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Acidogenic Fermentation of Food Waste for the Production of Short-Chain Fatty Acids: The Impact of Inoculum Type and Inoculum Heat Pretreatment

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Abstract: Acidogenic fermentation is an emerging biotechnology that allows for the utilization of food waste as a feedstock to produce high-value products such as short-chain fatty acids (SCFAs), effectively offering a tangible solution for food waste management as well as resource recovery. The objectives of the current study were to identify the ideal inoculum, waste-activated sludge (WAS) or anaerobic digester sludge (AD), for the acidogenic fermentation of food waste at room temperature, as well as to evaluate the impact of heat pretreatment of these inoculums on fermentation performance. The maximum hydrolysis yield of 399 g sCOD/kg VS $_{added}$ was obtained when untreated AD was used as the inoculum, whereas the pretreated AD inoculum provided the highest SCFA yield and conversion efficiency of 238 g $sCOD_{SCFA}$/kg VS $_{added}$ and 71%, respectively. Heat pretreatment had a detrimental impact on the WAS inoculum, leading to lower hydrolysis and SCFA yields, but exerted a positive influence on the AD inoculum. The microbial community showed that heat pretreatment negatively impacted the abundance of non-spore-forming hydrolytic and acidogenic microorganisms. Overall, this study demonstrates the critical role of inoculum type and heat pretreatment in optimizing the acidogenic fermentation process, laying the groundwork for future refinements in SCFA production from food waste through inoculum design.

Keywords: acidogenic fermentation; food waste; inoculum; heat pretreatment; short-chain fatty acids; organic waste management

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1. Introduction

Rapid growth in global population and urbanization have led to an increase in the demand for commodity chemicals and energy, placing stress on the available resources as well as raising concerns about climate change and energy security. This necessitates the identification and development of new processes that rely on renewable resources as feedstock to support our growing demands [1,2]. For instance, organic materials in domestic and industrial waste have the potential to be transformed into biofuels and biochemicals [3,4]. The use of organic waste materials as feedstock has two benefits: (i) reducing the amount of waste entering the environment, and (ii) enabling the recovery of valuable resources that would have been lost. In recent years, the food waste fraction of municipal solid waste (MSW) has emerged as a promising feedstock due to its high abundance (30–60% of MSW), biodegradability, and carbon content derived from carbohydrates, proteins, and lipids [2,5]. In particular, there has been an increased focus on the conversion of food waste into short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which have a wide

range of applications in various industries (i.e., chemical, pharmaceutical, and cosmetic) and also serve as a precursor for different bioproducts and biofuels such as butanol [6,7].

Acidogenic fermentation is an anaerobic bioprocess by which food waste can be converted into SCFAs. This process utilizes a mixed microbial community which eliminates the need for sterile conditions and costly pure-culturing of strains [8,9]. Acidogenic fermentation involves a series of three sequential biochemical reactions such as hydrolysis, acidogenesis, and acetogenesis. During hydrolysis, extracellular hydrolytic enzymes break down complex polymeric substrates into soluble monomers. Subsequently, in the acidogenesis and acetogenesis steps, these soluble monomers are transformed into SCFAs [8,10]. The efficient production of SCFAs necessitates the optimization of these biochemical reactions which are significantly influenced by key operating parameters such as the inoculum, pretreatment, pH, temperature, and fermentation time.

Only a few studies have investigated the effect of different inoculums on the acidogenic fermentation of food waste. Most of the studies, presented in Table 1, relied on the utilization of anaerobic inoculums to achieve efficient SCFA production, owing to the abundance of fermentative microbes [1,11,12]. For instance, refs. [1,12] demonstrated SCFA yields of 228 g COD/kg VS and 205 g COD/kg VS, respectively, using an anaerobic inoculum from food waste at mesophilic temperature. However, some studies have also suggested that the use of an aerobic inoculum also enhances SCFA production due to its higher hydrolytic activity which increases the production of hydrolysates and consequently enhances acidogenic efficiency [8,13,14]. In this context, ref. [14] reported the highest SCFA yields (i.e., 365 g COD/kg VS) from food waste using an aerobic inoculum at mesophilic temperature. Despite these promising results, determining the ideal inoculum for efficient SCFA production remains a challenge due to the variability in operating conditions in these studies (e.g., pH, food waste composition, inoculum-to-substrate ratio, etc.). Thus, a comprehensive comparative investigation is essential to understand the impact of aerobic and anaerobic inoculum on the acidogenic fermentation of food waste.

Table 1. Impact of inoculum type on hydrolysis and acidogenesis of food waste in the literature.

Substrate	Inoculum Type/ Pretreatment	Reactor Type	Temperature	pH	Hydrolysis Yield (g sCOD/ kg VS_{fed})	Acidification Yield (g COD $_{SCFA}$/ kg VS $_{added}$)	SCFA Concentration (g/L)	Hydrogen Yield (mL H_2/ gm VS $_{added}$)	Reference
Urban biowaste	AD/thermal	Batch	37 °C	NC		228			[1]
Food waste	AD/thermal	Batch	37 °C	Initial 9.3	12	284			[15]
Food waste	AD/thermal	Batch	52–56 °C	Initial pH 7		700			[16]
Food waste	AD/thermal	Batch	37 °C	NC		205		105	[12,14]
Synthetic food waste	WAS/thermal	Batch	39 °C	6 6.5 7				62 * 73 * 42 *	[13]
Food waste	Primary sludge	Plug flow reactor	35 °C 18 °C	pH 6.5/NC		365 490			[14]
Food waste	Mesophilic AD	Batch semi-continuously fed	35 °C/55 °C	4.5 5.5 6.5		576 668 643		5 2.5 1.3	[17]
Food waste	AD	Batch	30 °C	4 5 6 NC	4.71 ** 1.14 ** 1.87 ** 2.4 **	124 § 651 § 918 § 337 §			[18]
	WAS	Batch	30 °C	4 5 6 NC	2.03 ** 1.07 ** 1.52 ** 1.25 **	206 § 445 § 481 § 229 §			
Food waste	AD	Batch	35 °C	7	24 g/L		17		[19]
			55 °C	7	28 g/L		10		
			70 °C	7	36 g/L		12		

Table 1. *Cont.*

Substrate	Inoculum Type/ Pretreatment	Reactor Type	Temperature	pH	Hydrolysis Yield (g sCOD/ kg VS_{fed})	Acidification Yield (g COD $_{SCFA}$/ kg VS $_{added}$)	SCFA Concentration (g/L)	Hydrogen Yield (mL H_2/ gm VS $_{added}$)	Reference
Food waste	WAS	LBR	22 °C	6.5	491	375			[8]
Food waste	AD/thermal	LBR	22 °C	7	693	649			[11]
Food waste	AD sludge	Batch	21 °C	5.5–6			27	395	[20]
Food waste	Enriched inoculum	LBR	21 °C	6	774	697			[21]
Food waste	AD sludge	Batch	22 °C	6.5	567	462			[9]

AD (anaerobic digestor sludge); WAS (waste-activated sludge); NC (not controlled); HRT (hydraulic retention time). * NL H_2/kg VS. ** g sCOD/g VS reduced. § mg/g VS removed.

