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Performance and genome-centric metagenomics of thermophilic single and two-stage anaerobic digesters treating cheese wastes

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ABSTRACT

The present research is the first comprehensive study regarding the thermophilic anaerobic degradation of cheese wastewater, which combines the evaluation of different reactor configurations (i.e. single and two-stage continuous stirred tank reactors) on the process efficiency and the in-depth characterization of the microbial community structure using genome-centric metagenomics. Both reactor configurations showed acidification problems under the tested organic loading rates (OLRs) of 3.6 and 2.4 g COD/L-reactor day and the hydraulic retention time (HRT) of 15 days. However, the two-stage design reached a methane yield equal to 95% of the theoretical value, in contrast with the single stage configuration, which reached a maximum of 33% of the theoretical methane yield. The metagenomic analysis identified 22 new population genomes and revealed that the microbial compositions between the two configurations were remarkably different, demonstrating a higher methanogenic biodiversity in the two-stage configuration. In fact, the acidogenic reactor of the serial configuration was almost solely composed by the lactose degrader *Bifidobacterium crudilactis* UC0001. The predictive functional analyses of the main population genomes highlighted specific metabolic pathways responsible for the AD process and the mechanisms of main intermediates production. Particularly, the acetate accumulation experienced by the single stage configuration was mainly correlated to the low abundant syntrophic acetate oxidizer *Tepidanaerobacter acetatoxydans* UC0018 and to the absence of aceticlastic methanogens.

1. Introduction

The dairy industry consists of several production divisions, each one of them generating considerable amounts of effluent wastewater streams. Especially, during the cheese making process, different types of residues are discarded at various steps of the production chain. Cheese whey permeate (WP) is a by-product originating from the cheese manufacturing process during the step of proteins recovery by ultrafiltration and/or diafiltration. It mainly contains lactose and therefore is often used to standardize the nutritional composition and taste of milk. However, in most cases, WP is not exploited and thus is considered as high strength wastewater (i.e. BOD_5/COD ratio is usually higher than 0.5) (Prazeres et al., 2012). It is estimated that the annual production of WP at global scale can be over 10^8 tons per year (Grba et al., 2002). A less known waste derives from the portioning and shaving phases of hard-cheese manufacturing process, and it consists in a cheese powder waste (CP), which mainly contains proteins and fats. Especially, in Italy, the production of two Protected Designation of Origin (PDO) hard-cheeses, Grana Padano and Parmigiano Reggiano, counted more than 182,000 t and 137,000 t, respectively in 2015 (ISMEA, 2016). These volumes suggest a considerable amount of waste derived from each cheese mould. From the above, it is obvious that the residues of dairy industry require an effective treatment before their disposal to the final recipients.

Several biological treatments have been proposed to process WP

Abbreviations: WP, whey permeate; CP, cheese powder; AD, anaerobic digestion; VFA, volatile fatty acid; CSTR, continuous stirred-tank reactor; TRS, total random sequencing; PG, population genome; R1, single stage reactor; R2, acidogenic reactor of the two-stage configuration; R3, methanogenic reactor of the two-stage configuration.

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including anaerobic digestion (AD), lactose hydrolysis, ethanol, hydrogen or lactic acid fermentations, enzyme production, and microbial fuel cells (Cota-Navarro et al., 2011; Prazeres et al., 2012; Schirru et al., 2014). Among them, AD for biogas production is considered as a sustainable solution for waste valorization and energy recovery. AD is a complex biological process involving different microbial consortia to break down organic matter into several by-products and finally to biogas, which is mainly composed by methane and carbon dioxide. This overall process consists of four steps namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone et al., 2008); the resulting methane can be used for electricity and thermal energy generation or by performing additional purification steps biomethane can substitute natural gas (Kougias et al., 2017).

Nevertheless, the high sodium content, acidic pH and low alkalinity of WP hampers its treatment by biological processes (Backus et al., 1988; Ghaly, 1996; Castelló et al., 2009). In order to overcome such technical challenges, different reactor configurations (Stamatelatou et al., 2014) were tested or co-digestion strategies were employed in order to efficiently degrade wastewater from cheese-making processes (Gelegenis et al., 2007; Hagen et al., 2014). The majority of these studies have been performed under mesophilic conditions and the few works reporting thermophilic reactor operation are focusing on simultaneous production of H_2 and CH_4 in two steps (Fernandez et al., 2015; Kisielewska et al., 2014). However, it is well known that thermophilic conditions, even if they are more sensitive to inhibitors, pose several advantages in biogas production, such as higher methane production rates and shorter hydraulic retention times (Harris and Dague, 1993; Wiegant et al., 1986; Zinder et al., 1984). To the best of our knowledge, information regarding thermophilic operation of anaerobic reactors fed exclusively with cheese wastewater and by-products for biogas production is missing.

Another crucial parameter, which determines the degradation efficiency of these wastes, is the microbial consortium involved in the AD process. Understanding the diversity and dynamics of such community will lead to process optimization by calibrating operational parameters and by enhancing preferred microbial pathways, which will result in higher CH_4 yields. A way to achieve this goal is via genome-centric metagenomics, which employs shotgun sequencing, *de novo* assembly of the obtained reads and binning of the scaffolds in population genomes (Campanaro et al., 2016).

This study aims to compare the efficiency of two thermophilic reactor configurations, single and two-stage continuous stirred tank reactors (CSTRs), on the anaerobic degradability of a mixture of cheese wastes, namely WP and CP. Furthermore, we analyzed the reactors' metagenomes via Total Random Sequencing (TRS) and metagenomic binning strategy. Functional analyses of the main population genomes (PGs) were also carried out, in order to highlight possible connections with the main intermediates produced along the process, such as volatile fatty acids (VFAs).

2. Materials and methods

2.1. Substrates characterization and feedstock preparation

The cheese whey permeate was obtained from Arla, Denmark, and stored at −20 °C, in 2 L bottles. The Grana Padano PDO cheese waste powder (from the portioning phase of manufacturing process) was obtained from Colla S.p.A., Italy, and stored in vacuum-sealed bags at 4 °C. Before usage, the whey permeate was thawed at 4 °C for 1–2 days. The feedstock was prepared by mixing the two substrates by hands and it was kept homogenized with a magnetic stirrer during the feeding times. The chemical composition of each substrate and mixed feedstock are shown in Table 1.

