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Biogas Production and Metagenomic Analysis in a New Hybrid Anaerobic Labyrinth-Flow Bioreactor Treating Dairy Wastewater

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Abstract: Increasing worldwide milk manufacturing and dairy processing resulted in producing more effluents, and thus effective management of wastewater is now the most important issue. This study used a new design of a pilot plant-scale hybrid anaerobic labyrinth-flow bioreactor (AL-FB) to increase the efficiency of anaerobic biodegradation and biogas productivity and improve anaerobic microflora performance. In addition, effluent recirculation was used to boost the treatment of dairy wastewater. Metagenomic analyses of the anaerobic microbial community were performed. It was found that an organic loading rate (OLR) of 4.0–8.0 g COD/L·d contributed to the highest CH_4 yield of 0.18 ± 0.01–0.23 ± 0.02 L CH_4/g COD removed, which corresponded to a high COD removal of 87.5 ± 2.8–94.1 ± 1.3%. The evenest distribution of the microorganisms' phyla determined the highest biogas production. In all tested samples, Bacteroidetes and Firmicutes abundance was the highest, and Archaea accounted for about 4%. Metagenomic studies showed that methane was mainly produced in acetoclastic methanogenesis; however, higher OLRs were more favorable for enhanced hydrogenotrophic methanogenesis. Effluent recirculation enhanced the overall treatment. Thus, at OLR of 10.0 g COD/L·d, the highest COD removal was 89.2 ± 0.4%, and methane production yield achieved 0.20 ± 0.01 L CH_4/g COD removed, which was higher by 25% compared to the achievements without recirculation.

Keywords: biomethane; dairy effluent; hybrid reactor; recirculation; microbial community

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1. Introduction

Currently, there is an increase in milk production worldwide, and dairy processing is now considered the largest source of food industrial wastewater, estimated at 192.5 × 106 m^3 in the EU [1–3]. According to the literature, anaerobic systems are suitable and cost-effective methods for the treatment of high-strength dairy wastewater [4–7]. Nowadays, in full-scale applications, many different types of anaerobic reactors are used, such as upflow anaerobic sludge blanket (UASB) reactors, anaerobic filters (AFs), and anaerobic fluidized bed reactors (AFBRs) [2,4,7,8]. However, the major limitation of their use in the treatment of dairy wastewater is the need for wastewater pre-treating due to a high content of suspended solids as well as fat, oil, and grease (FOG), which inhibit the development of anaerobic microflora [4,9,10]. Reactors with suspended biomass are more recommended for dairy wastewater treatment, but they can be operated only at low loading rates [7,11,12]. Moreover, all of the typical reactors have poor biomass retention resulting from high concentrations of FOG in dairy wastewater [2]. Therefore, in recent years, searching for new designs of hybrid anaerobic reactors that combine the positive features of individual anaerobic reactors for the treatment of dairy wastewater has been a challenge [7].

The new constructions of anaerobic hybrid digesters must both enhance biogas production and overall treatment efficiency and be easy to scale up to full-scale applications [1]. According to the newest studies, higher biogas production efficiency can be reached by increasing free electron flow via mediated/direct interspecies electron transfer (MIET/DIET) [13–18]. Some authors have also suggested DIET is the essential mechanism for interspecies electron transfer in UASB reactors [19–21]. However, UASB reactors are typically operated at high OLRs to enhance the biogas production rate, but a high concentration of organic load increases the rate of acidogenesis, resulting in volatile fatty acid (VFA) accumulation and a sharp drop in pH leading to reactor failure [7,22,23]. In novel hybrid reactors, the acidogenic and methanogenic phases are separated to isolate each metabolic pathway that requires specific conditions for smooth and efficient operation [7,24–26]. In addition, the optimization of operating conditions has the primary effect on the successful performance of hybrid reactors, among which OLR is the most important in forming the appropriate groups of anaerobic microorganisms [7,27,28]. The phyla Firmicutes, Bacteroidetes, and Synergistetes have been identified as major consortia that cooperate in maintaining the appropriate environmental niche for efficient and stable methane production [27]. In addition, other phyla such as Chloroflexi, Actinobacteria, and Proteobacteria are commonly recognized in anaerobic digesters [29,30].

Recently, the performance of a structured-bed hybrid baffled reactor (SBHBR) with anaerobic and oxic/anoxic chambers has been designed to enhance simultaneous organic matter and nitrogen removal in dairy wastewater [31]. Hybrid anaerobic reactors with different inert media were successfully used to increase the efficiency of dairy wastewater treatment [32,33]. A hybrid anaerobic baffled reactor also ensured efficient digestion of dairy wastewater and promoted the growth of acetoclastic and hydrogenotrophic methanogenesis in the reactor, represented mainly by Methanosaeta and Methanobacterium species, as well as other communities such as Syntrophomonadaceae and Syntrophaceae microorganisms [34]. The potential of granular activated carbon supplementation to increase the anaerobic degradation of dairy wastewater was investigated by Logan et al. [35]. They found stable and efficient methane production and high activity of electroactive microorganisms such as Synergistes and Geobacter, as well as the methanogens Methanolinea and Methanosaeta. Some additives were also applied to promote DIET in anaerobic digestion (AD) [36–38]. However, future systems that optimize anaerobic reactor design and operational parameters have yet to be definitively defined. Thus, further research should be conducted on current reactor technologies to enhance biogas production and overall treatment efficiency and ensure proper microbial community formation.

In this study, the combination of an anaerobic contact process with the UASB reactor and a settling tank was designed as an anaerobic labyrinth-flow bioreactor (AL-FB). The special construction of the AL-FB provided the separation of acidogenesis and methanogenesis processes and was designed to retain biomass, providing the longer retention time of anaerobic biomass and a larger exchange surface between the liquid and gas phases and minimizing problems related to sludge flotation.

The aim of this study was to develop a new design of an anaerobic reactor for the efficient treatment of dairy wastewater and biogas production. Metagenomic analyses of the anaerobic microbial community were performed to determine the impact of reactor design and operational parameters on microbial community formation.

2. Materials and Methods

2.1. Materials

Dairy wastewater was used as a feedstock for AD and was collected from a retention tank at the wastewater treatment plant in the dairy industry processing about 450,000 L of milk daily, producing a wide range of dairy products (Dutch- and Swiss-type cheeses, mozzarella, cheese spreads, curd cheeses, creams, yogurts, butter, UHT milk, powdered milk). Hydraulic retention time (HRT) in the retention tank was 24 h. The physicochemical characteristics of dairy wastewater used in this study are presented in Table 1.

Table 1. Characteristics of dairy wastewater used in this study.

Parameter	Unit	Value
COD	mg O_2/L	21,300 ± 1750
BOD_5	mg O_2/L	14,290 ± 1020
TP	mg P/L	112 ± 31
TN	mg N/L	915 ± 97
total solids	mg/L	1734 ± 150
pH	-	7.01 ± 0.31

Dairy wastewater used in this study contained a high concentration of organic compounds of 21,300 ± 1750 mg O_2/L as COD and 14,290 ± 1020 mg O_2/L as BOD_5 (Table 1). The phosphorus concentration amounted to 112 ± 31 mg/L, while TN content was as high as 915 ± 97 mg/L, which created a balanced carbon-to-nitrogen (C/N) ratio of about 23 (Table 1). According to the literature, a C/N ratio between 20 and 35 is recommended for the optimization of methane production [39,40]. The average pH was a neutral value of 7.01 ± 0.31, which was favorable for AD (Table 1). The high organic content in dairy wastewater promoted the application of anaerobic digestion as a method of treatment. The anaerobic reactor with a new design was used on a semi-technical scale.

Anaerobic suspended sludge (ASS) for hydrolyzer inoculation derived from a full-scale anaerobic digester fed with an excess aerobic activated sludge from a full-scale aerobic dairy wastewater treatment plant. The operation parameters of the digester were OLR of approx. 2 kg volatile solids (VSs)/m^3·d, HRT of 20 h, a temperature of 42 °C, and sludge concentration of approx. 4200 ± 35 g TSs/L. The ASS characteristics are presented in Table 2.

Table 2. Characteristics of anaerobic granular sludge (AGS) and anaerobic suspended sludge (ASS) used for reactor inoculation.

