Supporting Information

Effects of the co-formulated antibiotics on sludge anaerobic digestion: transformation, microbial stress response, and community evolution

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Support information includes the following:

Number of Pages: 14

Number of Figures: 6

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Additional Methods

Key enzyme activity determination

Protease activity was quantified following the azocasein hydrolysis method (Goel et al., 1998). Briefly, 3.0 mL of fermentation substrate was mixed with 1.0 mL of 0.5% azocasein solution and incubated at 37°C for 90 min. The reaction was terminated by adding 2.0 mL of 10% trichloroacetic acid. The mixture was centrifuged at 4,500 rpm for 20 min. Subsequently, 2.0 mL of the supernatant was combined with 2.0 mL of 2.0 mol/L NaOH, and absorbance was measured at 440 nm using a spectrophotometer (Cary 60, Agilent Technologies, USA).

α-Glucosidase activity was determined via p-nitrophenyl-α-D-glucopyranoside (pNPG) hydrolysis (Nybroe et al., 1992). A 1.0 mL aliquot of fermentation substrate was mixed with 1.0 mL of 0.1% pNPG and 2.0 mL of 0.2 mol/L Tris-HCl buffer (pH 7.6), followed by incubation at 37°C for 60 min. The reaction was terminated by immersing the sample in a boiling water bath for 3 min. After centrifugation at 4,500 rpm for 20 min, the supernatant absorbance was measured at 410 nm.

Determination of acetate kinase (Allen et al., 1964): A reaction mixture containing 1.5 mL substrate storage solution, 1.75 mL hydroxylamine solution, and 0.5 mL of 0.1 mol/L adenosine triphosphate (ATP) was prepared. Subsequently, 0.75 mL of cell extract and 0.5 mL deionized water were added. The mixture was incubated at 29°C for 30 min, after which 0.5 mL of 10% TCA was added to terminate the reaction. Precipitates were removed by centrifugation (4,500 rpm, 20 min, 4°C). The supernatant was mixed with 2.0 mL of 1.25% $FeCl_3$ solution, and absorbance was measured at 540 nm.

Determination of coenzyme F420 (Delafontaine et al., 1979): A 10 mL aliquot of the fermentation mixture was homogenized with 20 mL of saline (0.85% NaCl) and centrifuged at 6,000 rpm for 15 min. The pellet was resuspended in 20 mL saline, centrifuged again, and finally suspended in 15 mL saline. The sludge was heat-treated at 95°C for 30 min, cooled to room temperature, and mixed with a 5:2 ethanol-to-substrate ratio. After 2 h of precipitation, the mixture was centrifuged at 6,000 rpm (4°C, 15 min). The supernatant pH was adjusted to 13.5 using 4 mol/L NaOH, followed by centrifugation at 10,000 rpm (4°C, 10 min). The solution was divided into two portions: one acidified to pH <3 with 6 mol/L HCl (reference) and the other diluted equivalently with ultrapure water. Absorbance was measured at 420 nm.

The concentration of coenzyme F420 was calculated by the following equation:

$$C = (A_1 - A_0) * \frac{\mathrm{f}}{\mathrm{s}} * L$$

C —— concentration of coenzyme F420 in sludge (mmol/L);

A_1 —— absorbance of sample at 420 nm;

A_0 —— absorbance of the reference sample at 420 nm;

f —— dilution ratio of sample;

L —— thickness of colorimetric dish (cm);

s —— extinction coefficient of F420, (L/(cm · mol)), when pH = 13.5, s = 54.3.

Measurement of reactive oxygen species production and cell viability

The intracellular reactive oxygen species (ROS) generation in anaerobic digested sludge was quantitatively analyzed through dichlorofluorescein (DCF) fluorescence spectrophotometry following established methodology (Jiao et al., 2022). Specifically, 5 mL aliquots of sludge samples underwent centrifugal separation at 10,000 rpm for 10 min, followed by triple washing cycles with 0.1 M phosphate buffer (pH 7.4). The resultant biomass pellets were subsequently reconstituted to initial volume using H2DCF-DA-supplemented phosphate buffer (50 μM final concentration) and subjected to dark-phase incubation at 35 ± 1°C for 30 min. Post-incubation centrifugation (10,000 rpm, 20 min) yielded supernatant removal, with subsequent resuspension in fresh buffer for fluorescence quantification via microplate spectrofluorometry. The emission wavelength is 485 nm and the emission wavelength is 520 nm.