2.2. Reactors' configurations and process parameters

The setup consisted of a single (R1) and a two-stage CSTRs (R2 and R3, respectively); each setup had a total working volume of 3 L. The working volume of the two-stage configuration was split between the acidogenic reactor (R2; 0.6 L) and the methanogenic reactor (R3; 2.4 L). Each reactor was filled with inoculum, obtained from Snertinge thermophilic biogas plant (Denmark), which is mainly fed with livestock manure (pig and cattle) and wastes from food industry. The inoculum had a pH of 8.1, total solids (TS) and volatile solids (VS) content of 31.71 ± 0.04 and 21.45 ± 0.05 g/L, respectively. The total volatile fatty acids (VFAs), total Kjeldahl Nitrogen (TKN) and ammonium nitrogen (NH_4^+-N) concentrations were 0.13 ± 0.02, 3.78 ± 0.01, 3.15 ± 0.01 g/L, respectively. The reactors were mixed by magnetic stirrers (stirring intensity equal to 150 rpm) and were kept at thermophilic conditions (55 ± 1 °C) using thermal jackets. R1 and R2 were fed four times per day with a mixture of whey permeate (WP) and cheese powder (CP), while R3 was fed with the effluent from R2. Each time the reactors were fed with fresh substrate, equal volume of effluent digestate was removed from the reactors by pneumatic pressure. The hydraulic retention time (HRT) was set at 15 days, for both configurations (split in 3 and 12 days in R2 and R3, respectively). The organic loading rates (OLRs) tested were initially 3.6 g COD/L-reactor day (Phase I), then, due to acidification problems, 2.4 g COD/L-reactor day (Phase II). Sodium bicarbonate addition in R1 and R3 was applied whenever the pH dropped below 6.5.

2.3. Analytical methods

The daily biogas production of R1 (single stage reactor) and R3 (methanogenic reactor of the two-stage configuration) were measured by an automated gas meter (Angelidaki et al., 1992). Total Solids (TS), Volatile Solids (VS), Chemical Oxygen Demand (COD),

Table 1
Substrates and feedstocks physico-chemical characteristics.

Parameter	WP	CP	Feedstock (Phase I)	Feedstock (Phase II)
pH	6.30 ± 0.20	5.10 ± 0.20	5.50 ± 0.20	5.50 ± 0.20
TS (g/L)	36.62 ± 3.35	854.03 ± 6.43	46.01 ± 4.89	34.55 ± 3.90
VS (g/L)	33.37 ± 3.00	826.70 ± 19.37	42.46 ± 3.21	31.89 ± 2.41
COD (g/L)	33.98 ± 2.99	1545.95 ± 36.13	54.02 ± 3.39	36.01 ± 2.54
Total VFA (g/L)	0.05 ± 0.01	0.53 ± 0.17	0.05 ± 0.01	0.04 ± 0.01
Lactic acid (g/L)	0.41 ± 0.01	0	0.41 ± 0.01	0.31 ± 0.01
TKN (g/L)	0.26 ± 0.05	69.81 ± 0.10	1.03 ± 0.05	0.77 ± 0.04
NH_4^+-N (g/L)	0	9.37 ± 0.02	0.10 ± 0.01	0.08 ± 0.01
Lipids (g/L)[a]	0	290.00[a]	3.20[a]	2.40[a]

[a] Data estimated considering the lipid content established by the PDO regulation in 100 g of Grana Padano cheese (https://www.granapadano.it/public/file/tabNutriGPit-5067-20094.pdf), whose powder waste was used in the experiment as indicated in the "Materials and methods" section.

pH, ammonium nitrogen (NH_4^+–N) and Total Kjeldahl Nitrogen (TKN) were measured according to the Standard Methods (APHA, 2005). Samples from reactors were collected for pH and VFA analyses twice per week. VFA samples were determined using a gas chromatograph (GC-2010; Shimadzu) with a flame ionization detector and FFAP column as described previously (Kougias et al., 2013). Lactic acid concentration was measured by HPLC (Agilent 1100) with a BioRad-Aminex HPX-87H column using 4 mM H_2SO_4 as eluent at a flow rate of 0.6 mL/min. The biogas composition was measured twice per week, using a gas chromatograph with a thermal conductivity detector (GC-TCD) as described by Kougias et al. (2014). The biochemical methane potential (BMP) of the substrate was determined following the protocol described by Angelidaki et al. (2009) and the measurements were carried out by a gas chromatograph with a flame ionization detector (FID), as described previously (Kougias et al., 2015). Macronutrients were measured via inductively coupled plasma optical emission spectrometry (ICP-OES), after the thermochemical pretreatment of 0.25 g freeze-dried sample. In particular, 9 mL of 65% nitric acid, 3 mL of 37% hydrochloric acid and 0.5 mL of 30% hydrogen peroxide were used to digest the sample, along with treatment in a microwave unit. The mixture was then centrifuged and diluted prior analysis. All analyses were performed in replicate samples.

2.4. Metagenomic sequencing and microbial communities analyses

Triplicate samples were taken at the reactors' steady state conditions of Phase II and genomic DNA was extracted from all reactors with RNA PowerSoil DNA Elution Accessory Kit (MOBIO Laboratories, Carlsbad, CA). DNA quality and quantity were analyzed using NanoDrop (ThermoFisher Scientific, Waltham, MA) and Qubit fluorimeter (Life Technologies, Carlsbad, CA). Samples were sequenced, using NextSeq 500 sequencing technology and Nextera XT kit (Illumina, San Diego, CA) for library preparation (150 + 150 bp). Quality filtering and adaptors removal were carried out using Trimmomatic software (v0.33) (Bolger et al., 2014). *De novo* assembly was executed by CLC Genomics workbench v. 5.1 (CLC Bio, Aarhus, DK, USA) using kmer 63 and bubble size 60. Gene finding was performed with Prodigal (v2.6.2) run in metagenomic mode (Hyatt et al., 2012). Genes were annotated according to KEGG using GhostKOALA (Kanehisa et al., 2016) and to EggNOG 4.5.1 using eggNOG-mapper (Huerta-Cepas et al., 2016). Scaffolds were binned into Population Genomes (PGs) using two strategies: MetaBAT (v0.25.4) (parameters –specific, -m 1500) (Kang et al., 2015) and the "Hierarchical Clustering followed by Canopy Profile selection" (HCCP) (Campanaro et al., 2016). In order to improve the binning strategies, the coverage profile of the scaffolds was determined by aligning the shotgun sequences derived from other projects on the assembly. Only samples providing a percentage of aligned sequences higher than 20% were considered (Kougias et al., 2016; Treu et al., 2016). Completeness and "contamination" of the PGs were estimated using "lineage wf" workflow of CheckM and the PGs obtained from the two approaches were compared using the "bin-compare" module of the same software (Parks et al., 2015). PGs identified using both binning strategies were retained from the HCCP procedure. For all the PGs, gene prediction was improved comparing results from two different software, Prodigal (Hyatt et al., 2012) and GeneMarkS (Besemer et al., 2001), and merging the final results with a perl script previously described (Campanaro et al., 2014). Functional analysis of the PGs was based on KEGG annotation via FOAM software (Prestat et al., 2014). Module completion ratio (MCR) in each functional module of the KEGG database was evaluated using MAPLE-2.3.0 (http://www.genome.jp/tools/maple/help.html). Taxonomical assignment for the PGs was achieved by comparing results from PhyloPhlAn (v0.99) (Segata et al., 2013), CheckM (Parks et al., 2015) and the 16S rRNA taxonomic assignment obtained according to the BLASTn results from the NCBI 16S ribosomal database. The presence of genomes belonging to the same species in the NCBI microbial genome database and in PGs recovered in previous assemblies was determined using Average Nucleotide Identity (ANI) calculation as previously described (Campanaro et al., 2017). Abundance of genes and PGs in different reactors was considered as directly related to scaffold coverage as previously described (Campanaro et al., 2014). Coverage values determined for PGs were visualized with MeV (Saeed et al., 2003).

