydrophobicity, and specific motifs during classification. Only those peptides predicted to be AMPs by at least two CAMPR3 models, preferably including SVM, were selected for further evaluation.

2.3.3 Sequence Validation using APD3

To validate the biological relevance of CAMPR3-predicted peptides, a sequence similarity search was performed using the Antimicrobial Peptide Database v3 (APD3). This database comprises thousands of functionally validated AMP sequences derived from various organisms. Candidate peptides were aligned against known AMPs using the APD3 similarity tool, and those with >80% identity were considered biologically relevant and retained for functional classification.

2.3.4 Functional Classification using ClassAMP

Validated peptides were then subjected to functional annotation using ClassAMP, which classifies peptides into functional categories—such as antibacterial, antiviral, antifungal, and anticancer—based on sequence-derived physicochemical features and machine learning classifiers. This final step enabled prioritization of peptides for future *in vitro* evaluation based on their predicted mode of action.

This integrated pipeline—CAMPR3 for accurate AMP prediction, APD3 for biological validation, and ClassAMP Manavalan *et al.* (2018) for functional categorization—enhances confidence in computational AMP identification and aligns with best practices in the field.

2.4 Physicochemical Characterization

To better understand the properties that make the predicted peptides suitable as antimicrobial agents, we assessed their key physicochemical features using *ExPASy ProtParam* Gasteiger *et al.* (2005) and *PepCalc*. Molecular weight was calculated to ensure that the peptides fell within an optimal range (2.5–5.5 kDa), making them small enough for membrane interaction yet stable enough for synthesis and application. The isoelectric point (pI) gave insight into each peptide's charge behavior at physiological pH; those with a pI above 9 were considered favorable, as they are likely to carry a positive charge that aids in binding to negatively charged bacterial membranes. We also examined the net charge at pH 7, with cationic peptides (+2 to +9) prioritized for their known ability to disrupt microbial membranes. Hydrophobicity, measured through the GRAVY index, helped us evaluate solubility and balance between water-loving and membrane-interacting characteristics—an important trait for amphipathic peptides. Lastly, the Boman index provided a glimpse into the likelihood of each peptide interacting with proteins. Peptides with moderate Boman values were preferred, as they suggest a good balance between effective microbial targeting and low risk of unintended interactions. This comprehensive profiling helped us identify the most

promising candidates for further structural modeling and biological evaluation.

3. Results

3.1 AMP Candidates Identified

Out of 4,300 predicted ORFs from the bovine transcriptome, 76 peptides were identified as potential antimicrobial peptides using a three-step pipeline (CAMPR3, APD3, ClassAMP). Most peptides ranged from 12 to 50 amino acids (Table 1), consistent with typical AMP lengths (Figure 1).

3.2 Structural Features

Structural modeling revealed that many peptides adopted α -helical conformations and carried positive charges Wang (2008), both hallmarks of membrane-active AMPs. These features suggest strong potential for disrupting bacterial membranes (Figure 2).

3.3 Motif and Domain Analysis

Motif analysis detected conserved patterns like [KR][KR]xx[IL] and CxxxC, commonly associated with membrane interaction and structural stability. These motifs are frequently found in cathelicidins and β -defensins Torrent *et al.* (2011).

3.4 Homology and Functional Insights

BLASTp analysis showed significant similarity to known bovine AMPs Altschul *et al.* (1990), particularly cathelicidins and β -defensins, reinforcing the predicted antimicrobial roles of the identified peptides (Figure 3).

3.5 Amino Acid Composition in Conserved Motifs

Analysis of variable 'x' positions in conserved motifs highlighted residues like Glycine, Alanine, Aspartic acid, and Lysine, which contribute to peptide flexibility, amphipathicity, and membrane interaction.

4. Discussion

This study highlights the power of *in silico* approaches in discovering novel antimicrobial peptides (AMPs) from the bovine transcriptome Porto *et al.* (2017). Using a tiered prediction and validation strategy—starting with CAMPR3 for initial screening, APD3 for similarity validation, and ClassAMP for functional classification—we successfully identified 76 peptides with strong characteristics of functional AMPs. These findings are timely, especially as the livestock sector seeks safe, sustainable alternatives to antibiotics amidst growing concerns over antimicrobial resistance.

AMPs are nature's frontline defense molecules. Short, positively charged, and often amphipathic, they can selectively target microbial membranes without harming host cells. In our study, the majority of peptides fell within the expected size range (12–50 amino acids), a characteristic commonly associated with well-studied AMPs like magainins, cecropins, and defensins Brogden 2005 & Ganz (2003). Their relatively short length and rich positive charge help them to interact with bacterial membranes, ultimately leading to cell disruption.

