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Multiple factors increase the degradation rate of tetracycline in anaerobic digestion

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ABSTRACT

Central composite design was combined with response surface methodology to optimize the process for the degradation of tetracycline by multifactor functioning of the carbon nitrogen ratio (C/N), pH, and temperature. The characteristics of environmental factors, relative abundance of bacteria and archaea, total abundances of tetracycline antibiotic resistance genes (T-ARGs), and degradation rate of tetracycline were determined in the anaerobic digestion (AD) process of the optimal test. The results showed that the optimal conditions of C/N, pH, and temperature were 33.56°C, 7.09°C, and 48.27°C, respectively, with a degradation rate of tetracycline of 96.66%, which was significantly higher than that of the check test and single factor experiment ($P < 0.05$; $P < 0.05$). Adjusting the initial environmental conditions of AD could enhance the degradation rate of tetracycline, not only because it could increase the abundances of microorganisms that degrade macromolecules but also change the main influencing factors of tetracycline degradation in each stage to make the relationship between environmental factors, T-ARGs and bacteria more coordinated, simplify the relationship between antibiotic resistance genes (ARGs) and bacteria, and make the T-ARGs concentrate on dominant bacteria to enhance the degradation of tetracycline.

Keywords: Statistical analysis; Antibiotic resistance genes; Response surface analysis; Metagenomics; Process optimization

1. Introduction

China is a large country for livestock and poultry breeding. Antibiotics are widely used in animal feed as drugs for treating disease in the prevention and promotion of animal growth. According to statistics, the total annual use of antibiotics in China is approximately 210,000 tons, 54% of which is used in livestock and poultry breeding. Most antibiotics cannot be absorbed by animals, and approximately 30%~90% of antibiotics are excreted into the environment in their original form along with faeces [1]. Contamination caused by antibiotics and related antibiotic resistance genes (ARGs) has been a research focus on environmental pollution and human health risks [2]. ARGs have been transferred from the environment to human commensals and pathogens due to the abuse of antibiotics and selection with antimicrobial agents, reducing the therapeutic potential of antibiotics for human and animal pathogens [3]. China actively responds to the global strategy of curbing bacterial drug resistance formulated by the World Health Organization

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and follows the "One Health" strategy to protect the health of people, animals, plants, food, and the environment (air, soil, and water) in a unified way. Therefore, there is great significance to explore the optimal method of antibiotic degradation in China.

At present, the degradation of antibiotic residues in livestock manure mainly adopts two methods: aerobic manure composting [4] and anaerobic digestion (AD) [5]. AD technology was first used to degrade livestock waste to produce energy, a necessary part of China's large and medium-sized farms. Later, researchers found that it could also degrade antibiotics [6]. However, as the application of antibiotics in the aquaculture industry increases, residual antibiotics in livestock faeces will inhibit AD, not only reducing methane-producing energy efficiency but also reducing the degradation of antibiotics, which is more induced in producing more ARGs in the microorganisms of AD [7]. Therefore, it is important to take certain technical measures to eliminate the inhibitory effect of antibiotics on AD and promote the degradation of antibiotics for the comprehensive utilization of AD technology.

Removing the inhibitory effect of antibiotics and improving the efficiency of AD are important to improve the degradation of antibiotics by AD. Adjusting the digestion conditions to promote the degradation of antibiotics and ARGs can change the bacterial community in different AD, especially ARG hosts, and cause the distinct fate of environmental characteristics, which enhances the adaptation of microorganisms to environmental factors and increases the degradation of antibiotics in AD [8,9]. The carbon nitrogen ratio (C/N) reflects the nutrient levels of the digestion substrate, and thus, the digestion system is sensitive to C/N feed [10]. The operational pH affects the digestive progress and products directly. The growth rate of microorganisms is significantly affected by pH changes, while AD microorganisms are very sensitive to temperature that affects hydrogen and methane production and the decomposition of organic material [11]. Thus, the efficiency of AD could be improved by adjusting the C/N, pH, and fermentation temperature of the initial environment in AD, and the initial environmental factors of C/N, pH, and temperature have synergistic effects on the biogas production of AD [11]. However, the synergistic effect of C/N, pH, and temperature on the degradation of tetracycline by AD and the relationship between environmental factors related to the performance of AD and the degradation rate of tetracycline have not been studied.

Therefore, the optimal degradation range of tetracycline by the C/N ratio, pH, and temperature was determined by a single-factor experiment. On this basis, the degradation process by the synergistic effect of the C/N ratio, pH, and temperature was optimized. The cellulose activities, volatile fatty acid (VFA) content, reducing sugar content, pH, and degradation rate of antibiotics were determined, and metagenomic analysis was used to analyse the community of bacteria and archaea and the abundance of tetracycline antibiotic resistance genes (T-ARGs) in the AD process. A network model, simple correlation analysis, factor analysis, and path analysis were used to study the degradation mechanism of AD by the synergistic action of the C/N ratio, pH, and temperature. The results can provide the best process for AD to degrade antibiotics and the theoretical basis for the study of the mechanism of antibiotic degradation.

2. Materials and methods

2.1. Materials for the test

Tetracycline (Tc, purity ≥97.5%) was purchased from Dr. Ehrenstorfer, Germany. Fresh pig manure and biogas slurry were collected from an ecological farm in Huafeng Town (Shunqing District, Nanchong City, Sichuan Province). No antibiotics were added to the fecal diet of the sampled pigs, and no antibiotics were given to the pigs within one month. The biogas slurry was taken from the pig manure fermentation pond of this farm, and no antibiotic contamination was found. The properties of pig manure and biogas slurry are shown in Table 1.

2.2. Experimental design

2.2.1. Single factor fermentation test

Glucose and urea were used to adjust the C/N [12], hydrochloric acid and sodium hydroxide were used to adjust the pH of the initial fermentation environment [13], and constant temperature was used to control the fermentation temperature in an incubator. Among them, the C/N ratio of the initial fermentation environment was 10, 20, 25, 30, 35, 40, 45 and 50 (the addition amount of glucose and urea was shown in Table 2). The pH value of the initial fermentation environment was adjusted as 4, 5, 6, 7, 8, 9, and the fermentation temperature was controlled as 25°C, 30°C, 35°C, 40°C, 45°C, and 55°C. In the single factor of C/N test, the initial pH was the natural pH of the substrate and the fermentation temperature was 25°C. In the single factor of pH test, the C/N was the natural C/N of the substrates and the fermentation temperature was 25°C. And in the single factor of temperature test, the C/N and pH were natural C/N and natural pH of the substrates.

