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Effect of Alkaline and Mechanical Pretreatment of Wheat Straw on Enrichment Cultures from *Pachnoda marginata* Larva Gut

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Abstract: In order to partially mimic the efficient lignocellulose pretreatment process performed naturally in the gut system of *Pachnoda marginata* larvae, two wheat straw pretreatments were evaluated: a mechanical pretreatment via cutting the straw into two different sizes and an alkaline pretreatment with calcium hydroxide. After pretreatment, gut enrichment cultures on wheat straw at alkaline pH were inoculated and kept at mesophilic conditions over 45 days. The methanogenic community was composed mainly of the Methanomicrobiaceae and Methanosarcinaceae families. The combined pretreatment, size reduction and alkaline pretreatment, was the best condition for methane production. The positive effect of the straw pretreatment was higher in the midgut cultures, increasing the methane production by 192%, while for hindgut cultures the methane production increased only by 149% when compared to non-pretreated straw. Scanning electron microscopy (SEM) showed that the alkaline pretreatment modified the surface of the wheat straw fibers, which promoted biofilm formation and microbial growth. The enrichment cultures derived from larva gut microbiome were able to degrade larger 1 mm alkaline treated and smaller 250 µm but non-pretreated straw at the same efficiency. The combination of mechanical and alkaline pretreatments resulted in increased, yet not superimposed, methane yield.

Keywords: alkaline pretreatment; wheat straw; lignocellulosic biomass; anaerobic digestion; larvae gut enrichment; methane; carboxylates

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1. Introduction

Conversion of lignocellulosic biomass (LCB) in anaerobic systems encounters two major hindrances: the absence of oxygen, which is essential for degradation of aromatic compounds such as lignin [1], and the large variation of the structural composition of agricultural wastes [2]. Hydrolysis is the rate limiting step of LCB conversion in anaerobic digestion processes [3,4]. Therefore, a previous treatment of the biomass is necessary before further processing in an artificial anaerobic system [5]. The great barrier to the biological conversion of LCB in anaerobic systems is lignin that embeds cellulose and hemicellulose in many plant cell walls [6]. Lignin prevents the access of microorganisms and enzymes to act on cellulose and hemicellulose and to convert these components into products with greater added value, such as carboxylates, ethanol, and biogas. The pretreatment of LCB is extensively studied and all types have been shown increase the yield of targeted products [7–12]. Alkaline treatments are good options of chemical pretreatment of LCB, since the OH radical separates lignin from hemicellulose and dissolves hemicellulose and cellulose by removing the hydrogen bond between them and consequently increases the digestibility of the lignin–hemicellulose complex [13–17]. The most common alkaline reagents used for LC biomass pretreatment are NaOH, KOH, and $Ca(OH)_2$. Pretreatment with NaOH has proven to be

very efficient, but it brings high costs to the process, can be toxic to the microorganisms, and NaOH is difficult to recycle [8,18]. Pretreatment with KOH costs even more than NaOH and it is as efficient or more efficient than NaOH pretreatment when biochemical methane potential (BMP) is considered [19]. The digestate from a biomass pretreated with KOH has the advantage that it can be used as fertilizer [20–22]. Alkaline pretreatment with $Ca(OH)_2$ has been shown to be an economically viable choice, since it does not require high temperatures [23,24], does not inhibit the subsequent bioprocesses [25], can be regenerated using lime kiln technology [26] and is cheaper [23]. The interaction between $Ca(OH)_2$ and CO_2 in aqueous solution can lead to the formation of $CaCO_3$ precipitate both chemically [27,28] and biologically [29,30], which might cause technical failures via fouling.

Mechanical pretreatments reduce the size of LCB particles and increase the relative surface area, which leads to considerable increases in methane production. Although cutting and crushing techniques are not the most commonly used by animals, they are the ones often applied as mechanical pretreatment of biomasses during anaerobic digestion [31]. A basic mechanical pretreatment is an intrinsic part of the substrate handling for effective feeding and also a prerequisite in stirred tank reactors to prevent mechanical failures. It is economically more viable than alkaline treatment or steam explosion, due to lack of need for high pressures or long residence times [32]. However, it was reported that the reduction of biomass size to less than 200 µm does not generate a significant additional increase in total methane production [33].

Nature has found ways to enable various animals supported by their symbiotic gut microorganisms to effectively convert materials rich in lignocellulose [12,34,35], thus making the energy conserved in LCB available for their survival and providing selective advantage in evolution by exploiting these alternative food sources. As an example, the larva of the beetle *Pachnoda marginata* has a digestive tract with very remarkable characteristics from a biotechnological point of view. Their gut system is composed of three main portions: foregut, midgut, and hindgut. In the foregut, all organic matter that is ingested by the larva is ground into small particles, which can be considered as an effective natural mechanical pretreatment. Subsequently, the food enters the midgut, which has a high pH as its main characteristic [36] and exerts an effective natural alkaline pretreatment. In addition, the enrichment cultures from the Pachnoda marginata midgut content in lignocellulosic biomass showed that bacteria capable of converting cellulose and hemicellulose into VFAs are present in this larva gut section and they are able to survive in alkaline artificial systems; however, hydrolysis remained to be the limiting factor for total biomass conversion [37]. The hindgut is known to harbor a great diversity of microorganisms, among them archaea, capable of producing methane [37] and bacteria such as *Promicromonospora pachnodae*, able to produce endoglucanases and xylanases [38], and *Dysgonomonas* sp., able to convert carbohydrates into different acids [37].

Some characteristics related to the inoculum seem to play a key role in the performance of anaerobic digestion of lignocellulose-rich biomass [39]. The inoculum source [40–42] combined with the substrate/inoculum ratio [37,43–45] are parameters often cited in the literature as one of the main characteristics shaping the anaerobic digestion process. Inoculum from ruminant animal manure and anaerobic sludge from different sources are the most commonly used in the anaerobic digestion of LCB [46–50]. Insects that feed on LCB have been shown to harbor a rich microbiota [51] with potential for application in biorefineries [52]. Although the microbiomes from insect larvae gut or insect gut have been studied [12,37,53–56], they are not applied in the anaerobic digestion of lignocellulosic biomass on a large scale or studied using different types of pretreated biomasses.

Enrichment cultures from the insect gut microbiota selecting features similar to those found in insect gut, when applied to engineered systems, can improve LCB degradation and increase production of desired compounds [1,53,56]. Therefore, in the present study, we aimed to assess the effect of alkaline and mechanical pretreatment of wheat straw on the microbial community, volatile fatty acids, and methane production of two larva gut

enrichment cultures from a former study [37] and one enrichment culture derived from a Hungarian soda lake sediment [57] as candidate.

2. Materials and Methods

2.1. Medium, Wheat Straw Pretreatment, and Experiment Setup

Three different enrichment cultures were tested as inoculum types: Midgut, Hindgut, and S37; two wheat straw sizes: 1 mm and 250 µm; with and without alkaline treatment: treated (T) and non-treated (NT)—of the wheat straw. All these different parameters were combined as shown in Figure S1. Negative controls were assembled: four bottles as abiotic controls, with wheat straw in different sizes and alkaline treatment and medium; and three bottles as biotic controls, with medium and the three different enrichment cultures separately. Wheat straw was used as the sole complex carbon source. The wheat straw was collected in Saxony, Germany. It was first ground using the SM 2000 machine (Retsch, Germany) to reduce the particles to 10 mm and then further ground in the same machine using a 1 mm sieve. After grinding the wheat straw, various straw sizes were observed. A 250 µm sieve was then used to separate the even smaller particle size fraction. The wheat straw was twice sterilized at 121 °C for 20 min to avoid the growth of spore forming bacteria. The raw wheat straw at size 1 mm was composed of 62.8% cellulose, 26.6% hemicellulose and 10.6% lignin. The total solids (TS) and the volatile solids (VS) of raw wheat straw at size 1 mm were 94.2%$_{\text{fresh mass}}$ and 95.5%$_{\text{TS}}$, respectively. The raw wheat straw of size 250 µm was composed of 60.3% cellulose, 32.7% hemicellulose, and 7% lignin. The TS and vs. were 94.1%$_{\text{fresh mass}}$ and 91.8%$_{\text{TS}}$, respectively. Enrichment cultures from the midgut and hindgut of the beetle larvae *Pachnoda marginata*, fourth transfer [37] and an enrichment culture from the Szarvas Hungarian soda lake, seventy-fifth transfer [57] were used as inoculum. The calcium hydroxide ($Ca(OH)_2$) wheat straw pretreatment was carried out directly inside the culture bottles. For alkaline treatment 8.2 mL $Ca(OH)_2$ (4.6 g·L^{-1}) was added to the culture bottles. The solubility of calcium hydroxide increases as the temperature decreases [58]. Pretreatment of biomass with $Ca(OH)_2$ at low temperatures has been shown to have an additional effect on methane production and COD (chemical oxygen demand) conversion efficiency in an anaerobic digestion system [23]. Therefore, this treatment was carried out in an incubator at 10 °C in static condition for 24 h. When the term treatment (T) is used during this study, we are referring to the alkaline treatment. The culture medium used was modified DSMZ 1036 pH 9.0 as described by Porsch et al. [57]. The cultivation was performed in 100 mL serum bottles, sealed with butyric rubber stoppers, in anoxic conditions, initial pH of 9.0 and kept in a 37 °C incubator. Each non treated (NT) culture bottle was filled with 0.5 g wheat straw, 48 mL DSMZ 1036 medium, and 2 mL inoculum. The alkaline treated culture bottles had the same composition plus 8.2 mL $Ca(OH)_2$ (4.6 g·L^{-1}).

