

Supplementary Materials

Discovery of potent inhibitors of rumen methane metabolism via an AI-assisted workflow

Xiong Xia, Liangzhen Zheng, Jinjie Zhou et al.

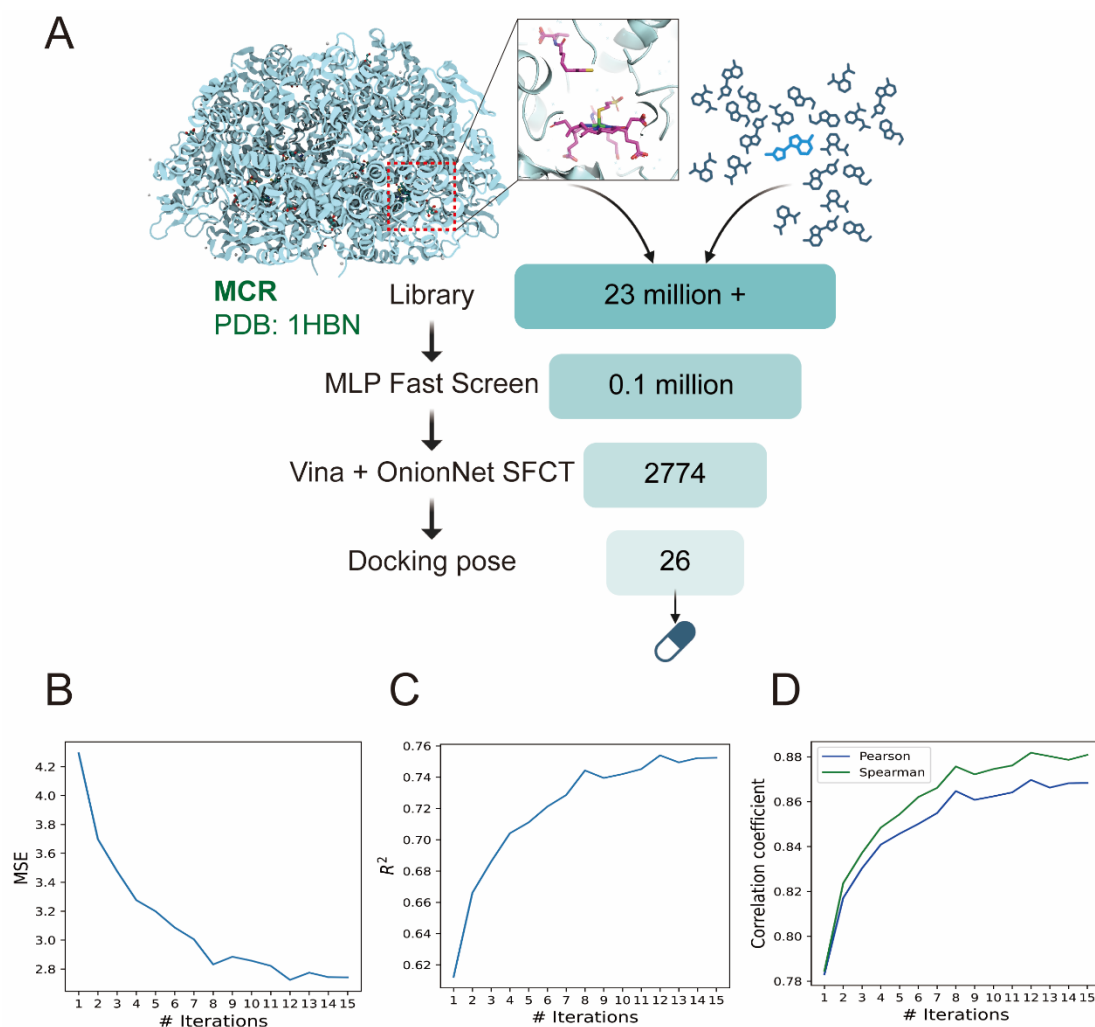


Figure S1. Workflow of AI-based ultra-fast virtual screening and model performance.

A) Workflow of AI-assisted ultra-fast virtual screening targeting MCR complex (PDB: 1HBN). Created in BioRender. Xia, X. (2027) <https://core.local.biorender.dev/api/short-link/6d0orqp>.

B-D) Changes in model performance across training iterations evaluated by mean squared error (MSE), Correlation determination, Pearson correlation coefficient and Spearman's correlation coefficient ρ .