2.2.2. Central composite design

Central composite design (CCD) is a mathematical method used to analyze the relationship among variable

Table 1
Chemical characterization of substrates used in the digestion experiments

Material	Pig manure	Inoculum
TOC[a] (%)	23.51 ± 0.32	36.53 ± 0.32
TKN[a] (%)	1.45 ± 0.02	1.59 ± 0.27
C/N	15.86 ± 0.22	20.01 ± 0.42
TS (%)	23.2 ± 0.2	8.51 ± 0.09
VS (%)	87.97 ± 0.7	70.35 ± 0.6
Cellulose[a] (%)	18.44 ± 0.2	ND[b]
Hemicellulose[a] (%)	13.07 ± 0.1	ND[b]
Lignin[a] (%)	10.82 ± 0.1	ND[b]

TOC, TKN, TS, and VS are abbreviations of total organic carbon, total Kjeldahl nitrogen, total solids, and volatile solids, respectively;
[a]Means dry weight basis;
[b]Means not determined.

Table 2
Specific addition amounts of glucose and carbamide

C/N ratio	Urea addition (g)	Glucose addition (g)
10	4.73	0
20	0	3.632
25	0	23.591
30	0	43.550
35	0	63.509
40	0	83.468
45	0	103.427
50	0	123.386

factors that is well suited for multi-factor tests and thus applied widely [14]. Because CCD can divide the factors into two subsets, one subset estimates the linear relationship and interaction of the two factors and the other estimates the effect of the response surface. This method also creates a wealth of information on the factors and experimental error [14]. In this study, CCD was applied to design the experimental conditions involving three factors that were C/N, pH and temperature. The single factor experiment is the climbing experiment of the CCD. Therefore, the results of the single-factor experiment were used to design the CCD. Where –1, 0 and 1 represent the minimum, intermediate and maximum values of single-factor experiments, respectively (Table 3). The control methods for adjusting C/N, pH and fermentation temperature were the same as the single-factor experimental design. Three replicates were set during the experiment.

The response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables on the response [15] This method is known as regression analysis and is used to obtain data, estimate parameters and establish relationships between test indexes and continuous variables [16]. The results and second-order polynomial coefficients were analyzed to optimize degradation rate of tetracycline in CCD.

2.3. AD method

A total of 56 g total solids (TS) of pig manure and 200 g inoculum were placed in a 1 L Erlenmeyer flask, and pure water was added to a total mass of 700 g, assuring that the TS content was 8%. Then 7 g of tetracycline was added to each AD flask so that the concentration of tetracycline in the AD was at 10% level, and set up a check test (without adding tetracycline, CK). AD was carried out under the experimental design C/N, pH and temperature by single factor experimental design and CCD for 35 d. And the AD device was designed independently by the Research Center for Recycling Agricultural Engineering [17], it is shown in Fig. 1. Three blank controls were operated to remove endogenous methane from the sludge [18,19].

2.4. Analysis methods

The polymerase chain reaction was used to determine the concentration of tetracycline, and the detailed

Table 3
The code of CCD

Number	Code		
	A	*B*	*C*
1	1	–1	1
2	0	0	1
3	1	0	0
4	–1	–1	–1
5	0	–1	0
6	–1	1	1
7	–1	0	0
8	–1	1	–1
9	1	–1	–1
10	0	0	0
11	0	0	0
12	–1	–1	1
13	0	0	0
14	0	0	0
15	0	0	0
16	1	1	–1
17	0	0	0
18	0	1	0
19	0	0	–1
20	1	1	1

A, *B* and *C* represent C/N, pH and fermentation temperature, respectively.

implementation method was referred to the instructions [20]. The metagenomic library-based technique was used to analyse the abundant of bacteria, archaea and T-ARGs in limited liability company of Sangon biological (Shanghai). The NCBI was used to align the genes of bacteria and archaea, and the parameter setting refers to Jiao et al. research [21]. The ARGs contained in the genomes in AD liquid were compared in the ARGs database (the Comprehensive Antibiotic Research Database, CARD), and then tetracycline antibiotic resistance genes (T-ARGs) were screened out [22].

TS, volatile solids (VS), VFA, and total Kjeldahl nitrogen were performed according to APHA Standard Methods [23]. The total organic carbon (TOC) was described in Cuetos [24]. The pH was measured by Model DLGA-1000 (Infrared Analyzer; Dafang, Beijing, China). The cellulose activities and reductive sugar content contents were determined as per the method described by Qi et al [25]. All samples were collected in triplicates, and the averages of the three measurements are presented.

2.5. Data analysis

The results were analyzed using the analysis of variance (ANOVA). The ANOVA, factor analysis and basic data of the path analysis were generated with IBM SPSS Statistics (Version 21, IBM Inc. 2012) software. The RSM was performed based on CCD using Design Expert Software (Version 8.0, Stat-Ease Inc., US). The network was analysed

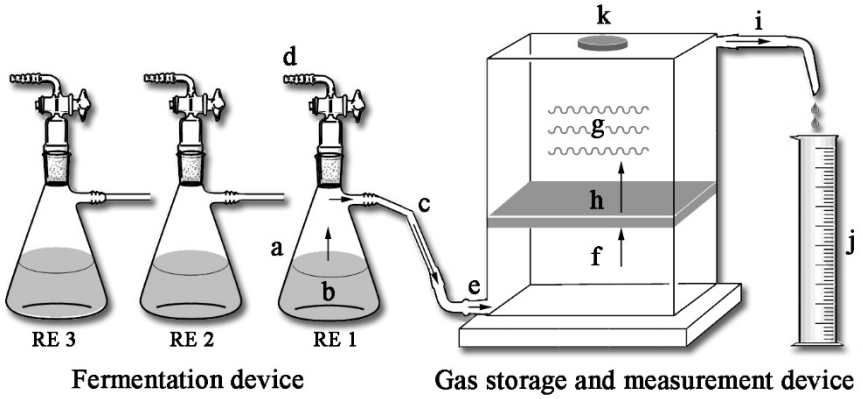

Fig. 1. Controlled constant temperature AD device. (a) Digester; (b) Biogas fluid and substrates; (c) Airway tube; (d) Taking biogas and sampling; (e) Air intake; (f) Biogas collecting bottle; (g) Water; (h) Piston; (i) Aqueduct; (j) Water collecting and measuring cylinder; (k) Water inlet.

by Gephi-0.9.2 and R 3.0, and the heat map of correlation analyse by R 3.0.