During mixed culture fermentation such as acidogenic fermentation, the microorganisms compete for the available nutrients, leading to the formation of undesirable by-products as well as the consumption of the target compound. For instance, during acidogenic fermentation, microbes consume the SCFAs to produce metabolites such as methane and hydrogen, thereby leading to reduced yield [1,22]. Therefore, to improve SCFA yields, it is vital to prevent SCFA loss to methanogens during mixed culture anaerobic digestion. Various methods such as heat pretreatment, pH adjustment, chemical inhibitors, and aeration have been employed to prevent this loss [18,23]. Among these strategies, heat pretreatment has been the most widely employed to prevent methanogenesis by eliminating the associated microbes due to their heat sensitivity [24]. However, methanogenesis can also be inhibited by the accumulation of SCFAs and the short fermentation time (<14 days) used for acidogenic fermentation. Thus, making the significance of heat pretreatment on SCFA production as well as its impact on the microbial community structure during acidogenesis unclear.

Temperature is one of the key parameters that may have a profound impact on acidogenic fermentation performance as well as its feasibility [14,19]. Operating bioreactors at higher temperatures increase energy demands, leading to elevated operational costs and decreased economic viability [25]. However, a few studies reported comparable yields even at a low temperature to those found in higher temperature operations, thus suggesting the potential to improve food waste solubilization and SCFA production by optimizing other operating parameters, such as inoculum [8,11,14]. A research gap exists in the exploration of the impact of different inoculums on acidogenic digestion performance at room temperature (22 °C), which is investigated in the current study.

The present study aims to enhance the acidogenic fermentation process of food waste at room temperature. To this effect, (i) the impact of two different inoculums i.e., aerobic and anaerobic, on food waste hydrolysis and SCFA production was evaluated, (ii) the effect of heat pretreatment of the inoculums (aerobic and anaerobic inoculum) on the process performance was assessed to identify its requirement and applicability in the acidogenic fermentation process, and (iii) a microbial community analysis was performed to further elucidate the impact of inoculum heat pretreatment on the microbial structure as well as diversity at the end of acidogenic digestion.

2. Material and Methods

2.1. Food Waste Characteristics

Food waste used in this study was collected from a local restaurant (Eats Café, Ottawa, ON, Canada) and supermarket (Walmart, Ottawa, ON, Canada) over 15 days. The collected food waste mainly consisted of vegetables, fruits, bread, and meat, along with some non-biodegradable materials such as glass, plastic cutlery, etc., which were manually segregated from the food waste. To facilitate storage, the larger food waste particles, such as meat, rotten vegetables, and bread, were chopped into small particles of approximately 1 cm in size using a mesh chopper (Starfrit, Ottawa, ON, Canada). The food waste was then thoroughly mixed in a large container and stored at −10 °C to prevent decomposition. Prior

to experimental use, the required quantity of food waste was removed from the freezer and thawed at 4 °C for 12 h and then blended in an electric blender with a 50 mm stainless steel blade to obtain a homogenous slurry. Table 2 summarizes the characteristics of the food waste slurry used in this study.

Table 2. Characteristics of food waste and inoculums used in this study.

Parameter	Food Waste Slurry	Centrifuged AD Inoculum	Centrifuged WAS Inoculum
TS (g/kg)	234.4 ± 0.42	102.3 ± 1.83	88.6 ± 1.88
VS (g/kg)	224.2 ± 0.39	60.4 ± 1.05	64.5 ± 0.96
VS/TS (%)	95.64 ± 0.01	59.1 ± 0.06	72.8± 0.50
TS (%)	23.4 ± 0.04	10.2 ± 0.18	8.9 ± 0.19
VS (%)	22.4 ± 0.04	6 ± 0.11	6.5 ± 0.10

2.2. Inoculum and Heat Pretreatment

To assess the impact of the inoculum, two types of inoculums were tested: anaerobically digested (AD) sludge and waste-activated sludge (WAS). The AD sludge was collected from an anaerobic mesophilic digester, while the WAS was obtained from the secondary tank of the Robert O Pickard Environmental Center (ROPEC) wastewater treatment facility in Ottawa, Ontario, Canada. Both inoculums were stored at 4 °C until further use. Before use, each inoculum was centrifuged to collect biomass and remove soluble organic matter. Inoculums with and without heat pretreatment were used in the batch acidogenic fermentation study. For heat pretreatment, the inoculum (centrifuged biomass) was suspended in 400 mL distilled water (working volume) and heated at 75 ± 5 °C for 15–20 min on a hot plate with a magnetic stirrer [11]. A manual thermometer (Fisherbrand, Ottawa, ON, Canada) was used to measure and maintain the temperature. Before experimental use, the inoculum suspension was allowed to cool down to room temperature (22–24 °C). The characteristics of the inoculums used in this study are shown in Table 2.

2.3. Batch Fermentation Study

The batch acidogenic fermentation study was carried out for 13 days. The experiments were performed in triplicate using 500 mL cylindrical glass reactors (QCVIALZ, MO, US) with a working volume of 400 mL. The reactors were operated at room temperature (22–24 °C). Twelve batch reactors were divided into groups of three and inoculated with heat-pretreated or untreated inoculum types. The experimental conditions are summarized in Table 3. The food waste with no inoculum served as the control. An inoculum-to-substrate ratio (ISR) of 5–8% has been shown to be efficient for hydrolysis and acidogenesis in the acidogenic fermentation process [17,26,27]. Thus, in the current study, each reactor was initially loaded with a food waste slurry at a volumetric load of 16 g VS/L and an ISR of 6%. There was no addition of additional nutrients as food waste is a highly degradable material rich in carbon and nutrients [2,28,29]. The initial pH was adjusted to 7 using 1N NaOH or 1N HCL. The total solids (TS) and volatile solids (VS) for each reactor were analyzed at the beginning of the experiment.

After loading and initial pH adjustment, each reactor culture and headspace was purged with nitrogen gas for 2–3 min. The reactors were then sealed and placed on a rotary shaker (VWR DS-500E Ortibal Shaker, Mississauga, ON, Canada) at 180 RPM. The shaker provided the necessary mixing to avoid the settling of coarse food particles without using an agitation device such as a magnetic stirrer or propeller. The off-gas of each reactor was measured daily throughout the 13 days of the fermentation period. A 50–100 mL glass syringe (Cadence Science, Crenston, Italy) was used to collect and measure the pressurized off-gas from the headspace of each reactor. The collected off-gas was further analyzed using a gas chromatograph (Agilent 990 Micro GC, Mississauga, ON, Canada) to quantify the composition. Additionally, sampling of the fermenting culture

was performed daily for the first five days (days 1–5) and then on alternate days (days 7, 9, 11, and 13). A 10 mL fermenting culture sample was withdrawn during each sampling period through the septum from a reactor for further analysis. Enhanced hydrolysis and acidogenesis activities have been reported at slightly acidic to neutral pH (6–7) during the fermentation process [8,9]. Previous studies have reported NaOH as an efficient pH regulator compared to other pH regulators to provide stable neutral pH conditions, owing to its high solubilization and acidification yield, and improve the buffering capacity of the system [30,31]. Refs. [30,31] investigated the effect of different pH regulators, i.e., NaOH, $CaCO_3$, $Ca(OH)_2$, Na_2CO_3, and KOH on the hydrolysis and acidification of food waste. They reported 2–3 times higher SCFA production with the NaOH regulator compared to the other pH regulators. Therefore, our study was conducted at a neutral pH, with adjustments made every 24 h using NaOH as a pH regulator to maintain a pH of 7 ± 0.5.

Table 3. Experimental conditions performed during batch fermentation study.

