Insights on anaerobic digestion foaming via association between bacterial metabolism and variations in microbiota

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Research

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Abstract

Background: Foaming in anaerobic digesters is considered a global concern due to significant impacts on process efficiency and operational costs. Although the importance of the organic loading rate on anaerobic foaming is now widely recognized, little is known about the key bacteria among the hundreds of species inducing foaming, especially the metabolite-microbiota correlation that influences foaming in anaerobic digesters.

Results: Here, we show that the organic loading rate promotes foaming and decreases the performances of bench-scale batch digesters. Metabolomics analysis revealed distinct changes in the metabolic phenotype, including mainly short-chain fatty acids and amino acids, decreasing the surface tension and inducing foaming. Furthermore, the correlation analysis revealed that *Clostridium* clusters were the main microbes contributing to these metabolite foaming incidents.

Conclusions: We provide the foaming microbes and metabolites in anaerobic digestion. Our findings elucidate the complex formation of foaming in anaerobic digestion and provide an effective early-warning for the control of foaming in full-scale digesters.

Background

Energy recovery from wastewater is widely considered a sustainable option for wastewater management and resource recovery [1]. The most common technology for energy recovery is anaerobic digestion (AD), which anaerobically converts organic matter into biogas through a series of biochemical steps [2]. The biogas that is generated can potentially offset anthropogenic greenhouse gas emissions and reduce reliance on fossil fuels. However, due to the sensitivity of anaerobic digestion, the accumulation of certain intermediates in digesters can lead to performance inhibition or process failure [3]. Among the inhibitors of performance, foaming incident is considered a critical problem that disrupts the operational stability in anaerobic digesters. Foaming in anaerobic digesters causes inefficient biogas recovery, creation of dead zones, reduction in effective volume, and blockage of devices in the digesters [4, 5]. In the United Kingdom, 56% of 16 surveyed anaerobic digesters in waste water treatment plants had experienced foaming[5]. In addition, 60.5% of 38 surveyed plants reported the incidence of AD foaming in Spain with seasonal (7 plants), intermittent (13 plants), and persistent (3 plants) frequency [4]. In a comprehensive survey of 3100 German biogas plants, 10.5% of operators in 327 biogas plants claimed that foaming is an important problem in operation [6]. A 10-week foaming event in a Swedish plant, producing 2,000 m^3 biogas per day, resulted in 40% biogas loss and $150,000 of additional cost. Hence, foaming is one of the bottlenecks that restricts digester potential.

Foaming during AD is a complex three-phase phenomenon with various constituent liquid, solids and gases, where several factors might cause foaming in practice. For example, little mixing leads to maldistribution of volatile solids and methane, causing foaming in full-scale anaerobic digesters [7]. However, excessive mixing might increase the entrapment of bubbles in the liquid, leading to foaming [8].

The main reasons for foaming related to mixing may be separation of solid and liquid and accumulation of surface-active substances on top of the digester. In addition, digesters operated under thermophilic conditions are effective in foaming minimization because high temperatures might decrease the surface tension and viscosity of the sludge [9]. A number of researchers have stated that the organic loading rate (OLR) is a key parameter for foaming in anaerobic digesters [10–12]. Excess organic compounds cannot be fully degraded and lead to the accumulation of hydrophobic or surface-active products, which might promote foaming. Subramanian and Pagilla reported foaming in anaerobic digesters with a shock load of 2.3 g volatile solids (VS)/(L·d) [12]. However, Brown suggested that anaerobic digesters undergo cumulative foaming incidences when the OLR is higher than 4.5 g VS/(L·d) [13]. Though the OLR is considered a critical cause of foaming in anaerobic digesters, there is still a lack of fundamental understanding for a clear correlation between foaming and OLR of digesters.

AD is a multistage biochemical process with different types of microbiota, and a number of researchers have investigated the cause of foaming from the aspect of microbiology. It is accepted that *Gordonia* species and *Microthrix parvicella* cause or contribute to AD foaming in anaerobic digesters [14, 15]. Due to hydrophobic properties, the filamentous microbiota adheres to biogas bubbles, then accumulate on the surface of digesters and decrease the surface tension of the solution in digesters [10]. Although there is some information on the relationship between the presence of filamentous microbiota and AD foaming, the mechanism by which surface-active byproducts produced by these species is still unclear. Surface-active compounds such as volatile organic acids (VFAs), found at the gas/liquid boundary interface, can decrease surface tension and enhance foaming potential [16]. These surface-active compounds include both substrates with surface active and biosurfactants produced during the metabolic activity of microbiota [9]. As initiators, biosurfactants are a prerequisite for foaming. However, the foaming in a complex AD system might depend on the type and concentration of biosurfactants. To date, evidence showing that biosurfactants, produced from the metabolic activity of microbiota contribute to foaming is lacking. In this study, we investigate the cause of foaming with different OLRs in bench-scale batch digesters. Metabolomics analysis was employed to reveal the metabolomics of foaming. Furthermore, the covariation between the metabolic phenotype and the microbiota was exploited to identify specific associations between key members of the microbiota and key metabolites during foaming. These results may contribute to a better understanding of the potential cause of foaming in anaerobic digesters, leading to control of foaming.

Methods

Experimental design

Bench-scale batch anaerobic digestion was carried out in 500-mL serum bottles with a working volume of 300 mL. The inocula were collected from both flocculent sludge (FS) treating domestic sewage and anaerobic granular sludge (AGS) treating protein factory wastewater in Qingdao, China. The inocula (characteristics shown in Table S1) were washed with phosphate buffer solution (PBS; 0.01 M, pH = 7.4) after crushing and sieving (200 mesh) to separate large particles. Before the experiment, both inocula

were cultured in synthetic wastewater (compositions shown in Table S1) at 37 °C with an OLR of 0.47 g VS/L·d for 14 days. During the experiment, a mixture of 0.2-g VS based inocula and 180-mL synthetic wastewater were cultured at 37 °C with 135 rpm. The OLR was set from 0.47 g VS/L·d to 18.68 g VS/L·d based on the hydraulic retention time (Table S1). The biogas yield, biogas composition, total solids (TS), VS, pH and VFAs were measured according to Feng et al [17]. The liquid in the bottles, sampled every two days, was precipitated and the supernatant fraction was used for the foaming test.

Methods And Apparatus For The Foaming Test

The aeration method was chosen to evaluate the foaming potential of solutions [18]. Schematic of the foaming apparatus is shown in Figure S1. A 30-mL sample was poured into a cone and aerated for 5 min with an air flow rate of 1 L/min. The foaming tendency was calculated from the volume of highest foam (mL) divided by the airflow rate (mL/min). The highest foam in the cone was measured within 5 min. The foaming stability was determined as the time, when the foam level remained at one-half the highest volume in the settling cone.

The surface tension of the samples was measured by a surface tension meter (BZY-2, Hengping, China) after the samples were heated with an air bath at 37 °C. The apparent viscosity of the liquid was measured by the capillary method using an Ubbelohde viscosimeter [19]. The apparent viscosity was calculated by the Eq. (1).

$$\eta_1 = \eta_0 \eta_r \quad (1)$$

$$\eta_r = \frac{\rho_{liquid} t_1}{\rho_{water} t_0} \quad (2)$$

where η_1 is the viscosity of testing solution (10^{-3} Pa·s), η_0 is the viscosity of distilled water at 37 °C (0.6947 × 10^{-3} Pa·s), η_r is the relative viscosity (dimensionless unit), ρ_{liquid} and ρ_{water} are the densities of liquid and water, respectively (g/mL), and t_1 and t_0 are the flow times of the test solution and water through viscometer, respectively (s).

Metabolic Profiling Analysis

The supernatants in the AGS digesters with OLRs of 0.47 g VS/L·d and 14.68 g VS/L·d were collected after foaming. The metabolic profiling of the samples was determined by LC-MS-MS [20]. Briefly, 300-μL samples were mixed with 300 μL of precooled methanol and 20 μL of an interior label (2-chloro-L-phenylalanine, purity ≥98%, Hengbai, China), followed by vortexing for 30 s and ultrasonic extraction in ice water for 5 min. The extraction was maintained for 2 h at -20 °C and centrifuged (13000 rpm) at 4 °C for 15 min to obtain the supernatant. The LC-MS-MS analyses were performed on an ultra-performance liquid chromatography system (UPLC) (1290, Agilent, USA) with a Q exactive orbitrap high-resolution tandem mass spectrometer (Thermo Fisher, USA). The detailed conditions for LC-MS-MS were shown in

the Supporting Information. Both positive and negative modes of electrospray ionization were employed. LC/MS raw data were processed using XCMS software according to the research [21]. The normalized data were then used to perform principal component analysis (PCA) and orthogonal to partial least squares-discriminate analysis (OPLS-DA). The variable important in projection (VIP) value ≥1.5, T-test $p < 0.05$ and fold change ≥ 3.0 were the criteria used to select important features.