Parameter	Unit	Value for AGS	Value for ASS
hydration	%	97.8 ± 0.30	98.1 ± 0.2
total solids	g/L	40.1 ± 1.2	29.9 ± 1.7
mineral solids	g/L	14.4 ± 0.9	8.6 ± 0.4
volatile solids	g/L	25.7 ± 1.1	21.2 ± 0.6
filtrate COD	mg O_2/L	830 ± 31.0	630 ± 27.0
filtrate TP	mg/L	99.3 ± 16.4	51.0 ± 7.2
filtrate TN	mg/L	148 ± 13.7	89.2 ± 10.1
pH	-	7.49 ± 0.13	7.27 ± 0.12

Anaerobic granular sludge (AGS) for methanogenesis tank inoculation derived from a full-size UASB reactor exploited in a full-scale dairy processing wastewater treatment plant. The operation parameters of the UASB reactor were an organic loading rate (OLR) of approx. 10 kg COD/m^3·d, HRT of 24 h, and sludge concentration of approx. 40 g total solids (TSs)/L. The AGS characteristics are presented in Table 2.

2.2. Study Organization

The study was divided into two stages as shown in Table 3.

In stage 1, the study was focused on testing the most effective OLR providing the highest organic compound removal and biogas production rate. The experiment was divided into five variants depending on the value of OLR calculated per volume of methanogenesis tank: variant 1, 4.0 g COD/L·d; variant 2, 6.0 g COD/L·d; variant 3, 8.0 g COD/L·d; variant 4, 10.0 g COD/L·d; and variant 5, 12.0 g COD/L·d. In each experimental variant, the reactor volume was replaced 20 times, and hence the duration of each variant and the hydraulic load of the reactor were different.

Table 3. The study organization and exploitation parameters in stages 1 and 2.

Variant	Stage 1			
	OLR (g COD/L·d)	Q (L/d)		HRT (d)
V1	4.0	15		5.3
V2	6.0	23		3.5
V3	8.0	30		2.7
V4	10.0	37		2.2
V5	12.0	45		1.8
Variant	**Stage 2**			
	OLR (g COD/L·d)	**Recirculation Degree (%)**	**Q (L/d)**	**HRT (d)**
V1	10.0	50	55	1.5
V2		100	74	1.1
V3		150	111	0.7
V4		200	148	0.5

In stage 2, the influence of the digestate liquid fraction recirculation on the efficiency of methane fermentation was examined. Four experimental variants differing in the degree of internal recirculation were tested: variant 1, 50%; variant 2, 100%; variant 3, 150%; and variant 4, 200%. As in stage 1, at OLR of 10 g COD/L·d (calculated per volume of methanogenesis tank), there was a significant decrease in AD efficiency; this OLR value was applied in stage 2.

2.3. Experimental Station Construction and Exploitation

The biogas production of dairy wastewater was performed in the AL-FB, in which the construction of the reactor forced a vertical wastewater flow (Figure 1).

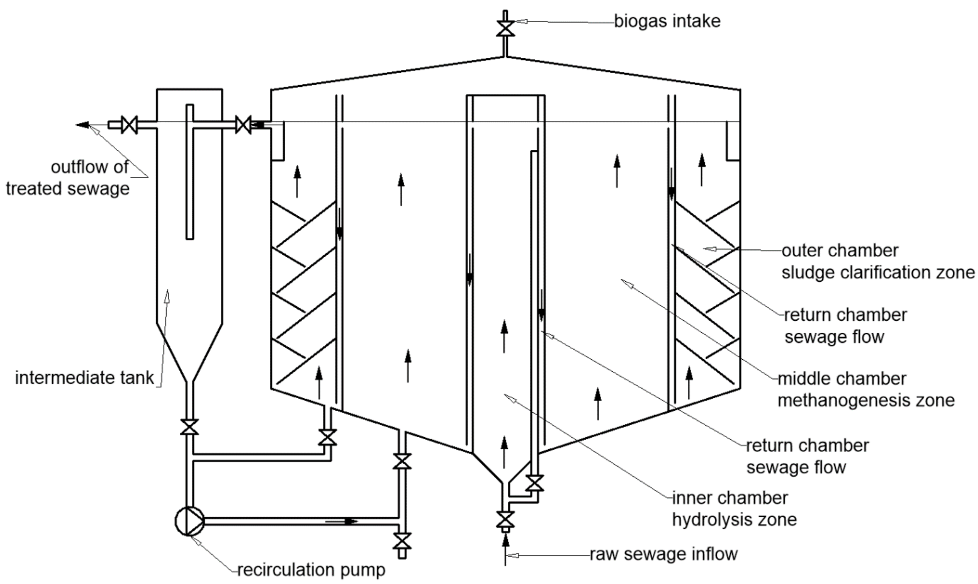


Figure 1. Scheme of anaerobic labyrinth-flow bioreactor.

The AL-FB consisted of three coaxially placed tanks with a total working volume of 178 L. The inner tank (working volume of 4 L, working height of 0.51 m, diameter of 0.1 m) was inoculated with ASS and was used as a hydrolyzer. The middle container (working volume of 78 L, working height of 0.51 m, diameter of 0.45 m) served as a methanogenesis tank and was inoculated with AGS, while the sedimentation process and clarification of digestate were in the external tank (working volume of 96 L, working height of 0.51 m, diameter of 0.5 m). Additionally, the final clarification of effluent was in the external effluent/sludge storage tank. The reactor was equipped with a peristaltic pump

(ALLWEILER ASH 15, Radolfzell, Germany) with a capacity of 1 L/min and pressure of 1 bar with an electric motor (0.12 kW, 12 rpm/min, IE1, 400 V, 50 Hz) connected to the inverter with thermistors (SV002iE5-C, 0.2 kW, 1.4 A).

Substrate (raw dairy wastewater) was pumped from the bottom of the central part of the hydrolyzer to the top (upward wastewater flow). Then it flowed down to the backflow chamber and up to the methanogenesis tank. Additionally, the effluent from the effluent/sludge storage tank was recirculated to the middle container (internal digestate recirculation). The mixture of digestate and effluent flowed into the second backflow chamber and then into the external tank equipped with a quadruple sludge trap with biogas discharge pipes. Biogas produced was collected in the gas chamber of the reactor and discharged through a pipe in the upper part of the AL-FB dome equipped with a gas flow meter. The clarified solids (biomass) were collected at the bottom of the external tank and pumped to the methanogenesis container or discharged out of the system. The effluent from the AL-FB was directed to the effluent/sludge storage tank through the sawtooth-shaped outlet.

The temperature inside the methanogenesis tank was maintained at the level of 37 ± 1 °C. To maintain the temperature, the AL-FB was insulated with a 50 mm layer of mineral wool and heated by a water jacked consisting of a hot water storage tank with a capacity of 40 L and an electric heater (2000 W).

2.4. Illumina MiSeq Sequencing

The samples of anaerobic granular sludge from V1, V3, and V5 were used for metagenomic analysis, due to the assumption that they would represent the most diverse microbial population. DNA extraction was performed using a FastDNA SPIN Kit for Soil (MP Biomedicals), and its purity (A260/280 and A260/230 ratios) and concentration were measured with a NanoDrop One spectrometer (Thermo Scientific, Waltham, MA, USA). Genomic DNA was then detected by 1% agarose gel electrophoresis. Metagenomic analysis of the 16S rRNA encoding gene was performed on the basis of the V3-V4 hypervariable region of the 16S rRNA gene by Genomed Laboratory (Poland). Specific primer sequences 341F and 785R were used to amplify the selected region of Archaea and Bacteria and prepare the library. PCR was performed using Q5 Hotstart High-Fidelity DNA Polymerase (NEBNext), under conditions consistent with the manufacturer's recommendations. Sequencing was carried out on a MiSeq sequencer, in paired-end (PE) technology, 2 × 250 nt, using the MiSeq Reagent Kit v2, according to the manufacturer's recommendations (Illumina). For sequence analysis, the 16S Metagenomics protocol was used, which provides a species-level classification of reads based on the Greengenes v13_5 reference sequence database modified by Illumina. The analysis consisted of automatic demultiplexing of samples, generating fastq files containing raw readings, and classification of paired-end readings in particular taxonomic categories.