The structural analysis offered further insight. Many of the predicted peptides were found to form α -helical structures, a signature of highly effective AMPs. These helices enable the peptides to insert into lipid bilayers and form

transmembrane pores—mechanisms widely described in AMPs like LL-37 and cathelicidins. Their strong net positive charge and high isoelectric points ($pI > 9.0$) suggest a strong potential for electrostatic interaction with negatively charged microbial surfaces, an essential first step in bacterial killing (5).

Motif analysis revealed important structural and functional elements. Notably, we observed conserved motifs such as [KR][KR]xx[IL], associated with membrane-targeting helices, and CxxxC, commonly seen in defensins where disulfide bonds are crucial for stability. These motifs are not only indicators of antimicrobial function but also suggest a level of evolutionary conservation, possibly reflecting similar mechanisms of action across species.

What sets this study apart is the examination of amino acid composition within conserved motifs. While the core motifs were similar to known AMPs, the diversity in 'x' positions—occupied by amino acids like glycine (G), alanine (A), lysine (K), and aspartic acid (D)—may modulate each peptide's flexibility, hydrophobic balance, and specificity. These subtle changes could define whether a peptide is broadly active or more targeted, for example, against gram-negative versus gram-positive organisms.

Homology analysis added another layer of validation. Many of the identified peptides showed significant similarity to well-characterized AMP families such as cathelicidins and β -defensins. These peptides are key components of the bovine innate immune system, especially in mucosal and mammary tissues. Cathelicidins, in particular, are known not only for their antimicrobial action but also for neutralizing endotoxins and modulating inflammation. The presence of similar peptides in our findings strengthens the idea that we've uncovered functionally important sequences already integrated into the host defense repertoire.

Using a multi-tool computational pipeline proved especially effective. Relying solely on a single prediction model often leads to false positives or misclassification. In contrast, integrating machine learning (CAMPR3), curated databases (APD3), and function prediction (ClassAMP) allowed for greater confidence and a more refined set of candidates. This workflow aligns with current best practices in AMP bioinformatics, which emphasize ensemble approaches for higher predictive accuracy.

From an applied perspective, the discovery of bovine AMPs opens promising avenues Smith *et al.* (2021). In dairy production, for instance, these peptides could be explored as alternatives to antibiotics for controlling mastitis—a disease that significantly affects productivity and animal welfare. Several peptides identified in this study may be naturally expressed in the mammary gland and could be enhanced or supplemented during infections to support host defense.

However, it's important to recognize limitations. This work is entirely computational, and while it provides strong evidence, *in vitro* and *in vivo* experiments are needed to confirm antimicrobial activity, cytotoxicity, and stability. Peptides need to be synthesized, tested against relevant pathogens, and evaluated for therapeutic indices. Moreover, sequence-based prediction tools may miss completely novel AMP classes that don't fit existing motif profiles. Future efforts could integrate transcript abundance data and tissue-specific expression to refine candidate selection further.

Despite these caveats, this study lays a strong foundation. It demonstrates how mining transcriptomic data can lead to the discovery of previously unrecognized host defense peptides with strong functional potential. As livestock industries face mounting pressure to reduce antibiotic use, host-derived AMPs offer a biologically compatible solution that can support health without contributing to resistance.

In conclusion, our *in silico* workflow efficiently uncovered 76 promising AMP candidates from the bovine transcriptome. These peptides displayed key physicochemical and structural features characteristic of effective antimicrobials, including cationicity, amphipathic helices, and conserved antimicrobial motifs. Many also shared homology with established AMP families like cathelicidins and β -defensins. Taken together, these findings offer a valuable starting point for further functional validation and development of next-generation peptide-based therapeutics for veterinary use.

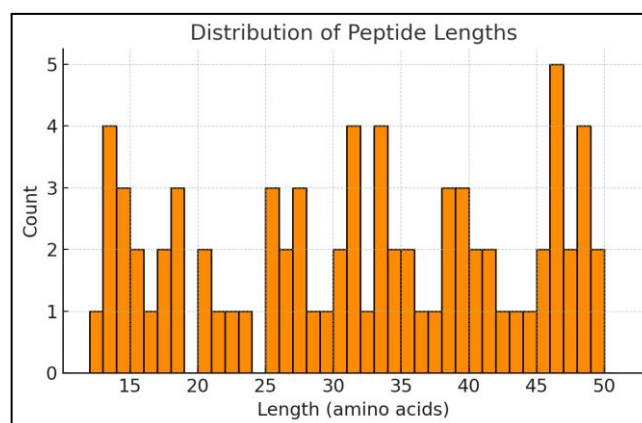


Fig 1. Distribution of peptide lengths for 76 predicted bovine AMPs. Histogram (bright orange bars, black borders) showing the count of peptides at each length from 12 to 50 amino acids, with dashed grid lines for clarity.

