Thermophilic Anaerobic Digestion of Second Cheese Whey: Microbial Community Response to H2 Addition in a Partially Immobilized Anaerobic Hybrid Reactor

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Date Submitted: 2021-09-22

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Record Type: Published Article

Submitted To: LAPSE (Living Archive for Process Systems Engineering)

Citation (overall record, always the latest version):	LAPSE:2021.0738
Citation (this specific file, latest version):	LAPSE:2021.0738-1
Citation (this specific file, this version):	LAPSE:2021.0738-1v1

DOI of Published Version: https://doi.org/10.3390/pr9010043

processes

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Thermophilic Anaerobic Digestion of Second Cheese Whey: Microbial Community Response to H_2 Addition in a Partially Immobilized Anaerobic Hybrid Reactor

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Keywords: cheese whey; anaerobic hybrid reactor; thermophilic anaerobic digestion; in situ hydrogen addition; microbial community

Citation: Lembo, G.; Rosa, S.; Mazzurco Miritana, V.; Marone, A.; Massini, G.; Fenice, M.; Signorini, A. Thermophilic Anaerobic Digestion of Second Cheese Whey: Microbial Community Response to H_2 Addition in a Partially Immobilized Anaerobic Hybrid Reactor. *Processes* **2021**, *9*, 43. https://doi.org/10.3390/pr9010043

Received: 3 December 2020
Accepted: 24 December 2020
Published: 28 December 2020

1. Introduction

The dairy industry is one of the main sources of industrial wastewater in Europe with cheese whey (CW) and second cheese whey (SCW) making up a large part [1]. SCW is a byproduct generated from the precipitation of CW proteins by means of heat (80–90 °C) with added organic acids and salts for the production of cottage and ricotta cheeses. Similar to CW, SCW is a highly pollutant dairy waste with a significant organic load (biochemical oxygen demand (BOD) ≈ 30 g/L, chemical oxygen demand (COD) 60–80 g/L, and lactose 40–50 g/L) but with lower levels of fat (0.5–8 g/L) and protein (0.5–8 g/L) and a higher salinity (7–23 mS cm^{-1}) [1].

The high organic loads of both SCW and CW represent severe disposal and pollution issues for the dairy industry and a huge opportunity for bioenergy and biochemicals production [2–4]. In particular, methane production by anaerobic digestion (AD) can be

an opportunity for small-medium dairies that cannot afford the high investment costs associated with the implementation of CW valorization technology. According to Mainardis et al., biogas dairy plants that use simple low-cost digesters, can provide most of the electricity and heat necessary for the plants, improving energy balance and reducing transport and management costs [5]. However, it is known that the high easily-fermentable organic content and low bicarbonate alkalinity of raw SCW, as well as of CW, unbalance the AD process towards an accumulation of volatile fatty acids (VFAs), and a decrease in the pH values far below the optimum value for the methanogens is often observed [6]. Different solutions have been suggested in order to control acidification and rule out the risk of failure for the AD process such as supplementation of alkalinity during the process or into the feed, use of the codigestion with a substrate having high buffering capacity (e.g., live-stock manure or slurry and sewage sludge), and use of two-stage reactor configuration [7–10]. Although the codigestion strategy can be a sustainable option for farms, its implementation is not economically and environmentally sustainable for dairies. Furthermore, when digesting CW in combination with pathogenic waste streams, health and safety issues could discourage their use. In Europe, only 7.1% of the milk produced is processed directly on farms, while the vast majority of raw milk is delivered to dairies (Eurostat, 2019). In addition, the two-stage configuration entails a higher investment cost making it economically unattractive [11].

Different one-stage reactor configurations have been used for methane production from CW. Among these, the continuously stirred tank reactor (CSTR) configuration has several advantages such as reasonable control, easy operation and cleaning, minor operating cost, and high removal efficiency [12]. However, a very low conversion per unit volume is obtained. Recently, Faisal et al. showed that the addition of high density polyethylene carriers to batch anaerobic digestion of agricultural wastes supported the formation of biofilm leading to significant increases in substrate utilization and biogas and biomethane production [13]. Similarly, Ramasamy et al. (2000) indicated that the incorporation of plastic media into a CSTR enhanced methane yield from dairy wastewaters more than 20% and without pH control [14].

Although relevant improvement in process performances were reported, no information concerning the impact of the biomass support material on the microbial communities was reported in these studies.

Recently, Treu et al. proposed the H_2 addition in CSTR used for CH_4 production from raw CW, as a strategy to manage the low pH value of the substrate [9]. The in situ H_2 addition, namely biogas in situ upgrading, is a technology generally used to increase the methane content in biogas, i.e., the introduced H_2 is combined with carbon dioxide (CO_2) produced in the process, to generate further CH_4 by the hydrogenotrophic methanogens. The diffusion of H_2 in the liquid phase is the limiting factor of this technology, i.e., due to the low H_2 solubility it is not bioavailable for microorganisms [15–18].

Moreover, the addition of H_2 could unbalance the equilibrium of the system. The additional H_2 represents an extra substrate for the microbial communities of bacteria and archaea involved in the AD process. It could cause a modification of the microbial community populating the biogas reactor. In particular, if hydrogenotrophic methanogens are unable to quickly remove the H_2, its partial pressure will increase leading to disruption of the syntrophic interaction occurring between H_2 producing microorganisms (heterotrophic community as acidogenic and acetogenic bacteria) and H_2 consuming microorganisms (autothrophic bacteria as homoacetogenic bacteria and archaea as hydrogenotrophic methanogens). This condition can lead to the accumulation of propionic and acetic acids and, consequently, a decrease of pH outside the optimal range for the methanogenic archaea produces adverse effects on the whole process. In contrast, due to the removal of CO_2, inhibition of methanogenesis could be generated by an increase in pH to values above 8.5 [19].

Little is known about how the H_2 addition could affect the microbial community responsible for the AD process, especially in concomitance with other operating parameters and reactors configurations.

The current study aimed to characterize a thermophilic (55 °C) anaerobic digestion (AD) performance and the microbial community structure, before and after in situ H_2 addition, in a novel hybrid gas-stirred reactor, namely a gas-stirred tank reactor (GSTR), fed with undiluted SCW. The GSTR hybrid reactor is the result of the CSTR modification, in which about one third of the reactor's working volume was filled with polymeric supports (high-density polyethylene, HDPE) and a gas recirculation was installed for homogenization of nutrients and as a strategy to improve H_2 solubility. The thermophilic condition was selected to exploit the acclimatization of the mixed microbial community used as inoculum and collected from a thermophilic (55 °C) anaerobic digestion plant. The microbial community of the effluent and the interstitial matrix inside the immobilized area were characterized by fluorescence in situ hybridization (FISH) and Illumina next-generation sequences (NGS) techniques. The impacts of hydrogen addition on the performance of methane production and on the modification of the microbial community structure were investigated.