Raw sequence data have been deposited at Sequence Read Archive (SRA) under the BioProject PRJNA394669 and the accessions SAMN07367931 - SAMN07367939.

3. Results and discussion

3.1. Reactors' process monitoring and configurations' comparison

Considering the first operation phase, during which the OLR was set to 3.6 g COD/L-reactor day, both configurations (single and two-stage) showed increased instability in terms of CH_4 yield (Fig. 1). Such instabilities can be mostly addressed to the difficulties in maintaining the CP well homogenized in the permeate fraction. This can provoke irregular organic daily loads, in particular of the insoluble

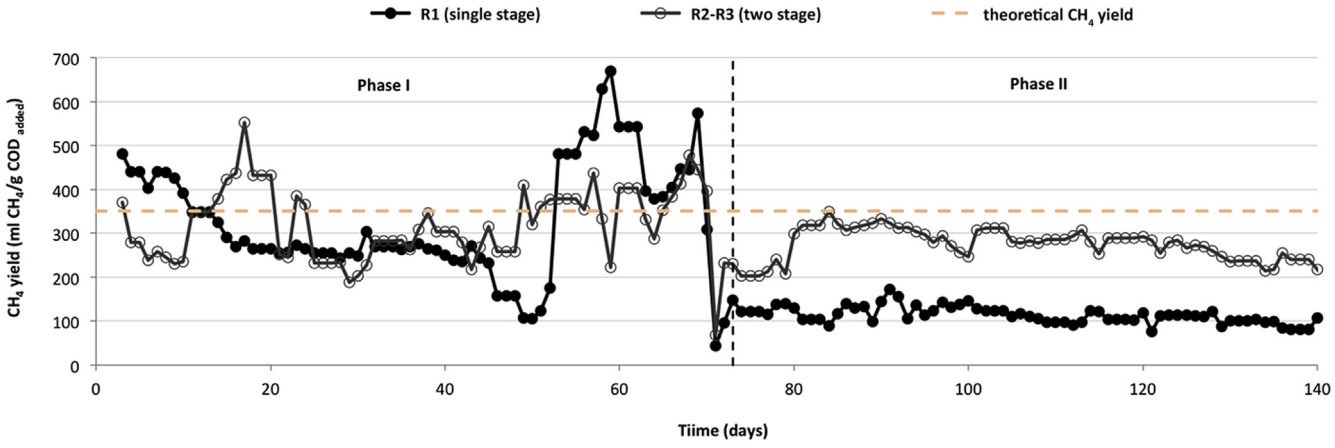

Fig. 1. CH_4 yield of the single stage (R1) and two-stage (R2-R3) configurations, during the two phases (I and II) investigated.

CP lipid fraction, which could have affected the methanogenic community. It has been previously found that lipid toxicity is higher under thermophilic conditions, especially concerning acetate-utilizing methanogens (Hwu and Lettinga, 1997). In addition, process over-acidification (Fig. 2) related to the feed characteristics could also be involved in the performance instability. Indeed, the acidic pH and the lack of buffer capacity of the whey permeate caused abrupt drops in pH, which could affect specific members of the microbial consortia involved in the AD food chain, resulting in slower metabolic activities (Ahring et al., 1995). Indeed, total VFAs concentration increased in R1 and R3, accumulating mostly acetate and butyrate (Fig. 2). This increment can inhibit specific taxa of acetogenic bacteria or methanogenic archaea, decelerating the whole process and affecting the methane yield. It is worth noting that the low concentration of propionate (less than 1 g/L) in R1 and R3 indicates an efficient syntrophic association between the propionate-degraders, which produce acetate and H_2, and the methanogenic communities (Kim et al., 2002; Van Lier et al., 1993).

Despite the addition of sodium bicarbonate to recover the pH in a suitable range for methanogenesis, the process remained unstable for both configurations. In addition to the lipid toxicity effect, the over-acidification that was concurrently experienced after day 66 in both R1 and R3 reactors could be also related to an inhibitory action provoked by the concentration of potassium in the feed (Table 2). It was previously observed that K^+ at concentrations higher than 0.4 g/L can inhibit particularly the aceticlastic methanogens (Kugelman and McCarty, 1965). This inhibition was also reported at higher extent under thermophilic conditions (Kugelman and McCarty, 1965).

