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The effect of introduction of chicken manure on the biodiversity and performance of an anaerobic digester

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ABSTRACT

Background: Ammonium stress is a prime limiting phenomenon that occurs during methane formation from poultry manure. It is caused by elevated ammonium nitrogen concentrations that result from substrate decomposition. The amounts of methane formed depend on the activity of methanogenic microbes.
Results: During the research reported in this paper, the response of a mesophilic consortium inhabiting a biogas reactor to rising load of poultry manure was investigated. The taxonomic composition of bacterial population was mostly typical, however syntrophic bacteria were not detected. This absence resulted in limitation of succession of some methanogenic microorganisms, especially obligate hydrogenotrophs. The methanogenic activity of the consortium was totally dependent on the activity of *Methanosaeta*. Inhibition of methanoganesis was noticed at ammonium nitrogen concentration of 3.68 g/L, total cessation occurred at 5.45 g/L. Significant amounts of acetic acid in the fermentation pulp accompanied the inhibition.
Conclusions: The effectiveness of the consortium was totally dependent on the metabolic activity of the acetoclastic *Methanoseata* genus and lack of SAOB did not allow hydrogenotrophic methanogens to propagate and lead to cessation of biogas production at an elevated ammonium concentration at which acetoclastic methanogens were inhibited.
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1. Introduction

Methane formation involves anaerobic microorganisms that catalyze decomposition of organic matter followed by generation of methane and carbon dioxide. It occurs in natural ecosystems, as well as in those artificially created by human. The process consists of four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis, all of which demand specific environmental conditions and involvement of different groups of microorganisms. Key parameters that determine the proper course of methane formation are: anaerobic atmosphere, temperature, pH, chemical composition of the fermented substrate (including C/N ratio), homogeneity of the fermented pulp, retention time of the substrate in the fermentation chamber, and lack of process inhibitors. Excess of ammonium nitrogen is one of such inhibitors [1].

The first phase of biogas formation is catalyzed by obligate and facultative anaerobes belonging to *Aerobacter*, *Alcaligenes*, *Clostridium*, *Escherichia*, *Flavobacterium*, *Pseudomonas*, *Lactobacterium*, *Lactobacillus*, *Micrococcus* and *Streptococcus* genera. The bacteria of the first phase of methanogenesis have been found to be relatively tolerant to deviations of temperature and pH. Bacteria involved in acetogenesis, classified in *Syntrophomonas* and *Syntrophobacter* genera, among others, have shown long generation times and significant sensitivity to shifts in process conditions. The final phase is catalyzed by obligately anaerobic Archeae. Depending on morphology these microorganisms are classified in *Methanobacterium, Methanospirillum, Methanococcus* and *Methanosarcina* genera. Methanogens are very sensitive to physico-chemical conditions of the environment, which makes maintenance of proper process conditions and kinetic balance between all the fermentation phases crucial for effective methane production [2]. Disturbances concerning even one of the primary phases may lead to inhibition of methanogens, and, in turn, result in losses in the effectiveness of biogas formation.

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Success of biogas formation depends to a great extent on the properties of the substrate used. This factor determines the quality and activity of the microorganisms involved. The abundance of poultry manure makes this material an attractive alternative to conventionally utilized substrates. However, its chemical composition, namely high ammonium nitrogen content, make its application in biogas production problematic [3].

Several mechanisms for ammonia inhibition are connected to the changes in the intracellular pH, increase of maintenance energy requirement, and inhibition of a specific enzyme reaction [4]. Ammonium ion NH_4^+ and free ammonia nitrogen (FAN) (NH_3) are the two principal forms of inorganic ammonia nitrogen in solution. The hydrophobic ammonia molecule may diffuse passively into the cell, causing proton imbalance, and/or potassium deficiency. The methanogens are the least tolerant and the most likely to cease growth due to ammonia inhibition [5]. Some research based on the comparison of methane production and growth rate indicated that the inhibitory effect was in general stronger for the acetoclastic than for the hydrogenotrophic methanogens [6,7,8], while others observed the relatively high resistance of acetate consuming methanogens to high total ammonia nitrogen (TAN) levels as compared to hydrogen utilizing methanogens [9,10].

In order to ensure effective biogas production, research has to be undertaken to either enable the elimination of ammonium nitrogen from the substrate, or develop microbial consortia tolerant to elevated NH_3 levels. Finally, the choice of co-substrate is essential for correcting the C/N ratio of the fermentation pulp. The value of this ratio is 1:10 in the poultry manure itself, and monofermentation requires it to be at least 1:25 [11].

Nonetheless, utilization of poultry manure through the means of biogas formation is an economically attractive solution to the problem posed by this burdensome and abundant waste. A breakthrough, similar to the one introduced by application of biogas plants to pig farms, seems possible if responses to the mentioned obstacles are found. In-depth knowledge concerning the course of physico-chemical changes during fermentation of poultry manure and the microorganisms involved is essential for the economical and technological viability of this difficult process. The aim of this work was to describe mesophilic methane monofermentation of poultry manure during which biogas production occurs solely via direct conversion of acetic acid into methane. Moreover, composition of a microbial consortium subordinated to the activity of *Methanosaeta* as a main methanogen was studied, and the susceptibility of the consortium to stress introduced by ammonium was investigated.