Microbiota Analysis

To address the potential of microbiota for foaming, Illumia MiSeq high-throughput sequencing was used for the microbiota analysis in both systems with OLRs of 4.67 g VS/L·d, 14.01 g VS/L·d and 18.68 g VS/L·d. For both systems with an OLR of 4.67 g VS/L·d, *Euryarchaeota* (30.35–46.10%) was the most abundant phylum (Figure S5a), which has been widely observed in anaerobic digesters. *Firmicutes* replaced *Euryarchaeota* as the abundant phylum at other OLRs, especially in the FS system (52.30%). Of the top 20 genera identified, *Methanothrix* and *Methanobacterium* were the dominant genera in both systems, with OLRs of 4.67 g VS/L·d and 14.01 g VS/L·d, respectively, accounting for 40% of the population (Figure S5b). *Clostridium IV* and *Clostridium sensu stricto* when the OLR was 18.68 g VS/L·d increased from 0.01–20% and 0.01–6% for the AGS and FS systems, respectively. Among these 20 genera, the correlations between reactor performance and genera were established based on Spearman's correlation coefficient (Figure S5c and S5d). *Clostridium* XIVb was positively correlated with foaming tendency in both the AGS and FS systems. To further analyze the genera with foaming potential, the genera with increasing significance (fold > 1.2) in each system after foaming were identified. The genera identified in both systems were recognized as microbiota potentially correlated with foaming. After foaming in the AGS and FS systems, 51 and 58 genera increased, respectively (Fig. 3a and 3b), with 21 genera identified in both systems (Table 1). The genera in both systems were classified as biosurfactant-producing bacteria, nitrogen-related bacteria and acid-producing bacteria (Table 1). Biosurfactant-producing bacteria such as *Klebsiella*, with most strains able to use citrate- and glucose-producing acid and 2,3-butanediol [27], can also produce VFAs. Notably, *Clostridium* has been reported to be related to foaming in active sludge systems producing biosurfactants [28], such as glycoproteins [29]. *Aminobacterium*, a typical nitrogen-related anaerobic bacterium, can degrade various amino acids when co-cultured with methanogens [30]. In addition, *Acidovorax* spp. are capable of heterotrophic denitrification of nitrate and may utilize acetate, propionate, and poly-β-hydroxybutyrate for denitrification [31]. Due to the accumulation of VFAs after foaming, many acid-producing bacteria significantly increased. *Anaerofilum*, *Oxobacter* and *Sporolactobacillus* are able to synthesize lactic acid, which is widely identified as a foaming agent [32].

Table 1
Metabolic function of potential foaming genera

Classification	Taxonname	Metabolic function
Biosurfactants-producing bacteria	*Klebsiella*	Production of acid, gas and 2,3-butanediol in glucose fermentation [27].
	Clostridium III	Production of ethanol and lactate in some carbohydrates formation [47].
	Clostridium IV	Synthesis of lactic acid and butyric acid in carbohydrates formation [48].
	Clostridium sensu stricto	Production of butyric acid with carbohydrate or cellulose biomass as carbon source [49].
	Clostridium XIVa	Acetate- and/or lactate-converting butyrate producers [50].
	Clostridium XIVb	Amino acid- and/or lactate-fermenting bacterium producers [51].
Nitrogen-related bacteria	*Aminobacterium*	Amino-acid-degrading bacterium [52].
	Acidovorax	Heterotrophic denitrification of nitrate utilizing acetate, propionate, and poly-β-hydroxybutyrate [53].
Acid-producing bacteria	*Aminomonas*	Acetate was the end-product formed from all these substrates [52].
	Anaerofilum	Production of lactate, acetate, ethanol and formate from glucose fermentation [54].
	Bacteroides	Production of short chain fatty acids with digesting complex polysaccharides [55].
	Bifidobacterium	Production of conjugated linoleic acid in either synthetic media or milk [56, 57].
	Enterococcus	Generation of butyrate, short chain fatty acids [58].
	Escherichia/Shigella	Relation with fatty acid composition of bacterial lipopolysaccharides [59].
	Oscillibacter	n-Valeric acid was the major end product from glucose [60].
	Oxobacter	The fermentation products from glucose are acetate, isobutyrate, isovalerate, valerate, lactate, and ethanol [61].
	Parabacteroides	Acid is produced from glucose [62].
	Petrimonas	Production of large amount of acetic acid and hydrolyzation of carbohydrates and organic acids in the presence of elemental sulfur [63].
	Sedimentibacter	Fermentation of pyruvate or of amino acids [64].

Classification	Taxonname	Metabolic function
	Sporanaerobacter	Acetate is the only fatty acid produced from glucose metabolism. It can degrade amino acids and peptides [65].
	Sporolactobacillus	Lactic acid is produced actively without liberation of gas from glucose [32].

Metabolomics profiles of soluble microbial products in the AGS system

To identify the potential initiators for foaming, metabolomics analysis was used to identify the specific components in soluble microbial products (SMPs) in the AGS system with OLRs of 0.47 g VS/L·d and 14.68 g VS/L·d. The PCA and OPLS-DA models showed SMPs differences between low OLRs and high OLRs (Figure S6). A total of 46 metabolites confirmed by secondary ion mass spectrometry are summarized in Figure S7. Then, VIP ≥ 1.0 together with fold change ≥ 3.0 analysis indicated that 29 metabolites were significantly (T-test, $p < 0.05$) changed for different OLRs (Fig. 3c). The altered metabolites were classified into 6 main categories: organic acids, polyketides, lipids, benzenoids, organooxygen compounds and organoheterocyclic compounds (Table S4).

Among these categories, organoheterocyclic compounds contained the most metabolites, which indicated that the OLR may have a significant impact on the organoheterocyclic compound metabolism of SMPs in the AGS system. Heterocyclic compounds exist naturally in anaerobic systems as electron carriers, energy storage molecules and nucleotides [33] related to bacterial metabolites. Organic acids and derivatives such as amino acids, are the potential foaming substances. The significantly changed compounds included lysine, N-methyl proline, 4-aminobutanoate, cysteinyl-cysteine, ethyl acetate and trans-aconitate. For example, the intensities of lysine in SMPs increased 77.76-fold in high-OLR digesters with foaming, indicating that lysine accumulated in the reactor with increasing OLR. These amino acids underwent biosynthesis processes to produce peptides and proteins as precursors for the biosynthesis of nucleotide sugar, phospholipids, and peptidoglycan [34]. Hydroxyphenyllactic acid is a tyrosine metabolite likely derived from phenolic or polyphenolic compounds [35]. Bacterial-derived hydroxyphenyllactic acid was found in bacterial overgrowth [35], suggesting that imbalances existed in microbiota after foaming. Lipids are well known to induce foaming in biogas plants. Lipids were also detected as end products in the anaerobic batch supernatants, in which the highest relative abundance lipid was cardiolipin, which is related to biofilm formation [36]. Bacteria can trigger the biosynthesis of sphingolipids under stress conditions induced by temperature and pH and then transform them into various ceramides to protect themselves [37]. This process was shown by the presence of dihydroceramide and ceramide in this study. Benzenoids are usually found in anaerobic digesters [38]. As a quinone substance, atovaquone is an important moiety of humic substances and is considered to serve as an electron shuttle [39].

