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# Assessment of Microbial Diversity during Thermophilic Anaerobic Co-Digestion for an Effective Valorization of Food Waste and Wheat Straw

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**Abstract:** In this study, predominant bacterial and archaeal populations and their roles during anaerobic mono-digestion of food waste (FW) and co-digestion of FW with straw pellets (SP) at thermophilic temperature (53 ± 1 °C) were assessed by Next Generation Sequencing (NGS) analysis at organic loading rates (OLRs) of 3.0 and 7.0 gVS/L/d. Depending on the seed; results revealed that *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were, respectively the most prevalent bacterial phyla at both OLRs investigated. On the other hand, *Euryarchaeota* was dominated by methanogens playing crucial role in biogas production and correlated mainly with the activities of *Methanobacteria* and *Methanomicrobia* at class level. Acetoclastic *Methanosaetae* was the predominant genus at OLR = 3.0 gVS/L/d; however, shared the same predominance with hydrogenotrophic methanogens Methanospirillium at the highest OLR. Although no clear effect in response to straw addition at OLR of 3.0 gVS/L/d could be seen in terms of methanogenic archaea at genus level, hydrogenotrophic methanogens revealed some shift from *Methanobacterium* to *Methanospirillium* at higher OLR. Nevertheless, no prominent microbial shift in the presence of wheat straw at increased OLR was likely due to adapted inoculation at start-up which was also demonstrated by relatively stable biogas yields during co-digestion.

**Keywords:** biogas yield; co-substrate; food waste; methanogens; next generation sequencing



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## 1. Introduction

Anaerobic digestion (AD) is a well-established process for the valorization of biomass due to sufficient energy production in the form of biogas. Although the substrates rich in organic content (e.g., food waste, agricultural residues, etc.) could be digested effectively under both mesophilic and thermophilic conditions; the stability of the process is generally a concern due to some operational challenges, such as high acidification potential, high organic loading rates (OLRs), high nitrogen content, etc. For example, high ammonia could inhibit anaerobic reactors dependent on the free form of ammonia ($NH_3$) especially at high pH and temperature values [1–8]. Moestedt et al. [9] reported a threshold concentration of ca. 1.0 g/L for free ammonia nitrogen maintaining the specific methane production in the reactors irrespective of the OLR which is a critical parameter impeding stability, performance, and the cost of AD process. The OLR represents the amount of chemical oxygen demand (COD) or volatile solids (VS) fed per unit volume of digester per day. A short HRT and a high OLR are generally requested in commercial biogas production plants, because in this way high-quantity waste treatment and sufficient biogas production can be achieved. However, if the AD is not properly operated, the process might be inhibited due to the accumulation of volatile fatty acids (VFAs) [8,10,11]. Accordingly, an increase in the concentration of propionic acid, a decrease in pH, and an increase in the $CO_2$ concentration in the produced biogas are the first signs if systems are overloaded. Besides, process imbalance was reported if propionic acid/acetic acid ratio is higher than 1.4 due to quite high OLR in anaerobic digester [12]. There were contradictory results about the impact of

OLR on AD process stability and corresponding biogas yield due to the highly complex nature of AD and its dependency on several parameters. For instance, Liu et al. [13] reported that the methane production from food waste at thermophilic condition was more efficient and stable at a higher OLR (compared to that at mesophilic condition); however Guo et al. [14] observed that the methane yield during mesophilic AD was more stable in response to increasing OLR from 1.0 to 2.5 gVS/L/d. Although the increase in OLR up to a certain level enhanced biogas production; irreversible failure of the process would occur beyond the optimum OLR value due to imbalances between hydrolysis, acidogenesis, acetogenesis, and methanogenesis stages [13]. On the other hand, inappropriate C/N ratio also leads to operational problems such as lower methane yield and longer digestion time. Accordingly, the C/N ratio in the range of 16–25 is recommended as the optimum condition for an effective anaerobic process [15]. Hence, the adjustment of the C/N ratio in the feedstock is generally required in large scale biogas plants especially when a single substrate is digested. In this context; optimizing the aforementioned operational parameters by the application of co-substrates have been gaining importance in recent decades for improving biogas production from various biomass sources (e.g., agricultural and lignocellulosic residues, livestock manure, food wastes) [8,16,17]. For example, co-digestion of food waste with cattle manure could improve the buffer capacity and led to increased acceptable OLRs in comparison with mono-digestion [15]. In other studies, co-digestion with green biomass, such as crop residues and different parts of plants, was reported to stimulate the AD of food wastes [5] whereas co-digestion with lignocelluloses was reported to overcome VFA accumulation and inhibition [18] by providing the optimum conditions, such as the proper nutrient supply, pH, and buffering capacity. Among the lignocellulosic biomass sources; straw (corn, rice, tobacco, wheat) pellets or briquettes are ideal for biogas production mainly in co-digestion, especially with nitrogen-rich substrates (e.g., food waste and chicken manure) [4,11]. Likewise, combining these materials with manure as substrate would also yield a positive effect since the straw is generally used as a bedding material. Hence, by utilizing wheat straw to produce renewable energy; straw disposal problems might be also solved [19]. Since the high content of lignin and the low content of trace elements of straw pellets or briquettes might limit microbial degradation, growth and activity, and thus methane yield despite their remarkable biogas potential, co-digestion is particularly recommended in order to provide a balanced carbon/nitrogen (C/N) ratio in the reactors especially when operated at thermophilic condition (i.e., high operating temperature speeds up ammonia inhibition) [11]. On the other hand, choosing the proper mixing ratio of the feedstocks is also crucial during co-digestion, and hence that should be optimized [8,20].

