Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-022-03659-8

ORIGINAL ARTICLE

Volatile fatty acid production from hydrolyzed sewage sludge: effect of hydraulic retention time and insight into thermophilic microbial community

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Received: 12 September 2022 / Revised: 2 December 2022 / Accepted: 12 December 2022

Abstract
The disposal of sewage sludge potentially reaches 50–60% of the total operation cost of a wastewater treatment plant. Given its high content of organic material, adopting effective technologies for sewage sludge treatment minimizes its environmental impact and the parallel conversion of the organics into recovered bio-products. Hence, the such stream can be viewed as a renewable carbon source to produce high-value products such as volatile fatty acids (VFA). Short-time (8 h) alkaline (pH 9–11) and thermal (70–85 °C) hydrolysis were applied to enhance the acidogenic fermentability of thickened sewage sludge. Mild thermal hydrolysis (70 °C) was chosen as the best performing method to increase the soluble chemical oxygen demand (COD_{SOL}) and boost the VFA production in the following dark fermentation process, designed at three different hydraulic retention times (4.0, 5.0, and 6.0 days). The highest acidification yield (0.30 g COD_{VFA}/g VS) and COD_{VFA}/COD_{SOL} ratio (0.73) were obtained at 6.0 days as hydraulic retention time. Microbial community analysis performed at the end of semi-continuous tests showed the occurrence of several fermentative bacteria (i.e., *Coprothermobacteraceae*, *Planococcaceae*, *Thermoanaerobacteraceae*) responsible for the fermentation of complex organic matters mainly into acetic, propionic, and butyric acids, which dominated the VFA spectrum.

Keywords Dark fermentation · Volatile fatty acids · Sewage sludge · Biorefinery · 16S rRNA gene

1 Introduction

Due to population growth and subsequent urbanization, the volume of sewage municipal sludge produced by wastewater treatment plants (WWTP) has increased in recent years [1]. The mandatory sludge disposal process can reach 50–60% of total WWTP operating costs [2]. These sludges can be considered a source to be valorized since they are characterized by a high content of protein and carbohydrates and are abundant in metropolitan areas equipped with sewer systems and WWTPs at the end. One of the routes potentially exploitable for sludge valorization is the anaerobic dark fermentation (DF) process by using mixed microbial communities, which allows to produce volatile fatty acids (VFA) from a variety of organic wastes [2]. As a result, WWTPs can be evaluated not only based on effluent quality but also based on recovered materials to strengthen the water sector's sustainability and move toward a circular economy [3]. DF is a stage of anaerobic digestion in which organic matter is digested in the absence of oxygen by a variety of organisms, mostly converting it to CO_2 and H_2 [4], via three process steps: hydrolysis, acidogenesis, and acetogenesis. Fermentative bacteria make VFA during the acetogenesis phase by metabolizing soluble compounds obtained during the hydrolysis phase. VFA are short-chain linear mono aliphatic compounds (2–5 carbon atoms) and interesting building

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Published online: 21 December 2022

blocks frequently used in the chemical industry due to their functional groups. Additionally, VFA can be employed as carbon sources in WWTPs for biological nutrient removal (nitrogen and phosphorus). While methanol is frequently used for this purpose, VFA can be just as effective and less expensive if made from the same WWTP sludge [4]. Other applications include chemical manufacturing, ester manufacture, and food colorant and additive solvents; butyric acid is utilized as an aromatic, pharmaceutic, or animal nutrition element, to mention a few. Presently, they are synthetized from crude oil, which contribute to the production of greenhouse gases and pollution.

If recovered from waste, VFA extraction from fermentation broth is a crucial step for the following VFA utilization, also in terms of process economy [5]. The methods explored to date include liquid–liquid extraction, membrane processing, adsorption, ion exchange, distillation, and evaporation. Some are more expensive or efficient than others and must be chosen according to both chemical and physical qualities as well as the required VFA concentration.

Given the high potential of such compounds, DF within WWTP facilities merits investigation since it may represent a sustainable and economically attractive method for the management of this waste, often limited to biogas production in an oversized digestion plant [6]. To investigate this possibility in a wide range of options, this study examines the effects of thermal and alkaline sewage sludge hydrolysis (separately and in combination) on the solubilization of organic matter and its subsequent use in the DF process to produce VFA. Fermentation batch tests were conducted in an equally wide range of temperatures (from psychrophilic to hyper-thermophilic conditions) using previously collected hydrolyzed sludge. Finally, a single temperature was chosen for the semi-continuous fermentation tests, in which three different hydraulic retention times (HRT; 4–5–6 days) were explored to compare VFA production (maximal VFA concentration, fermentation rates, and yields) and composition. The high-throughput sequencing of 16S rRNA gene was performed on samples taken at the beginning and at the end of semi-continuous tests to describe microbial community composition and dynamics in response to different operating conditions.

2 Materials and methods

2.1 Source and characteristics of the sewage sludge

Sewage sludge was collected from the Treviso WWTP (northeast Italy) and specifically from the static thickener after secondary settling of activated sludge. Fresh aliquots of sludge were taken and immediately utilized in each foreseen tests, since the whole experimental plan was conducted in the laboratory adjacent to the Treviso WWTP. Its characteristics are summarized as follows: total solids (TS) 36.1 ± 0.5 g TS/kg; volatile solids (VS) 26.6 ± 0.2 g VS/kg; soluble chemical oxygen demand (COD_{SOL}) 0.6 ± 0.1 g COD_{SOL}/L; ammonia 163 ± 22 mg N-NH_4^+/L; phosphorus 65 ± 9 mg P-PO_4^{3-}/L; total Kjeldahl nitrogen (TKN) 33 ± 3 g N/kg TS; and total phosphorus (P_{TOT}) 17 ± 1 g P/kg TS.

2.2 Configuration and process set up

The pre-treatment procedure was conceived to solubilize organic materials (volatile solids; VS) and thereby raise the liquid phase soluble chemical oxygen demand (COD_{SOL}). Alkaline tests were performed by adding 3.0 M NaOH solution to pH 9.0 (A1) and 11.0 (A2). Thermal pre-treatment experiments were conducted in the oven at temperatures (T) of 70 °C (T1) and 85 °C (T2). The use of T instead of external chemical agents has been chosen as a possible cost saving and environmentally friendly approach in a perspective of the technology scale up. A combined pre-treatment was also tested: the pH value was set to 9.0 and the T was maintained at 70 °C (A1-T1) and 85 °C (A1-T2). Each test was completed in duplicate, over 8 h, in the 1.0 L borosilicate glass bottles.

