The effect of the source of microorganisms on adaptation of hydrolytic consortia dedicated to anaerobic digestion of maize silage

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Abstract

The main aim of this study was to evaluate the effect of the source of microorganisms on the selection of hydrolytic consortia dedicated to anaerobic digestion of maize silage. The selection process was investigated based on the analysis of changes in the hydrolytic activity

and the diversity of microbial communities derived from (i) a hydrolyzer of a commercial agricultural biogas plant, (ii) cattle slurry and (iii) raw sewage sludge, during a series of 10 passages. Following the selection process, the adapted consortia were thoroughly analyzed for their ability to utilize maize silage and augmentation of anaerobic digestion communities.

The results of selection of the consortia showed that every subsequent passage of each consortium leads to their adaptation to degradation of maize silage, which was manifested by the increased hydrolytic activity of the adapted consortia. Biodiversity analysis (based on the 16S rDNA amplicon sequencing) confirmed the changes microbial community of each consortium, and showed that after the last (10th) passage all microbial communities were dominated by the representatives of *Lactobacillaceae, Prevotellaceae, Veillonellaceae.*

The results of the functional analyses showed that the adapted consortia improved the efficiency of maize silage degradation, as indicated by the increase in the concentration of glucose and volatile fatty acids (VFAs), as well as the soluble chemical oxygen demand (sCOD). Moreover, bioaugmentation of anaerobic digestion communities by the adapted hydrolytic consortia increased biogas yield by 10-29%, depending on the origin of the community. The obtained results also indicate that substrate input (not community origin) was the driving force responsible for the changes in the community structure of hydrolytic consortia dedicated to anaerobic digestion.

1. Introduction

Over the past decades the biogas production technology has been focused on the development and optimization of systems characterized by high rate of anaerobic digestion of energy crops and solid agro-industrial wastes [1]. The anaerobic digestion process consists of two phases. In the first one, the substrate is converted mainly to soluble organic compounds such as volatile fatty acids (VFAs), which are then used in the second phase by methanogens

48 to produce biogas [2]. These two phases can be carried out simultaneously in a single
49 bioreactor or separately, in a two-step system, which consists of two connected bioreactors.
50 Due to their simplicity, many full-scale biogas plants in Europe utilize single-step systems.
51 However, two-step technologies may ensure optimal conditions for each of the processes,
52 which is especially crucial, as hydrolysis of complex polysaccharides such as cellulose or
53 lignocellulose is often the rate-limiting step of the entire biogas production process. The
54 production of VFAs proceeds much faster than the conversion of VFAs to methane, thereby
55 causing acid accumulation, often leading to a drop in pH below 6, and consequently, to the
56 inhibition of methanogenesis [3].

57 Two-stage anaerobic digestion allows to overcome the problem of imbalance between
58 the acidogenesis and methanogenesis processes. Moreover, the two-stage technology enables
59 the separation of solid and liquid phases and thus maintaining of a high rate of biogas
60 production. Therefore, the use of the two-stage systems may lead to a higher overall reaction
61 rate and biogas yield, and for this reason they are considered more effective than the
62 conventional single-step technologies [4,5]. Moreover, some authors reported that splitting
63 and separate optimization of hydrolysis/acidogenesis and methanogenesis could enhance the
64 overall reaction rate, maximize biogas yields, and make the process easier to control, both
65 under mesophilic and thermophilic conditions [4,5].

66 One of the most important problems encountered at commercial agriculture biogas
67 plants is the start-up of new anaerobic reactors. According to Ahring [6], without efficient
68 hydrolytic microbial consortium, the start-up period of thermophilic anaerobic digestion can
69 be prolonged to one year before it enters a steady state. Selection of microorganisms
70 responsible for different stages of anaerobic digestion, from the initial inoculum may by very
71 long when is carried out during the start-up of anaerobic bioreactors. This often generates
72 high operating costs for biogas plants. Therefore, the search for efficient consortia sources,

their selection and adaptation, as well as the choice of the main substrate, are the most important factors affecting the successful start-up of anaerobic digestion.

The substrate used for anaerobic digestion is vary between the biogas production plants. Maize silage is one of the most popular energy crops and is widely used in agricultural biogas facilities as a substrate for the biogas production, and it represents 73% mass of the plant biomass processed in the biogas plants [7]. To increase biogas production from maize silage, various methods can be used, including chemical, physical and biological pretreatment. Biological methods are a good alternative to the physical and chemical methods as they are cost-effective and allow for lower energy use [8]. In nature, plant biomass is degraded by hydrolytic enzymes produced by microorganisms, including bacteria and fungi. Biologically stable and controllable microbial consortia with a high hydrolytic activity isolated from such environments, seem to be more valuable and effective than individually strains [9].

Many studies have shown that hydrolytic microbial consortia are very often isolated from different natural environments. Wongwilaiwalin et al. [10] developed stable thermophilic, lignocellulose-degrading microbial consortium (MC3F) from sugarcane bagasse compost, which can degrade up to 75% of rice straw, 70% of corn stover, and 60% bagasse in 7 days. Haruta et al. [11] obtained a consortium (MC1) from composting materials (sugarcane dregs, chicken feces, dried straw, pig feces, and cattle feces). These stable community degraded 60% of rice straw, 88% of cotton, 70 % of corn stalk and 79% of filter paper. The biodiversity and varying hydrolytic efficiency of bacterial consortia isolated from different environments indicates the importance of the initial inoculum composition and its effect on the entire biogas production process.

Additionally, natural consortia are able to survive under a wide range of environmental conditions. Nowadays, remains a challenge to recognize and optimize degradation of maize silage by natural mesophilic bacterial consortia. Natural consortia are very diverse, both in terms of microbial biodiversity and cellulolytic activity [12]. Thus, the

selection, adaptation, stabilization and characterization of the natural consortia may play a key role in improving the degradation of the substrate and create an efficient way leading to the enhancement of biogas production during anaerobic digestion.

Although a number of studies have addressed to hydrolytic microbial consortia used for enhancing the mesophilic anaerobic digestion of maize silage, only few have focused on the actual changes in hydrolytic activity and biodiversity of microorganisms during the selection and adaptation processes. .

The purpose of this study was to evaluate the influence of the source of microbial community on the selection and stabilization of hydrolytic consortia designated for utilization of maize silage. Additionally, the effect of bioaugmentation of a stable methanogenic community with the adapted hydrolytic consortia on biogas production from maize silage was investigated.

2. Materials and Methods

2.1. Source of microorganisms and substrate

Hydrolytic microbial consortia were isolated from: (i) two mixed hydrolyzers of an agricultural biogas plant located in Miedzyrzec Podlaski (Poland), where maize silage is used as a substrate, (ii) cattle slurry from farms in Trzebieszow Pierwszy (Poland) and (iii) raw sewage sludge from the wastewater treatment plant "Czajka" in Warsaw (Poland).

Methanogenic consortium for anaerobic digestion was obtained from the fermenter tank of an anaerobic digester from the biogas plant in Miedzyrzec Podlaski (Poland).