Parallel cellular viability assessments were performed through CCK-8 colorimetric bioassays. Triplicate 15 mL sludge aliquots underwent sequential phosphate buffer (0.01 M, pH 7.4) washing prior to CCK-8 reagent incubation (10μL/well, 35 ± 1°C, 30 min). Metabolic activity quantification was achieved through optical density measurements at 450 nm (reference wavelength 600 nm) employing calibrated microplate photometry. Control cohort measurements established baseline ROS production levels and cellular viability indices, serving as reference values for experimental comparisons.

EPS extraction method

A heat extraction method was employed to extract extracellular polymeric substances (EPS) from activated sludge. The process began with centrifuging a sludge suspension in a 50 mL tube at 4,000 g for 5 minutes. Subsequently, the sludge pellet was diluted with preheated NaCl solution (70 °C) to restore its original volume of 50 mL. The diluted sludge suspension underwent sonication for 1 minute, followed by centrifugation at 4,000 r/min for 10 minutes. The organic matter present in the supernatant was considered as the loosely bound

EPS (LB-EPS) of the biomass. The remaining sludge pellet was resuspended in NaCl solution to its original volume of 50 mL. This suspension was then warmed to 60 °C in a water bath for 30 minutes, after which it was centrifuged at 4,000 r/min for 15 minutes. The resulting supernatant was collected and regarded as the tightly bound EPS (TB-EPS) of the sludge. The EPS content was characterized by analyzing different parameters. Both the LB-EPS and TB-EPS extractions were analyzed for proteins and polysaccharides. Proteins were quantified using an anthrone - based method.

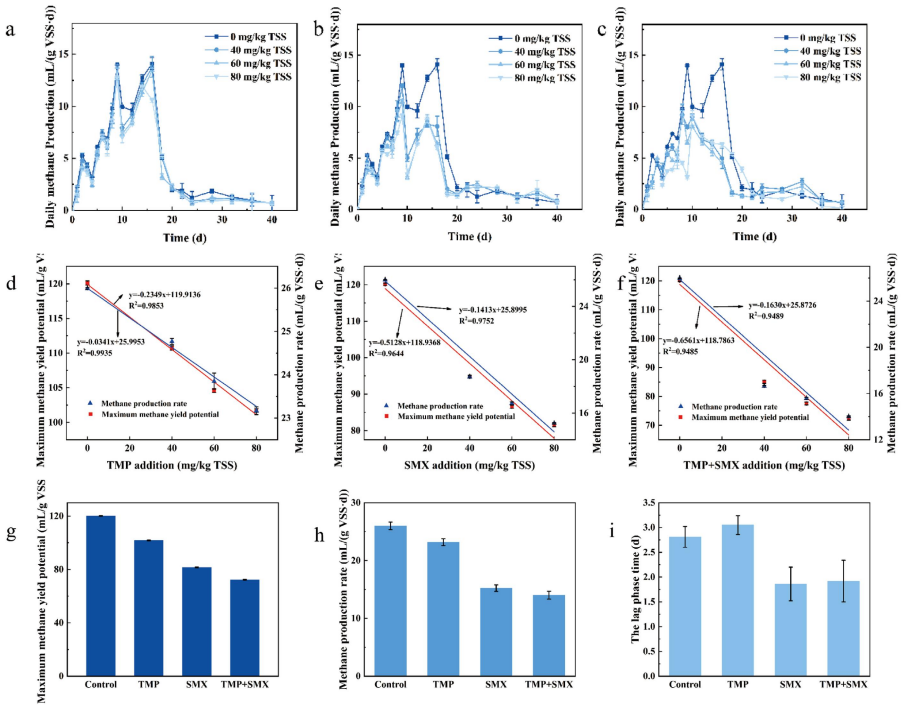

Fig. S1. Impact of TMP (a), SMX (b), and TMP+SMX (c) on daily methane production; correlations of antibiotic concentrations with maximum methane yield potential and production rate (d-f); and comparison of kinetic parameters (g-i).