Thermally pretreated sludge (T1) was routinely produced and used as feedstock for the following batch DF tests (conducted in duplicate). These tests were performed in the 1.0 L borosilicate glass bottles (working volume 0.8 L) at four distinct temperatures (20, 37, 55, and 70 °C) ranging from slightly psychrophilic to hyper-thermophilic conditions. To monitor the performance of the batch DF tests (maximum duration of 10 days), a liquid sample was regularly taken for VFA, COD_{SOL}, and pH analyses.

Semi-continuous stirred tank reactor (sCSTR) were then utilized for the related DF process, which was conducted in the 2.0 L borosilicate glass bottles, stored in an oven to keep thermophilic condition (55 °C) and with magnetic stirring. Three runs were monitored for approximately 45 days, at three distinct hydraulic retention times (HRT), equal to 4.0, 5.0, and 6.0 days. The three reactors were fed with thermally pre-pretreated sludge (70 °C, 8 h), once per day. In each reactor, a volume of 0.2 L of inoculum was also added at the beginning of the run; the inoculum was taken from a parallel thermophilic pilot-scale anaerobic digestion reactor, usually fed with food waste and sewage sludge mixture at a medium–high organic loading rate (OLR; 4–5 kg VS/m^3 d). A fixed aliquot (according to the chosen HRT) was withdrawn once per day. After each withdrawal and feeding event, the bottles were opened and kept under N_2 flux for 10 min to re-establish anaerobic conditions. To monitor the characteristics of the sCSTR effluents, COD_{SOL}, VFA, and pH were analyzed three times per week, approximately.

Nutrients (ammonium and phosphate) concentrations were quantified on a weekly basis. At the end of the three sCSTR runs, alkalinity was also quantified.

2.3 Analytical methods

The analyses were carried out according to the Standard Methods for the determination of TS and VS, COD_{SOL}, alkalinity, ammonia, phosphate, TKN, and P_{TOT} [7]. The VFA was analyzed using an Agilent 6890 N gas chromatograph (GC) equipped with a flame ionization detector (FID) at a temperature of 250 °C. An Agilent J&W DB-FFAP fused silica capillary column (DB-FFAP; 15 m length, 0.53 mm ID, 0.5 mm film) was utilized as stationary phase. Hydrogen was utilized as carrier. The inlet was set in split mode, with a split ratio of 20:1. The instrument was designed to operate with ramp temperature, from 80 to 100 °C, at 10 °C per minute. Before GC analysis, each sample was centrifuged for 10 min at 4,500 rpm and the supernatant filtered through 0.2 µm acetate-cellulose filters porosity (Whatman).

2.4 Calculation and statistical analysis

In the semi-continuous experiments (in sCSTR), data were processed according to the adopted frequency of sampling. To better represent VFA distribution, the molar ratio between odd numbered acids and the total VFA was quantified. The nutrients' concentration, which was related to ammonia and phosphate release in the medium, was quantified by means of the COD:N:P ratio expressed in grams. The substrate solubilization was calculated by the ratio between the COD_{SOL} (as net concentration subtracted to the initial COD_{SOL}; COD_{SOLin}) and the initial VS of the sludge (VS_{in}), as reported elsewhere [8]:

$$\text{Solubilization} = \frac{COD_{SOL} - COD_{SOLin}}{VS_{in}}$$

The specific rate was calculated by the ratio between the VFA concentration (in the steady state period) subtracted to COD_{VFAin} and the VS_{in} multiplied by the applied HRT [9]:

$$\text{Specificrate} = \frac{COD_{VFA} - COD_{VFAin}}{HRT \cdot V_{Sin}}$$

The VFA yield was quantified by the ratio between the produced VFA (as net concentration subtracted to the initial VFA; COD_{VFAin}) and the VS_{in} [8]:

$$Yield_{VFA} = \frac{COD_{VFA} - COD_{VFAin}}{V_{Sin}}$$

The COD solubilization and the specific rate were calculated in the three sCSTR by considering the COD_{SOLin} and VS_{in} of sewage sludge before pre-treatment step. The fermentation yield was calculated in both batch tests and sCSTR run, by considering the COD_{VFAin} and VS_{in} of sewage sludge after pre-treatment step.

All the parameters characterizing the performances of each reactor were expressed as mean values (with standard deviation) and calculated after steady states were reached. The analysis of variance (ANOVA) and Tukey HSD post hoc tests were conducted to detect the significance of the results. Shapiro–Wilk test was performed to check whether the considered data were normally distributed. Bartlett test was used to determine the homoscedasticity between the several groups considered. The null hypothesis (H_0) was rejected when the calculated p-values were less than or equal to level of significance (α). Statistical analyses were performed using the open-source program, R (The R Foundation for Statistical Computing, version 4.0.3).

2.5 Microbial community analysis

Anaerobic sludge samples (10 mL) were taken at the beginning and at the end of sCSTR operation, in all the three HRT investigated. The DNA extraction was performed by using Dneasy PowerSoil Pro Kit (QIAGEN, Germantown, MD) and utilized as the template for the amplification of the V1–V3 region of 16S rRNA gene of Bacteria (27F 50-AGA GTTTGATCCTGGCTCAG-3′; 534R 50-ATTACCGCG GCTGCTGG-30) following the procedure for library preparation and sequencing described in Crognale et al. [10]. Bioinformatics analyses were carried out using QIIME2 v. 2018.2 [11], following the procedure previously reported [12]. High-throughput sequencing yielded a total of 51,652 sequence reads after quality control and bioinformatics processing that resolved into 275 ASVs. Datasets are available through the Sequence Read Archive (SRA) under accession PRJNA830363.