Maize silage obtained from a farm in Mikanow (Poland) was homogenized in a blender, mixed with low-mineral water (Eden Springs, Poland) and used as a substrate for the selection of hydrolytic consortia and analysis of anaerobic digestion process.

2.2. Experimental set-up for the selection of hydrolytic microbial consortia

123 The selection of hydrolytic microbial consortia was carried out in 500 mL bottles . These lab-
124 scale hydrolyzers were filled with 0.5% (v/v) of total solids (TS) of the aforementioned
125 samples, 3% (v/v) of TS maize silage. Mineral water was added up to the final volume of 400
126 mL. The selection process consisted of a series of 10 passages. Hydrolysis of maize silage in
127 each passage lasted for 72 hours and was carried out at 30 °C. Samples for the estimation of
128 hydrolytic activity were taken at the end of each passage. Samples for microbial community
129 analysis were taken at the end of the passages: 1, 3, 6 and 10 and stored at -20 °C until DNA
130 extraction.

131 **2.3. Estimation of the hydrolytic activity**

132 Semi-quantitative analysis of the hydrolytic activity, was determined by the modified method
133 by Teather and Wood [13] using Congo-Red Dye as an indicator of hydrolytic (cellulolytic
134 activity) of microorganisms. Supernatants from the cultures (8000 rpm, 10 min), obtained
135 after each passage, were injected into metal cylinders placed on CMC-Congo Red agar plates
136 (containing 1% Congo Red Dye) and incubated for 96 hours at 30 °C. A clear zone around a
137 metal cylinder indicated the hydrolytic activity of the tested consortium.

138 The hydrolytic activity of the consortia after each passage was determined based on
139 the assessment of the amount of reducing sugars (equivalent of glucose) generated during the
140 enzymatic hydrolysis of insoluble cellulose (from maize silage). Reducing sugars
141 concentration was estimated spectrophotometrically, by measuring the absorbance at 540 nm
142 after the addition of 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of the
143 centrifuged cultures [14].

144 **2.4. Hydrolysis of maize silage**

145 Laboratory-scale degradation of maize silage by the adapted ABH, CS and WTP
146 consortia was carried out in 1 L bottles GL 45 (Schott Duran, Germany). The reaction mixture
147 containing 3% TS maize silage was inoculated with 10% (v/v) of the selected consortia. The

148 pH was adjusted to 7.2 using sodium bicarbonate. The hydrolysis was carried for 120 hours at
149 30 °C. Physical and chemical analyses were performed at the beginning of the experiment and
150 every 24 hours until 120 hours. The following parameters were assessed for each sample:
151 soluble chemical oxygen demand (sCOD), volatile fatty acids (VFAs) concentration and the
152 total reducing sugar (glucose) released due to the hydrolytic activity of the consortia.

153 **2.5. Batch assay of the anaerobic digestion**

154 The effect of bioaugmentation of anaerobic digestion by the adapted hydrolytic consortia was
155 investigated in laboratory-scale anaerobic batch experiments, which were performed in
156 reactors consisting of 1 L glass bottles GL 45 (Schott Duran, Germany) connected with
157 Dreschel-type scrubbers. To each reactor, a 1 L Tedlar gas bag (Sigma, Germany) was
158 attached to collect biogas. The bioreactors were filled with the liquid phase from the
159 fermenter tank of an anaerobic digester containing the methanogenic consortium innoculate
160 [18 g volatile solids per liter ($g_{vs}\ L^{-1}$)], maize silage (9.6 $g_{vs}\ L^{-1}$) and supplemented with 100
161 mL of the adapted hydrolytic consortia. Low-mineral water was added up to the final volume
162 of 900 mL. The pH in each bioreactor was adjusted to 7.2 using sodium carbonate. Anaerobic
163 batch assays were run at 37 °C for 21 days without refeeding. Physical and chemical analyses
164 were carried out at the beginning of the experiment and after 7, 14 and 21 days. The
165 experiment was performed in triplicate.

166 **2.6. Analytical methods**

167 To monitor the hydrolysis of maize silage and the anaerobic digestion process, the following
168 parameters were determined: sCOD, VFAs concentration, the total reducing sugar (glucose)
169 concentration and volume of the biogas produced. The VFAs concentration and sCOD were
170 determined using Nanocolor® kits (Machery-Nagel GmbH, Germany). The total
171 concentration of reducing sugar was determined by the method developed by Miller (1959).

The volume of the produced biogas was monitored by Milligascounter MGC-1 (Ritter, Germany).

2.7. DNA extraction and PCR amplification

Total DNA from the selected consortium was isolated using the method described by Dziewit et al. [15]. Briefly, the samples were centrifuged (8000 rpm, 4 °C, 15 min), pellet was resuspended in lysis buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% (w/v) CTAB] and the cells were disrupted in a 5-step bead-beating protocol, supported by freezing and thawing procedure. Final DNA purification from protein, humic substances, etc., was carried by cesium chloride density gradient ultracentrifugation. The concentration and quality of the purified DNA was estimated by means of the NanoDrop 2000 instrument (NanoDrop Technologies) and gel electrophoresis.

The whole community DNA was used as a template for amplification of bacterial hypervariable V3–V4 regions of the 16S rDNA with the primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21, described by Klindworth et al. [16]. The reaction mixture (50 µL) contained 100 ng of the template DNA, primers and 0.02 U of Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Bacterial 16S rDNA fragments were PCR amplified in a thermocycler (Biorad) with 20 cycles. PCR conditions were as follows: initial denaturation (5 min at 96 °C), cycles consisting of denaturation (30 s at 96 °C), annealing (50 s at 58 °C), extension (25 s at 72 °C), and a final extension step (5 min at 72 °C). The PCR products were analyzed by horizontal gel electrophoresis (2% agarose with ethidium bromide in 1x TAE) and then purified with Agencourt AMPure XP beads (Beckman Coulter).

