

## Article

# Restoring the Stability of Long-Term Operated Thermophilic Anaerobic Digestion of Maize Straw by Supplying Trace Elements

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**Abstract:** Maize straw has been widely used for the production of energy through anaerobic digestion, but biogas production can be hindered by a lack of trace elemental nutrients. To address this issue, a lab-scale anaerobic plug flow reactor was continuously operated at 55 °C for 300 days, with a hydraulic retention time of 42 days and an organic loading rate of 2.1 g total solids/(L·day). Results from this study showed that between days 101 and 194, the methane yield slightly decreased from  $0.26 \pm 0.04$  to  $0.24 \pm 0.03$  L/g volatile solids (VS), but significant volatile fatty acid accumulation was observed by reaching up to  $2759 \pm 261$  mg/L. After trace elements were added to the reactor, the methane yield increased to  $0.30 \pm 0.03$  L/g VS, with 53% methane content. Around 62% of the total chemical oxygen demand and volatile solids were broken down into methane. Volatile fatty acid levels dropped and stabilized at around  $210 \pm 50$  mg/L, indicating restored process stability. The addition of trace elements increased the abundance of Firmicutes and decreased Synergistetes in bacteria while simultaneously increasing the abundance of *Methanosarcina* in archaea. In conclusion, trace element supplementation was experimentally found to be necessary for stable thermophilic anaerobic digestion of maize straw.

**Keywords:** thermophilic anaerobic digestion; maize straw; trace elements; long-term operation; stability



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## 1. Introduction

Lignocellulosic biomass, which includes farm and forest waste, energy crops, and grasses, is the most sustainable natural bioresource on Earth [1]. China generated around  $8.6 \times 10^8$  tons of straw products in 2020, the majority of which was from maize harvests [2]. On average, 580 million tons of processed byproducts are produced annually, of which <40% are exploited [3]. Therefore, it is important to promote the multifaceted exploitation of agricultural products and byproducts. Maize straw can be valorized as a source of sustainable energy through anaerobic digestion (AD). Under anaerobic conditions, the maize straw, which serves as an organic substrate, is converted to biogas, i.e., a mixture of mainly carbon dioxide and methane, and digestate by a wide diversity of micro-organisms [4].

To treat and maximize the value of maize straw, anaerobic digestion has proven to be an effective and promising technology. Hence, it is capable of producing clean energy (biogas) with minimum odor issues, reducing the overall volume of waste that is disposed of, and lowering emissions of greenhouse gases [5]. Nevertheless, aside from overcoming the tough biodegradability of lignocellulosic substrates, including maize straw, the absence of necessary nutrients frequently impairs the efficiency of biogas and methane production during the mono-digestion of maize straw by AD in industrial biogas plants [6,7]. Thus, reactor instability and accumulation of volatile fatty acids (VFAs) have been observed by prior investigations after a specific treatment period and a process failure if no external

nutrition and buffering agents were given [8,9]. An alternative method to optimize the nutrient balance of maize straw for AD feedstocks is via supplementation of trace elements. Irrespective of reactor design, trace element deficiency was considered a common cause of failure in AD systems during straw digestion [10,11]. The impacts of deficiency of TEs on the stability of mesophilic anaerobic digestion of maize straw have been reported through long-term operation experiments [12]. However, whether the thermophilic anaerobic system also requires addition of trace elements for maize straw has not been investigated. An adequate quantity of trace elements not only encourages the degradation of VFAs [13] but also increases the effectiveness of VFA conversion [14]. Furthermore, trace elements are crucial for the development of methanogens and are the primary determinant of how effectively anaerobic digestion converts organic matter to methane [15].

Of note, the accumulation of VFAs often leads to system acidification and failure [16]; therefore, it is necessary to determine whether or not trace elements can be added to alleviate the acidification caused by maize straw mono-digestion. In addition to that, long-term mono-digestion of maize straw may also result in biological failure due to the paucity of these essential trace nutrients [16]. The obvious concern is centered on the question of whether or not the anaerobic digestion of maize straw and long-term operation without co-digestion and trace element supplementation would be feasible. In AD, the removal of each ton of chemical oxygen demand (COD) requires 200–450 g of Fe, 6–54 g of Co, and 6–49 g of Ni [17,18]. Based on the industrial-grade metal element prices, the cost of removing each ton of COD ranges from CNY 0.2 to 1.4, making it a low-capital investment method.

Optimizing the levels of trace elements necessary for methane production to simultaneously reduce costs and minimizes the risk of trace element release into the environment, requiring in-depth awareness of the impacts and efficacy of trace elements in anaerobic digestion [19]. Previous researchers have studied the effects of trace elements on different substrates, such as food waste [20], stillage [21], manure [22], and highly concentrated organic wastewater [14], mostly in traditional reactors. Meanwhile, few studies exist on the impact of adding trace elements to straw crops for mono-digestion to preserve long-term biogas yields.

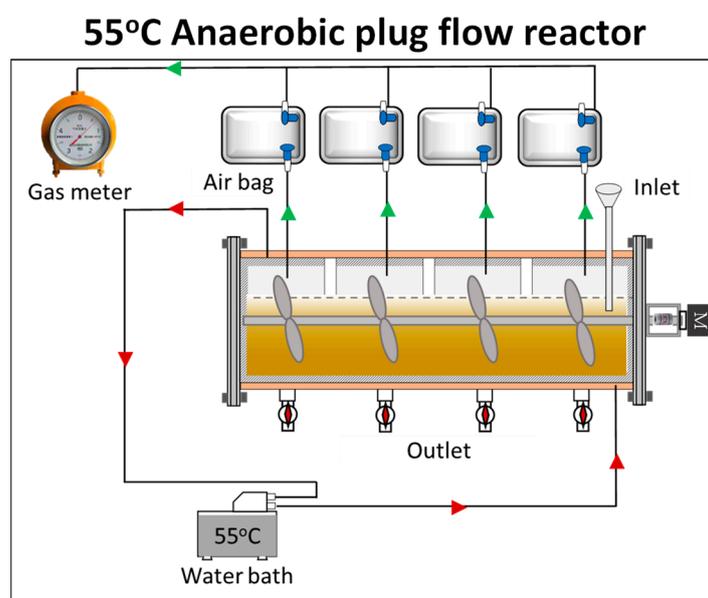
When considering the substrate composition, amount of substrate to be treated, and process economy, a plug flow reactor was used because it is known for its lower cost, more stable performance, and ability to withstand environmental stress [23]. Moreover, researchers have also investigated the effectiveness of plug flow reactors on organic substrates with solid content in the range of 11–14% total solids (TS) [24,25]. Biogas production in a plug-flow reactor may be more favorable than in a typical reactor [26], and the role of trace elements in a plug-flow reactor may be different, but this is unknown at this time. Most studies mainly employ a mesophilic temperature (37 °C) to study the digestion of crop energy, and to the best of our knowledge very few studies have reported on thermophilic anaerobic mono-digestion of crop energy. Microorganisms in anaerobic digestion are extremely responsive to variations in temperature [27], and high temperature could indeed enhance hydrolysis and physical degradation of the substrate, increase the decomposition of organic matter, and boost methane yields during aerobic fermentation [28].