2. Materials and methods

2.1. Fermentation

Continuous methane fermentation was conducted in CSTR - BTP2-control bioreactors (Umwelt- und Ingenieurtechnik GmbH Dresden) with 15 L working volume. Poultry manure, used as substrate, was obtained from a poultry farm (Łukasz Rożniakowski; Skrzynki, Poland; 52°16′03,53″N 16°42′22,87″E) operating in a cage system. Postfermentation pulp used as an inoculum for the process was taken from an agricultural biogas plant (Biogaz Działyń Sp. z o.o.; Działyń, Poland; 52°35′39, 28″N 17°29′28,18″E), fed with cattle manure, cattle slurry and corn silage. The installation worked properly reaching 85% of its nominal power and the methane concentration in raw biogas was around 52–55% proving the correctness of the process. The experiment in CSTR was conducted at a constant temperature of 39°C ± 1°C, with a constant stirring rate of 60 rpm. Initial organic loading rate was 1 g/L day and was elevated by 0.5 g/L day on a weekly basis to a final rate of 2.5 g/L day.

Basic substrate characteristics are given in Table 1. It should be pointed out that poultry manure is an unstable substance. During one month storage in refrigerator, the concentration of ammonium nitrogen increased tenfold which is related to the fact that chickens, as uricotelic animals, excrete nitrogen mostly in the form of uric acid. Undergoing enzymatic transformations, this compound is gradually converted into ammonium and ammonia [12].

Table 1
Properties of the raw materials used.

Substrate	TS (%)	SD σ	oTS (% TS)	SD σ	pH (-)	SD σ	NH^+_4 (g/L)	SD σ
Postfermentation pulp (inoculum)	7.14	0.11	69.26	0.72	7.85	0.02	1.738	0.1
chicken manure (fresh)	25.65	0.14	65.91	0.64	6.97	0.09	0.950	0.2
chicken manure (After one month of storage)	26.72	0.21	64.72	0.63	9.14	0.17	10.775	0.2

TS: total solids; oTS: organic total solids.

2.2. Analytical procedures

2.2.1. Dry matter, organic dry mater, pH, ammonium nitrogen

The analyses were conducted according to Polish standards: dry matter in 105°C (PN-75 C-04616/01), dry organic matter and ash in 550°C (PN-Z-15011-3). The pH was analyzed potentiometrically with Elmetron CPC-401 meter (according to PN-EN 12176:2004), ammonia (NH_3) deterimination was determined through distillation in the presence of MgO and titration with 0.1 H_2SO_4.

2.2.2. Biogas production and composition

The composition and volume of the produced biogas was measured every 24 h. Gas composition was determined when at least 1 L had been produced. The concentrations of methane, carbon dioxide, hydrogen sulphide, ammonia and oxygen in the produced biogas were determined using certified Geotech gas analyzer GA5000 [certificates ATEX II 2G Ex ib. IIA T1 Gb (Ta1/410°C doþ 50°C), IECEx, CSA and calibration certificates UKAS ISO 17025]. The analyzer allowed measurement of gases in the following ranges: O_2 0–25%; CO_2 0–100%; CH_4 0–100%; NH_3 0–1000 ppm and H_2S 0–10,000 ppm. Volumetric biogas production and methane content of biogas were calculated using MS Excel. Interpretation of the results enabled to determine if the process was running properly during the experiment. Gas-monitoring system was calibrated each week using calibration gases provided by Messer Company, using the following concentration of gases: 65% of CH_4 and 35% of CO_2 (both in the same mixture), 500 ppm of H_2S and 100 ppm of NH_3. For O_2 sensor calibration, typical synthetic air was used.

2.2.3. HPLC analysis

Samples were centrifuged, filtered with a 0.45 μm-membrane syringe-driven filter (Milipore) and analyzed by HPLC (Agilent Technologies 1200 series) equipped with a refractive-index detector. Analyzes were performed isocratically at a flow rate of 0.6 mL/min using Rezex ROA-Organic Acid H^+ column (Phenomenex) at a constant temperature of 40°C. 0.001 M H_2SO_4 was a mobile phase. Identification and quantitation of compounds such as volatile fatty acids was carried out with an external standard method, using peak surface for calculation. ChemStation for LC 3D systems (Agilent) was used to analyze the chromatographic data.

2.2.4. sCOD — chemical oxygen demand

Lovibond® COD kits (dichromate method) were used for sCOD analyzes. The samples were centrifuged at 15000 rpm and filtered through a 0.45 μm syringe filters prior to analysis.

2.3. Molecular methods

2.3.1. DNA extraction

Total genomic DNA was isolated from 200 and 400 mg of fresh samples transferred to Lysis tubes included in the NucleoSpin® Soil Kit (Macherey-Nagel). The isolation procedure was conducted according to manufacturer instructions, two times with application of SL1 and SL2 buffers. DNA was extracted from two samples and then pooled. Double-stranded DNA quantity was measured with Dynamica Spectrophotometer and quality of the extracted DNA was determined by measuring the absorbance ratios between 230, 260 and 280 nm as well as in 1% agarose gel stained with 1× concentrated GelGreen dye (Immuniq, Biotium).

2.3.2. PCR-DGGE

Amplification of V3 variable region from 16S rDNA was done with 357FGC and 518R primers set [13]. PCR mixture (50 μL) contained 100 ng of template DNA, 5.0 μL 10× buffer with 2.5 mM $MgCl_2$, dNTP, each primer and U Run Polymerase (A&A Biotechnology). Polymerysation was run in Nexus Thermacycler (Eppendorf) in program consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 60 s followed by a final extension at 72°C for 7 min. Each sample was run in duplicate, checked in 1.8% agarose gel and pooled before acrylamide electrophoresis.