The batch experiment lasted 45 days and samples from the liquid and gas phase were collected every 5 days. Every culture condition consisted of three biological replicates and negative and positive controls were also established.

2.2. Analytical Methods

Every 5 days, the pressure of the culture bottles was measured with a high-resolution digital manometer (LEO 5, Keller, Switzerland) and then 1 mL of gas was sampled and analyzed by gas chromatography (Perkin Elmer GC equipped with HayeSep N/Mole Sieve 13X columns and a thermal conductivity detector).

To determine the total gas volume, methane volume and the specific methane yield, the equations described by Schroeder et al. [37] were used. The new headspace volume after each liquid sampling was considered for calculations during the whole experiment.

Organic acids were measured by high performance liquid chromatography (HPLC) after sampling 1 mL of cultivation broth. First, 100 µL of liquid was used to determine the pH, using a mini pH meter (ISFET pH meter S2K922, ISFETCOM Co., Ltd., Hidaka, Japan). The remaining 900 mL sample were centrifuged, filtered, and analyzed using HPLC

(Shimadzu Scientific Instruments, US) according to the protocol described in detail by Logroño et al. [59]. The pellets were stored at −20 °C for further DNA extraction. After liquid and gas samplings, bottles were degassed down to 0.009 bars and placed back into the incubator at 37 °C. The determination of total solids (TS) and volatile solids (VS) of wheat straw were performed as described by Logroño et al. [59]. The determination of lignin, hemicellulose, and cellulose of the raw wheat straw was determined in duplicates by Van Soest et al. [60] forage analysis.

2.3. Microbial Community Analysis

Samples for microbial community analysis were taken on day 5, 15, and 30 of cultivation. The frozen pellets were thawed and DNA was extracted using the NucleoSpin®Soil Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) using the buffers SL2 and SX. The DNA quantity was measured with a NanoDrop® ND-1000 UV-Vis spectral photometer (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA quality was assessed by gel electrophoresis (0.8% agarose).

The bacterial and methanogenic community structures were analyzed by terminal restriction fragment length polymorphism (T-RFLP). For bacterial community analysis, the DNA concentration was diluted to reach 10 ng μL^{-1}. The bacterial 16S rRNA gene polymerase chain reaction (PCR) was carried out in 12.5 µL reaction mixture containing 6.25 µL of MyTaq™ Mix (BIOLINE), 0.7 µL (5 pmol μL^{-1}) UniBac 27F-FAM (5′ GAG TTT GAT CMT GGY TCA G 3′), 0.7 µL (5 pmol μL^{-1}) Univ 1492r (5′ TAC GGY TAC CTT GTT ACG ACT T 3′), 1 µL DNA, and 3.85 µL H_2O. The PCR cycles were the following: 95 °C for 1 min, 95 C for 15 s, 58 °C for 15 s, 10 s at 72 °C, 29 cycles of 15 s at 95 °C, 15 min at 72 °C, and finally the product was cooled down to 8 °C before removal from the thermocycler. PCR products were cleaned with the SureClean Kit (BioLine) according to the manufacturer's instructions. A total of 40 ng purified PCR products were digested with 0.2 µL (2U) restriction enzyme *Rsa I* (New England Biolabs GmbH, Frankfurt/Main, Germany), 1 µL CutSmart Buffer, and 8.8 µL H_2O at 37 °C overnight. Restriction fragments were purified by ethanol precipitation using 2.5 µL (125 mM) EDTA pH 8.0 and 30 µL absolute ethanol. Samples were incubated for 5 min at room temperature and centrifuged at 15,000 rpm for 30 min. The supernatant was carefully removed and 95 µL 70% ethanol (freshly prepared) was added to each sample. They were centrifuged for a second time at 15,000 rpm for 20 min, the supernatant was carefully removed, and pellets were dried for 15 min in a desiccator. DNA samples were prepared for T-RFLP analysis using 9.75 µL HiDi formamide (Applied Biosystems GmbH, Weiterstadt, Germany) containing 0.25 µL ROX-Standard MapMarker® 1000 (BioVentures Inc., Murfreesboro, TN, USA) per sample.

The mcrA gene fragments were amplified via PCR, mixing 6.25 µL of MyTaq™ Mix (BIOLINE), 0.7 µL (5 pmol μL^{-1}) of forward primer mlas (5′ GGT GGT GTM GGD TTC ACM CAR TA 3′; Eurofins MWG Operon, Ebersberg, Germany), 0.7 µL (5 pmol μL^{-1}) of reverse primer mcrA-rev_FAM (5′ CGT TCA TBG CGT AGT TVG GRT AGT 3′; Eurofins MWG Operon) [61], 1 µL DNA and 3.85 µL H_2O. The PCR cycles were the following: 95 °C for 3 min, 20 s at 95 °C, 20 s at 48 °C, 15 s at 72 °C with a ramp rate of 0.1 °C·s^{-1}, 4 cycles of 20 s at 95 °C, 20 s at 95 °C, 20 s at 55 °C, 15 s at 72 °C, followed by 24 cycles of 20 s at 95 °C, 10 min at 72 °C, and finally it was cooled down to 8 °C before removal from the thermocycler. Purified PCR products (40 ng each) were digested with 0.2 µL (2U) restriction enzyme *BstNI* (New England Biolabs GmbH, Frankfurt/Main, Germany), 1 µL CutSmart Buffer, and 8.8 µL H_2O at 60 °C for 2 h. The purification of the restriction fragments and the preparation of DNA samples for T-RFLP analysis followed the same method as previously described for the 16S rRNA gene; however, the ROX-Standard used was GeneScan 500 ROX™ (Applied Biosystems GmbH, Weiterstadt, Germany).

The fluorescent terminal-restriction fragments (T-RFs) length were determined using the GeneMapper V3.7 software (Applied Biosystems). In order to remove background noise, a cut-off value of six and four times the standard deviation was used in the case

of mcrA and 16S rRNA genes, respectively. The *mcrA*-derived T-RFs were taxonomically assigned by using a T-RF database generated by Bühlingen and co-workers [62].

The diversity indices: richness, diversity of order, and evenness of order 1, were calculated based on the R Script available by Lucas et al. [63]. The ClusBinMatrix (TRFs table) used for the calculations were first filtered to 1% and then TRFs that appeared only once among triplicates were removed.

2.4. Scanning Electron Microscopy (SEM)

In preparation for SEM analysis, treated and untreated wheat straw samples from control, midgut, and hindgut cultures were dehydrated in a graded ethanol–water solution series at concentrations 30, 50, and 70% for 15 min each. After that, the samples were kept in 70% ethanol in properly sealed petri dishes to prevent drying and were placed in the refrigerator at 4 °C for 48 h to give a soft fixation effect. Then, the ethanol series was continued with concentrations of 80, 90, 95, and 100%, also for 15 min each. In preparation for drying, the samples were immersed for 15 min in a solution of 50% ethanol and 50% hexamethyldisilazane (HMDS) and another 30 min in 100% HMDS inside a fume hood. Afterward, they air-dried for 24 h inside the fume hood at room temperature. Once air-dried, the samples were placed in sealed petri dishes and kept in a vacuum chamber in order to properly remove volatile substances and make them vacuum-compatible. The so prepared samples were mounted onto SEM stubs using sticky carbon tape and subsequently sputter-coated with an approximately 30 nm thick layer of gold–palladium (80%:20%) in order to make the surface electrically conductive, lest charging under the electron beam occur.