Results

The OLR promotes anaerobic foaming

Both types of anaerobic sludge were cultured in digesters with an OLR of 0.47 g VS/L·d. When methane concentration and methane yield in all bottles reached 50% and 200.00 mL CH_4/g Glu, respectively, the OLRs in different bottles were adjusted to 0.47 g VS/L·d, 4.67 g VS/L·d, 14.01 g VS/L·d and 18.68 g VS/L·d to induce foaming. There was no observed foaming in the reactors during the steady stage, as well as in the reactors with OLRs of 0.47 g VS/L·d and 4.67 g VS/L·d during the whole process (Figure S2). However, foaming was obvious in both sludge reactors with high OLRs (14.01 g VS/L·d and 18.68 g VS/L·d) from the 2nd day to the 10th day (Figure S2). To describe the foaming propensity in the reactors quantitatively, we determined the foaming propensity of the liquid during AD. Similar to the phenomenon observed in the reactors, the foaming tendency in both the FS and AGS systems was approximately 2 mL-foam/(mL-air·min^{-1}) for the OLRs of 0.47 g VS/L·d and 4.67 g VS/L·d for 10 days (Fig. 1). In the AGS system, the foaming tendency in the reactors with OLRs of 14.01 g VS/L·d and 18.68 g VS/L·d initially increased and then decreased, with peak values of 40 mL-foam/(mL-air·min^{-1}) and 50 mL-foam/(mL-air·min^{-1}) for 14.01 g VS/L·d and 18.68 g VS/L·d, respectively on the 4th day (Fig. 1a). In the AGS system, the foaming tendency increased with an increasing OLR on a specific day, indicating that a high OLR promoted anaerobic foaming. There was a similar tendency in the FS system (Fig. 1b). However, the foaming tendency in the AGS system was higher than that in the FS system on a specific day. Because foaming is related to the surface tension, the parameter was measured in the reactors. As expected, each surface tension with an OLR of 4.67 g VS/L·d, 14.01 g VS/L·d or 18.68 g VS/L·d was significantly ($p < 0.05$) lower than the surface tension with an OLR of 0.47 g VS/L·d both in both the AGS and FS systems (Fig. 1c). In addition, similar to the tendency of foaming, the surface tension with OLRs of 0.47 g VS/L·d and 4.67 g VS/L·d exhibited negligible variation during the whole fermentation process in both the AGS and FS systems (Figure S3). The surface tension in both systems decreased with increasing OLR on a specific day, as it decreased from 70.80 mN/m to 56.32 mN/m with the OLR increasing from 0.47 g VS/L·d to 18.68 g VS/L·d in the AGS system (Figure S3a). The aqueous system with low surface tension is prone to foaming [22, 23]. We also analyzed the liquid viscosity due to the fact that it plays a dominant role in the ageing of anaerobic foaming. When both types of sludge were cultured with different OLRs for 10 days, the apparent viscosity was increased with the increasing of OLR (Fig. 1d). The apparent viscosity with an OLR of 4.67 g VS/L·d, 14.01 g VS/L·d or 18.68 g VS/L·d was significantly ($p < 0.05$) higher than the apparent viscosity with an OLR of 0.47 g VS/L·d in the AGS system. Overall, our results suggest that a high OLR promotes anaerobic foaming both in the AGS and FS systems, where the tendency was dependent on the inoculated sludge.

Performance Of Reactors With Different Olrs

Given that OLR influences the performance of anaerobic reactors, we examined the main gaseous products for reactors with different OLRs. The methane production in both systems decreased significantly ($p < 0.01$) with increasing OLR on the 2nd, 4th, and 6th days (Fig. 1e, Figure S4a and S4b). For example, in the AGS system, the methane yield on the 2nd day was 175.54 mL CH_4/g Glu, 53.05 mL CH_4/g Glu, 13.12 mL CH_4/g Glu and 5.33 mL CH_4/g Glu for the OLRs of 0.47 g VS/L·d, 4.67 g VS/L·d,

14.01 g VS/L·d and 18.68 g VS/L·d, respectively. However, no methane was produced in either system when the OLR reached 14.01 g VS/L·d or 18.68 g VS/L·d at the end of the digestion. In addition, a clear tendency for a decreased methane concentration with an increasing OLR was also observed, as shown in Fig. 1f and Figure S4b. Similarly, the differences in methane production and content were dependent on the inoculated sludge. For example, the methane content with an OLR of 0.47 g VS/L·d was highest among the reactors, 57.42% for AGS and 54.56% for the FS system. In contrast, hydrogen production increased with an increasing OLR, especially from the 2nd to the 6th day in the FS system (Figure S4c and S4d). In the FS system, the hydrogen production was 0 mL H_2/g Glu, 13.56 mL H_2/g Glu and 16.03 mL H_2/g Glu for 4.67 g VS/L·d, 14.01 g VS/L·d and 18.68 g VS/L·d on the 6th day, respectively. The pH value in these digesters was lower than 5.0 (Figure S4e and S4f), indicating insufficient buffering capacity in the digester for a high OLR. Hence, a high OLR resulted in incomplete degradation of organic matter and the accumulation of biosurfactants [24], which can cause a decrease in methane yield and promote anaerobic foaming.

As previous results indicated that the accumulation of VFAs might induce anaerobic foaming [25], we analyzed the concentration of VFAs, the main aqueous products in AD. The total VFAs (TVFAs) concentration in both systems showed a substantial increase with increasing OLR ($p < 0.01$) (Fig. 2). TVFAs continually accumulated in the reactors, which may lead to an inhibitory effect on methane yield. In addition, acetic acid was the dominant component in both systems after 10 days of fermentation (Fig. 2c and 2d). In the AGS system, after 10 days of fermentation, propionic acid was the dominant acid, with values of 58.01%, 82.61% and 88.24% for OLRs of 4.67 g VS/L·d, 14.01 g VS/L·d and 18.68 g VS/L·d, respectively (Fig. 2c). However, propionic acid was dominant only when the OLR was 18.68 g VS/L·d in the FS system, with n-butyric acid dominant when the OLRs were 4.67 g VS/L·d or 14.01 g VS/L·d (Fig. 2d). Overloading causes the increase of VFAs and a decrease in pH, which can lead to an imbalance in the growth of acetogenic bacteria and methanogens [26]. In addition, the carboxylic groups of VFAs could actually decrease the surface tension of the liquids in digesters to increase the foaming tendency [18]. The protein in the digester was also determined (Fig. 2b). After fermentation, the total protein concentrations increased consistently from 95.10 mg/L to 938.8 mg/L and 79.30 mg/L to 1204.00 mg/L for the AGS and FS systems, respectively, with the OLR increasing from 0.47 g VS/L·d to 18.68 g VS/L·d.

Discussion

To explore the correlation between foaming tendency and digester performance, a Pearson correlation index was identified (Fig. 4). The foaming tendency in the anaerobic digester exhibited a significantly ($p < 0.05$) negative correlation with surface tension and methane yield. However, the foaming tendency in the anaerobic digester showed a significantly positive correlation with the concentration of TVFAs, butyric acid, proteins and apparent viscosity ($p < 0.05$). These factors may be routine indicators useful to optimize fermentation. Generally, the drainage rate of foaming might decrease with increasing liquid viscosity, which plays a dominant role in foam aging processes [40]. Low drainage velocity of lamellae

can enhance foaming stability by sufficient limiting Ostwald ripening and coalescence [41]. However, there was no visual foaming stability in this research since the solid phase, which was mainly generated by the accumulation and deposition of metabolites, was removed (Movie S1). In the full-scale anaerobic digesters, high concentrations of particles and metabolites, such as proteins and polysaccharides, were attached to the bubble surface, causing high surface viscosity. The viscosity in the solution can enhance the mechanical strength of the liquid film of the foam and increase the anti-disturbance capacity of foaming. In addition, Petrovski indicated that only surfactants with scumming populations can form stable foaming [42]. Unstable foaming was generated without hydrophobic cells or particles in solution, which was proven in activated sludge systems and flotation theory [42, 43]. Therefore, foaming incidents are the result of complex environmental factors and are not due only to the inherent features of typical organisms. Similarly, the VFAs with foaming incidents has also been reported in other studies. For example, He et al. reported VFAs accumulation with foaming in an AD of food waste, and the VFAs concentration was maintained at a high level, in the liquid layer (12.36 g/L) during foaming [25]. The higher protein concentration in AGS leaded to the lower surface tension. Collivignarelli and Cosenza proved that the foaming formation may be favored by the presence of soluble microbial product and, in particular, by the presence of soluble proteins [44, 45].