In order to overcome the problems due to the feed characteristics, which led to process instabilities in terms of methane production (Phase I, Fig. 1), the OLR was reduced to 2.4 g COD/L-reactor day (Phase II, Fig. 1). The decrease of the OLR managed to improve the efficiency of the two-stage configuration, which maintained a stable profile in terms of CH_4 yield (300 ± 33 mL CH_4/g COD_{added}),

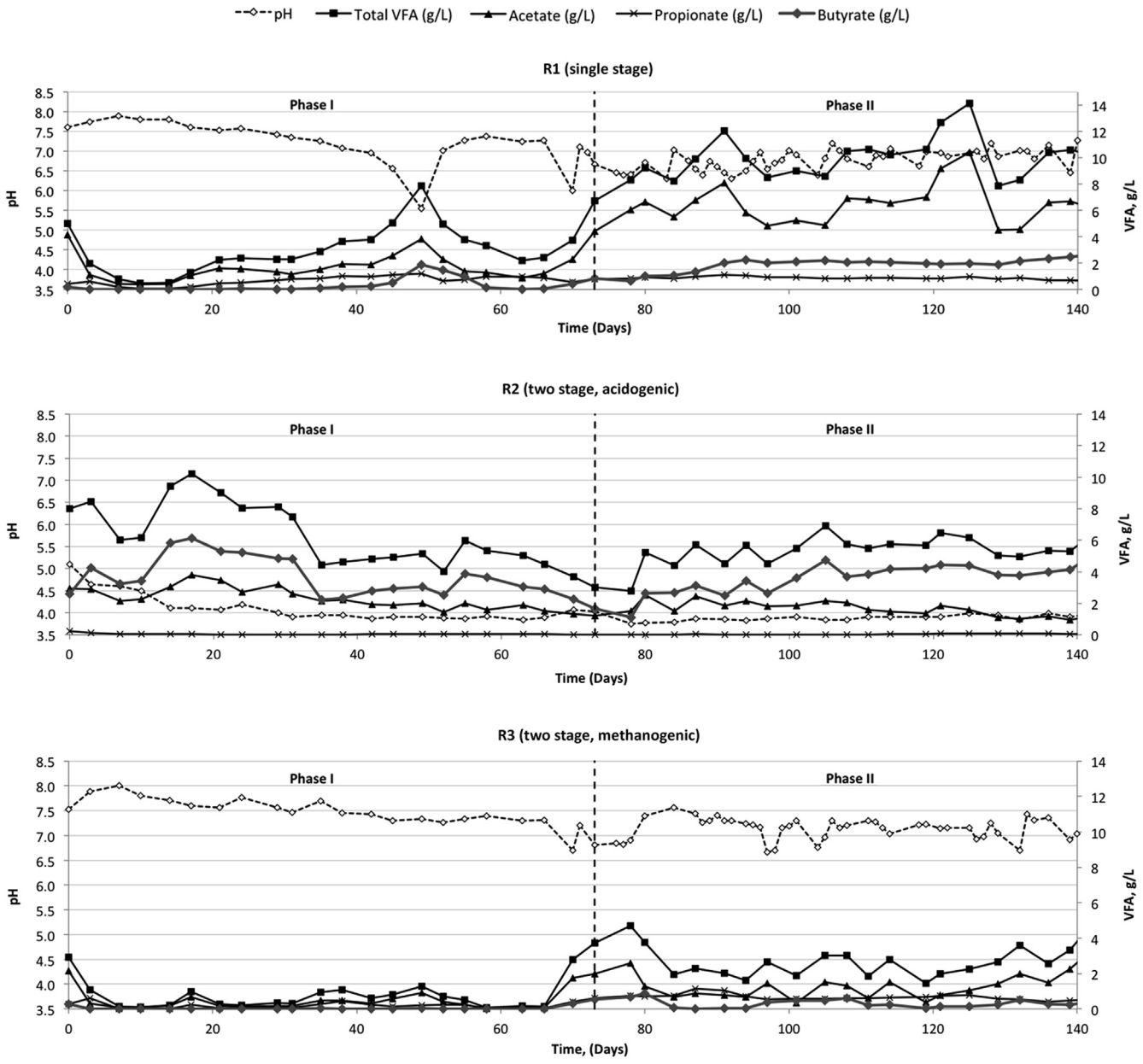

Fig. 2. pH and VFAs profiles of the single stage (R1) and two-stage (R2-R3) CSTRs, during Phase I and Phase II.

Table 2
Most abundant macronutrients in the feedstock, expressed in g/Kg of dry matter, at Phase I and II.

Element	Concentration (Phase I)	Concentration (Phase II)
Ca	10.47 ± 0.31	7.86 ± 0.23
K	22.19 ± 1.54	16.66 ± 1.16
Mg	1.61 ± 0.01	1.21 ± 0.01
Na	6.91 ± 0.25	5.19 ± 0.19
P	7.33 ± 0.37	5.50 ± 0.28
S	2.10 ± 0.04	1.58 ± 0.03

corresponding to a maximum of 95% of the theoretical value (Angelidaki et al., 2011) (Fig. 1). On the contrary, the single stage reactor exhibited pH instability, showing a further increase in acetate concentration up to 10 g/L (Fig. 2). Despite the addition of sodium bicarbonate for the maintenance of pH values over 6.5, the reactor was performing at an inhibited steady state condition (100 ± 16 mL CH_4/g COD_{added}), reaching a maximum of 33% of the theoretical CH_4 yield. The higher process efficiency of the two-stage configuration could be mainly due to the pre-acidification step occurring in R2. Similar findings were also found in a mesophilic CSTR-UASB system digesting diluted cheese whey (Diamantis et al., 2014). The acidogenic reactor (R2) maintained a stable pH (i.e.