2. Materials and Methods

2.1. Substrate Characteristics

In Italy, 4.9 Mt of cheese whey are produced annually and about 1 Mt is used to produce ricotta, a typical soft cheese of the Mediterranean region, generating a second cheese whey (SCW) called "scotta". Moreover, the manufacturing of ricotta cheese is also widespread in Latin America and in USA, where it is referred to as requeson and ricottone, respectively.

SCW is periodically collected at Formaggi Boccea, a small dairy factory located in Rome, Italy, providing a thermophilic (55 °C) biogas plant fed with SCW. The wastewater was stored at −20 °C and thawed before use. Undiluted SCW was fed into the GSTR reactor. Due to the fluctuations of the residual organic load derived from the production process, slight variations in its composition were observed. The main physical and chemical characteristics of the SCW are presented in Table 1.

Table 1. Main characteristics of second cheese whey (SCW) used in this study.

Parameters	Range
pH	5.90–6.20
Lactose (gL^{-1})	40–60
Total solid (TS) (gL^{-1})	47–64
Volatile solid (VS) (gL^{-1})	40–54
COD (gL^{-1})	45–70
Proteins (gL^{-1})	0.45–0.90
NH_4^+ (gL^{-1})	0.10–0.12
Total volatile fatty acids (TVFAs) (gL^{-1})	1.5–2.5

2.2. Reactors Setup and Operation

The experiment was carried out in a novel hybrid gas-stirred tank reactor (GSTR) with a working volume of 49 L, at thermophilic temperature (55 ± 1 °C) and atmospheric pressure. Figure 1 shows the scheme of the reactor and the overall equipment design. Reactor mixing and homogenization of nutrients was ensured by continuous gas recirculation. A vacuum pump took the biogas from the reactor headspace and injected it into the bottom of the GSTR. Biofilm media carriers were used as packing material (Scubla MBBR 800, HDPE) with a specific surface area of 800 m^2/m^3. The immobilized area was completely submerged in the middle part of the reactor and occupied a volume of 15 L. Media carriers were enclosed in a mesh bag to prevent washing out in the outlet stream. Biogas coming from both gas recirculation and gas outlet lines were dehumidified by a chiller and the con-

densed water was returned into the digester. The GSTR was inoculated with thermophilic (55 °C) sludge obtained from the Formaggi Boccea biogas plant. Undiluted second cheese way (SCW) was fed in a continuously way from the bottom using a peristaltic pump. The hydraulic retention time (HRT) was 15 days, and the organic loading rate (OLR) was on average within a range of 2.18 ± 0.14 g COD L^{-1} d^{-1} and 2.4 ± 0.12 g COD L^{-1} d^{-1}.

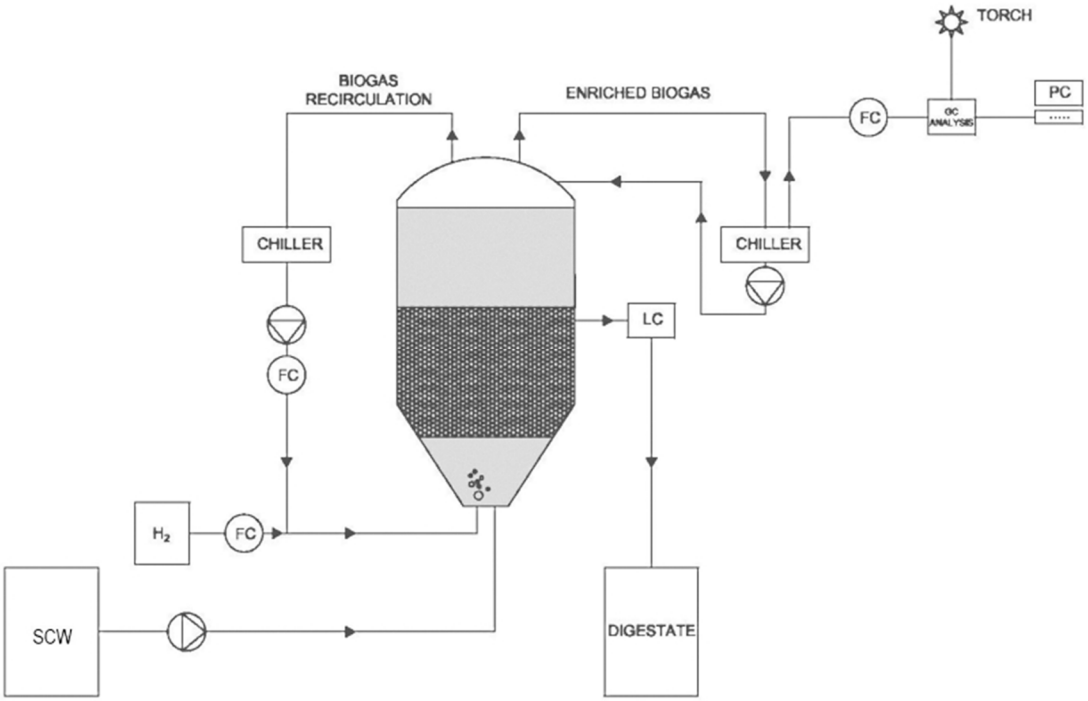


Figure 1. Schematic representation of the hybrid gas-stirred tank reactor (GSTR) and the experimental equipment. LC, liquid control; FC, flow control; SCW, second cheese whey.

Initially, a biogas recirculation flow rate of 59 L L_r^{-1} d^{-1} was applied. When a stable AD process was achieved, pure H_2 (≥99%) obtained from an electrolyzer (DBS, model PG-H2 100), was continuously injected into the reactor from the bottom by the biogas recirculation line. In order to avoid an increase in hydrogen partial pressure, the stoichiometric ratio H_2/CO_2 used in this experimental phase was lower (2.7:1 UP1 and 2:1 for UP2, UP for upgrading phase) than needed for hydrogenotrophic methanogenes (4:1). The starting H_2 flow rate was 1.8 L_{H_2} L_r^{-1} d^{-1} and gas recirculation flow rate was maintained at 59 L L_r^{-1} d^{-1}.This experimental phase is denoted as Upgrading 1 (UP1). Thereafter, the H_2 flow rate was decreased to 1.32 L_{H_2} L_r^{-1} d^{-1} and gas recirculation flow was increased to 118 L L_r^{-1} d^{-1}. This experimental phase is indicated as UP2. Both UP1 and UP2 lasted for 30 days.