2.8. Sequencing library preparation and amplicon sequencing

To prepare the libraries for sequencing, approximately 250 ng of amplified DNA (pooled from the PCR replicates) was used with the Illumina TruSeq DNA Sample Preparation Kit

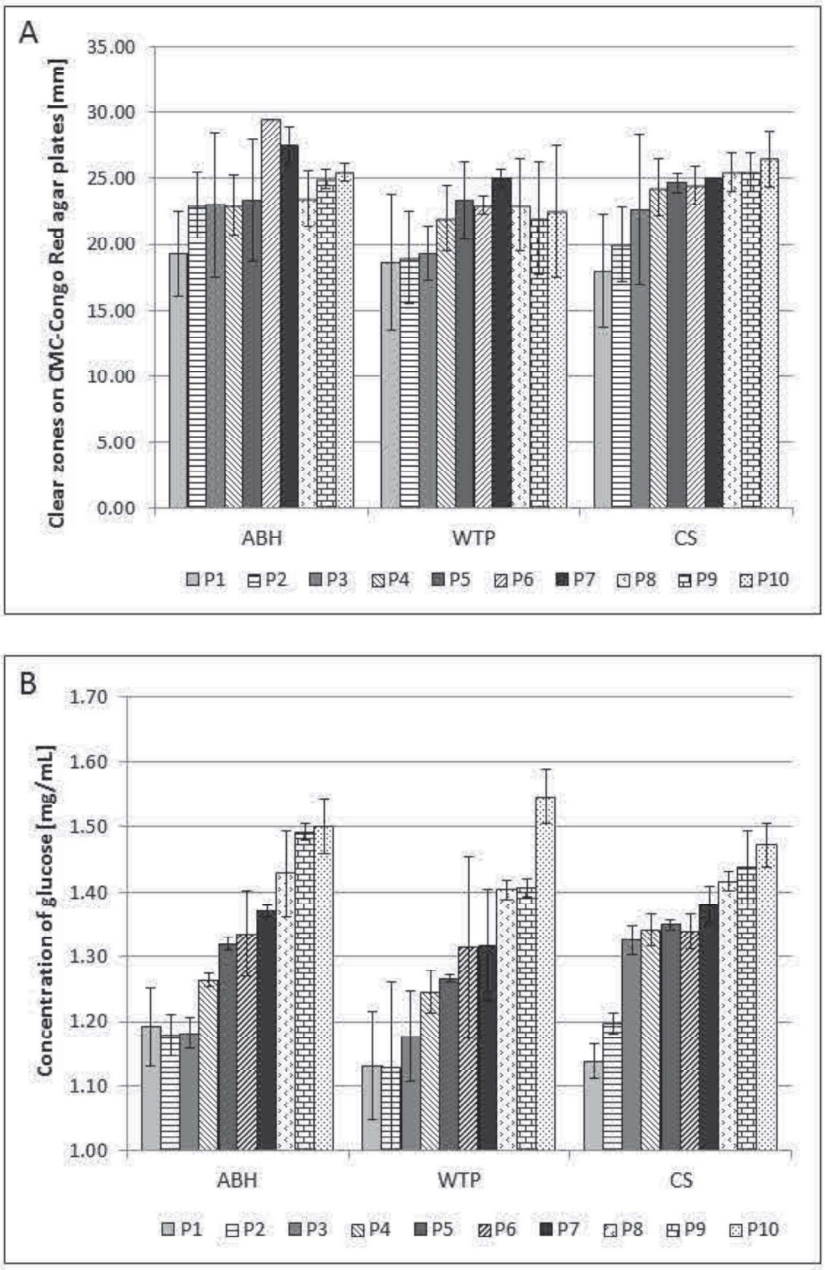
197 according to the manufacturer's protocol, except that the final library amplification step was
198 omitted. Libraries were verified using the 2100 Bioanalyzer (Agilent) High-Sensitivity DNA
199 Assay and KAPA Library Quantification Kits (Illumina).
200 Amplicon DNA sequencing was performed using the Illumina MiSeq Platform (MiSeq
201 Illumina Kit V3) with a 300 bp read length. Computational analyses were performed in
202 similar manner as described by Nelson et al. [17], using a local computing environment with
203 the Quantitative Insights in Microbial Ecology (QIIME, v1.9.0) pipeline [18]. Briefly, raw
204 sequences were processed with the Cutadapt software enabling trimming of the nucleotides
205 corresponding to the sequence of adapters and primers used for PCR amplification and library
206 preparation. In next step, the sequences were merged and combined into a single fastq file, in
207 order to ensure an even treatment and comparison for QIIME analyses. This resulted in the
208 generation of 2.4 mln sequences with the mean length of 422 nucleotides. Chimera detection
209 was performed using usearch61 [19] with subsequent filtering from sequences. Operational
210 taxonomic units (OTU) were d*e novo* assigned with uclust [20], clustered at 97% similarity
211 against the SILVA version 111 reference OTU alignment [21]. A representative sequence for
212 each OTU was selected and then the taxonomic assignment was made using the RDP
213 Classifier v2.2 [22]. Additional filtering for sequence errors was performed with
214 filter_otus_from_otu_table.py script by removing OTUs appearing in fewer than 3 samples
215 and represented by less than 0.0005% of the total sequences.
216 Taxonomic structures were prepared based on OTU tables specific for bacterial
217 amplicons, with a family level default. Sequences that were not assigned at the family level
218 were named in accordance with the lowest possible taxonomy. A principle coordinates
219 analysis (PCoA) plot was constructed to visualize the dissimilarity of samples at different
220 stages of selection.
221 Raw sequences of 16S rDNA amplicons obtained in this study (15 libraries) were
222 deposited in the SRA (NCBI) database under Bioproject accession number PRJNA350818.

3. Results and discussion

Hydrolytic microbial consortia were obtained after successive subcultivations of various environmental sources of microorganisms: (i) an effluent from a hydrolyzer tank from an agricultural biogas plant (ABH), (ii) cattle slurry (CS) and (iii) raw sewage sludge from a wastewater treatment plant (WTP) on medium containing maize silage. Selection and adaptation of the hydrolytic consortia was monitored during a series of 10 passages carried out every 72 hours at 30 °C on a fresh portion of maize silage medium. The performed experiments were aimed to find out: (i) the effect of the source of microorganisms on the hydrolytic activity of the selected consortia, (ii) biodiversity changes within these consortia and (iii) the effect of the selected consortia on degradation and anaerobic digestion of maize silage.

3.1. The effect of the source of microorganisms on the hydrolytic activity of the adapted consortia

To determine the changes in the hydrolytic activity of the analyzed consortia during each step of adaptation (each passage), basic physiological tests on the CMC-Congo Red medium agar plates were performed. After 96 hours of cultivation, the level of cellulolytic (hydrolytic) activity of studied the consortia was semi-quantitatively assessed by measuring the diameter of the produced clear zones (Fig. 1A).



241

242 **Fig. 1. Hydrolytic activity of the consortia during the selection process.** Analysis of the
243 changes: clear zones diameter on CMC-Congo Red agar plates (A) and concentrations of the
244 released glucose (B).