The temperature of the plug flow reactor was set at 55 °C for this investigation of the long-term performance of the anaerobic mono-digestion of maize straw. To this end, the mass balances of some parameters, including COD balance and TS and volatile solids (VS) balance, in biogas production of the maize straw were evaluated. Furthermore, a material flow assessment was carried out to determine the performance of this technology in converting maize straw. The novel contribution of this work lies in investigating the long-term anaerobic mono-digestion of maize straw in a plug flow reactor and assessing the supplementary role of trace elements in the restoration of system acidification.

## 2. Materials and Methods

### 2.1. Reactor Design and AD Experiment

The construction and design of a horizontal plug flow reactor for a continuous anaerobic digestion process was carried out. The entire volume of the reactor is 30 L, and the working volume is 21 L. A timer that worked for five minutes every half an hour was used to achieve the intermittent stirring at 60 rpm/min. The thermophilic conditions were maintained by circulating water from the heater water through a water pump (Sensen HQB-2200, China). A thermostatic water bath with a thickness of around three centimeters was used to maintain the temperature at 55 °C. To prevent any further loss of heat, the reactor was shielded with a bubble insulator made of aluminum foil that measured 6 mm in thickness. The reactor was set to a semi-continuous feeding mode, with an input and output of 500 g each day. The reactor's hydraulic retention time (HRT) was set to 42 days, and, following that, a 300-day experiment was performed. The schematic representation of the plug flow reactor system is illustrated in Figure 1. Biogas production and composition were monitored every day, and the digestate was extracted for the biochemical property analysis every 3 or 5 days.



**Figure 1.** The schematic representation of the plug flow reactor system.

### 2.2. Substrate and Inoculum

The maize straw utilized in the aforementioned study was obtained from the China Agricultural University's Shang Zhuang Experimental Station in Beijing, China. After being returned, the maize straw was initially cut into small segments with a length below 10 cm using a chopping knife and left to air-dry at room temperature. Subsequently, it was introduced into a continuous grinding machine (HBM-103B, 2840 rpm) and a crusher (800A, 35,000 rpm) to be crushed into a mixture of rod-shaped and powdered particles with a length of approximately 1 cm. The maize straw was then sieved through an 18# mesh sieve (1.0 mm size). To prepare the substrate, the maize straw was mixed with a liquid fraction of centrifuged cattle manure digestate from an biogas plant to guarantee that the TS of the mixture was 10–15% and soaked at 55 °C for one day. Table 1 summarizes the most important characteristics of the substrate and the inoculum used during the investigations. Meanwhile, the reactor's organic loading rate (OLR) was set to 2.1 g TS/(L·day) for long-term operation. The quantitative method of trace metals to be used in this experiment was discussed in a previous study, and the addition of trace elements was conducted in accordance with the same study [12].

**Table 1.** The main characteristics of the substrate and the inoculum.

Parameters	Unit	Maize Straw	Inoculum
TS	%	91.0 ± 0.2	2.1 ± 0.1
VS	%	88.3 ± 0.1	1.2 ± 0.1
pH	/	/	7.73 ± 0.13
TVFAs	mg/L	/	0.2 ± 0.1
TAN	mg/L	/	1.5 ± 0.1

TVFAs: total VFAs; TAN: total ammonia nitrogen.

### 2.3. Analytical Methods

pH, total nitrogen, TS and VS, ammonium nitrogen, and other biochemical parameters were determined according to the American Public Health Association (APHA) methods. Particulate COD (PCOD) is defined as the remaining fraction of total COD (TCOD) after subtracting soluble COD (SCOD). The VFAs and the components of CH<sub>4</sub> and CO<sub>2</sub> in the biogas were tested by gas chromatography (Shimadzu GC-2010 plus, Kyoto, Japan) and gas chromatography (Shimadzu GC-8A, Japan), respectively.

### 2.4. Data Analysis

A regression curve was built to characterize the total biogas and methane production data obtained experimentally through a cumulative gas production model referred to per unit of volume of the substrate. The cumulative gas production was only performed for the stable state period. To this end, the Gompertz equation model (1) was used.

$$P = P_0 \exp \left\{ -\exp \left[ \frac{R_{\max} e}{P_0} (\lambda - t) + 1 \right] \right\} \quad (1)$$

where P is the cumulative methane production at time t in mL. P<sub>0</sub> is the maximum methane production potential in mL. R<sub>max</sub> is the maximum methane production rate in mL/d. λ is the lag phase duration in days. t is the experimental time in days. e is the constant (approximately 2.7183).