2.5. Analytical Methods

The parameters such as COD, total phosphorus (TP), and total nitrogen (TN) were analyzed using cuvette tests in a DR 2800 spectrophotometer with mineralizer (HACH Lange, Düsseldorf, Germany). Total solid (TS) and volatile solid (VS) concentrations and mineral solids (MSs) were measured by gravimetric method (part of EPA Standard Method 2540). Determination of biochemical oxygen demand (BOD_5) was carried out according to PN-EN 1899-1 using an OxiTop Control system (WTW, Bartoszyce, Poland). The ratio of free organic acids (FOS) to total inorganic carbonate (TAC)—FOS/TAC—was determined by a titration method (Tritlab AT 1000, Hach, Düsseldorf, Germany). The pH was determined with a VWR 1000 L pH meter (Weilheim, Germany). Contents of TN, TP, and COD in the filtrate were determined with the spectrophotometric method using a Hach DR6000 spectrometer (Hach, Loveland, CO, USA).

A digital gas flow meter (XFM17S, Aalborg Instruments & Controls, Inc., Orangeburg, NY, USA) measured the instant flow rate and total biogas flow. The biogas composition was analyzed every 24 h using a gas chromatograph (GC, 7890A Agilent Technologies, Santa Clara, CA, USA). The device was equipped with a thermal conductivity detector (TCD), two Hayesep Q columns (80/100 mesh), two molecular sieve columns (60/80 mesh), and a Porapak Q column (80/100). The temperatures of the injection and detector ports were 150 °C and 250 °C, respectively. Helium and argon were used as carrier gases at a 15 mL/min flow. The volumetric methane production rate (VMPR) was calculated per volume of the methanogenesis tank.

2.6. Calculation Methods

Gross energy (*GE*) gained as the total energy from the production of biogas was calculated as follows:

$$GE = Y_{CH4} \cdot HV_{CH4} \quad (1)$$

where Y_{CH4} is a methane yield (m^3/d) and HV_{CH4} is a heating value of methane (kWh/m^3).

Consumption of energy (*CE*) for pumping wastewater and recirculation was calculated as follows:

$$CE = P_p \cdot T_w \quad (2)$$

where P_p is the power of pumping system (kW) and T_w is the pump operating time (h/day).

The net energy output (*NE*) was calculated as follows:

$$NE = GE - DE \quad (3)$$

where *GE* is the gross energy (kWh/d) and *DE* is the energy demand for pumping and recirculation (kWh/d).

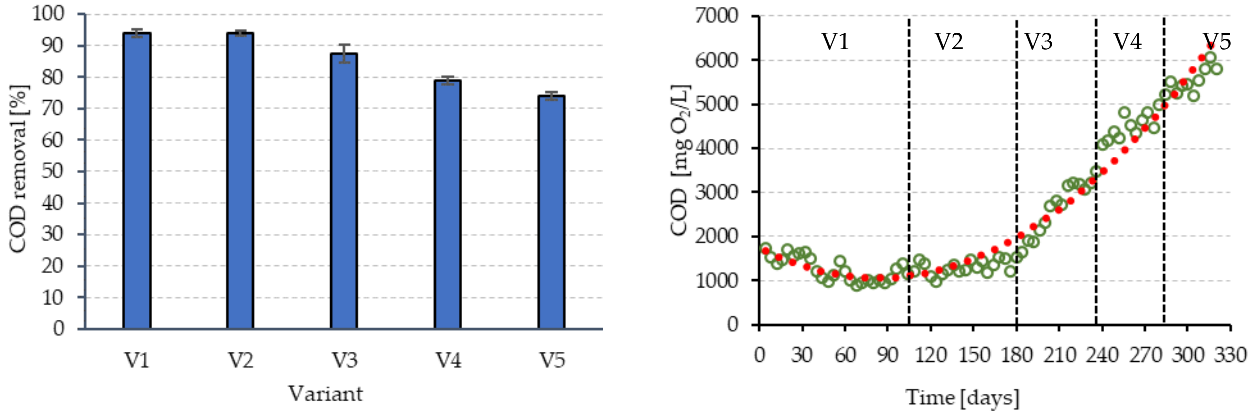
2.7. Statistical Methods

The Statistica 13.1 PL software package (StatSoft, Inc., Tulsa, OK, USA) was used for the analysis. The homogeneity of variance in groups was determined using Levene's test. Tukey's HSD test was applied to determine the significance of differences between the series. A confidence level of 95% was used in the statistical analyses, and the variables were considered significant for the evaluated process when $p < 0.05$.

3. Results and Discussion

3.1. AD Performance and Wastewater Treatment Efficiency

In stage 1, the AL-FB was operated with increasing OLR from 4.0 g COD/L·d to 12.0 g COD/L·d in five experimental variants (Table 3). The highest COD reduction of over 87% was noted at OLRs between 4.0 g COD/L·d and 8.0 g COD/L·d with an average COD concentration in the effluent of 1279.6 mg O_2/L (Figure 2, Table 4). The application of a higher load of organic compounds in dairy wastewater influenced the reduction in COD removal to the value of approx. 74% and an average COD concentration in the effluent of 5521.0 mg O_2/L in V5 (Figure 2, Table 4). However, increasing OLR involved an increase in COD load removal, which was 3.76 ± 0.1 g COD/L·d at 4.0 g COD/L·d and rose to 8.89 ± 0.1 g COD/L·d at the highest OLR (Table 4). The FOS/TAC ratio was found to be less than 0.4 for OLRs from 4.0 g COD/L·d to 8.0 g COD/L·d, which ensured a proper buffering capacity in the AL-FB chambers (Figure 3). Within this OLR range, the pH in the methanogenesis tank remained at an average level of pH 7.19 ± 0.11 (Figure 3). Dosing a larger organic load into the anaerobic reactor resulted in an increase in the FOS/TAC ratio over 0.4 in variant 4 and over 0.5 in variant 5, as well as a decrease in pH to the value of 6.49 at the highest OLR, indicating that the reactor was overfed (Figure 3) [41,42]. Biogas production remained at a high level within OLRs from 6.0 g COD/L·d to 12.0 g COD/L·d (Table 4). The highest average productivity of 121.7 ± 2.9 L/d was noted at the highest OLR, but there were no differences in variants 4–5 ($p > 0.05$) (Table 4). The highest methane production of 101.4 ± 1.9 m^3/d was achieved at an OLR of 10.0 g COD/L·d (Table 4). Methane concentration in biogas ranged from 73.0 ± 1.9% at the OLR of 4.0 g COD/L·d to 59.3 ± 3.0% at the OLR of 12.0 g COD/L·d (Figure 4). Biogas and methane yields were the highest at OLRs ranging from 4.0 g COD/L·d to 6.0 g COD/L·d and amounted respectively to 0.32 ± 0.02 L/g COD removed and 0.23 ± 0.02 L/g COD removed (Table 4). The yields decreased with increasing organic load to the value of 0.23 ± 0.01 L biogas/g COD removed and 0.13 ± 0.01 L CH_4/g COD removed in the last variant (Table 4). Similarly, the yields calculated per gram of COD inlet dropped with increasing OLR (Figure 4). Nutrient removal was low and is shown in Figure 3. Phosphorus was not removed effectively regardless of the load, while the highest nitrogen removal of 14.42 ± 1.9% was noted in variant 1.



(a) (b)

Figure 2. The efficiency of anaerobic dairy treatment in stage 1: (**a**) organic compound removal as COD with a standard deviation in experimental variants V1–V5; (**b**) COD concentration in the effluent during the experimental time (variants V1–V5).