2.3. Analytical Methods

H_2, CH_4, CO_2, N_2, and O_2 percentages in biogas were analyzed by online Micro Gas Chromatograph Varian (GC4900). Two columns of 10 m were used. The first, MS 5A, had a stationary phase with molecular sieves capable of separating the permanent gases of low molecular weight, H_2, CH_4, N_2, and O_2. The second column was a Poraplot U, used as a stationary phase divinyl benzene able to separate CO_2. Argon was used as the carrier gas. The biogas and H_2 flow rates were monitored by digital online flow meters (EL-Flow select series, Bronkhorst High-Tech B.V, Ruurlo Netherland).The flows of the different gases were calculated on the basis of the percentage compositions of the individual gas present in the biogas. Volatile fatty acids (VFAs), lactic acid, alcohols, and sugars were analyzed by a HPLC Thermo Spectrasystem, equipped with a UV detector (λ = 210 nm)

and a refraction index detector, using the isocratic method of analysis at 75 °C with Column Rezex ROA-Organic Acid H^+ (8%), size 300 × 7.8 mm Phenomenex, USA.

2.4. Calculation

Performance and efficiency of the methanation process were expressed as the methane evolution rate (MER), i.e., the rate of CH_4 production from the injected H_2, and H_2 conversion efficiency (η_{H_2}). The MER (Equation (1) expresses the increase in the specific CH_4 production rate (L_{CH_4} $L_r{}^{-1}d^{-1}$) caused by H_2 injection as compared with the CH_4 production rate in the control condition. It is calculated as follows (Equation (1)):

$$MER = CH_{4UPs} - CH_{4AD} \quad (1)$$

where UPs are the upgrading periods (with H_2 addition) and AD is the control period (without H_2) The H_2 gas-liquid mass transfer rate, namely r_t (L_{H_2} $L_r{}^{-1}$ d^{-1}), and the efficiency of H_2 utilization, namely η_{H_2} (%), were calculated according to Equations (2) and (3), respectively:

$$R_t = H_2 \text{ in flow rate} - H_2 \text{ in output gas} \quad (2)$$

$$\eta_{H_2} = \frac{H_2 \text{ in flow rate} - H_2 \text{ in output gas}}{H_2 \text{ in flow rate}} \cdot 100 \quad (3)$$

where H_2 in flow rate and H_2 in output gas are the volumetric H_2 flows entering and leaving the reactor. It was assumed that all the H_2 transferred to the liquid phase was ultimately converted to CH_4 or used for other microbial metabolic pathways or employed for microbial growth [15,17].

The H_2 rate converted to biomethane (L $L_r{}^{-1}$ d^{-1}) was calculated according to Equation (4):

$$\text{rate to biomethane} = 4 \times (CH_4 \text{ in UP output gas} - CH_4 \text{ in AD output gas}) \quad (4)$$

where 4 is the stoichiometric coefficient according to Equation (4) $H_2 + CO_2 = CH_4$, CH_4 in UP output gas (L_{CH_4} $L_r{}^{-1}$ d^{-1}) is the rate of CH_4 produced in UP experimental phases, and CH_4 in AD output gas (L_{CH_4} $L_r{}^{-1}$ d^{-1}) is the rate of CH_4 produced in AD phase.

2.5. Sample Collection for the Microbial Community Analysis

Samples for the microbial community analysis were collected at the end of AD and UP phases from both the liquid medium and the interstitial matrix between the HDPE supports. After the AD phase, the GSTR was opened to collect the interstitial matrix; its dark-grey color and the presence of flocs indicated the formation of a biofilm. Before the UP phases, the GSTR was flushed with nitrogen to restore the anaerobic condition and was operated under the same previous operating conditions until N_2 was no longer detected. Samples were collected in duplicate, aliquoted (10 mL) and stored at −20 °C for different uses (i.e., DNA extraction and fluorescence in situ hybridization (FISH) analysis). They are recognized as EF-AD or EF-UP2 (AD, anaerobic digestion phase; UP2, H_2 upgrading at the end of UP2 phase; EF, effluent,) and IM-AD or IM-UP2, (IM, interstitial matrix).

2.6. Illumina Sequencing

Genomic DNA was extracted usinga Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit (EURxLtd. Gdansk Poland), in accordance with the manufacturer's recommendations. The quantity and quality assessment of the extracted DNA were performed using spectrophotometry (Eppendorf BioPhotometer plus). All DNA samples were stored at −20 °C until use.

Library preparation was performed utilizing a 460bp amplicon corresponding to the hypervariable V3-V4 region of 16S rRNA using universal primers (S-D-Bact-0341F and S-D-Bact-0785R for bacteria and archaea domains). Sequencing was performed at the Department of Agricultural Sciences, University of Naples Federico II (Portici, Italy) using

the Illumina MiSeq platform. From the generated reads, demultiplexing, quality filtering, trimming, merging, and operative taxonomical units (OTUs) picking were performed using QIIME pipeline [20].

The taxonomical assignment was performed with the Greengenes database at a 97% similarity. The bioinformatics analysis of all sequences generated more than 220,000 reads, with an average of 26,800 counts per sample. The results reported are focused on the most abundant members of the microbial community having a relative abundance $\geq$ 0.5%. Raw sequences data were deposited at the Sequence Read Archive (SRA) under the BioProject PRJNA681387 with the accession numbers SAMN1695146 and SAMN16951461. Shannon–Weaver (H) and Pielou's evenness (E) indices were calculated using the relative abundance of sequences obtained from NGS sequencing at the family level.

2.7. Total Microbial Abundance and Bacteria and Archaea Detection

DAPI fluorescent staining and fluorescence in situ hybridization (FISH) were used to evaluate the microbial abundance and bacteria and archaea relative abundance, respectively. The collected samples were fixed (4% w/v paraformaldehyde fixative solution for 6 h, at 4 °C), as described in Amann, 1990, and stored (−20 °C) [21] until use. In order to detach microbial cells from inorganic particles, a cleaning sample procedure was performed immediately after thawing and before analysis, using the density gradient medium OptiPrep™ (Axis-Shield PoCAs, Oslo, Norway) [22]. Total microbial abundance (cells mL^{-1}) was determined in the effluent and interstitial matrix by direct cell counting after DAPI staining (4′,6-diamidino-2-phenylindole) (1 μg mL^{-1}) [22] using the epifluorescence microscopy AXIOSKOP 40 (Carl Zeiss, Germany) equipped with a ZEISS HXP 120v Light Source and 1000× magnification.

The FISH analysis (in triplicate) was carry out using samples collected from effluent and interstitial matrix focusing on the relative abundance (%) of archaea and bacteria. Analyses were performed as described in Amann et al., 1995 [23] and oligonucleotide probes for the *Bacteria* (EUB338 II,III) and *Archaea* (ARC915) domains were used [24].