Despite of the fact that straw, straw pellets, and briquettes are the attractive biomass sources for high energy generation; microorganisms need a high accessibility to degrade these materials. Hence, co-digestion would also allow the activation of cellulose-degrading bacteria. Since the substrate is highly available for these bacteria, their enzyme production potentially increases. Moreover, co-digestion with other wastes is also recommended for better adaptation of microorganisms to inhibitory substances [21]. It is also considered that microbial relationships and metabolic functions need to be clarified for understanding the existing microbial interactions between bacteria and methanogens and how these cultures promote or hinder the performance of AD processes especially at high OLRs. Since biogas generation is carried out by a complex microbial community through multiple biochemical reactions that take place in successive stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) during AD of organic materials; the OLR has substantial impact on the microbial community involved in the process and eventually on the biogas yield. Guo et al. [14] reported that the microorganisms in a mesophilic digester were observed at higher predominance and diversity when compared to that of in a thermophilic digester, which was associated with acetoclastic methanogens (i.e., that convert acetate into $CH_4$ and $CO_2$) in the more stable mesophilic AD. In fact, many previous reports pointed out that the sensitivity to inhibitory compounds of the acetoclastic methanogens are much higher

than that of hydrogenotrophic methanogens and thus more likely terminating methane production [21]. Westerholm and Schnürer [11] also reported that anaerobic microbial cultures responsible for several pathways involved in degradation of various substrates is continually being updated with recent improvements in molecular techniques and cultivation studies. Thanks to these molecular methods such as next generation sequencing (NGS), it is understood that microbial species in anaerobic environments might be very diverse depending on the conditions [22] and the predominance of different phyla can have significant impact on process stability [6]. Hence, identification of microbial diversity using promising culture-independent molecular methods has gained increasing interest in recent years to foresee the possible operational problems during anaerobic co-digestion of various biomass sources.

The objective of this study was therefore to assess the predominance of bacterial and archaeal communities and their role on biodegradation of food waste alone and with straw pellets by NGS analysis using the biomass samples taken from thermophilic anaerobic mono- and co-digesters, respectively, both operated at lower OLR of 3.0 gVS/L/d as well as a higher OLR of 7.0 gVS/L/d regarding the load of food waste in the reactors.

## 2. Materials and Methods

The biomass samples used in this particular study were taken from lab-scale Continuously Stirred Tank Reactors (CSTRs) treating food waste or food waste co-digested with straw pellets. This paper will focus on the microbial consortium analyses which will be connected to specific methane yield and methane productivity achieved, whereas details of operation data and process performance are presented elsewhere [23].

### *2.1. Substrates and Inoculum Sources*

The source-sorted food waste was collected from households and its slurry form was provided by a large-scale biogas plant (Borås Energi & Miljö, Borås, Sweden). The food waste slurry had the following influent chemical composition: ~12, C/N ratio; ~13% *w*/*w*, total solids ($TS_{in}$); ~11% *w*/*w*, volatile solids ($VS_{in}$); 86%, $VS_{in}$ of $TS_{in}$; and 5 kg/ton, Total-N. Wheat straw pellets (SP) (particle size of around 2 mm) were used as the co-substrate with dry matter and volatile solids content of 91% and 86%, respectively (Laga BioEnergy, Laholm, Sweden). The inoculum used, with 3.28% TS and 1.98% vs. was provided by the same full-scale biogas plant (Borås Energi & Miljö, Borås, Sweden) to start up the digestion process.

### *2.2. Bioreactors and Operating Conditions*

Two anaerobic CSTRs were operated simultaneously, one with food waste (FW) slurry alone (mono-digester; R1) and the other with the addition of 20% (VS basis) straw pellets (SP) (co-digester; R2) in semi-continuous mode at thermophilic conditions for almost a year in 3 periods. Each reactor had a total volume of 5 L and a working volume of 3 L. During Period 1, the digesters were operated with only FW at a hydraulic retention time (HRT) of 30 d and OLR of 3.0 gVS/L/d. This period has lasted for 3 HRTs aiming to achieve a stable process at steady state conditions. After this period, R1 was running further at the same conditions with only FW as substrate, while to R2, 20% (VS basis) SP was added, meaning that the loading rate in this reactor has increased to 3.6 gVS/L/d. Both reactors were then running additionally under a period of 3 HRTs (Period 2). During the last part of the experiment (Period 3), the OLR was increased gradually in both reactors at a rate of 0.5 gVS/L/d per week until it reached an OLR of 7.0 gVS/L/d in R1, while finally an OLR of 8.4 gVS/L/d in R2 was achieved, there proportionally 20% (VS basis) SP was added to FW in each step. Meanwhile, the HRT decreased to 14 days in line with the increase in OLR. The temperature was controlled to maintain 53 °C by circulating water from a water bath into the water jacket of the reactor. The content of the digester was continuously mixed by impellers at 90–110 rpm to avoid the floating of particulate materials. The produced gas volume was determined continuously by a µFlow volumetric gas flow

meter (Bioprocess Control, Sweden). Furthermore, the process imbalance was monitored by VFA/Alk ratio in order not to exceed the critical value of 0.35 [24]. No deliberate influent pH control was carried out, and sufficient buffering capacity was achieved despite smaller pH variations [25]. During operation, the reactors were monitored with conventional process parameters. The parameters analyzed were biogas production and composition, methane production, fatty acids concentration, nitrogen levels, pH and alkalinity, as they are presented elsewhere [23].