A similar COD removal efficiency of about 90% at the OLR of 5 kg COD/m^3 was obtained by Ince [43] in a system of anaerobic reactors with separation of acidogenesis and methanogenesis, treating dairy wastewater. A hybrid UASB reactor in a lab-scale study was used by Banu and co-workers [44] to treat dairy wastewater. The reactor was operated at an OLR varying from 8 to 20 kg COD/m^3·d for a period of 110 days. The maximum COD removal and biogas production was obtained at the lowest OLR, and when the OLR rose to 19.2 kg COD/m^3·d, the COD removal decreased to 84%. In turn, Kavitha and co-workers [45] achieved a poor COD removal of 44.3% in a UASB reactor with modifications and improvements, treating dairy wastewater with inlet COD concentrations ranging from 1090 to 1415 mg O_2/L. Dębowski and co-workers [46] used magneto-active packing to enhance the anaerobic digestion of synthetic dairy wastewater in a moving biofilm reactor, in which methanogenesis was separated from the acidogenic phase. At the OLR of 3.9–7.5 kg COD/m^3·d, the removal of organic substances was 86.6% with the maximum methane yield of 0.23 L/g COD removed. The authors postulated that the appropriate design of the reactor in combination with stimulating factors (e.g., magnetic field) allowed for achieving more biogas productivity and higher COD removal. In the presented study, a specific reactor construction made it possible to achieve the same methane yield at OLRs ranging from 4 kg COD/m^3·d to 6 kg COD/m^3·d. In turn, Jürgensen and co-workers [47] obtained a slightly higher methane yield of 0.264 L/g COD and 91% of COD removal, but in lower OLRs from 1.3 kg COD/m^3·d to 4.3 kg COD/m^3·d in a hybrid reactor combining a continuous stirred tank reactor (CSTR) with an anaerobic baffled reactor (ABR) treating dairy effluent. Dareioti and Kornaros [48] obtained a higher methane yield and COD removal efficiency up to 0.9 L/kg COD removed and 84.4% in a two-stage system of CSTRs with a separate methanogenic stage at an OLR of 3.58–7.15 kg COD/m^3·d enhanced. However, in experiments, cheese whey was co-digested with ensiled sorghum and liquid cow manure, which, according to the literature, improves the treatment process [48].

Table 4. Removal of organic compounds and production of biogas and CH_4 in stage 1.

Variant	OLR (g COD/L·d)	COD		Biogas Production			CH_4 Production			
		Removal (%)	Load Removal (g COD/L·d)	(L/d)	(L/g COD_{inlet})	(L/g $COD_{removed}$)	(L/d)	(L/g COD_{inlet})	(L/g $COD_{removed}$)	VMPR (L/L·d)
1	4.0	94.1 ± 1.3	3.76 ± 0.1	71.8 ± 2.7	0.23 ± 0.09	0.31 ± 0.03	53.0 ± 0.6	0.17 ± 0.05	0.23 ± 0.02	0.7 ± 0.01
2	6.0	93.8 ± 0.8	5.63 ± 0.1	112.3 ± 1.9	0.24 ± 0.05	0.32 ± 0.02	79.6 ± 0.8	0.17 ± 0.01	0.23 ± 0.01	1.02 ± 0.01
3	8.0	87.5 ± 2.8	7.00 ± 0.2	112.3 ± 1.4	0.18 ± 0.07	0.27 ± 0.02	93.6 ± 1.0	0.13 ± 0.03	0.18 ± 0.01	1.2 ± 0.02
4	10.0	78.9 ± 1.4	7.89 ± 0.1	117.0 ± 1.3	0.15 ± 0.03	0.24 ± 0.02	101.4 ± 1.9	0.11 ± 0.09	0.16 ± 0.01	1.3 ± 0.03
5	12.0	74.1 ± 1.3	8.89 ± 0.2	121.7 ± 2.9	0.13 ± 0.08	0.23 ± 0.01	93.6 ± 0.9	0.09 ± 0.06	0.13 ± 0.01	1.2 ± 0.02

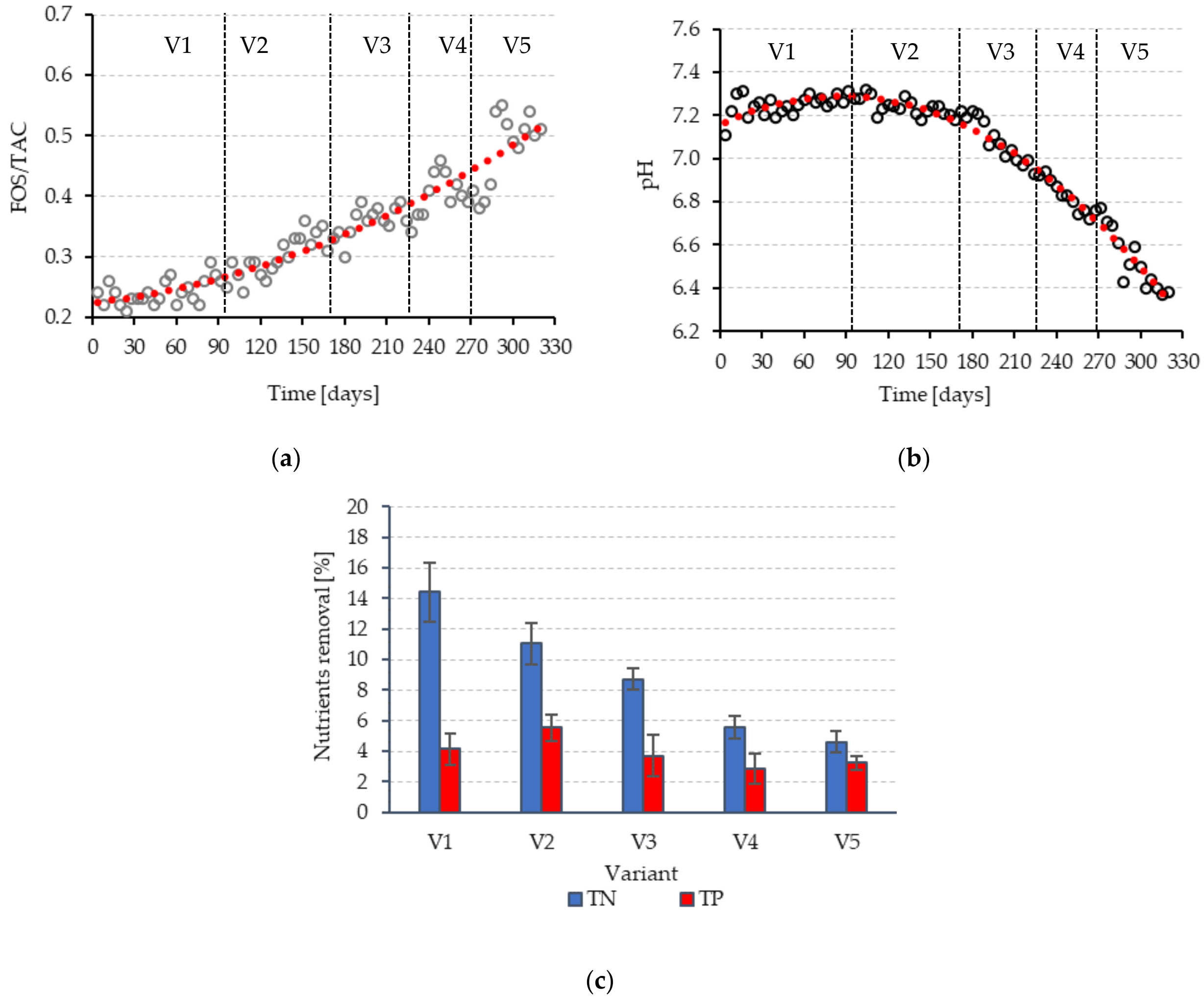


Figure 3. The efficiency of anaerobic dairy treatment in stage 1: (**a**) FOS/TAC ratio in the methanogenesis tank during the experimental time (variants V1–V5); (**b**) pH in the methanogenesis tank during the experimental time (variants V1–V5); (**c**) nutrient removal (nitrogen and phosphorus) with a standard deviation in experimental variants V1–V5.