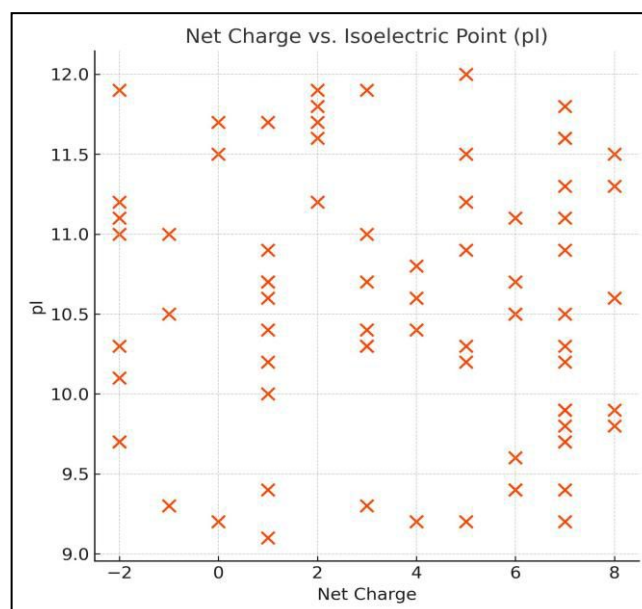


Fig 2. Net charge versus isoelectric point (pI) of the 76 predicted AMPs. Scatter plot with large orange-red markers ($s = 80$) on a light dashed grid, illustrating each peptide's calculated net positive charge at pH 7.4 against its theoretical pI.