To identify the specific association between key members of the microbiota and foaming in the digester, we analyzed the correlation of microbiota and the metabolic phenotype. Figure 5 shows the functional relationships between the potential foaming microbiota and metabolites in the AGS system with an OLR of 14.68 g VS/L·d based on the Pearson correlation coefficients ($|R|>0.5$, top 200 relationships in rank). The predominantly enriched genera, including *Clostridium* clusters XIVb, *Methanolinea*, *Paludibacter*, *Sporolactobacillus*, *Clostridium* clusters IV and *Clostridium sensu stricto*, were negatively associated with the concentrations of increased metabolites, such as 2-propylpiperidine (correlations in cyan) and positively correlated with the concentrations of 29 metabolites, such like 1-O-galloylglycerol (correlations in red). In addition, *Bacteroides*, *Sporolactobacillus*, *Clostridium* cluster IV and *Clostridium* sensu stricto possessed the same significantly positive correlations with metabolites ($p < 0.05$). However, 10 of the 22 genera identified with related foaming belonging to the phylum *Firmicutes* did not show similar correlation trends, which was also proven by the loading plot (Figure S8). In addition, the co-occurrence network showed that *Sporolactobacillus*, *Bacteroides* and *Clostridium* cluster IV were in the main microbiota-related positions (Figure S9). The relative abundances of *Sporolactobacillus*, *Clostridium* clusters IV and *Clostridium sensu stricto* showed trends opposite to *Tissierella*, *Sporosarcina*, *Syntrophomonas*, *Clostridium* clusters XIVa and *Anaerofilum*. The results also revealed clear correlations between other genera and metabolites. For example, *Bacteroides* belonging to the phylum *Bacteroidetes* were strongly positively correlated with a series of metabolites. The results suggested that metabolites related to these microbiomes were short-chain fatty acids and amino acids. These changes were the result of the modulation of microbiota, especially phyla *Firmicutes* and *Bacteroidetes*, which are closely related to hydrolysis and acidification at a high-OLR.

Clostridium clusters keep up the close relationships with amino acids, which have been identified as the predominant function in the digestive tract of humans and animals [21]. Our study showed a significantly

($p < 0.05$) positive association of lysine, N-methyl proline, 4-aminobutanoate and cysteinyl-cysteine with the relative abundance of genus *Clostridium* clusters IV and *Clostridium sensu stricto*, which suggested that high OLR might influence the amino acid metabolism of *Clostridium* clusters in anaerobic digesters, contributing to foaming. Previous research has also reported that *Actinobacteria* and *Synergistetes* increased after foaming, which is partly related to amino metabolism [25]. The correlation of microbiota and metabolic phenotype is essential for the laboratory-scale explanation of foaming and full-scale management of AD. Intuitively, it might be useful to predict foaming through the monitoring of key aqueous products or/and the abundance of specific bacteria that are likely to cause foaming. For example, the fluorescence in situ hybridization analyses were used to identify the dominant bacteria associated with bulking incidents in activated sludge [46]. However, in previous studies, few attempts were made to identify the key bacteria or metabolites that lead to foaming in the AD, thus limiting the value of these studies in the prediction and control of foaming. Therefore, explorations of other highly sensitive indicators and multiple formations, assessments of their quantitative correlations with foaming potential, and rapid and effective methods for the detection of key bacteria should be considered for universally applicable indices in future studies.

Conclusions

In summary, the study demonstrates the increasing OLR promotes foaming with decreasing surface tension and increasing apparent viscosity of solution. Additionally, increasing OLR decrease performances of bench-scale batch digesters, especially the methane production. Metabolomics analysis revealed distinct changes in the metabolic phenotype, including mainly short-chain fatty acids and amino acids. Results from 16S rRNA gene sequencing identified biosurfactants-producing bacteria, nitrogen-related bacteria and acid-producing bacteria as foaming potential microorganism. Furthermore, the metabolite-microbiota correlation revealed that *Clostridium* clusters were the main microbes contributing to these metabolite foaming incidents.

List Of Abbreviations

OLR: Organic loading rate; AD: Anaerobic digestion; VFAs: Volatile organic acids; FS: Flocculent sludge; AGS: Anaerobic granular sludge; TVFAs: Total VFAs; SMPs: Soluble microbial products.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw 16S rRNA gene sequence data used in the present study has been deposited in the NCBI database under project ID PRJNA678425.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Feng Y., Feng L.J. and Yuan X.Z. designed the research project. Xu P.C, Tian R.K, Wang W.L and Duan J.L. contributed to the start-up and operation of the reactor. Sun X.D., Ma J.Y, Wang Q, Xiao F., Li X.Y. and Duan J.L. analyzed all the data. Duan J.L. wrote the manuscript. All authors discussed, read, and approved the final manuscript.

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Figures

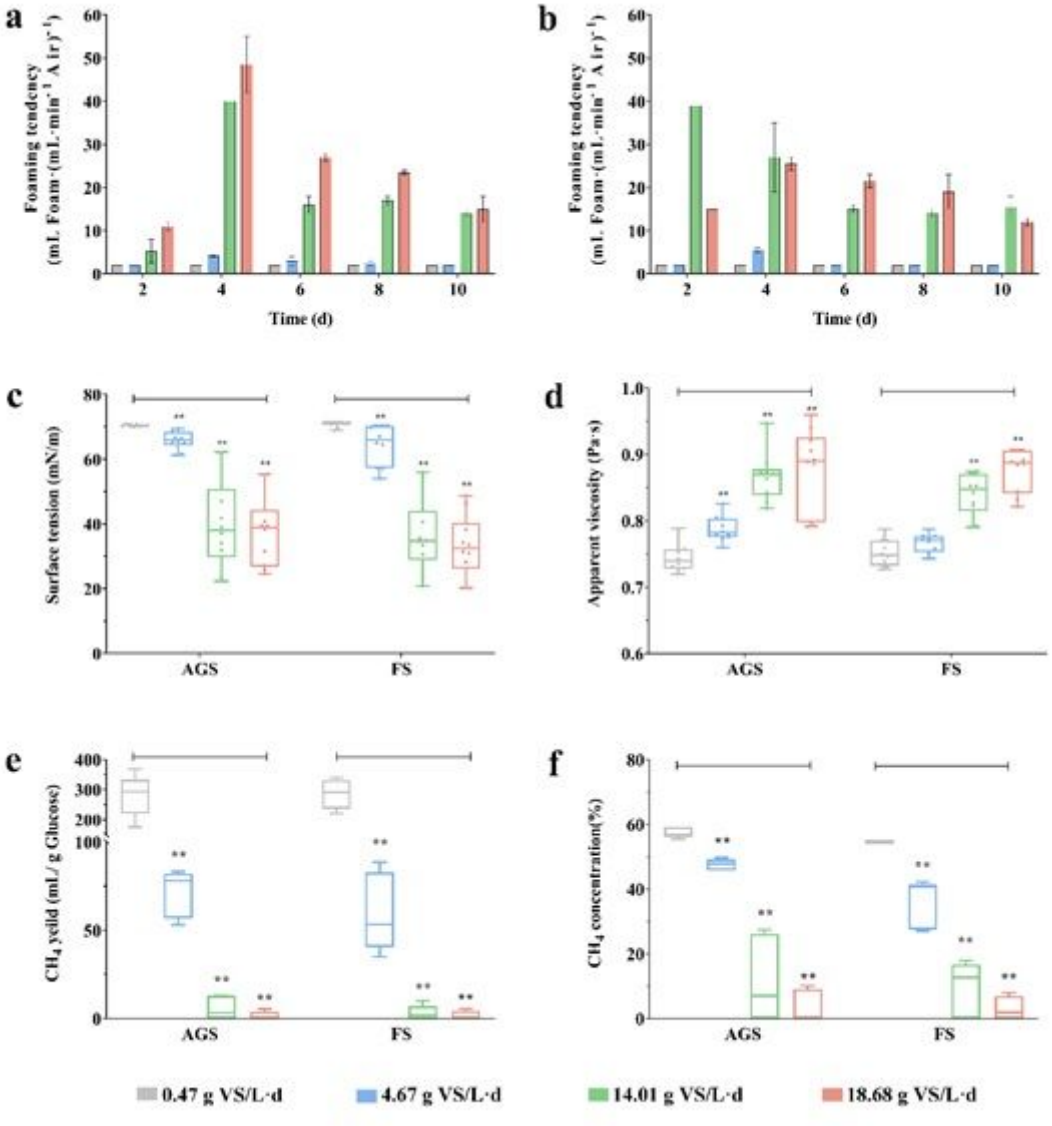
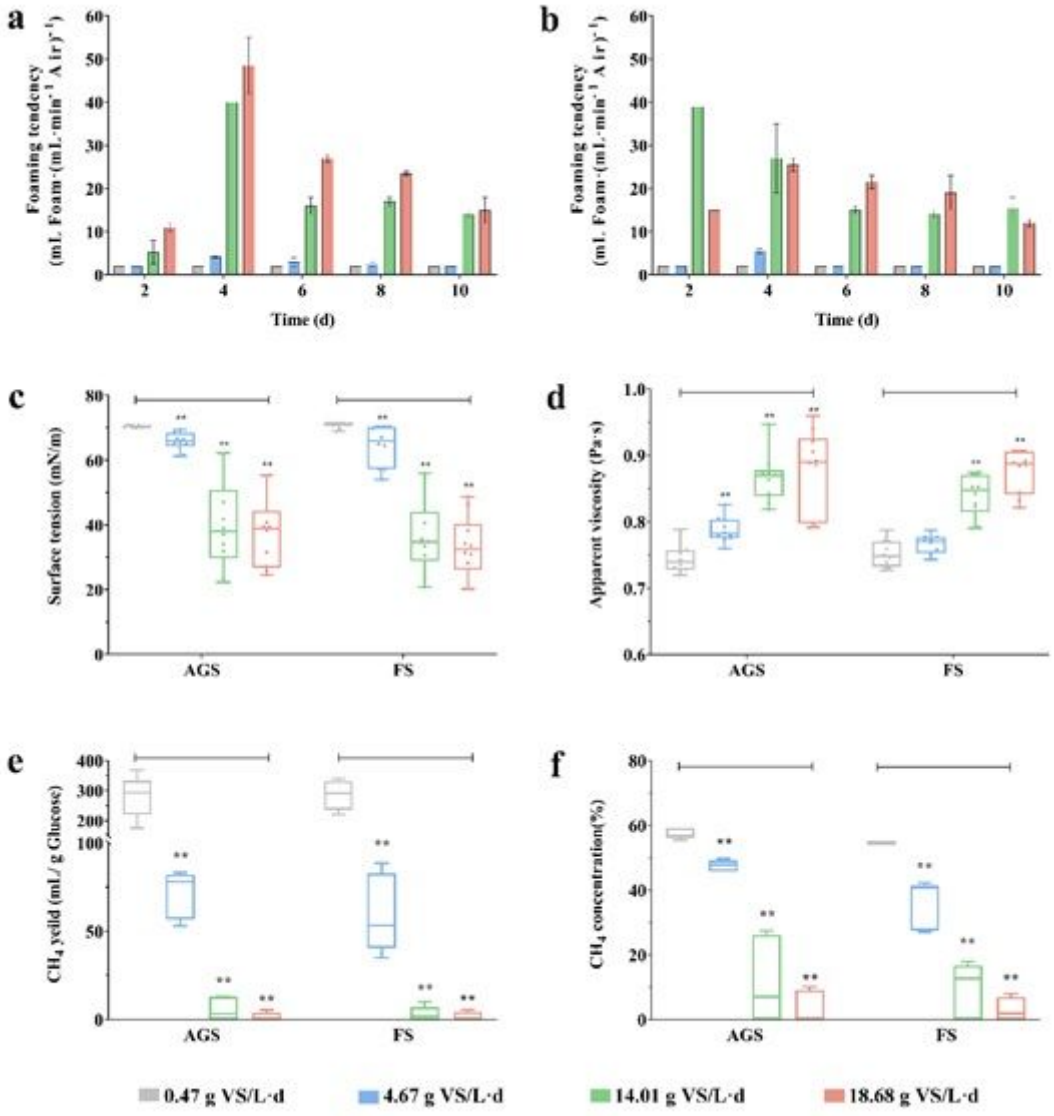