245 The initial diameter of the clear zones after first passage (P1) was 18-19 mm for all
246 consortia. The size of the clear zones has increased and reached the maximum diameter of

247 29.5 mm, 26.5 mm and 25.0 mm for ABH (after P6), CS (after P10) and WTP (after P7),
248 respectively. These results indicate that all the selected consortia had hydrolytic (cellulolytic)
249 activity and it was higher than that of the initial consortium after adaptation for used substrate.
250 Literature data on the Congo red clearing zone assay, describes the results only for pure
251 strains of bacteria. The results obtained in this study indicated that the level of hydrolytic
252 activity of the analyzed consortia, corresponds to that described in the literature as a high for
253 the pure strains. For example, Gupta et al. [23] obtained 28-50 mm clear zones for
254 cellulolytic bacteria isolated from guts of termite, caterpillar, bookworm and snail, and Liang
255 [24] – 20-30 mm clear zones for organic-rich soil isolates. Our earlier studies on hydrolytic
256 strains (belonging to the following genera: *Bacillus, Ochrobactrum, Providencia*), which
257 produced clear zones reaching from 15 to 44 mm in diameter. These strains were isolated
258 from a hydrolyzer tank from an agricultural biogas plant, cattle slurry and manure and were
259 dedicated for degradation of maize silage [25].
260 As previously stated, the diameter of the hydrolyzing zone produced by bacteria on
261 CMC-Congo Red plates it is only semi-quantitative method for assessing the hydrolytic
262 activity of bacteria and it may not accurately reflect their actual hydrolytic activity. For this
263 reason the hydrolytic activity of the selected consortium was evaluated by analyzing the
264 changes in the concentration of glucose released during maize silage degradation. Cleavage of
265 the β-1, 4-glycosidic bonds by microbial enzymes leads to the hydrolysis of cellulose,
266 resulting in the release of glucose or oligosaccharides. The amount of reducing sugars
267 produced during the degradation of maize silage indicates the pretreatment effectiveness
268 [26,27]. For the studied consortia, the initial concentrations of glucose in the first passage of
269 all cultures were similar (1.13-1.19 g/L). During the selection process, the concentration of
270 glucose gradually increased, reaching the highest value of 1.50 mg/mL, 1.58 mg/mL and 1.47
271 mg/mL in the passage 10 for ABH, WTP and CS, respectively (Fig. 1B). The increase was
272 equivalent to the release of 26% (ABH), 37% (WTP) and 29% (CS) more glucose molecules

compared to first passage and indicates a higher hydrolytic activity of the selected consortia. Additionally, the fastest increase in the concentration of glucose was observed for CS (1.34 g/L after the third passage (P3)). CS had a higher increase at the beginning, which means it reached its peak reducing sugar yield earlier than the other two consortia. This trend was also observed in the clearing zone assay and thus suggested that cattle manure can be a good source of microorganisms for fast-acting and stable hydrolytic consortia.

Moreover, another aspect of the source of hydrolytic microorganisms are the nutrients it contains. The micronutrients contained in the source can enhance the metabolism of microorganisms and hydrolytic activity, and consequently, biogas yield [28,29]. However, the concentrations of micronutrients in different sources of microorganisms were rarely mentioned. In the case of using maize silage as a substrate, the concentration of nitrogen could be low with simultaneous excess of carbon, what may result in lower biogas production [30]. When cattle manures is used as source of microorganisms, extra nitrogen, present in the initial sample, is provided to meet the needs of the hydrolytic consortium. However, after the first passage, cattle slurry no longer constitutes a source of nutrients, what may have caused further decrease in the biodiversity of the consortium.

The results of CMC-Congo Red agar plates assay as well as the changes in concentrations of glucose released indicate that hydrolytic activity increased during experiments for all consortia and suggest the selection of specialized bacteria and adaptation for enhanced degradation of maize silage.

3.2. The effect of the source of inoculum on the diversity changes during the adaptation of the hydrolytic consortia

Biodiversity of the microbial consortia was analyzed based on high throughput gene sequencing amplicons of hypervariable fragment V3-V4 of bacterial 16S rDNA. In the studied samples, 3 to 8 bacterial phyla constituting at least 1% of whole microbial community

were detected. The most abundant phylum was *Firmicutes* with the average count 60% (ranging from 6% to 93%) followed by *Bacteroidetes* with 24% (ranging from 1% to 64%) of total microbial sequences. Members of the *Firmicutes* and *Bacteroides* phyla play the most important role in the hydrolysis of the plant biomass and in the secondary fermentation [31].

Considering the microbial structure at family level, the results clearly shows the adaptation of the microbial communities (Fig.2).

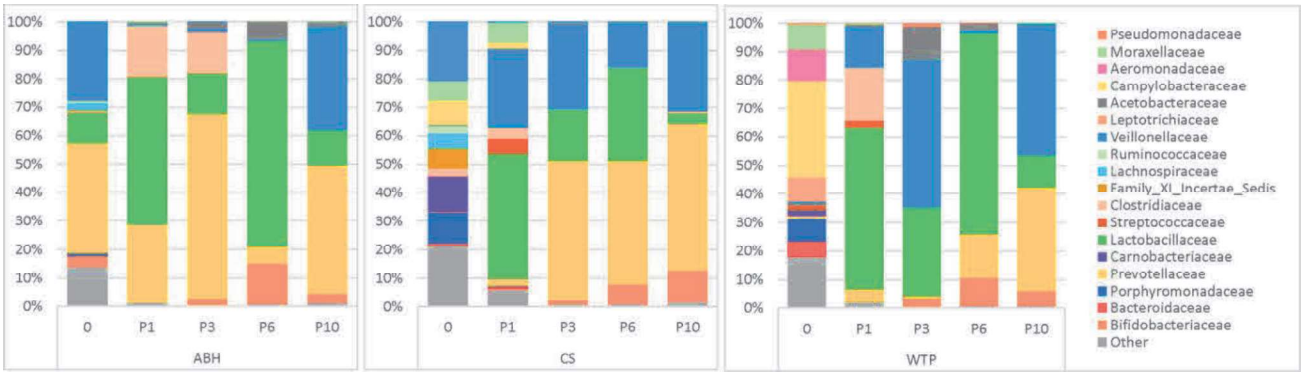


Fig. 2. Bacterial community structure dynamics. The bar chart shows the relative abundance of bacterial families with abundance > 5% in at least one variant. Inoculum communities were denoted with 0 while the numbers 1, 3, 6, 10 represent the subsequent passages as described in the Materials and Methods section. ABH – agricultural biogas hydrolyzer, CS – cattle slurry, WTP – wastewater treatment plant.

The number of families (with >1% abundance) present in the inoculum samples were reduced from 17 (CS), 12 (WTP) and 10 (ABH) to 4-5 families in each sample in the last passage. For both ABH and WTP samples, a decrease in the dominating families (in the percentage content of the families dominating in the initial sample) was already seen after the first passage. For the remaining consortium CS, the reduction to 4-5 families occurred at passage 3. This quick alteration of the microbial community structure can indicate a fast response of the microorganisms to the changes in conditions and selection of specialized best-adapted bacteria. Confirmation of the adaptation of microbial communities was presented by

Principal Coordinates Analysis (PCoA) of the Bray-Curtis dissimilarity indices (Fig. 3). These data indicate diversity differences between the communities and also within the consortia after successive passages. The original sources of ABH, CS and WTP were significantly different, while the consortia adapted after 10 passages have a very similar structure (Fig. 3).