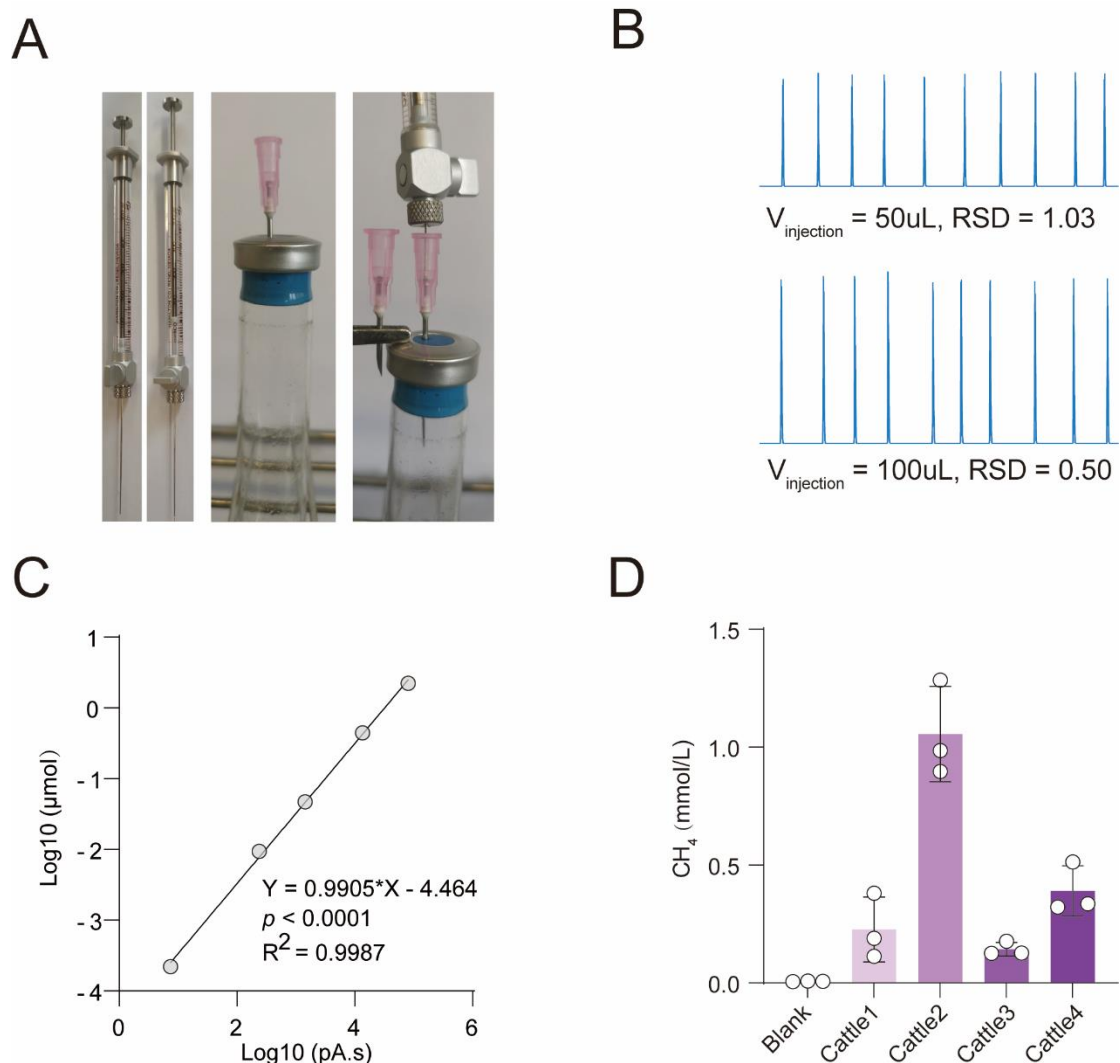


Figure S2. Methane detecting based on gas-tight syringes and manual injection.

A) Operation of the gas-tight syringe. An 18 G needle was inserted halfway into the stopper to serve as a guide, preventing bending of the Hamilton needle during insertion.

B) Assessment of manual injection repeatability. Chromatograms of 10 consecutive injections of standard methane gas at two injection volumes are shown. Manual injection exhibited reproducibility comparable to automated systems, with peak area precision (RSD < 1.5%).

C) Calibration curve for methane quantification.

D) Methane production after 72 h of *in vitro* fermentation of rumen microbiome from different cattle. Data are presented as mean $\pm$ SEM (n = 3 biological replicates).