### *2.3. Analytical Methods*

The substrates (FW and SP) and samples from each reactor were collected and analyzed weekly during the semi-continuous experiments. The total solids (TS) and thereafter volatile solids (VS) were determined according to the National Renewable Energy Laboratory (NREL) protocol [26] after drying the samples to constant weight at 105 °C and thereafter by ignition at 550 °C until constant weight.

Analysis of biogas regarding methane and carbon dioxide content was carried out using gas chromatographs (GC). An Auto System, Perkin Elmer, Waltham, MA, USA equipped with a packed column (Column 8000 PKD, Perkin Elmer, Waltham, MA, USA) and a thermal conductivity detector (Perkin Elmer, Waltham, MA, USA), with an injector temperature of 150 °C was used. Nitrogen served as the carrier gas with a flow rate of 20 mL/min at 60 °C.

### *2.4. Microbial Community Analysis*

Total DNA was isolated from 1 mL sludge samples using PureLink Genomic DNA (GDNA) extraction kits (Invitrogen, Renfrewshire, UK) according to the recommended procedure by the manufacturer followed by concentration measurements using NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 16S rRNA genes were sequenced following the Illumina MiSeq method (Illumina, Inc., San Diego, CA, USA). The V4-V5 hypervariable region of the 16S rRNA gene was reproduced with region-specific primers and sequence analysis and the identification of operational taxonomic units (OTUs) were obtained using the methods suggested by Cole et al. [27] and DeSantis et al. [28].

## 3. Results

### *3.1. Effects of Straw Addition during Anaerobic Digestion of Food Waste at Low and High OLRs*

After reaching steady state conditions at an OLR of 3.0 gVS/L/d with FW in both reactors (Period 1), the addition of SP was started, and consequently the OLR was increased to 3.6 gVS/L/d in R2, while R1 was continued to feed with only FW at OLR of 3.0 gVS/L/d (Period 2). The process was operated under these conditions until it reached a new steady state. Then, during Period 3, the OLR was increased gradually in both reactors reaching the highest value of 7.0 gVS/L/d in R1 (with only FW), and, respectively 8.4 gVS/L/d with the addition of 20% SP (VS basis) to FW in R2.

As expected, the TS and vs. in the substrate in R2 increased with the presence of straw. In line with this, the TS and vs. in the effluent from R2 were also higher compared to the reference reactor, R1, running with only FW. During Period 2, the total VFA levels were low, between 0.2–0.4 g/L and the pH was stable at around 8.0 in both reactors. The methane content in the produced biogas fluctuated between 71–72% $CH_4$ for the reactors [23].

The results of the specific methane production (SMP—expressed as NmL $CH_4$ produced/gVS feed), as well as the volumetric methane production (VMP—expressed as NL $CH_4$ produced/L reactor capacity (RC)/day) obtained during the operation of the two reactors are shown in Figures 1 and 2, respectively. Both reactors showed the same trend in the SMP (Figure 1). The addition of straw had no effect on the specific methane production and no significant statistical differences ($t$-test $p > 0.05$) were observed in R2 (co-digester with FW + 20% SP) compared to that in R1 (mono-digester with only FW). The average SMP values in Period 2 were 478 ± 23 and 488 ± 38 NmL $CH_4$/gVS in R1 and R2, respectively (Figure 1). These results are in good accordance to what other studies has

reported for mixed food waste, for example Zamanzadeh et al. [29] reported an SMP of 480 mL $CH_4$/gVS.

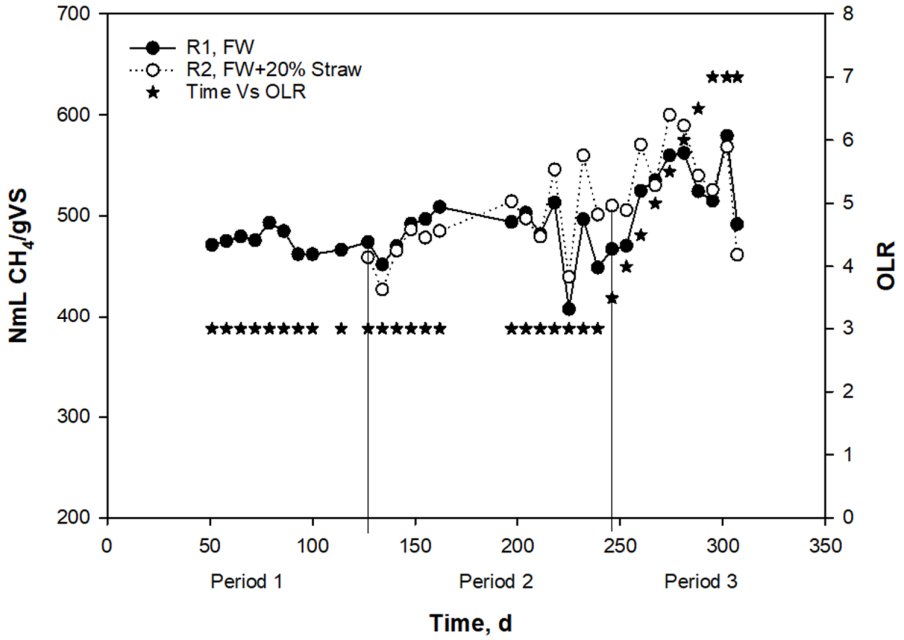


**Figure 1.** Specific methane production (SMP) obtained in R1 and R2 (target OLRs from FW were 3.0 gVS/L/d in Period 1 and Period 2 whereas 7.0 gVS/L/d in Period 3).