Experiment	Designation	Inoculum	Substrate	Heat Pretreatment Applied to Inoculum	pH
1	Control	No inoculum	Food waste	-	7
2	AD pretreated (heated)	AD sludge	Food waste	Yes	7
3	AD untreated	AD sludge	Food waste	No	7
4	WAS pretreated (heated)	Waste-activated sludge	Food waste	Yes	7
5	WAS untreated	Waste-activated sludge	Food waste	No	7

2.4. Analytical Procedure

Total solids (TS) and volatile solids (VS) were analyzed according to standard methods (EPA, 2001). The soluble chemical oxygen demand (sCOD) and SCFA analysis were performed on collected samples. For sCOD analysis, the sample was initially filtered with a vacuum filter using a 0.45 µm pore size filter membrane, followed by analyzing it using a COD reagent tube (SCP Science, Baie-d'Urfé, QC, Canada). To analyze SCFAs, the culture samples were filtered through 0.45 µm and 0.25 µm filter membranes using a vacuum filter. High-performance liquid chromatography (HPLC) was employed to determine the SCFAs present in the filtered sample. The mobile phases used in HPLC comprised 100% Acetonitrile and 2.5 mM methane sulfonic acid. The HPLC system (Thermo Scientific Ultimate 3000 RSLC, Mississauga, ON, Canada) was equipped with a column (Acclaim™ OA 5 micro m, 4 × 150 mm) maintained at 30 °C and the mobile phase at a fixed flow rate of 1 Ml/min. The detector's absorption wavelength was set at 210 nm to measure the concentrations of the three main SCFA components: acetate, propionate, and butyrate. The off-gas composition analysis involved injecting 200 nanoliters of the off-gas into a gas chromatograph (Agilent-990 Micro GC) equipped with a thermal conductivity detector (TCD) and a CP-PORABOND Q 1 m × 0.8 mm carboxen column. Argon was used as the carrier gas. The gas chromatograph detected hydrogen, nitrogen, methane, and carbon dioxide. The injection port temperature was set at 40 °C, and the column temperature was set at 80 °C.

2.5. Performance Indicator

The cumulative sCOD production was measured for the samples which depicts the amount of soluble organic matter produced in the reactor over time. It was calculated by multiplying the volumes of the culture with a measured concentration of sCOD [8]. Further, the performance of the batch fermentation process was assessed based on four different indicators: (1) hydrolysis yield, (2) SCFA or acidification yield, (3) SCFA: sCOD ratio, and (4) hydrogen yield.

(i) Hydrolysis yield estimates the conversion efficiency of the particulate organic substrate to soluble organic matter. It can be calculated as follows:

$$\text{Hydrolysis yield } (\text{mg cum sCOD/kg VS}_{\text{added}}) = \frac{\textit{Total cumulative SCOD produced } (\text{mg cum sCOD})}{\textit{Initial VS}_{\textit{added}} \textit{ to the reactor } (\text{kg})} \quad (1)$$

where: total cumulative sCOD produced (mg cum sCOD) = final cumulative sCOD of fermented culture–initial cumulative sCOD of unfermented culture.

$$\text{Initial VS}_{\text{added}} \text{ to the reactor} = \text{VS of inoculum (kg)} + \text{VS of food waste (kg)}$$

(ii) The SCFA yield was estimated based on total SCFAs produced in milligram COD equivalents to the initial VS (gram) added to the bioreactor.

$$\text{Hydrolysis yield } (\text{mg cum sCOD/kg VS}_{\text{added}}) = \frac{\textit{Total SCFA produced } (\text{mg COD SCFA})}{\textit{Initial VS}_{\textit{added}} \textit{ to the reactor } (\text{kg})} \quad (2)$$

where: total cumulative SCFAs produced (mg COD SCFA) = final total SCFAs of fermented culture–initial total SCFAs of unfermented culture.

(iii) The ratio of SCFAs to sCOD (%) indicates the extent to which soluble organic matter is transformed into SCFAs. This ratio is determined by dividing the SCFA yield by the hydrolysis yield.

(iv) Hydrogen yield was estimated based on the total hydrogen gas produced in the reactor compared to the amount of initial VS added to the reactor.

$$\text{Hydrogen yield } (\text{LH}_2\text{/kg VS}_{\text{added}}) = \frac{\textit{Total hydrogen production } (\text{L})}{\textit{Initial VS}_{\textit{added}} \textit{ to the reactor } (\text{kg})} \quad (3)$$

where:

$$\text{total hydrogen production } (\text{L}) = \sum_{d=0}^{d=t} \text{hydrogen percent in off gas}(\%) \times \text{Volume of off gas produced in reactors headspace } (\text{L}).$$

2.6. Microbial Community and Statistical Analysis

To investigate the impact of inoculum type and heat pretreatment on microbial structure, the fermented biomass from each condition was collected at the end of the fermentation process. The centrifuged fermented biomass was used for microbial community analysis based on 16S Rrna genes. This analysis was conducted by Metagenom Bio Life Sciences Laboratory in Waterloo in Canada. DNA extraction was performed on each sample using Sox DNA Isolation Kit, followed by a polymerase chain reaction (PCR) performed in triplicates for 25 µL of each sample following the manufacture's guidelines. The PCR mixture consisted of 0.5 µL of 10 mM Dntp, 2.5 µL of 10× Taq buffer, 0.25 µL of BSA, 5 µL of 1 µM forward primer (Pro341F: CCTACGGGNBGCASCAG), 5µL of 1 µM reverse primer (Pro805R:GACTACNVGGG TATCTAATCC), 5 µL DNA, 0.2 µL of Taq DNA polymerase, and 6.55 µL of PCR water (ref for primers). The DNA was then initially denatured at 95 °C for 50 s, followed by multiple cycles of heating and cooling for denaturing (95 °C for 30 s), annealing (30 °C for 30 s), and extending (72 °C for 50 s), and final extending at 72 °C for 10 min, and finally resolved using a 2% TAE agarose gel. The PCR products were purified with gel, quantified with Qubit dsDNA HS Assay kit (Thermo Fisher Scientific Inc.), and sequenced using the MiSeq Reagent Kit v2 (2 × 250 cycles). Taxonomic analysis was performed on FASTQ using DADA2 and QIIME 2 for sequence processing, including chimera filtering and taxonomic identification. Chimera filtering was performed to filter amplified sequence variants, and a naive Bayesian classifier developed in QIIME 2 was used to identify the taxonomy of the representative sequences. ANOVA in conjunction with the Tukey post-hoc test was used to analyze the statistical significance between experimental conditions. A p-value <0.05 was used as a threshold to determine statistical significance.

3. Results and Discussion

3.1. Hydrolysis of Food Waste

3.1.1. Impact of Inoculum Type

The impact of two different types of untreated (non-heat-pretreated inoculums (anaerobic AD and aerobic WAS sludge)) inoculums on food waste hydrolysis/solubilization was investigated by examining the changes in the daily cumulative sCOD production (mg), as shown in Figure 1A. The cumulative sCOD production obtained with untreated AD inoculum was 2542 ± 208 mg sCOD, which was significantly higher ($p < 0.05$) than that obtained with the WAS inoculum (2166 ± 132 mg sCOD), indicating higher hydrolysis with the use of the AD inoculum. A similar trend was also observed for the hydrolysis yield (Table 4). The maximum hydrolysis yield of 399 ± 14 g sCOD/kg VS $_{added}$ was obtained with untreated AD inoculum which was 17% higher ($p < 0.05$) than the untreated WAS inoculum. Moreover, the untreated AD inoculum showed a higher hydrolysis rate, resulting in a reduced fermentation time. For instance, the untreated WAS inoculum produced a cumulative sCOD of 2241 ± 105 mg sCOD on day 13, whereas the untreated AD inoculum achieved a similar cumulative sCOD two days earlier (i.e., day 11). This enhanced hydrolysis yield and rate with untreated AD inoculum could be attributed to the higher relative abundance of fermentative microorganisms [12,28]. On the contrary, studies in the literature reveal that WAS performs better for polyphenolic and lignocellulosic substrates [16,32]. For instance, ref. [16] reported that the solubilization of olive mill wastewater was 35% higher with aerobic WAS (i.e., 15.1 mM COD/kg VS) than with the anaerobic digester sludge (i.e., 11.2 mM COD/kg VS). Similarly, ref. [32] achieved greater hydrolysis of rice straws using aerobic inoculum compared to anaerobic cow compost. This higher hydrolysis yield with WAS was mainly attributed to the presence of cellulose-hydrolyzing microorganisms such as *C. stercorarium* and *C. pasteurianum*, producing compounds such as acetate, lactate, and ethanol. Conversely, AD sludge is dominated by *Acidamiococcaceae*, *Clostridiaceae*, and *Planococcaceae*, which primarily hydrolyze and ferment carbohydrates, proteins, and lipids [5,12,33]. This suggests that aerobic WAS performs better for lignocellulosic substrates like rice straws or agro-industrial waste. However, for carbohydrate-rich substrates such as food waste (carbohydrates—44%, proteins—14%, and lipids—30%), an anaerobic inoculum may be more effective, as demonstrated in this study [7,34].