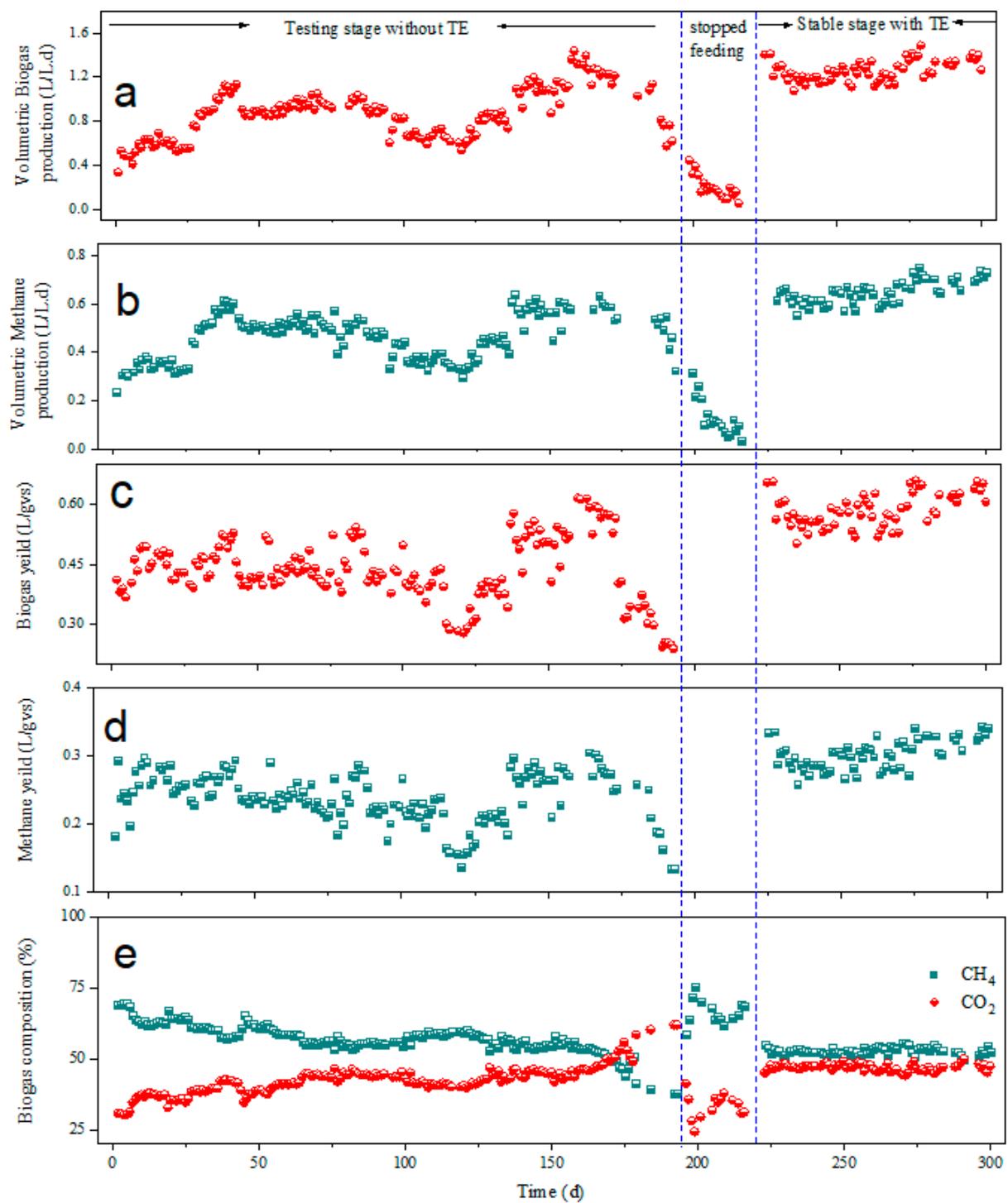
## 3. Results and Discussion

### 3.1. Biogas Production

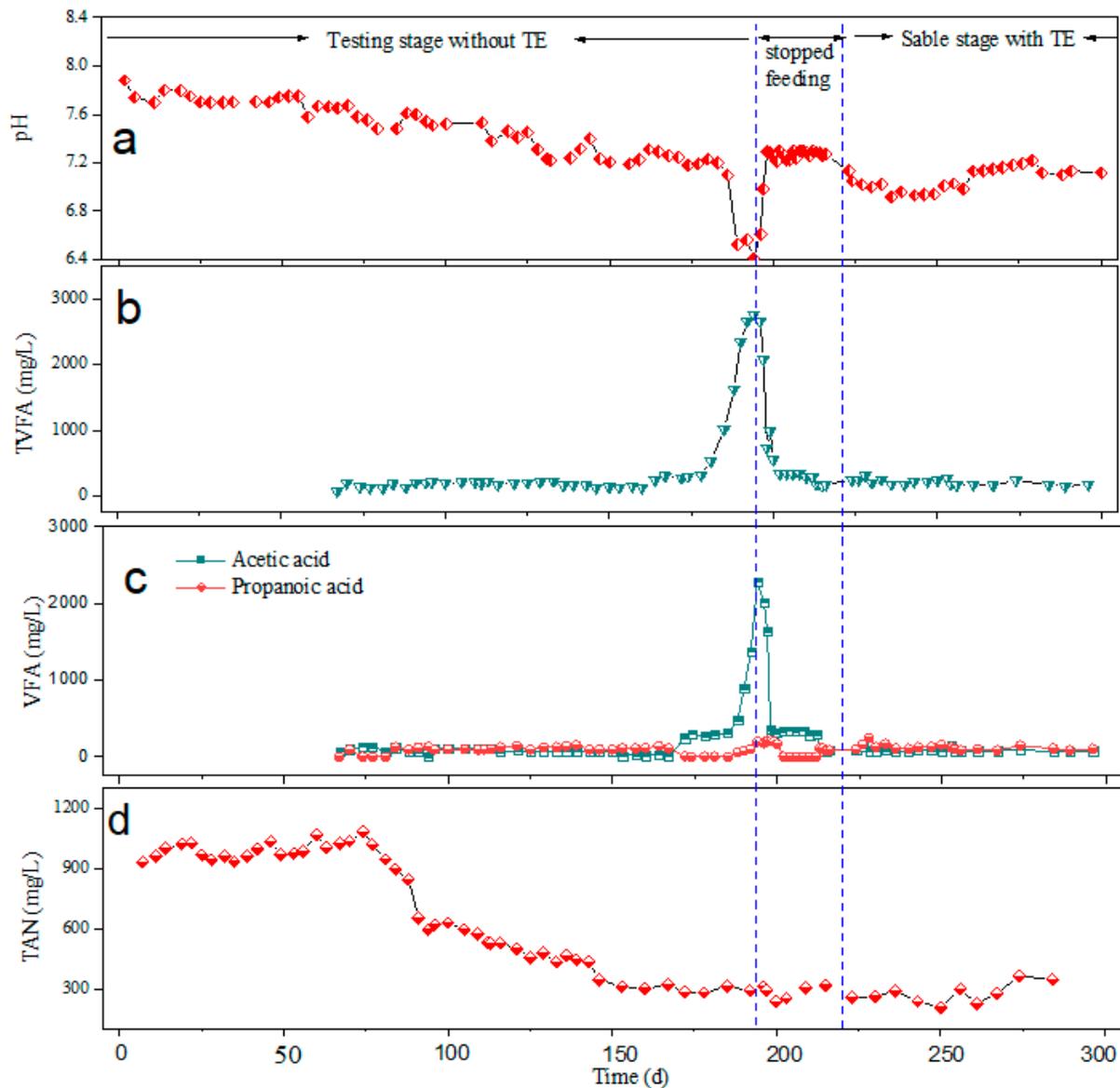
Figure 2 illustrates the daily volumetric biogas yield, gas composition, pH, and VFAs from the reactor during the long-term AD process. The digestibility of the straw was evaluated. The operation parameters and reactor performance are summarized in Figures 2 and 3. The volumetric biogas and methane increased gradually during the initial phase and then increased rapidly from 0.33 ± 0.02 and 0.24 ± 0.03 L/(L·d) to a maximum of 1.12 ± 0.04 and 0.62 ± 0.03 L/(L·d) within 42 days; this might be a result of the thermophilic hydrolytic bacterial population that accelerated the hydrolysis process [29]. This was followed by a gradual reduction in the volume of both biogas and methane production. This must have occurred from a decrease in the supply of convertible organic matter inside the biogas production system as observed by other studies [30]. During this period, a slightly stable period could be achieved, and only a small fluctuation occurred within days 95–136. The system's biogas production increased drastically again to its maximum, which was then followed by a drastic decrease in production after 193 days of operation. During this phase, methanogenesis might have been inhibited, resulting in the precipitous decline. Therefore, the reactor was no longer being fed on the 194th day for 25 days. In parallel, the VFA concentration (Figure 3c) continued to increase leading to its accumulation, hence causing the system's instability. The reactor was then fed after supplementing with trace elements [12] for additional days (223–300) to check the stability of the process under this condition. The biogas and methane production were kept stable with an average of 1.25 ± 0.1 and 0.65 ± 0.1 L/(L·d), respectively, at the end of this period. Generally, the actual volumetric biogas and methane produced after supplementation of trace elements