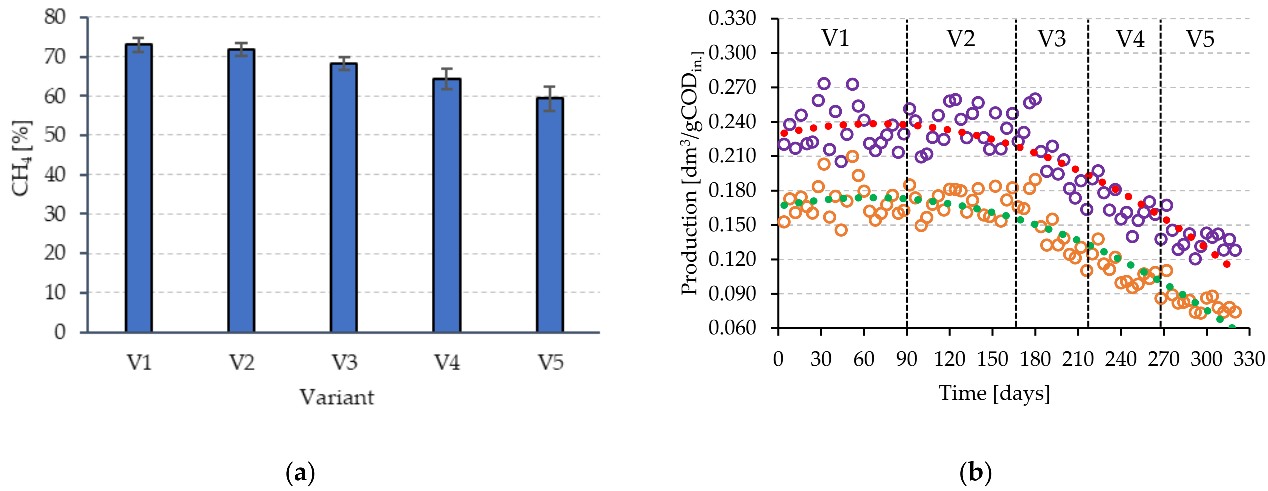
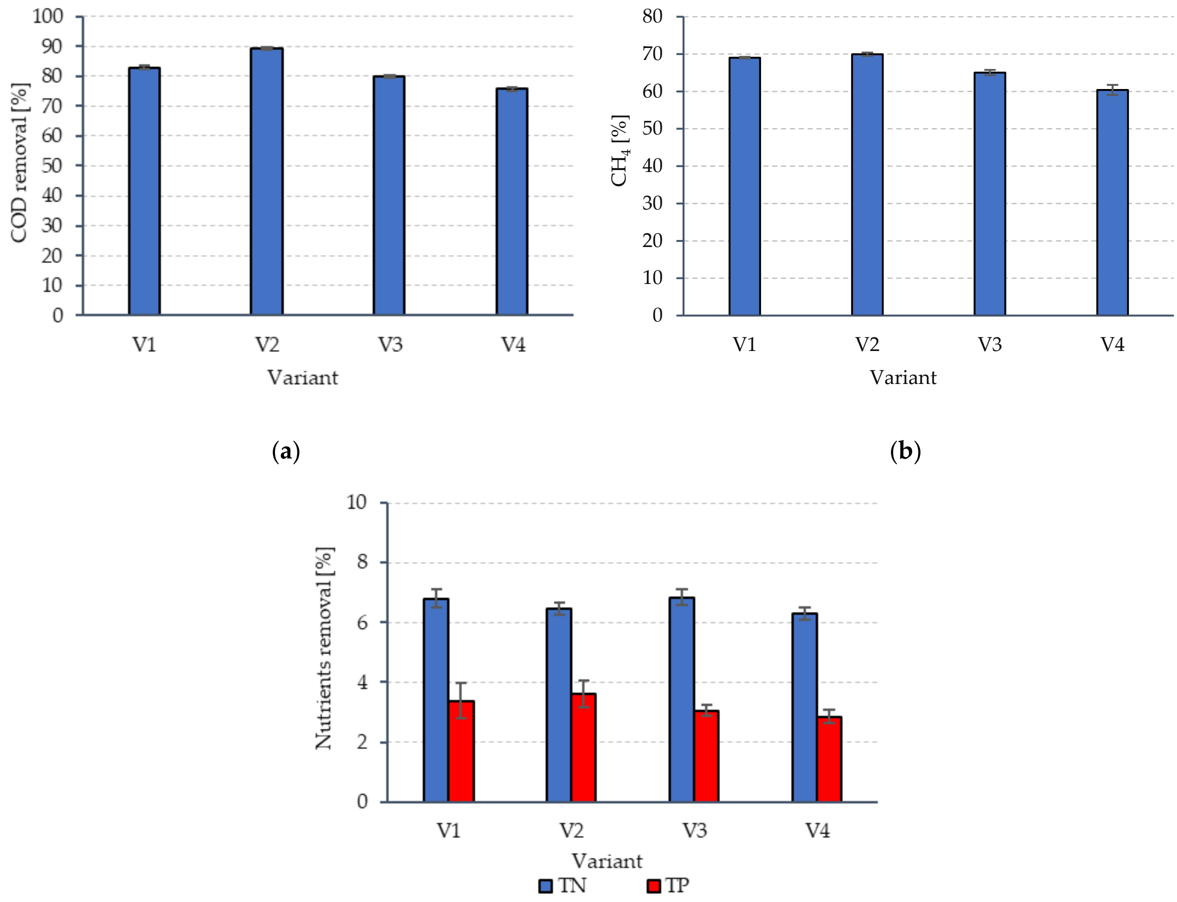


Figure 4. The efficiency of anaerobic dairy treatment in stage 1: (**a**) CH_4 concentration in biogas with a standard deviation in experimental variants V1–V5; (**b**) biogas and methane yield per gram of COD inlet during the experimental time (variants V1–V5).

Taking into account the influence of the OLR on the course of the AL-FB, the anaerobic digestion of dairy wastewater was the most effective at OLRs ranging from 4.0 g COD/L·d to 8.0 g COD/L·d. In variant 4, COD removal, biogas, and methane yield significantly decreased; thus, in the second stage of the study, the experiments were conducted at a constant OLR of 10 g COD/L·d to enhance anaerobic digestion by recirculation. The study was performed for four variants (Table 3). The highest COD removal of 89.2 ± 0.4% was achieved in variant 2 with 100% recirculation, which was 10.3% higher than that in experiments without recirculation (Table 5, Figure 5). In variants 1 and 3, the COD removal also exceeded 80% (Table 5, Figure 5). The recirculation degree of 200% in the last variant deteriorated the removal of organic compounds under the level achieved in stage 2 (Figure 5, Tables 4 and 5). The highest organic load removal of 8.92 ± 0.04 g COD/L·d was achieved in variant 2 and was 1.03 g COD/L·d higher than that in experiments without recirculation (Tables 4 and 5). The pH values in all experimental variants were close to neutral, ranging from pH 6.83 ± 0.06 in variant 4 to 7.06 ± 0.04 in variant 2, which was within the optimum range for methanogens [49,50]. Recirculation provided a better reduction in volatile fatty acid (VFA) concentration in the liquid fraction of digestate, thus avoiding the accumulation of VFAs, increasing pH and alkalinity, and resulting in better process stability [51,52]. During the whole experiment, the FOS/TAC ratio was within the range of 0.35–0.4, indicating the presence of a proper buffering capacity in the methanogenesis tank [41,42].



(**a**) (**b**) (**c**)

Figure 5. The efficiency of anaerobic dairy treatment in stage 2: (**a**) organic compound removal as COD with a standard deviation in experimental variants V1–V5; (**b**) CH_4 concentration in biogas with a standard deviation in experimental variants V1–V5; (**c**) nutrient removal (nitrogen and phosphorus) with a standard deviation in experimental variants V1–V5.

Table 5. Removal of organic compounds and production of biogas and CH_4 in stage 2.