3 Results and discussion

3.1 Sewage sludge pre-treatment and hydrolysis

The characteristics of sewage sludge after 8-h pre-treatment are depicted in Fig. 1. If compared to the performance of alkaline treatment (A1 and A2), thermal pre-treatment (T1 and T2) caused a greater solids' reduction. Hence, T1 and T2 pre-treatment were more effective in the solubilization of organics. According to these results, the COD_{SOL} values were greater in both T1 and T2 tests (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$), where the COD_{SOL} increased from 0.6 to roughly 10.0 g/L, with no significant difference between the two thermal pre-treatments (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). For the alkaline

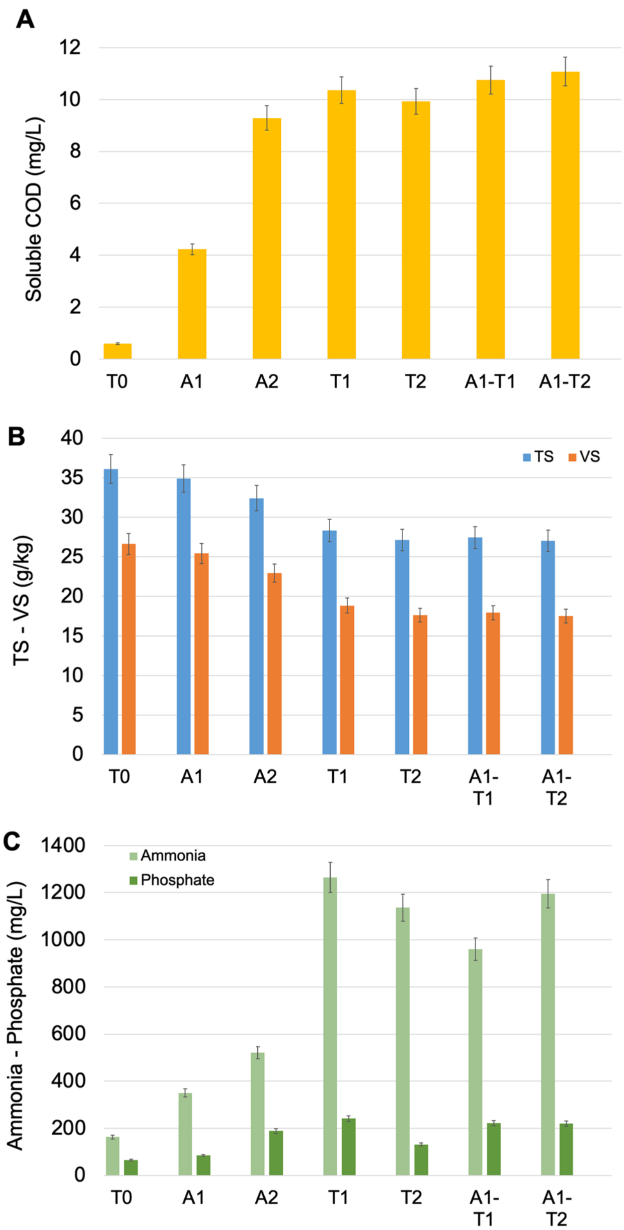


Fig. 1 Summary of the main characteristics of the thickened sewage sludge after the different applied pre-treatment: soluble COD (**A**); TS and VS (**B**); ammonia and phosphate (**C**)

pre-treatment, only pH 11.0 (A2) showed to be effective in solids solubilization. When treated at pH 9.0 (A1), the COD_{SOL} increased from 0.6 to 4.2 g/L, which was more than 50% lower if compared to the performances achieved in A2, T1, and T2. The combination of alkaline (pH 11.0) and heat pre-treatment (70 °C) led to no significant improvements compared to the thermal pre-treatment (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). Hence, the COD_{SOL} cannot be increased by more than 10–11 g/L, at least under the mild conditions tested, in comparison to the thermal hydrolysis reported elsewhere [13]. Accordingly, under both 70 °C and 85 °C, thermal pre-treatment alone can be sufficient for a good solubilization grade, without the need of chemicals' addition. The alkaline pre-treatment caused a lower release of ammonia and phosphate (350–521 mg $N\text{-}NH_4^+$/L and 85–188 mg $P\text{-}PO_4^{3-}$/L in the effluent respectively) compared to the thermal and combined pre-treatment, where ammonia was close to or greater than 1000 mg $N\text{-}NH_4^+$/L, and phosphate was always greater than 200 mg $P\text{-}PO_4^{3-}$/L. Because of the high solubilization grade achieved, the thermal pre-treatment was selected as the best method to boost the following acidification process.

Among others, heat treatment has been described in the literature as an effective method for sludge disintegration [14]. In particular, the colloidal and flocculent structures can be destroyed, causing a release of the organic matter, and facilitating the following anaerobic fermentation process. As additional benefit, the dehydration performance of the sludge can be substantially improved. Literature studies reported a wide range of temperature (60–270 °C); however, the heat pre-treatment at lower temperatures (60–70 °C) has been recognized as the most suitable to increase the rate of sludge hydrolysis and its degree of decomposition [15]. Higher temperatures may lead to agglomeration of larger particle size, such as the formation of high molecular weight heterogeneous polymers above 180 °C [16]. Such polymers are refractory and difficult to degrade; in addition, they can also inhibit the microbial anaerobic fermentation of the organic matter. Unlike high temperatures, the pre-treatment at low temperatures has been described in the literature as an effective method to improve the organics' solubilization and to promote the enzymatic hydrolysis reaction, without the risk of generating non-biodegradable compounds [14]. Alkaline pre-treatment method has been also extensively discussed in the literature in terms of hydrolysis of extracellular polymer structure (EPS) of sludge flocs, leading to the disruption of the cell wall and its macromolecular substances [17]. The effectiveness of the alkaline process is strictly related to the reaction time and alkaline dosage, which in turn affect the reaction of the additives with the EPS and lipids of the cell membrane [14]. The NaOH dosage to reach pH 9.0 (A1) was probably not sufficient to obtain a good level of sludge flocs disintegration and solubilization; in addition, the 8-h reaction time was not effective under both pH in terms of nutrients' release, probably because of the only partial destruction of the EPS matrix and cell wall achieved.

Morgan-Sagastume et al. [18] also found that physical-thermal pre-treatments were the most effective methods to support the acidification of municipal sludge. Basically, even though high levels of nutrients are not recommended for a certain type of application (e.g., microbial synthesis of polyhydroxyalkanoates from fermented waste streams [19]), their release may drive the choice of downstream technology for their potential recovery [3], as additional benefits to the VFA recovery from liquid streams.

3.2 Acidogenic dark fermentation in batch tests

The batch DF tests were carried out with thermally pretreated (T1) sewage sludge to evaluate the effect of the temperature on the VFA production and eventually the time needed for the maximum VFA level achievable. In addition to the observed increase of the COD_{SOL}, the thermal pretreatment (T1) probably led to a suppression or inhibition of methanogens, promoting the accumulation of VFA without their consumption [14].