Figure 1

Foaming parameters of different reactors. Foaming tendency in (a) AGS and (b) FS systems; (c) surface tension values and (d) apparent viscosity values of solutions in AGS and FS systems; (e) specific methane production in AGS and FS systems; (f) methane concentration in AGS and FS systems. The statistical significance was estimated by ANOVA with an unpaired t test. The differences were considered significant at $p < 0.05$, and are referred to as *$p < 0.05$, **$p < 0.01$.



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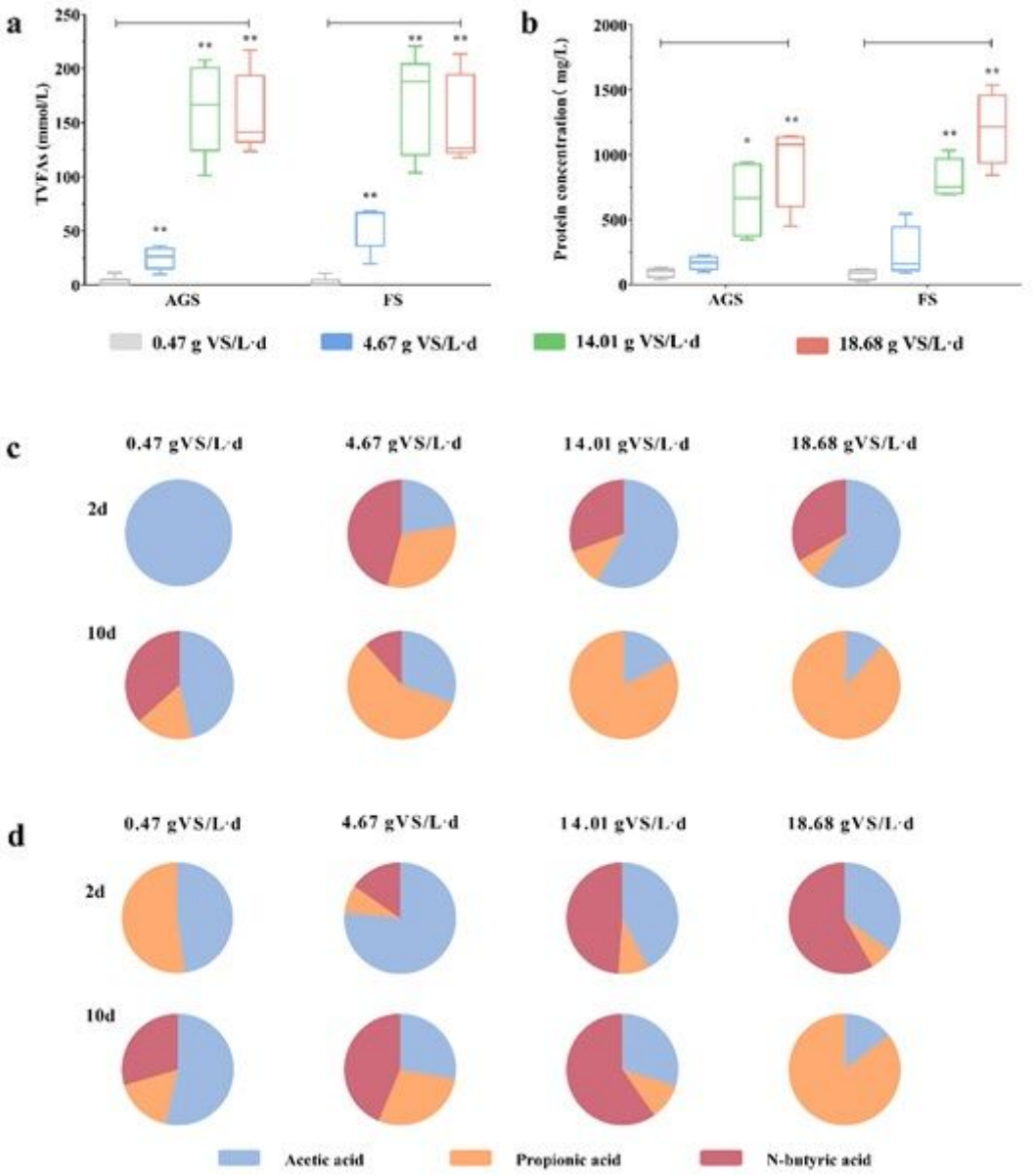