The oligonucleotide probes (50 ng μL^{-1}), labeled with carboxyfluoresce in (FAM) or indocarbocianine (Cy3) dyed at the 5′-end, were purchased from MWG AG Biotech, Germany. All the hybridizations were carried out in combination with DAPI staining to estimate the proportion of cells targeted by the specific probes out of the total cells. Slides were mounted with a few drops of Vecta Shield (Vector Laboratories, USA) and seen using Zeiss epifluorescence microscope. The average of cells for each target probe (15 fields for each slide) was used to evaluate the percentage of the positive signals versus DAPI-positive cells. Microbial abundance of bacteria and archaea in the samples (cells mL^{-1}) was calculated using the relative abundances obtained by FISH technique and the microbial abundance detected after DAPI staining.

3. Results

3.1. Reactors Performance

The daily course profiles of process performances of all experimental phases are shown in Figure 2 and the steady operation condition process performance data are summarized in Table 2.

During the AD period, starting from the 10th day to the end of this phase, a stable methane production rate of 0.79 ± 0.04 L_{CH_4} L_r^{-1} d^{-1} was reached. The pH of the reactor was around 7, indicating a strong buffer capacity of the system, although no pH control was applied. The average CH_4 content in the biogas was 55.5 ± 0.8% and the average specific methane yield (SMY) was 0.329 ± 0.02 L_{CH_4} g COD^{-1}, which corresponded to 94% of the maximum theoretical value obtainable by the conversion of organic matter into methane (i.e., 0.350 L_{CH_4} g COD^{-1}) [9,19]. The substrate was almost completely consumed, as confirmed by the low average value of lactose (270 ± 28 mg L^{-1}) detected in the reactor effluent, indicating an efficient performance of the AD process. The acetic acid was the unique VFA found during the AD process, moving from an average value

of 210 ± 5.6 mg L^{-1} during the first 10 days to that of 37 ± 2.4 mg L^{-1} at the end of AD. Moreover, in order to verify the effect of biogas recirculation on the soluble metabolite distribution along the GSTR, an HPLC analysis was also performed on the lower and upper liquid fractions. The average lactose and acetic acid concentrations of 270 ± 28 mg L^{-1} and 45 ± 10 mg L^{-1}, respectively, were measured indicating a good mixing of the reactants.

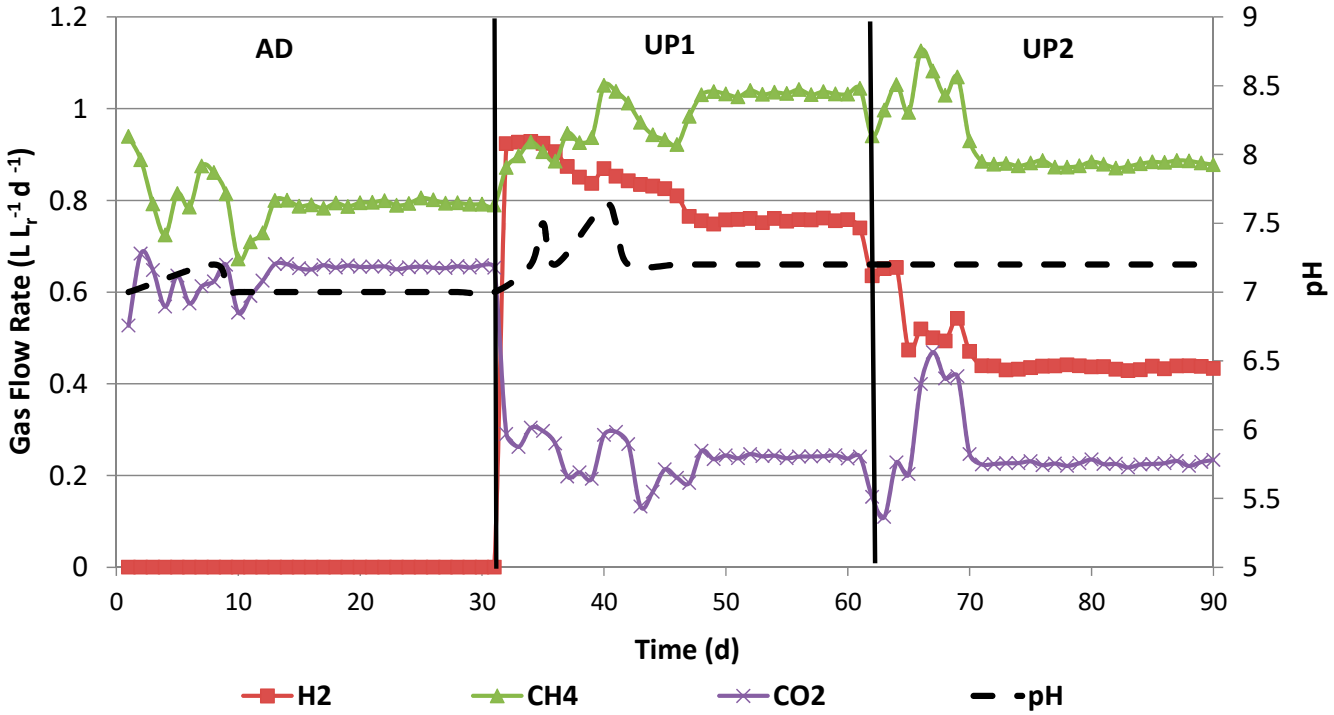


Figure 2. Daily course profiles of process performances during the three experimental phases.

Table 2. Process performances at the end of the three experimental phases.

Parameters	AD	UP1	UP2
OLR (g COD $L_r^{-1}d^{-1}$)	2.4 ± 0.12	2.18 ± 0.14	2.28 ± 0.25
H_2 in flow rate (L L_r^{-1} d^{-1})	-	1.76 ± 0.01	1.32 ± 0.01
Biogas recirculation rate (L L_r^{-1} d^{-1})	59 ± 5	59 ± 5	118 ± 5
Biogas production rate (L L_r^{-1} d^{-1})	1.42 ± 0.06	2.05 ± 0.07	1.65 ± 0.18
H_2%	-	39.7 ± 3.0	28.4 ± 2.7
CH_4%	55.5 ± 0.8	48.2 ± 3	56.0 ±1.9
CO_2%	45.1 ± 1.65	11.6 ± 1.62	14.8 ± 3.07
H_2 Flow rate (L L_r^{-1} d^{-1})	-	0.81 ± 0.06	0.47 ± 0.07
CH_4 Flow rate (L L_r^{-1} d^{-1})	0.79 ± 0.04	0.99 ± 0.06	0.92 ± 0.08
r_t (L L_r^{-1} d^{-1})	-	0.95	0.85
η_{H_2} (%)	-	54	65
Specific methane yield (SMY) (L_{CH_4} g COD^{-1})	0.329 ± 0.02	-	-
MER (L_{CH_4} $L_r^{-1}d^{-1}$)	ND	0.20	0.13
Lactose (mg L^{-1})	270 ± 28	225 ± 72	209 ± 10
Acetic acid (mg L^{-1})	45 ± 10	53 ± 19	38 ± 5.2