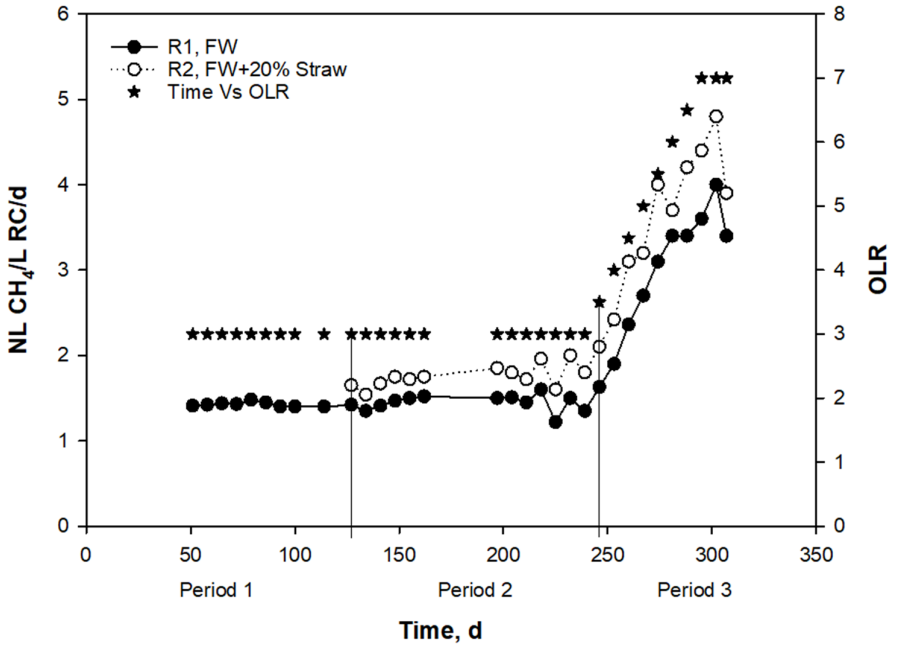


**Figure 2.** Volumetric methane production (VMP) obtained in R1 and R2 (target OLRs from FW were 3.0 gVS/L/d in Period 1 and Period 2 whereas 7.0 gVS/L/d in Period 3).

The volumetric methane production (VMP, NL/L/d) determined in the reactors is shown in Figure 2. There was a significant (*t*-test $p > 0.05$) difference in VMP between these two reactors, i.e., R1 (FW), and R2 (FW+20% SP). The VMP increased from 1.4 ± 0.07 to 1.8 ± 0.14 NL/L/d, corresponding to an increase of 29% when 20% SP was added to FW compared to when only FW was used as substrate (Figure 2).

After a gradual increase in the OLR to a final value of 7.0 gVS/L/d regarding FW an additional steady state period at this high load was investigated (Period 3). At these higher OLRs, the SMPs obtained were 529 ± 45 and 519 ± 54 NmL $CH_4$/gVS in R1 and R2, respectively (Figure 1). Additionally, higher VMPs, i.e., 3.7 ± 0.3 and 4.4 ± 0.5 NL/L/d, in R1 and R2, respectively, could be observed compared to that under Period 2 (Figure 2).

However, the total concentration of VFAs was 3.1 g/L in R1 and 3.9 g/L in R2 which were much higher compared with the total VFA levels observed during Period 2 [23]. High levels of VFAs can lead to a decrease in the pH and thereby cause unfavorable conditions in the digester. However, depending on the buffer capacity in the reactor liquid, a pH change can be hindered to different extent. The buffer capacity in an anaerobic digester mainly consists of the bicarbonate/carbon dioxide buffer system but also other ions can contribute such as ammonia [30,31]. Nevertheless, the pH was stable, at around 8.0 even at these high OLRs due to a high buffering capacity, with an alkalinity of 18.4 g $CaCO_3$/L, in both reactors [23]. Hence, the high buffering capacity could balance the high concentration of VFAs at these high OLRs of 7.0 gVS/L/d and 8.4 gVS/L/d in R1 and R2, respectively.

In summary, the addition of straw did not increase the SMP when co-digested with FW, because of the lower BMP from straw as compared to that of food waste. However, an increase in the VMP was observed in the presence of 20% of SP during both Period 2 and 3 independently on the OLR and HRT used, hence giving a better utilization of the reactor volume.

Samples taken from the seed (inoculum) as well as from R1 and R2, respectively by the end of Period 2 and Period 3 were then assessed for microbial consortium analysis. R2 was shown to experience some turbulence by the end of Period 3, which is expected as higher amount of straw, i.e., 1.4 gVS/L/d was added to an already high load (7.0 gVS/L/d) food waste [23].

### *3.2. Assessment of Microbial Diversity*

With recent advances in molecular techniques, more insights into anaerobic microorganisms and their response to changing operating conditions are being gathered for ensuring high process efficiency and stability. Although different approaches can be used, next-generation sequencing (NGS) method has arisen as the most effective method of deciphering DNA sequences recently. The use of a high-throughput Illumina MiSeq to perform genomic analysis is widely considered to represent a promising culture-independent method to determine the microbial community structure in anaerobic digesters. In this context, a high methanogenic inoculum concentration is crucial for a healthy process as well as using a seed source with high microbial diversity is one of the most important factors in order to be correlated with high functional redundancy. Although the prevailing microbial community at the time of the change in the organic loadings is utmost important, the microbial response was also found out to be dependent on other operating conditions (e.g., temperature, substrate composition, etc.) [32,33]. Hence, the interaction between microbial community structure and operating parameters is vital in order to understand the process performance and to observe maximum biogas yield when digesting organic-rich substrates [11].