For micro-imaging of the samples, a Zeiss Merlin VP Compact (Carl Zeiss Microscopy, Oberkochen, Germany) field-emission scanning electron microscope was used. The electron acceleration voltage and beam current amounted to 2 kV and 250 pA, respectively. For image collection, an Everhard–Thornley type secondary electron detector was used.

2.5. Statistical Analysis

Statistical analyses were done by means of analysis of variance (ANOVA). For multiple comparisons, Tukey's post hoc test was used with a confidence level of 0.05. Graphpad (Graphpad Software, Inc., San Diego, CA, USA) was used to analyze the data. Non-metric multidimensional scaling (NMDS) analysis using the Bray–Curtis dissimilarity index was applied for showing the microbial community similarity relationships among midgut, hindgut, and Hungarian soda lake cultures, as well as upon different treatments of the same inoculum. The data set was reduced by removing the T-RFs below 1% abundance and the T-RFs that were present only in one of the triplicates.

3. Results and Discussion

3.1. Physical-Chemical Parameters

In this study, two alkali tolerant communities enriched from the midgut (sample names start with M) and hindgut (sample names start with H) of the *P. marginata* larva were tested for anaerobic digestion of chemically pretreated (denoted with T, while NT means lack of chemical treatment) wheat straw ground to two different sizes (1 mm and 250 μm). A third enrichment culture established in our laboratory, from a soda lake in Hungary, was used as a candidate culture [57] denoted with samples name starting with S37. The major focus of this study was the investigation of the microbiology of the enrichment cultures obtained from the gut of *Pachnoda marginata* larvae as a function of various pretreatments of substrate. The biogas and VFA production were used as an approximation of the conversion of the biomass and to estimate the biogas and methane yield. Due to the different experimental set-ups, our results are not directly comparable to standard BMP tests.

The highest volume cumulative biogas of 233.7 mL, 228.0 mL, and 213.0 mL was observed at 30 days of cultivation in the hindgut cultures with pretreated and non pretreated small straw particles and pretreated big straw particles, respectively (Figure 1B).

This was followed by enrichment cultures digesting alkaline treated smaller sized straw S37_250µm_T (202.1 mL) (Figure 1C) and M_250µm_T (198.4 mL) (Figure 1A). The biogas yield improvement when compared with untreated wheat straw ranged from 30.4% up to 82%. Memon and Memon [64] digested alkaline pretreated wheat straw (6% KOH during 4 days) and improved the biogas yield by 45% compared with non-treated wheat straw. No significant difference was found from 30 to 45 days of cultivation in the same culture, indicating that wheat straw digestion ceased already at 30 days of cultivation. Only in the cultures H_1mm_T and H_1mm_NT at 45 days there was still a significant slow increase in cumulative biogas production. This suggests that the microbiota present in the hindgut inoculum is more effective and can degrade fractions of the straw not available for the microbiota of the other enrichment cultures. The larva of the *P. marginata* beetle is able to naturally digest up to 63% of the ingested biomass [65]. The enrichment cultures from the *P. marginata* gut [37] and the *P. marginata* larva gut [38] harbor a microbial community able to degrade cellulose and hemicellulose and consequently hydrolyze them into smaller molecules for the cascade of microbial interactions during anaerobic digestion to be performed. Momoh and Ouki [66] reported in their study that large lignocellulosic biomass particles generate a higher affinity but lower initial rate of hydrolysis, while smaller particles generate a lower affinity but a higher initial rate of hydrolysis. Sträuber et al. [67] reported that in cultures with pretreated wheat straw, being bioaugmented or not, there was no difference in the total methane production. Bioaugmentation accelerated the beginning of the process, but the microorganisms added to the culture did not act in some specific way on the wheat straw fibers, thus not contributing to the increase in total methane production. However, the inoculum from the alkaline lake had a positive effect on the production of VFAs in pretreated wheat straw cultures when compared to the standard inoculum [67]. In Chandra et al. [68] low biogas production using either wheat or rice straw, small particles ceased already at 20 days of cultivation.

In general, the cultures needed 5 days to start methane production, with the highest activity occurring between the fifth and twentieth day of cultivation. The treatment with the highest specific methane yield up to 30 days of cultivation was H_250µm_T (215.2 mL g^{-1}_{VS}) (Figure 1E) followed by S37_250µm_T (190.7 mL g^{-1}_{VS}) (Figure 1F) and M_250µm_T (182 mL g^{-1}_{VS}) (Figure 1D). The highest increase in specific methane yield was found when non-pretreated wheat straw (1mm_NT) was compared to the most pretreated wheat straw (250µm_T) at 30 days of cultivation. It represented 191.6% and 148.9% increase in the midgut and hindgut cultures, respectively.

At 30 days of cultivation (Figure S2), in the cultures inoculated with hindgut enrichment, the alkaline pretreatment provided higher methane production in cultures with wheat straw 1 mm size. There was 99.3% increase in methane production in 1mm_NT compared to 1mm_T, while in 250µm_NT compared to 250µm _T the increase was only 23.5%. In the midgut cultures at 30 days of cultivation, specific methane production increased by 121.4% in 1mm_NT compared to 1mm_T, while in 250µm_NT compared to 250µm _T it increased only by 34.6%. In soda lake (S37) cultures, the specific methane production increased by 125.7% in 250µm_NT compared to 250µm_T, while it increased by 109.4% in 1mm_NT compared to 1mm_T. The data and the statistical analysis are provided in the Tables S1–S3 in the Supplementary Material. A similar straw (same region but different year of production) was tested in our previous studies and the average methane yield was 154 [65] and 173 mL_N CH_4 g^{-1}_{VS} [66]. The enrichment cultures used in these studies were not performing well regarding biogas yield, but they were still very effective when used as bioaugmentation cultures to enhance the methane yield with the standard inoculum.