Table 4. Performance parameters in untreated and pretreated inoculums.

	Control	AD Untreated	WAS Untreated	AD Pretreated	WAS Pretreated
Cumulative sCOD production, mg sCOD	1475 ± 317	2542 ± 208	2166 ± 132	2171± 150	1260 ± 161
Hydrolysis yield, g sCOD/kg VS $_{added}$	243 ± 62	399 ± 14	366 ± 17	333 ± 38	194 ± 23
Acetate, mg COD	51 ± 5	78 ± 30	85 ± 20	153 ± 16	18 ± 2
Propionate, mg COD	80 ± 11	229 ± 50	108 ± 40	335 ± 89	45 ± 13
Butyrate, mg COD	505 ± 30	851 ± 40	671 ± 80	1036 ± 59	698 ± 60
Total SCFA production, mg COD_{SCFA}	636 ± 55	1158 ± 150	864 ± 30	1525 ± 65	761 ± 25
SCFA yield, g COD_{SCFA}/kg VS $_{added}$	104 ± 9	182 ± 3	131 ± 5	238 ± 6	117 ± 2
SCFA/sCOD, (%)	43	46	36	71	60
Hydrogen yield, L_{H2}/kg VS $_{added}$	20 ± 4	34 ± 5	32 ± 2	18 ± 2	20 ± 4

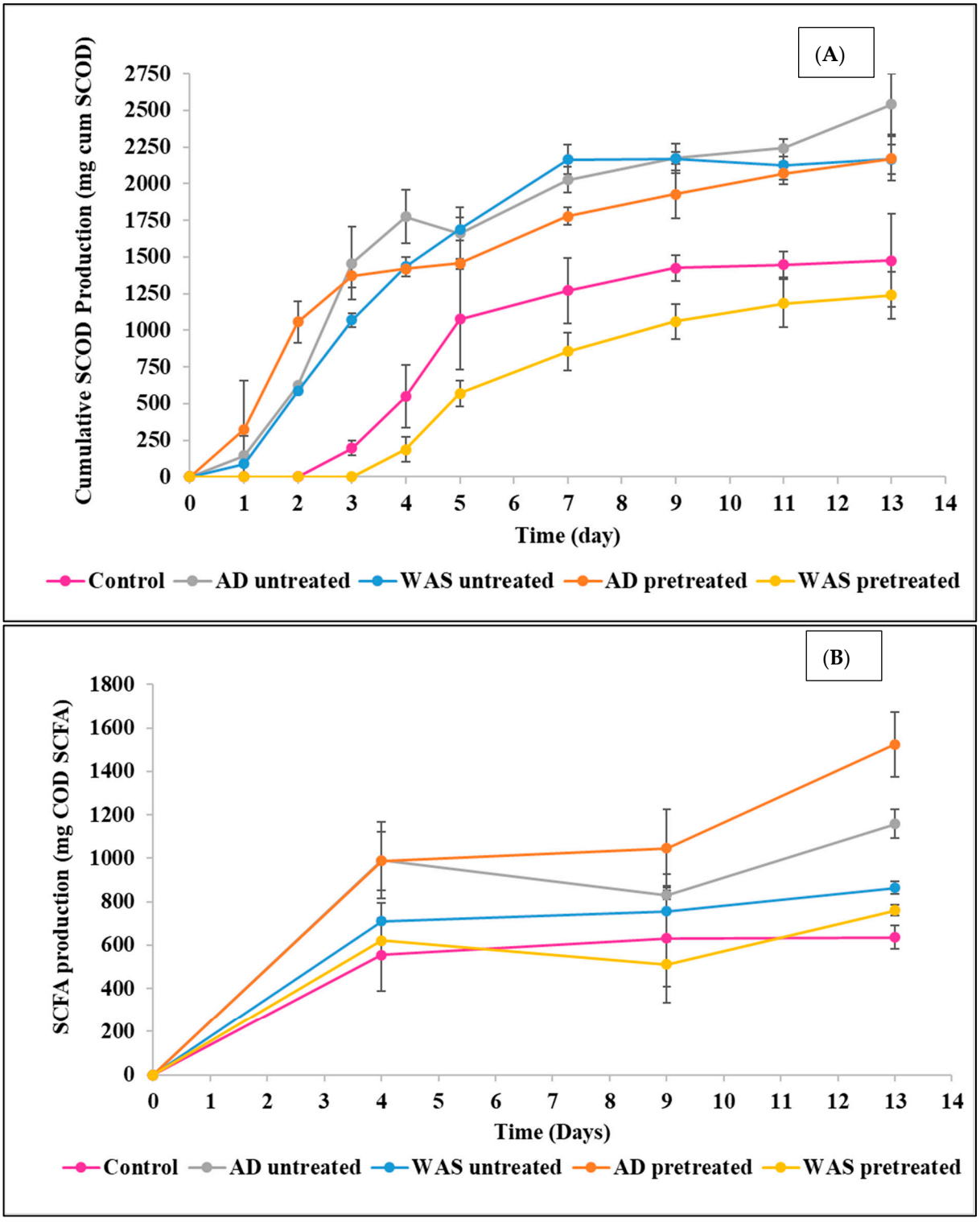

Figure 1. (**A**) Cumulative sCOD production in the reactors with untreated and pretreated inoculums, and (**B**) SCFA production in the reactors with untreated and pretreated inoculums.

3.1.2. Effect of Heat Pretreatment

The heat pretreatment of AD and WAS inoculums had negative effects on food waste hydrolysis, resulting in a lower cumulative sCOD production compared to the untreated inoculums (Figure 1A and Table 4). The cumulative sCOD produced for the pretreated AD inoculum (2171 ± 150 mg sCOD) was 14% lower ($p < 0.05$) than for the untreated

AD. Similarly, the pretreated WAS inoculum also showed a reduced cumulative sCOD production of 1260 ± 161 mg sCOD, which was 41% lower ($p < 0.05$) than the untreated WAS. Additionally, the effect of heat pretreatment on the solubilization of food waste was demonstrated by evaluating hydrolysis yield, as given in Table 4. A hydrolysis yield of 333 ± 38 g sCOD/kg VS $_{added}$ was achieved with pretreated AD inoculum, which was 16% lower ($p < 0.05$) than that obtained with untreated AD inoculum (399 ± 14 g sCOD/kg VS $_{added}$). The impact of heat pretreatment was more profound on the WAS. Notably, the hydrolysis yield of the pretreated WAS was two-fold lower (194 ± 23 g sCOD/kg VS $_{added}$) in comparison to the untreated WAS inoculum (Table 4). This shows that the heat pretreatment had a more adverse impact on WAS than AD. Various studies have reported a higher relative abundance of heat-sensitive and non-spore-forming fermentative microbes such as *Comamonas* and *Pseudomonas* in WAS which were eliminated after heat pretreatment, resulting in a reduced hydrolytic performance [24,35,36]. For instance, ref. [24] reported a 40% greater hydrolysis yield with the untreated WAS compared to the heat-pretreated WAS. Furthermore, the lower hydrolytic performances of pretreated inoculums were also attributed to the reduced microbial diversity [23,37].