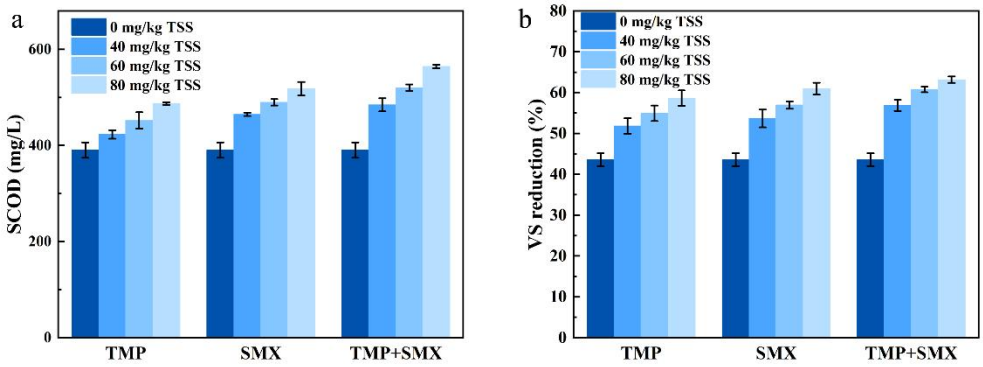

Fig. S2. Effects of antibiotics on SCOD concentration (a) and VS reduction (b) at 3 d