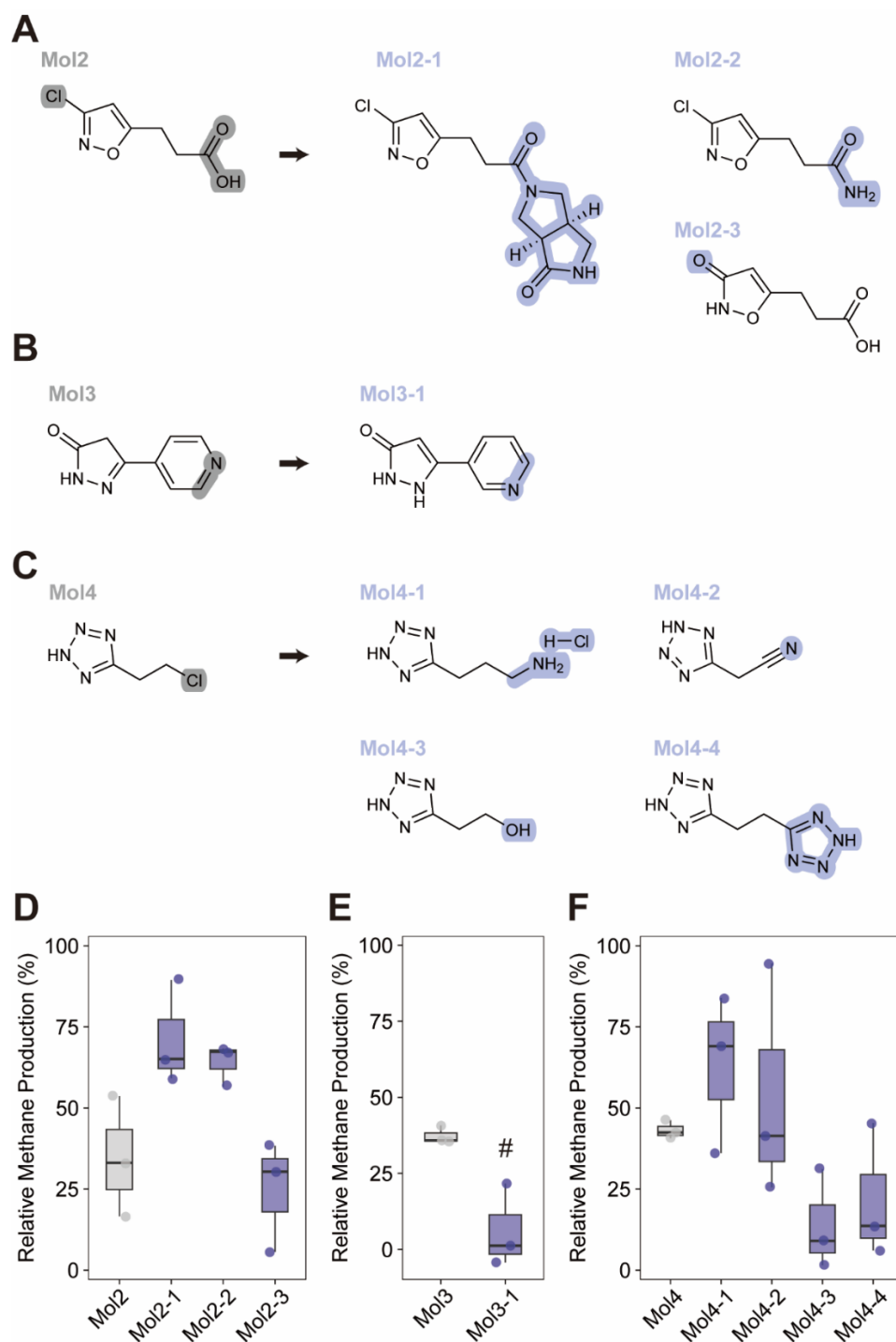


Figure S3. Optimization of hit compounds identified by virtual screening.

A–C) Rational structural optimization of Mol2, Mol3, and Mol4. Gray represents the group planned for modification, and purple indicates the modified group.

D–F) Effect of optimized derivatives of Mol2, Mol3, and Mol4 on methane production at 98.31 μ M after 72 h of *in vitro* ruminal microbial fermentation. Statistical significance was determined by t-test comparing to originated molecule group with Bonferroni post hoc test (compared with control, # $p < 0.1$). Data were shown in boxplot, and each dot represents a technical replicate, and assays were performed in triplicate ($n = 3$).

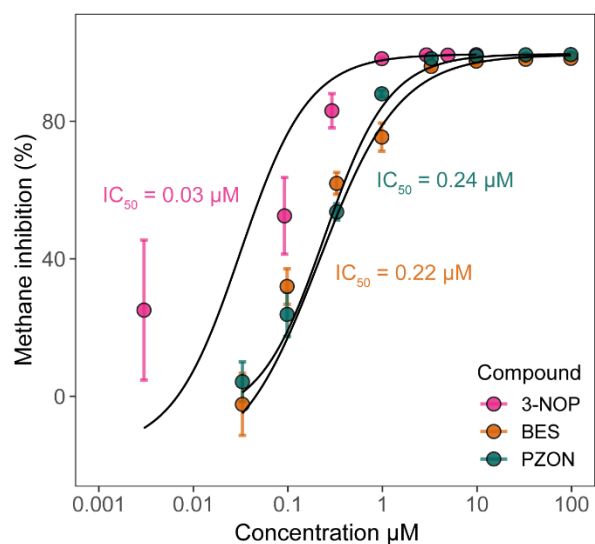


Figure S4. Benchmark comparison of the anti-methanogenic activities of PZON, BES, and 3-NOP under identical experimental conditions. Inhibition data across a range of concentrations were fitted using a four-parameter log-logistic model. Data are presented as mean $\pm$ SEM, based on triplicate assays ($n = 3$).