Heat pretreatment of the inoculums also impacted the hydrolysis rate. For instance, the pretreated AD inoculum yielded a cumulative sCOD of 2171 ± 200 mg sCOD on day 13, whereas an equivalent amount of cumulative sCOD (2174 ± 39 mg sCOD) was achieved on day 9 when the untreated AD inoculum was used. The pretreated WAS inoculum mirrored the observations made for the pretreated AD inoculum, which suggests that the inoculums exposed to heat shock exhibited an extended lag time and consequently a lower hydrolysis rate. The observations of the current study are in agreement with previous studies demonstrating a reduced hydrolysis rate after heat pretreatment [36,38,39], thus further confirming the adverse impact of heat pretreatment on the solubilization of food waste.

3.2. Impact on SCFA Production

3.2.1. Effect of Inoculum Type

The soluble products from FW hydrolysis serve as the substrate for SCFA production [8]. Figure 1B illustrates the total SCFA production from food waste with respect to the inoculum types and control. Figure 2 shows the variation in pH during fermentation before pH adjustment to neutral in the inoculum types. SCFA production in the reactor was evident by the variation in pH. SCFA production in the untreated AD and WAS inoculums caused a sudden drop in pH. For untreated AD inoculum, a sudden decline in pH was observed from an initial 7 to 4.2 on the first day of fermentation, with an SCFA production of 990 mg COD_{SCFA}. The further untreated AD inoculum showed a gradual rise in pH till day 6 and then pH stabilized to around 6.5 till the end of fermentation. A similar trend was observed for pH in the untreated WAS inoculum. Such pH variation could be the result of a higher buffering capacity of the system with an increase in SCFA production, which tends to resist pH changes [2,40,41]. Ref. [40] reported a sudden drop in pH from 7.5 to 5.8, followed by a gradual increase toward the neutral conditions during the acidogenic fermentation of cheese whey. The maximum SCFA production obtained with the untreated AD and WAS inoculums was 1158± 150 mg COD_{SCFA} and 864 ± 30 mg $COD_{SCFA,}$ respectively, at the end of fermentation, which was significantly higher than that found in the control reactor ($p < 0.05$). The positive impact of the untreated AD inoculum on acidogenesis was further elucidated by assessing the SCFA yield (g COD_{SCFA}/kg VS $_{added}$), presented in Table 4. A higher SCFA yield of 182 ± 3 g COD_{SCFA}/kg VS $_{added}$ was achieved while using the untreated AD inoculum, which was 38% higher than that obtained with the untreated WAS inoculum (131 ± 5 g COD_{SCFA}/kg VS $_{added}$). This increase in SCFA yield with the AD inoculum might be attributed to the higher relative abundance of fermentative and acidogenic bacteria that convert soluble organics into SCFA [18,28,37]. Furthermore, the higher hydrolysis yield observed for the AD inoculum would have provided increased availability of hydrolysates to be converted into SCFAs by the acidogens, thereby improving the

SCFA yield. On the contrary, previous studies have demonstrated that the WAS inoculum provides greater acidification during the acidogenic fermentation of slowly biodegradable feedstocks like rice straws or agro-industrial waste [16,32]. For instance, ref. [16] achieved an SCFA yield of 12.1 mM COD/kg VS with aerobic WAS, which was 55% higher than that obtained with AD sludge (i.e., 7.8 g COD/g VS) during olive mill wastewater fermentation. These results indicate that substrate type and composition significantly affect the selection of an appropriate inoculum for higher SCFA production. Hence, for easily biodegradable organic waste like food waste, the AD inoculum results in higher SCFA production, as shown in the current study.

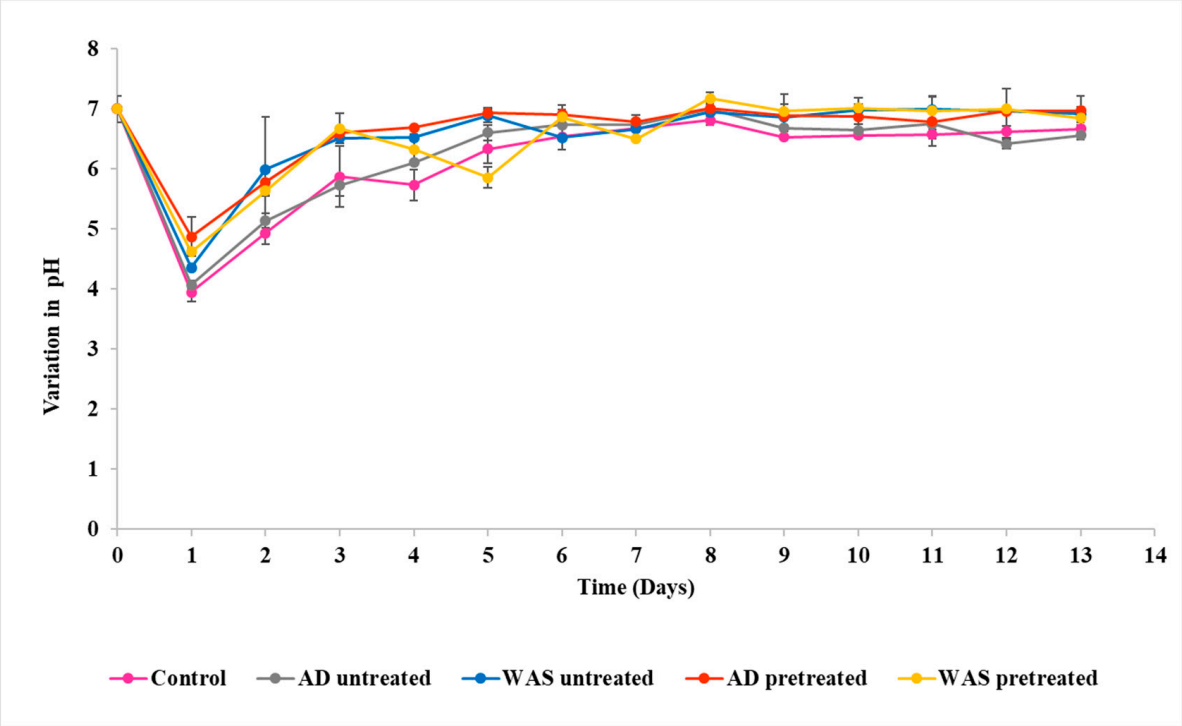

Figure 2. Variation in pH in different reactors before adjustment with buffering agent (NaOH).

The degree of sCOD conversion to SCFA (%) was investigated by determining the SCFA/sCOD ratio and is provided in Table 4. Irrespective of the inoculum, the degree conversion was observed to be lower, i.e., 46% for the untreated AD and 38% for the untreated WAS. This low conversion could be attributed to the presence of diverse fermentative microbes, such as *Lactobacillus*, *Pseudomonas*, and *unclassified Clostridium* sp., in the native inoculum. These microbes compete with the acidogens for the available sCOD to produce by-products such as lactate, alcohol, and hydrogen, thus reducing the amount of SCFA produced, which in turn translates into reduced conversion efficiencies, as observed in the case of the untreated inoculums [18,42]. The conversion efficiencies can be improved by inhibiting the undesired microbes, which could be achieved by heat-pretreatment of the inoculums.