In general, the VFA concentration increased in all the batch tests over the first 4–6 days, with a modest increase or total stabilization occurring after that. To quantify the occurred acidification, the obtained VFA concentrations had to be compared to the COD_{SOL} values: a high VFA/COD_{SOL} ratio indicated a successful of the DF process. The temperature had a significant impact on the anaerobic process, as the greatest VFA/COD_{SOL} value was seen in thermophilic conditions (55 °C): 0.82 ± 0.03 (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). Furthermore, when compared to the other tested temperature, the thermophilic test had the highest fermentation yield, which was 0.29 ± 0.03 g COD_{VFA}/g VS_{in} (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). Mesophilic condition (37 °C) was also favorable for the acidification of pre-treated sludge (0.75 ± 0.02 COD_{VFA}/COD_{SOL}; 0.21 ± 0.02 g COD_{VFA}/g VS_{in}). In the DF tests conducted at slightly psychrophilic condition (20 °C), the fermentation activity was still present since VFA accounted for 60% of the COD_{SOL}. Hence, this condition could be considered as acceptable in a vision of compromise between moderate acidification and low energy requirement (compared to high-temperature DF process) in the context of a full-scale application. As a result of the relatively low fermentation yield achieved (0.15 ± 0.02 g COD_{VFA}/g VS_{in}), this condition can be evaluated viable or not as part of a larger discussion, in which the mass and energy balances are also evaluated (out of the scope of this work). The acidogenic fermentation process proved to be incompatible with a hyper-thermophilic environment (70 °C; 0.44 ± 0.02 and 0.09 ± 0.01 g COD_{VFA}/g VS_{in} respectively for the COD_{VFA}/COD_{SOL} ratio and yield). According to all collected data, thermophilic condition (55 °C) was chosen as the optimum for the acidogenic fermentation process to be investigated in the foreseen sCSTR. Among other parameters discussed above, thermophilic trials showed the greatest VFA concentration (7.8 ± 0.4 g COD_{VFA}/L; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$).

Literature data on the batch DF of sewage sludge showed different outcomes, partially in agreement to the results obtained in this study. If not pretreated, sewage sludge required longer reaction time to reach the maximum VFA concentration, compared to this investigation [20, 21]. Garcia-Aguirre et al. [20] reported a stable VFA concentration of 8.3 g COD_{VFA}/L (with 0.94 COD_{VFA}/COD_{SOL}) in 10 days as reaction time, under mesophilic condition; in this case, the acidification process was boosted by alkaline condition (pH 10). Lower reaction time (6 days) was required under thermophilic conditions [21]. In a more recent work [22], the authors explored the VFA production in mesophilic (30 °C) batch mode from thermally pre-treated sludge (120 °C, 15 min), without any pH adjustment. The thermal pre-treatment positively affected the acidification process, with a stable VFA level around 10 g COD_{VFA}/L, achieved after 10 days of reaction time. Compared to this study, a lower level of VFA was achieved by Mineo et al. [23]: the maximum concentration was close to 0.4 g COD_{VFA}/L, achieved in 8 days of reaction time. Despite this low VFA level, a certain acidification was obtained, since the final COD_{VFA}/COD_{SOL} was close to 0.50. However, the low TS value of the sewage sludge (4.3–4.8 g TS/kg) did not allow to obtain a VFA concentration comparable to the values reported in this study.

Table 1 summarizes the main parameters obtained in the batch fermentation tests conducted at four different temperatures.

Table 1 Summary of the main parameters (average data and standard deviation) of the acidogenic fermentation liquids in batch tests

Parameter	Unit	Temperature (°C)			
		20.0	37.0	55.0	70.0
COD_{SOL}	g/L	8.8 ± 0.2	8.9 ± 0.2	9.5 ± 0.5	10.0 ± 0.2
COD_{VFA}	g/L	5.4 ± 0.6	6.7 ± 0.3	7.8 ± 0.4	4.4 ± 0.1
COD_{VFA}/COD_{SOL}	g/g	0.60 ± 0.04	0.75 ± 0.02	0.82 ± 0.03	0.44 ± 0.02
$Yield_{VFA}$	g COD_{VFA}/g VS_{in}	0.15 ± 0.02	0.21 ± 0.02	0.29 ± 0.03	0.09 ± 0.01
Time*	d	6	6	5	4

*Required time for the achievement of the maximum VFA concentration

3.3 Acidogenic dark fermentation in semi–continuous stirred tank reactor (sCSTR)

Semi-continuous tests were carried out at the chosen thermophilic temperature (55 °C) for three distinct HRT (4.0, 5.0, and 6.0 days, respectively) and filled with the same inoculum as described above. Literature examples of continuous and semi-continuous DF processes mainly described the sewage sludge fermentation as co-substrate, under different thermal regime and at an HRT range similar to this study [8, 24]. Generally, high HRT are associated to high reactor's volume (when HRT is equal to sludge retention time, SRT); hence, the choice of a narrow range of relatively low HRT has been also driven by a cost-saving approach in a perspective of full-scale implementation.

All the experiments demonstrated that the biomass required only a short acclimation time before reaching a stable VFA synthesis, most likely due to the persistence of fermentative bacteria in the pretreated sludge. The start-up period was almost identical in all three conditions (approximately 10–14 days). In the steady-state periods, the COD_{SOL} was not significantly different in all three cases, with values of 10.0 ± 0.5 g/L at 4.0 and 5.0 days as HRT, and 9.4 ± 0.3 g/L at 6.0 days as HRT (Fig. 2). In practice, the HRT had no significant impact on the solubilization of the organic matter because the DF stage was applied on an already solubilized sludge (70 °C, 8 h). On the contrary, the HRT had a substantial impact on the fermentation activity: the highest amount of VFA was produced in the sCSTR run conducted at HRT 6.0 days (7.3 ± 0.3 g COD_{VFA}/L; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$), followed by the run conducted at HRT 5.0 days (6.5 ± 0.2 g COD_{VFA}/L; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$) and HRT 4.0 days (5.7 ± 0.3 g COD_{VFA}/L; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$) (Fig. 2). Therefore, the highest COD_{VFA}/COD_{SOL} was obtained at 6.0 days of HRT (0.72 ± 0.02), followed by 0.66 ± 0.004 and 0.56 ± 0.05 at 5.0 and 4.0 days of HRT respectively (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). Similar trend of COD_{VFA}/COD_{SOL} was also observed in a previous work, where the mixture of sludge and food waste was utilized as feedstock in DF process after a thermal pre-treatment step [8]. Despite of the presence of FW (a highly putrescible substrate), the HRT of 6.0 days was similarly found to be more appropriate among the others explored (5.0 and 4.1 days) for VFA production. Under this condition, the obtained effluent was characterized by a stable VFA production with limited amount of non-VFA soluble COD (0.91 COD_{SOL}/COD_{VFA}). In addition, the sludge utilization as co-substrate favored the stability of the process, avoiding pH fluctuations and drops, usually observed in acidogenic fermentation of highly putrescible feedstock.