3. Results and discussion

3.1. Cooperation of multi-factors on the degradation of tetracycline

Suitable C/N and pH and temperature could significantly improve the degradation of tetracycline by AD ($P < 0.5$). When the C/N ratio, pH and temperature was 35°C, 7°C and 45°C in their single factor experiments, the degradation rate of tetracycline was the highest, their maximum degradation rate was 93.27%, 92.34% and 93.21%, respectively, all of them was significantly higher than that of CK ($P < 0.05$) (Fig. 2). According to the results of single factor experiment, the optimal ranges of C/N ratio, pH and temperature were 25°C–40°C, 6°C–8°C and 35°C–55°C, respectively, and they were designed in CCD. The RSM was used to optimize the result of CCD, the model of relationship of tetracycline degradation rate and C/N, pH and temperature is shown in Eq. (1):

$$Y = -21.2039 + 1.9833A + 17.29134B_2 + 0.97751C_3 + 0.0001667AB + 0.004833AC + 0.060375BC - 0.03315A^2 - 1.42455B^2 - 0.0163C^2 \quad (1)$$

where A is the C/N ratio, B is the pH, C is the fermentation temperature, and Y is the degradation rate of tetracycline. The analysis of variance of the regression equation shows that the regression equation model was extremely significant (Table 4). The regression equation model ($R^2 = 0.9219$) showed a high degree of fit, and the regression equation model could be used to study the effect of multi-factor synergism on the degradation of antibiotics by anaerobic fermentation. The interaction effect of the model is shown in Fig. 2.

The optimization results showed that the C/N, pH and temperature of the optimal fermentation conditions were 33.56°C, 7.09°C, and 48.27°C, respectively, and the degradation rate of tetracycline was 96.66%, which was higher than that of CK ($P < 0.05$) and the single factor tests ($P < 0.05$). To verify the model, 33.5°C, 7.0°C, and 48°C C/N, pH and temperature of AD were carried out to degrade tetracycline, and the experimental results showed that the tetracycline degradation rate was 97.03%, which was not significantly different from the predicted results ($P > 0.05$). It was further proven that the RSM method could accurately predict and analyze the actual tetracycline degradation efficiency. Appropriate C/N could improve the utilization of substrates by microorganisms in the AD system, and an appropriate initial pH value could improve the acid-base balance in the process of AD, while an appropriate fermentation temperature improved the activity of microorganisms in the process of AD and promote the growth of microorganisms, which indicated that adjusting the C/N and pH of AD substrates and controlling the temperature in the process of AD can improve the activity of microorganisms and increase their adaptability to the environment in different ways [11]. Therefore, the synergistic effect of C/N, pH and temperature could significantly improve the degradation of tetracycline by AD ($P < 0.05$).

3.2. Cooperation of multiple factors on the characteristics of environmental factors

The tendencies of degradation rate of tetracycline in the AD process of CK and optimal test (OPT) were almost the same, and the degradation rate of tetracycline in the first 5 d was the highest, which was significantly higher than that in the following stages ($P < 0.05$). The antibiotic degradation rate of OPT for 0–5 d and 6–10 d in AD was significantly higher than that of CK ($P < 0.05$), but no significant difference was observed in the later stage of fermentation ($P > 0.05$). The relative abundances of all kinds of T-ARGs are shown in Table 5. In other words, after adjusting the initial fermentation environment, the tetracycline degradation rate of OPT differed from that of CK in the early stage, because antibiotics were also degraded by hydrolytic action in the AD process. The initial fermentation environment treated by OPT conditions was not only conducive to tetracycline degradation by microorganisms in the hydrolysis stage but also promoted the hydrolysis of tetracycline.

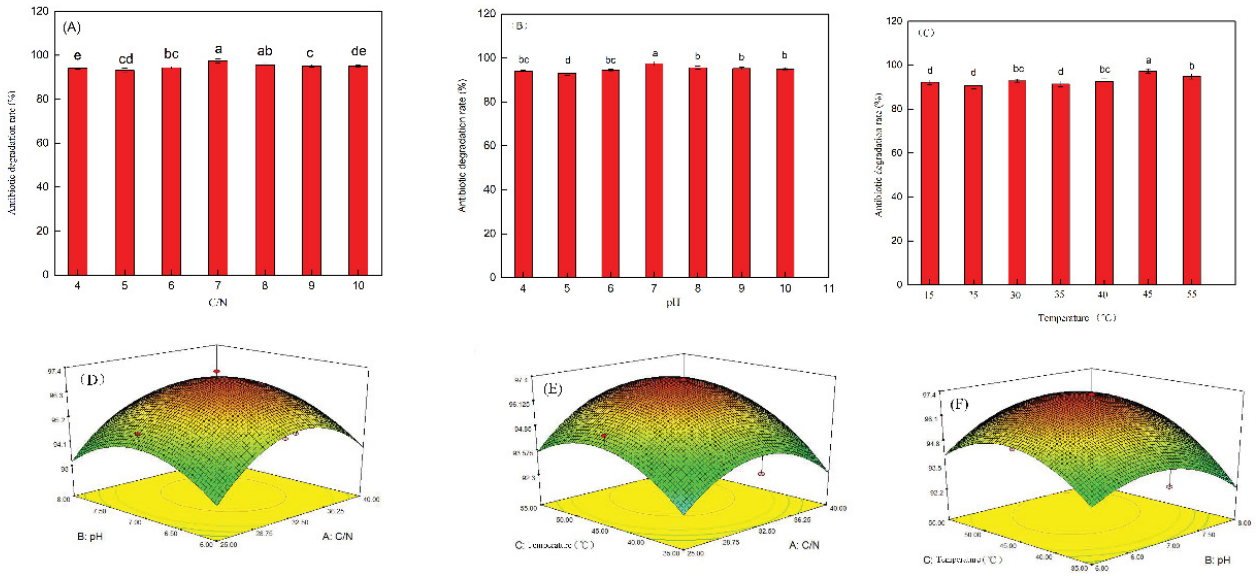

Fig. 2. Effects of C/N, pH and fermentation temperature on the degradation of tetracycline by anaerobic fermentation. (A–C) show the effect of C/N, pH and temperature on degradation rate of tetracycline, respectively. (D–F) represent the interaction effect of pH and C/N, C/N and temperature, temperature and pH, respectively.

Table 4
Analysis of variance of CCD model

Source	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	1.04E+02	9	1.15E+01	13.12	0.0002
A–A	1.65	1	1.65	1.88	0.2007
B–B	0.049	1	0.049	0.056	0.8181
C–C	9.12	1	9.12	10.38	0.0091
AB	1.25E-05	1	1.25E-05	1.42E-05	0.9971
AC	1.12	1	1.12	1.27	0.2857
BC	2.92E+00	1	2.92	3.32	0.0985
A^2	9.56	1	9.56	10.88	0.008

A, *B* and *C* indicate C/N, pH and fermentation temperature.