3.2.2. Effect of Heat Pretreatment

The total SCFA produced during food waste fermentation using the heat-pretreated inoculums is illustrated in Figure 1B. Similar to inoculum type, the accumulation of SCFAs with heat-pretreated AD and WAS inoculums caused a sudden drop in pH from neutral pH to acidic, followed by a slow increase toward 6.5, then remaining stable till the end of fermentation (day 13) (Figure 2). Ref. [41] observed a similar drop in pH from 9.5 to 5 in the first 6 days and then an increase toward neutral pH during grass and tobacco dust fermentation. Notably, the total number of SCFAs produced using the pretreated AD

were 1525 ± 65 mg $COD_{SCFA,}$ which was significantly higher (31%) than for the untreated AD (1158 ± 150 mg COD_{SCFA}). However, SCFA production with the pretreated WAS inoculum was 761 ± 25 mg COD_{SCFA}, which is 13% lower ($p < 0.05$) than that obtained using the untreated WAS (864 ± 30 mg COD_{SCFA}). A similar pattern was observed for SCFA yield (refer Table 4), with the highest SCFA yield of 238 ± 6 g COD_{SCFA}/kg VS_{added} obtained for the pretreated AD inoculum, but a 12% decrease in SCFA yield was noted for the pretreated WAS inoculum. The increase in SCFA yield for the heat pretreated AD inoculum was attributed to a higher relative abundance of heat-resistant and spore-forming SCFA producers [23,35,37]. The reduced acidogenic performance of the pretreated WAS inoculum might be due to a lower abundance of heat-resistant SCFA producers. Studies in the literature have shown that aerobic inoculums are more sensitive to heat pretreatment due to the presence of heat-sensitive and non-spore-forming hydrolytic and fermentative bacteria in greater abundance [14,35,37]. For instance, [35] reported 31% higher fatty acid production with an untreated aerobic inoculum than a heat-pretreated one during glucose fermentation.

Additionally, an improvement in conversion efficiency (SCFA/sCOD ratio) was observed for the pretreated AD and WAS inoculum. For example, the pretreated AD inoculum provided the highest SCFA/SCOD ratio of 71%, which was 25% higher than that obtained with the untreated AD inoculum. Similarly, the pretreated WAS also achieved a 24% higher conversion of sCOD compared to the untreated WAS. This improvement in acidogenesis upon heat pretreatment can be attributed to the selection of heat-resistant and/or spore-forming acidogens while eliminating heat-sensitive and/or non-spore forming SCFAs consuming fermentative microbes [23,35,37]. The microbial community analysis also showed that most of the SCFA producers were spore-forming and heat-resistant (as discussed in Section 3.3). Therefore, pretreatment caused the selection of spore-forming and heat-resistive fermentative acidogens which enhanced the conversion of sCOD to SCFA, thereby resulting in elevated fatty acid production with the pretreated AD inoculum.

3.3. SCFA Composition

Figure 3 depicts the distribution of individual SCFAs as a percentage of the total number of SCFAs produced throughout anaerobic digestion. Regardless of inoculum type as well as pretreatment, butyrate was the predominant product, accounting for 73–77% of the total SCFAs by the end of the fermentation (day 13). This observation aligns with prior research, which demonstrated that butyric acid fermentation predominantly takes place in a pH range of 6–7 [18,28]. Propionate was the second most abundant SCFA, followed by acetate. However, the use of the AD inoculum led to a profound increase in the butyrate concentration, reaching up to 1000 mg COD/kg VS_{added}, especially upon pretreatment of the inoculum. Meanwhile, a maximum concentration of 698 ± 60 mg COD/kg VS_{added} was achieved for the WAS inoculum. The observations in the present study indicate that heat pretreatment of inoculums impacts the individual concentrations of SCFAs but does not markedly shift the core metabolic pathways. Both untreated and heat-pretreated inoculums predominantly undergo butyric acid fermentation at pH 7, as reported in the literature [28,35]. This trend aligns with findings from [32], who emphasized that although the overarching metabolic pathway remains stable, heat pretreatment can influence the dynamics of SCFA production. Furthermore, irrespective of inoculum type or pretreatment, the decline in acetate and propionate percentage was observed in SCFA composition with an increase in fermentation time (as shown in Figure 3). On the other hand, the butyrate percentage increased with fermentation time for both inoculum types and pretreated inoculums. For instance, no butyrate was measured on day 0 but it reached up to 68% for the pretreated AD inoculum. This increase in butyrate production along with a decline in acetate and propionate production indicates the conversion of lower-carbon carboxylate to high-carbon carboxylate through chain elongation [20,43].

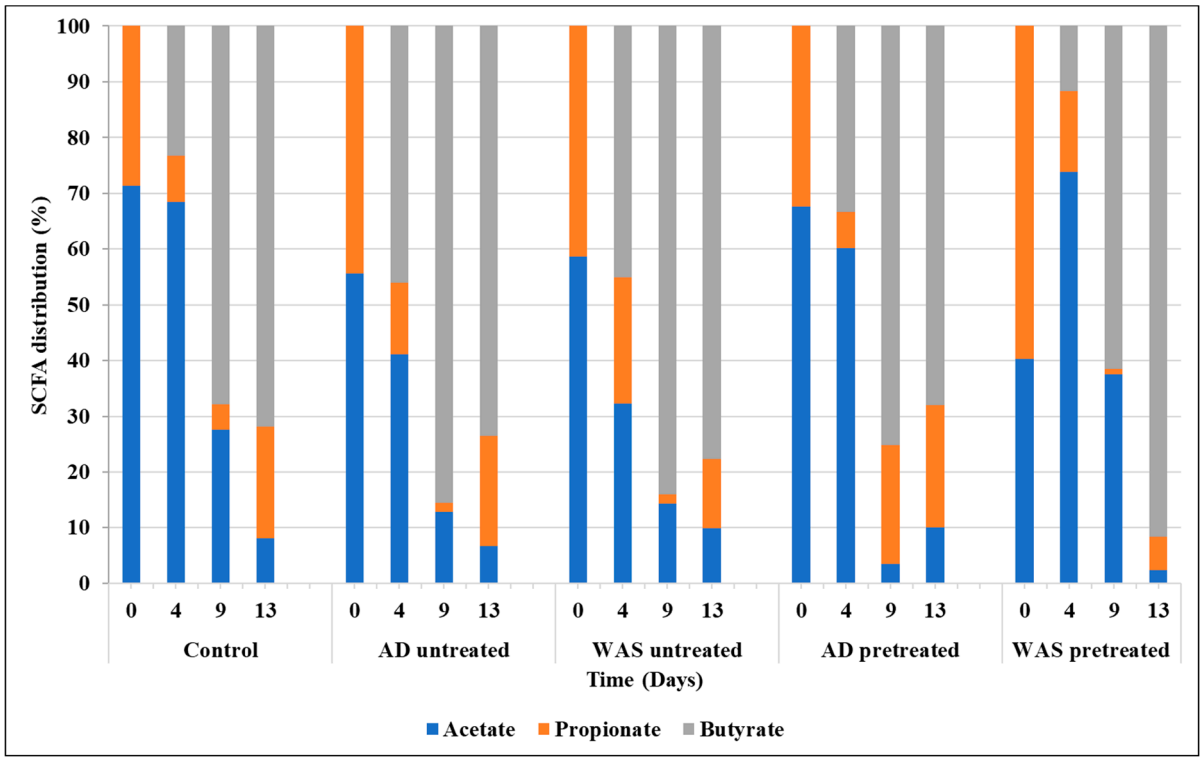

Figure 3. Distribution of SCFA components with untreated and pretreated inoculums.