were within the ranges of 1.2–1.5 and 0.4–0.8 L/(L·d), respectively. Overall, the volumetric biogas and methane yields increased by 67.2% and 67.7%, respectively, after the addition of trace elements. Our result accords with a study by Zhao et al. [31], who reported that the biogas volume increased by 43.4% when Fe, Co, and Ni were supplemented in the digester. Likewise, Liu et al. [32] reported 61.8%, 55.7%, and 50.8% increments of biogas volume after the addition of Fe, Co, Ni, Mo, and Se in a maize stover substrate. The biogas yield (Figure 2c) also followed a similar pattern to the daily volumetric. The average biogas and methane yields during the first 26 days were  $0.44 \pm 0.04$  L/gVS and  $0.26 \pm 0.03$  L/gVS, respectively. Average biogas yield increased by 4.5% from 26 days to 194 days of operation, while the average methane yield during these periods remained unchanged. After supplementing with trace elements, the average biogas and methane yield increased to  $0.58 \pm 0.04$  L/gVS and  $0.30 \pm 0.03$  L/gVS, which is approximately 32% and 16% increment, respectively. In previous studies on thermophilic AD of maize straw, the methane yield reached levels of 0.18–0.22 L/gVS [33], which were comparable to levels before the addition of trace elements but significantly lower than the methane yield after the addition of trace elements. This further supports the positive effect of adding trace elements to methane production from maize straw. To this end, it can be inferred that trace elements are indeed essential factors in promoting the metabolism of methanogens [34]. The trace elements have a strong connection to the metabolic processes of microorganisms and are among the most important variables in ensuring the reaction's stability [35]. Reports show that the key trace elements needed to increase the activity of the methanogens studied thus far in the anaerobic digestion were Fe, Co, and Ni [35–37], and the absence of them will have an impact on maize straw anaerobic digestion [38,39]. A sufficient quantity of trace elements can activate the activity of relevant enzymes, such as F420, thereby enhancing the conversion of VFAs to methane and alleviating the acidification process in the reactor [12]. As a result, we can conclude that thermophilic mono-digestion of maize straw supplemented with trace elements may provide significant benefits to the economy by increasing biogas generation for a specific quantity of maize straw.

Biogas composition is presented in Figure 2e. The average methane content in the biogas was 65% during the first 26 days; in the subsequent days, the methane content was 58% and 56% for 27–100 and 101–194 days, respectively. During 169 days of operation, the methane content was within the range of 50–69%. After 169 days of operation, methane decreased rapidly to  $37.0 \pm 1.5\%$ , while the proportion of  $\text{CO}_2$  evolved to  $62.0 \pm 1.8\%$ . This could be attributed to the accumulation of VFAs [40]. The methane content gradually increased when the reactor was not fed for some days, and the methane content measured ranged from 51% to 72%. This may be because the pH was slightly raised, returning to the ideal pH range of 7–8 following a minor reduction [41] for anaerobic digestion. Shortly after re-feeding, alongside supplementation of trace elements the process began to show signs of stability, with the methane content reaching  $53 \pm 1\%$  by the end of the operation. Examining the variations in digestate pH revealed that pH values slightly fluctuated throughout the operation, though they were within the appropriate pH conditions for anaerobic digestion, showing sufficient buffering abilities [42], until day 189, where the pH dropped below 7.0. Here, the reduction in pH could be related to an accumulation of organic acids caused by methanogens' failure to convert organics to biogas and methane [43]. After the reactor feeding ceased, the pH returned to around 7.3 and remained stable for a while, and afterwards the addition of trace elements initially decreased the pH again but gradually increased shortly afterwards and remained within the range of 7.00–7.22 until the end of the operation, which could be explained as good bacterial and methanogen cooperation [44].



**Figure 2.** Gas production during the anaerobic digestion process. (a) Volumetric biogas production; (b) volumetric methane production; (c) biogas yield; (d) methane yield; (e) biogas composition.