Obtained products were analyzed by denaturing gradient gel electrophoresis (DGGE) using the D'Code System (Bio-Rad). The gradient of denaturants in the gel ranged from 30 to 70% and gels size was 16.0 × 16.0 cm and 1.0 mm thick with the electrophoresis buffer 1×TAE (Serva) maintained at 60°C during electrophoresis under 50 V. After 14 h, the electrophoresis was stopped and gels were stained in 1×TAE with 3× GelGreen stain (Immuniq, Biotium).

Similarities between DGGE patterns were calculated using band presence or absence via pairwise similarity of the banding patterns for the different samples, and clustering of patterns was calculated using the unweighted pair group method using average linkages (UPGMA) with GeneTools software (TK Biotech, Syngen).

2.3.3. Library preparation and sequencing

The microbiota composition was determined using 16S rRNA gene amplicon MiSeq-based high throughput sequencing (Illumina, CA, USA). Sequences of primers targeting V3-V4 hypervariable region of 16S rRNA were as follows: 16S_F: 5′ – CCTACGGG NGGCWGCAG – 3′ and 16S_R: 5′ – GACTACHVGGGTATCTAATCC – 3′. These primers also contained the overhang adapter sequences attached to the 5′ end of primers, compatible with the MiSeq flow cell adapters (Illumina, CA, USA).

Amplification of hypervariable regions (V3 and V4) of 16S rRNA was performed to characterize the taxonomic diversity present in samples. PCR reaction containing 2.5 μL of genomic DNA (5 ng/μL), 5 μL of each primer (1 μM) and 12.5 μL of 2× KAPA HiFi HotStart ReadyMix kit (KAPA BIOSYSTEMS, USA) was run on a ProFlex PCR System thermal cycler (Applied Biosystem, MA, USA). Cycling conditions were as follows: initial denaturation at 95°C for 3 min; 25 cycles: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min.

The second amplification was performed using the PCR product from the first reaction as a template in order to index the samples for multiplexing. This reaction contained 5 μL of product from first PCR reaction, 5 μL of P5 and P7 indexes (Nextera Index v2 Kit, Illumina), 25 μL of 2× KAPA HiFi HotStart ReadyMix kit (KAPA BIOSYSTEMS, USA) and 10 μL of nuclease-free water. Cycling conditions were similar to the first PCR amplification but with the number of cycles reduced to 8.

The amplified fragments with tags and adapters were purified using AMPure XP beads (Beckman Coulter Genomic, CA, USA) after each PCR reaction. Amplicon concentrations were quantified by Qubit fluorometer (Invitrogen, Carlsbad, CA, USA), normalized to 4 nM and pooled prior to sequencing. To control the purity of DNA libraries, sterile water was used.

The 10 pM library containing pooled indexed samples with spike-in PhiX control DNA was loaded onto the MiSeq sequencing platform. 2 × 300 pair end sequencing was performed using the MiSeq Reagent Kit ×3 (600 cycles).

2.3.4. Sequencing data analysis

The raw dataset containing pair-ended reads with corresponding quality scores was merged using PEAR software and trimmed with quality lower than 30 and converted to Fasta format using FASTX – Toolkit [http://hannonlab.cshl.edu/fastx_toolkit/]. The header of each read was relabeled with an index number followed by a sample ID [14].

Purging the dataset from chimeric reads and constructing de novo Operational Taxonomic Units (OTU) with a minimum identity of 97% were conducted using the UPARSE pipeline [15]. Taxonomy was assigned to representative OTU sequences using uclust implemented in the assign_taxonomy script from Quantitative Insights into Microbial Ecology (QIIME) software package (1.8.0) with 90% of identity [16]. The Greengenes (13.8) 16S rRNA gene collection was used as a reference database [17].

2.3.5. Data availability

All raw sequence data had been deposited in NCBI Sequence Read Achieve (SRA) under the BioProject number PRJNA390251. The experiment numbers for all samples examinated in NGS are SRX2941980-SRX2941984.

3. Results

3.1. Physico-chemical analyses of experiment

The fermentation was run for 50 d. Periodic increments of the organic loading rate (OLR) resulted in increased biogas production (Fig. 1 and Fig. 2). This trend was observed until day 26, after which biogas production began to decline dynamically, although the OLR was kept constant at 2.5 g/L d. On the 45th d, the biogas production efficiency was only 35% of the maximum production rate observed at the same OLR (on the 26th d). It should be pointed out that the highest daily methane content in the produced biogas (66.8%) was observed on the 26th d of the process. After day 26, the methane content started to decrease, reaching 42.6% on the 45th d, the last day when the process was performed at a constant OLR of 2.5 g/L d. It is worth mentioning, that because of a relatively high dry matter content of the chicken manure used as feed 25.65% (Table 1), the hydraulic retention time (HRT) was 68 days during the stage performed at the highest OLR level. This excluded the possibility of the washout effect on methanogens (Fig. 2).