#### 3.2.1. Hydrolytic and Acidogenic Bacteria

Microbial results of the samples [i.e., taken from the inoculum at start-up, as well as from the digested biomass treating food waste alone (control reactor; R1) and with the co-substrate (co-digester with 20% SP; R2) at phylum level are shown in Figure 3a.

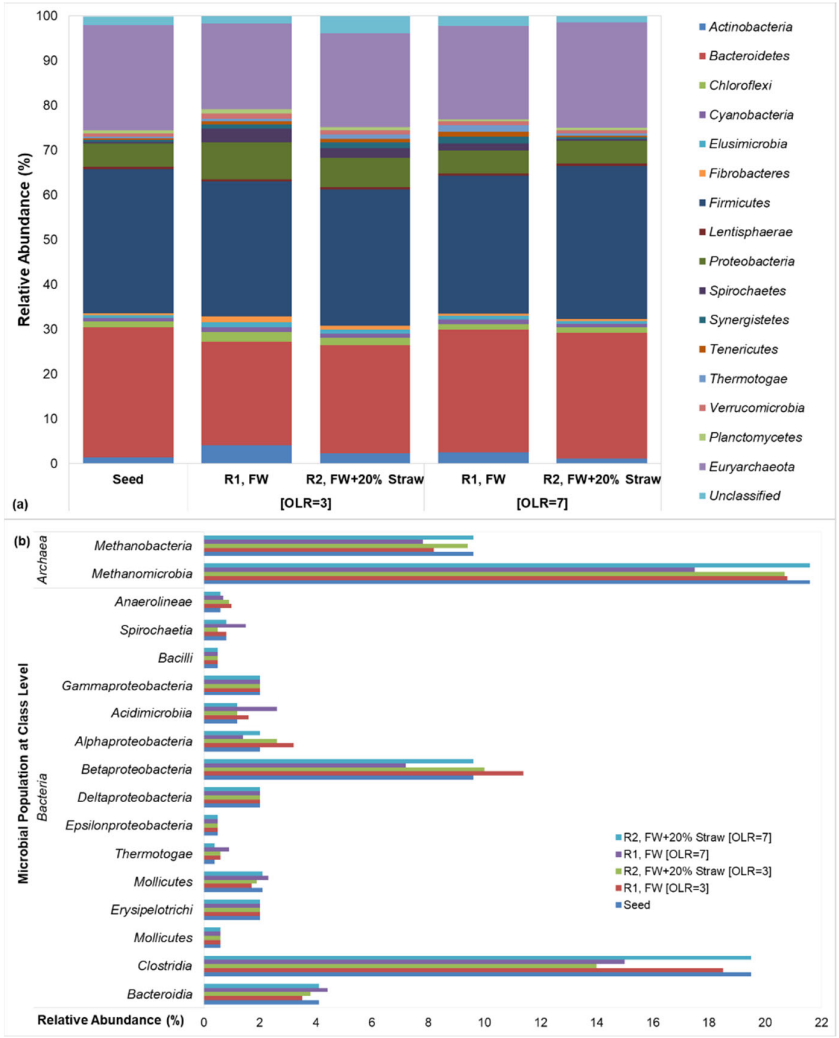


**Figure 3.** Relative abundance of microbial communities at (**a**) phylum level (**b**) class level at the end of Period 2 and Period 3 (target OLRs from FW were 3.0 and 7.0 gVS/L/d, respectively).

NGS results revealed that *Firmicutes* and *Bacteroidetes* were the first two predominant phyla whereas *Proteobacteria* was identified as the third abundant bacterial phylum with relatively small abundance of *Actinobacteria*. In accordance with Mirmohamadsadeghi et al. [8] who reported that the degradable fraction of food wastes was mainly carbohydrates ($C_6H_{12}O_6$), proteins ($C_{13}H_{25}O_7N_3S$), and lipids ($C_{12}H_{24}O_6$); members of the *Firmicutes* and *Bacteroidetes* were identified at high abundances in this study. Because, these predominant phyla has special carbohydrate degrading enzymes for successful degradation of complex substrates (e.g., starch and cellulose) [34,35]. Moreover, *Firmicutes* have important role for the degradation of several biomass sources such as lignocelluloses. Because, *Firmicutes* have the ability to produce various cellulolytic enzymes or cellulosome complexes. On the other hand, *Bacteroidetes* belong to the order of *Bacteroidales*, could also degrade large macromolecules (e.g., cellulose) in the biogas reactors. The impact of operating conditions (e.g., temperature, OLR, etc.) has also been investigated in several studies and it was reported that OLR and hydraulic retention time (HRT) had a critical impact on most of the predominant phyla (e.g., *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Thermotogae*, *Cloacimonetes*, and *Euryarchaeota*) involved in the AD process [33,36,37]. Besides, other common impact observed was the operating temperature, and it was shown that *Firmicutes* were detected at increased predominance instead of *Bacteroidetes*/*Proteobacteria* at thermophilic conditions compared to those at mesophilic ones [11]. Similar findings were also found in this study so that *Firmicutes* were detected at higher abundance compared to