Figure 2

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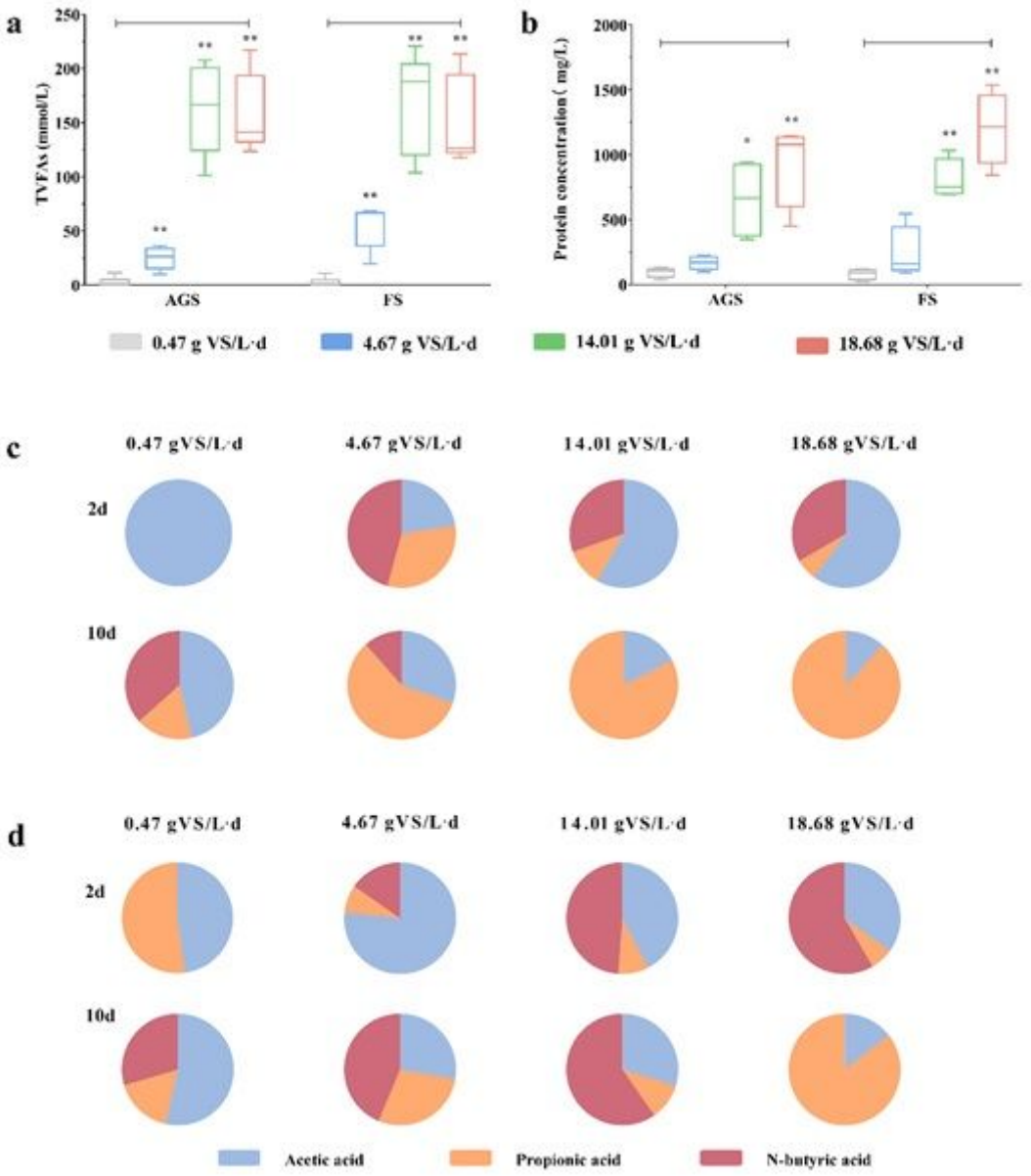

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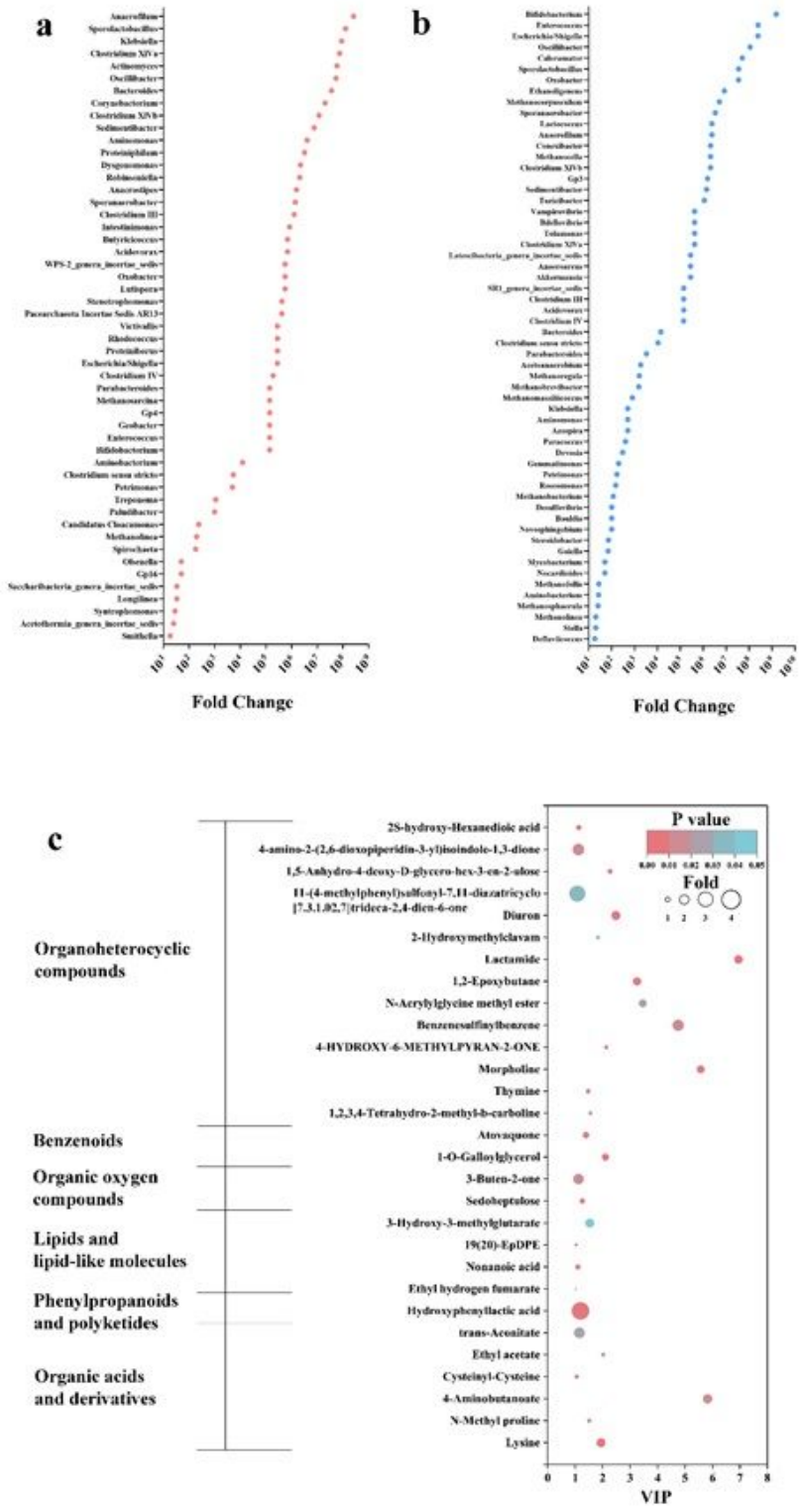
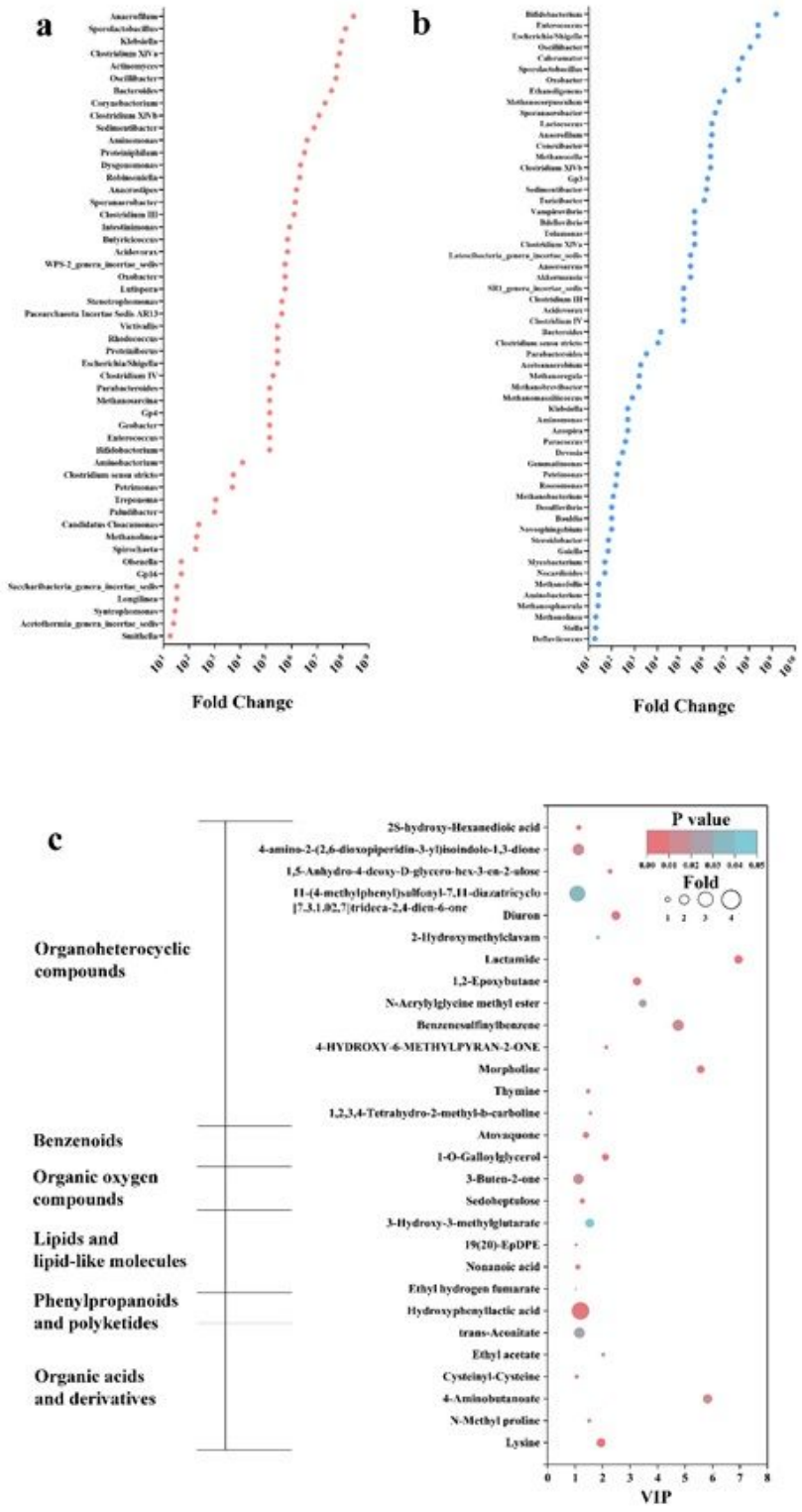