On the 26th d of the process, when inhibition of methanogenesis was observed, the concentration of ammonium nitrogen (NH_4-N) in the fermentation pulp was 3.68 g/L (Fig. 3). This value, however, cannot be interpreted as the level at which ammonium inhibition began to occur because this was not the case in this experiment and the experiment was not designed to verify it. Daily addition of large amounts of fresh chicken manure caused rapid increase of NH_4-N that precludes the definition of the actual inhibitory level. The concentration of this form of nitrogen was observed to rise systematically and reached 5.45 g/L in 44th d. Moreover, decreasing biogas production efficiency was accompanied by increasing concentration of sCOD (Fig. 3). The rate of sCOD accumulation was at its highest after day 33. The analysis of volatile fatty acids in the soluble fraction revealed that acetic acid concentration was greatly increased during this stage of the process. It is a logical consequence, as acetogenesis follows hydrolysis and acidogenesis and proceeds methanogenesis during the course of fermentation.

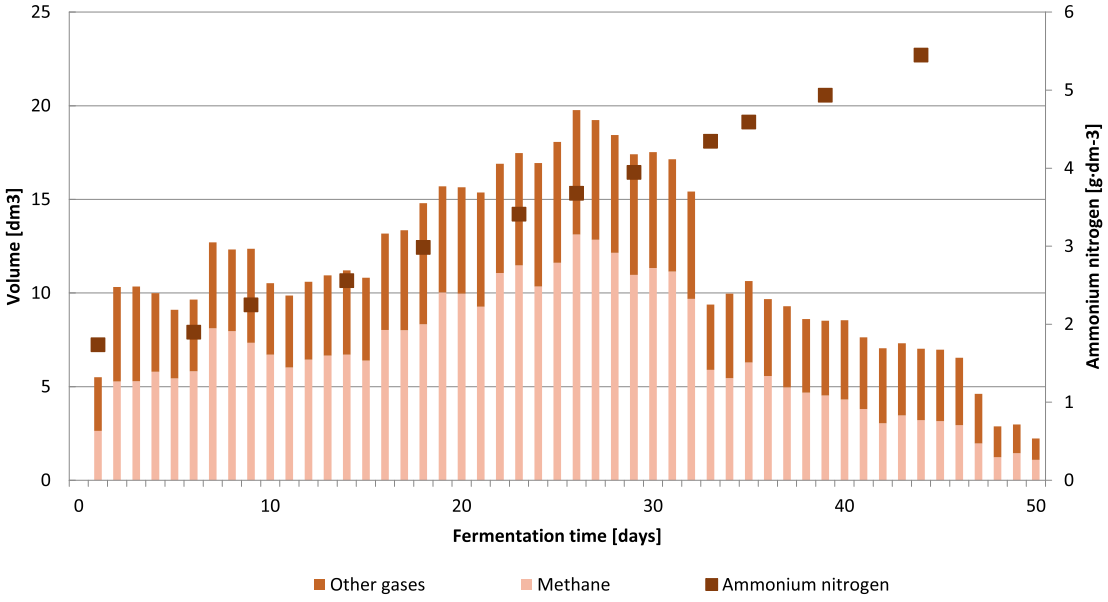

Fig. 1. Biogas production, composition, and organic load of the reactor (OLR); VS: volatile solids.

Concentrations of acetic, propionic and butyric acids measured on the 46th d, were 9.28, 0.33 and 0.47 g/L respectively. The increasing concentration of VFA, however, did not decrease the pH level. The reason for this was the high alkalinity of poultry manure related to the large amount of ammonium nitrogen released during the degradation of this substrate (Table 1). Even though methanogenesis was inhibited, the high alkalinity of ammonium nitrogen resulted in a FOS/TAC value of 0.37 on the 39th d of the process. In processes run on conventional substrates, without ammonium inhibition such a value would indicate optimum supply of organic matter [18].

It is worth top mention that inhibition of a fermentation process results from two inhibiting forms of nitrogen – ammonium and ammonia. pH determines the balance between the two forms. It is known that ammonia inhibition starts at the concentration of 0.15 g/L and ammonium inhibition at 3.0 g/L [19]. During the experiment the pH in the fermenter was in the range between 7.41 and 7.98. In such conditions, the proportion between the two forms was 93–96% of ammonium and 4–7% ammonia. Thus, if the ammonium concentration measured at the moment of inhibition was 3.68 g/L, the ammonia concentration was 0.17 g/L, a value that corresponds with literature data.

3.2. Community structure in DGGE fingerprint analyses

PCR-DGGE enabled the analysis of the changes in the taxonomic profile of Bacteria and Archaea in the consortium during the fermentation process. The results of the analyses are shown in Fig. 4 and Fig. 5 presents the UPGMA dendrogram obtained after similarity analysis of the samples. The obtained profile and the layout of the dendrogram indicate dynamic changes of the microbiota present in the fermentor. Reported changes are a consequence of the differences in the microbial composition between poultry manure and inoculum which were used during the fermentation. The DGGE profile obtained at the start of the process reflects a community structure that is characteristic of the inoculum sample, whereas the profile obtained on the 2nd d is strongly influenced by the introduction of poultry manure. It also could be an illustration of rapid adaptation of the consortium to process conditions during the first two days. In this case, it should be emphasized that bacteria and especially fermenters can acclimate quickly to new environmental conditions because of their relatively high growth rates, while proton-reducing acetogens and methanogens show significantly slower growth [20]. Compared to the other samples