3.4. Hydrogen and Methane Production

It is important to assess the conversion of food waste to hydrogen and methane as it directly affects the accumulation of SCFAs in the digesting slurry. During the acidogenic fermentation of food waste, hydrogen was produced, whereas no methane was formed. The hydrogen yield achieved with the untreated inoculums at the end of fermentation (day 13) is shown in Table 4. The hydrogen yield obtained with the untreated AD and WAS inoculums was 34 ± 5 L H_2/kg VS $_{added}$ and 32 ± 2 L H_2/kg VS $_{added,}$ respectively, which was significantly higher ($p < 0.05$) compared to the control (20 ± 4 L H_2/kg VS $_{added}$). The increased yields observed for the untreated inoculums could be attributed to the presence of a varied group of fermentative microbes including known hydrogen producers such as *Enterobacter* sp. and *Pseudomonas* sp. as well as bacteria belonging to the *Venelloilceae* family. These bacterial communities are known hydrogen producers [37,39]. The absence of methane production, observed in the current study, can be attributed to the inherent culture conditions during acidogenic fermentation, such as organic acid accumulation and short solid retention time, preventing the proliferation of methanogens [23,44].

Further, as shown in Table 4, heat pretreatment of the AD and WAS inoculums caused a significant reduction in hydrogen yield. The hydrogen yield obtained with the pretreated AD inoculum was 18 ± 2 L H_2/kg VS $_{added}$, which was 47% lower ($p < 0.05$) than that obtained with the untreated AD inoculum. Similarly, the pretreated WAS inoculum achieved 20 ± 4 L H_2/kg VS $_{added}$, which was 37% lower ($p < 0.05$) than that obtained with the untreated WAS inoculum. This outcome can be attributed to the inhibition of certain hydrogen-producing microbial species that are heat-sensitive and non-spore-forming. Specifically, strains like *Pseudomonas* and members of the *Venelloilceae* family, which have been indicated in the literature as crucial hydrogen producers, were observed to be particularly affected by heat pretreatment [37,39,45]. It is evident that heat pretreatment of inoculums negatively impacts the microbial consortium responsible for efficient hydrogen production, leading to lower hydrogen yield but a concomitant improvement in the conversion of available sCOD to SCFA. These observations are in agreement with the observations of [24]. They observed a reduction in hydrogen yield when a heat-pretreated inoculum

was used for hydrogen production. Furthermore, an increase in fatty acid accumulation, acetate, and butyrate was also observed by the researchers with the use of heat-pretreated inoculums [24]. Thus, heat pretreatment has a beneficial impact on SCFA production, especially with an AD inoculum, by eliminating heat-sensitive non-spore-forming hydrogen producers, leading to improved SCFA yield.

3.5. Microbial Community Composition

3.5.1. Microbial Community Composition in Control and Untreated Inoculums

The microbial communities present in the control and experimental runs at the end of the fermentation period (13 days) are shown in Figure 4A,B. The most dominant phylum found in the control was *Proteobacteria* (65%), followed by *Firmicutes* (35%). *Proteobacteria* are majorly responsible for hydrolyzing and fermenting food waste [11,45]. *Firmicutes* are mainly acidogens that convert food waste components (carbohydrates, protein, and lipids) to SCFAs, i.e., acetate and butyrate [28,33]. However, the microbial community composition varied with the use of different inoculums. The relative abundance of *Proteobacteria* was reduced by 14% and 9% with the untreated AD and WAS inoculums, respectively, while fermentative microorganisms such as *Firmicutes*, *Bacteriodota*, and *Actinobacteriota* increased by 13% with the untreated AD and 9% with the WAS inoculum. This finding supports the higher SCFA yields obtained for inoculums in comparison to the control. The heat pretreatment of the inoculums further increased the abundance of fermentative microbes like *Firmicutes* and *Bacteriodota*. Several microbes belonging to *Bacteriodota* are known to survive adverse conditions and are capable of hydrolyzing carbohydrates and protein to produce SCFAs (acetate and propionate) [5,12,33]. Additionally, *Firmicutes* such as *Clostridia* are also spore-forming and stress-resistant fermentative bacteria [1,12,33]. The pretreated AD inoculum showed a significantly higher abundance of *Firmicutes* and *Bacteriodota*, 47% and 18%, respectively. Similarly, the pretreated WAS inoculum also showed a high relative abundance of heat-resistant and spore-forming communities (around 50% of *Firmicutes* and *Bacteriodota*). This observation supports the higher acidification with pretreated inoculums, leading to improved SCFA yield.

At the genus level, Figure 4B shows variations in microbial relative abundance due to the influences of the inoculum and pretreatment. In the control fermentation, *Unclassified Enterobacteriaceae* dominated, representing 51% of the microbial population. Other notable genera included *Unclassified Veillonellaceae* (21%), *Unclassified Enterobacterales* (13%), and *Unclassified Acidaminococcaceae* (9%). *Enterobacteriaceae* and *Enterobacterales*, from the *Proteobacteria* phylum, hydrolyze food waste components and produce fatty acids like acetate, propionate, and butyrate, along with hydrogen [11,45]. *Acidamiococcaceae* primarily converts carbohydrates to acetate and hydrogen, whereas *Veillonellaceae* ferments fibers, yielding propionate and acetate [45,46]. These findings indicate that the inherent bacterial communities present in food waste facilitate the efficient breakdown and transformation of food waste components into SCFAs.

However, the addition of inoculums increased the microbial diversity which led to high hydrolysis and acidogenesis in comparison to the control. In untreated AD fermentation, the microbial composition was diversified with the presence of *Unclassified Planococcaceae* (8%), *Unclassified Oscillospiraceae* (7%), *Unclassified Prevotellaceae* (4%), *Acidaminococcus* (2%), *Unclassified Acidaminococcaceae* (6%), *Solobacterium* (2%), and *Unclassified Clostridiaceae* (3%). On the other hand, untreated WAS fermentation showed a distinct variation in microbial communities and consisted of *Pseudomonas* (14%), Morganella (2%), *Eubacterium* (2%), *Lactococcus* (2%), *Lactobacillus* (3%), *unclassified lactobacillus* (2%), and *unclassified Clostridiaceas* (8%). *Pseudomonas* breaks down complex organic materials and ferments them, producing acetate, butyrate, and hydrogen [45,47]. *Lactococcus* and *Lactobacillus* are non-spore-forming microbes that ferment carbohydrates and proteins into products like lactate, acetate, and ethanol [5,48]. These results indicate that untreated inoculums, while rich in SCFA-producing bacteria, also host microbes that might consume

the hydrolysates to produce alternative products like hydrogen and lactate. This explains the lower conversion efficiency (SCFA/SCOD ratio) obtained for the untreated inoculums.