Figure 3

The identification of potential foaming genera and metabolites. The increasing fold change of genera in (a) AGS and (b) FS systems after foaming. Only bacterial genera that significantly changed (increasing fold change > 1.2) after foaming and with relative abundance higher than 0.01% are represented. (c) The fold change of metabolites rank in the relative scale of circle and markers are scaled by log (Fold change). P values are highlighted in blue and red.



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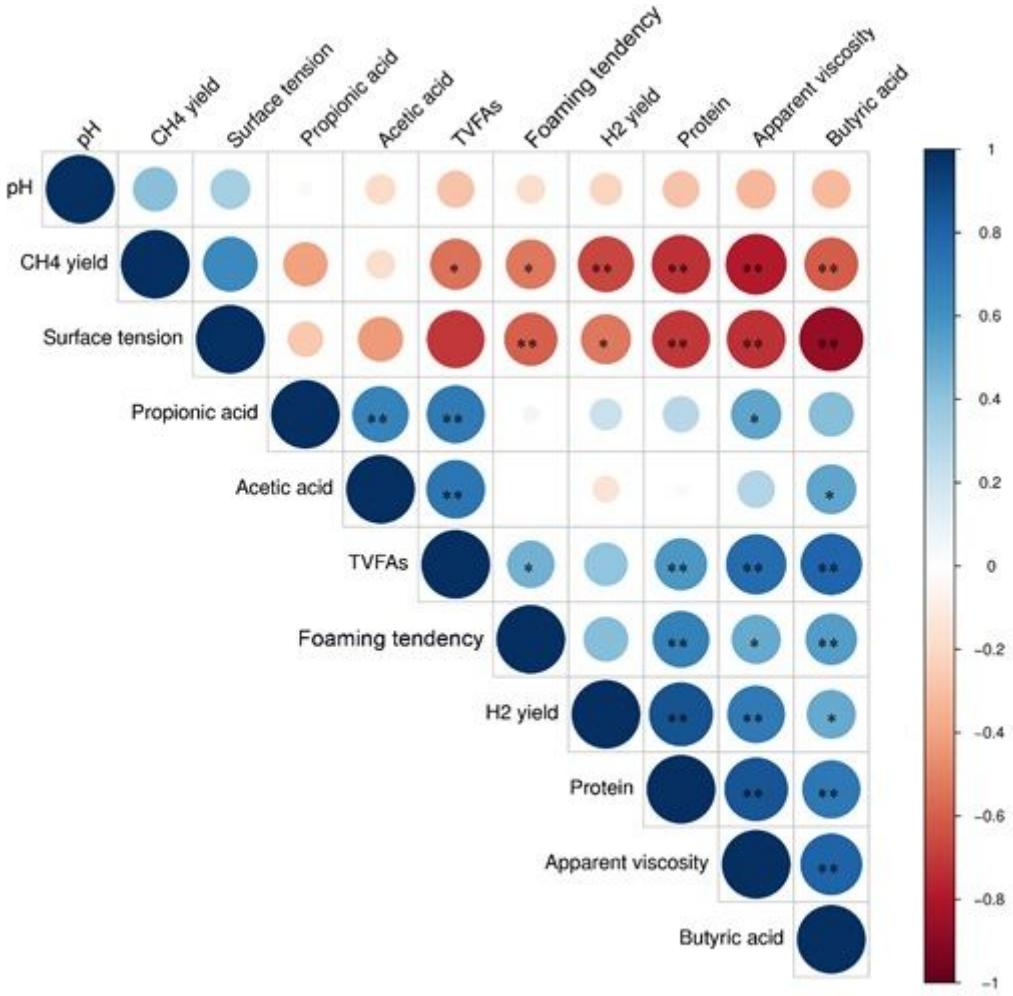
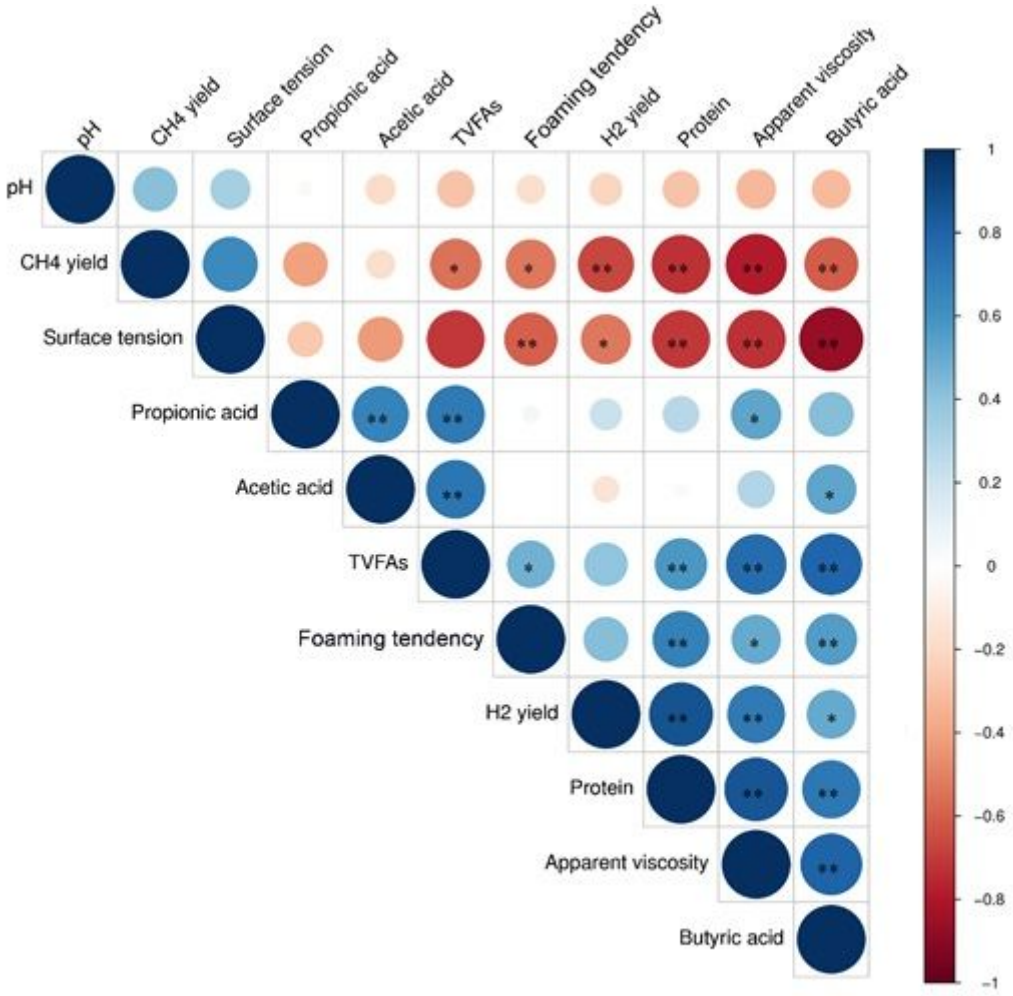


Figure 4

Correlation between the foaming index and digester performance in AD. The differences were considered significant at $p < 0.05$, and are referred to as *$p < 0.05$, **$p < 0.01$.