The variation characteristics of T-ARGs abundances in OPT and CK were different during AD. The T-ARGs abundance reached a maximum on the 25th day in CK, while it appeared on the 5th day in OPT (Fig. 3). Antibiotics could induce microorganisms to produce ARGs, and a suitable initial fermentation environment could accelerate the production of ARGs during AD. Previous studies also proved that measures to promote the degradation of antibiotics would increase the abundance of ARGs ($P < 0.05$). Thus, the maximum abundance of T-ARGs appeared in OPT earlier and was significantly higher than that of CK.

On the 5th day of AD, the cellulase activity and reducing sugar content were lower than those of the initial fermentation environment in CK, while they were higher than those of the initial fermentation environment in OPT. Subsequently, the cellulase activity and reducing sugar content increased gradually in CK, while those in OPT gradually decreased. On the 25th day of AD, the change trend of cellulase activity and reducing sugar content remained the same in OPT (Fig. 3). The results showed that adjusting the initial fermentation environmental conditions had a great influence on the cellulase activity and the reducing sugar content in the initial stage of AD because tetracycline degradation was accelerated by adjusting the initial environment, the inhibitory effect of antibiotics was reduced, and the start-up of AD was accelerated. In the early stage of fermentation, cellulase activity increased, cellulose decomposed the fermentation substrate to produce reducing sugars, and the content of reducing sugars increased [26]. With the progress of the reaction, the content of reducing sugars decreased because they were decomposed into methane and carbon dioxide. Then, the content of reducing sugar produced tended to remain stable with its decomposition rate; thus, the reducing sugar content remained stable, and the cellulase activity also remained stable [27].

The minimum pH value appeared on the 15th day of AD in CK, while it appeared on the 5th day in OPT and was earlier than that of CK (Fig. 3). Adjusting the initial fermentation conditions increased the cellulase activity and reducing sugar content in the early stage of fermentation, resulting in the early decomposition of macromolecular substances into small molecular acidic substances, and thus the acidogenic stage occurred in advance in AD [28]. The content of VFA on the 5th day of AD in OPT was higher than that of CK. Our results indicate that the synergistic effect of factors has a great influence on the environmental factors in the acidification stage of AD, so the degradation rate of antibiotics in the acidification stage in the OPT gap away from CK.

3.3. Cooperation of multiple factors on the characteristics of microorganisms

The relative abundance of Proteobacteria and Fibrobactere in OPT was significantly higher than that in CK (Fig. 4; $P < 0.01$). Phascolarctobacterium is a fermentation bacterium at the hydrolysis stage that could degrade organic matter. *Pusillimonas* decompose lipids and is also the main hydrolytic bacteria in the process of AD [29]. The relative abundances of Phascolarctobacterium and *Pusillimonas* in OPT were significantly higher than those in CK ($P < 0.01$; $P < 0.05$). Our results indicated that adjusting the initial fermentation environment could remove the inhibition of tetracycline on the decomposition of cellulose

Table 5
Relative abundance of T-ARGs during AD process

Treatments	Relative abundance of T-ARGs (%)	Days				
		5	10	15	25	35
CK	tet	0.003967	0.003442	0.002413	0.002099331	0.001487562
	tet30	0	0	1.76E-06	3.8314E-05	7.35863E-06
	tet31	0	7.55E-06	7.62E-06	7.20099E-06	0
	tet32	0.0073	0.008076	0.005173	0.002558026	0.0048096
	tet33	8.39E-05	0.000127	0.000336	2.70787E-05	5.88203E-05
	tet34	0.000361	0.000668	0.002535	0.001432471	0.000501315
	tet36	0.004525	0.004233	0.003624	0.006873877	0.011277148
	tet37	0.036383	0.034234	0.025417	0.006656824	0.013694394
	tet38	8.56E-05	7.9E-05	0.000419	0.00010576	0.000317513
	tet39	0.001434	0.001276	0.00607	0.000185357	0.000246638
	tet40	0.00738	0.004721	0.003065	0.001086432	0.001187039
	tet41	9.96E-05	8.85E-05	0.000393	0.000484884	5.22781E-05
	tetA	0.000269	0.000284	0.002786	0.0026015	0.002437579
	tetB	6.41E-06	2.18E-05	0.000596	0.001160016	0.00107788
	tetC	0.000647	0.000413	0.00109	0.002030111	0.001231429
	tetD	0	8.18E-06	9.43E-05	8.32844E-05	7.25953E-05
	tetE	7.57E-06	5.8E-06	0.000279	7.9122E-06	9.65879E-05
	tetG	8.65E-05	8.77E-05	0.001219	0.006981182	0.004960071
	tetH	4.94E-06	4.42E-05	0.000166	0.000112735	3.30658E-05
	tetK	8.67E-05	0.00014	0.000158	0.000140298	8.05335E-05
	tetL	0.002617	0.002083	0.001573	0.001323024	0.000911143
	tetM	0.005421	0.004797	0.003937	0.004779613	0.005451387
	tetO	0.01132	0.00983	0.005664	0.002308545	0.005078703
	tetPA	0.000546	0.000882	0.003296	0.001475796	0.00212593
	tetPB	0.04757	0.043775	0.033397	0.019270386	0.026006801
	tetQ	0.04198	0.045029	0.029667	0.00884205	0.015795276
	tetS	0.002911	0.002498	0.001322	0.000383552	0.003502815
	tetT	0.032057	0.028515	0.025823	0.025732421	0.031261164
	tetV	0.00015	0.000243	0.000244	0.000264084	3.11216E-05
	tetW	0.053463	0.044625	0.030345	0.008896672	0.013124507
	tetX	0.001366	0.001769	0.001405	0.0014946	0.000431587
	tetY	4.02E-05	4.3E-05	0.000105	8.03673E-05	1.83075E-05
	tetZ	1.86E-05	3.04E-05	0.000166	0.000240723	6.42459E-05

(Continued)