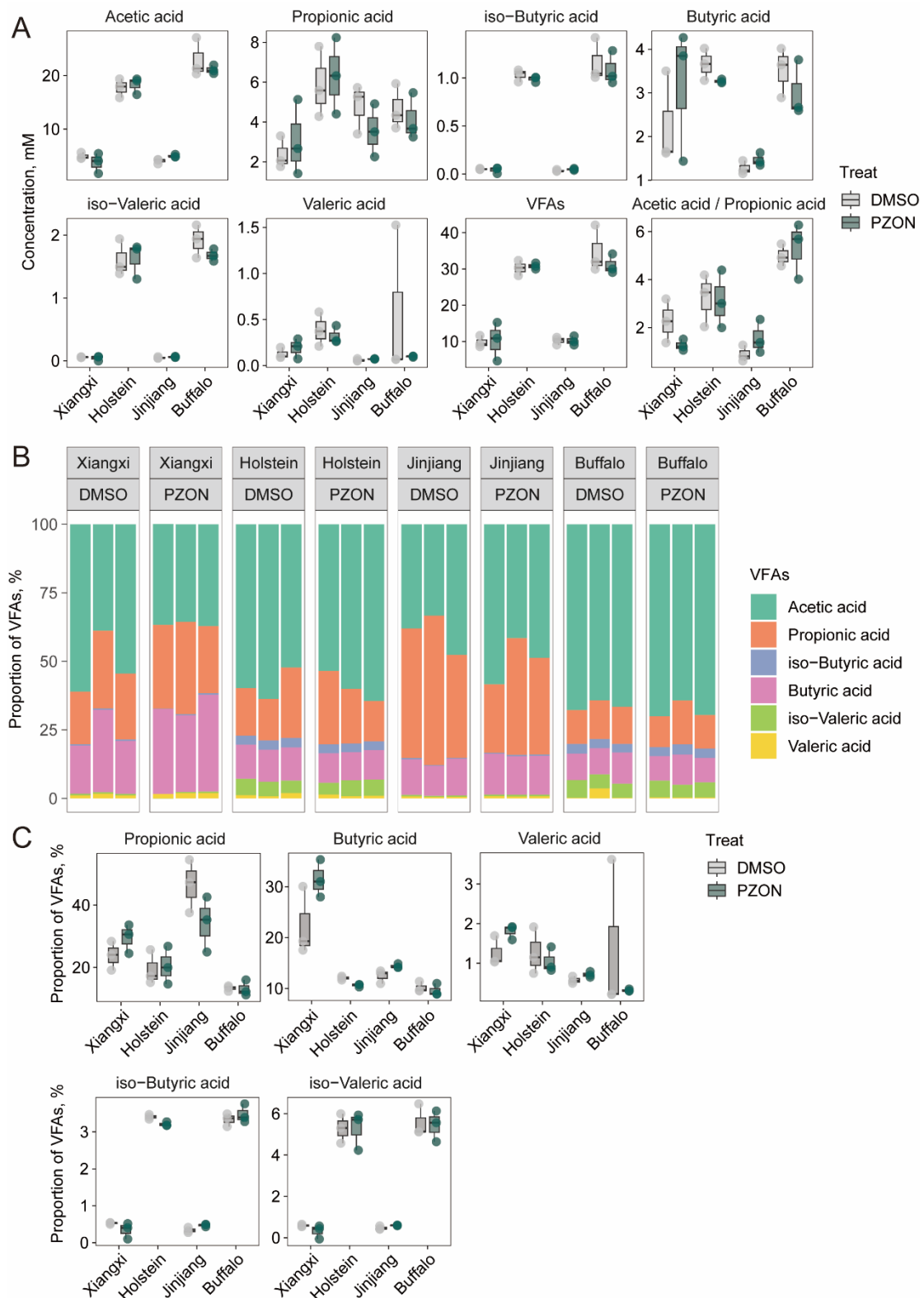


Figure S5. Impact of PZON on the VFAs profile during 72 hours *in vitro* fermentation of rumen microbiome.

A) Concentrations of VFAs and acetate-to-propionate ratio after 72 h.

B) Stacked bar plot showing the relative proportions of individual VFAs after 72 h.

C) Relative proportions of propionate, butyrate, iso-butyrate, iso-valerate, and valerate within total VFAs after 72 h.

Total VFAs comprise acetate, propionate, iso-butyrate, butyrate, iso-valerate, and valerate. Statistical significance of A) and C) were determined by two-way ANOVA with Bonferroni post hoc test under each group. In each panel of A) and C) PZON effect showed $p > 0.05$ comparing to DMSO control (data not show). Data were show in boxplot, and each dot represents a technical replicate, and assays were performed in triplicate ($n = 3$).