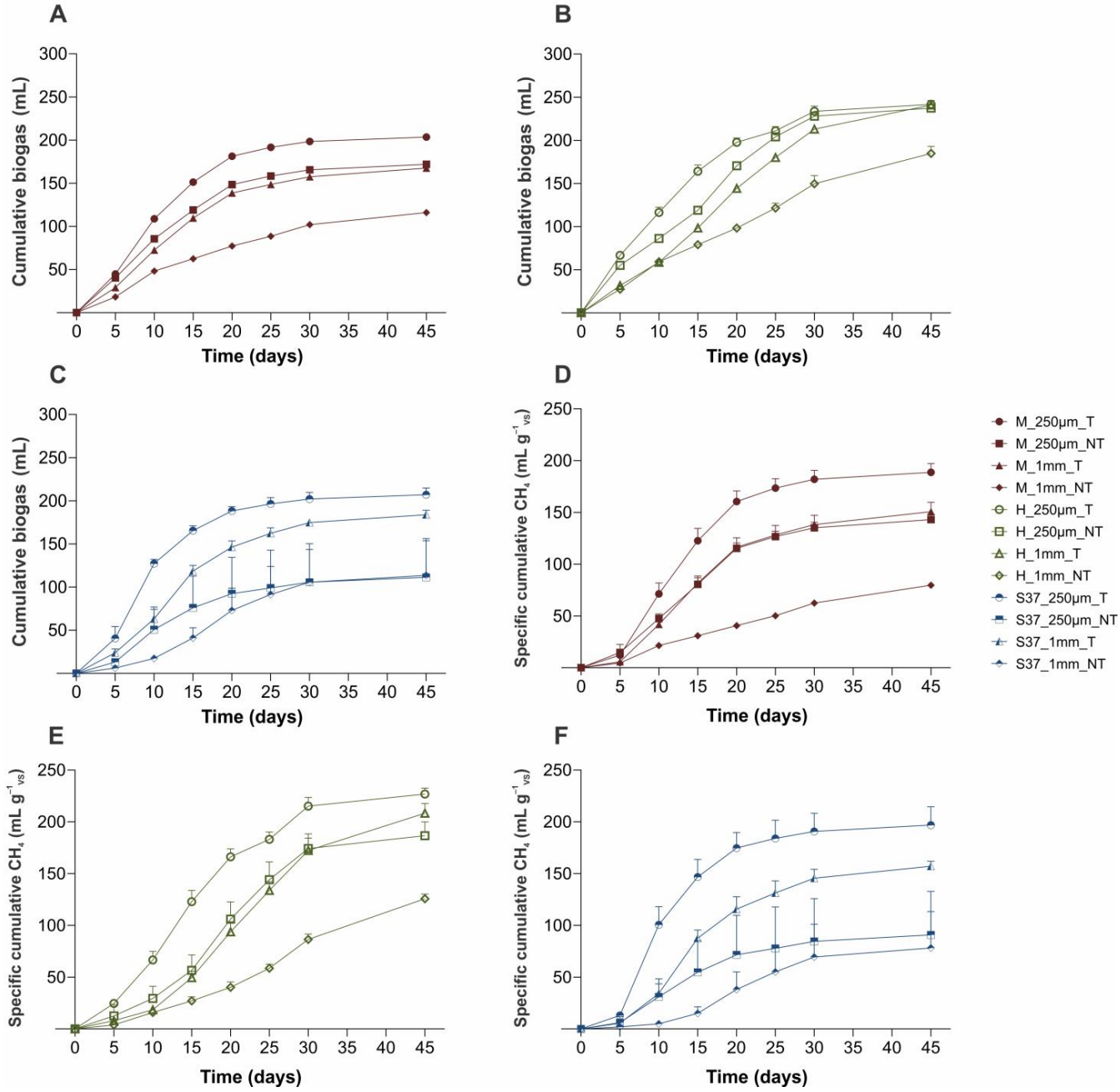

Figure 1. (**A**–**C**) Cumulative biogas; (**D**–**F**) Specific cumulative methane; over 45 days of cultivation in the midgut (M) and hindgut (H) larva cultures and Szarvas soda lake (S37) culture. The error bars represent the standard deviation of the mean of $n = 3$ (invisible error bars are smaller than the symbol).

There was no significant difference ($p > 0.05$) in specific methane production during 45 days of cultivation between 250µm_NT and 1mm_T treatments in the hindgut and midgut cultures. This finding suggests that for the enrichment culture microbiota from both larval gut compartments, the wheat straw size reduction had the same effect as the alkaline pretreatment with $Ca(OH)_2$. According to the studies of Moset et al. [19] where they combined alkaline and mechanical pretreatment, mechanically pretreated wheat straw did not require large additions of alkaline compounds, since the reduction of particle size already increases the biogas yield considerably. In all cultures, the worst straw conversion into methane was in 1mm_NT, the least pre-treated condition, with methane yield ranging from 62.4 mL g^{-1}_{VS} to 86.5 mL g^{-1}_{VS} in the three different inocula at 30 days

of cultivation. These values are considerably lower than some found in the literature. When starved pig slurry inoculum was used to digest untreated wheat straw (1 mm), 176 [69] and 205 mL g^{-1}_{VS} [70] were reached. Sträuber et al. [67] digesting untreated wheat straw (1 mm) with S37 inoculum reached 183 mL g^{-1}_{VS}. Sambusiti et al. [71] digested untreated wheat straw (1 mm) with cattle and poultry manure + waste activated sludge and reached 204 mL g^{-1}_{VS}. Jaffar et al. [22] using activated sludge from WWTP for digesting untreated wheat straw (5–10 mm) reached 184 mL g^{-1}_{VS}. Using digestate of a biogas plant during the anaerobic digestion of untreated wheat straw (0.65 mm), Zeback et al. [72] reached 261 mL g^{-1}_{VS}. However, lower methane yields were also reported such as 84.3 mL g^{-1}_{VS} [73] and below 100 mL g^{-1}_{VS} [74]. However, it should be emphasized that our enrichment culture set-up was different and, therefore, it is not directly comparable to standard BMP tests.

When wheat straw is subjected to only one of the treatments, either mechanical or alkaline, the increase in specific methane production, when compared to doubly pretreated (mechanically and alkaline) wheat straw at 30 days of cultivation is on average 33% lower for midgut cultures and 24% lower for hindgut cultures.

It is not new that alkaline pretreatment of LCB increases methane production in anaerobic systems [74]. The alkaline solution containing calcium hydroxidecan acts in different ways depending on the characteristics of the substrate. In the case of easily digestible biomass waste, it acts as a buffer, increasing the stability of the anaerobic digestion process, and in the case of lignocellulosic waste, it alters the fiber structure and reduces the amount of lignin, cellulose, and hemicellulose [75]. Sträuber et al. [67] improved methane production by 36% via pretreating wheat straw (10 mm) with $Ca(OH)_2$ for 24 h at 22 °C. Cern et al. [23] improved methane production by 37.3% via pretreating grass using similar conditions as in our study. Reilly et al. [76] combined $Ca(OH)_2$ pretreatment (7.4% $Ca(OH)_2$ for 48 h) with size reduction (3 mm) of wheat straw and after 30 days of digestion reached 335 mL g^{-1}_{VS} methane production, representing an increase of about 22% when compared to untreated straw of the same size. Other lignocellulose-rich organic wastes have also been pretreated with $Ca(OH)_2$ and showed an increase in methane yield. Mustafa et al. [77] working with sugarcane bagasse achieved 220 mL g^{-1}_{VS} methane yield, representing an increase of 66.3% when compared to the untreated biomass. The methane yield of alkaline pretreated rice straw in the study of Guan and collaborators [78] was 274.7 mL g^{-1}_{VS}, representing an increase of 57.6% when compared to the untreated biomass. Gu et al. [79] digested pretreated rice straw (8% $Ca(OH)_2$) and reached 330.9 mL g^{-1}_{VS}, representing an increase of ~32% when compared to the untreated biomass. Interesting that they found the pretreatment containing the most cellulose was the treatment with 5% $Ca(OH)_2$, but the methane yield was 10% lower than the pretreatment with 8% $Ca(OH)_2$. The pretreatment of wheat straw with NaOH has also been widely studied and shows positive results. Taherdanak and Zilouei [80] achieved 404 mL g^{-1}_{VS} methane yield after 30 days of digestion of wheat plant + straw + grains submitted to alkaline treatment with NaOH (75 °C), representing an increase of 54.5% when compared to non-pretreated biomass. After wheat straw sodium hydroxide pretreatment, similar improvement in methane production (52.7%) was achieved by Chandra et al. [81]. Janke et al. [82] digested alkaline (NaOH) pretreated sugarcane filter cake and reached 222 mL g^{-1}_{VS} CH_4, an increase of 20% when compared to non pretreated biomass. Contrary to the findings of this study, Chandra et al. [68] achieved lower methane production rates when reducing straw size from 1.5 mm to 300 µm (4.7% increase) than from 1.5 mm to 750 µm (38% increase). Even though mechanical pretreatment usually has lower methane yields when compared to alkaline pretreatment or steam explosion, it is still the most suitable because it is more economically feasible [32].

The pretreated small straw particle had the highest methane concentration for all three cultures. The methane concentration at 30 days of cultivation varied between 41% (M_1mm_NT) and 61% (H_250µm_T) (Figure 2D). Biogas with methane concentration

between 50 and 60% is considered good [83], but methane production can vary greatly according to the characteristics of the biomass in question [84].