Table 5 Continued

Treatments	Relative abundance of T-ARGs (%)	Days				
		5	10	15	25	35
OPT	tet	0.00196	0.002388	0.002521	0.004541609	0.001480888
	tet30	6.96E-05	0	5.56E-05	0	0
	tet31	2.98E-05	0.000174	3.06E-05	3.5559E-06	0
	tet32	0.005035	0.00544	0.004806	0.006637798	0.006166533
	tet33	0.000352	4.77E-05	0.000155	0.000272791	2.45604E-05
	tet34	0.001185	0.0019	0.00157	0.00059923	0.00161139
	tet36	0.002595	0.009987	0.016094	0.007301448	0.006823993
	tet37	0.019425	0.023932	0.027234	0.030470907	0.018547219
	tet38	0.000716	0	0.000391	0.000405837	9.82982E-06
	tet39	0.001457	0.007116	0.001135	0.003659412	0.000199632
	tet40	0.003177	5.93E-05	0.001279	0.007608715	0.001354893
	tet41	0.000164	0.000826	0.000235	0.000320573	9.42698E-05
	tetA	0.000353	0.001109	0.000261	0.000911715	0.001398303
	tetB	0.000142	0.000149	0.00075	4.20838E-05	0.000253693
	tetC	0.00067	0.001786	0.000952	0.000749437	0.000598124
	tetD	3.19E-05	4.04E-06	1.18E-05	1.87992E-06	0.000670413
	tetE	0.000171	1.26E-05	0.000456	3.10722E-05	5.18146E-06
	tetG	0.000625	0.004287	0.001994	0.000339235	0.001934917
	tetH	0.00017	0.000448	0.000129	6.0303E-05	0.000131479
	tetK	0.000326	0	6.04E-05	7.74484E-05	4.05E-06
	tetL	0.001248	0.000131	0.000767	0.00015656	0.000299977
	tetM	0.006051	0.011051	0.008353	0.006730568	0.011091184
	tetO	0.005007	0.009172	0.008304	0.008828161	0.005251209
	tetPA	0.003971	0.00111	0.002275	0.000995683	0.000459068
	tetPB	0.030288	0.04069	0.036541	0.039067465	0.022197748
	tetQ	0.018606	0.007585	0.011996	0.025044277	0.014052748
	tetS	0.001231	0.001624	0.002153	0.003037932	0.000987431
	tetT	0.019029	0.028567	0.036744	0.034430201	0.026958327
	tetV	0.000373	8.33E-05	0.000102	0.000234376	7.54989E-05
	tetW	0.024005	0.015392	0.021029	0.039525649	0.018842659
	tetX	0.000549	0.000815	0.001079	0.001940875	0.000791868
	tetY	0.000161	0.000333	0.000123	0.000140799	0.000132906
	tetZ	0.000144	0.000338	0.00016	0.000138174	4.29612E-05

and enhance the utilization of glucose, acetate, propionate and butyrate, reinforce the hydrolysis of macromolecular substances by hydrolytic microorganisms in the process of AD, and then promote subsequent fermentation.

Bathyarchaeota, Korarchaeota, and Lokiarchaeota are the dominant archaea in the fermentation process. The relative abundances of Bathyarchaeota, Thorarchaeota, Euryarchaeota, Methanosaeta, and Methanosphaerula in OPT were significantly lower than those in CK ($P < 0.05$). The results showed that tetracycline does not inhibit the growth of archaea in AD. Archaea has special components of the cell wall and cell membrane; their cell wall contains unique pseudopeptidoglycan, and the cell membrane contains unique ether bonds and branched lipid chains, which could help them resist pressure from extreme environments [30].

3.4. Driving factors for cooperation of multiple factors on the degradation rate of tetracycline

3.4.1. Driving effect of environmental factors during AD process

Factor analysis can explore the effect of environmental factors on the performance of AD [31]. According to the extraction method, in which the initial eigenvalue is greater than 1, the OPT extracted three common factors, while CK extracted two common factors. The three common factors in OPT could explain 98.342% of the information of cellulase activity, VFA content, reducing sugar content, pH, T-ARGs, bacterial diversity and archaea diversity, while the two common factors extracted by CK only explain 78.504% of these factors (Table 6). F_1 was determined by cellulase activity, VFA content, reducing sugar content, pH and archaea diversity,

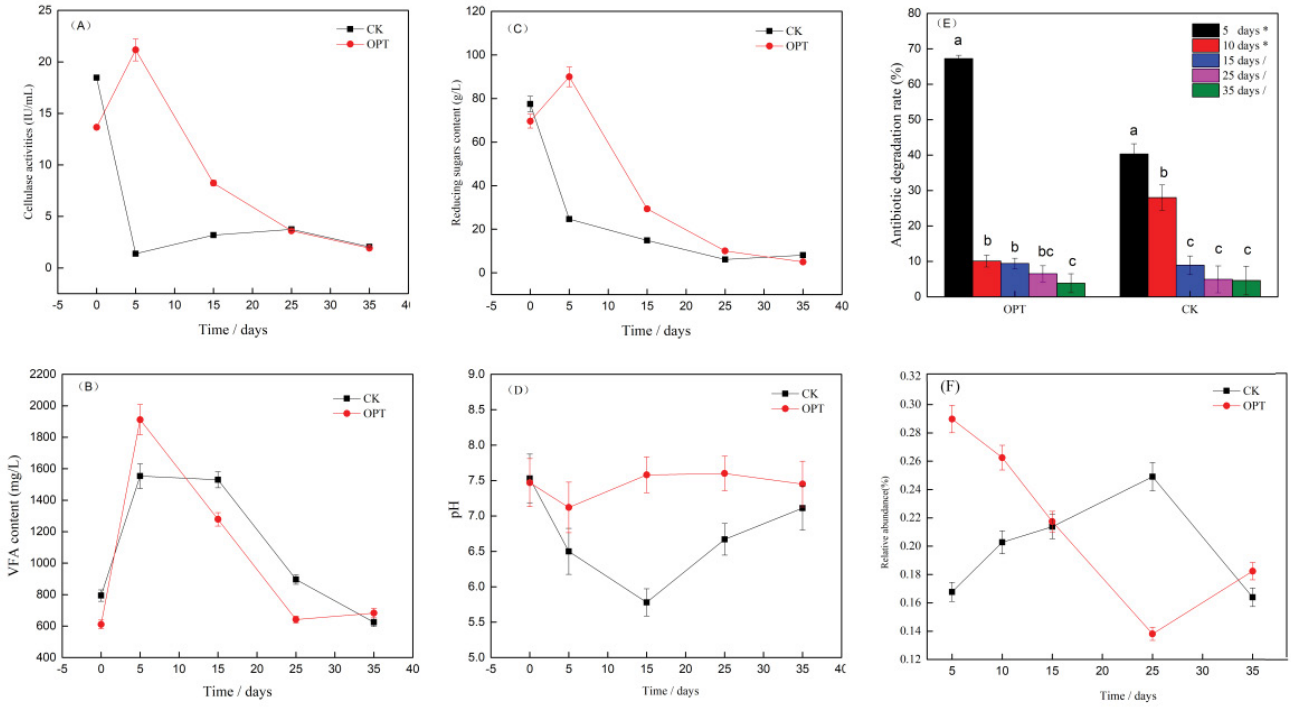

Fig. 3. Cooperation of multiple factors on the environmental characteristics of anaerobic fermentation process. (A–F) represent characteristic of CA, VFA content, RS content, pH, degradation rate of tetracycline and T-ARGs, respectively.

while F_2 was determined by T-ARGs, and bacterial diversity in CK. F_1 was determined by cellulase activity, reducing sugar content, pH and T-ARGs, while F_2 was determined by VFA content and archaea diversity, and F_3 was determined by bacterial in OPT (Table 7).