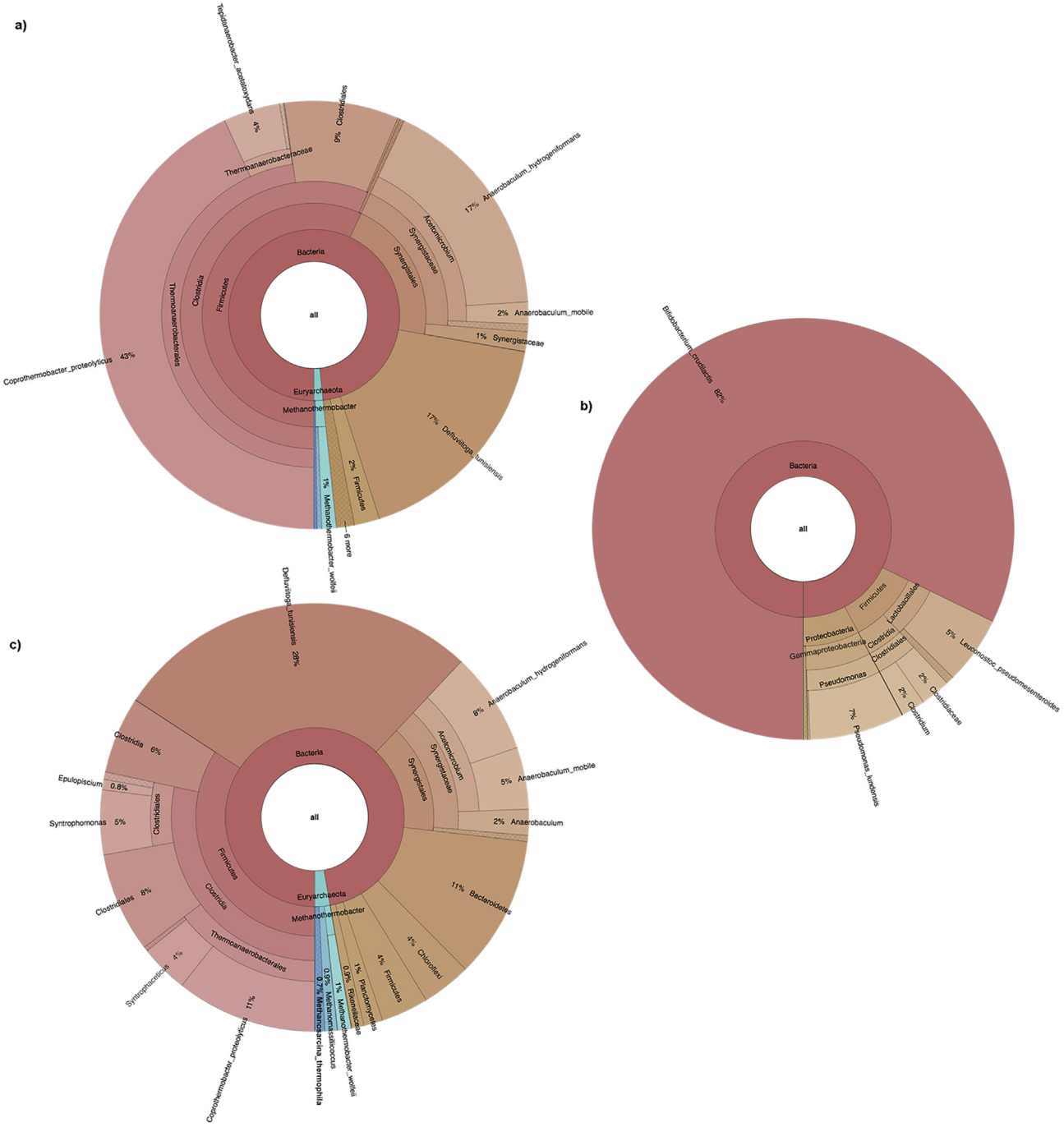

Fig. 3. Microbial community composition of the CSTRs: single stage configuration R1 (a), acidogenic R2 (b) and methanogenic R3 (c) reactors of the two-stage configuration. The percentages indicate the relative abundance of the PGs assigned to the taxonomic groups as described in Material and Methods section.

3.9 ± 0.1) all along the duration of the process. Moreover, the concentration of VFAs was stabilized after the OLR reduction (Phase II) having values between 4.5 and 7 g/L (Fig. 2).

Butyrate was the intermediate compound showing the highest accumulation level (3.5 ± 1.1 g/L), followed by acetate (1.6 ± 0.4 g/L), whereas propionate was almost absent. The VFAs composition of R2 could have also favored the two-stage performance since butyrate and acetate were found to be the best precursors for methane production (McCarty and Smith, 1986; Stronach et al., 1986; Speece, 1996). Moreover, the lack of propionate in this reactor probably contributed to avoid potential toxicity effects on the methanogenic community of R3 (Barredo and Evison, 1991).

3.2. Genome-centric metagenomics and predictive functional analyses

The obtained assembly had a total length of more than 160 Mbp (N50: 5442) indicating that almost the total community was represented in the assembled scaffolds (since it included 86%–99% of the reads, depending on sample). To assign a putative functional role to the species present, a genome-centric approach was applied to the assembly. The binning strategy assigned the scaffolds to the microbial species and identified 50 PGs, 19 of them having estimated completeness higher than 90% and average contamination around 2%. The success of the applied strategy is highlighted by the high percentage of scaffolds that were assigned to a genome, with only few left unassigned (less than 40%). The presence of previously sequenced genomes identified in the present project was verified by comparing the reconstructed PGs of this study with the genome sequences deposited in the Microbial Genome Resources (NCBI) and reported in previous genome-centric investigations (Kougias et al., 2016; Treu et al., 2016; Mosbaek et al., 2016; Stolze et al., 2016; Vanwonterghem et al., 2016). From this analysis, it was found that 12 PGs were assigned to known species, 16 were identified in previous genome-centric studies, and 22 PGs were newly identified. The new PGs were mostly assigned to *Clostridia* class and