This result is among the highest SMY obtained from AD of synthetic or raw dairy wastewaters in hybrid anaerobic biofilm reactors (0.03–0.359 L_{CH_4} $gCOD^{-1}$) [12,25]. However, these studies were conducted at mesophilic temperature. So far, there have been few studies on single-stage thermophilic anaerobic digestion using only dairy wastewaters [4]. Yang et al. achieved a maximum yield of 0.360 L_{CH_4} g COD^{-1} using a 5 L CSTR reactor under an HRT value of 7.5 days and using diluted CW (10 g COD L^{-1}) [26]. Other authors have recorded an unstable AD process with poor thermophilic (55 °C) CH_4 production yield

(0.100 L_{CH_4} g COD^{-1} ± 0.21) and a remarkable accumulation of acetic acid 10 ± 1 g L^{-1} using a 3 L CSTR configuration with an OLR of 2.4 g CODL $^{-1}$ d^{-1} and an HRT of 15 days using cheese whey permeate and cheese waste powered as substrates [27]. Fernandez et al., obtained a SMY of 0.315 ± 6.6 L_{CH_4} g COD^{-1} during a thermophilic AD of deproteinized CW in a 25 L anaerobic sequencing batch reactor (ASBR) at an HRT of 8.3 days and an OLR of 4.6 ± 0.3 g COD L^{-1} d^{-1} [28]. Although different HRT and OLR values were used, the yields obtained were comparable to ours, suggesting that the GSTR, used in this study, maintained the characteristics of a CSTR for the homogenization of nutrients in the liquid phase. However, the implementation of supports for the immobilization of the biomass makes it comparable to an ASBR whose peculiar feature is to uncouple the SRT (solid retention time) from the HRT.

The UP1 started at day 30 and, after 12 days, a stable methane production process was reached and lasted until the end of the experimental phase (60 days). During the steady state of UP1, the CH_4 production rate was 0.99 ± 0.06 L_{CH_4} L_r^{-1} d^{-1}, which represented an increase of 25% as compared with the AD phase. The calculation of the efficiency of H_2 utilization (η_{H_2}), suggests that 54% of H_2 added is utilized by the microbial community.

However, the incomplete H_2 consumption led to a dilution of CH_4 concentration as compared with that obtained in AD, indeed the composition of the outflow biogas in the UP1 phase was 48.2% CH_4, 39.8 H_2, and 11.6% CO_2. Considering the high unconverted percentage of H_2 observed in UP1, it was assumed that the H_2 injection rate of 1.8 L_{H_2} L_r^{-1} d^{-1} used in UP1 was too high. Therefore, the H_2 inlet flow was decreased to 1.32 L_{H_2} $L_r^{-1}d^{-1}$ in the UP2 phase. Moreover, in order to increase the H_2 dissolution in the liquid phase, the gas recirculation rate was increased from 59 to 118 L_{H_2} $L_r^{-1}d^{-1}$.

During the steady state of UP2 (reached after day 10) the CH_4 flow rate was equal to 0.92 ± 0.08 L_{CH_4} L_r^{-1} d^{-1} and the percentage of CH_4 in the biogas went up to 55%. The pH increased, reaching a value of 7.3–7.4. The CH_4 production rate was 17% higher as compared with AD and no accumulation of acetic acid (38 ± 5 mg L^{-1}) or lactose (208 ± 10.5 mg L^{-1}) were observed. The H_2 efficiency value increased to 65%, but the MER value (0.13 L_{CH_4} L_r^{-1} d^{-1}) was lower than the one calculated in UP2 (0.20 L_{CH_4} L_r^{-1} d^{-1}). According to the stoichiometric reaction $4H_2 + CO_2 = CH_4$, MER values of 0.21 and 0.24 L_{CH_4} L_r^{-1} d^{-1}were expected, respectively.

These differences could be partly explained by the use, in UP1 and UP2, of OLRs slightly lower than the one used in AD, involving in an overestimation of the CH_4 formation rate from SCW. In addition, taking into account that the amount of acetate as H_2 equivalent can be neglected, two further metabolic pathways of the H_2 added other than the production of CH_4 took place inside the GSTR reactor. For example, during the methanation process, a distinct part of both H_2 and CO_2 are metabolized to produce biomass by the archaea community [29]. The results of the FISH analysis seem to confirm this hypothesis; the Archaea abundance was more than doubled after the H_2 dispersion phase in EF-UP2 and it increased in IM-UP2. However, sulphate-reducing bacteria also utilize H_2 as a substrate to reduce sulphate to hydrogen sulphide. In this study, the hydrogen sulphide content of biogas was not monitored.

Briefly, although the purpose of this study was not to improve the efficiency of the in situ biomethanation process and, although higher CH_4 concentration values were achieved in a published paper, the above results demonstrated that it was possible to convert H_2 to CH_4 in this novel GSTR with partially immobilized biomass by using HDPE supports.

3.2. Total Microbial Abundance and Bacteria and Archaea Detection

Total microbial abundance (DAPI staining), as well as abundance in bacteria and archaea (FISH technique associated with DAPI staining) under different experimental conditions are shown in Figure 3.