In this study, the pH of the fermentation effluent was quite similar in each reactor. Initial pH values around

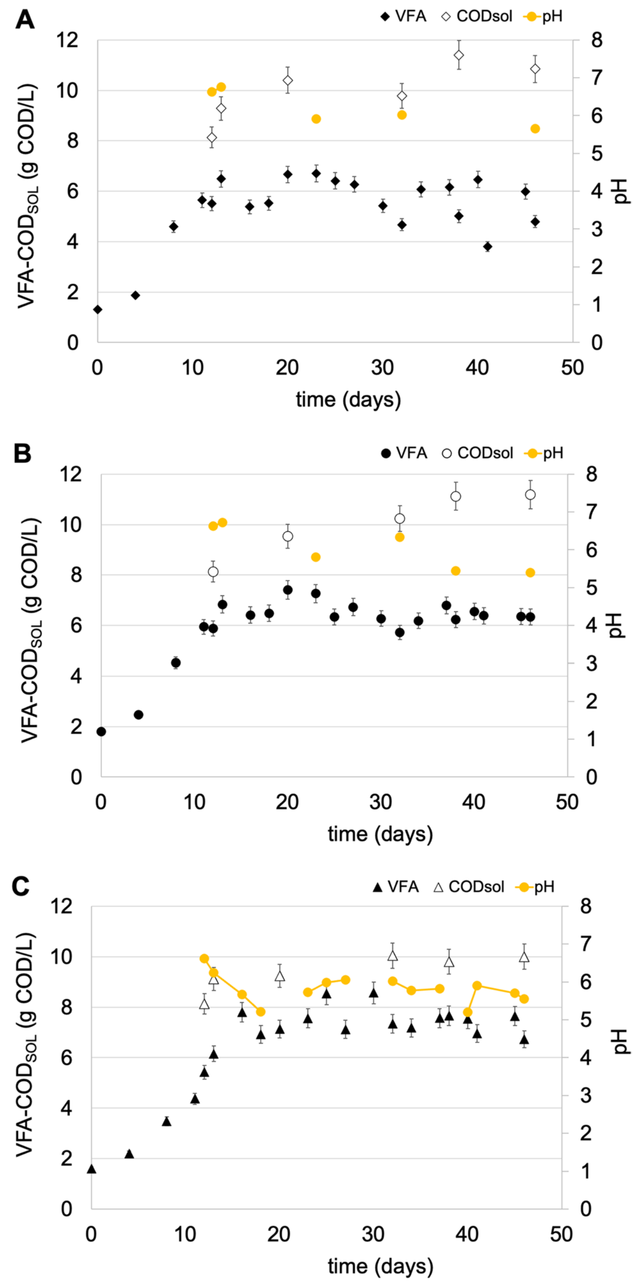


Fig. 2 Trends of VFA, COD_{SOL}, and pH in three sCSTR runs carried out at HRT of 4.0 (**A**), 5.0 (**B**), and 6.0 (**C**) days

6.5–6.7 decreased with the increase of acidogenic fermentation activities and VFA concentration. The sludge buffering capacity was not affected by the different HRT adopted since in all the three reactors the steady-state pH was spontaneously maintained around 5.5 and never below 5.2, sufficiently high to prevent any inhibition. The alkalinity values in the final effluent of the three sCSTR runs confirmed the good buffering capacity in each test, without any remarkable effect related to the adopted HRT. More specifically, the alkalinity in the final effluent was 1920 ± 65, 2048 ± 34, and 1976 ± 85 mg $CaCO_3$/L, respectively in the sCSTR conducted at 4.0, 5.0, and 6.0 days as HRT; these values were comparable with literature data describing VFA-rich streams where the pH was buffered by the recirculation of alkalinity-rich digestate [25].

In terms of rate and yield, the obtained data revealed an inverse relationship of the HRT with the fermentation yield and specific rate (Fig. 3). As general knowledge, the microbial activity tends to decrease as the HRT increases; the low HRT acts as key parameter for a selection of high-rate biomass. Based on the produced VFA (estimated as an average value throughout the stability periods), the fermentation rate decreased with the increase of the HRT. Consequently, the hydraulic regime had a significant impact on biomass kinetics, with the greatest fermentation rate obtained at 4.0 days as HRT (39 ± 2 mg COD_{VFA}/g VS_{in} h; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). With the increase of the HRT up to 5 days, a net decrease in the specific fermentation rate was observed (the mean fermentation rates obtained at 5.0 days and 6.0 days as HRT were not significantly different; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). On the other hand, the fermentation yield exhibited an opposite behavior: the best acidification performance was obtained at 6.0 days as HRT (0.30 ± 0.02 mg COD_{VFA}/g VS_{in}; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$), and the lowest one at 4.0 days as HRT (0.22 ± 0.01 mg COD_{VFA}/g VS_{in}; ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). These results revealed that the lowest HRT led to a selection of a mixed consortia with a high fermentation rate, but with a lower efficiency in the conversion of organic matter into VFA, compared to the consortia selected at higher HRT. According to the highest fermentation yield recorded in the sCSTR conducted at 6.0 days as HRT, a maximum COD_{VFA}/COD_{SOL} ratio of 0.72 ± 0.02 was attained (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). Hence, the 30% approximately of the solubilized COD remained unconverted. This was due to the nature of the organics in the sewage sludge, which is typically more difficult to be fermented into VFA if compared to other putrescible substances such as primary sludge and/or food waste [18]. This result certainly indicated the necessity of additional improvement in the process, possibly maintaining a relatively low-cost of the technology [26]. However, compared to the full-scale established anaerobic digestion, which has its limits in the fully exploitation of the bioresources' potential (the biogas is usually produced in oversized designed plants), the DF can be seen as a part within a multi-step biorefinery technology chain, where the organic substrates can be converted into added-value marketable products other than biogas alone [3].