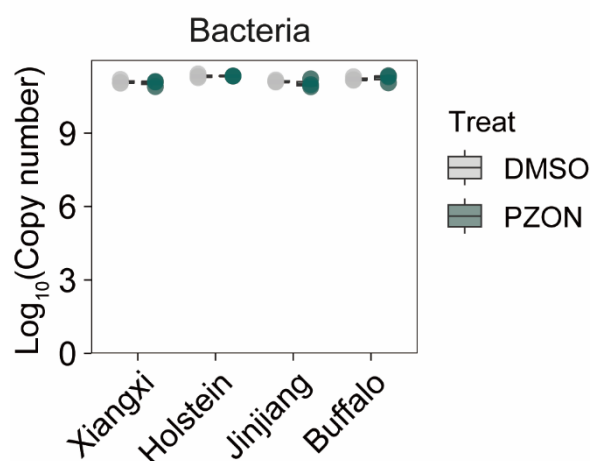


Figure S6. Absolute abundance of bacteria in cultures of rumen microbiome for 72 h. Absolute abundance was estimated using the qPCR assay. Statistical significance was determined by two-way ANOVA with Bonferroni post hoc test under each group. PZON effect showed $p > 0.05$ comparing to DMSO control (data not show). Data were show in boxplot, and each dot represents a technical replicate, and assays were performed in triplicate ($n=3$).

