fermentation

MDPI

Article

Efficient Two-Stage Meso- and Thermophilic Anaerobic Digestion of Food Waste from a Microbial Perspective

Katsuaki Ohdoi [1], Yoshihiro Okamoto [2], Tomonori Koga [2], Haruka Takahashi [2], Mugihito Oshiro [2], Toshihito Morimitsu [3], Hideki Muraoka [3], Yukihiro Tashiro [2,*] and Kenji Sakai [2,*]

[1] Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan; ohdoi.katsuaki.5r@kyoto-u.ac.jp
[2] Graduate School of Bioresource and Bioenvironmental Science, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan; oshiro@agr.kyushu-u.ac.jp (M.O.)
[3] Momoyama Recycling Center R&D Division, Vioce Co., Ltd., 2822-6 Tsukatsuki, Momoyama-cho, Kinokawa 649-6112, Japan
* Correspondence: tashiro@agr.kyushu-u.ac.jp (Y.T.); kensak@agr.kyushu-u.ac.jp (K.S.)

Abstract: Two-stage meso- and thermophilic anaerobic digestion (TSMTAD) of food waste was examined and its microbiological structure was investigated. The first stage was designed for the primary storage of perishable food waste and the second stage for central biogas production. Mesophilic storage with initial neutralization and inoculation of lactic acid bacteria (LAB) resulted in an accumulation of lactic acid (21–23 g/L) with a decreased pH, in which bacterial members in facultative hetero-fermentation-type LAB dominated. Repeated fed-batch storage showed stable accumulation of lactic acid, retaining 89.3% (av.) carbon and preventing the growth of exogenous food pathogens. When the second stage of TSMTAD was compared with direct single-stage anaerobic digestion (SSAD) at 55 °C, the amount of methane accumulated was 1.48-fold higher (896 NmL/g-vs.). The methane yield of the original food refuse was 6.9% higher in the case of TSMTAD. The microbial community structures of both cases were similar, consisting of a sole thermophilic hydrogen-assimilating methanogen, *Methanothermobacter thermautotrophicus*. However, the abundance of bacteria belonging to two functional groups, H_2 CO_2 and acetic acid producer, and syntrophic acetate-oxidizing bacteria increased in TSMTAD. This may change the metabolic pathway, contributing to the stimulation of methane productivity.

Keywords: food waste; anaerobic digestion; biogas production; two-stage meso- and thermophilic anaerobic digestion (TSMTAD); two-stage anaerobic digestion (TSAD); methane production; microbial community structure

check for updates

Citation: Ohdoi, K.; Okamoto, Y.; Koga, T.; Takahashi, H.; Oshiro, M.; Morimitsu, T.; Muraoka, H.; Tashiro, Y.; Sakai, K. Efficient Two-Stage Meso- and Thermophilic Anaerobic Digestion of Food Waste from a Microbial Perspective. *Fermentation* **2024**, *10*, 607. https://doi.org/10.3390/fermentation10120607

Academic Editor: Giulia Bozzano

Received: 13 September 2024
Revised: 17 November 2024
Accepted: 22 November 2024
Published: 28 November 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Among the United Nations' Sustainable Development Goals (SDGs, https://sdgs.un.org/goals (accessed on 27 February 2023)), waste and wastewater treatment (SDG6) poses a particular challenge. A tremendous amount of food waste, including kitchen refuse, is created worldwide [1], with small portions generated by individual households every day. The key issue of waste is its unhygienic disposal, leading to disease. A solution would require frequent waste collection and treatment to maintain a clean living environment. However, waste also holds nutritional value for microorganisms [2]. Many countries have already recognized this unique attribute of waste and have put it to use through the on-site microbial treatment of food waste via both aerobic [3] and anaerobic digestion [4].