**Figure 3.** Stability performance of the reactor. (a) pH; (b) TVFA; (c) VFA; (d) TAN.

### 3.2. Process Stability Performance

Figure 3 shows a closer examination of the alterations in the maize straw digestate's properties over the entirety of the AD process, such as VFA and TAN values. VFAs (Figure 3b), on the other hand, function as precursors that may indicate the biochemical condition of syntrophic anaerobic consortia. The predominant VFAs were acetic acid and propionic acid, which are the most prevalent acids in anaerobic digestion, and these comprise more than 90% of TVFA. This observation corroborated the outcomes of other investigations [7,45]. In this study, the VFAs were evaluated after 66 days of operation, and it can be observed that the TVFA was stable and then peaked on the 194th day to a maximum of  $2759 \pm 261$  mg/L. Acetic acid increased up to  $2287 \pm 183$  mg/L, while the concentration of propionic acid was low at  $196 \pm 20$  mg/L, and other acids included were up to  $276 \pm 14$  mg/L. In general, acid inhibition occurs in an anaerobic digestion system when the acetic acid concentration exceeds 2000 mg/L [46]; therefore, in this study, acetate concentrations were accumulated to a level of inhibition. Reports on thermophilic operations reveal that, under conditions of low VFA concentration, a collection of hydrogenotrophic methanogens prevails, while the accumulation of VFA induces the growth of acetolactic methanogens [47]. Another study by Jiang [48] proposes that the specific methanogenic

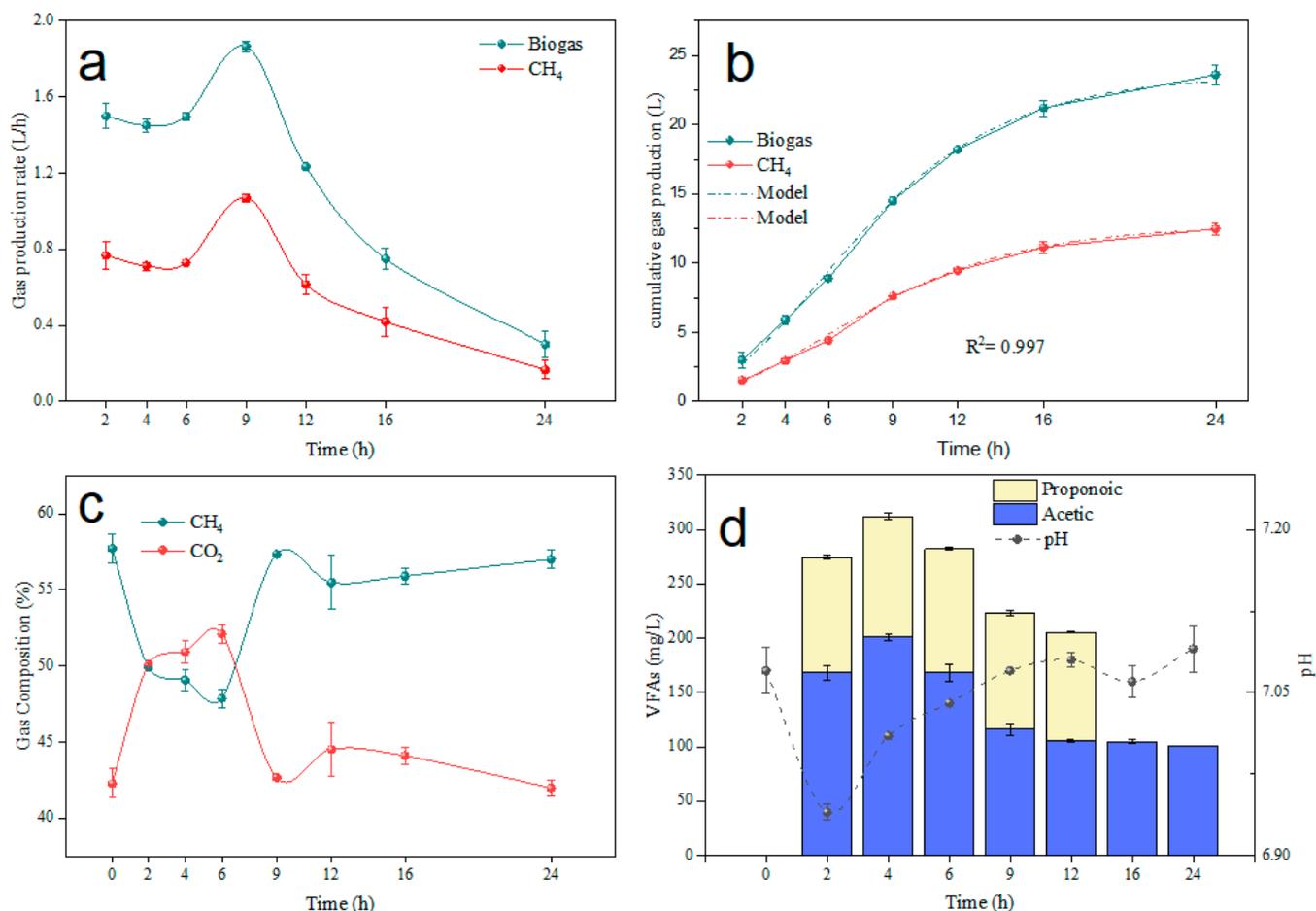
pathway is dependent on the acetate degradation status in the anaerobic digestion reactor. This suggests that, in this study, the growth of acetolactic methanogens was inhibited, hence the accumulation of acetate, whereas the bacterial responsible for propionate degradation were also predominant, leading to their low concentration. Moreover, it has been stated previously that VFAs are closely related to dissolved hydrogen [49,50]. This further implies that dissolved hydrogen directly or partially affected the prevalence of hydrogenotrophic methanogens relative to VFA degradation [47]. This could explain the low biogas and methane production in the system during this period. In other words, the accumulation of VFAs within 194 days induced an episodic partial blockage of the action of hydrolyzing fermentative bacteria. However, after more than 20 days of not feeding the reactor, VFAs dropped to 174 mg/L. It is possible that hydrolysis bacteria activity had resumed, and VFA production by acidogenesis and acetogenesis bacteria had begun. On the contrary, upon the addition of trace elements, it was observed that VFAs were stable until the end of the experiment, which was below 250 mg/L. This phenomenon suggests that adding trace elements might greatly boost the effectiveness of VFA utilization, which may be associated with an increase in methanogenic activity [12,51].