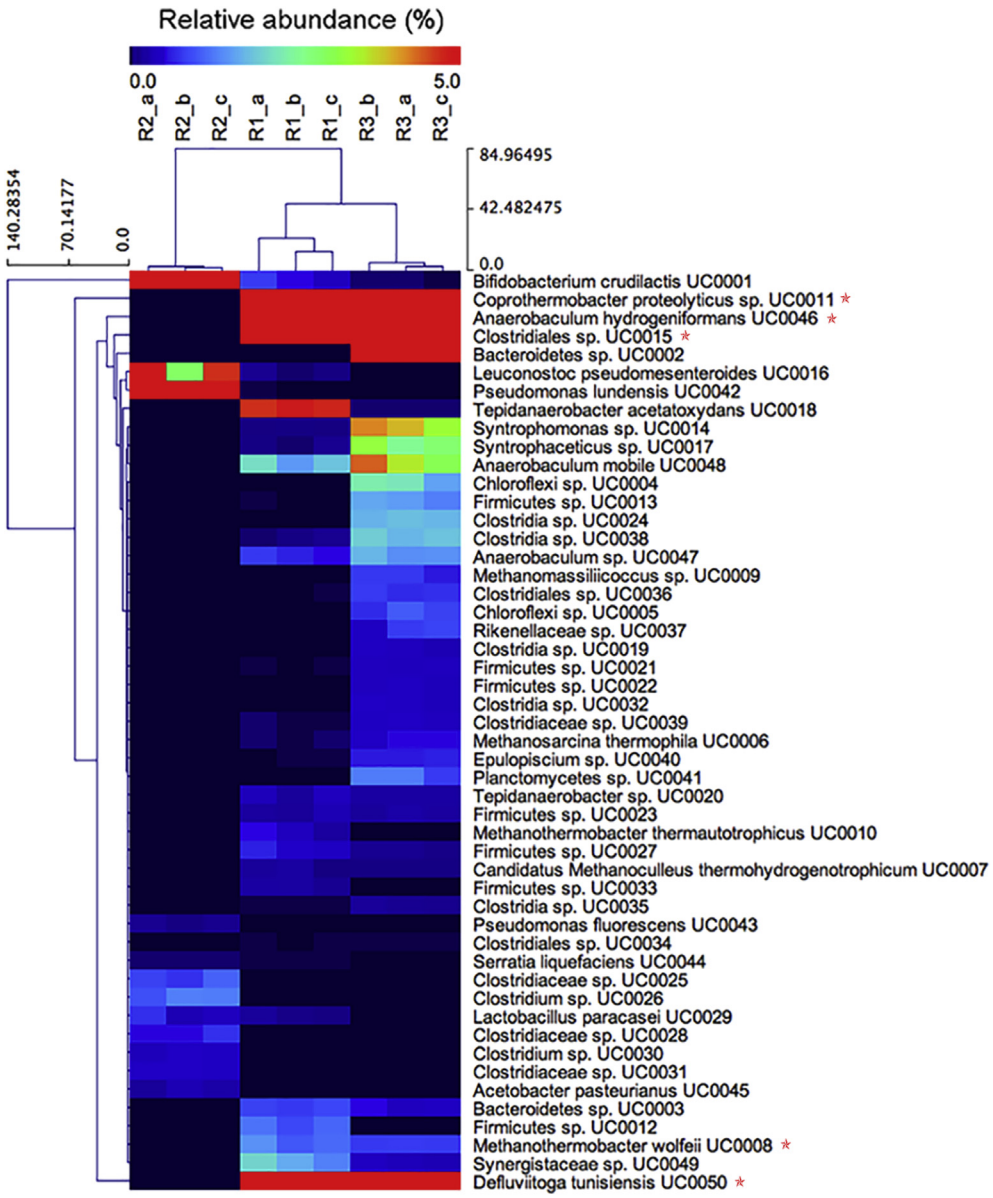

Fig. 4. Heat map of relative abundance of the 50 PGs identified in the study (R1: single stage; R2: acidogenic reactor of the two-stage; R3: methanogenic reactor of the two-stage; a, b, c: replicates). The red stars indicate the common "microbial core" between R1 and R3.

none of them was classified at species level.

Investigation of the proteins function and identification of the metabolic pathways encoded in the PGs, allowed to draw some correlations between the abundance of the PGs and the presence of key metabolic intermediates. For practical reasons, only the highly abundant and most complete PGs were selected in this analysis (coverage >3 and completeness >70%). Subsequently, by selecting the pathways having completeness higher than 70%, it was possible to obtain a "single-species level" metabolic reconstruction (Fig. A4).

The microbial consortium established in the reactors under investigation was a result of the physico-chemical characteristics of the feed, namely acidic pH and presence of lactose as the main carbon source. Indeed, the bacterial community of the current study was found to be remarkably different compared to previously investigated AD microbiomes, even if the same thermophilic inoculum was used (Bassani et al., 2015; Campanaro et al., 2016) (Fig. 3).

The predictive functional analysis based on KEGG annotation, showed that the higher biodiversity found in the two-stage configuration (including both *Bacteria* and *Archaea*) (Fig. 3) resulted in a higher number of ABC transporters (27) for amino acids and lipo-oligosaccharides intake, as well as genes involved in amino acids and fatty acid metabolisms (65). Thus, it can be assumed that a wider range of intermediate compounds were available as feed for the microbial consortia of the serial CSTRs.

3.2.1. Single stage reactor (R1)

The single stage reactor R1 showed a dominance of three phyla: *Firmicutes* (57%), *Synergistetes* (21%) and *Thermotogae* (17%) (Fig. A1a). It is worth noticing the absence of the phylum *Bacteroidetes*, which is generally one of the most abundant phylum in biogas reactors (around 10%), frequently involved in the hydrolytic step of AD (Campanaro et al., 2016; Fontana et al., 2016; Luo et al., 2016; Stolze et al., 2016). Interestingly, each phylum identified in R1 was represented by a single species, highlighting the effect of feedstock on the bacterial population established in the reactor (Fig. 3a).

Despite the differences between the configurations, the two methanogenic reactors, R1 and R3, shared a "microbial core" composed by four *Bacteria* species (Fig. 4): *Coprothermobacter proteolyticus* sp. UC0011, *Defluviitoga tunisiensis* UC0050, *Anaerobaculum hydrogeniformans* UC0046 and *Clostridiales* sp UC0015. These PGs were previously associated to the hydrolytic step of the thermophilic anaerobic digesters (Campanaro et al., 2016; Maune