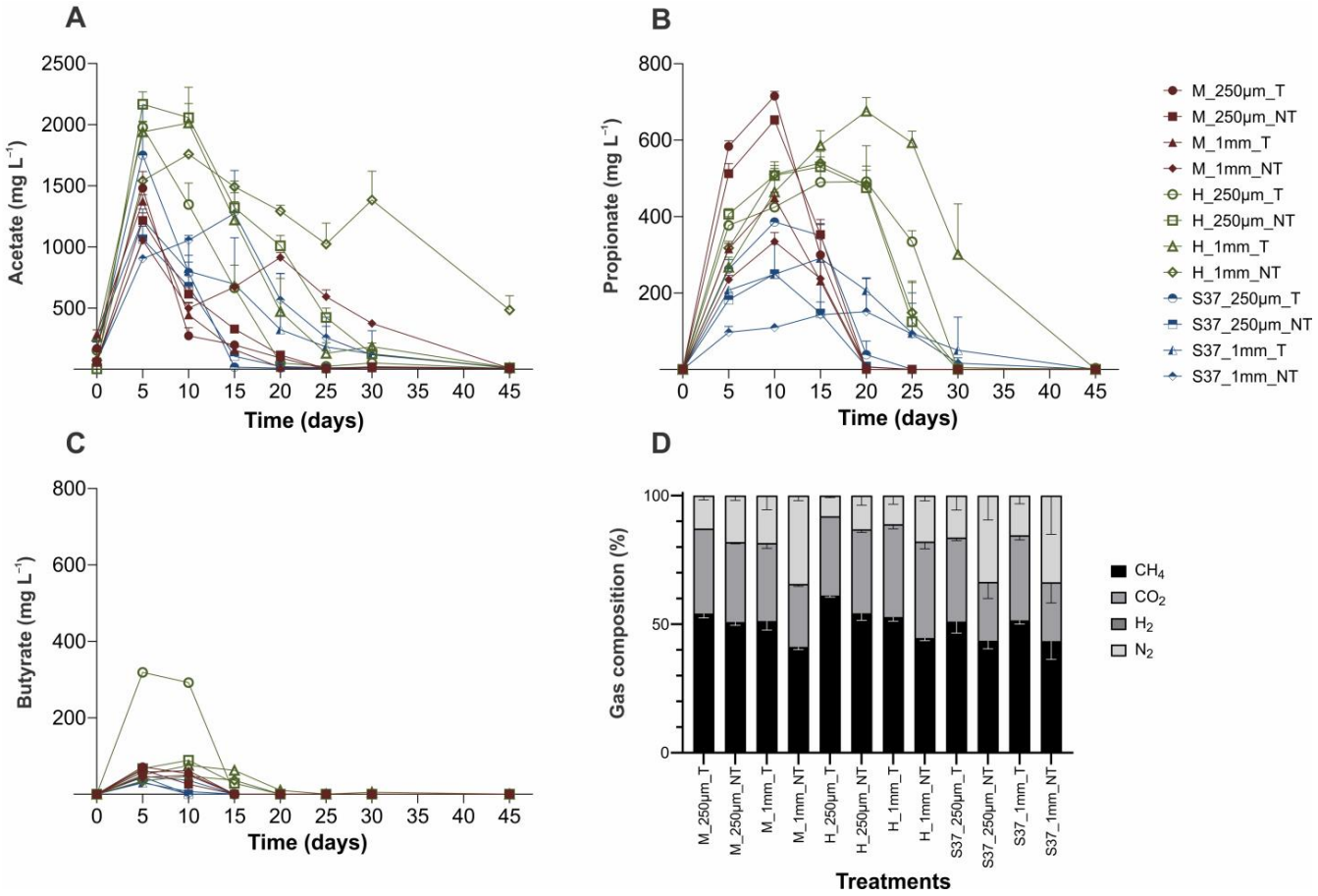

Figure 2. Volatile fatty acids concentration over 45 days of cultivation. (**A**) Acetate; (**B**) Propionate; (**C**) Butyrate. (**D**) Gas concentration at 30 days of cultivation. The error bars represent the standard deviation of the mean of $n = 3$ (invisible error bars are smaller than the symbol).

In almost all cultures, the peak of acetate concentration was registered at 5 days of cultivation, except for H_1mm_NT (10 days) and S37_1mm_NT (15 days). The highest acetate concentration was recorded in the culture H_250µm_NT (2166.8 mg L^{-1}) at 5 days of cultivation, but there was no statistically significant difference between cultures H_250µm_T and H_1mm_T at the same sampling point (Figure 2A). Volatile fatty acids, which are basically composed of acetic acid, propionic acid, butyric acid, and valeric acid, are important intermediates in the anaerobic digestion processes. It is observed that the accumulation of VFAs, mostly acetate, in the early days of anaerobic digestion is often reported in the literature [85,86], most likely due to the readily available organic compounds in wheat straw and the lag time that acetate consumers (acetoclastic methanogens and syntrophic acetate oxidizing bacteria (SAOB) with hydrogenotrophic methanogens) required to start converting acetate to methane. Sträuber et al. [67] observed 61% increase in volatile fatty acid (VFA) production during the anaerobic digestion of pre-treated wheat straw with $Ca(OH)_2$ when compared to untreated straw.

At 10 days of cultivation, the highest propionate concentration was recorded in the midgut cultures M_250µm_T (715.2 mg L^{-1}) and M_250µm_NT (652.8 mg L^{-1}), while the peak in the hindgut culture H_1mm_T (675.8 mg L^{-1}) was observed at 20 days of cultivation. From day 20, no propionate was detected in midgut cultures, while the other treatments showed no more propionate after 30 days of cultivation, except for the H_1mm_T treatment that ceased production only after 45 days of cultivation (Figure 2B).

Butyrate production ranged from 29.7 to 88.9 mg L^{-1} in the first 10 days of cultivation, and after 15 days of cultivation, no more butyrate was detected. The exception was the culture H_250µm_T, in which the concentration was 318.7 and 292.1 mg L^{-1} at 5 and 10 days of cultivation, respectively. In the hindgut cultures, butyrate production ceased after 20 days of cultivation (Figure 2C). Lower concentrations of propionic and butyric acid have been observed during anaerobic digestion of pretreated wheat straw than those found in the study by Sträuber et al. [67]. Although it is reported in the literature [33,87] that extreme reduction of straw particles (1 mm–0.35 mm) can promote acidification and consequent inhibition of anaerobic digestion (AD), the microbial community established from the gut content of *P. marginata* larvae overcame this issue. It is believed that because the archaeal community includes acetoclastic organisms such as *Methanosarcina* and the bacterial community contains SAOB [37], as soon as acids are produced in the system, acetoclastic methanogens readily convert them into methane or SAOB into H_2, CO_2 and formate, thus avoiding acidification during the AD process. The temporal imbalance between acidogenesis/acetogenesis and methanogenesis might be attributed to the gut origin of the enrichment cultures. In the gut system, the favored products from the animal's perspective are VFAs, while methane is more a side product, which might led to the selection of better acid than methane producers.

The pH of all cultures dropped from 9.0 to 7.4 (±0.4) at 5 days of cultivation (Figure S3). The lowest pH was registered at 5 days for H_250µm_T cultures (7.0).

3.2. Scanning Electron Microscopy

Wheat straw control samples with alkaline treatment ($Ca(OH)_2$) displayed crystal-like structures (Figure 3C,D), which are most likely calcium hydroxide crystals from the pretreatment. Crystal-like structures were not present in images of the control samples without alkaline pretreatment (Figure 3A,B). These crystals were not dissolved during the 45 days of cultivation, most probably due the constant, stabilizing pH around 9.0.

Micrometer-sized holes were observed in the fiber of the alkaline treated wheat straw. Most likely, delignification occurred during pretreatment, an event also observed in the study by Asghar et al. [88] after alkaline and thermal wheat straw pretreatment. Taherdanak and Zilouei also took scanning electron microscopy images before and after pretreatment of the wheat straw with NaOH and could see that some of the lignin was removed, increasing the porosity and accessibility of the biomass, thus increasing digestibility compared to the untreated wheat plant including straw and grains [80].

In general, more microbial colonization was observed on wheat straw in cultures that were alkali pretreated in all three types of cultures (H, M, and S37). Furthermore, in these cultures, more formation of extracellular polymeric substances (EPS) was observed (Figure 3G,H). Biofilms are found in various habitats in large numbers and are the main life form of bacteria and archaea [89]. The organization of microorganisms in biofilms can generate synergistic or antagonistic interactions, promoting the emergence of properties that are not predictable when cells are analyzed individually, generating interactions through signaling molecules and horizontal gene transfer [90]. EPS is a highly differentiated and functional matrix and among its many functions, has the function of protecting cells against toxic compounds and contact damage [91]. Calcium has been reported to be a crucial cofactor involved in EPS synthesis [75]. When calcium is present in the system, it binds with EPS, altering the responses of the microorganisms in the system [92–94]. Moreover, EPS can be degraded and contribute to increased methane production in anaerobic systems [95]. Microbial colonization and formation of EPS was also observed on wheat straw fibers not pretreated with calcium hydroxide, but visually in smaller amounts when compared to pretreated straw (Figure 3E,F).

Comparing results obtained with fluorescent optical microscopy analyses, previously performed in our laboratory on samples from wheat straw enrichment cultures from midgut and hindgut of *P. marginata* (results not shown) with SEM analysis, it can be concluded that

part of the microorganisms attached to the wheat straw were methanogens based on F_{420} autofluorescence.