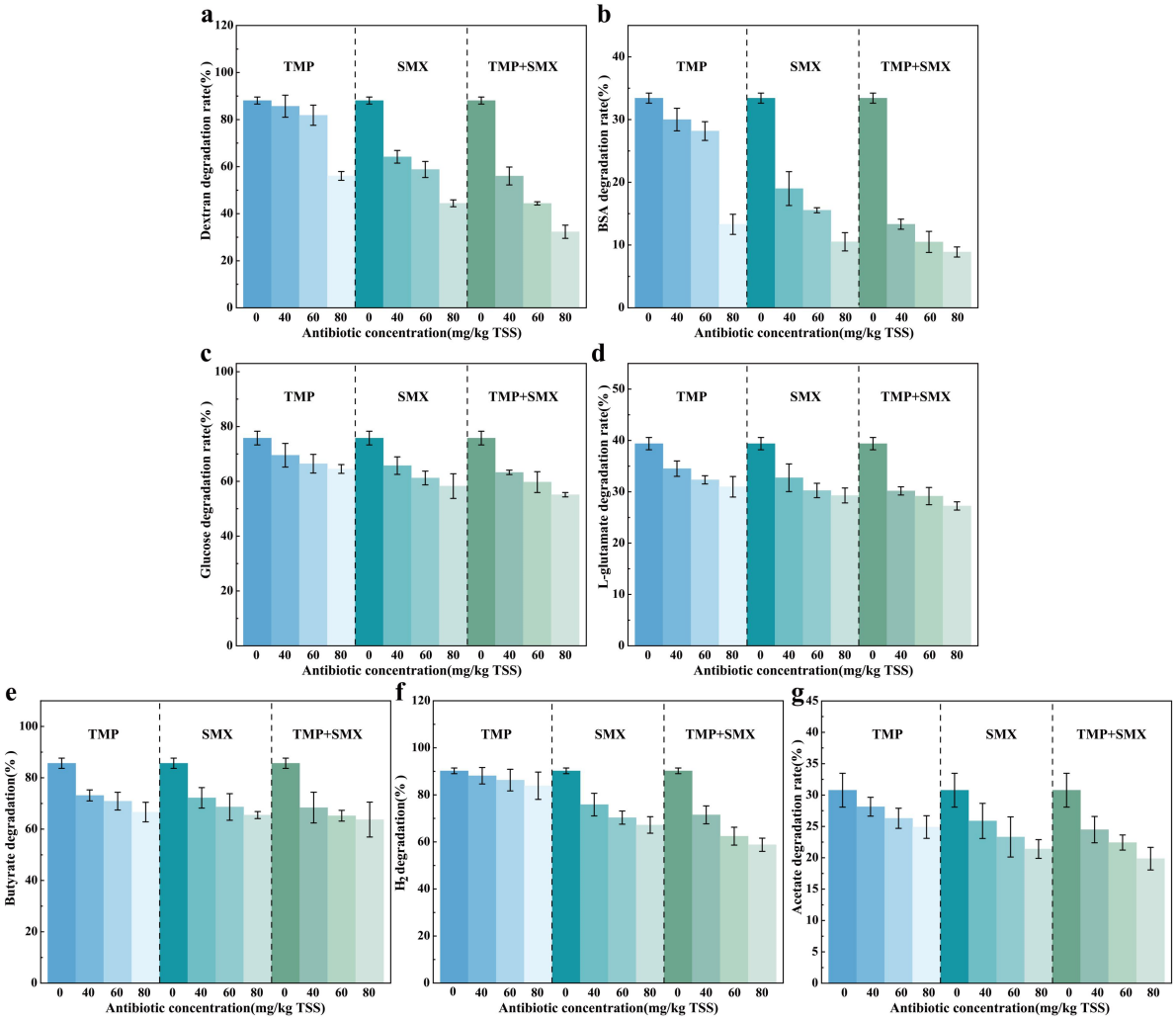

Fig. S3. Effects of TMP and SMX on model substrate degradation of anaerobic digestion: Dextran(a), BSA(b), glucose(c), L-glutamate(d), Butyrate(e), Hydrogen(f), and Acetate (g)

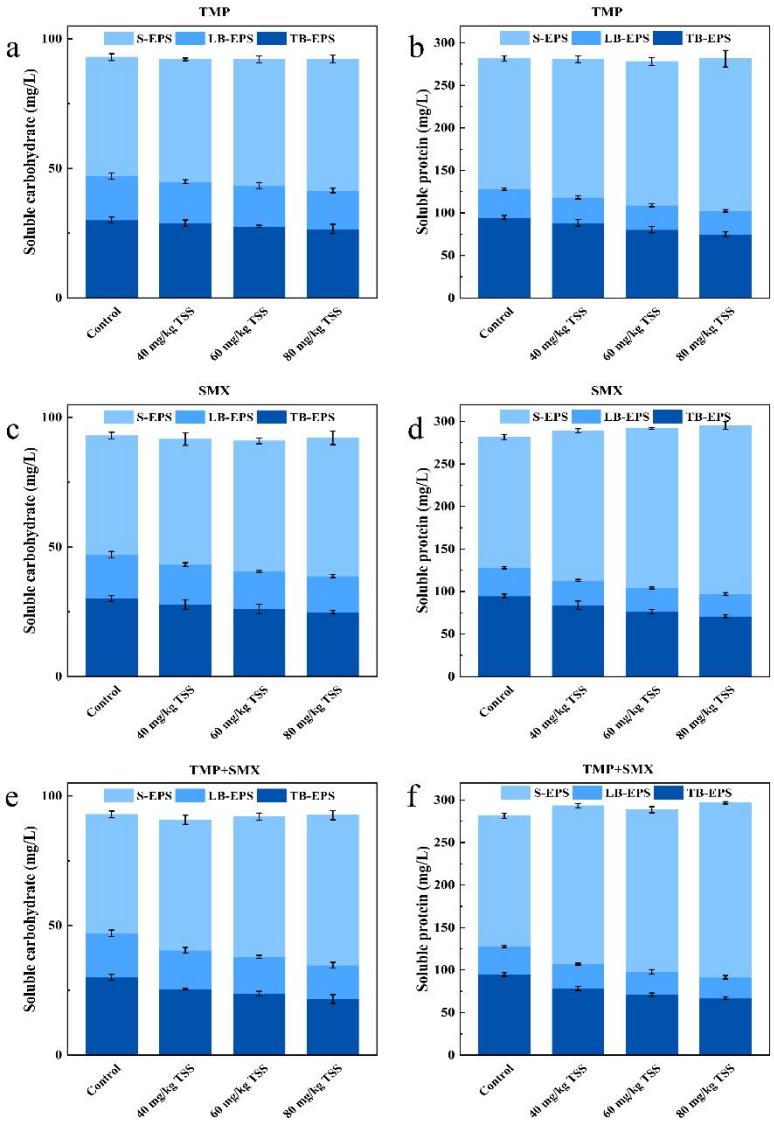

Fig. S4. Soluble carbohydrate concentrations (a)(c)(e) and soluble protein concentrations (b)(d)(f) in the EPS