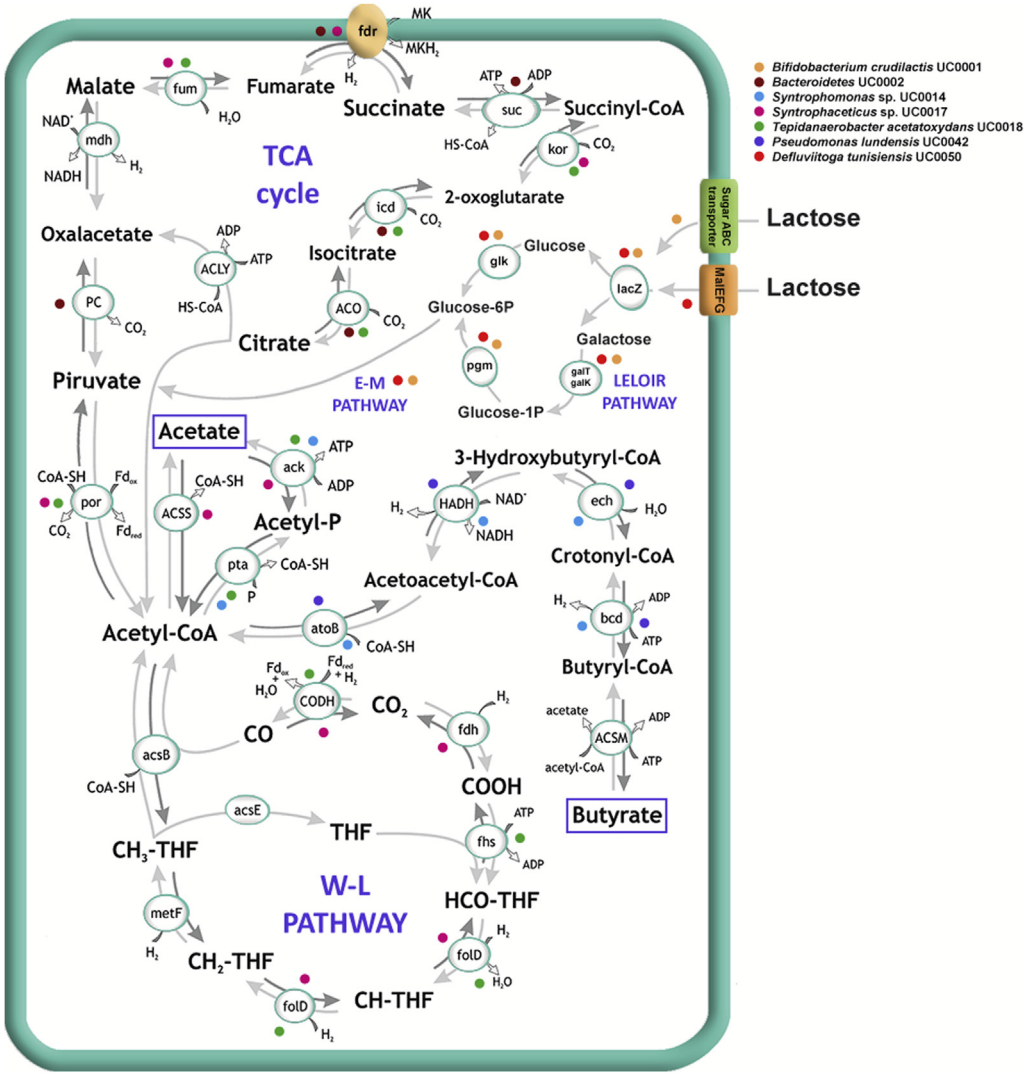

Fig. 5. Metabolic reconstruction of the most important PGs discussed. The main VFA metabolisms (acetate and butyrate), E-M, Leloir, W-L pathway and TCA cycle, are represented. The genes present in the corresponding PG are indicated with colored dots.

and Tanner, 2012; Maus et al., 2016; Sasaki et al., 2011). In R1, the species *Defluviitoga tunisiensis* UC0050 and *Clostridiales* sp. UC0015 (belonging to *Thermotogae* and *Firmicutes* phyla, respectively) were the main responsible for lactose fermentation. In particular, the EggNog functional analysis predicted in both species the presence of all the subunits composing the MalEFG transporter, which allows the import of disaccharides (including lactose) in the cell. The presence of this transporter in *Defluviitoga tunisiensis* was also supported by previous studies (Maus et al., 2016). The continuous lactose fermentation led to the production of acetate (Fig. 5); however, the absence of aceticlastic methanogens in R1 and the low abundance of syntrophic acetate oxidizing bacteria, such as *Tepidanaerobacter acetatoxydans* UC0018, resulted in accumulation of acetic acid, and concomitantly, deterioration of the AD process. *Tepidanaerobacter acetatoxydans* was previously isolated from an ammonium-rich, mesophilic CSTR fed with silage (Westerholm et al., 2011). The functional analysis of UC0018 showed a complete Wood–Ljungdahl (W-L) pathway (Fig. A4 and A5) in which the reducing equivalents generated from the glycolysis are re-oxidized by the reduction of two molecules of CO_2 to acetate. The EggNog analysis confirmed the presence of genes encoding the enzymes involved in the pathway, from the CO_2 reduction to the acetate formation (Fig. 5). It is worth noticing that an inefficient H_2 removal by H_2-scavengers, such as hydrogenotrophic methanogens, could have affected the metabolic activity of *Tepidanaerobacter acetatoxydans* UC0018, from acetate-oxidizer to acetate-producer (Müller et al., 2015), participating to the acetate accumulation occurred in R1.

Focusing on the archaeal community in R1, the abundance was approximately around 2% (Fig. A2a), similarly to values found in reactors fed with other substrates (Luo et al., 2016; Stolze et al., 2016). The reactor was dominated by one class of methanogens namely *Methanobacteria*, which represented the 87% of the total archaeal population (Fig. A3a). The instability of the pH in R1 frequently led to over-acidification, reaching values lower than 6.5. It could be hypothesized that this effect reduced the biodiversity of the methanogenic community, which generally grow in a pH range of 6.5–8.5 (Weiland, 2010). This unfavorable environment allowed mostly the growth of one species, *Methanothermobacter wolfeii* UC0008 (Fig. 4), which can utilize H_2/CO_2 or formate as the sole carbon and energy source (Wasserfallen et al., 2000). However, even if in lower abundance, it was also found another hydrogenotrophic methanogens, *Methanothermobacter thermautotrophicus* UC0010 (Wasserfallen et al., 2000) (Fig. 4). The difficulties exhibited by R1 to maintain the pH in the optimum range, led to a partial inhibition of the methanogenic hydrogenotrophic activity, hampering syntrophic associations for acetate disposal. Moreover, it is worth highlighting that the absence of acetate-scavengers, such as aceticlastic methanogens, can be the consequence of a lipid toxicity effect, which is more evident in a single stage configuration than in a two-stage design. Moreover, the lack of acetate-utilizing methanogens in R1 can be one of the principal explanations for the higher acetate concentration reached in this reactor.

3.2.2. Two-stage, acidogenic reactor (R2)

The bacterial selection imposed by the feed was particularly evident in the acidogenic reactor of the serial configuration (R2), which specifically represented the hydrolytic and acidogenic phases of the AD process. In this reactor, *Actinobacteria* was the most abundant taxon (82%) (Fig. A1b) even though it generally accounts as minor fraction in AD reactors (Campanaro et al., 2016; Fontana et al., 2016). This phylum was composed by a single species, *Bifidobacterium crudilactis* UC0001, a PG very similar to species previously isolated from raw milk and raw milk cheeses (Delcenserie et al., 2007). UC0001 was the main responsible for lactose fermentation in the reactor, showing a complete pathway for galactose degradation, including the enzyme β-galactosidase (*lacZ*) responsible for lactose hydrolysis (Fig. 5 and Fig. A4). The ability of *B. crudilactis* to import lactose in the cell and then acidify it was also described by Delcenserie et al. (2007).

The reactor R2 was additionally populated by other species present in lower abundance, such as *Pseudomonas lundensis* UC0042 and *Leuconostoc pseudomesenteroides* UC0016 (7% and 5%, respectively) (Fig. 3b).