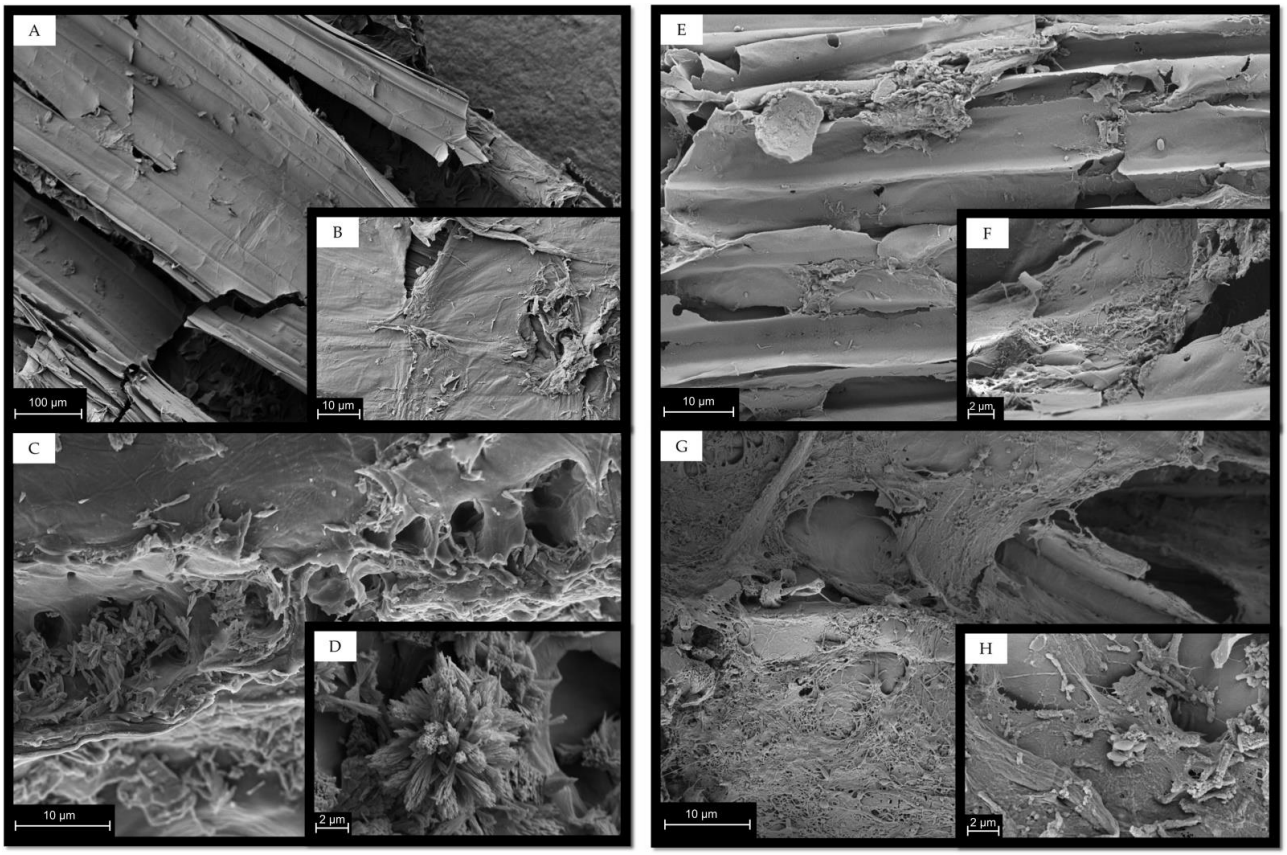

Figure 3. Scanning electron microscopy images of the wheat straw after 45 days of anaerobic digestion, negative control cultures (NC), and hindgut cultures (H). (**A**) NC_1mm_NT—100 µm zoom in, non treated wheat straw; (**B**) NC_1mm_NT—10 µm zoom in, detailed non treated wheat straw; (**C**) NC_1mm_T—10 µm zoom in, crystal-like structures; (**D**) NC_1mm_T—2 µm zoom in, detailed crystal-like structures; (**E**) H_1mm_NT—10 µm zoom in, wheat straw without alkaline pretreatment in hindgut culture; (**F**) H_1mm_NT—5 µm zoom in, detailed wheat straw without alkaline pretreatment in hindgut culture; (**G**) H_1mm_T—10 µm zoom in, EPS network; (**H**) H_1mm_T—2 µm zoom in, detailed EPS network and microorganisms.

3.3. Microbial Community

Terminal-restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA gene amplicons was used to assess the bacterial community composition of 12 different treatments at 5, 15, and 30 days of cultivation and non-metric multidimensional scaling (NMDS) analysis was applied to follow bacterial community dynamics (Figure 4A). In our previous work [37], we showed the bacterial and methanogenic community compositions of the larval gut and the first transfers of enrichment cultures on wheat straw. It was observed that after a few transfers the microbial community composition was already stabilized. Since one of the treatments (1mm_NT) was considered in this experiment to have similar conditions used during the establishment of the enrichment cultures, we assumed this as the initial community. A clear separation of midgut and hindgut cultures was observed at 30 days of cultivation. It is evident that the H_250µm_NT bacterial community was the most distinct from the other cultures. It is noted that the different treatments also clustered separately within the samples derived from the same inoculum at 30 days of cultivation. Even though the 250µm_NT and 1mm_T cultures had similar volatile fatty acid and specific methane production profile, the bacterial community dynamics are different between these two cultures from the same inoculum. In the study by Sträuber et al., the mi-

crobial community of the cultures with alkaline pretreated wheat straw clustered together regardless of the inoculum used, but they differed from the cultures with non-alkaline pretreated wheat straw [67]. The microbial community structure also shifted over time. Three different clusters were formed in the midgut and hindgut cultures (Figure S4A,B) but not in the soda lake cultures (Figure S4C), where only 5 days of cultivation samples were grouped separately. In co-digestion of wheat straw pretreated with H_2O_2 and cow manure, Song and Zhang [73] also observed the microbial community dynamics over 35 days of cultivation, where three different clusters were formed, beginning (day 1), middle (days 7 to 21), and late samples (days 28 to 35). The S37 samples also grouped separately according to the given straw pretreatment, indicating that the different combinations of treatments for this inoculum shaped the community in different directions (Figure 4B).

The richness analysis of the bacterial community over 30 days of experiment showed that in the midgut culture samples the richness increased over time except for 250µm_T treatment (Figure 4C). In the hindgut culture samples, the richness was highest at 30 days of cultivation in the alkaline treated cultures (250µm_T and 1mm_T). In the lake culture samples, the highest richness was observed at 15 days of cultivation, but with no statistically significant difference compared to samples at 30 days of cultivation ($p > 0.05$). In the M_250µm _T_NT, H_250µm _T, and H_1mm_NT, the diversity of the bacterial community decreased at 15 days of cultivation and increased again at 30 days of cultivation, and in most cases, the community was more diverse at the beginning than at the end of the 30 days of experiment (Figure 4D). In the M_1mm_T_NT and H_1mm_NT cultures, the diversity increased over the 30 days of cultivation, with the M_1mm_NT culture being significantly more diverse at 30 days than at 5 days. The evenness analysis for the microbial community showed no significant difference for any pretreatment, on any of the sampling days, in any of the three inocula (Figure 4E).

3.4. Methanogenic Community Structure

To assess the methanogenic community composition of various treatments at 5, 15, and 30 days of cultivation, terminal-restriction fragment length polymorphism (T-RFLP) of *mcrA* gene amplicons was used. NMDS analysis was used to better understand the dynamics of the methanogenic community.

The larva gut cultures clustered together and separated from the lake culture samples, indicating that the methanogenic community present in the cultures from the larva gut differed from those in the alkaline lake in Hungary (Figure 5A). When the methanogenic communities were analyzed separately over time (5, 15 and 30 days of cultivation) for hindgut and soda lake inoculum (Figure S5B,C), no clear change in community dynamics was observed in contrast to the bacterial communities. However, in the midgut cultures, three clusters were formed, one at 5 days, one at 15 days, and one at 30 days of culture (Figure S5A).

Among the three different inocula used in this experiment, the richness of the methanogenic community was higher in the lake cultures, and for these, the highest richness was found at 5 days of cultivation for most of the treatments. The cultures from the larvae gut had similar richness in all samples; however, in the midgut cultures the richness was higher at 30 days of cultivation while in the hindgut cultures at 15 days of cultivation (Figure 5B).

There was no significant difference in the diversity of the methanogenic community within the same inoculum in the different pretreatments and different sampling days, as well as between the midgut and hindgut cultures. When comparing midgut and hindgut cultures with the lake cultures, significant differences were observed (Figure 5C). The evenness analysis of the methanogenic community showed that it increased significantly, especially in the M_250um_T_NT and H_250_NT cultures, when comparing samples at 5 days and at 30 days of cultivation with the same inoculum and treatment. For M_1mm_NT, there was a significant increase in the evenness from 5 to 15 days of cultivation (Figure 5D). The community dynamics of the methanogenic communities in lignocellulose-degrading methanogenic enrichment cultures have not been largely investigated according to our

best knowledge. Song and Zhang [73] found that the main change in the methanogenic community during the digestion of pretreated wheat straw with cow manure, was a decrease in acetoclastic methanogens and increase in hydrogenotrophic methanogens.