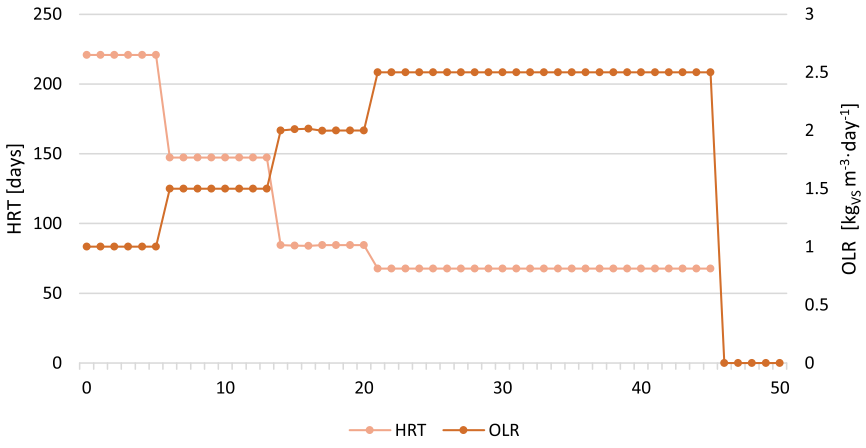

Fig. 2. Changes of Hydraulic Retention Time and Organic Loading Rate during the process.

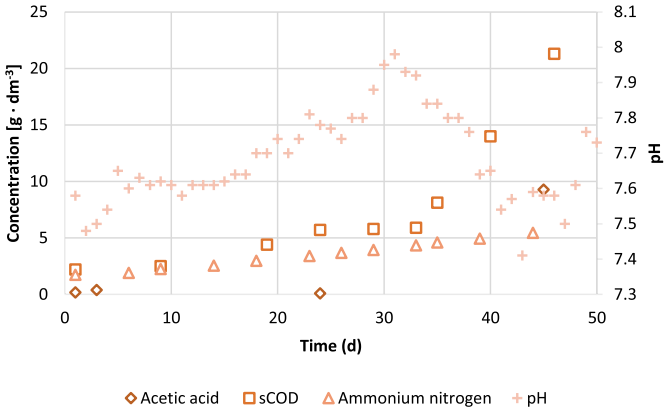

Fig. 3. Changes in ammonium nitrogen, sCOD, and acetic acid concentration during fermentation progress; FW: fresh weight.

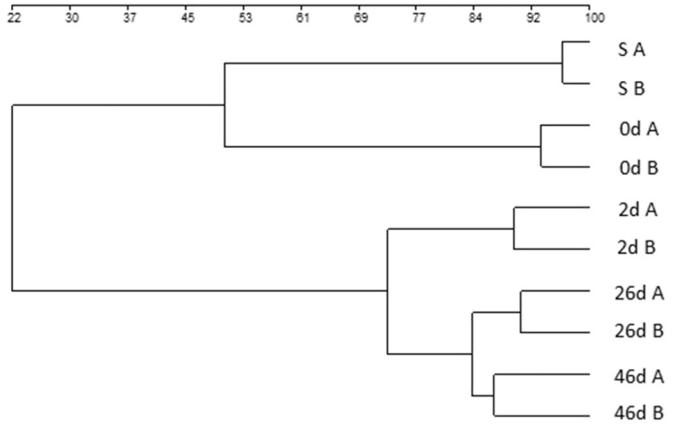

Fig. 5. UPGMA tree constructed basing on the PCR-DGGE profiles of microbiota occurred in substrate (S) and in samples from reactor in each day of the assay (0 d, 2 d, 26 d, and 46 d where numbers indicate the day of sampling); indexes A and B indicate weight of fresh material used for genomic DNA isolation, 200 and 400 mg, respectively.

analyzed, greatest phylogenetic diversity was detected in the sample obtained on the 2nd d of the process. 24 d later (on the 26th d), accompanied by a 60% rise in ammonium nitrogen concentration, significant rearrangement of the microbial population was observed. Since then, the consortium appeared to have had stabilized, as indicated by the similarity of the profiles observed on the 26th and 46th d.

3.3. Overview of the microbial community composition determined by metagenomic sequencing

Changes in the composition of the fermentation consortium during the process were investigated using metagenomic sequencing. Bacteria constituted the majority of the microbiota, being more abundant than Archaea in all the samples analyzed (Fig. 6). *Methanosaeta* spp. were the most represented archeal species. Other detected methanogens belonged to *Methanosarcina* and *Methanobrevibacter* genera (Fig. 7a). Archaea were the most variable population of all the microbiota in terms of relative quantity. Methanogens multiplied rapidly, constituting 1% of total microbiota on the day of the beginning of the process and 10% on the 2nd d of fermentation (Fig. 6). While the archeal population of the post-fermentation pulp used as inoculum was dominated by *Methanosarcina*, *Methanosaeta* sp. became the most abundant representatives of this population. *Methanosaeta* constituted nearly 100% of all *Archaea* on day 2 of the process, while on the 26th d, the relative quantity of this group decreased only slightly to 98% (Fig. 7a).