Although a previous study demonstrated the importance of thermal pre-treatment for increasing COD_{SOL} and VFA content [4], thermal hydrolysis at temperatures greater than 150 °C could not be economically sustainable and, from the technical point of view, no significant benefit in acidification performance could be obtained: the fermentation yield was even lower if compared to this study (0.22 g COD_{VFA}/g VS_{in}), and the VFA was in the range 7.5–9.5 g COD_{VFA}/L. On the other hand, the absence of the pre-treatment may limit the potential of the acidogenic fermentation of sewage sludge: Presti et al. [27] obtained a fermented stream with a VFA concentration slightly greater than 2.0 g COD_{VFA}/L.

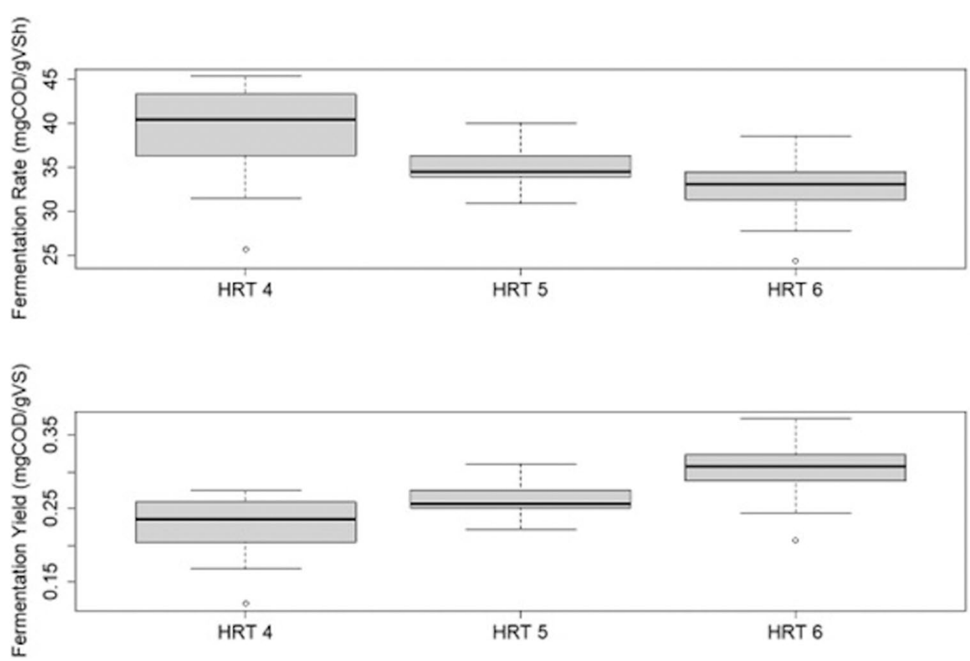


Fig. 3 Boxplot of the fermentation rate and yield in the three investigated HRT

Table 2 Summary of the main parameters (average data and standard deviation) of the acidogenic fermentation liquids in the three sCSTR runs

Parameter	Unit	HRT (days)		
		4.0	5.0	6.0
COD_{SOL}	g/L	10.0 ± 0.5	10.0 ± 0.5	9.4 ± 0.3
COD_{VFA}	g/L	5.7 ± 0.3	6.5 ± 0.2	7.3 ± 0.3
COD_{VFA}/COD_{SOL}	g/g	0.56 ± 0.05	0.66 ± 0.04	0.72 ± 0.02
Alkalinity	mg $CaCO_3$/L	1920 ± 65	2048 ± 34	1976 ± 85
Acetic acid	g COD/L	1.8 ± 0.1	1.9 ± 0.1	2.2 ± 0.1
Propionic acid	g COD/L	0.8 ± 0.06	1.0 ± 0.07	1.1 ± 0.1
Butyric acid	g COD/L	1.8 ± 0.1	2.0 ± 0.1	2.3 ± 0.2
Valeric acid	g COD/L	0.7 ± 0.07	0.8 ± 0.04	0.9 ± 0.03
Caproic acid	g COD/L	0.7 ± 0.08	0.8 ± 0.06	0.8 ± 0.05
Specific Rate	mg $COD_{VFA}/(g\ VS_{in}\ h)$	39 ± 2	35 ± 2	33 ± 2
$Yield_{VFA}$	g $COD_{VFA}/g\ VS_{in}$	0.22 ± 0.01	0.26 ± 0.02	0.30 ± 0.02
COD:N:P	g	100:5.3:2.9	100:6.6:2.8	100:7.7:3.5

Table 2 summarizes the main outcomes from the three investigated HRT.

3.3.1 VFA composition in the sCSTR runs

In all the performed runs, the start-up phase was characterized by a progressive increase in acetic acid, which occurred in conjunction with a gradual drop in caproic acid. This behavior was most likely caused by the adaptability of the culture as well as the gradual creation of fermentation pathways that led to the production of the typical products of the acetogenesis process (i.e., acetic acid, hydrogen, and carbon dioxide). This fact was probably favored by the utilization of pretreated sludge and, therefore, the presence of a partially hydrolyzed organic matter. Once the stability periods was reached, both acetic and caproic acids exhibited stable trends in their concentrations. Propionic, butyric, and valeric acids also exhibited stable trends, with minor changes in the first week of operation, independently from the adopted HRT. The stability aspect is of crucial importance for the effectiveness of the process, not only for VFA concentration but also in terms of composition. In particular, the VFA spectrum is pivotal because each VFA has a distinct market scenario and use, such as building blocks for the chemical industry or as precursors for the synthesis of reduced compounds in conventional organic chemistry.