Variant	OLR (g COD/L·d)	COD		Biogas Production			CH_4 Production			
		Removal (%)	Load Removal (g COD/L·d)	(L/d)	(L/g COD_{inlet})	(L/g $COD_{removed}$)	(L/d)	(L/g COD_{inlet})	(L/g $COD_{removed}$)	VMPR (L/L·d)
1	10.0	82.8 ± 0.6	8.28 ± 0.06	140.4 ± 6.2	0.18 ± 0.08	0.27 ± 0.01	93.6 ± 4.8	0.12 ± 0.06	0.19 ± 0.01	1.2 ± 0.01
2		89.2 ± 0.4	8.92 ± 0.04	156.0 ± 6.2	0.20 ± 0.08	0.29 ± 0.01	109.2 ± 3.6	0.14 ± 0.06	0.20 ± 0.01	1.4 ± 0.01
3		80.0 ± 0.5	8.00 ± 0.05	124.8 ± 7.3	0.16 ± 0.06	0.25 ± 0.02	78.0 ± 2.7	0.10 ± 0.07	0.16 ± 0.01	1.0 ± 0.05
4		75.7 ± 0.6	7.57 ± 0.06	101.4 ± 6.1	0.13 ± 0.05	0.21 ± 0.01	62.4 ± 1.9	0.08 ± 0.02	0.13 ± 0.01	0.8 ± 0.01

In stage 2, the most effective biogas and methane production of 156.0 ± 6.2 L biogas/d and 109.2 ± 3.6 L CH_4/d, respectively, were obtained in variant 2 with 100% recirculation and were respectively 33.5% and 45.2% more compared to the achievements without recirculation (Tables 4 and 5). A 200% degree of recirculation negatively affected the production of biogas (which reached 124.8 ± 7.3 L/d) and methane (78.0 ± 2.7 m^3/d), which were below the levels achieved in stage 1 (Table 5). During the anaerobic digestion of dairy wastewater, the biogas and methane yields were the highest when the recirculation degree ranged from 50% to 100% (Table 5). The maximum methane yield of 0.20 ± 0.01 L/g COD removed was obtained with 100% recirculation, which was 25% higher than the production obtained in stage 1 at the OLR of 10 g COD/L·d (Table 5). The concentration of methane in biogas ranged from 60.4 ± 1.4% in variant 4 to 70.0 ± 0.4% in variant 2 (Figure 5). In the whole experiment, nitrogen removal remained at about 6–7%, while phosphorus removal was at about 3% (Figure 5).

The study showed that the new construction of the anaerobic reactor and 100% degree of recirculation of the digestate liquid fraction significantly enhanced the anaerobic digestion of dairy wastewater. Barber and Stuckey [53] described an anaerobic reactor that was similar in design assumptions. A baffled anaerobic reactor was designed as a series of baffles forcing wastewater flow through the series of compartments from inlet to outlet. This construction provided longer biomass retention time, lower sludge yield, the ability to partially separate acidogenesis and methanogenesis, better resilience to hydraulic and organic shock loadings, and better substrate accessibility to methanogens, consequently resulting in high treatment efficiencies at low HRT (2–6 h) and high OLR (0.4–28 kg/m^3·d) as well as increasing the overall stability of the reactor. According to the literature, recirculation provides some benefits during wastewater treatment, such as improving contact between the methanogenic bacteria and substrate components, greater reactor homogeneity, and shortening the time in which maximum methane yield is achieved [54,55]. Recirculation of the digestate liquid fraction within the anaerobic digester is mostly used in dry anaerobic digestion [54,56–58]. Pezzolla and co-workers [52] obtained better biogas production during the anaerobic digestion of pig slurry with increasing frequencies of a liquid fraction of digestate recirculation. Chan and co-workers [59] achieved maximum methane production in 9 days with recirculation compared to 11 days without it. Recirculation of a liquid fraction of the digestate in a system of CSTRs for corn stover anaerobic digestion enhanced methane and biogas yield by 2.3% and 10.8%, respectively [46]. Methane production increased by 26% during the anaerobic digestion of food waste in a hybrid solid–liquid reactor with recirculation compared to a conventional two-phase anaerobic digester without recirculation [60]. However, some problems may occur after the long-term operation with digestate recirculation, such as the accumulation of VFAs, ammonia nitrogen, non-biodegradable intermediates, and other inhibitory substances [61].

3.2. Microbial Compositions in Anaerobic Digester

To achieve efficient and stable AD, a diverse microbiome is required. The design, the chemical and physical composition of the feedstock, and the operational parameters of the reactors such as OLR, HRT, pH, and temperature have the greatest impact on the biogas-producing microorganisms [62]. The specific environmental conditions enhance the formation of different groups of microorganisms, and the stability of AD plays a key role in maintaining a delicate balance in microbial populations, which consequently ensures high biogas production [63]. Metagenomic analyses provide insight into the taxonomic diversity and the physiological potential of microbiomes, among which the identification of the biogas-producing microbiomes is the most important [64]. Links between bacterial and archaeal populations and AD process parameters have been studied by several authors [64–67]. However, these correlations did not directly and clearly identify factors that promote AD or act as a barrier [63]. Nowadays, the most important issue is to identify the diversity and composition of individual microorganisms forming under different environmental conditions in order to better understand their behavior in response to AD process disturbances, and the main goal of these studies is to effectively improve the efficiency and stability of AD [63,67]. Thus, there is now a high potential for microbial-based management in AD to determine a set of microbial indicators.

A total of 23 taxonomic phyla were observed in anaerobic granular sludge samples collected from methanogenesis tanks operated at OLRs of 4 g COD/L·d, 8 g COD/L·d, and 12 g COD/L·d, represented by V1, V3, and V5, respectively (Figure 6).

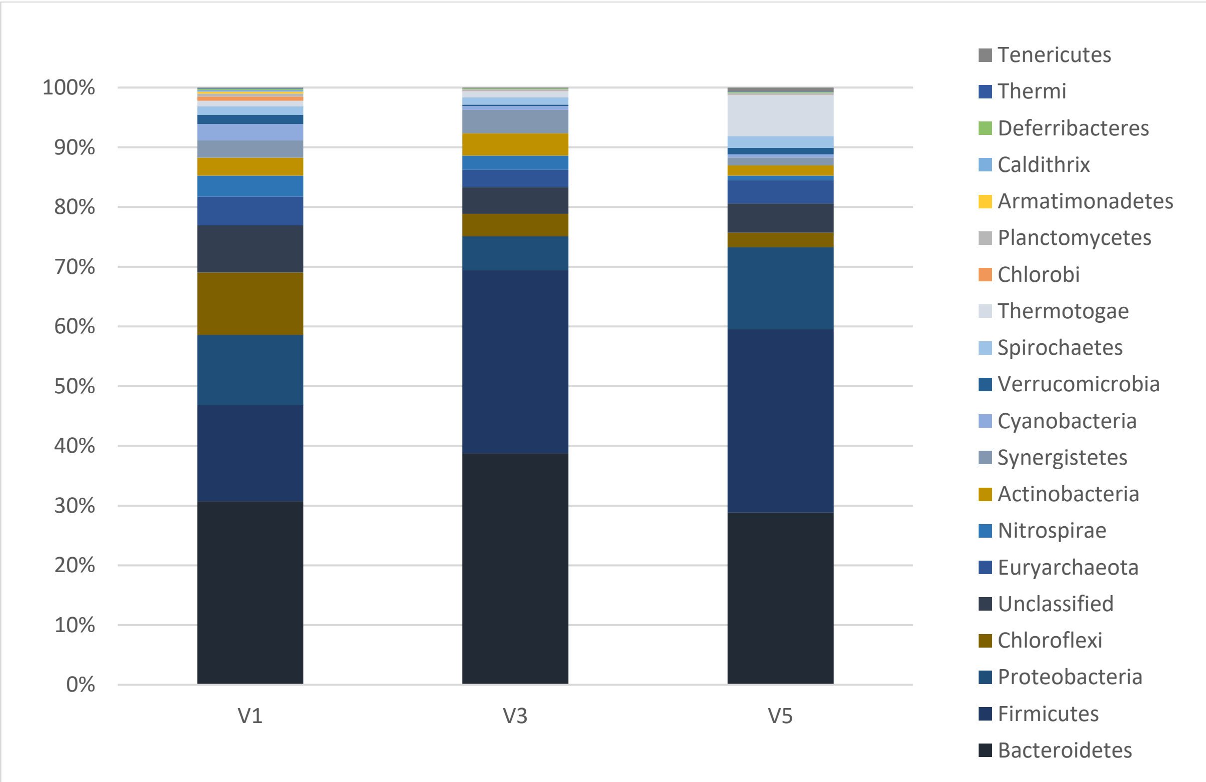


Figure 6. The abundance of phyla in anaerobic granular sludge in V1, V3, and V5 in stage 1.