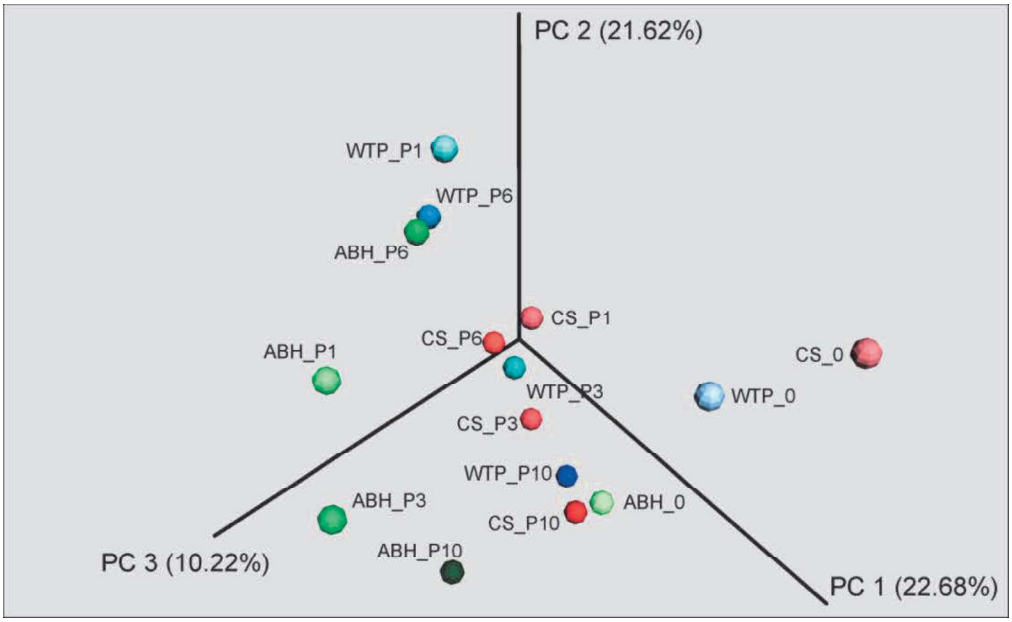


Fig. 3. Principal Coordinates Analysis (PCoA Bray-Curtis) of differences in bacterial diversity observed during the selection of microbial communities. Spheres representing each sample were colored according to their origin as follows: agricultural biogas hydrolyzer, ABH – green, cattle slurry, CS – red, wastewater treatment plant, WTP – blue. The intensity of colors increases with the passage number.

3.2.1. Agricultural biogas hydrolyzer (ABH) community

The inoculum originating from the agricultural biogas hydrolyzer was dominated by the representatives of *Prevotellaceae* (39%), *Veillonellaceae* (28%) and *Lactobacillaceae* (11%) families. These groups of bacteria are characterized by a strong hydrolyzing activity towards polysaccharide compounds resulting in high production of volatile fatty acids (VFA)

334 [32,33]. Since the ABH inoculum was obtained from a full-scale biogas plant utilizing maize
335 silage as a substrate, the initial microbial community was already adapted to maize
336 degradation and was characterized by a strong hydrolyzing activity. During the cultivation
337 experiments, at the next two analyzed time-points, we observed shifts between the dominating
338 bacteria *Lactobacillaceae* and *Prevotellaceae* with the relative abundance of 52% and 27% at
339 passage 1 and 15% and 64% at passage 3, respectively. Additionally, the family
340 *Clostridiaceae* emerged from below 1% to 18% (ABH1) and 14% (ABH3). Members of this
341 family are well-known for their high cellulolytic activity [34] and thus release of simpler
342 sugars from polysaccharides, which eventually leads boosted the growth of *Lactobacillaceae*
343 (72%) and *Bifidobacteriaceae* (15%) in our study. Bacteria belonging to these families are
344 known to produce lactic acid as one of the main products of carbohydrate fermentation
345 degradation [35,36]. Interestingly at the end of the experiments, the abundance of dominant
346 bacterial families, *Prevotellaceae* (45%), *Veillonellaceae* (36%) and *Lactobacillaceae* (13%),
347 almost reflected that of inoculum but with reduced importance of low abundant families.

348 *3.2.2. Cattle slurry (CS) community*

349 Most of the sequences identified within the CS inoculum were affiliated with non-cellulolytic
350 *Pseudomonadaceae* (21%), *Carnobacteriaceae* (12%), *Campylobacteriaceae* (8%) and
351 *Moraxellaceae* (7%), as well as microorganisms capable of fermentation of carbohydrates:
352 *Porphyromonadaceae* (12%), *Lachnospiraceae* (5%) and probably Family XI Incertae Sedis
353 (7%) [37,38,39]. After the first passage a drastic (1100-fold) increase in the abundance of
354 *Lactobacillaceae* to the level of 44% of all the bacterial sequences was observed. Second
355 most abundant family became *Veillonellaceae* (27%) with over 90-fold greater coverage in
356 microbial structure. Moreover, all of the previously dominating bacteria (except
357 *Moraxellaceae*) were diminished to the level below 0.5%. This drastic shift indicates very fast
358 changes in bacterial community structure in response to substrate pressure. In the passage 3,

359 hemicellulose- and pectin-degrading *Prevotellaceae* (49%) gain dominance, which is in
360 accordance with the results of a previously showed in a high-concentrate diet experiment [33].
361 As a result of the improved cellulose degradation, an increase in abundance of fast growing
362 carbohydrate fermenters, which often produce lactic acid, namely *Lactobacillaceae* (33%)
363 and *Bifidobacteriaceae* (7%) was observed after the passage 6. Similarly to the ABH
364 community, after the passage 10 the microbial community structure was again dominated by
365 *Prevotellaceae* (51%) and *Veillonellaceae* (31%), but instead of lactobacilli,
366 *Bifidobacteriaceae* (11%) remained the third most abundant family. Moreover, in the case of
367 the CS consortium thereafter the passage 3 we observed the most stable microbial structure
368 (calculated as the smallest difference between the dominant families in the P3-P10 passages)
369 of all studied consortia. This observation can explain more stable performance of CS
370 community in the CMC-Congo clearing zones measurement and glucose concentration.

371 *3.2.3. Wastewater treatment plant (WTP) community*

372 The inoculum originating from the wastewater treatment plant was composed of many non-
373 cellulolytic families such as *Campylobacteraceae* (34%), *Aeromonadaceae* (11%),
374 *Moraxellaceae* (9%), and *Leptotrichiaceae* (9%). The families which could be affiliated to
375 polysaccharide and VFA utilization included *Porphyromonadaceae* (8%) and *Bacteroidaceae*
376 (5%) [38]. In the selection experiment after substrate change to maize silage, we observed the
377 dominance of *Lactobacillaceae* (57%) with approximately 440-fold increase compared to the
378 initial community. The change in the bacterial community structure was similar to that
379 detected in the previously described variants. However, the second and the third most
380 abundant families became *Clostridiaceae* (19%) and *Veillonellaceae* (15%) which can explain
381 differences in CMC-Congo clearing zones and glucose concentration tests. In the next
382 analyzed step, the selection of bacteria also occurred in a slightly different way. After passage
383 3, members of *Veillonellaceae* (52%), *Lactobacillaceae* (31%) and *Acetobacteraceae* (12%)

384 dominated in the community. Members of the genus *Acetobacter* primarily contribute to
385 acetate production [40].
386 After passage 6, members of *Lactobacillaceae* again outcompeted other bacteria with the
387 relative abundance of 71%, but at the end of experiment their number was reduced to 12%.
388 Passage 10 was dominated by *Veillonellaceae* (46%) and *Prevotellaceae* (36%).