Among *Bacteria*, *Firmicutes* (mainly *Lactobacillales* and *Clostridiales*) were the most abundant in the substrate. In the inoculum, significant presence of *Bacterioidetes* and candidate division WWE1 (only from the family *Cloacamonaceae*) was observed (Fig. 7b). During each stage of the fermentation, the phylum *Bacterioidetes* was highly represented (16–25%), and the phylum *Firmicutes* was dominant (31–58%). The later phylum was very diverse, but *Clostridiaceae* family constituted the majority of the members of this taxon (Fig. 7c–d). Among *Clostridia*, rising representation of *Caldicoprobacteraceae*, accompanied by diminishing representation of candidate division *Tisserellaceae*, was observed (Fig. 7d). Increased representation of bacteria belonging to phylum *Tenericutes* was registered at high ammonium concentration (Fig. 7b). Generally, the bacterial population was less susceptible to

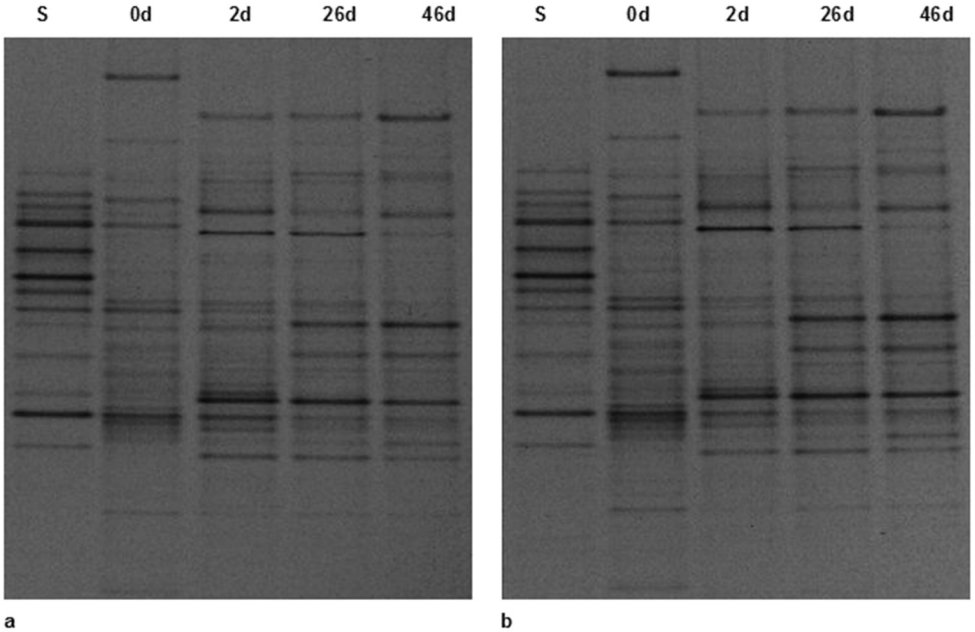

Fig. 4. PCR-DGGE fingerprints of microbiota occurred in substrate (S) and in samples from reactor in each day of assay (0 d, 2 d, 26 d, and 46 d where numbers indicate the day of sampling); (a) products amplified on DNA isolated from 200 mg of fresh material; (b) products amplified on DNA isolated from 400 mg of fresh material.