Compared with CK, OPT show more diverse influencing factors in the process of AD, which indicated that each factor has more precise action in different stages of AD because the appropriate initial fermentation conditions could make the relationship between environmental factors, bacteria, and archaea more harmonious [32]. The tetracycline degradation rates on the 5th, 15th, and 25th days were mainly affected by T-ARGs and bacteria, while they were affected by cellulase activity, VFA content, reducing sugar content, pH, and archaeal diversity on the 10th and 35th days in the process of AD in CK. On the 5th day, the degradation rate of tetracycline was mainly affected by cellulase activity, reducing sugar content, pH and T-ARGs, while it was mainly affected by bacterial diversity on the 10th day and 35th day, and mainly affected by VFA content and archaeal diversity on the 15th to 25th day in the process of AD in OPT. The results showed that the main factors affected the degradation rates of tetracycline were different in different AD stages, mainly because the main factors affected the performance of AD were also different in different AD stages [33]. The total factor scores showed that environmental factors and characteristics of microorganisms had great influence on the degradation rates of tetracycline in the initial stage of AD in both OPT and CK. This phenomenon could be explained by the variation characteristics of environmental factors, the relative abundances of T-ARGs fluctuating greatly in the initial stage of AD (Fig. 3), and the hydrolysis of antibiotics accelerating in the hydrolysis stage of AD [34]. The suitable initial fermentation conditions changed the characteristics of the environmental factors in the early stage of AD, made they more harmonious to accelerate the start of AD [31]. In OPT, the environmental factors showed the greatest influence on the 5th day of AD, which was earlier than that in CK, because of the cellulase activity and reducing sugar content, pH and T-ARGs had a positive effect on the degradation of tetracycline on the 5th day of AD in OPT. While in CK, all environmental factors had a negative effect on the degradation rates of tetracycline on the 5th day but showed a positive effect on the 10th day of AD (Table 8).

In the process of AD, environmental factors have a direct influence and indirect influence on the performance of AD [35]. The effects of environmental factors (cellulase activity, reducing sugar content, VFA content and pH) and the relative abundance of T-ARGs on the tetracycline degradation rates were studied by path analysis, and the results are shown in Table 9. All of the total correlation coefficients of cellulase activity, pH, T-ARGs, and bacterial diversity with the degradation rates of tetracycline were higher than their direct correlation coefficients in OPT. All of the total correlation coefficients of reducing sugar content, T-ARGs, and bacterial diversity were lower than their direct correlation coefficients in CK. Environmental factors in the AD process, such as pH, VFA, dissolved organic carbon, and TOC, can affect the abundance of ARGs by affecting the characteristics of microorganisms in the AD process to affect the degradation of antibiotics [36].

Table 6
Analysis of variance of factor analysis for environmental factors

	Component	Initial eigenvalues					
		Total	Percentage of variance	Total percentage of variance	Total	Percentage of variance	Total percentage of variance
OPT	x_1	3.263	46.612	46.612	3.263	46.612	46.612
	x_2	2.005	28.642	75.254	2.005	28.642	75.254
	x_3	1.616	23.088	98.342	1.616	23.088	98.342
	x_4	0.116	1.658	100			
	x_5	1.00E-13	1.05E-13	100			
	x_6	1.00E-13	1.01E-13	100			
	x_7	–1.00E-13	–1.03E-13	100			
CK	x_1	4.026	57.516	57.516	4.026	57.516	57.516
	x_2	1.469	20.988	78.504	1.469	20.988	78.504
	x_3	0.989	14.132	92.635			
	x_4	0.516	7.365	100			
	x_5	1.00E-13	1.02E-13	100			
	x_6	–1.00E-13	–1.00E-13	100			
	x_7	–1.00E-13	–1.01E-13	100			

x_1, x_2, x_3, x_4, x_5, x_6, x_7 and y instead of cellulase activity, VFA content, reductive sugar content, pH, T-ARGs, bacterial diversity, archaea diversity and tetracycline degradation rate.

Table 7
Rotating component matrix

Treatments	Index	Index		
		1	2	3
OPT	x_1	0.858	0.325	–0.351
	x_2	–0.492	0.703	0.507
	x_3	0.895	0.441	–0.07
	x_4	0.839	–0.385	–0.293
	x_5	0.684	0.545	0.479
	x_6	0.464	–0.251	0.847
	x_7	–0.311	0.838	–0.445
CK	x_1	0.909	–0.002	
	x_2	0.894	0.173	
	x_3	0.844	–0.086	
	x_4	–0.716	–0.261	
	x_5	–0.042	0.979	
	x_6	–0.508	0.628	
	x_7	–0.956	–0.097	

x_1, x_2, x_3, x_4, x_5, x_6, x_7 and y instead of cellulase activity, VFA content, reductive sugar content, pH, T-ARGs, bacterial diversity, archaea diversity and tetracycline degradation rate. F, b_i, $r_{jk}b_k$, and r_{ij} stand for factors, direct path coefficients, indirect path coefficients and correlation coefficients, respectively. "*" and "**" means there are a significant correlation at the 0.05 level and 0.01.

The environmental factors in the OPT treatment had a positive effect on the degradation rate of tetracycline through T-ARGs and microbial diversity. However, some environmental factors have negative effects on the degradation rate of tetracycline due to their mutual restriction in CK. For example, the reducing sugar content in CK presented a negative effect on the degradation rate of tetracycline through the diversity of archaea. Therefore, we concluded that adjust the initial fermentation environment make the relationship among environmental factors, T-ARGs, bacteria and archaea more coordinated, to make them more positive role in the degradation of tetracycline.