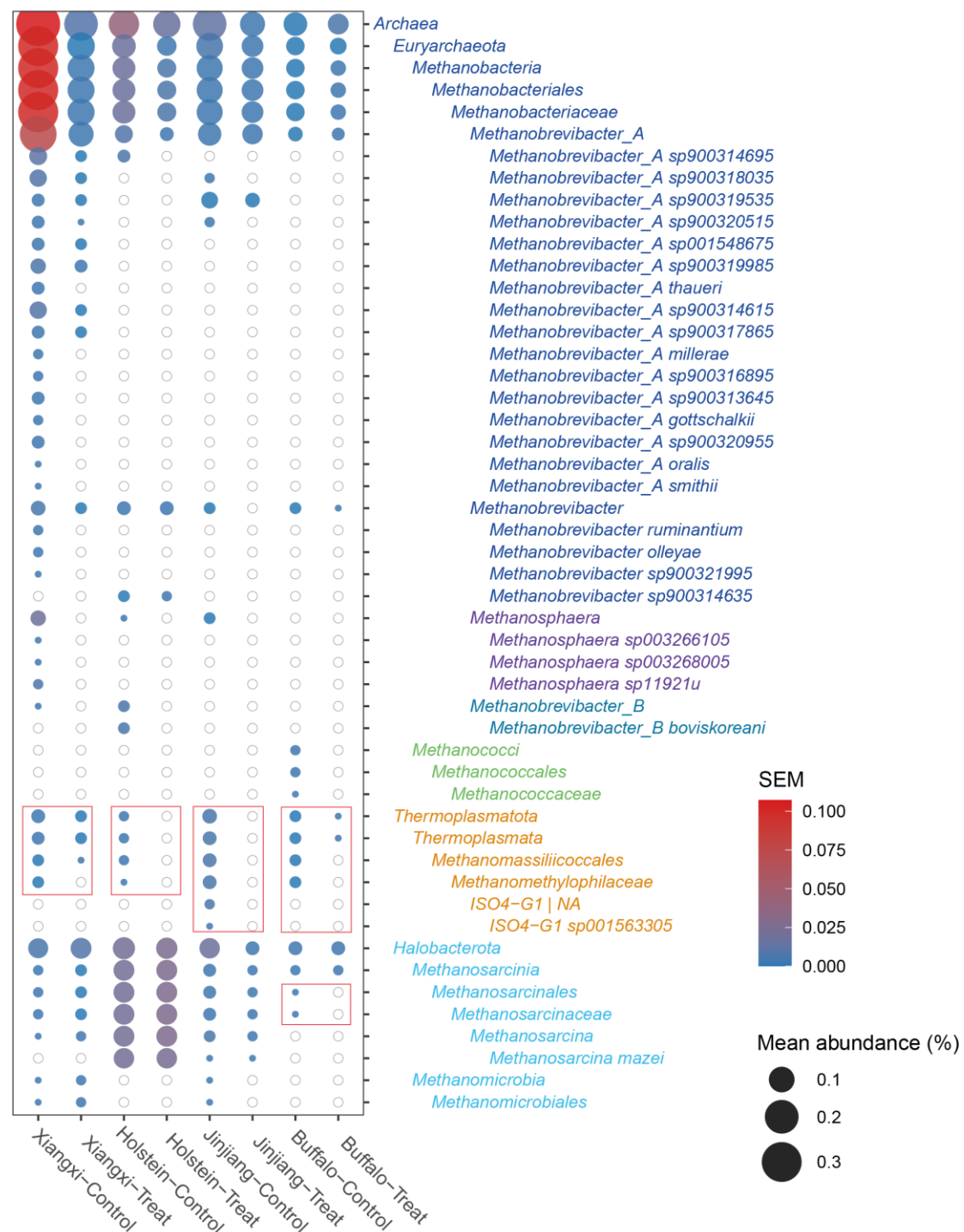


Figure S7. Distribution of methanogenic archaeal taxa across four independent rumen microbial communities under control and PZON-treated conditions. Bubble plot showing the relative abundances of methanogenic archaeal taxa identified by metagenomic sequencing in four independent rumen microbial communities (Xiangxi cattle, Holstein cattle, Jinjiang cattle and Buffalo) following 72 h of in vitro fermentation in the absence (Control) or presence (Treat) of 2.95 μ M PZON. The archaeal communities comprised multiple methanogenic lineages, including hydrogenotrophic (*Methanobrevibacter_A*, *Methanobrevibacter*, *Methanosphaera* and *Methanococcaceae*), methylotrophic (*Methanomassiliicoccales*), and low-abundance acetoclastic (*Methanosarcinaceae*) methanogens, demonstrating that PZON was evaluated in complex rumen communities containing diverse methanogenic taxa. Each column represents one experimental

group of metagenomic samples ($n = 3$ biological replicates per treatment), and each row represents an archaeal taxonomic lineage. Bubble size corresponds to the mean relative abundance of each archaeal taxon; hollow circles indicate taxa with a mean relative abundance of $<0.01\%$. Bubble color represents the corresponding standard error of the mean (SEM) among biological replicates. Red boxes highlight representative methylotrophic and acetoclastic methanogenic archaeal lineages after in vitro fermentation between control and PZON-treated groups.

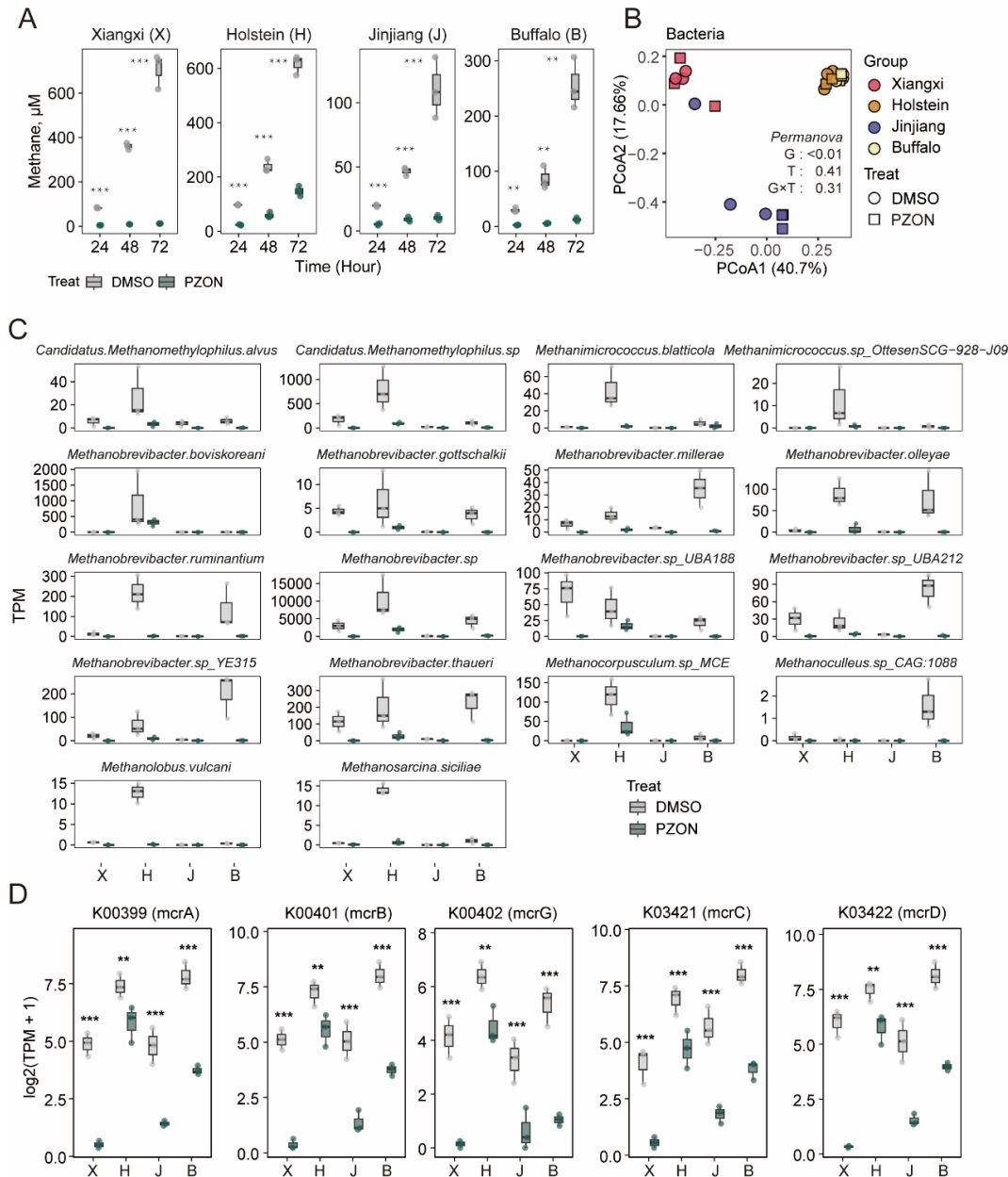


Figure S8. Transcriptomic characterization of microbial responses to targeted MCR inhibition by PZON during *in vitro* rumen fermentation.