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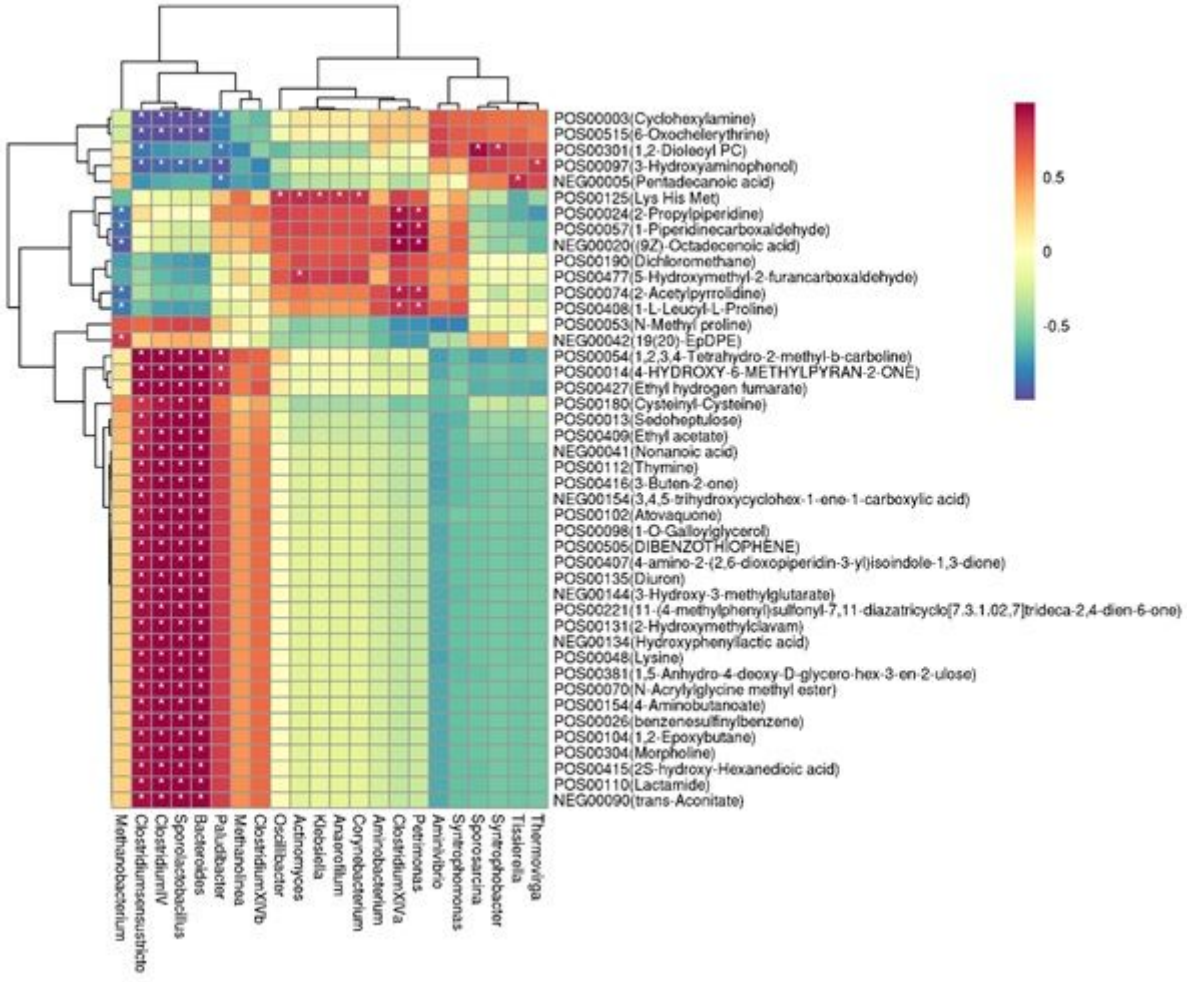
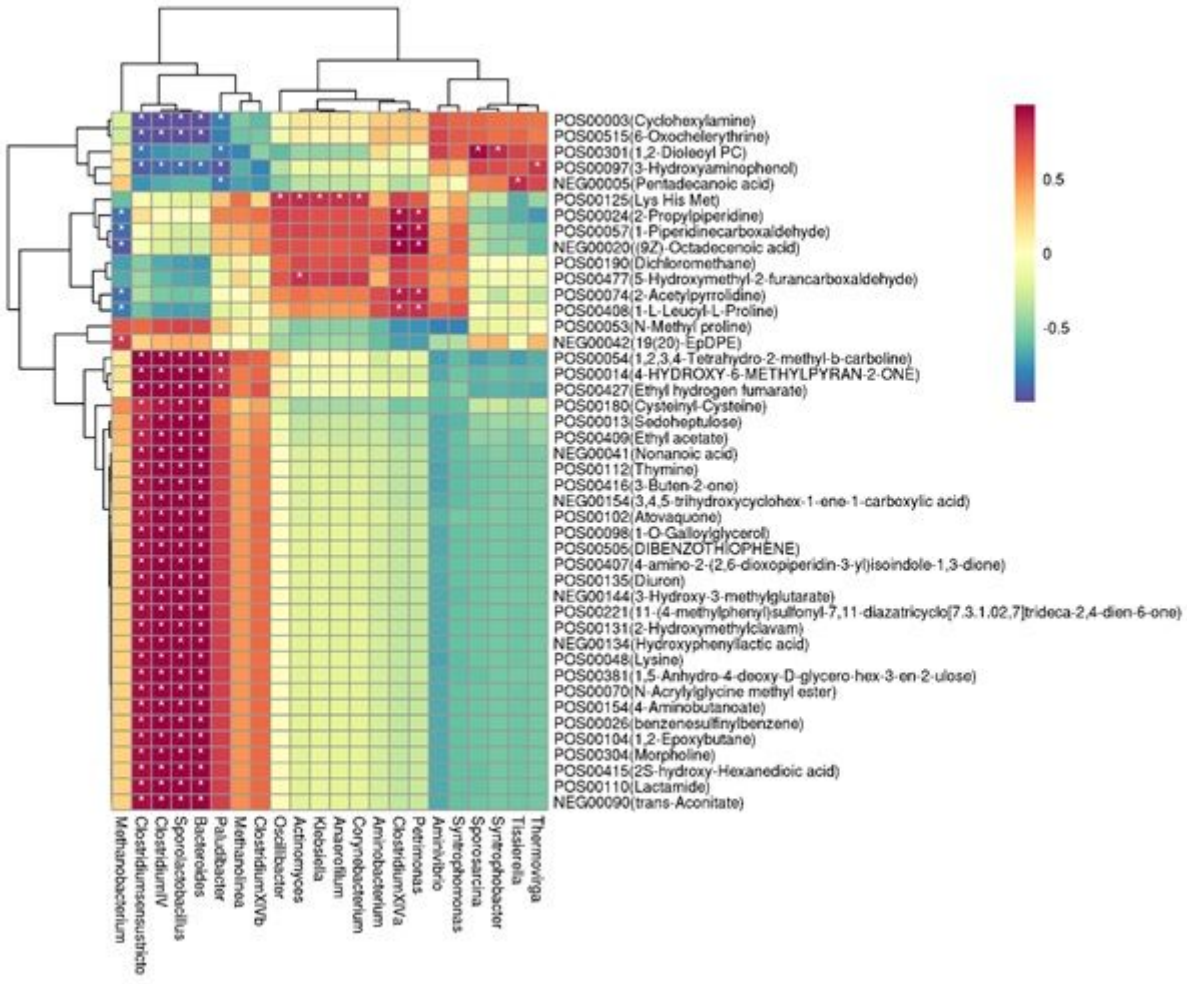


Figure 5

Correlation analysis of certain microbiota genera and altered metabolites. Heat map summarizing the Pearson correlations (|R|>0.5, Top 200 relationships in rank) of microbiota genera and metabolites which explaining the difference between OLR with 0.47 g VS/L·d and OLR with 14.68 g VS/L·d. The differences were considered significant at $p < 0.05$, and are referred to as *$p < 0.05$, **$p < 0.01$.



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