3.4.2. Driving effect of ARGs and microorganisms during the AD process

The pressure of antibiotics can promote the production of ARGs by microorganisms. If the ARGs are negatively correlated with the degradation rate of antibiotics, tetracycline has selective pressure on the ARGs [37], while they are positively related to the degradation rate of antibiotics, they promote the degradation of antibiotics [38]. If the abundance of the bacteria has a significant positive correlation with the abundance of the ARGs, the bacteria are considered to be the potential host of the ARGs [39]. The correlation analysis between the relative abundance of T-ARGs and the antibiotic degradation rate is shown in Fig. 5A, and the relative abundance of T-ARGs and the relative abundance of bacteria are shown in Fig. 5B and C. The results showed that there were 30 significant correlation networks between T-ARGs and bacteria in OPT; among them, there were 26 significant positive correlation networks and significant negative correlation networks ($P < 0.05$). There were 77 significant correlation networks between T-ARGs and bacteria, including 50 significant positive correlation networks and 27 significant negative correlation networks in CK (Fig. 5B and C). More seriously, the number of significant positive correlations between T-ARGs and the tetracycline degradation rate in OPT was

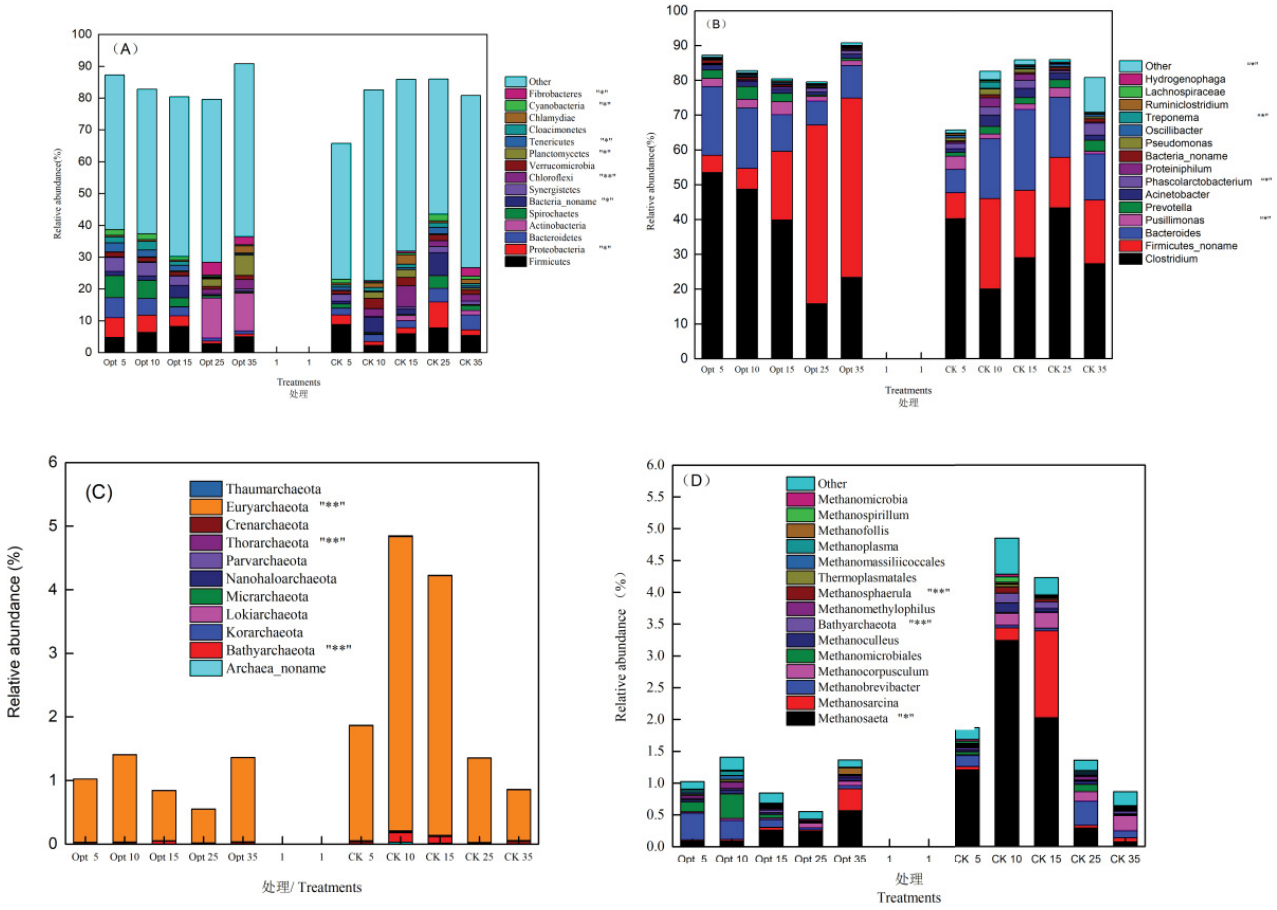

Fig. 4. Cooperation of multi-factors on the characteristics of microorganisms in the process of anaerobic fermentation. (A, B) represent relative abundance of bacteria in phylum level and genus level and (C, D), represent relative abundance of archaea in phylum level and genus level.

less than that in CK. However, the total abundances of T-ARGs was positively correlated with the degradation rate of tetracycline in OPT, while the total abundances of T-ARGs was negatively correlated with the degradation rate of tetracycline in CK. It may be because: (1) the numbers of significant negative correlations between T-ARGs and tetracycline resistance genes in CK was higher than that in OPT, the selection pressure of antibiotics on T-ARGs in CK was higher than that in OPT, which meant that regulate the initial fermentation conditions reduces the selection pressure of antibiotics to some ARGs; (2) adjusting the initial fermentation environment could greatly simplify the relationship between T-ARGs and bacteria in the process of AD, but strengthen the relationships between T-ARGs and dominant bacteria in the AD, the numbers of connections between T-ARGs and dominant bacteria in OPT was lower than that in CK, to put to good use of dominant bacteria on the degradation of antibiotics; (3) OPT enhanced the degradation of tetracycline through the total action of T-ARGs in process of AD, rather than through the single action of a single class of T-ARGs. Therefore, adjusting the initial fermentation environment could induce dominant bacteria to produce T-ARGs, and give full play to the coordination function of T-ARGs, so that the T-ARGs could become a force and play a greater role in degrading antibiotics.

Table 8
Factor score of environmental factors effect tetracycline degradation rate in AD

Treatments	Time/Days	F_1	F_2	F_3	F
OPT	5	1.627	0.550	–0.448	0.813
	10	–0.209	0.527	0.998	0.284
	15	–0.788	0.737	0.487	–0.0437
	25	–0.809	–0.123	–1.525	–0.765
	35	0.179	–1.691	0.488	–0.288
CK	5	–0.184	–0.615		–0.235
	10	1.747	0.104		1.027
	15	–0.269	0.461		–0.058
	25	–0.700	1.328		–0.124
	35	–0.593	–1.279		–0.610

F_1, F_2, F_3 and F instead of Factor 1, Factor 2, Factor 3, and total factor.