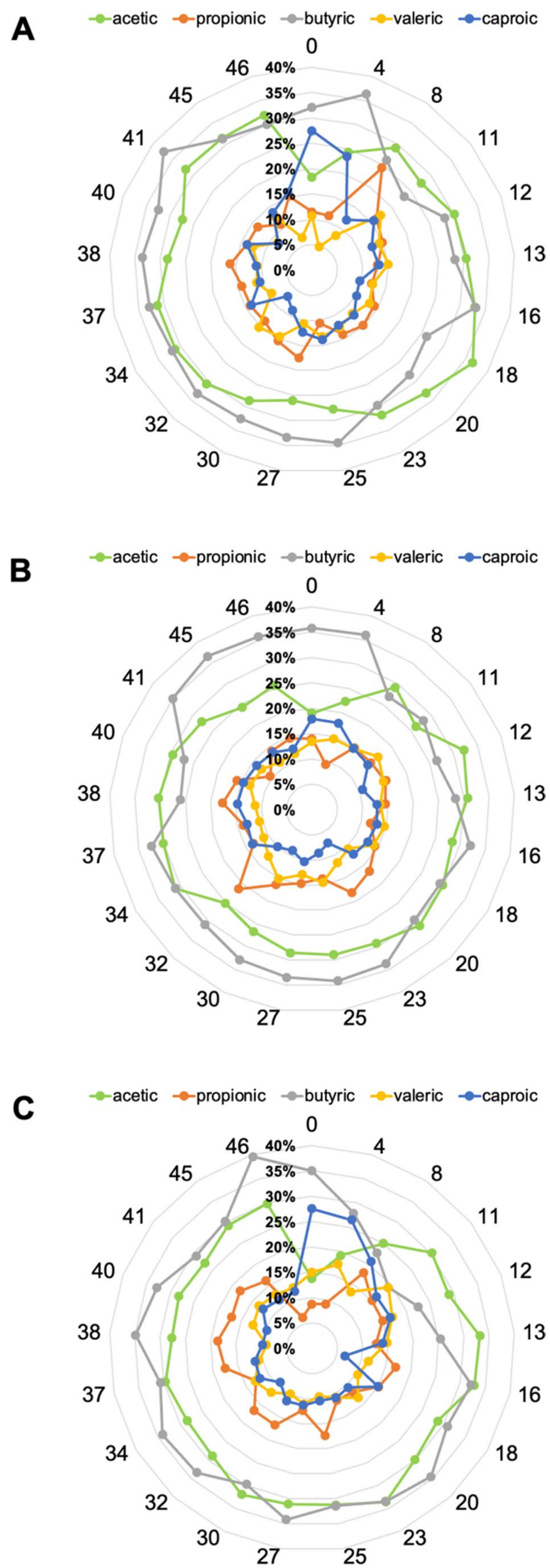
Between the three HRT investigated, no significant differences in VFA composition in the stability periods were observed (ANOVA test, $\alpha = 0.05$). The trends of each VFA (as COD fraction) clearly demonstrated a net predominance of VFA containing even carbon atoms (acetic and butyric acids) if compared to the others (Fig. 4). In this study, the composition of the acidified effluents was comparatively consistent with previous studies conducted with sewage sludge: around 28–33% acetic acid, 16–18% propionic acid, 25–30% butyric acid, and 21–27% valeric acid [4, 13]. By considering literature examples and this study, even if different thermal regime has been applied as pre-treatment, it is appropriate to claim that a certain impact of the temperature on VFA composition was observed. High-temperature pre-treatment may increase the fraction of shorter chain VFA (up to butyric acid) while decreasing the proportion of longer chain VFA (from valeric to heptanoic acid), especially if the pre-treatment is conducted at temperature higher than the typical values of mesophilic and thermophilic environment.

The description of the impact of high-temperature pre-treatment (in relatively mild condition) on acidogenic fermentation performances and/or fermentation products is lacking in the literature, at least for sewage sludge, often subjected to high-pressure thermal hydrolysis process (CAMBI™; [28]). In addition, CAMBI™ process could be costly (sludge pre-dewatering, pre-heating and then thermally hydrolyzed up to 175 °C and 6 bar) and not particularly necessary as pre-treatment for VFA production in the following DF process.

3.4 Microbial community composition

At the end of three distinct semi-continuous tests, the composition of the microbial communities thriving in the reactors was analyzed via high-throughput sequencing of the 16S rRNA gene of *Bacteria* (Fig. 5). A general selection and enrichment of fermentative bacteria was observed from the beginning up to the end of the tests. The most striking difference was the strong reduction of *Clostridium* sensu stricto *1*: 48.3% in the inoculum and 0.4%, on average, in

Fig. 4 Trends of each VFA (as COD percentage) observed at HRT ▸ of 4.0 days (**A**), 5.0 days (**B**), and 6.0 days (**C**); the numbers from 0 to 46 represent the days of the process from inoculum; the numbers from 0 to 40% represent the COD fraction of each single VFA



the reactors (Fig. 5). This shift can be most likely due to the not survival of some indigenous sludge microbes through high-temperature thermal pre-treatment and the mesophilic growth conditions usually preferred by members of *Clostridiaceae* [29, 30].

Overall, the biodiversity indexes calculation revealed a high bacterial diversity in the reactors, regardless the imposed HRT, with Dominance (D) index in the range 0.07–0.11, Simpson (1-D) 0.89–0.93, and equitability (J) 0.62–0.72.

Most likely, the abundance of soluble organic matters stimulated the growth of kinetically efficient fermentative bacteria, as indicated by the predominance of phyla *Firmicutes* and *Coprothermobacteraeota* (Fig. 5), in agreement with the acidogenic fermentation detected in the sCSTR. The phylum *Firmicutes* was mainly represented by the orders *Bacillales*, *Clostridiales*, and *Thermoanaerobacterales* (collectively accounting for an average 80.5% of total reads). These taxa can produce cellulases, lipases, proteases, other extracellular enzymes and hydrolyse complex organic matter providing the ability to efficiently use the substrates available in the anaerobic digestion of the sewage sludge [22, 31]. In fact, members of *Planococcaceae* (e.g., *Kurthia*, *Rumellibacillus*), herein accounting on average for 52.6% of total reads, are able to produce acids (mainly acetic) from a variety of carbohydrates. They were previously described in gut microbiome in agreement with their occurrence also in the inoculum and, most likely, in the sewage sludge used in this study [32]. The VFA production observed during the semi-continuous tests can be also due to the fermentative activity of members of genera *Thermoanaerobacter* and *Romboutsia* representing on average 23.1 and 2.0% of total reads (Fig. 5) [33]. In line with previous reports in anaerobic thermophilic reactors [34], the biomass in all three sCSTRs was also characterized by the presence of the proteolytic genus *Coprothermobacter* (on average 16.3% of total reads) most likely involved in the fermentation of proteins and sugars.