*Bacteroidetes* which were, respectively in the range from 30% to 34% and from 23% to 28% in the digesters. Hence, these results implied that the *Firmicutes* and *Bacteroidetes* acted as the main phyla resulted in an efficient food waste and wheat straw degradation capability in these thermophilic anaerobic digesters investigated. This was also proven by the most abundant bacterial order in the digester which was *Clostridiales* (up to 20%) belonging to the phylum of *Firmicutes* while the second predominant order was *Bacteroidales* (up to 5%) in this study. Venkiteshwaran et al. [38] reported *Bacteriodetes*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria* as the common bacterial phyla which were active in acidogenesis process. Xia et al. [39] also reported that both orders of *Bacteroidales* and *Clostridiales* contain prominent bacteria with the ability to degrade cellulose. However, *Bacteroidales* typically do not have the enzyme complex of cellulosomes. On the other hand, cellulosomes are mostly found in *Clostridiales*. Another common bacterial class found was *Mollicutes* (~2%) under *Firmicutes*. Similar to the results of our study, hydrolytic and acidogenetic bacteria under *Firmicutes* or *Bacteroidetes*, were found to be enriched at increased organic loadings [33,37]. On the other hand, during mesophilic AD of food waste at increasing OLR (3.0–7.0 gVS/L/d) and HRT (15–20 d), a dynamic sequence was seen in different bacterial phyla (*Firmicutes* and *Actinobacteria*) [33]. Although Liu et al. [36] also observed that predominance of *Bacteroidetes* increased with an increase in OLR during mesophilic digestion operated at high TS content, in contrast they found that the relative abundance of *Firmicutes* decreased which could be attributed to the incubation temperature. Yu et al. [40] also reported that the microbial community structure in an anaerobic reactor treating rice straw was affected by the operating temperature. Here, *Firmicutes* to *Bacteroidetes* were identified at a higher ratio when the process was running at higher temperature. Similar to the results of this particular study, the levels of *Firmicutes* increased, while *Chloroflexi* decreased in abundance when the retention time was reduced from 20 to 3 days in a thermophilic AD reactor treating lignocelluloses [41]. In another study, the predominance of *Clostridium* genus (phylum *Firmicutes*) increased, whereas *Bacteroidetes* decreased by the addition of cellulose and xylan to a reactor degrading wastewater sludge [42].

Furthermore, the family *Veillonellaceae* (14.3%) (i.e., proposed to group anaerobic Gram-negative cocci that belongs to the phylum *Firmicutes*) and *Tissierellaceae* (13%) (i.e., that is the most important *Clostridia*-related member) were identified as the most abundant family members in this study. Similar findings were reported by Marchandin and Jumas-Bilak [43]. Previously, a relationship between VFA accumulation and the increase in these *Tissierellaceae* family members was detected and it was reported that when the co-digestion of food waste with other substrates (e.g., cow manure) reached higher organic loading rates; reactor acidification is incited with the abundance of *Tissierellaceae* at family level [44]. The analysis of the bacterial community also showed the presence of nitrite oxidizing bacteria (NOB) from *Proteobacteria* phylum at considerable ratios (i.e., *Betaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* were detected at class level) as shown in Figure 3b. The genera *Nitrobacter*, *Nitrococcus* and *Nitrospina* belong to the classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* within the phylum *Proteobacteria* [45]. Westerholm and Schnürer [11] also reported that *Proteobacteria* had the ability to degrade cellulose. Besides, although acetogenesis and syntrophic acid degradation were often carried out by *Clostridium* and *Acetobacterium* under the phylum *Firmicutes*, it was observed that the phylum *Proteobacteria* was also involved. It was reported that animal manure and sludge are usually treated in co-digesters with the addition of different straw sources (e.g., corn, rice, tobacco, wheat) and compatible with our findings; the two orders *Clostridiales* (phylum *Firmicutes*) and *Bacteroidales* (phylum *Bacteroidetes*) often dominated in these processes. On the other hand, it was also reported that the phyla *Proteobacteria*, *Chloroflexi*, and *Fibrobacteres* increased in response to addition of lignocellulosic materials, with some variation depending on co-digestion material and ambient conditions. However, although these aforementioned three phyla were identified relatively at high abundances; no apparent increase was observed in this study. Moreover, similar to the findings of this study; metagenomic studies have also confirmed the involvement of the phylum *Actino-*

*mycetes* next to *Proteobacteria*, *Firmicutes*, *Chloroflexi*, and *Bacteroidetes* in the degradation of lignocellulose. Here, the existence of CAZymes (Carbohydrate-Active Enzymes) in the microbial consortia, which were adapted to lignocellulosic materials, played a critical role [11,40,46,47]. Similarly, Westerholm and Schnürer [11] also reported that when the protein level in an anaerobic digester treating food waste increased, the predominance of the families *Porphyromonadaceae* and *Caldicoprobacteraceae* also increased, proposing the direct or indirect involvement of these microbial cultures in protein hydrolysis.

#### 3.2.2. Acetoclastic and Hydrogenotrophic Methanogens

Acetoclastic and hyrogenotophic methanogens under the predominant phylum *Euryarchaeota* accounted for up to 24% at phylum level in the bioreactors operated in this study. *Euryarchaeota* has been reported as the commonly detected microbial cultures in thermophilic digesters where considerable biogas yields were obtained, since the genera of this phylum produce methane ($CH_4$) as an integral part of their metabolism [11]. Evans et al. [48] also reported this phylum as one of the four phyla of the domain archaea which comprises a physiologically diverse group all known methanogens. Accordingly, acetoclastic (*Methanosarcina* and *Methanosaeta*) and hydrogenotrophic (*Methanobacterium*, *Methanobrevibacter*, and *Methanospirillum*) methanogens are reported to be essential for the last step of methanogenesis. However, $H_2$ and $CO_2$, or formate are the only carbon sources for hydrogenotrophic methanogens whereas acetate is the usual substrate for acetoclastic methanogens to produce methane via acidogenesis and acetogenesis. Hence, limited substrates are available for the methanogens to utilize in the industrial process of AD [49,50]. Among the methanogenic classes, members of the *Methanobacteria* and *Methanomicrobia* were detected in bioreactors with high loadings in relation to operating conditions [11]. Similarly, the most abundant classes were *Methanomicrobia* (up to 22%) followed by *Methanobacteria* (up to 10%) in the CSTRs operated in the scope of this study (Figure 3b).