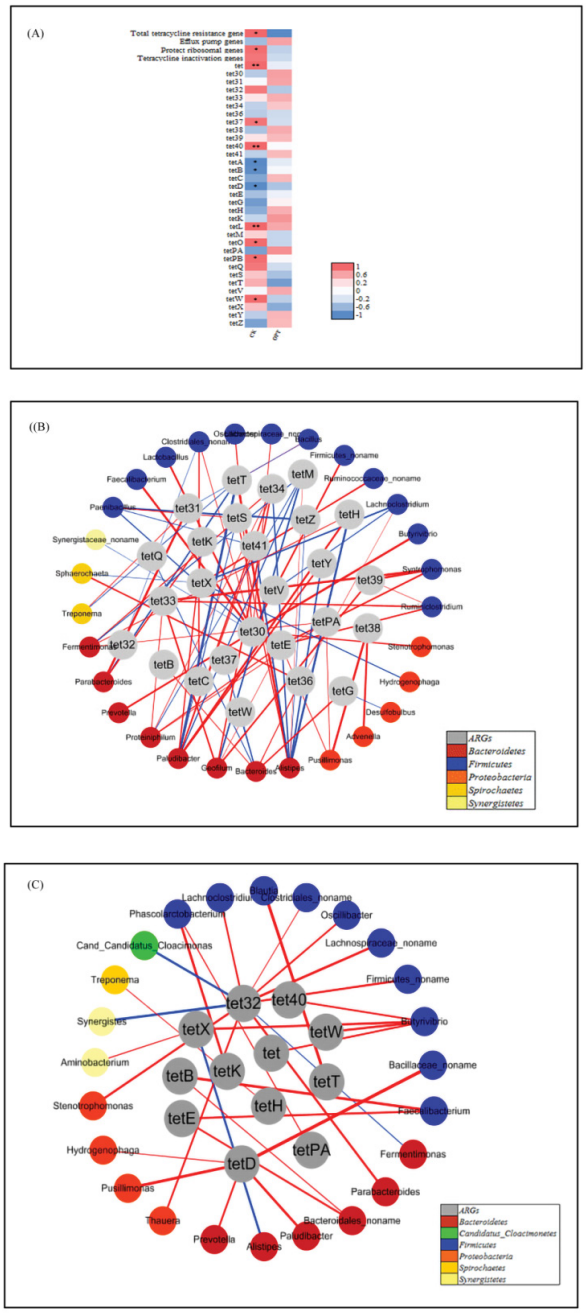

Fig. 5. Relationship among ARGs, bacterial characteristics and degradation rate of antibiotics. "*" and "**" means there are a significant correlation at the 0.05 level and 0.01. A, B and C stand for relationship of ARGs and degradation rate, relationship of ARGs and bacterial in CK, and relationship of ARGs and bacterial in OPT.

4. Conclusions

The synergistic effect of factors could significantly improve the degradation of tetracycline by AD ($P < 0.01$). The optimal fermentation conditions of C/N, pH, and fermentation temperature were 33.56°C, 7.09°C, and 48.27°C, respectively. Under these conditions, the degradation rate of antibiotics was 96.66%. The degradation rate of tetracycline in OPT was different from that in CK in the early stage of AD. The synergistic action of factors could improve the abundance of bacteria degrading macromolecules to make the environmental factors related to AD reach the best state. The results of statistical analysis showed that the synergistic effect of factors could enhance the degradation of tetracycline by changing the main influencing factors of each stage in AD, inducing dominant bacteria to produce T-ARGs, making the relationship between T-ARGs and bacteria more coordinated.

Table 9
Direct and indirect path coefficient of the effect of environmental factors on the degradation rate of tetracycline

Treatments	Path	b_i	$r_{jk}b_k$		r
OPT	$x_1 \to y$	0.801	$x_1 \leftrightarrow x_4 \to y$	0.050	
			$x_1 \leftrightarrow x_5 \to y$	0.128	0.986**
			$x_1 \leftrightarrow x_7 \to y$	0.007	
	$x_4 \to y$	0.077	$x_4 \leftrightarrow x_1 \to y$	0.522	
			$x_4 \leftrightarrow x_5 \to y$	0.065	0.631
			$x_4 \leftrightarrow x_7 \to y$	0.024	
	$x_5 \to y$	0.220	$x_5 \leftrightarrow x_1 \to y$	0.753	
			$x_5 \leftrightarrow x_4 \to y$	0.046	0.706
			$x_5 \leftrightarrow x_7 \to y$	0.002	
	$x_7 \to y$	0.047	$x_7 \leftrightarrow x_1 \to y$	0.121	
			$x_7 \leftrightarrow x_4 \to y$	–0.034	0.143
			$x_7 \leftrightarrow x_5 \to y$	0.008	
CK	$x_3 \to y$	1.264	$x_3 \leftrightarrow x_4 \to y$	0.011	
			$x_3 \leftrightarrow x_5 \to y$	0.013	0.883*
			$x_3 \leftrightarrow x_7 \to y$	–0.479	
	$x_4 \to y$	–0.024	$x_4 \leftrightarrow x_3 \to y$	–0.593	
			$x_4 \leftrightarrow x_5 \to y$	0.031	0.747
			$x_4 \leftrightarrow x_7 \to y$	0.386	
	$x_5 \to y$	–0.107	$x_5 \leftrightarrow x_3 \to y$	–0.281	
			$x_5 \leftrightarrow x_4 \to y$	0.007	–0.434
			$x_5 \leftrightarrow x_7 \to y$	–0.052	
	$x_7 \to y$	0.602	$x_7 \leftrightarrow x_3 \to y$	–0.875	
			$x_7 \leftrightarrow x_4 \to y$	–0.015	–0.278
			$x_7 \leftrightarrow x_5 \to y$	0.009	

x_1, x_2, x_3, x_4, x_5, x_6, x_7 and y instead of cellulase activity, VFA content, reductive sugar content, pH, T-ARGs, bacterial diversity, archaea diversity and tetracycline degradation rate. F, b_i, $r_{jk}b_k$, and r_{ij} stand for factors, direct path coefficients, indirect path coefficients and correlation coefficients, respectively. "*" and "**" means there are a significant correlation at the 0.05 level and 0.01.

Ethical approval

This work does not involve non-human primate or endangered or protected species. The pig manure was collected from the pig's natural waste, during which the pig has no pain.

Authorship contribution statement

Shuzhen Zou: Methodology, Software, Formal analysis, Investigation, Writing - original draft, Writing - review and

editing, Visualization, Funding acquisition. Xiaoyu Luo: Methodology, Software, Formal analysis, Investigation, Writing - original draft, Writing - review and editing, Visualization; Yun Tang: Methodology, Software, Formal analysis, Investigation, Funding acquisition. Hairong Tu: Investigation, Data curation, Writing - review and editing. Kunyue Zhang: Investigation, Data curation, Writing - review and editing. Di Kang: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review and editing, Supervision.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper, and all of the authors consent this manuscript to participate and publish.

Availability of data and materials

The authors declare that the main data supporting the findings of this study are available within this Article and in the Supplementary Information files. Extra data supporting the findings of this study are available from the corresponding author upon reasonable request.

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