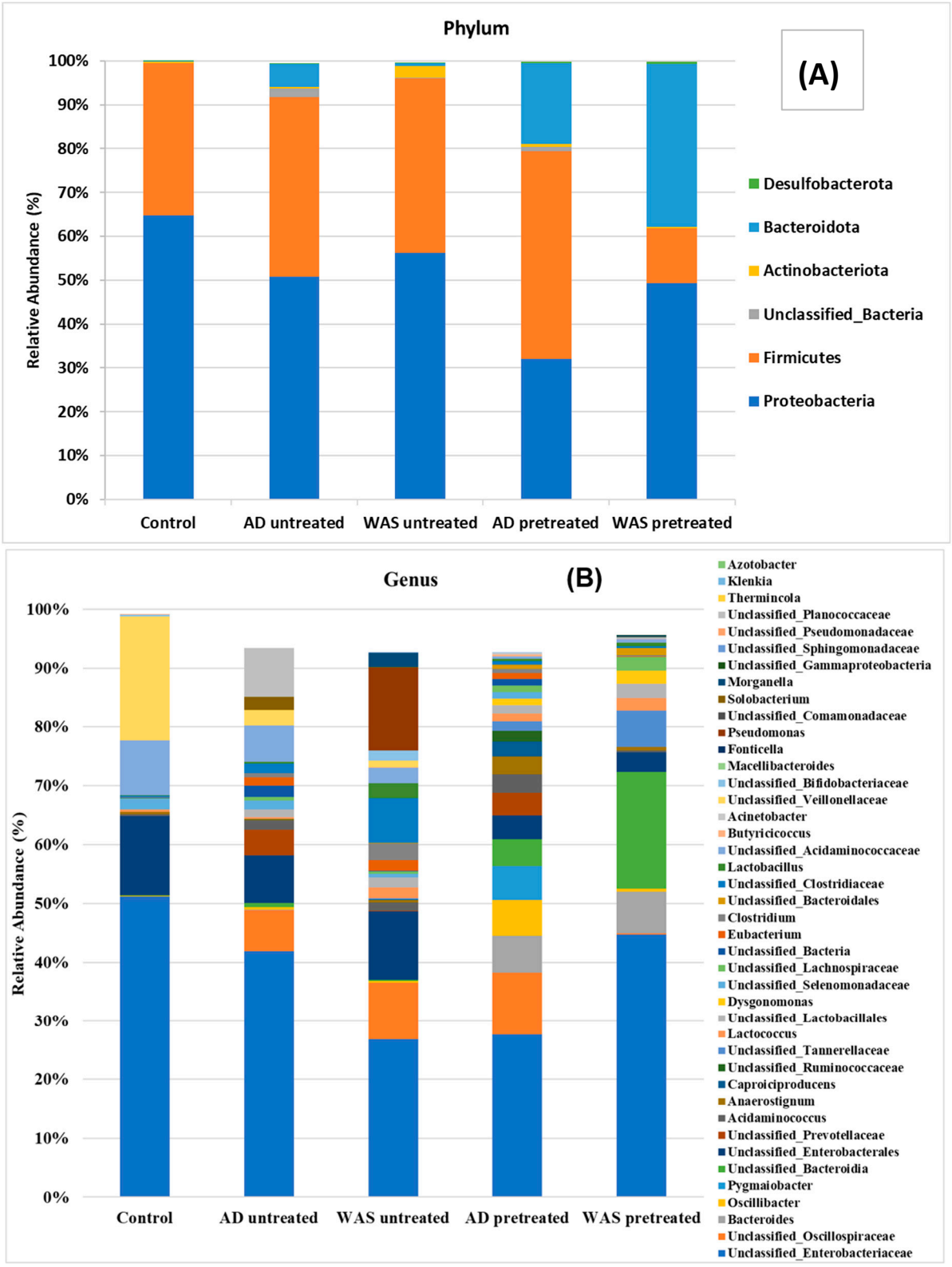

Figure 4. Relative abundance of microbial community in the untreated and pretreated inoculums at the (**A**) phylum and (**B**) genus level.

3.5.2. Microbial Community Composition in Treated Inoculums

The heat pretreatment favored the selection of microorganisms which were capable of forming endospores or resisting heat shock. The heat pretreated AD fermentation showed a greater abundance of spore-forming and heat-resistive fermentative microbes, i.e., *Clostridia* (41%) and *Bacteroidales* class (24%). Unclassified *Clostridia*, *Clostridium*, *Ruminococceae*, *lachnospiraceae*, *Oscillibacter*, and *Oscillospiraceae* belong to the *Firmicutes-Clostridia* class which ferment food waste to acetate and butyrate [1,5,15]. Moreover, the pretreated WAS fermentation was also dominated by *Bacteroidales* (39%) and *Clostridia* (16%). This suggests that heat pretreatment caused the elimination of heat-sensitive and spore-forming SCFA consumers and increased the abundance of spore-forming/heat-resistive fermentative SCFAs producing microorganisms, which aligns with the high conversion efficiency and enhancement of the SCFA yield of pretreated AD inoculums. Selecting the fermenting biomass has the potential to enhance the SCFA yield, and this approach has been widely used in various related studies [8,11]. Ref. [8] performed two-stage enrichment of an aerobic inoculum during food waste fermentation in a leachate bed reactor (LBR). They reported no changes in the microbial community and observed an increase in the abundance of fermentative microorganisms such as *Clostridium* by 30% through enrichment. Similarly, ref. [11] demonstrated an increase in the abundance of hydrolytic and acidogenic microbes through subsequent enrichment of an anaerobic inoculum, resulting in a 35–50% increase in hydrolysis and acidification yield compared to an anaerobic inoculum (non-enriched). Ref. [21] obtained a high hydrolysis and acidification yield of 774 g sCOD/g VS and 697 g SCFA/g VS using an enriched anaerobic inoculum. Hence, future studies should focus on enhancing targeted species obtained through heat pretreatment of an inoculum and use that enriched inoculum to improve process yield.

4. Conclusions

The results establish the impact of inoculum type and heat pretreatment on the acidogenic fermentation of food waste. The use of AD sludge (untreated) resulted in greater solubilization and acidogenesis (SCFA production) of food waste in comparison to the WAS (untreated). A hydrolysis yield of 399 g sCOD/kg VSadded and an SCFA yield of 182 g CODSCFA/kg VSadded was obtained for AD, sludge which was significantly higher than that obtained for WAS. The overall SCFA yield was further improved by heat pretreatment of the AD inoculum to achieve a maximum SCFA yield of 238 $\pm$ 6 g CODSCFA/kg VS added, which corresponded to a 30% increase over the untreated AD inoculum. This increase in SCFA production with heat pretreatment was due to the increased abundance of spore-forming/heat-resistive SCFA-producing fermentative microorganisms. Consequently, the maximum conversion of available sCOD to SCFA was also obtained for the heat-pretreated AD inoculum. However, heat pretreatment of the AD inoculum showed a lower hydrolysis rate compared to AD sludge (untreated), resulting in a longer hydrolysis time. On the other hand, heat pretreatment had a significant effect on the WAS inoculum, causing a decline in hydrolysis and SCFA yield by 78% and 10%, respectively. This indicates that the WAS inoculum was highly affected by heat pretreatment due to the higher presence of heat-sensitive and non-spore-forming hydrolytic and fermentative bacteria, resulting in a lower hydrolysis and SCFA yield in the heat pretreated WAS inoculum. Inoculum type or heat pretreatment had negligible impacts on SCFA composition, indicating that butyrate was the dominant SCFA component, amounting to 41–57% of the total SCFAs. Furthermore, heat pretreatment caused the inhibition of crucial hydrogen-producing microbial species in the AD and WAS inoculums, resulting in a 37% and 47% decline in hydrogen yield after heat pretreatment of AD and WAS sludge compared to the respective untreated inoculums. Future studies could focus on the optimization of operating parameters, such as the inoculum-to-substrate ratio and organic loading rates, that could significantly impact the SCFA yield and process efficiency.

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