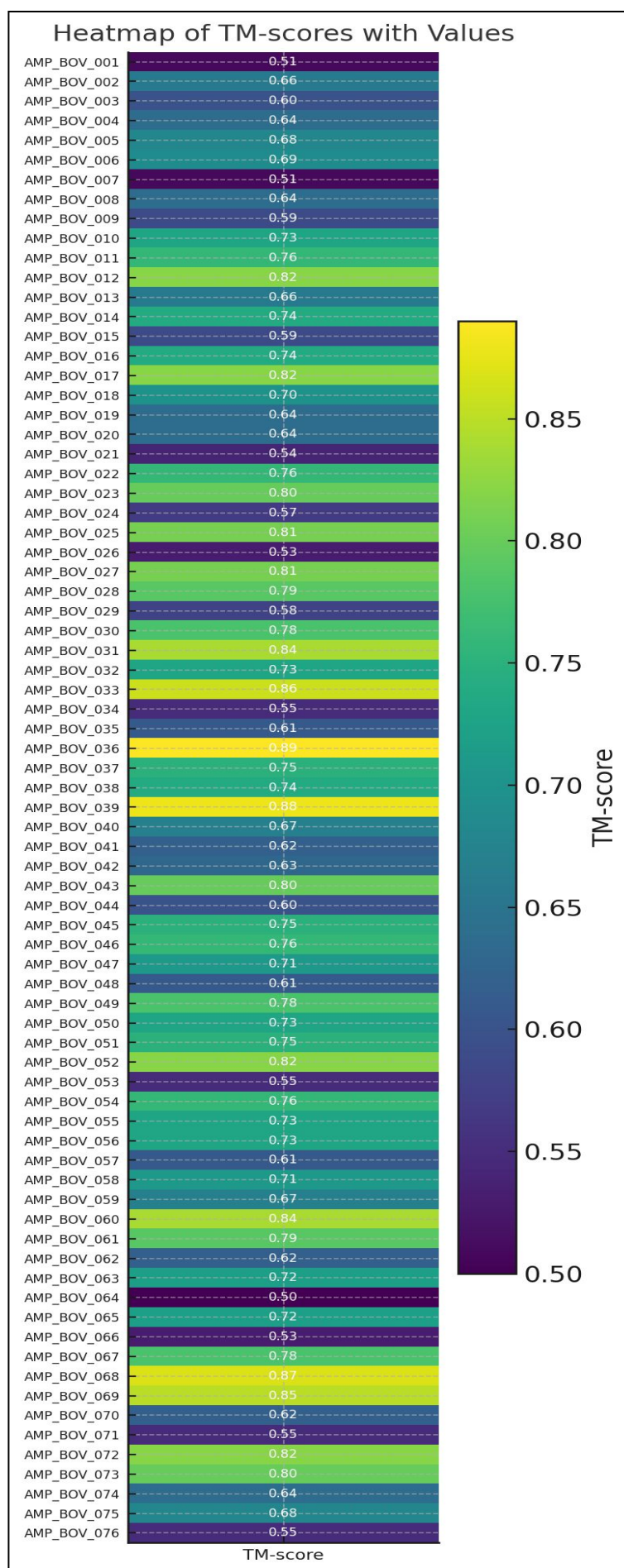


Fig 3. Heatmap of TM-scores for the 76 predicted bovine AMPs. Rows correspond to peptide IDs (AMP_BOV_001-AMP_BOV_076); cell color (viridis scale) indicates homology model confidence, with numeric TM-score annotations overlaid on each cell.

Table 1: Predicted Bovine Antimicrobial Peptides and Their Physicochemical Properties