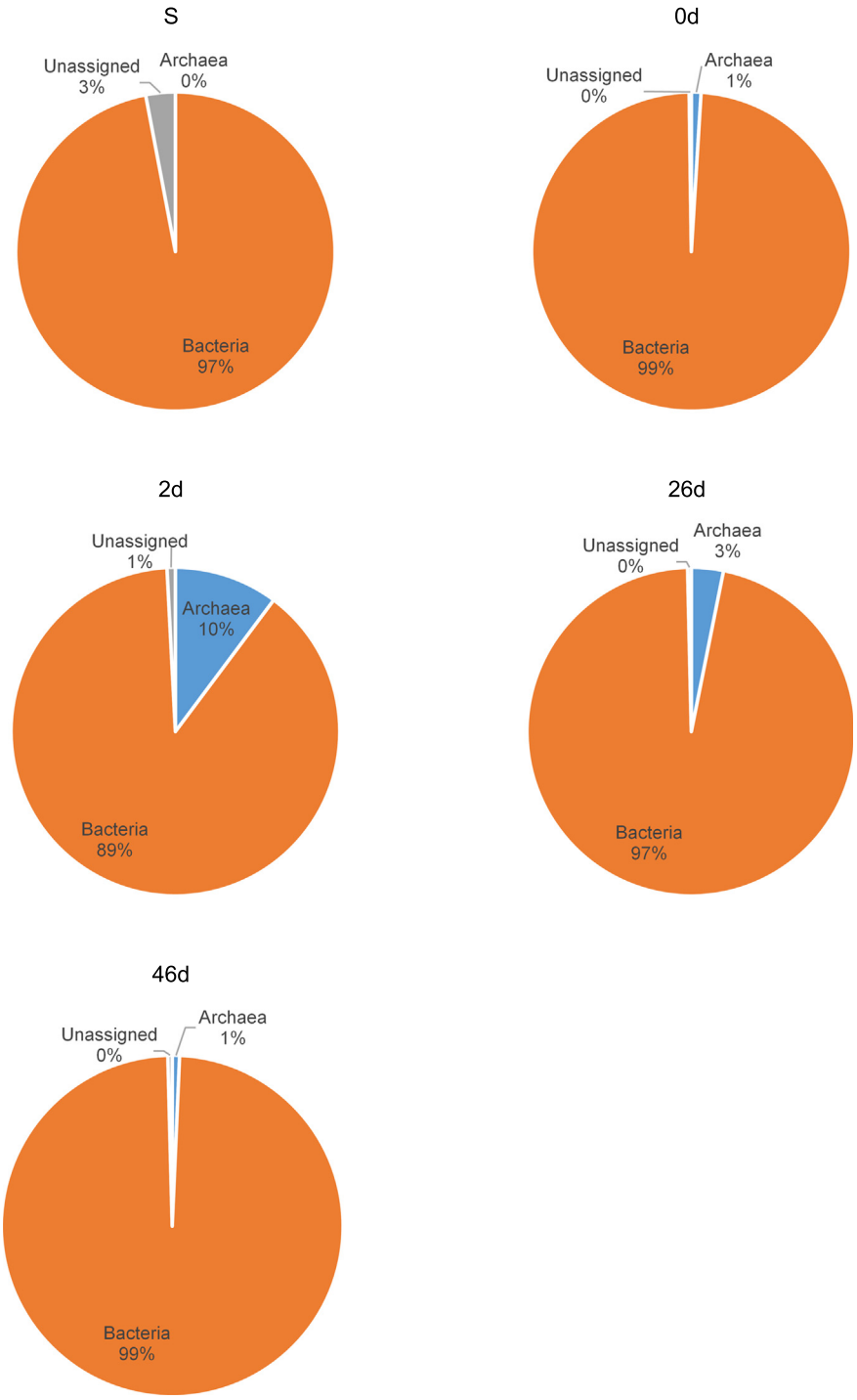

Fig. 6. Composition of the microbial population of the substrate (S) and the reactor (numbers indicate the day of sampling) at the level of domain.

variations in taxonomic composition than the archeal population. Between the beginning of the process and the 46th d, vivid similarities among bacterial taxons were noticeable. At the phylum level, *Firmicutes* constituted between 52 and 57%, *Bacteriodes* — 16.5 and 17.5%, candidate division WWE1 — 8.4 and 10% of the total bacterial representation (Fig. 7b).

4. Discussion

As a substrate for methane formation, poultry manure is very rich in nitrogen. Ammonium nitrogen is of special concern as it was proven to be an inhibitor of the activity of methanogenic microorganisms [21]. Acetoclastic methanogens were pointed out for their significant role in biogas formation in bioreactors operated at increased ammonium concentrations [6,7,22]. Although, methanogens from the genus *Methanosaeta* are known for the production of methane through the acetotrophic pathway, *Methanosarcina* are mentioned more often as typical for environments characterized by high ammonia concentration. Frequent occurrence of *Methanosarcina* may, on the one hand, be attributed to their metabolic flexibility which results from the ability to form methane through both direct acetotrophy and hydrogenotrophy [23]. The latter ability allows utilization of hydrogen