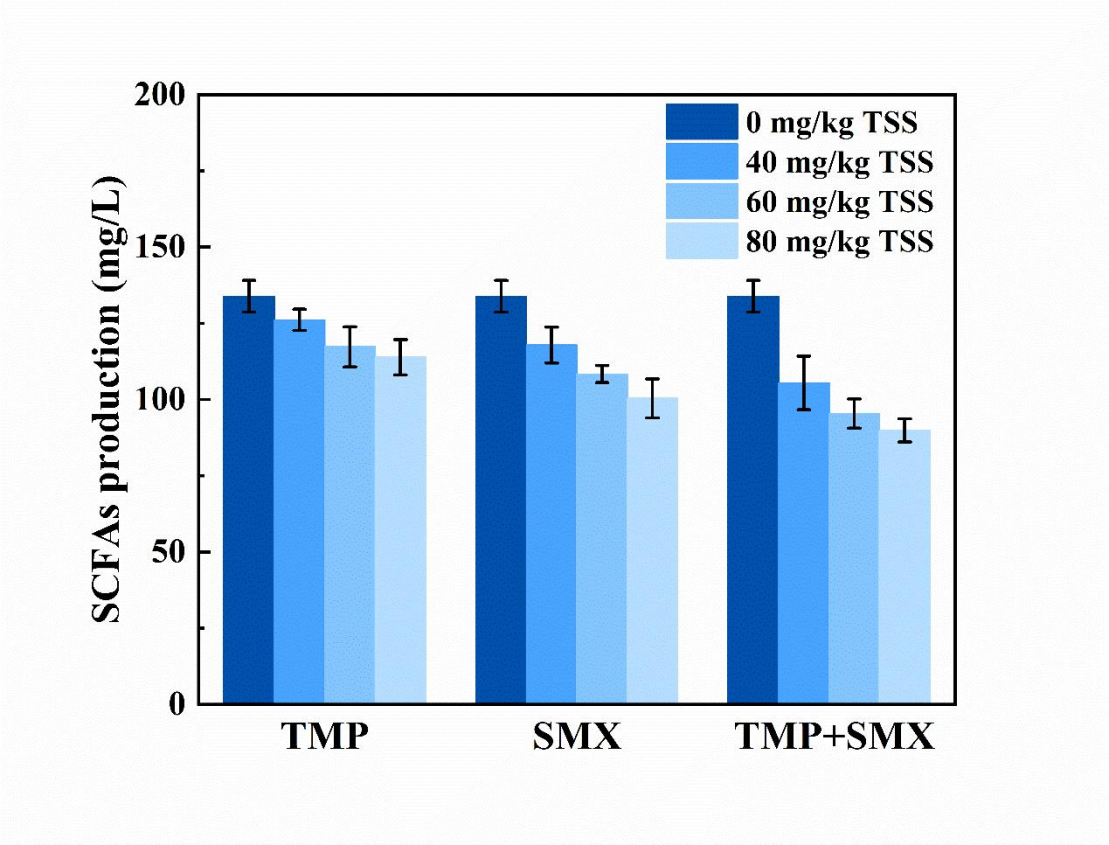

Fig. S5. Effects of TMP and SMX on SCFAs production use glucose and L-glutamate as substrates