Anaerobic digestion comprises a sequence of microbial degradation processes of complex organic matter under anoxic conditions. During these processes, macromolecules are broken down through a series of microbial transformation steps, including hydrolysis/acidogenesis, acetogenesis, and methanogenesis, to produce methane, carbon dioxide,

hydrogen, and ammonia as end products; this is sometimes denoted as methane fermentation [5]. Anaerobic digestion has received considerable attention due to the fact that carbon and energy can be recovered as methane in biogas fuel, in addition to stabilizing waste and wastewater. On the contrary, the abortive anaerobic digestion of food waste results in the emission of methane, a greenhouse gas, into the atmosphere, which is related to an issue on climate action in SDGs (SDG13). Research on efficient treatment systems has been conducted to improve energy recovery via systems [6]. In addition, recent analytical advances in microbial community structure and function have enabled a precise understanding of the complex transformation steps in dynamic mixed-culture systems [7,8]. In particular, the two-stage anaerobic digestion (TSAD) of food waste has been investigated and summarized [9]. Therein, the microbes at the hydrolysis/acidogenic step fundamentally differ from the subsequent acetogenic and methanogenic microbes in their physiology and environmental conditions [10]. Thus, separate operation of each stage, including the control of pH and temperature, is an advantage of two-stage AD systems.

During a study of an open but controlled transformation of biomass by a mixed culture system (meta-fermentation), Tashiro et al. [11] found that intermittent pH neutralization of food waste under open conditions resulted in the selective proliferation of lactic acid bacteria (LAB), with or without seed inoculation, resulting in the accumulation of lactic acid, which was effective to repress non-LAB in the food waste accordingly. Considering that small amounts of food waste are created daily by households, we hypothesized that this simple system of food waste control could be applicable for primary storage in noble TSAD, where the first stage of the simple hydrolysis/acidogenesis process would be operated at multiple regional storage sites, with the second stage of biogas production conducted at a centralized location (Figure 1). This type of "hub-and-spoke" logistics has several merits [12] in terms of the energy required to collect food waste, which will be discussed later.

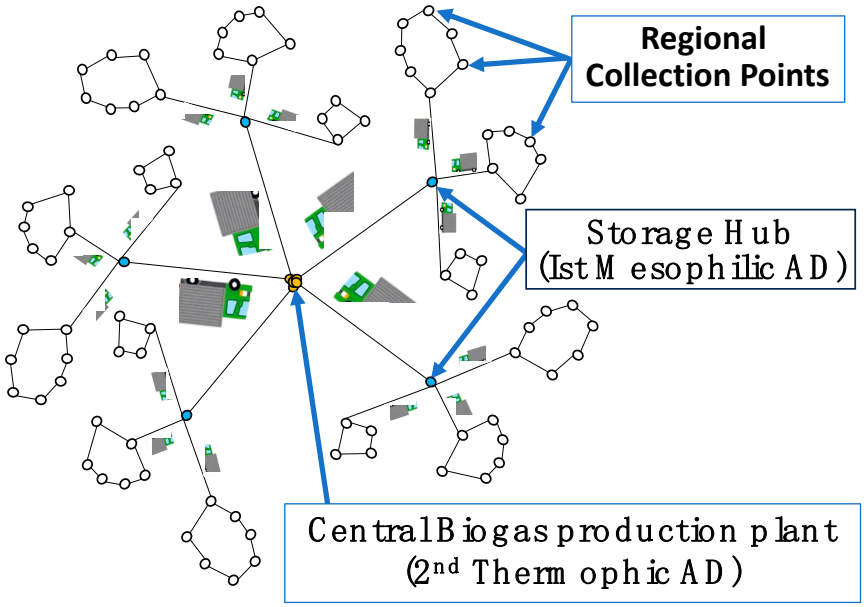

Figure 1. Schematic representation of treatment system of food waste by TSMATD based on the "hub-and-spoke" model. The first stage (primary storage) and the second stage of AD (biogas production) correspond to hub and demand nodes, respectively.