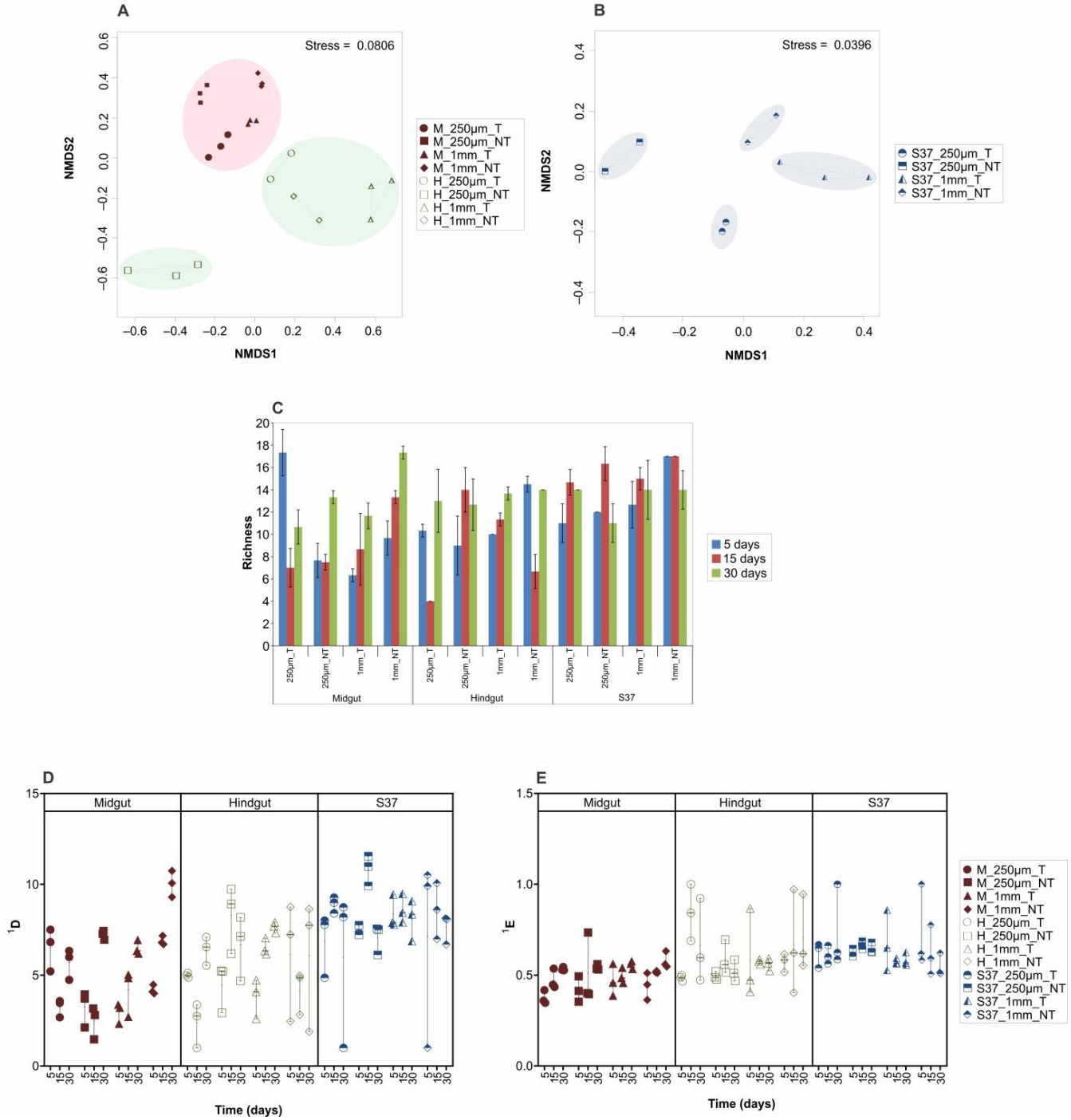

Figure 4. Bacterial community dynamics and diversity. The non-metric multidimensional scaling (NMDS) plot of T-RFLP profiles of bacterial 16S rRNA gene amplicons obtained with restriction enzyme *RSa* I. Plot based on Bray–Curtis dissimilarity index showing the similarity relationships of the (**A**) midgut and hindgut cultures, (**B**) Szarvas soda lake cultures. Microbial communities at 30 days of cultivation. (**C**) Richness. (**D**) Diversity of order 1. (**E**) Evenness of order 1. Colors and symbols were used to indicate the different cultures as follows: red: midgut, green: hindgut, blue: S37. To differentiate between treatments across cultures the following coding was used: circles symbols: 250µm_T, square symbols: 250µm_NT, triangle symbols: 1mm_T, rotated square symbols: 1mm_NT.

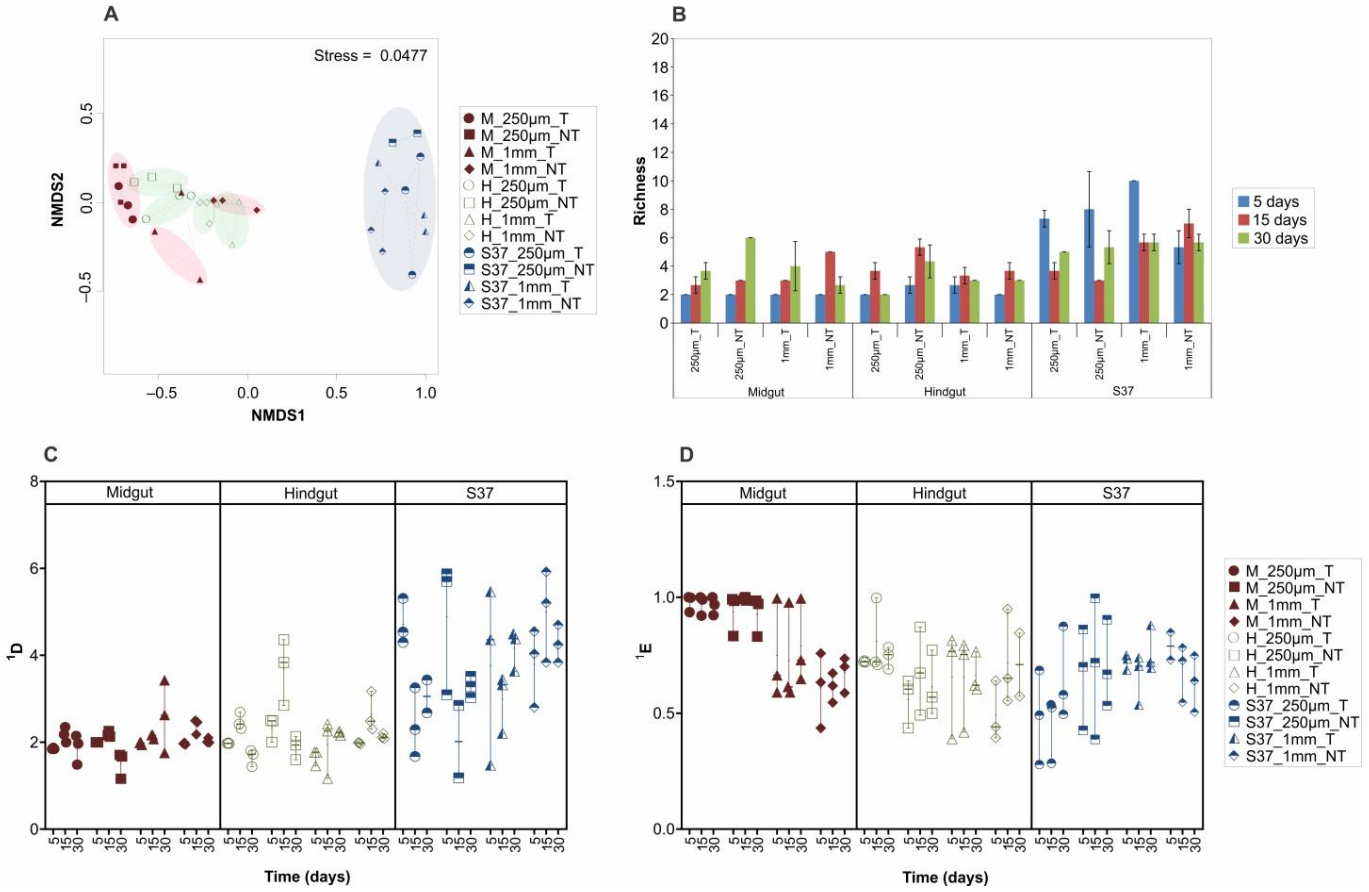

Figure 5. Methanogenic community dynamics and diversity. (**A**) The NMDS plot of the T-RFLP data obtained by *BstN* I restriction enzyme. Plot based on Bray–Curtis dissimilarity index showing the similarity relationships of the midgut, hindgut and Szarvas soda lake cultures. Microbial communities at 30 days of cultivation. (**B**) Richness. (**C**) Diversity of order 1. (**D**) Evenness of order 1. Colors and symbols were used to indicate the different cultures as follows: red: midgut, green: hindgut, blue: S37. To differentiate between treatments across cultures the following coding was used: circles symbols: 250µm_T, square symbols: 250µm_NT, triangle symbols: 1mm_T, rotated square symbols: 1mm_NT.