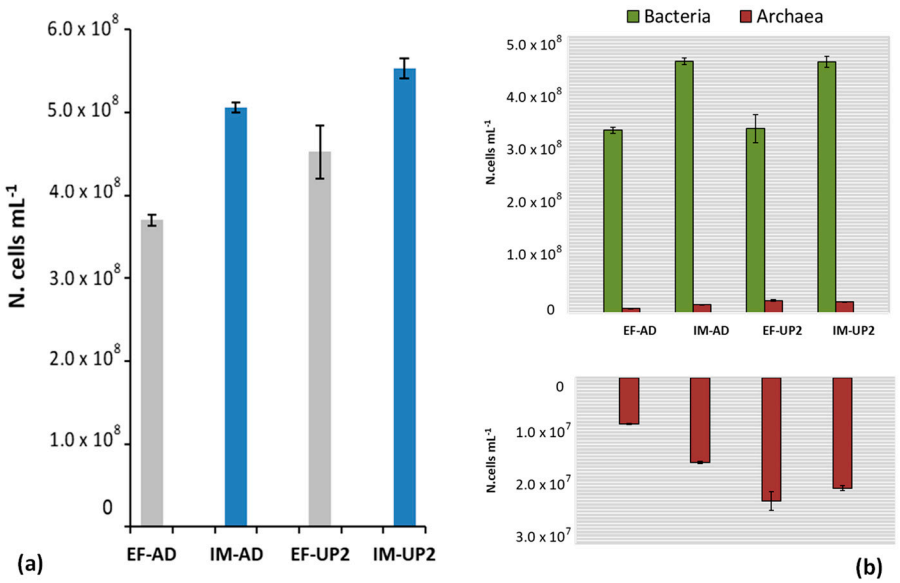


Figure 3. Microbial abundance (N. cells mL^{-1}) under different experimental conditions is reported. (**a**) Total microbial abundance detected in the effluent (EF, gray bars) and in the interstitial matrix (IM, blue bars), before (AD, anaerobic digestion) and after (UP2) H_2 addition, using DAPI staining; (**b**) Abundance of bacteria (green) and archaea (red) in the effluent (EF) and interstitial matrix (IM), before and after H_2 addition (AD and UP2, respectively) obtained by FISH technique and DAPI staining. At the bottom, the archaea are shown on a lower scale to highlight the differences.

The highest and the lowest total microbial abundances were detected, respectively, in IM-UP2 with $5.53 \times 10^8 \pm 1.25 \times 10^7$ cells mL^{-1} and in EF-AD with $3.7 \times 10^8 \pm 6.24 \times 10^6$ cells mL^{-1} (Figure 3a). In both effluent (EF) and interstitial matrix (IM), the total microbial abundance increased after H_2 addition by 22% and 9%, respectively. Both IM-AD and IM-UP2 had a total microbial abundance higher than the corresponding effluent phase (EF-AD and EF-UP2) by 37% and 22%, respectively, highlighting the positive effect that the presence of immobilizing supports exerted on the microbial community. The FISH investigations showed a strong predominance of bacteria in the microbial communities of all experimental conditions (Figure 3b), while archaea contribution to the microbial communities, ranged between 2.2% and 5.2%, in line with the concentration usually detected in microbial communities during an AD process [30]. More specifically, before H_2 addition the bacterial cells detected were 89% and 91% in EF-AD and IM-AD, respectively, while at the end of UP2 phase their concentration decreased to 80% and 84%, respectively. The H_2 addition positively affected archaea populations, especially in EF-UP2 effluent samples, for which a concentration of 5.2% corresponding to 2.23×10^7 cells mL^{-1} of archaea was detected, with an increase of 134% as compared with EF-AD which showed a concentration of 0.83×10^7 cells mL^{-1} (Figure 3b). The percentage of archaea in the interstitial matrix, before and after H_2 addition, was 3.1% and 3.7%, respectively, corresponding to a 43% increase in the interstitial matrix after the H_2 addition. Moreover, the presence of HDPE supports generated a favorable habitat for the microbial community, as evidenced both by the total microbial (Figure 3a) and bacteria abundances (Figure 3b) in both experimental phases. After the H_2 addition, an increase in total microbial (Figure 3a) and archaea abundances (Figure 3b) in the interstitial matrix was observed. It can be hypothesized that these supports, promote the contact of hydrogen with microbial cells and also provide a habitat refuge in which the disturbance of the spatial disposition of AD populations with different cell leaching have been limited. Other authors [31] observed an increase in the archaea component when a porous support was offered to a mesophilic anaerobic microbial community as a refuge habitat, although the present study is among the first to refer to a thermophilic condition.

3.3. Illumina Sequencing

The microbial communities were analyzed by Illumina-based 16S sequencing. The representativeness (%) of the microbial communities' composition at the different taxonomic levels tended to decrease from the phyla (96%) toward the species (25%) levels, showing 85% (families) and 50% (genera) of representativeness. The low assignment at genera and species levels suggested the presence, in the microbiome, of numerous unexplored or undescribed taxa.

3.3.1. Bacteria Communities

The relative abundance of *Bacteria* at the phylum level is shown in Figure 4.

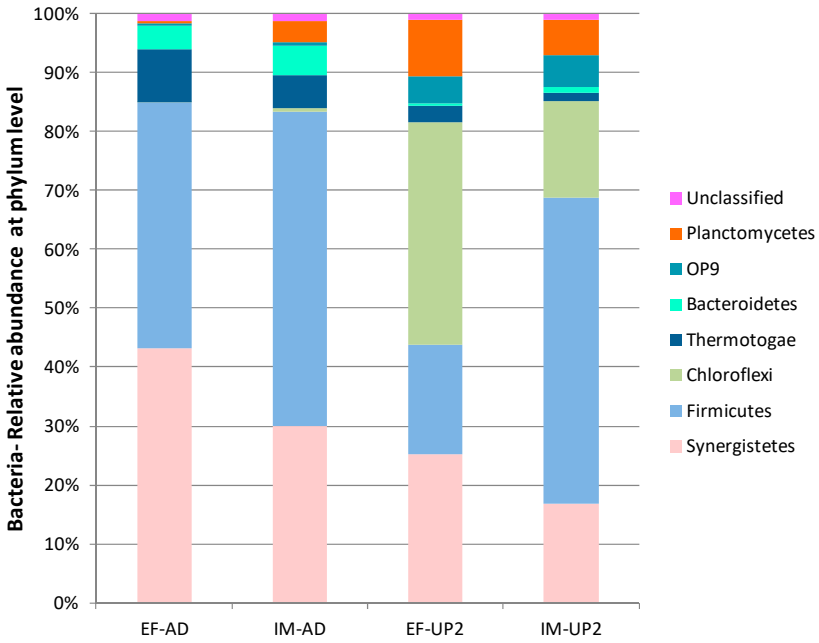
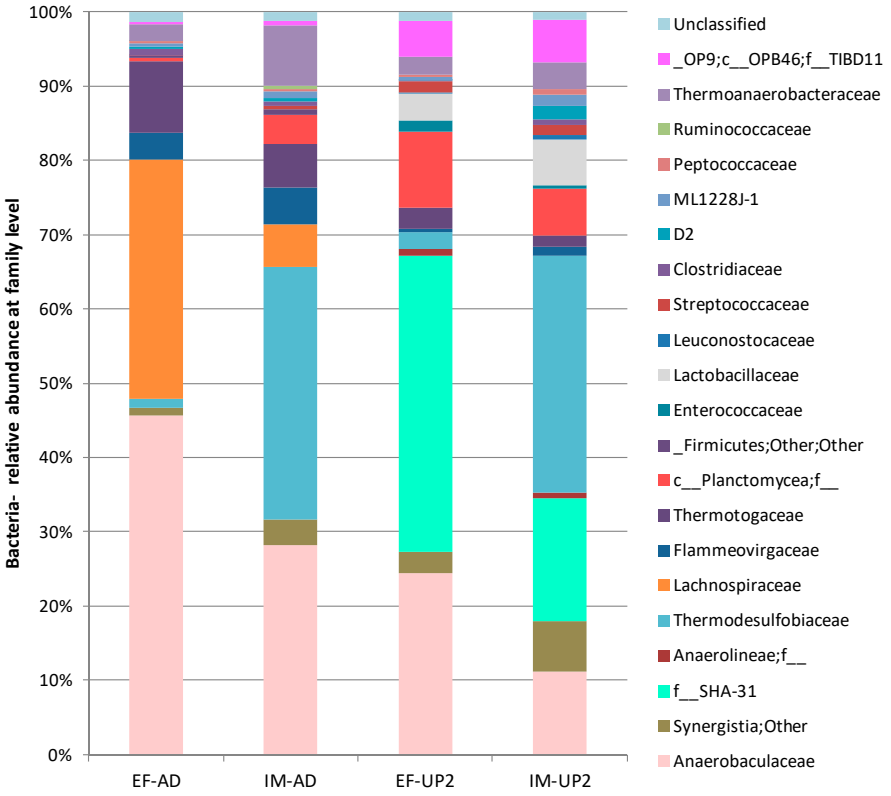