In this study, we evaluated the two-stage meso- and thermophilic anaerobic digestion (TSMTAD) of food waste. The first step was controlled with simple pH neutralization under mesophilic open conditions, as a primary storage method for collected food waste, and then, the performance for biogas production by the second stage of thermophilic anaerobic digestion of the primary stored food waste was compared with that of single-stage

anaerobic digestion (SSAD). Regarding the temperature, as far as we know, treatment under mesophilic and thermophilic conditions, rather than under mesophilic and mesophilic, thermophilic and thermophilic, or thermophilic and mesophilic conditions, has not been reported [6,9,13,14]. In addition to the methane productivity of TSMTAD, the microbial community structure of the system was evaluated.

2. Materials and Methods

2.1. Design of TSMTAD System

A conceptual representation of the collection and treatment system for food waste by the TSMTAD is provided in Figure 1. The first step enables primary storage at hub sites within regional urban areas within which to operate fed-batch-type storage. The storage tank is equipped with a feeding–mixing and pH control apparatus to operate the fed-batch-type storage. For the reproducibility of this study, the primary storage step was performed at 30 °C. The second step comprised thermophilic anaerobic digestion to produce biogas operated at 55 °C at a central site.

2.2. Fed-Batch Storage of Food Waste Under Mesophilic Open Conditions with pH Control

Food waste in the form of vegetable cuttings were collected from a factory, consisting of 94.2% water, 0.5% ash, COD(Mn) 430,000 ppm, nitrogen 2,100 ppm, carbon 30,100 ppm, and a C/N ratio of 14.3. Water content was determined by drying the sample at 105 °C for 24 h. The analyses of COD(Mn), nitrogen, and carbon were outsourced to the Sumika Chemical Analysis Center. Nitrogen and carbon were quantified using a carbon–nitrogen analyzer, while COD(Mn) was measured according to the method established by the Ministry of the Environment, Japan. The minced food waste (70 mL) was placed in a 500 mL bottle and statically incubated at 30 °C. A small amount (1 mL) of a mixed seed from Japanese pickle (Olive Bran Bed, ANK Co. Ltd., Iizuka, Japan) was added on day 0. One type of compost sample and three types of Japanese pickles were preliminarily tested as commercial seeds. The Japanese pickle was selected based on their abundance of LAB and selective productivity of lactic acid in minced kitchen refuse medium [11] (Supplementary Table S1).

An additional sample of food waste (70 mL) was added for every day of incubation (490 mL after 7 days) when the content was gently homogenized with or without pH neutralization using 10 M sodium hydroxide. A small portion of the sample was periodically withdrawn to conduct an analysis of the chemical composition and microbial community structure.

2.3. Effect of Repetition on Fed-Batch Storage of Food Waste

Repeated fed-batch storage was performed as follows: the minced food waste (70 mL) was placed in 500 mL bottle and statically incubated at 30 °C. At day 0, a small amount (1 mL) of mixed seed from Japanese pickle selected above was added and the content was neutralized with gentle homogenization using spatula. An additional 70 mL of minced food waste was added after 24 h and homogenized in a bottle. Every two days, 136.5 mL of the content was withdrawn. The fed-drawn batch process was repeated five times for a total of 10 days.

2.4. Second Stage of Anaerobic Digestion

In the second stage of TSMTAD, three digesters with an effective volume of 10.0 L were used to test biogas production. As a control, no stored food waste obtained from the same factory was used in the other three digesters after mincing. Each digester was kept at 55 °C using a rubber heater connected to a temperature controller (TRZ-303; Toho Electronics Inc., Sagamihara, Japan), and the temperature of each digester was recorded using a temperature logger (TR-52i; T&D Corporation, Matsumoto, Japan). Each digester was continuously stirred at 180 rpm using a stirring machine without any pH adjustment (MSMS-BLh600; Kato Stainless Kagaku Co., Ltd., Tokyo, Japan). Seed sludge was obtained