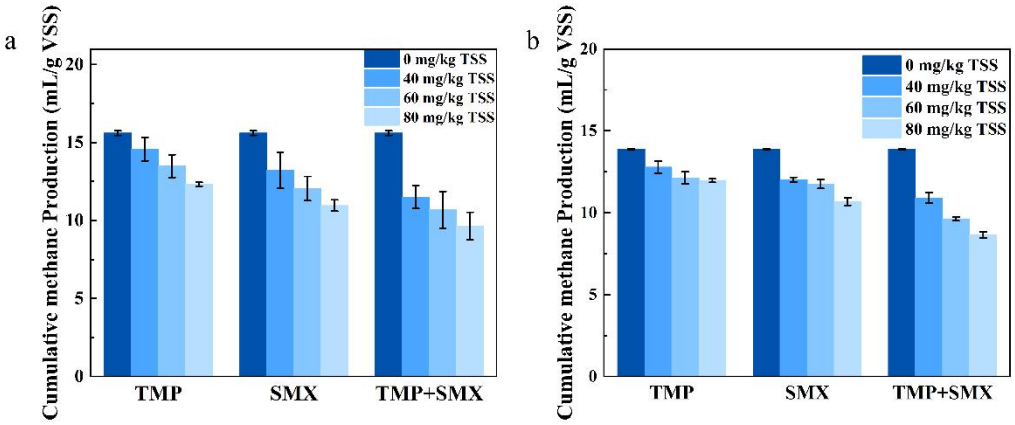

Fig. S6. The cumulative methane production of hydrogenotrophic methanogenesis (a) and acetoclastic methanogenesis (b) process

Table S1 The main properties of WAS and inoculum sludge

Parameter	Unit	WAS	ADS
pH		6.70 ± 0.10	6.70 ± 0.10
TS	g/L	29.90 ± 0.20	19.11 ± 0.12
VS	g/L	13.93 ± 0.21	8.04 ± 0.15
SCOD	g/L	0.43 ± 0.01	0.44 ± 0.01
TCOD	g/L	17.79 ± 0.37	12.72 ± 0.35
Total polysaccharides	g/L	2.84 ± 0.13	1.68 ± 0.12
Total proteins	g/L	8.54 ± 0.52	6.85 ± 0.07

Table S2 Substrates for solubilization, hydrolysis, acidogenesis, acetogenesis, hydrogenotrophic methanogenesis, and acetoclastic methanogenesis tests

Steps	Substrates
Solubilization	250 mL WAS
Hydrolysis	250 mL synthetic wastewater (4.60 g/L Dextran + 3.20 g/L BSA)
Acidogenesis	250 mL synthetic wastewater (4.60 g/L glucose + 3.20 g/L L-alaine)
Acetogenesis	250 mL synthetic wastewater (3.00 g/L sodium butyrate)
Hydrogenotrophic methonogenesis	250 mL WAS + 40% V_{H2}, 10%V_{CO2}, 50%V_{N2}
Acetoclastic methanogenesis	250 mL synthetic wastewater (3.00 g/L sodium acetate)

Table S3 Simulated (using modified Gompertz equation) methane production from anaerobic digestion in the presence of antibiotics at different concentrations.

Antibiotic concentration (mg/kg TSS)	Kinetic model parameters											
	Mm (mL/g VSS)			Rm (mL/ (g VSS·d))			λ (d)			R^2		
	TMP	SMX	TMP+SMX	TMP	SMX	TMP+SMX	TMP	SMX	TMP+SMX	TMP	SMX	TMP+SMX
40	110.7	94.8	85.0	24.7	18.7	16.6	2.9	2.0	1.9	0.9998	0.9997	0.9994
60	104.6	86.5	77.5	23.8	16.7	15.5	3.0	1.9	1.9	0.9998	0.9996	0.9994
80	101.7	81.5	72.3	23.1	15.2	14.0	3.0	1.8	1.9	0.9999	0.9995	0.9991

Table S4 Analysis of microbial population diversity in reactor sludge samples

	Sample	Ace	Chao	Shannon	Simpson	Coverage
Bacteria	Control	953.358	924.541	4.767	0.034	0.998
	TMP	1075.524	1049.775	4.700	0.029	0.998
	SMX	1086.995	1060.307	4.525	0.024	0.996
	TMP&SMX	913.991	892.697	4.363	0.022	0.997
Archaea	Control	31.882	31.333	1.557	0.344	0.999
	TMP	32.535	31.750	1.647	0.305	1.000
	SMX	31.822	35.000	1.648	0.281	1.000
	TMP &SMX	27.672	26.333	1.754	0.256	1.000

Supplementary References

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