A) Methane production during 72 h *in vitro* fermentation of rumen microbiome cultures. Statistical significance was assessed by three-way mixed ANOVA (Treat $\times$ Group $\times$ Hour), followed by Benjamini-Hochberg FDR correction. ** $p < 0.01$, *** $p < 0.001$.

B) Principal coordinate analysis (PCoA) of bacterial community transcriptional profiles based on Bray–Curtis dissimilarities calculated from species-level metatranscriptomic community profiling. PERMANOVA was used to test for treat and group effects.

C) Transcript abundance of the 18 methanogenic archaeal species significantly decreased following PZON treatment identified by MaAsLin2 analysis (corresponding to Fig. 4I). Boxplots show transcript abundance distributions across biological replicates ($n = 3$ per group).

D) Expression of individual MCR subunits. Boxes show median and interquartile range; points show individual samples ($n = 3$). Statistical significance was assessed by three-way mixed ANOVA (Treat $\times$ Group), followed by Benjamini-Hochberg FDR correction. ** $p < 0.01$, *** $p < 0.001$. TPM, transcripts per million.

Table S1. Candidate compounds selected from AI-driven virtual screening and purchased for experimental validation

Compound ID	Composite score (0.8×SFCT + 0.2×Vina)	Vina score (kcal/mol)	SFCT score	Supplier	Catalog No.	CAS	PubChem CID	Molecular weight (Da)	SMILES / InChI
Mol1	2.148	-7.1	4.459	Chembridge	4011499	1185508-90-0	25220854	175.2	<chem>Nc1[c][c][c]c(C2=NNC(=O)[C]2)[c]1</chem>
Mol2	1.843	-5.7	3.729	ChemDiv	Y509-9032	80403-82-3	715815	175.6	<chem>OC(=O)CCc1cc(Cl)no1</chem>
Mol3	2.081	-6.5	4.226	ChemDiv	8011-7794	101421-76-5	13268957	161.2	<chem>O=C1[C]C(c2[c][c]n[c][c]2)=NN1</chem>
Mol4	2.111	-4.2	3.689	ChemDiv	8015-2124	18755-46-9	1381993	132.6	<chem>ClCCc1nn[nH]n1</chem>
Mol5	1.946	-5.1	3.707	Enamine	Z959893238	98021-37-5	12200478	138.1	<chem>NC(=O)c1ccc(N)nn1</chem>
Mol6	2.366	-3.3	3.783	Targetmol	T0022	107-35-7	1123	125.1	<chem>NCCS(O)(=O)=O</chem>
Mol7	1.847	-5.1	3.584	Vitas-M	STK606830	75989-19-4	3161864	157.1	<chem>O=C(O)CCc1cc(O)no1</chem>
Mol8	2.062	-4.8	3.778	ChemDiv	Y507-1790	18595-97-6	2060087	142.2	<chem>Nc1nc(CCCO)n[nH]1</chem>
Mol9	2.068	-7.9	4.561	ChemDiv	Y509-9260	92712-48-6	706933	214.2	<chem>FC(F)(F)c2cccc(c1nn[nH]n1)c2</chem>
Mol10	1.865	-4.8	3.532	Targetmol	T5246	2583-24-6	53440600	144.1	<chem>O=C(O)[C][C]/[C]=[C]C(=O)O</chem>
Mol11	1.917	-6	3.896	MCE	HY-32820	17556-41-1	11148246	194.1	<chem>C1=CC(=CC=C1C(C(F)(F)F)O)F</chem>
Mol12	1.971	-5.9	3.938	Enamine	Z3300803809		76221257	181.2	<chem>NC(=O)/C=[C]/c1[c][c]c(C(=O)O)o1</chem>
Mol13	1.958	-4.3	3.522	Enamine	EN300-7586972	55947-46-1	23673600	142.1	<chem>[Na+].[O-]S(=O)(=O)CC#C</chem>
Mol14	2.069	-5.2	3.887	Enamine	Z1205656971	100508-42-7	23440167	142.1	<chem>O=C(O)CCc1nn[nH]n1</chem>
Mol15	1.956	-5.9	3.92	Enamine	Z90667813	50907-20-5	333943	164.1	<chem>Fc2cccc(c1nn[nH]n1)c2</chem>
Mol16	2.134	-6.1	4.193	ChemDiv	8019-4527	119192-12-0	14443415	176.2	<chem>Oc2ccc(Cc1nn[nH]n1)cc2</chem>
Mol17	1.644	-5	3.305	MCE	HY-Y1139	111-16-0	385	160.2	<chem>OC(=O)CCCCC(O)=O</chem>
Mol18	1.73	-4.8	3.362	Enamine	Z119989852	618-30-4	234853	146.5	<chem>OC(=O)c1ccc(Cl)o1</chem>
Mol19	1.644	-5	3.305	MCE	HY-W005241	6338-41-6	80642	142.1	<chem>OCc1ccc(o1)C(O)=O</chem>
Mol20	1.711	-5.3	3.463	ChemDiv	R092-0037	585-70-6	68511	191.0	<chem>OC(=O)c1ccc(Br)o1</chem>
Mol21	1.869	-5.6	3.736	ChemDiv	Y507-1507	169213-73-4	10877483	166.1	<chem>OCc1cc(n[nH]1)C(F)(F)F</chem>
Mol22	1.803	-5.4	3.604	Vitas-M	STL294800	80242-24-6	11804811	136.1	<chem>OC(=O)c1cc(c[nH]1)C#N</chem>
Mol23	1.914	-5.3	3.717	Vitas-M	STL311978		135635370	155.1	<chem>OC(=O)C=Cc1nc(O)n[nH]1</chem>
Mol24	1.871	-5.1	3.614	ChemDiv	0325-0352	456-22-4	9973	140.1	<chem>OC(=O)c1ccc(F)cc1</chem>
Mol25	1.857	-5.5	3.696	Vitas-M	STL383205	937-27-9	11846406	155.1	<chem>OC(=O)c1ccc([nH]1)C(O)=O</chem>
Mol26	1.81	-5.6	3.663	Chembridge	4034053	1185320-28-8	45791217	155.2	<chem>CNC(=O)c1ccc(CO)o1</chem>