389 In the above experiments, adaptation of three communities towards consortia highly
390 specialized in degradation of maize silage was observed. Although the community structure of
391 the initial consortia was very different, from the third passage the dominance of bacteria
392 belonging to six families: *Lactobacillaceae, Prevotellaceae, Veillonellaceae, Clostridiaceae,*
393 *Bifidobacteriaceae* and *Acetobacteraceae* was observed . This could be an indicator of
394 selection and qualitative stabilization of the bacterial communities but, on the other hand, the
395 relative abundance of the above-mentioned bacterial families varied between the passages,
396 excluding quantitative stability. In the adapted consortia, members of the families
397 *Lactobacillaceae, Prevotellaceae, Veillonellaceae* were the most important as they were
398 characterized by the highest average count (Fig. 2). In all the variants, adaptation the selection
399 process began with an increase in the relative abundance of fast growing *Lactobacillaceae*,
400 specialized in oligosaccharide degradation and production of short chain fatty acids (SCFA),
401 mainly lactic acid [35]. Enrichment of the consortia with members of this family was
402 previously observed in studies where agricultural materials were used for anaerobic digestion
403 [41,42]. Wirth et al. [43] obtained interesting results which reported that indigenous
404 microorganisms of maize silage include representatives of the genera *Lactobacillus* and
405 *Acetobacter*.

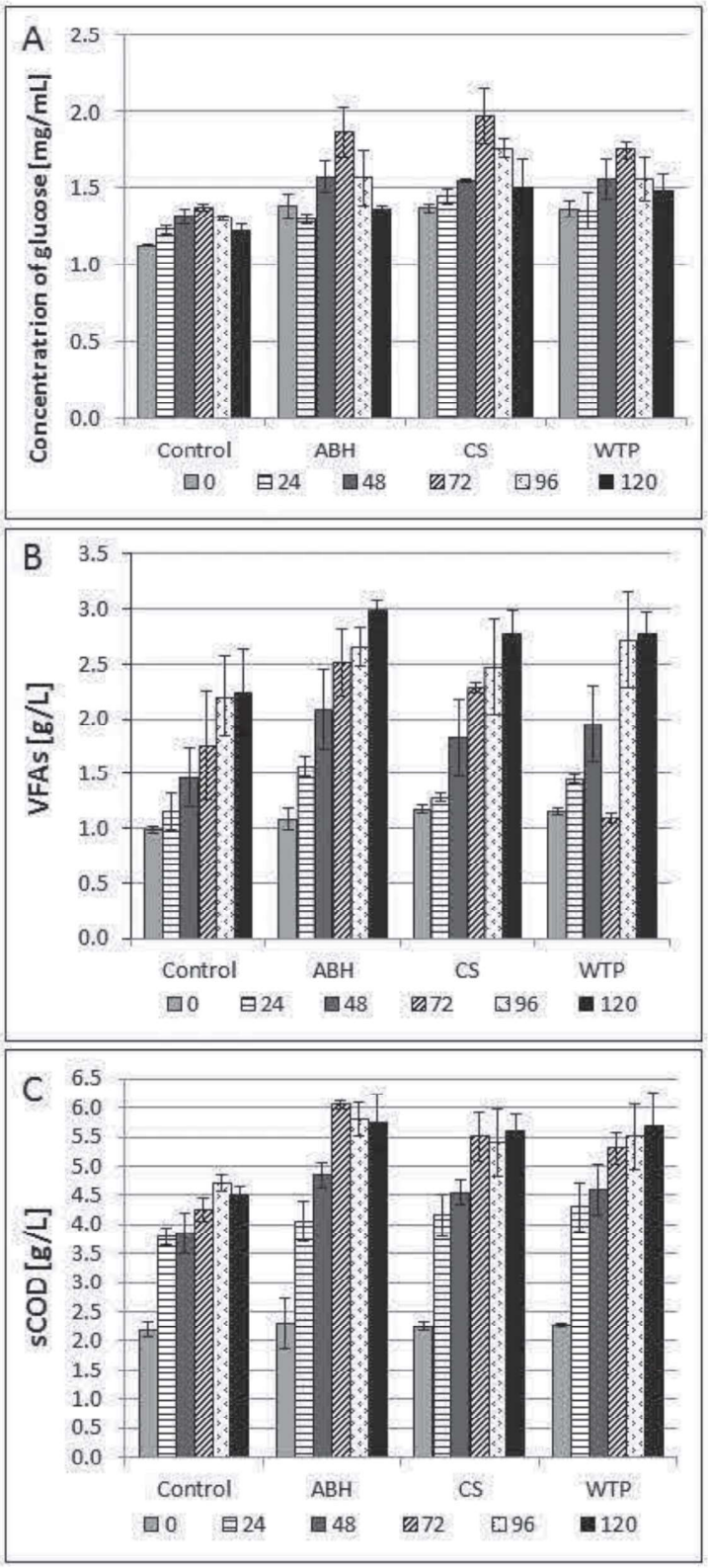
406 Our results of selection process of the analyzed consortia was driven by substrate input
407 rather than the composition of the initial inoculum, which differed with the origin of the
408 sample. After cultivation on maize silage, the multiple families present in sources samples

(even very low abundant <0.1%) in the subsequent passages emerged 3-6 families involved in maize silage degradation. De Francisci et al. [44] also observed that selection process caused changes in the bacterial population by substrate input but not the sample origin. In turn, Porsch et al. [45] indicated that only the initial enrichment step significantly influence the adaptation and changes of microbial community structure of wheat straw. The subsequent passages did not affect the structure and activity of the community.

3.3. Functional analysis of the adapted consortia

3.3.1 Hydrolysis of maize silage

To analyze the hydrolytic potential of the adapted consortia, 120-hour anaerobic batch cultures on maize silage were performed. The control variant consisted of maize silage/low mineral water medium, which was treated exactly as the culture variants, except for the inoculation with hydrolytic consortia. The efficiency of maize silage hydrolysis was expressed as a function of concentration of the produced (released) glucose and VFAs, as well as the level of sCOD (Fig 4.).



423

424 **Fig. 4. Solubilization of maize silage by the adopted hydrolytic consortia.** Changes in the

425 concentration of: glucose (A) and VFAs (B), as well as the level of sCOD (C) were analyzed.