The abundance of Archaea in the anaerobic granular sludges was 4.85%, 2.95%, and 3.92% in V1, V3, and V5, respectively. Unclassified reads constituted only less than 0.5% at this level in all samples. Bacteria dominated, even if the anaerobic granular sludge samples were collected from the methanogenesis tank. The abundance of Archaea in the two-stage system operated in this study was similar to that observed in solid-state anaerobic digestion [68] and full-scale anaerobic digesters operated in municipal wastewater treatment plants [69].

Most of the Archaea were Euryarcheota, and among them, the domination of Methanomicrobia (1.5% in V1, 1.6% in V2, 2.6% in V5) and Methanobacteria (3.3% in V1, 1.3% in V3, 1.2% in V3) was observed. The abundance of the former increased with the increase in OLR, and the abundance of the latter decreased with an increase in OLR (Figure 7). The abundance of Archaea was similar in all operating variants; therefore, bacteria providing the substrate for Archaea were probably more responsible for observed differences in biogas production. These different supplies of substrates could affect the activity of Archaea and thus the efficiency of biogas production. Among methanogens in all tanks, the most abundant species was acetoclastic Methanosaeta concilii. Methanosaeta grow in stable habitats characterized by low concentrations of acetate, due to their affinity for acetate [70,71]. McHugh and co-workers [72] investigated six different samples of anaerobic sludges, treating wastewater containing simple and complex compounds with low- and high-strength treatments at psychrophilic (10–14 °C), mesophilic (37 °C), and thermophilic (55 °C) temperatures. The authors observed domination of Methanosaeta spp. irrespective of wastewater type or operating conditions, showing underlying potential for effective growth of Methanosaeta sp. in different environments and ensuring efficient operation of an anaerobic bioreactor [72].

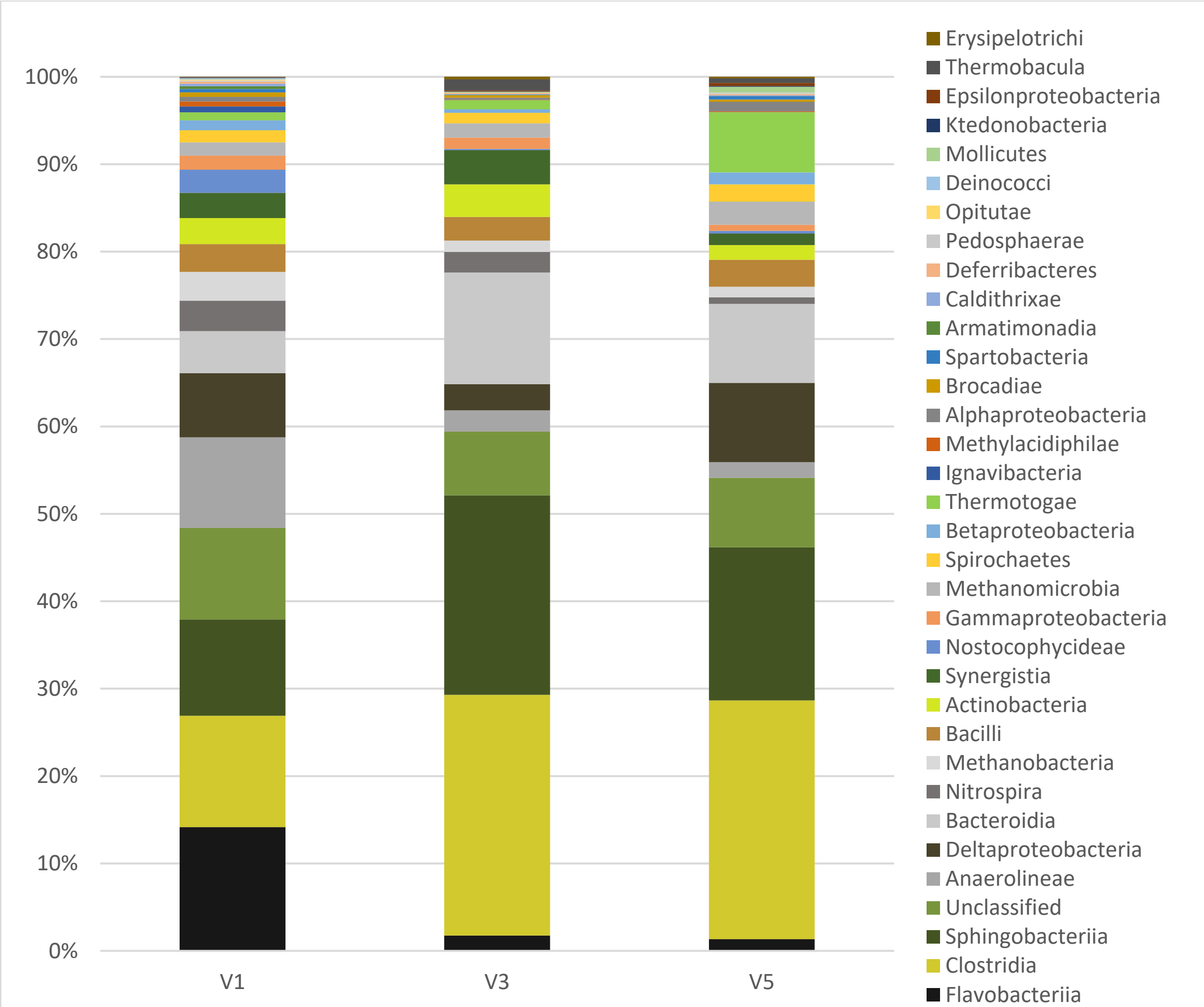


Figure 7. The abundance of classes in anaerobic granular sludge in V1, V3, and V5 in stage 1.

At the phylum level, Bacteroidetes were the most abundant in V1 (30.7%) and V3 (38.7%) (Figure 6). In V5, the abundance of Firmicutes increased so that they constituted the majority (30.7%); however, the abundance of Bacteroidetes was still high (28.8%). The abundance of Firmicutes was 16.1% in V1, and it was the second most abundant phylum. In V3, the abundance of Firmicutes increased to 30.7% and remained at that level also in V5 (which was mentioned above). Firmicutes are characterized by the ability to degradation of different kinds of organic compounds under various conditions [73]. The domination of Firmicutes was observed in a reactor with high acidification conditions (at OLRs of 11.0, 12.9, and 14.0 g COD/L·d) during the anaerobic digestion process of two-phase olive mill residue [74]. However, some other studies reported by the same authors showed that Firmicutes were the predominant bacteria at a low OLR [75]. This is in contradiction with the observations in the present study, where the abundance of Firmicutes increased at higher OLRs. It turns out that such statements on the phylum level are probably too generalized, due to phyla being represented by numerous microorganisms, which can be replaced one by another. Bacteroidetes are responsible for hydrolytic and fermentative reactions in a range of organic substrates such as municipal waste and manure organic materials with complex carbohydrates [76–80].

In V1, the abundance of phyla was characterized by an even distribution, except for the domination of Bacteroidetes (Figure 6). The abundance of Proteobacteria and Chloroflexi was about 10%. Next, Euryarcheota, Nitrospirae, Actinobacteria, and Synergistates had an abundance of about 3–5%. In V3, the domination of Bacterioidetes and Firmicutes (both at about 30%) above other phyla was significantly marked. The phyla Proteobacteria, Synergistates, Chloroflexi, Actinobaceria, Nitrospirae, and Euryarcheota (the abundance in this order) constituted 3–5%. In V5, the domination of Bacterioidetes and Firmicutes was also clearly marked; however, the abundance of Proteobacteria increased to 13%. In V5, the abundance of Thermotogae was 6.87%, which constituted less than 1% in V1 and V3. Only the abundance of Euryarcheota (3.91%) and Chloroflexi (2.41%) exceed 2%, and the abundance of other phyla was less than 2% in V5. The conditions in V1 (low OLR, neutral pH) were more favorable for a more diverse community than the more restricted conditions in V5 (high OLR, acidic pH), which caused most of the species to be less abundant. Therefore, an increase in OLR reduced microbial richness and the even distribution of populations. This evenest distribution of phyla in V1 ensured high biogas production.