Regardless the imposed HRT, the high-throughput sequencing showed that the main taxa in the reactors were related to the degradation of complex organic matter like proteins and carbohydrates, which were subsequently fermented to produce acetic, propionic, and butyric acids as main products. Therefore, the operating conditions applied in this work strongly selected a microbial community involved in the fermentation of sewage sludge for VFA production, with no differences in terms of biodiversity in the three stability periods, in line with no differences in

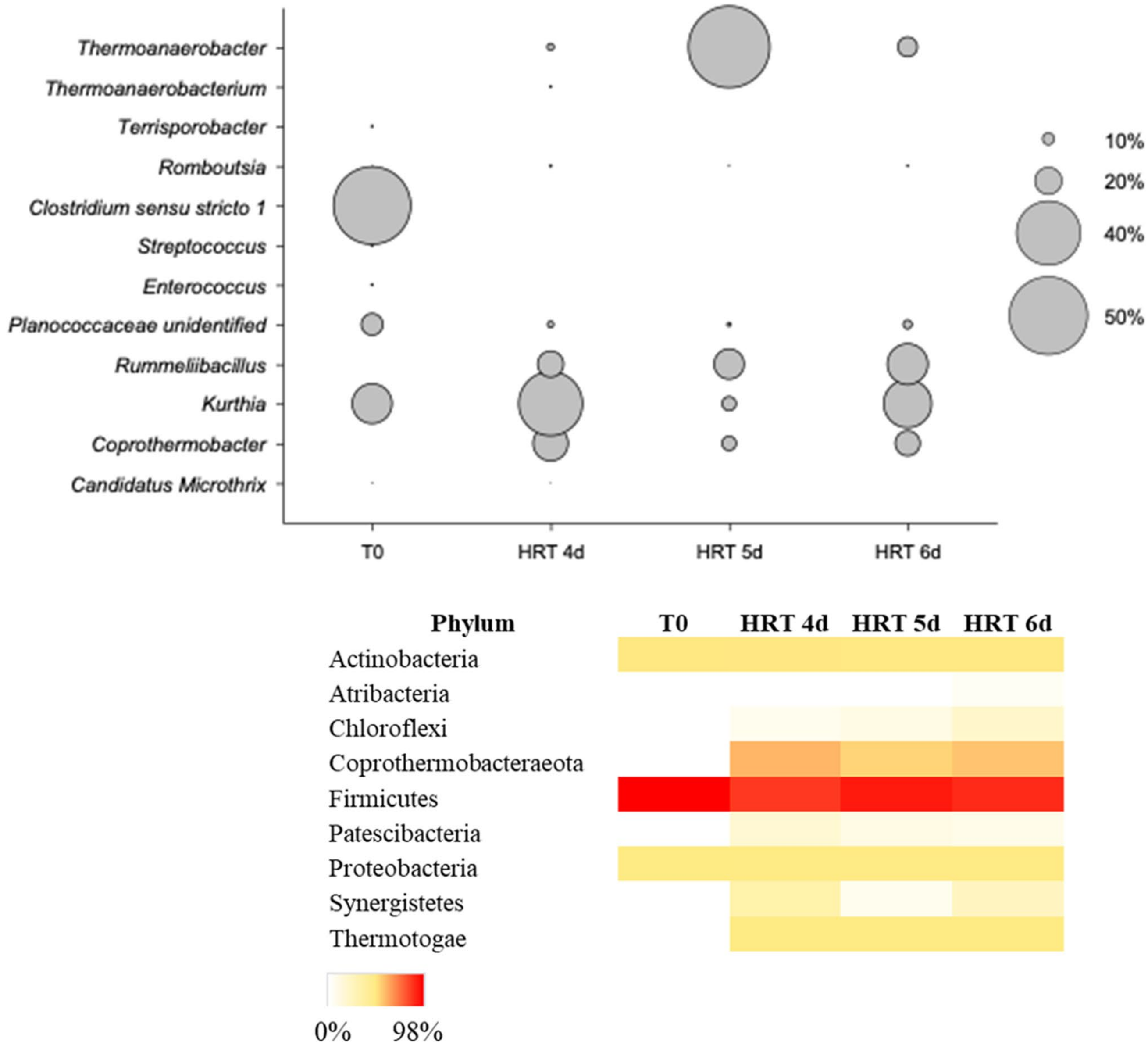


Fig. 5 Bubble plot depicting the relative abundance (as percentage of total reads) of the main taxa at genus level (≥ 1% in at least one sample) at the beginning (T0) and at the end of the tests in the biomass of reactors at different HRT; sequence frequency heat-map of bacterial communities at phylum level (the colour intensity shows the relative abundance; white min 0%, red max 98.1%)

VFA composition. Nevertheless, the imposed HRT most likely exerted different pressure on microbial metabolic activity thus reflecting differences in terms of VFA fermentation rate and yield.

4 Conclusion

The urgency related to the sewage sludge management is due to its large production and the necessity to develop recovery options in compliance with the current European and national legislation, and with the forthcoming transition toward the principles of a circular economy. The synthesis of VFA from this precious carbon source has been investigated by exploring different options related to its pre-treatment and to the operating conditions of the process (e.g., HRT). This work demonstrated how thermally hydrolyzed sewage sludge can be utilized as a possible feedstock to sustain the VFA generation under thermophilic condition. Optimal solubilization of the organic matters was achieved at 70 °C (without addition of chemicals), being the COD_{SOL} increased by approximately ten times if compared to raw sludge. Among the three distinct investigated HRT, the DF process showed higher acidification performances (VFA concentration of 7.3 g COD_{VFA}/L; acidification yield 0.30 g COD_{VFA}/gVS_{in}; COD_{VFA}/COD_{SOL} ratio 0.72) at 6.0 days as HRT. *Coprothermobacteraceae*, *Planococcaceae*, and *Thermoanaerobacteraceae* were the most abundant selected microorganisms, responsible for the accumulation of acetic, propionic, and butyric acids, which dominated the VFA spectrum.

Abbreviations *CSTR*: Continuous stirred tank reactor; *DF*: Dark fermentation; *FID*: Flame ionization detector; *GC*: Gas chromatograph; *HRT*: Hydraulic retention time; *OLR*: Organic loading rate; *SRT*: Sludge retention time; COD_{SOL}: Soluble chemical oxygen demand; *VFA*: Volatile fatty acids; *TKN*: Total Kjeldahl nitrogen; *TS*: Total solids; *VS*: Volatile solids; *WWTP*: Wastewater treatment plant

Acknowledgements The hospitality of Alto Trevigiano Servizi (ATS) S.r.l. is gratefully acknowledged.

Author contribution Conceptualization, methodology, writing—original draft preparation, resources, supervision: Marco Gottardo; methodology, formal analysis and investigation, writing—original draft preparation: Simona Crognale; formal analysis and investigation: Barbara Tonanzi; supervision, resources: Simona Rossetti; formal analysis and investigation: Ludovica D'Annibale; conceptualization: Joan Dosta; funding acquisition, writing—original draft preparation: Francesco Valentino.

Funding This work was partially supported by DAIS—Ca' Foscari University of Venice, within the IRIDE program.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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