426 The concentration of glucose increased from 1.12 g/L to 1.37 g/L in the control variant,
427 from 1.38 g/L to 1.85 g/L for ABH, from 1.37 g/L to 1.97 g/L for CS and from 1.36 g/L to
428 1.75 g/L for WTP after 72 hours. These were the highest glucose concentrations obtained
429 during the experiment. Longer hydrolysis resulted in a decrease in glucose concentration in all
430 the cultures, what is probably caused by the consumption of sugars by microorganisms (Fig.
431 4). The concentration of VFAs was increased in all variants during 120 hours. In the control
432 increased concentrations of VFAs from 0.99 g/L to 2.24 g/L were observed. in the variants
433 where adopted consortia were added the concentrations of VFAs increased from 1.08 g/L to
434 2.99 g/L for ABH, from 1.17 g/L to 2.78 g/L for CS and from 1.15 g/L to 2.77 g/L for WTP. (
435 The obtained results showed an increase in the amount of reducing sugars (glucose) and
436 VFAs formed/released during the degradation of maize silage. The production of volatile fatty
437 acids may also play an important role in enhancing the degradation of maize silage. Zhang
438 and co-workers [29] suggested that mild acids can loosen the structure of maize silage leading
439 to an improved overall rate of hydrolysis due to increased accessibility of the substrate for
440 hydrolytic enzymes which would further benefit production of biogas.
441 The initial value of sCOD in all the cultures was similar (2.2-2.3 g/L) and it increased
442 throughout the experiment. The highest level of sCOD was observed after 72 hours for ABH
443 (6.05 g/L), and 120 hours for CS and WTP (5.6 g/L and 5.7 g/L, respectively). In the control,
444 the highest level of sCOD was obtained after 96 hours (only 4.7 g/L) and then it decreased.
445 Faster increase in sCOD maybe an indicator of the accelerated maize silage degradation.
446 The observed increase in sCOD as well as glucose and VFAs concentrations probably
447 resulted from the metabolic activity of the indigenous microbiota of maize silage. Ensiling is a
448 well-known technology for preserving lignocellulosic material. Microbial silage starters
449 (lactic acid bacteria) are very often added during the process in order to ensure rapid
450 acidification or the formation of specific metabolites which inhibit the growth of other
451 organisms, which may cause spoilage of plant matter [46,47].

Cultivation of hydrolytic consortia in natural media has been previously described by many researches. In 2010, Guo et al. [48] obtained a lignocellulose-degrading composite microbial system (XDC-2) from soil amended with composted agricultural and animal waste. The concentration of reducing sugars obtained after the degradation of rice straw and corn stalk was 1.3 g/L and 2.4 g/L, respectively. Yuan et al. [49] showed that the VFAs concentration and sCOD increased rapidly during 4 days (120 hours) of pretreatment of corn stalk using the MC1 consortium, and their values differed depending on the initial concentration of the substrate.

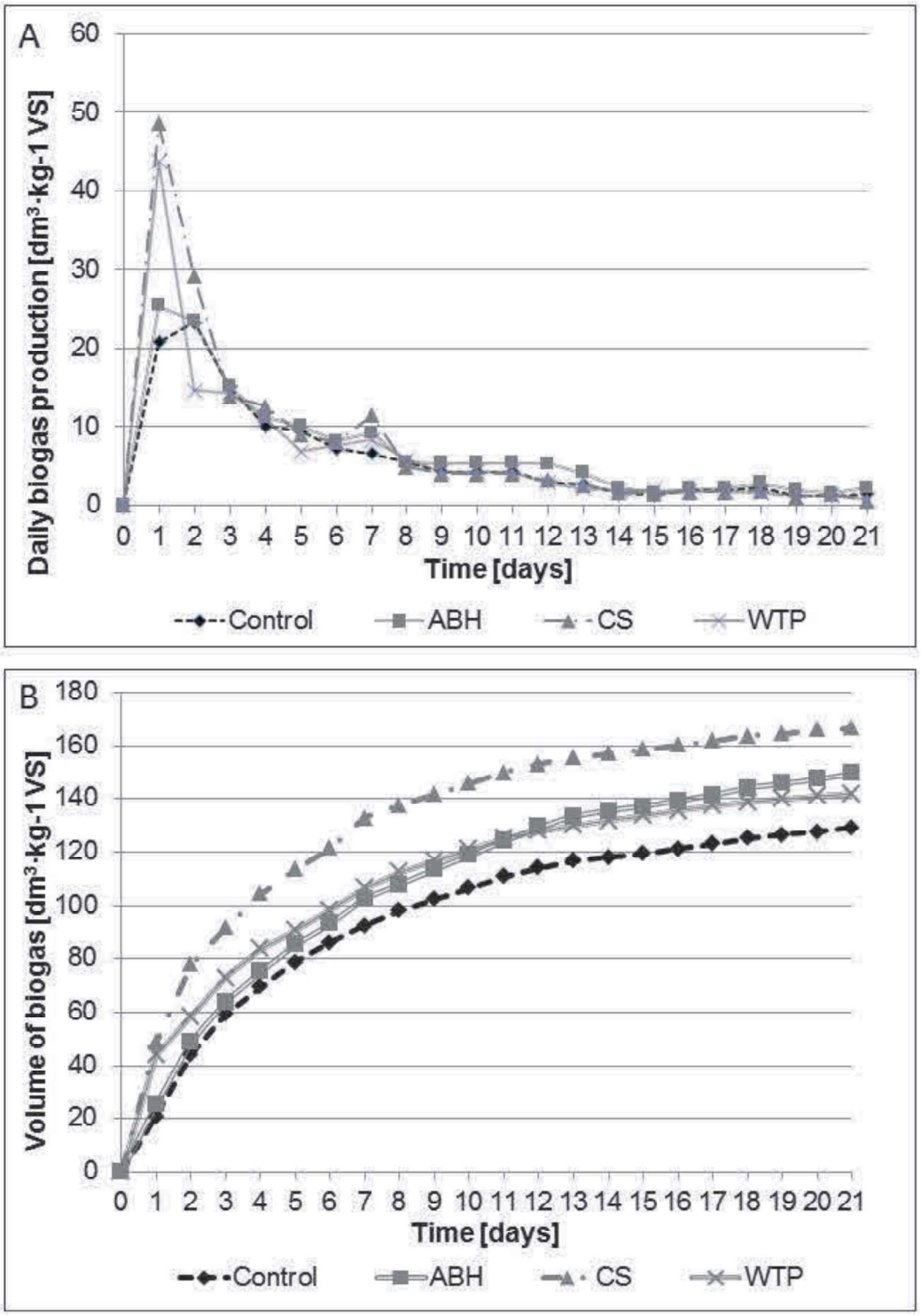
Our results also indicated an increase in VFAs and glucose concentrations, as well as the level of sCOD during hydrolysis. This was mainly due to the degradation of insoluble macro-molecular organic compounds of maize silage by hydrolytic activity of analyzed consortia. The intermediate products of hydrolysis, could probably be utilized by other groups of microorganisms during the next phase of anaerobic digestion.