Another aspect to be considered is the TAN. Throughout the experiment, the ammonia concentration remained lower than the stated inhibitory limit of 3500 mg/L [52]. However, the highest TAN concentration occurred on the 19th day, recording  $1173 \pm 75$  mg/L. With trace element supplementation, TAN in the system slightly increased towards the end of the experiment. The observed maximum biogas and methane yield, as well as the stable operation of the anaerobic digestion system, supported non-inhibitory TAN levels in this system.

### 3.3. The Complete Degradation of Maize Straw in a Feeding Cycle

Another series of investigations recorded the hourly gas output in the digester operating under the same conditions as the long-term but with the addition of trace elements. Degradation studies lasting 24 h within a single feeding cycle are commonly used to assess feedstock decomposition capabilities [53]. In Figure 4, it was discovered that there was no consistency in gas output during 24 h. The maximum amount of gas was produced in the first hours, and there was a surge in gas production at more or less regular intervals. More gas was produced during the first 9 h of anaerobic digestion. The cumulative gas production was determined. Biogas reached 23.6 L, with methane at 12.4 L. No lag phase occurred. Notably, 90% of biogas was generated between hours 1 and 16. Additionally, the composition of biogas changed over time. The hydrolysis and acidification were more active in the first 6 h, and the pH decreased to below 7.0, which significantly increased the CO<sub>2</sub> concentration. Nevertheless, the CH<sub>4</sub> content was maintained at roughly 55.4% after 9 h of digestion.

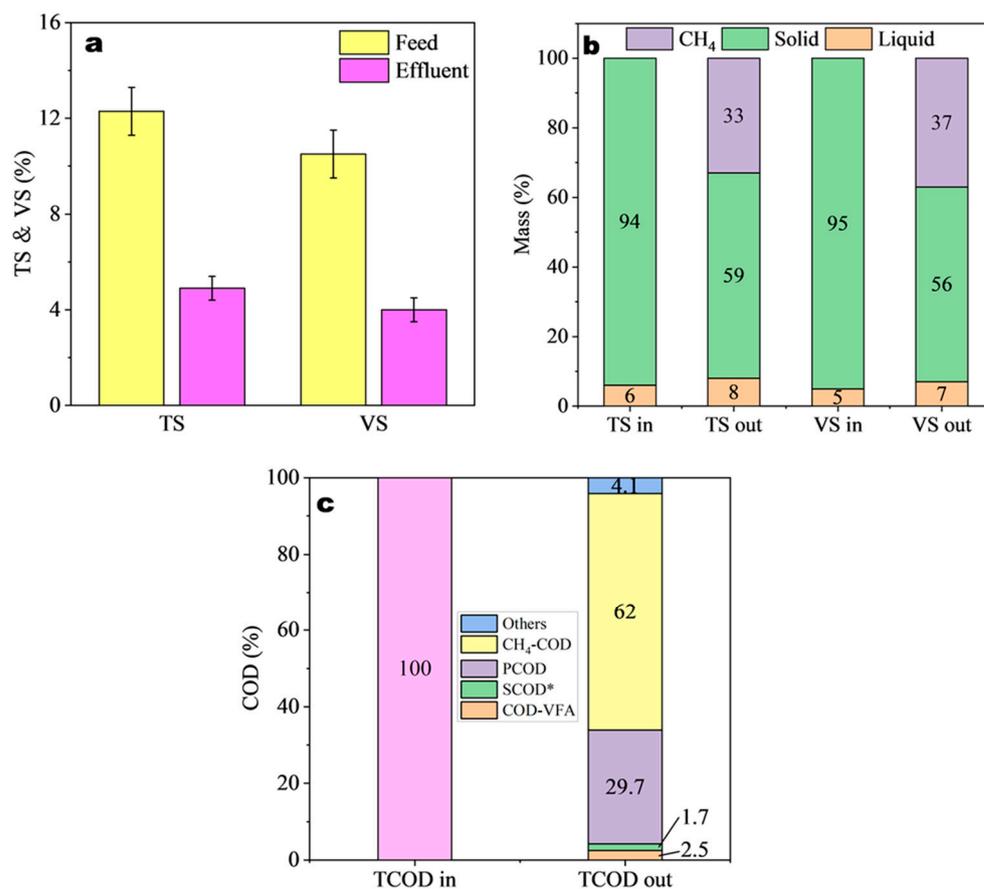
In Figure 4d, the VFA concentration increased and eventually decreased to  $101 \pm 12$  mg/L, which is consistent with recent research on food waste [54] and synthetic waste with coffee powder at 55 °C [55]. VFAs accumulated significantly in the first 6 h, reaching a peak of over 300 mg/L, leading to a significant decrease in pH. The accumulation and degradation processes of VFAs were nearly complete around 16 h, supporting more than 85% of the maximum cumulative methane production at this point. These findings indicate that organic matter was decomposed effectively with a single feed cycle.



**Figure 4.** Biogas production and VFA variations over 24 h. (a) Gas production rate; (b) cumulative gas production; (c) gas composition; (d) VFAs.

### 3.4. Material Flow and Mass Balances in the AD

The mass balances of different parameters, including COD, VS, and TS as well as TS and VS removed, are presented in Figure 5. The variability of TS and VS concentration is frequently connected to a microbial population in the reactor. Thus, this could be largely described as microbial growth and reproduction [42]. Further, changes to TS and VS in AD represent substrates transforming to methane and carbon dioxide via microbial metabolism, resulting in a stable drop in TS and VS [56]. Herein, the change in the absolute mass of the substrate before and after anaerobic treatment, TS, and VS was calculated for the stage of adding trace elements. Moreover, the degradation rates of TS and VS in anaerobic digestion are important indicators with which to evaluate the status of the anaerobic system [57]. Figure 5a depicts the TS and VS variations in the anaerobic digestion of maize straw in this study during the stable period. During this period, the final TS and VS degradation rates were 60.4% and 61.9%, respectively. In other words, a higher TS and VS degradation rate may result in increased methane production. In addition to that, the mass balances of TS and VS bioconversion were calculated based on the fractions of gas, liquid, and solid as represented in Figure 5b. It is clear that the majority of the maize straw's digestate at this time was solid, with TS and VS results of 59% and 56%, respectively. The liquid digestate was only 8% and 7%, whereas 33% and 37% were converted into biogas of the TS and VS, respectively. The rate of transformation of organic matter per COD was seen when the COD was around 124 g/L and the TS and VS were approximately 12.7% and 11.9%, respectively. This indicates that the amount of bioenergy produced per unit of substrate utilized is higher in the maize straw that has been enriched with trace elements.