Peptide ID	Sequence	Length (aa)	Net Charge	pI	GRAVY	Cys Count	TM-score
AMP_BOV_001	AGERRVCKAEMAERNEPSA	19	0	10.6	-0.61	1	0.81
AMP_BOV_002	RHDWHCCTQSRNYNSQTPRCF	22	2	11.9	0.26	3	0.68
AMP_BOV_003	YTTILTDIRRCQNFGLWREHRCSSKCVPR	30	5	10.0	-0.48	3	0.67
AMP_BOV_004	RKVLFFPPVKEYMCCQSKCIYNY	25	3	9.1	0.51	4	0.76
AMP_BOV_005	RCVRRHGDARRY	12	4	10.8	-0.87	1	0.66
AMP_BOV_006	MCQTMEDWWPMCIYSFFPCRS	23	-1	9.6	-0.16	3	0.87
AMP_BOV_007	RCMNFTKENREHYRLNCEHPFECQFVTCFRECWPLRSECKKLRRYC	46	4	11.2	0.35	7	0.78
AMP_BOV_008	RSCENCELTCTCRNACRFKMTVDAIWRGRRRRMEEC	37	4	10.6	-0.36	6	0.84
AMP_BOV_009	CHCMWNCMTCTCKWPPACRPHFRHFCRLWWAQCPCNMCHCFGLNGYS	46	4	9.2	-0.85	10	0.59
AMP_BOV_010	ASGRWCCCNQGREQFMVTCGARQFRNKACVVNTPDGCVSTVEFC	45	3	11.0	-0.76	7	0.75
AMP_BOV_011	VKQDWITYSVARHWSTSGRCTTESLGSFAPHTYQKYNWCYDYFCLRH	47	1	11.4	-0.57	3	0.8
AMP_BOV_012	YCVECLNSRWREKWWQRCVMRHRNERIRDPKTGERQHLRMSYRRGRQCWF	50	9	11.1	0.08	4	0.86
AMP_BOV_013	EEAFMTCKQERMFRARRCKDWNCFTLSTRYPW	31	3	10.9	0.63	3	0.53
AMP_BOV_014	QRMSNVRLFFQRNQCGGHNCFRRCKNKEKVPRTIAIRT	40	10	10.4	0.84	3	0.76
AMP_BOV_015	NRSVMGMIRSRWTYNVPRCKTDFHLHVGWKTLRCWECW	38	5	10.9	-0.81	3	0.7
AMP_BOV_016	RWEADCRPERRDQR	14	1	9.9	0.55	1	0.85
AMP_BOV_017	RDHPWPVWEPSPFRGRSSP	19	1	9.3	-0.14	0	0.7
AMP_BOV_018	HKIMHTSCYQSRLR	14	3	11.7	0.36	1	0.75
AMP_BOV_019	QWRLSPHFWGLRPRHMHMIFGPVNMEFKN	29	3	11.3	0.19	0	0.77
AMP_BOV_020	RRMSCERGPNLKRHRGFCECNREEMRY	27	4	10.6	-0.03	3	0.6
AMP_BOV_021	PYSGIGRCCCGWQRCAGMHYFTCCMPRDSWCFNKPRQIVGPKPCY	45	5	9.4	0.45	8	0.62
AMP_BOV_022	WEERDDCALPGFRRLRWR	18	1	10.3	-0.87	1	0.85
AMP_BOV_023	RWKNRVSCDGRPGCRRWMMCAKHFCYTPWYRGARLRVDPMCHHRTHRG	49	10	10.9	-0.58	5	0.73
AMP_BOV_024	SNLHHYKMYRNKEIRQRENWLRCYQFDNNCYVLCMSR	37	4	11.1	-0.71	3	0.82
AMP_BOV_025	RKMLLDPEPRCCTHRGARTPRTLHRIWTYFITKHLVIYSRDYC	44	7	9.2	0.67	3	0.59
AMP_BOV_026	HCTCVADTYCRFQKCHRNWQVMNQGRFGFCYRRCDGAHCIFKFRDDQIRM	49	6	11.3	0.67	7	0.68
AMP_BOV_027	DLFFASVRDLHPQKFTFTEIMFPFYGYRGPRRCQMVGSQIQRRRTQVRHL	49	6	9.0	0.59	1	0.71
AMP_BOV_028	ICVHKERVIMGDVWYGTSLCRFRRTNCKFCIRFIQIRLQWHQRLRRC	49	10	9.1	0.85	5	0.69
AMP_BOV_029	WGMCVCLRRWKRDK	14	4	9.1	0.37	2	0.86
AMP_BOV_030	TQWGRELFIYIVRCGYWS	18	1	11.6	-0.74	1	0.77
AMP_BOV_031	RRPARFCPCREETHDVA	17	1	11.5	0.08	2	0.56
AMP_BOV_032	YNSCTCIRPAFCCKCLIPLEQ	21	1	11.0	0.46	5	0.72
AMP_BOV_033	GRAYTKCRLVHDCQPRIPGMKSCRVTNKVSKRHHFWRLCCKRIC	45	12	11.3	0.95	6	0.79
AMP_BOV_034	PPHCIRRTYQIPER	15	3	11.4	-0.13	1	0.77
AMP_BOV_035	TINCGFKPRQRNQRRAGRWDRCRY	26	6	11.3	0.57	2	0.52
AMP_BOV_036	REKHRRPCDDQGRML	17	4	11.7	-0.82	2	0.81
AMP_BOV_037	EMHCRPNHEYCEWRRGCMKCNFPRIKRL	29	4	11.4	-0.86	4	0.63
AMP_BOV_038	TPPRKCAHSQSWCTFP	16	2	10.9	0.2	2	0.58
AMP_BOV_039	CTRERCQNERIWRDCCFDDRCWQLPVRVYHYHLLQSPICLAIGRY	45	3	12.0	0.51	5	0.81
AMP_BOV_040	QMLNSVLSRHMDCCVHR	17	1	11.7	-0.72	2	0.52
AMP_BOV_041	RARYNQIWRRCQHNNIFTYQDCRWHRRCRCRPHCRWYVCRMAH	44	10	11.8	0.28	6	0.71
AMP_BOV_042	HREYDGRMNRRNVQDDQVRCIRY	25	3	11.3	-0.24	1	0.54
AMP_BOV_043	RRFFYRVERPLRRMKDEQTCMIWRMCCDL	30	4	9.6	-0.15	3	0.51
AMP_BOV_044	CVSVWKKIICEHCRHDAWLSRLQVAEDWQKRFQFQCTM	39	2	9.5	0.91	5	0.82
AMP_BOV_045	AMFDKQFKRCYCNMYNQLQYFARHRQRHCHNSDRLRQSCRLGCECW	46	7	9.6	0.65	6	0.86