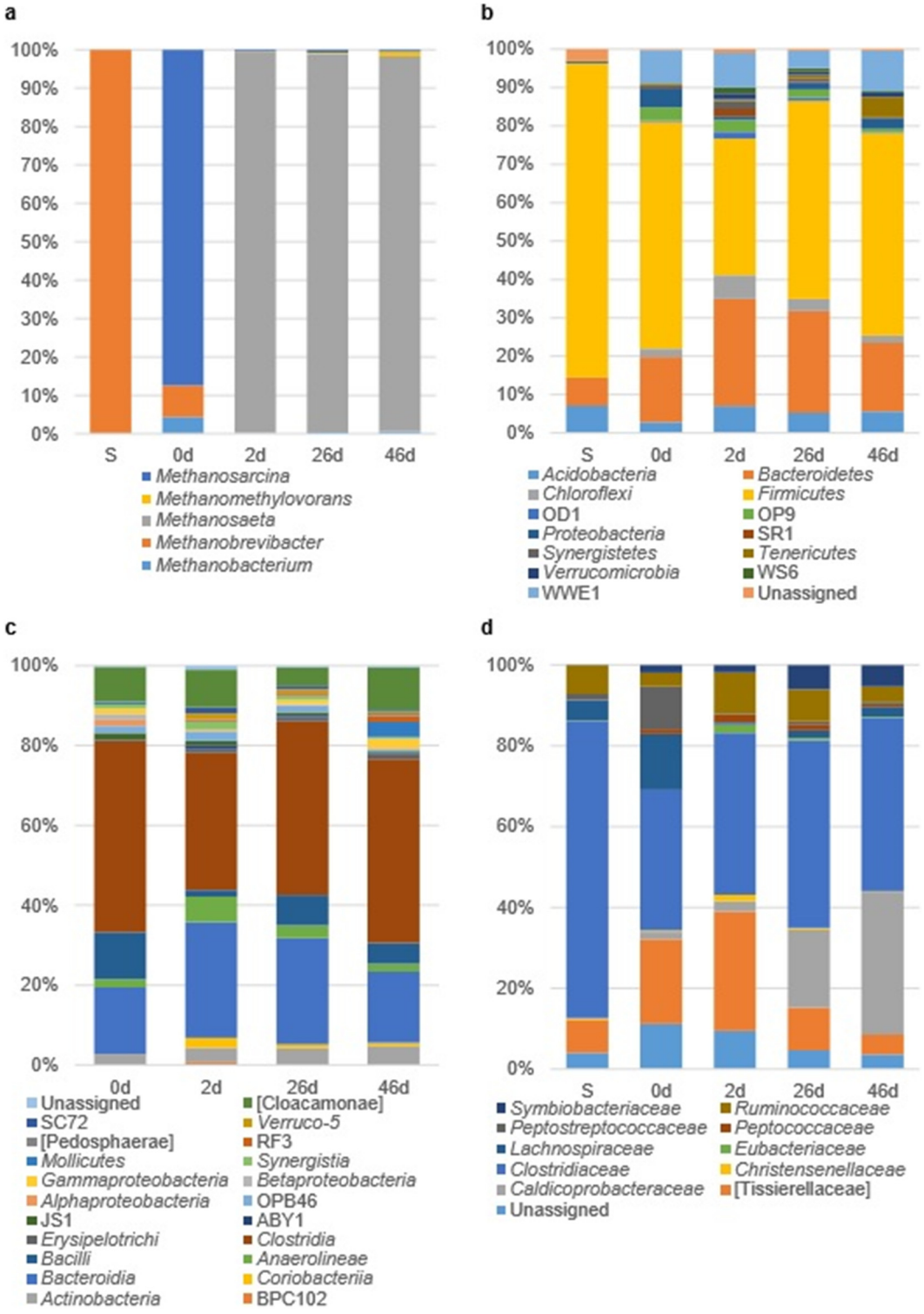

Fig. 7. Composition of the microbial population of the substrate (S) and the reactor (numbers indicate the day of sampling). (a) Structure of Archaea at the level of genus; (b) Structure of Bacteria at the level of phylum; (c) Structure of Bacteria at the level of order; (d) Structure of Clostridia at the level of family.

formed during oxidation acetate. On the other hand, *Methanosarcina* are known for their relatively high tolerance of elevated ammonia concentrations [24]. During the investigated process, *Methanosarcina* were detected only on the day of bioreactor inoculation, when the concentration of ammonium was 1.8 g/L. As the process progressed, and levels of ammonium increased, *Methanosarcina* were superseded by *Methanosaeta.* Moreover, methanogens of the latter genus rapidly became the most represented of *Archaea,* and dominated the archeal population even when ammonium levels reached 5.45 g/L on the 46th d of the process.

Karakashev et al. [25] and Westerholm et al. [26] showed increased role of the activity of syntrophic acetate oxidizing bacteria (SAOB) which triggered strengthening of the syntrophic route of methane synthesis at elevated ammonium levels. This route is characteristic for hydrogenotrophic methanogens, and, in scope of data available on the metabolism of microbes in ammonium-rich environments [25,27,28],

it seems typical in consortia showing efficient biogas production. Although, presence of DNA of hydrogenotrophic *Methanobrevibacter* and *Methanosarcina* was confirmed in the substrate and inoculum, respectively, analyzes of samples obtained during fermentation indicated significant inhibition of these microorganisms. It seems plausible that acetoclastic methanogens belonging to *Methanosaeta* genus were better adapted to process conditions, which allowed them to metabolize acetate rapidly. Moreover, according to the results obtained by Karakashev et al. [25], who correlated the abundance of SAOB with the absence of *Methanosaeta*, the presence of this methanogen in the described digester could indicate the lack of SAOB. SAOB are characterized by slow growth. As such they are prone to the washout effect and careful choices regarding the HRT are necessary in order to ensure enough time for the their propagation [29,30]. All these findings and factors were a likely cause for the diminished role of hydrogenotrophic methanogenesis in the described digester.

Species typically classified as SAOB, a group of specialized microorganisms, are the thermophilic *Thermacetogenium phaeum* and *Pseudothermotoga lettingae*, the thermotolerant *Tepidanaerobacter acetatoxydans*, and the mesophilic *Clostridium ultunense* and *Syntrophaceticus schinkii* [26]. None of these species were detected according to the metagenomic analyzes performed. At the genera level only *Clostridium* was detected in the described digester. Sun et al. [31] emphasized that all SAOB species were not detected or detected at significantly low level in a digester operating with acetoclastic methanogenesis. The conclusion was supported by Real-time quantitative PCR which results suggested that in a syntrophically operated digester there were up to 7.2 or 11.0 log copies per ml and none or 4.4 log copies less of *Clostridium ultunense* or *Syntrophaceticus schinkii* respectively in digesters dominated by acetoclastic methanogens. At the same time in all the digesters the number of total bacterial copies detected was 12.0 to 13.0 log level [31]. Such a wide quantitative disproportion between bacteria in general and SAOB could indicates that more sensitive and selective methods are necessary for estimation of the presence and activity of SAOB in complex populations.

The analyzed consortium showed relatively high susceptibility to changes in ammonium concentration that manifested itself in major qualitative and quantitative rearrangement when ammonium concentration reached of 3.5 g/L Hao et al. [23] and Su et al. [32] did not observe such significant reaction of consortia up to ammonium concentrations of 7.0 and 5.97 g/L, respectively.

Taking into account the supposed lack of effective syntrophic biogas production mechanisms, a result of SAOB underrepresentation in the studied consortium, cessation of methanogenesis at 5.45 g/L ammonium concentration seems justified. Inhibition of methane production was accompanied by systematic sCOD increase. Similar observations were reported by Niu et al. [1]. Under steady state operation, *Methanosarcina mazei* Go1 represented 11.1% of the total methanogenic population prior to ammonia inhibition, after which (c.a. 3.7 g/L ammonium) this species was no longer detected, and significant VFA accumulation was observed.

Interpretation of the dynamics of microbial communities during fermentative processes demands taking various variables into account. Besides the availability of nitrogen and the form in which it is introduced, factors such as substrate characteristics and its autochtonic microbiome, fermenter type, temperature, substrate supply and its retention time are of importance [32,33,34,35].

5. Conclusions

Nonetheless, based on the results, it can be summarized that the studied consortium was unique in two major aspects: Its effectiveness was totally dependent on the metabolic activity of the acetioclastic *Methanoseata* genus; Lack or diminished role of SAOB did not allow hydrogenotrophic methanogens to propagate and lead to cessation of biogas production at an elevated ammonium concentration at which acetoclastic methanogens were inhibited.

Conflict of interest

The authors declare that they have no conflict of interest.

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