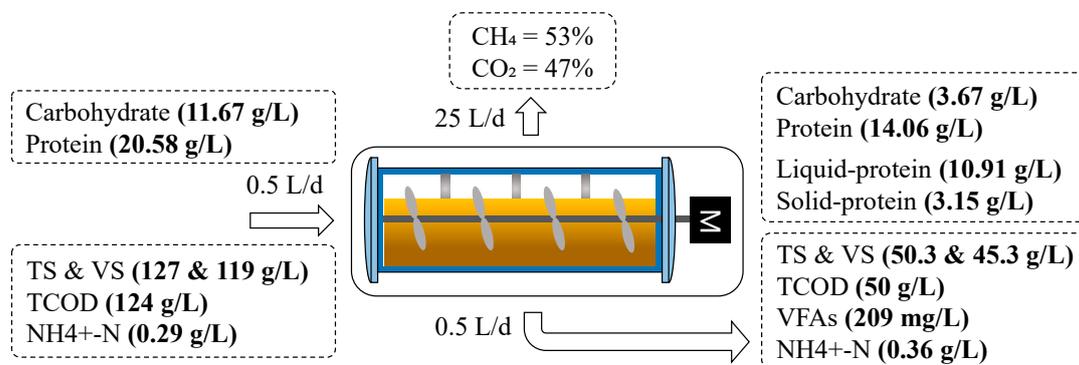


**Figure 5.** TS/VS removal, mass balances based on TS/VS, and COD.

The COD mass balance that is displayed in Figure 5c describes the percentage of non-degraded organic matter that was estimated as TCOD, and the mass percentage of the influent was 100% (124 g/L) while the system was in a stable state. The anaerobic process resulted in approximately 62.0% of the TCOD that had been hydrolyzed out being degraded and transformed into methane gas, and this occurred after the operation. It is important to note that the COD of methane gas was determined by applying the Buswell–Mueller formula with a theoretical coefficient of 0.35 L CH<sub>4</sub>/g COD. This was carried out to arrive at the final result. In the effluent, the levels of PCOD and COD-VFA were 29.7% and 2.5%, respectively. The portion of PCOD that persists after AD may consist of hard-to-degraded lignin and part of cellulose protected by lignin in maize straw. In comparison, there was only 1.7% of SCOD\* (excluding COD-VFA) that was still present.

During the stable operation time of the digester, i.e., when trace elements were being introduced, an evaluation of the material flow of the maize substrate was carried out. It can be seen in Figure 6 that the influent was fed at a volume of 0.5 L/d as the substrate. This influent had an initial TCOD concentration of 124 g/L, as well as TS and VS concentrations of 127 and 119 g/L, respectively. The quantities of protein and carbohydrates in the substrate were also examined and found to be around 11.67 and 20.58 g/L, respectively. The organic matter was transformed into biogas at a rate of 25 L/d in the system, which contains 53% methane and 47% carbon dioxide. The effluent had a TCOD concentration that was lower than 50% (50 g/L); the TS and VS concentrations were 50.3 and 45.3 g/L, respectively; and the TVFA concentration was 209 mg/L. At the same time, the quantity of carbohydrates and proteins that were present in the effluent dropped to 3.67 and 14.06 g/L, respectively. During protein degradation in the system, most protein remained in the liquid phase, which was about 10.91 g/L, whereas 3.15 g/L was present in solid form. It was noted that about 68.6% of carbohydrates were removed, while protein decomposition was around 31.79%. Therefore, among the main organic components producing methane, carbohydrates

accounted for the largest proportion, while protein was the smallest. Several studies reported that throughout anaerobic digestion, carbohydrates decompose more effectively and faster than protein [53,58], and the rate of hydrolysis constant for carbohydrates is 0.5–2/d, which is greater than the rate constant for protein, that is 0.25–0.8/d [54]. In contrast, because ammonium nitrogen is produced during protein biodegradation [53], it has been established that when ammonium nitrogen concentrations are above 1500 mg/L, inhibitory effects in an AD system can be triggered [59]. Meanwhile, ammonium nitrogen in our study was within 300–1173 mg/L, which is way below the range of the inhibition. Of note, we can strongly infer that a lower protein decomposition rate in this study might be a major factor in the system's failure to significantly accumulate ammonium nitrogen. The concentration of TAN in the effluent rose significantly to 0.36 g/L, which is attributed to the anaerobic biological conversion of proteins that make up the substrate into amino acids and eventually ammonia [60]. Overall, only 6.52 g/L of protein was degraded (over 31.79%), while 53.0% was dissolved, and the undissolved protein was 15.3%.



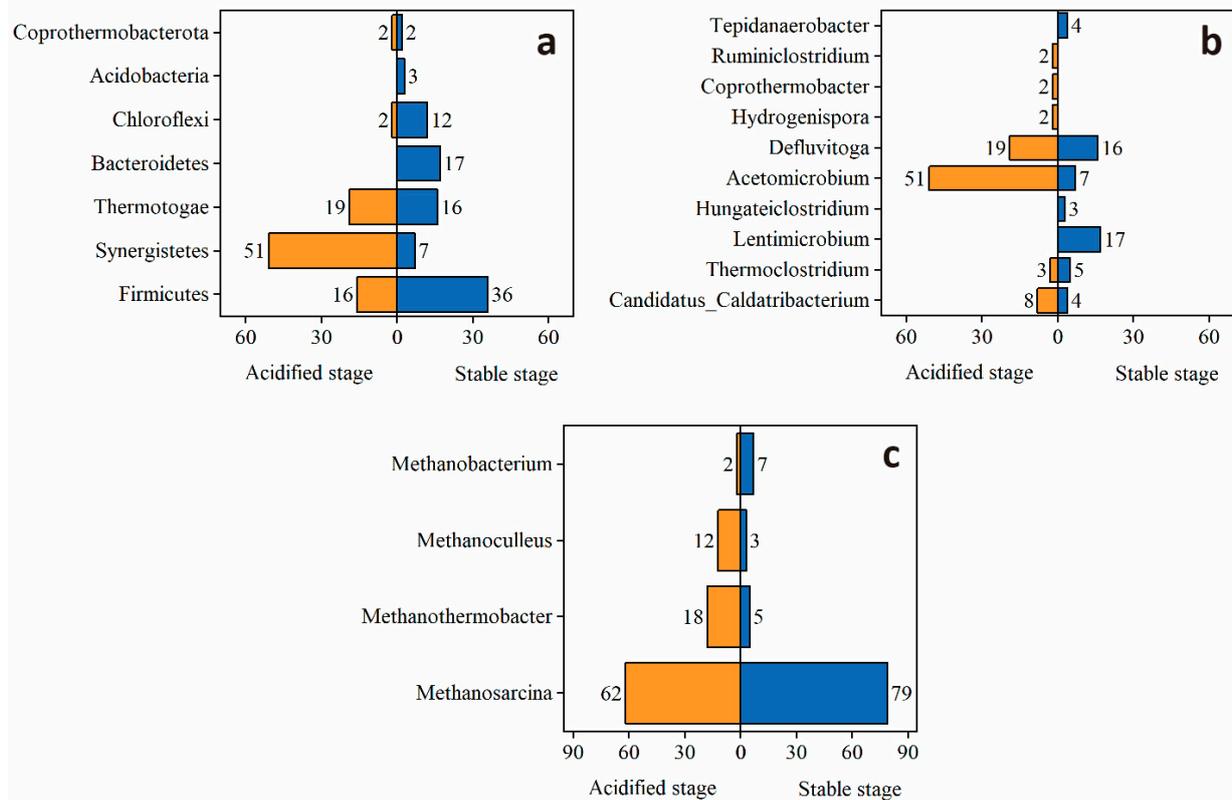
**Figure 6.** Material flow during the stable state of AD.