Figure 4. Relative abundances of the bacteria community at the phylum level at the end of AD (anaerobic digestion) and UP2 (H_2 injection) phases. Communities with a relative abundance $\geq$ 0.5% (in at least one sample) are reported. EF, effluent and IM, interstitial matrix.

The microbial communities during the AD phase were dominated by *Synergistetes* (43% and 30% in EF and IM, respectively) and *Firmicutes* (42% and 53% in EF and IM, respectively). Representatives of *Thermotogae* and *Bacteriodetes* phyla were also abundant in microbial communities of the AD phase, representing 9% and 4% in the EF sample and 6% and 5% in the IM sample, respectively. Minor representative phyla in the AD phase were identified only in the IM sample, i.e., *Planctomycetes* (4%), *Chloroflexi* (1%), and OP9 (1%). After H_2 injection, relevant changes in the microbial community structures were observed both for effluent and interstitial matrix samples. The *Synergistetes* and *Firmicutes* phyla still represented a considerable fraction of UP2 communities, although their relative abundance decreased considerably as compared with the AD samples. In particular, in the EF-UP2 samples, *Synergistetes* represented 25% of the bacterial community, while *Firmicutes* represented 19%. A different trend was observed in the IM-UP2 sample, in which the abundance of *Synergistetes* further decreased to 17%, while the *Firmicutes* accounted for 51% of the whole community. The high increase in the relative abundance of *Chloroflexi* phylum was the most relevant observed change affecting the microbial community structure during the UP2 phase. Representatives of this phylum accounted for 37% and 16% of the bacterial community in EF-UP2 and IM-UP2 samples, respectively. Moreover, an increase in the relative abundances of *Planctomycetes* (9% and 6% in EF and IM, respectively) and *OP9* (5% and 5% in EF and IM, respectively) was also observed. In contrast, the relative abundances

of *Thermotogae* and *Bacteroidetes* decreased, representing 3% and 1.5% in EF-UP2 and 0.5% and 1% in IM-UP2 samples, respectively.

The detailed results of the bacterial communities at the family level are shown in Figure 5.



Index	EF-AD	IM-AD	EF-UP2	IM-UP2
H'	1.62	2.01	2.06	2.38
E	0.55	0.68	0.65	0.75

Figure 5. Relative abundances of the bacteria community at the family level at the end of AD (anaerobic digestion) and UP2 (H_2 injection) phases. Communities with a relative abundance ≥0.5% (in at least one sample) are reported. EF, effluent and IM, interstitial matrix. Table shows Shannon–Weaver (H') and Pielou's evenness (E) diversity indices.

Anaerobaculaceae was the unique family within *Synergistetes* detected in all samples, regardless of the experimental phase or the sampling point, thus, suggesting its functional role in the anaerobic digestion process of SCW. This family was characterized at the species level as *A. hydrogeniformans*, a moderately thermophilic NaCl requiring fermentative bacterium, attesting that it was suitable for the saline and the milk derived substrates. The same microorganism was identified by Treu et al. (2019) [9] during thermophilic anaerobic digestion of cheese whey. Among the *Firmicutes* phylum, the highest number of families was identified. In particular, members of the *Lachnospiraceae* family were dominant in the EF-AD sample (30%) and had a lower relative abundance in the IM-AD (5%) sample. This family was not detected in the UP2 samples. Moreover, members of the *Thermoanaerobacteraceae* family were detected with a higher relative abundance in the IM-AD sample (8%) than in the EF-AD (2%) sample. These microorganisms were stable in EF-UP2 sample (2%) but showed a relevant decrease in the IM-UP2 sample (3%) as compared with the IM-AD sample. The genus *Thermacetogenium* was identified as a unique genus detected in all samples; it is characterized as an acetate-oxidizing syntrophic microorganism [32]. Members of the *Thermodesulfobiaceae* family were dominant in the interstitial matrix of both the AD and UP2 phases, accounting for 32% and 29% of the

microbial community, respectively. Previous studies have highlighted how these families carried out an acidogenic hydrolytic activity [33,34]. *Coprothermobacter* was identified among the *Thermodesulfobiaceae* family as the dominant genus with a relative abundance of 32% and 29% in samples IM-AD and IM-UP2, respectively. It is worth noting that its presence in anaerobic digesters is often related to configurations using biofilm supports [35]. *Coprothermobacter* is a bacterial genus that includes anaerobic thermophilic members that are proteolytic and produce acetate, H_2, and CO_2. Moreover, its involvement in the syntrophic acetate oxidization (SAO) pathway during thermophilic AD processes has been previously suggested [36,37]. A new phylogenetic affiliation has been proposed for this taxa according to its overall phenotypic properties as well as to a phylogenetic analysis supporting its placement in a distinct deeply rooted novel phylum [38]. The SHA-31 family of the class of *Anaerolineae* was revealed among the *Chloroflexi* phylum as the unique member characteristic of the UP2 experimental phase. Relative abundances corresponding to 36% and 15% of the microbial community were found in EF and IM samples of UP2 phase, respectively. Several authors [39–41] considered members of the *Anaerolineae* family to be a "semi-syntrophic" microorganisms in anaerobic systems due to their involvement in heterothrophic carbohydrate degradation and in interspecies electron transfer mechanism in mutualistic cooperation with methanogens.