The functional analysis of *Pseudomonas lundensis* UC0042 revealed a complete β-oxidation pathway (Fig. A4). In particular, it exhibited a set of proteins correlated to acetyl-CoA production and its subsequent conversion into butanoyl-CoA (Fig. 5 and Fig. A7), explaining the presence of butyrate at high concentration in R2. This metabolic activity was also supported by Morales et al. (2005), who identified a high production of butyrate in milk inoculated with *P. lundensis*. The metabolic reconstruction of this species also supported the presence of pathways involved in amino acids degradation, namely methionine, histidine, tyrosine and leucine. A deeper analysis performed using EggNogg annotation, showed that this PG encoded 21 proteases, among which some secreted (e.g. *subtilisin*). These results suggested a crucial role of *Pseudomonas lundensis* UC0042 in the degradation of proteins present in the feeding substrate.

This reactor also showed a higher abundance of *Clostridiaceae* spp. and, in particular, of *Clostridium* spp. (Fig. 3b), which could have contributed to the sugars degradation in R2 (Hung et al., 2011). The remarkably different microbial composition exhibited by this reactor, indicates a higher specialization in the hydrolytic and acidogenic activities, which might have contributed to the higher process efficiency of the two-stage configuration.

As expected, no *Archaea* were found in R2 since the pH was prohibitive for methanogenic growth (3.9 ± 0.1) (Weiland, 2010) (Fig. A2b).

3.2.3. Two-stage, methanogenic reactor (R3)

The methanogenic reactor of the serial configuration (R3) presented the highest microbial richness when compared to the other reactors (Fig. A1c). This divergence could be mainly due to the feed of R3, which was mainly composed by the VFAs produced in the acidogenic reactor (R2), resulting in different metabolic activities and functionalities in the two configurations.

Among bacterial population, *Bacteroidetes* phylum (11% abundance) was included and represented by only one species, *Bacteroidetes* sp. UC0002 (Fig. 3c). Differently from other PGs belonging to the *Bacteroidetes* phylum in previous studies (Campanaro et al., 2016; Luo et al., 2016; Stolze et al., 2016), the predictive functional investigation performed on *Bacteroidetes* sp. UC0002 did not classify it to the hydrolytic step of the AD food chain but assigned a role in amino acids intake and degradation, as well as in fatty acids biosynthesis. Moreover, from the KEGG functional analysis was suggested an ability of *Bacteroidetes* sp. UC0002 to perform the reductive TCA cycle, by encoding five fundamental enzymes (*fdr*, *suc*, *icd*, *ACO*, *PC*) (Fig. 5 and Fig. A8).

It is worth noticing the high abundances of *Syntrophomonas* sp. UC0014 and *Syntrophaceticus* sp. UC0017 in R3 (Fig. 3c). UC0014 was one of the PGs (together with *P. lundensis* UC0042 in R2) having a complete β-oxidation pathway (Fig. A4). This suggested an involvement of *Syntrophomonas* sp. UC0014 in the syntrophic oxidation of the butyrate produced in the acidogenic reactor R2 into acetate (Fig. 5 and Fig. A9). Considering *Syntrophaceticus* sp. UC0017, its functional analysis revealed a complete Wood–Ljungdahl (W-L) pathway, which can be likely utilized in

the oxidative direction (Fig. 5 and Fig. A10). It was demonstrated that strictly related species such as *S. schinkii* can oxidize acetate to hydrogen and/or formate, whose usage by hydrogenotrophic methanogens makes the reaction energetically favored (Manzoor et al., 2015).

It is interesting to note the specific presence (~4%) of *Chloroflexi* spp. in R3 (Fig. 3c). Despite the metabolic functions of bacteria belonging to this group are still poorly characterized, few studies established the potential role of *Chloroflexi* spp. in carbohydrates degradation (Ariesyady et al., 2007).

Even though the archaeal abundance was comparable between the two configurations (approximately 2%; Fig. A.2a, A.2c), R3 had a more biodiverse population of methanogens, that were assigned to three different classes (Fig. A3), namely *Methanobacteria* (38%), *Methanomicrobia* (34%), *Thermoplasmata* (28%). They correspond to a core population of three equally abundant methanogens: *Methanothermobacter wolfeii* UC0008, *Methanosarcina thermophila* UC0006 and *Methanomassiliicoccus* sp. UC0009 (Fig. 4). *Methanomassiliicoccus* species can produce methane by reducing methanol with hydrogen (Dridi et al., 2012) but they can also utilize methylamines to perform methylotrophic methanogenesis (Poulsen et al., 2013). *Methanosarcina thermophila*, can instead utilize acetate, methanol, methylamines and H_2/CO_2 (Zinder et al., 1985). The H_2 generated from the β-oxidation pathway performed by *Syntrophomonas* sp. UC0014 can be transferred to hydrogenotrophic methanogens, such as *Methanothermobacter wolfeii* UC0008 and the generalist *Methanosarcina thermophila* UC0006, which act as H_2 sinks and support the syntrophic association between species. Utilization of H_2 by the *Archaea* shifts the reaction equilibrium towards acetate oxidation, increasing the energy gain of the bacterial species (Sieber et al., 2010; Stams and Plugge, 2009).

The higher biodiversity in methanogenic taxa could be one of the reasons why R3 was more efficient, since a wider range of metabolic intermediates can be converted to methane. This could avoid the accumulation of specific compounds such acetate, which concentration was found to be remarkably increased in the single stage reactor configuration R1.

4. Conclusions

In conclusion, this study allowed gaining new insights on the anaerobic digestion of dairy wastes, identifying constraints about reactor configurations and highlighting the main metabolic players of the complex microbial consortia. The superior performance of the two-stage configuration was mainly due to the pre-acidification step, which allowed a better distribution of the microbial species in suitable habitat for their metabolic activities. In particular, *Bifidobacterium crudilactis* UC0001 was the main lactose degrader in the acidogenic phase of AD, whereas a wider set of methanogenic archaea (*Methanothermobacter wolfeii* UC0008, *Methanosarcina thermophila* UC0006 and *Methanomassiliicoccus* sp. UC0009) was responsible for the higher methane yield reached by the serial configuration. Moreover, it was evident that the acetate concentration exhibited by the single stage reactor was mainly due to the lack of aceticlastic methanogens and to the partial inhibition of the methanogenic hydrogen scavengers *Methanothermobacter wolfeii* UC0008 and *Methanothermobacter thermautotrophicus* UC0010, hampering the activity of the low abundant syntrophic acetate oxidizer species such as *Tepidanaerobacter acetatoxydans* UC0018.

The findings of this work can be further utilized for development of reactor designs aiming high rate/yield methane production from dairy wastewater and serve as complementary foundation for deciphering the biogas microbiome.

Author contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.02.001.

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