Since straw was used as the co-substrate, the lignin-derived compounds (i.e., one of the main components of straw), have been found to be inhibitory to methanogens depending on concentration. In this context, hydrolytic activity reduced, and substantial changes were observed within both archaeal and bacterial populations. However, adaptation of the microbial cultures makes the degradation of these compounds possible by the members of the family *Synergistaceae*, combined with hydrogenotrophic methanogens [51]. Since, the inoculum used was taken from the same large-scale digester of the investigated industry and most likely due to adaptation to the aforementioned compounds; no apparent inhibition was observed on methanogenic orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* that were identified at high abundance ratios (between 7% and 13%) in our study. Similarly, Westerholm and Schnürer [11] also reported that methanogens which were mostly observed in usual AD processes, belonged to the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* (phylum *Euryarchaeota*). Methanogenic community shift related to ammonia concentration has been found often in connection with the increased operating temperatures and it was reported that positive correlations between high temperature and improved predominance of *Methanobacteriales* (often *Methanothermobacter*) and/or *Methanomicrobiales* (often *Methanoculleus*) were observed. Increase in the hydrogenotrophic *Methanomicrobiales* was also observed during loading by pulsed feeding that favored propionate consumption, most likely through hydrogen utilization. Similarly, these methanogens were also identified in the bioreactors operated at high ammonia concentrations and at high OLRs [11]. According to Lerm et al. [52] and Xu et al. [33], although acetate-utilizing methanogens were critical for an effective methane generation in a steady-state AD process at increasing OLR; hydrogenotrophic methanogens (e.g., representatives of the orders *Methanomicrobiales* and *Methanobacteriales*) became more important and dominant in case of overloading and acidification. On the other hand, methanogenic partners in syntrophic acetate oxidation (SAO) were also suggested to be members of these hydrogenotrophic *Methanobacteriales* and *Methanomicrobiales* (often the

genus *Methanoculleus*) [11]. Since the most abundant class was identified as *Clostridia* (about 20% at OLR = 8.4 gVS/L/d with FW and 20% PS) in this study; it could be concluded that syntrophic acetate oxidation (SAO), acetate-oxidizing bacteria and hydrogenotrophic methanogens worked in a syntrophic manner for methane production. Because one of the genera that bacterial species currently known to be capable of SAO, belong to *Clostridium* [11]. It was also reported that although typical filamentous acetotrophic methanogens are favored at low acetate concentrations, they disappear at high inhibitory compounds like ammonium and sulfide [50]. Because, acetate-utilizing methanogens offering thin filaments with a great surface seemed to be more sensitive to inhibitory concentrations than hydrogenotrophic methanogens which grow as rods or consist of thick clumps. In this context, *Methanosarcinaceae* were more flexible to grow on various substrates (e.g., acetate, hydrogen, and methanol), while *Methanosaetaceae* members use solely acetate [11]. In our study, the families *Methanosarcinaceae* and *Methanosaetaceae* (i.e., respectively 7.3% and 8.6%) and *Methanobacteriaceae* and *Methanospirillaceae* (i.e., respectively 6.3% and 7.3%) were all identified at high predominance ratios (OLR of 8.4 gVS/L/d with FW and 20% SP). Although *Methanobacteriaceae* and *Methanospirillaceae* indicated some reduction in the relative abundance as OLR increased; straw addition at each OLR did not indicate any particular impact on these two family members. On the other hand, *Methanosaetaceae* indicated some decrease from 11.5% to ca. 8.5% as OLR increased with 20% SP addition. Karakashev et al. [53] also observed that the acetate oxidation to $H_2/CO_2$ with methane production by hydrogenotrophic methanogens should be the ruling pathway when *Methanosaetaceae* was not found in the system. Under thermophilic conditions, rodlike or coccoid hydrogenotrophic methanogens are commonly advantageous, while sometimes thermophilic *Methanosarcinae* can be detected, but thermophilic *Methanosaetae* cannot be found. Moreover, it was indicated that the archaeal community structure was closely correlated with the VFA concentration in a thermophilic anaerobic digester [54]. When VFA and $NH_3$ concentrations are found at high levels in the digesters treating nitrogen-rich substrates like food waste, the dominance of *Methanosarcinaceae* was observed, while in the digesters with low levels of VFA and $NH_3$, *Methanosaetaceae* dominated [50]. Accordingly, among the methanogenic archaea; *Methanosaetaceae* which are the family of the *Methanosarcinales* in taxonomy, was found at the highest level at thermophilic co-digesters operated in this study (i.e., up to ~11.5% and 9%, respectively at OLRs 3.6 and 8.4 gVS/L/d with FW and 20% SP). Hence, *Methanosaetaceae* was still the predominant acetoclastic methanogen at family level even at the highest OLR studied. Relatively high biogas yields at the highest OLR value and 20% straw amount also demonstrated no inhibition that could be also attributed to the usage of the adapted inoculum sludge at start-up of thermophilic co-digester. On the other hand, in addition to existing environmental conditions; the methanogenic communities degrading the carbohydrate-rich substrates indicated different structures primarily dependent on the co-substrate source. For example, *Methanosarcina* or *Methanosaeta* often dominated during co-digestion of straw with cow manure or digestion of straw alone [11,37,40,47,54–56]. However, when nitrogen level, operating temperature, OLR, and/or carbohydrate availability increased; it was observed that hydogenotropic methanogenic archaea, involving *Methanoculleus*, *Methanothermobacter*, and *Methanobacterium*, contributed more. Xu et al. [33] also reported that the abundance of *Euryarchaeota* (e.g., family members of *Methanosarcinaceae* and *Methanosaetaceae*) increased during AD of food waste at increasing OLR (3.0–7.0 gVS/L/d) and HRT (15–20 d) at mesophilic condition.