The two most abundant methanogenic families were Methanomicrobiaceae and Methanosarcinaceae in all cultures (Figure 6). They were also previously reported to have a good share of the methanogenic community in the enrichment cultures from *P. marginata* larva gut [37]. The family Methanosarcinaceae is a versatile group that can utilize methyl compounds, CO_2/H_2 or even acetate to produce methane [96]. Methanomassiliicoccacae with H_2-dependent methylotrophic methanogenesis [97,98] was detected in the midgut and hindgut cultures at 15 days of cultivation with non-alkaline treated wheat straw and at 5 days of cultivation in S37 cultures with alkaline treated wheat straw. They were most probably present in other cultures in low abundance below the detection limit of T-RFLP. Members of the Methanomassiliicoccacae family are found in biogas systems in low abundance [99] and in higher abundance in cow manure [100,101] and rumen [102]. They are also present in anaerobic systems with high ammonia levels and high organic load [103–105].

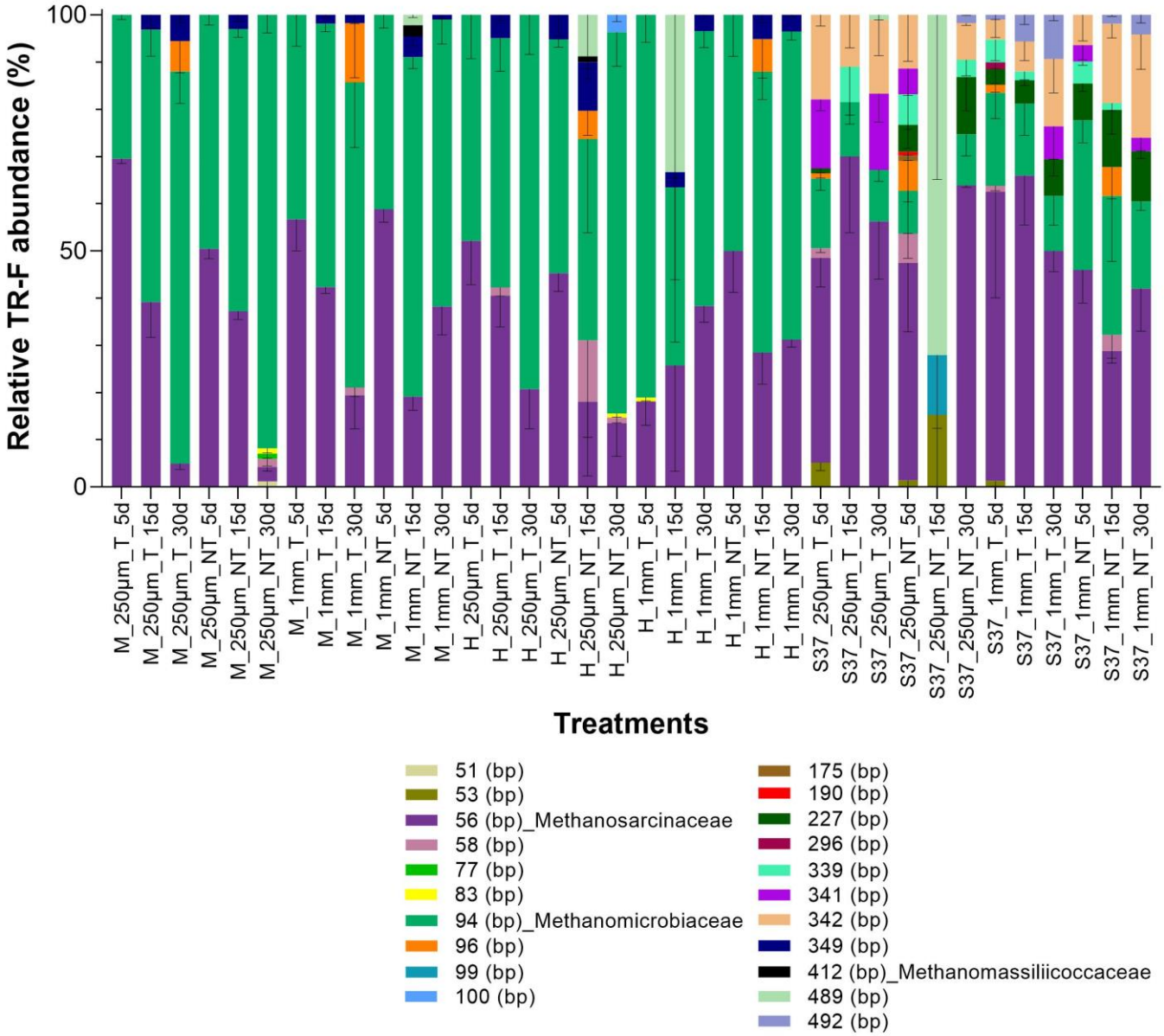

Figure 6. Methanogenic community composition analyzed by T-RF abundance (n = 3) of the archeal gene *mcrA* digested with the restriction enzyme *BstN* I at 5, 15, and 30 days of cultivation in midgut (M), hindgut (H), and Szarvas soda lake (S37) cultures.

4. Conclusions

The wheat straw pretreatment had a positive effect on all enrichment cultures, independent of the inoculum source. Using the enrichment culture from the gut microbiome of *P. marginata* for the anaerobic digestion of mechanically and chemically pretreated wheat straw, the highest production of acids and methane was achieved. The methanogenic community was composed of hydrogenotrophic and versatile methanogens capable of acetotrophic, hydrogenotrophic, and methylotrophic methanogenesis. The composition and dynamics of the microbial communities from the three different inocula played an important role during the conversion of wheat straw into VFAs and methane. The Hungarian soda lake microbial community was less effective compared to the gut enrichment cultures and more dependent on the alkaline treatment to reach the highest methane yield. In contrast, the microbial community of the larval gut enrichments responded better to the mechanical treatment alone in comparison to the Hungarian lake cultures. The alkaline pretreatment has acted on the wheat straw fibers and promoted EPS growth. It is evident

that microorganisms from the intestine of *P. marginata* were able to better convert LCB under the given conditions, when compared to the soda lake cultures. In the cultures inoculated with the larvae gut enrichment, the combination of two pretreatments did not bring the combined increase in methane yield.

Considering the moderate methane yields and the VFA accumulations observed in this study a direct application of these enrichment cultures are not recommended. However, they might be utilized for anaerobic fermentation aiming at VFA production or in two-stage systems more comparable to the compartmentalization of larva gut. However, such studies should be combined with economic analyses to confirm the viability of the results.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation9010060/s1, Figure S1: Experiment scheme; Figure S2: Specific cumulative methane at 30 days of cultivation; Figure S3: pH over 45 days of experiment; Figure S4: NMDS plot for 16S; Figure S5: NMDS plot for mcrA; Table S1: Specific cumulative methane at 30 days of cultivation data; Table S2: The three-way ANOVA analysis of specific cumulative methane at 30 days of cultivation; Table S3: Tukey's multiple comparisons test of specific cumulative methane at 30 days of cultivation.

Author Contributions: Project concept, M.N., W.L. and B.G.S.; methodology, M.N., W.L. and B.G.S.; contribution to the bioinformatic analysis and discussion of the results, M.N., M.S., H.B.İ., H.H., W.L. and B.G.S.; writing—original draft preparation, B.G.S.; writing—review and editing, M.N., M.S., H.B.İ., H.H., W.L. and B.G.S.; visualization, B.G.S.; supervision, M.N. and H.H.; project administration, M.N.; funding acquisition, M.N. and H.H.; All authors have read and agreed to the published version of the manuscript.

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