3.3.2. The influence of the adapted consortia on degradation and anaerobic digestion of maize silage

The selection process of microbial communities to degrade maize silage was confirmed both in experiments testing hydrolytic activity and bacterial community structure based on 16S rDNA survey. In the next step of our experiments we used the adapted consortia in order to verify if and how the hydrolytic ABH, CS and WTP consortia may affect the activity of methanogenic community of biogas plant fermenter tank and ultimately its efficiency of biogas production.

To determine the effect of augmentation of the anaerobic digestion communities by ABH, CS and WTP consortia on the efficiency of maize silage anaerobic digestion (biogas production), a batch cultures experiment was carried out. The analyzed hydrolytic consortia were added to the anaerobic digester only once at the beginning of the experiment. The liquid

phase from the fermenter tank of anaerobic digester from a biogas plant was used as a the source of methanogenic consortium. During 21 days simulation of maize silage anaerobic digestion process, VFAs concentration, sCOD and the daily biogas production were measured. The physical and chemical parameters affect the anaerobic digestion process. The first phase of simulation of anaerobic digestion was hydrolysis. According to the literature, hydrolysis does not take more than 5 days, so it was decided to determine VFAs, and sCOD only after 7 days, and at the end of entire process (21 days). The obtained results show that VFAs concentration and sCOD increased during the anaerobic digestion in all batch assays (Tab. 1). The concentration of VFAs increased from 5.88 g/L to 10.29 g/L, from 5.61 g/L to 10.06 g/L, and from 6.12 g/L to 10.11 g/L , for the ABH, CS and WTP variants respectively), after 7 days. In the control variant VFAs concentration increased from 5.37 g/L to 9.33 g/L during the same time, and then decreased to 8.96 g/L after 21 days.. It was observed that VFAs concentrations in culture ABH, CS and WTP, remained at high level even after 21 days, and amounted 10.37 g/L, 12.06 g/L and 10.71 g/L, respectively. The initial value of sCOD (18.43 – 18.80 g/L) was increased (to 22.50 g/L for ABH, 23.47 g/L for CS and 23.20 g/L for WTP, after 7 days. After 21 days, the values of this parameter had increased to 24.93 g/L for ABH, 25.90 g/L for CS and 26.10 g/L for WTP. In the control, sCOD was increased from 18.63 g/L to 20.83 g/L after 7 days and to 24.63 g/L after 21 days. Concentrations of VFAs were higher by 10%, 48%, 8% after 21 days for ABH, CS and WTP, respectively, compared to the control. The level of sCOD was higher by 4%, 6% and 9% after 21 days, for ABH, CS and WTP, respectively, than in the control. Our results suggest that bioaugmentation of maize silage by hydrolytic consortia contributes to an improvement in decomposition (liquefaction) of this substrate during anaerobic digestion. The analysis to the daily and cumulative biogas production (CBP) revealed that the hydrolytic consortia (ABH, CS and WTP) increased the efficiency of biogas production from maize silage (Fig. 5A and Fig. 5B).



503

504 **Fig. 5. The efficiency of biogas production during anaerobic digestion of maize silage: A)**

505 **daily biogas production and B) cumulative biogas production.**

CBP for anaerobic digestion of maize silage with the addition of hydrolytic consortium was 150 dm^3/kg of VS and 166.5 dm^3/kg of VS and 141.8 dm^3/kg of VS for ABH, CS and WTP, respectively. The production of biogas in the experiment without bioaugmentation (control) was 128 dm^3/kg of VS. The results showed that the high production of biogas was maintained for 7 days. After this time, in the variants with hydrolytic consortia, biogas production was higher by about 10%, 43% and 15% (for ABH, CS and WTP, respectively) than in the control variant. This was probably caused by the increased efficiency of maize silage hydrolysis related to the presence of the analyzed consortia during the entire anaerobic digestion process.

The obtained results indicate that biogas production increased by 16%, 29% and 10% after bioaugmentation of the methanogenic consortia with ABH, CS and WTP, respectively.

The results of physical and chemical analyses show that the accumulation of intermediate products (VFAs) and soluble substrate (as indicated by the increased level of sCOD) after hydrolysis facilitated by the hydrolytic consortia ABH, CS and WTP, may be beneficial for the anaerobic digestion process and it was correlated with the improved efficiency of biogas production (Fig. 5B). Moreover the addition of the adapted hydrolytic consortia to the anaerobic digestion process increased the activity of methanogenic consortium. Furthermore, effective hydrolysis means a short digestion time and this may increase the overall biogas production efficiency [50].This could bring significant economic benefits for increasing the biogas production efficiency or treatment capacity of anaerobic digesters because of the shortened digestion time.

4. Conclusions

In this study the effect of the source of microorganisms on the selection and adaptation of hydrolytic microorganisms was analyzed. The obtained results showed that subsequent passages of hydrolytic consortia using maize silage as substrate led to the selection of the

representatives of three families of bacteria: *Lactobacillaceae, Prevotellaceae* and *Veillonellaceae*, which dominate in the investigated communities after the adaptation process. This indicates that the substrate, not the community origin was the main driving force in the adaptation of hydrolytic consortia.

Following adaptation, hydrolytic capabilities of all three consortia have improved, as indicated by the increased concentration of the released glucose, VFAs, and the level of sCOD in the culture. Moreover, bioaugmentation of anaerobic digestion using the adapted hydrolytic consortia increased the efficiency of biogas production by up to 29%. This proves that subsequent passaging of hydrolytic consortia on an appropriate substrate, such as maize silage, prior to their application in biogas plant hydrolyzers, may facilitate their adaptation and increase their hydrolytic activity.

Future work should involve the selection and characterization of the key hydrolytic microorganisms found in the analyzed consortia, as well as investigation of the mechanism of their activity, which leads to the enhancement of biogas production during anaerobic digestion.

Author contributions

KP: planned and performed the selection, hydrolysis and anaerobic digestion experiments, as well as most of the chemical analyses, DNA isolation and wrote the manuscript; AP: was involved in planning of the metagenomics approach, performed the deep sequencing and bioinformatics analysis, and was involved in writing of the manuscript; ASob and LL: designed and supervised the metagenomics and bioinformatics approaches; ASklo: is the head of a group, and was involved in consultation and article correction; LD: is the head of a project; planned and directed the studies and was involved in consultation and article preparation.

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711 **Table legends**

712 **Table 1. The physical and chemical characteristics of digester during anaerobic**
713 **digestion.**

	VFAs			sCOD		
	[g/L]			[g/L]		
	0 days	**7 days**	**21 days**	**0 days**	**7 days**	**21 days**
Control	5.37±0.82	9.33±0.43	8.96±0.71	18.63±0.35	20.83±0.31	24.63±0.5
ABH	5.88±0.19	10.29±0.74	10.37±0.53	18.43±0.55	22.50±0.7	24.93±0.86
CS	5.61±0.13	10.06±0.13	12.06±0.05	18.80±0.21	23.47±0.26	25.90±0.35
WTP	6.12±0.11	10.11±0.46	10.71±0.58	18.47±0.53	23.20±0.21	26.10±0.5

714