In this study, the NGS analysis at genus level revealed that *Methanospirillum* and *Methanobacterium* (hydrogenotrophic methanogens) as well as *Methanosaeta* and *Methanosarcina* (acetoclastic methanogens) were the prevalent members of archaeal community (Figure 4).

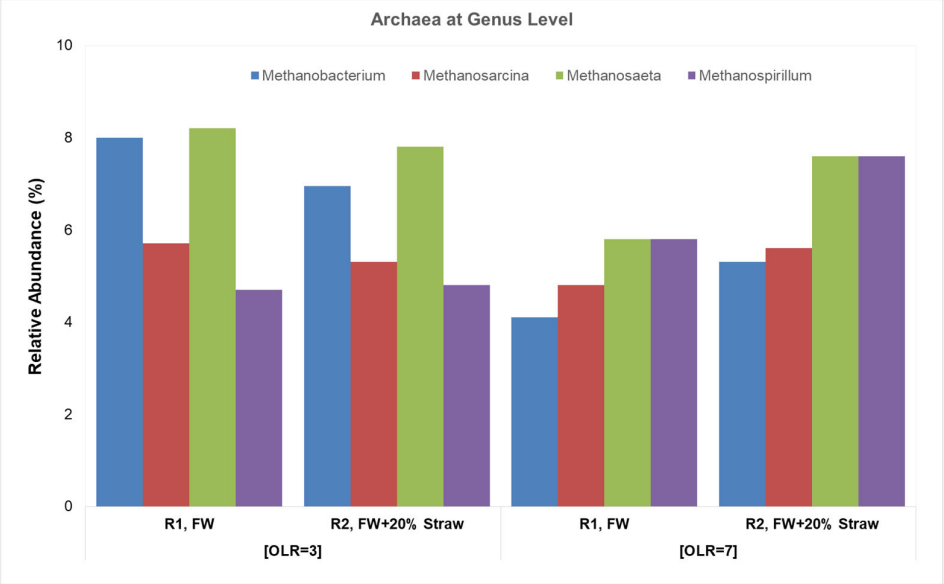


**Figure 4.** Relative abundance of archaeal communities at genus level at the end of Period 2 and Period 3 (target OLRs from FW were 3.0 and 7.0 gVS/L/d, respectively).

Among them, *Methanosarcina* sp. are able to use both the acetoclastic and the hydrogenotrophic methanogenesis pathways. Demirel and Scherer [50] reported that *Methanosaeta concilii* was found to be the dominant acetotrophic methanogen in the digesters with low acetate concentrations, while *Methanosarcina* sp. was determined to be the most abundant aceticlastic methanogen in unstable co-digesters with high acetate concentrations. Similarly, they have also found that long start-up periods indicated lower levels of archaea, with an abundant population of *Methanosarcina* species. Moreover, high acetate concentrations favor *Methanosarcineae* consisting of many irregular cell clumps formed to protect the cells against harmful chemical agents. It was also reported by de Vrieze et al. [57] that *Methanosarcina* sp. were more tolerant to specific inhibitors of the acetoclastic pathway, compared to *Methanosaeta* sp. Despite no prominent change in the genus *Methanosarcina* was seen in this study; increase in this genus was frequently reported in response to an increasing OLR due to its ability for efficient acetate degradation and its stability during stress conditions. Several studies also suggested that members of the *Methanosarcina* were crucial for maintaining an effective methane production under increasing OLR. Besides, this genus could use both the hydrogenotrophic and acetoclastic pathways for methane formation because of its moderate tolerance to ammonia, and thus possibly acted as a hydrogen scavenger in SAO [11,57] or mediated the entire process, i.e., both acetate oxidation and subsequent methanogenesis [53]. Moreover, if anaerobic reactors had high VFA levels and low pH values then they had comparatively low levels of *Methanosarcinales*, emphasizing the significance of this methanogen for efficient biogas production. Hence, the predominance of *Methanosarcinales* was crucial for an adequate start-up of a thermophilic process, especially when exposed to high acetate levels [11,14,46,57–59]. Guo et al. [14] also reported that high functional redundancy in the bacterial consortium was due to acetoclastic methanogens which were observed at higher abundance and diversity in more stable digester. Compatible methane yields in our study also indicated steady-state operation at thermophilic reactors probably due to the fact that the balance between acetoclastic (*Methanosaeta* and *Methanosarcina* species) and hydrogenotrophic (*Methanobacterium* and *Methanospirillium* species) methanogens could be kept in anaerobic digesters at the highest OLR applied with straw addition despite the shift in the relative abundance from *Methanobacterium* to *Methanospirillium* species.

## 4. Conclusions

In the present study, microbial community structure and predominance profile during anaerobic digestion of food waste without and with straw pellets were investigated at different OLRs as one of the most crucial drivers on the overall shift. According to metagenomic data by Illumina MiSeq NGS technology, *Firmicutes*, *Bacteroidetes*, and *Euryarchaeota* acted as the main phylum with efficient anaerobic degradation of food waste and straw. Competitive advantage of *Firmicutes* could be related to high methane productivity most probably due to their significant role in successful hydrolysis of such complex substrates. Furthermore, both hydrogenotrophic and acetoclastic methanogens played crucial role during co-digestion of the carbon- and nitrogen-rich substrate with the cellulose-rich biomass source.

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