The phylum Bacteroidetes was represented by classes Flavobacteria, Sphingobacteria, and Bacteroidia at all applied conditions. The abundance of Flavobacteria was the highest in V1 (14.1%); in V3 and V5, its abundance was 1.7% and 1.3%, respectively (Figure 7). Flavobacteria were recognized as bacteria responsible for the degradation of carbohydrates and proteins [81], which were also observed in an acidogenic reactor [82]. The presence of Sphingobacteria and Bacteroidia, in the present study, was characterized by a similar tendency; however, they differed in numbers. These classes were most abundant in V3 (22.8% and 12.7%, respectively), and then their abundance decreased in V5 (17.5% and 9%, respectively) and was the lowest in V1 (11% and 4.8%, respectively) (Figure 7). Class Bacteroidia was recognized as important hydrolyzers and fermenters, which can grow in extreme pH conditions [83,84].

The phylum Firmicutes was represented by classes Clostridia and Bacilli. The abundance of Clostridia was 12.7% in V1 and about 27% in V3 and V5 (Figure 7). Clostridia are mostly recognized as cellulose-hydrolyzing and protein-hydrolyzing bacteria [85]. These bacteria also perform acidogenesis and produce short-chain fatty acids, CO_2, and H_2. The class Bacilli was mainly represented by Lactobacillales, the abundance of which in all samples was about 3%. Lactobacillales utilize sugars as a carbon source and produce either mainly lactate (homofermentation) or lactate, acetate, and ethanol (heterofermentation). Lactobacillus sp. is often used in biological pre-treatment or as an additive in the anaerobic digestion of complex substrates [86,87]; therefore, its natural presence in the anaerobic digestion community is favorable for stable methane production.

The abundance of the phylum Chloroflexi was the highest in V1 (10.3%); then, increased OLR markedly reduced its abundance to 2.4% in V3 and 1.7% in V5 (Figure 6). Chloroflexi grow during stable anaerobic digestion performed at high HRT [88] and are responsible for the fermentation of hydrocarbons and amino acids. In the present study, Chloroflexi was also observed in the digester with the high HRT. The phylum Chloroflexi was dominated by the class Anaerolineae. Nakasaki and co-workers [89] conducted anaerobic digestion of oil-related substrates and observed that Anaerolineae was the most dominant group of bacteria during all experiments.

Among Proteobacteria, the most abundant were Deltaproteobacteria (7.3% in V1, 3% in V3, and 9.1% in V5), Gammaproteobacteria (1.6% in V1, 1.3% in V3, and 0.7% in V5) and Betaproteobacteria (1.1% in V1, 0.4% in V3, and 1.4% in V5) (Figure 7). The phylum Proteobacteria was commonly found in digesters treating dairy wastewater [77,90,91].

The abundance of 3.7% of Actinobacteria was the highest in V3, followed by 3% in V1, and the lowest, 1.7%, in V5 (Figure 6). This phylum is involved in the degradation of complex carbohydrates and proteins [92]. Gulhane and co-workers [93] pointed out that Actinobacteria and Bacteroidetes were growth promoters of syntrophic bacteria and acetoclastic archaea.

The abundance of Synergistates represents the same tendency as Actinobacteria (3% in V1, 3.9% in V3, and 1.3% in V5) (Figure 6). This phylum was dominated by Synergistia, to which belong syntrophic acido- and acetogens involved in amino acid fermentation [94]. Probably due to interspecies hydrogen transfer between Synergistia and methanogens, Nakasaki and co-workers [95] observed that the abundance of Synergistales highly correlated with the rate of methane production from oil during the anaerobic digestion for treatment of synthetic lipid-rich wastewater.

As mentioned at the beginning of this section, Thermotogae was observed mainly in V5 (constituted almost 7%). Members of this phylum were observed in mesophilic anaerobic reactors [96] where they were responsible for complex polysaccharide fermentation and hydrogen production [97], which might promote interactions with hydrogenotrophic methanogens. Goux and co-workers [98] observed a high abundance of Thermotogae in a reactor at pH 5.7, which is in consensus with the present study, in which they were observed at pH 6.49.

The community of anaerobic granular sludge in all variants of experiments was represented by various hydrolytic and fermentative bacteria. Based on the microbial composition, it might be assumed that the methane was mainly produced in acetoclastic methanogenesis; however, the increased presence of Methanomicrobia and Thermotogae at the highest OLR suggests that this condition enhanced hydrogenotrophic methanogenesis. The study revealed classes that were sensitive to increasing OLR, which were Flavibacteriia, Anaerolineae, Nitrospira, Methanobacteria, and Gammaproteobacteria, and classes that preferred increasing OLR, which were Clostridia, Methanomicrobia, and Thermotogae.

3.3. Energy Balance of the Anaerobic Digester

The energy gain assessment of anaerobic digestion is presented in Table 6. The net energy output gained from methane production was increased with methane yield. In stage 1, the highest net energy output of 90.35 kWh/d was obtained in variant 2, which was assessed as the best technological variant. Similarly, in variant 2, there was a net energy gain of 116 kWh/d.

Table 6. Energy balance of the anaerobic digester depending on the stage and variant of the experiment (Y_{CH4}—methane yield, HV—the heating value of methane, P_p—power of pumping system, T_w—pump operating time, GE—the gross energy, DE—energy demand for pumping and recirculation, NE—net energy output).

Stage	Variant	Y_{CH4} (m^3/d)	HV (kWh/m^3)	GE (kWh/d)	P_p (kW)	T_w (h/d)	DE (kWh/d)	NE (kWh/d)
1	V1	6.8 ± 0.6	9.17	62.17	12	0.25	3.00	59.17
	V2	10.3 ± 0.6		94.91		0.38	4.56	90.35
	V3	10.3 ± 1.0		94.54		0.50	6.00	88.54
	V4	9.8 ± 0.9		90.05		0.62	7.44	82.61
	V5	9.6 ± 0.7		60.52		0.75	9.00	51.52
2	V1	12.4 ± 0.6	9.17	114.07	12	0.92	11.04	103.03
	V2	14.3 ± 0.6		130.76		1.23	14.76	116.00
	V3	10.3 ± 0.7		94.73		1.85	22.20	72.53
	V4	7.6 ± 0.5		69.78		2.47	29.64	40.14

4. Conclusions

A new reactor concept was designed to combine the benefits of the anaerobic contact process with the UASB reactor and a settling tank in dairy wastewater treatment. Experimental studies were conducted in the pilot plant-scale hybrid anaerobic labyrinth-flow bioreactor to assess anaerobic digestion performance, biogas productivity, and the structure of microorganisms.

The research was carried out in two stages. In the first stage, the anaerobic labyrinth-flow bioreactor was operated with increasing OLR from 4.0 g COD/L·d to 12.0 g COD/L·d in five experimental variants. The anaerobic digestion was the most effective at OLRs ranging from 4.0 g COD/L·d to 8.0 g COD/L·d. The highest COD reduction was more than 87%, and biogas and methane yields reached maximum values of 0.32 ± 0.02 L/g COD removed and 0.23 ± 0.02 L/g COD removed, respectively. The highest methane production reached 101.4 ± 1.9 L/d, while methane concentration in biogas ranged from 68.12 ± 1.3% to 73.0 ± 1.9%. This evenest distribution of phyla at the lowest OLR ensured high biogas production. The determination of the microbial community showed a selection of classes more sensitive to overloading (e.g., Flavibacteriia) than others (e.g., Thermotogae).

In the second stage, effluent recirculation was used to improve the treatment. The reactor was operated at a constant OLR of 10.0 g COD/L·d and different degrees of internal recirculation. The maximum methane production yield of 0.20 ± 0.01 L CH_4/g COD removed was achieved with 100% recirculation, which was 25% more compared to the achievements without recirculation.

This study showed that the new construction of an anaerobic reactor and the use of recirculation significantly enhanced the anaerobic digestion of dairy wastewater. Sludge flotation and biomass washout were avoided during the study, proving that the installation was powerful for treating dairy wastewater rich in fat, oil, and grease. The most favorable technological variant was characterized by a positive energy balance. These findings could play an important role in scaling the new hybrid reactor.

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