Notes, Screening criteria for the 26 candidate compounds:

Stage 1: Composite scoring. All molecules were ranked using a combined score of OnionNet-SFCT (weight 0.8) and AutoDock Vina (weight 0.2), following the weighting scheme

recommended in the original OnionNet-SFCT publication (Liangzhen Zheng, et al, Improving protein–ligand docking and screening accuracies by incorporating a scoring function correction term, Briefings in Bioinformatics, 2022, bbac051). Compounds with a composite score below 2.5 were retained.

Stage 2: Visual inspection of binding poses. For each compound passing the scoring threshold, the predicted binding pose with the target (MCR and coenzyme F430) was examined. Only molecules forming stable polar interactions at both termini of the ligand with the receptor and the cofactor were selected for experimental validation.

Table S2. Compounds selected from structure-based optimization and purchased for experimental validation

Compound ID	Supplier	Catalog No.	CAS	PubChem CID	Molecular weight (Da)	SMILES / InChI
Mol1-1	Chembridge	5994275	54945-13-0	609542	205.2	<chem>[O-][N+](=O)c1ccc(cc1)C1=NNC(=O)C1</chem>
Mol1-2	Advanced library	Z56174893		4039462	176.2	<chem>Oc1ccccc1C1=NNC(=O)C1</chem>
Mol1-3	Vitas-M	STK508807	264208-45-9	4778792	178.2	<chem>Fc1ccc(cc1)C1=NNC(=O)C1</chem>
Mol2-1	Chembridge	29975242		163308015	283.7	<chem>Clc1cc(CCC(=O)N2C[C@@H]3CNC(=O)[C@@H]3C2)on1</chem>
Mol2-2	Vitas-M	STL251611		56723819	174.6	<chem>NC(=O)CCc1cc(Cl)no1</chem>
Mol2-3	Vitas-M	STK606830	75989-19-4	3161864	157.1	<chem>OC(=O)CCc1cc(O)no1</chem>
Mol3-1	Vitas-M	STK687807	90280-24-3	5302800	161.2	<chem>Oc1cc(n[nH]1)-c1cccnc1</chem>
Mol4-1	Advanced library	Z1407007328	273400-20-7	68476498	163.6	<chem>Cl.NCCc1nnn[nH]1</chem>
Mol4-2	Vitas-M	STK689874	13616-36-9	10820485	109.1	<chem>N#CCc1nnn[nH]1</chem>
Mol4-3	Vitas-M	STK722211	26670-16-6	141316	166.2	<chem>C(Cc1nnn[nH]1)c1nnn[nH]1</chem>
Mol4-4	Vitas-M	STK682545	17587-08-5	285162	114.1	<chem>OCCc1nn[nH]n1</chem>

Table S3. Primers used for quantitative PCR

Primer pair ID	sequence	purpose
uniMet1-F	CCGGAGATGGAACCTGAGAC	Quantitative determination for methanogen
uniMet1-R	CGGTCTTGCCCAGCTCTTATTC	
16S-331F	TCCTACGGGAGGCAGCAGT	Quantitative determination for bacteria
16S-797R	GGACTACCAGGGTATCTAATCCTGTT	