### 3.5. Microbial Community Analysis and Diversity of Bacteria and Archaea

Microbial communities are shown in Figure 7a,b,c for bacteria phyla level, genus level, and archaea genus level during the acidified and stable stages. The phyla with relative abundances greater than 1% in the substrate during the entire operation were Firmicutes, Coprothermobacterota, Chloroflexi, Synergistetes, and Thermotogae. Synergistetes was dominant during the mono-digestion process. The relative abundance changes in bacterial communities at the genus level showed that seven genera with a relative abundance greater than 1% were detected in the acidified stage, dominated by *Acetomicrobium*, *Defluviitoga*, and *Candidatus\_Caldatribacterium*. The addition of trace elements altered the composition of the bacteria. At the phylum level, the dominant bacteria were Firmicutes, Bacteroidetes, Thermotogae, Chloroflexi, and Synergistetes. Firmicutes accounted for the largest proportion followed by Bacteroidetes. Previous studies have stated that the bacteria *Bacteroidetes* and *Clostridia* (Firmicutes) are mostly abundant in cellulose substrates as these bacteria are effective in degrading cellulose into organic acids [61]. Moreover, a study reported that biogas yield is correlated with the abundance of Firmicutes, which is consistent with the results of the current work [62]. The significant increase in Firmicutes species indicates that they have good adaptability to environmental changes. The present study found that the increased diversity of bacteria following the addition of trace elements was favorable for methane production in anaerobic digestion.

In the case of Archaea, the diversity of archaea was significantly lower than that of bacteria. *Methanosarcina* and *Methanothermobacter* were the major genera during the acidified stage. The results of the current study indicated that the addition of trace elements changed the community composition of the archaea, especially the proportion of *Methanothermobacter*. Compared with the acidified stage, the proportion of *Methanothermobacter* decreased from 17.97% to 4.53%, perhaps because *Methanothermobacter* was not sensitive to the addition of trace elements. Meanwhile, *Methanosarcina* increased by 17%, perhaps

because *Methanosarcina* was sensitive to the addition of trace elements. Archaea's fundamental purpose in anaerobic digestion is to produce methane by consuming organic acids, hydrogen, and carbon dioxide. The addition of trace elements to the archaea community did not change dominant archaea. This is comparable to prior research that found that adding Co and Se to a trace-element-deficient substrate did not alter the dominant archaea relative to the control [51].



**Figure 7.** Bacteria ((a) phylum, (b) genus) and archaea ((c) genus) community of anaerobic digestion in acidified and stable stage.

### 3.6. The Comparison of Effects of Adding Trace Elements to Maize Straw and Other Substrates

In this study, the purpose of adding trace elements was to enhance the activity of relevant metabolic processes, thereby accelerating the conversion of VFAs to methane. This alleviates acid accumulation, allowing the reactor to operate stably, with a focus on restoration. In some studies involving AD of other substrates, challenges such as methane inhibition due to high ammonia levels and acid accumulation at high OLR may arise. In such cases, supplementation of trace elements can enhance microbial activity, leading to a significant improvement in methane production. For instance, in the AD of chicken manure, which often experiences elevated ammonia concentrations, methane production is inhibited. By adding Co and Ni etc., during continuous operation at an OLR of 3.6 g-VS/(L·d), the methane yield increased by 117% [63]. In comparison, during the stable phase of trace element addition in this study, the methane yield only increased by 16%. The AD of pig and cattle manure showed methane yield increases of only 17–26% [64,65] and 7–25% [66,67], respectively, after adding trace elements, indicating modest improvements possibly due to low loading conditions without methane production inhibition. Therefore, the addition of trace elements tends to have a relatively small impact during stable and normal AD processes. However, when the reactor is experiencing acidification or methane production inhibition, the addition of trace elements often has surprising rescuing and stabilizing effects.

#### 4. Conclusions

Thermophilic anaerobic digestion is often viewed as less stable, especially when treating some 'pure' substrates like maize straw. In this study, the long-term operation of a thermophilic anaerobic mono-digestion of maize straw in a plug-flow reactor was examined. The results demonstrated the effectiveness of adding trace elements to maintain process stability. With the introduction of trace elements, accumulated VFAs were converted into methane, then the methane production reached  $0.30 \pm 0.03$  L/gVS, and VFAs remained at a low level. The COD mass balance results further showed that most of the biodegradable TCOD had been converted. A total of 29.7% of TCOD consists of hard-to-degraded PCOD, possibly corresponding to lignin. Additionally, the addition of trace elements increased the abundance of Firmicutes and decreased Synergistetes in bacteria while simultaneously increasing the abundance of *Methanosarcina* in archaea. These findings suggest that for industrial anaerobic digestion of maize straw, adding trace elements should be considered a mandatory strategy to maintain stability for thermophilic AD.

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