According to both Shannon–Weaver (H') and Pielou's evenness (E) indices, the IM-UP2 sample showed the highest diversity (H' = 2.38 and E = 0.75), suggesting that the H_2 injection had a positive impact in the presence of HDPEs supports. Moreover, a comparison of indices between EF-AD (H' = 1.62 and E = 0.55) and EF-UP2 (H' = 2.06 and E = 0.65) highlighted that microbial diversity was increased also in the effluent by the hydrogen addition. At the same time, the higher diversity in the IM-AD sample (H' = 2.01 and E = 0.68) as compared with the EF-AD sample (H' = 1.62 and E = 0.55) indicated that the immobilization strategy was also able to increase the microbial diversity.

3.3.2. Archaea Communities

Archaea microbial communities were represented by the unique phylum of *Euryarchaeota*. The overall relative abundances of all methanogens were very low during the AD phase, ranging from 0.1% in EF-AD to 0.40% in the IM-AD. Subsequently, during H_2 addition, the methanogenic population increased up to 0.64% in the effluent and up to 1.42% in the interstitial matrix. Figure 6 shows the relative abundances of archaea at the family level.

Members of *Methanobacteriaceae* family dominated in both experimental phases, as well as in effluent and interstitial matrix, showing values of relative abundances higher than 74%. Among this family, the hydrogenotrophic *Methanothermobacter thermoautrophicus* was found to be highly abundant in the AD and UP2 phases. The dominance of the hydrogenotrophic pathway during anaerobic digestion of cheese whey was reported by Treu who suggested that this pathway was driven by the saline characteristics of a substrate [9]. Moreover, members of the *Methanosarcina* family were detected in all samples except for the EF-AD sample. In particular, the relative abundance in EF-AD was 16%, increasing in EF-UP2 (25%) and lowering in IM-UP2 (11%). *Methanosarcina* are known as generalist methanogenic archaea that are able to use hydrogen/dioxide carbon, acetic acid, and methylamines [42] as substrates. Therefore, addition of an extra substrate, namely hydrogen, caused the enrichment of the *Methanosarcina* community.

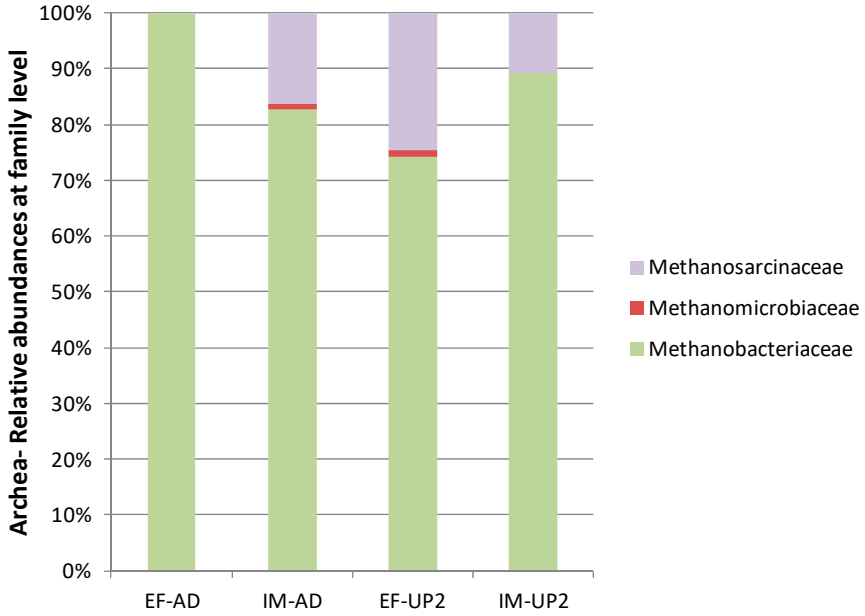


Figure 6. Relative abundances of the archaea community at the family level at the end of AD (anaerobic digestion) and UP2 (H_2 injection) phases. Communities with a relative abundance $\geq$0.5% (in at least one sample) are reported. EF, effluent and IM, interstitial matrix.

4. Conclusions

The partially immobilized gas-stirred tank reactor (GSTR) used in this study was suitable for thermophilic anaerobic digestion (AD) of undiluted second cheese whey (SCW). A methane yield of 0.329 $\pm$ 0.17 L_{CH_4} $gCOD^{-1}$, corresponding to 94% of the maximum theoretical value, was obtained without pH control. Moreover, H_2 injection (UP1 phase) increased the CH_4 production rate by 25% as compared with that obtained during conventional AD (AD phase) without compromising the efficiency of organic matter removal.

Data on microbial abundance showed an increase in bacteria communities at the end of both the AD and UP2 experimental phases in the interstitial matrix. Moreover, the H_2 addition (UP2) increased the archaea communities in both effluent and interstitial matrix as compared with the AD phase. From a functional point of view, we identified a common microbial "core" in all samples represented by members of the *Anaerobaculaceae* family, strictly correlated to the SCW metabolism. In addition, in samples collected from the interstitial matrix at the end of both experimental phases, a new microbial core was identified in members of the *Thermodesulfobiaceae* family. Finally, H_2 addition caused the enrichment of a peculiar microbial core represented by members of the SHA-31 family. A common core of the methanogenic microbial community was identified in the hydrogenotrophic members of the *Methanobacteriaceae* family in AD and UP experimental phases. Moreover, the versatile members of *Methanosarcinaceae* were enriched by the H_2 addition. All these functional microbial cores obtained a balanced AD process in both experimental phases, as shown by results of the GSTR performances at the steady state. Therefore, the whole microbial communities were able to adapt to H_2, as well as to the HDPE supports, thus, maintaining all the metabolic syntrophic relationships over the experimental time.

Author Contributions: Conceptualization, S.R., M.F., and A.S.; methodology, G.L. and V.M.M.; investigation, G.L. and A.S.; data curation, G.L. and A.S.; writing—original draft preparation, G.L.; writing—review and editing, G.L., S.R., A.M., G.M., and A.S.; visualization, S.R., G.M., and A.S.; supervision, M.F. and A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research has received national funding from AdP ENEA-MISE Project "Ricerca di sistema elettrico" (2019–2021).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AD	Anaerobic digestion
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
CW	Cheese whey
EF	Effluent
FISH	Fluorescent in situ hybridization
GSTR	Gas-stirred tank reactor
HDPE	High-density polyethylene
HRT	Hydraulic retention time
IM	Interstitial matrix
MER	Methane evolution rate
NGS	Next-generation sequencing
OLR	Organic loading rate
SCW	Second cheese whey
SMY	Specific methane yield
TS	Total solid
TVFA	Total volatile fatty acid
UP	Upgrading phase
VFA	Volatile fatty acid
VS	Volatile solid

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