AMP_BOV_046	KSLARGTCRGLSLKWHERISGCKKYDGCGLRLDLRVQLQRCRK	44	9	11.7	0.62	4	0.66
AMP_BOV_047	SRWDQACRIIPQRWIRPNKRCRMIFFLYGVMTLVLCTH	44	8	10.2	-0.88	4	0.52
AMP_BOV_048	NGGDRRDIEYSFMPL	16	-1	11.2	-0.84	0	0.82
AMP_BOV_049	CVCKRMKKPCHCLNCSNPRCMTSNGDWFVWNGCEWQQCPDPQVHWA	47	2	9.2	0.45	8	0.84
AMP_BOV_050	CWRFEPAVANRGF	13	1	9.2	-0.2	1	0.63
AMP_BOV_051	GCCLMEQHRRTYCIPLHFGCGIQFTDHPHRMMHAWM	37	1	9.9	0.35	4	0.65
AMP_BOV_052	EICMVRLNTKVRRIKPCENRGYCYK	27	6	10.1	-0.68	3	0.57
AMP_BOV_053	NIDQKAARSNDVCWHKQTPMMMLAHCKGVTQP	33	2	11.6	-0.2	2	0.74
AMP_BOV_054	YRRPDPQQTDRAWCAHDRKRWTRCCEH	28	4	11.8	0.69	3	0.55
AMP_BOV_055	NHDVHIRRRRQEFNEYGGERHHWSGCRISWSGGRHLMENW	42	2	9.9	0.67	1	0.78
AMP_BOV_056	CQFPRNQCKRCRANWKVVMVEYHRVCPNRW	29	6	11.6	0.99	4	0.58
AMP_BOV_057	KTWATRERTGAGQWHLQNVCMGS	23	2	10.7	0.73	1	0.65
AMP_BOV_058	DLFERPRDVTSMCRECHFFRWGRARRCCGKM	33	5	11.6	0.47	4	0.67
AMP_BOV_059	RQWIEWMLYIRVSSRRPRNNIWCVENDTL	31	3	10.8	-0.44	1	0.64
AMP_BOV_060	RMVLCKPPRGAFACKRNCVYLG	25	3	9.1	-0.18	4	0.74
AMP_BOV_061	RYRKYITVIRNRHDCNNREA	20	5	10.7	-0.93	0	0.52
AMP_BOV_062	YLRKIFLDEVRR	13	2	10.9	-0.72	0	0.71
AMP_BOV_063	FCPLRTFCLNRR	13	3	11.4	-0.26	3	0.57
AMP_BOV_064	FVPPRSFVYNKGNPRDRNCRINRKGENFF	30	5	11.2	0.5	1	0.7
AMP_BOV_065	SVPYNCFERWTCREGRQTER	21	2	9.9	0.46	2	0.79
AMP_BOV_066	EEVGVEERRRVRKRFHGLRHIVLSRRHEHCTRANPRGEFFPNWHMR	47	7	10.9	0.31	1	0.57
AMP_BOV_067	LRICNEVYWNTCSACQCDELQWVRANKARNRGMKRWDFNQF	43	2	11.7	0.25	6	0.8
AMP_BOV_068	GKIKPYFRTRTRCINACMLRPTCNCEVTMCCWV	34	4	10.5	-0.67	6	0.53
AMP_BOV_069	RDTAEDSSWGPPTNRIRRCRHCFQDPYHQLFNIPIRGFCGIRDI	48	4	11.0	-0.74	4	0.53
AMP_BOV_070	CAGIGCELNKAICPHIGICVVYPDICGMCLKMCFF	35	0	11.0	0.67	7	0.69
AMP_BOV_071	PEGWQHSHSTYCHWEHYGRMPFISKPPNRNWLRLGRSSK	43	4	10.8	0.34	3	0.87
AMP_BOV_072	CGFDKILPPNWTKAYRSPVCFRDQPRYHSCCERNREILRDY	43	5	11.9	0.32	4	0.74
AMP_BOV_073	CKYCWDHLEMLRHGSCTRDYDFYFKEQKAQEPPIRSC	42	0	9.9	0.4	4	0.57
AMP_BOV_074	SNIECRMYHIKCLDRSW	18	1	9.2	-0.86	2	0.79
AMP_BOV_075	CCCGDRMKCIEQRRYNMCHLCIACSYMMRKTMCARGGNDLRNCCRSIS	49	7	10.7	-0.59	10	0.82
AMP_BOV_076	RLEKRRFTAFTCTC	14	3	10.8	-0.74	2	0.57

5. Conclusion

This study demonstrates the effectiveness of a structured *in silico* pipeline for identifying novel antimicrobial peptides from the bovine transcriptome. By integrating machine learning prediction, sequence validation, and functional classification tools, we were able to uncover 76 promising AMP candidates that exhibit key structural and physicochemical traits associated with antimicrobial activity. Many peptides shared motifs and homology with known bovine AMPs, such as cathelicidins and β -defensins, suggesting a likely role in innate immunity. These findings not only contribute to the growing database of host defense peptides in cattle but also provide a foundation for the development of peptide-based alternatives to antibiotics in livestock health. Further *in vitro* and *in vivo* validation is essential to confirm their therapeutic potential, but this approach offers a scalable, species-specific strategy for discovering effective and sustainable antimicrobial solutions.

6. Acknowledgements

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