from a thermophilic anaerobic digester located in Kinokawa City (Wakayama Prefecture, Japan). The density, pH, and concentration of total solids (TSs) and volatile solids (VSs) of the seed sludge were 1.019 kg/L, 7.55, 4.04%, and 2.44%, respectively. After feeding the seed sludge, each digester was incubated; 12 h later, the primary stored or non-stored minced kitchen refuse substrate was fed into each digester. A batch thermophilic anaerobic digestion test was conducted over 35 days. The quantity of generated biogas in each digester was measured and recorded by using gas volume and flow meter (BPC Go and μFlow, BPC Instruments AB, Lund, Sweden). Both the BPC Go and μFlow used volumetric techniques to measure gas volume and flow rate. The BPC Go consists of a base unit and a flow cell unit with a resolution choice of 2 mL or 9 mL and is connected to a PC via Ethernet. In this study, the 9 mL flow cell unit was used. The μFlow contains a 9 mL flow cell and connects to a PC via a data logger. The accumulated biogas yield and flow rate were recorded every 15 min for each piece of equipment.

2.5. Estimation of Biogas Yield

Based on the results of the batch test described above, biogas yield was estimated as follows: because 1/20 volume of the substrate was fed into and the same volume of digested liquid was discharged from the digester every day, the total biogas yield from the second anaerobic digestion with an HRT of 20 days was estimated using the following equation:

$$B_{HRT20} = \sum_{n=1}^{35} B_{BATn} \times \left(\frac{19}{20}\right)^{n-1}$$

where B_{HRT20} is the estimated daily biogas yield per VS fed from anaerobic digestion with an HRT of 20 d, and B_{BATn} is the biogas yield per VS fed on the nth day obtained from the batch test.

The methane yield of biogas obtained from anaerobic digestion with an HRT of 20 d was also estimated using the methane concentration obtained from the batch test. The methane yield obtained from anaerobic digestion with an HRT of 20 d was estimated using the following equation:

$$M_{HRT20} = \sum_{n=1}^{35} B_{BATn} \times \left(\frac{19}{20}\right)^{n-1} \times MC_{BATn}$$

where M_{HRT20} is the estimated daily methane yield per VS fed from anaerobic digestion with an HRT of 20 d, and MC_{BATn} is the methane concentration of biogas obtained on the nth day from the batch test.

2.6. Chemical Analysis

The concentration of total solids (TSs) was determined by drying at 105 °C for 24 h in the constant-temperature drying oven (EO-300B; As One Corporation, Osaka, Japan), while that of volatile solids (VSs) was measured based on the ignition loss of TS at 600 °C for 1 h in an electric muffle furnace (FUW222PB; Advantec Toyo Kaisha, Ltd., Tokyo, Japan). The total elemental carbon and nitrogen contents were analyzed using a MACRO CORDER JM1000CN (J-SCIENCE, Kyoto, Japan) instrument. The organic acids (lactic acid, acetic acid, propionic acid, (iso-)butyric acid, and formic acid) in the digestate were measured using an HPLC system (Organic Acid Analyzer, Shimadzu, Kyoto, Japan), as reported previously [11]. The composition of the biogas from each digester was analyzed using a gas chromatograph (GC-3200D; GL Sciences Inc., Tokyo, Japan).

2.7. Microbial Community Structure Analysis

The microbial community structure in the sludge was analyzed by PCR amplicon sequencing of the partial 16S rRNA gene (V4 region). Total DNA was extracted from the sample (0.25 g) using a DNeasy® PowerSoil® Kit (QIAGEN, Tokyo, Japan), following the manufacturer's instructions. DNA sequencing was performed after the two-stage PCR

amplification of the extracted DNA template. The procedures including PCR conditions, extraction of PCR amplicons, measurement of PCR amplicons, and sequence analysis using the Illumina MiSeq system (Illumina Inc., San Diego, CA, USA) were performed according to a previous study [14]. Bioinformatic analysis of the sequence data was performed using Usearch V8.1.1861 and QIIME2 [15]. Species related to each operational taxonomy unit (OTU) was deduced from the unique sequences of each amplicon sequence variant (ASV) using EzBioCloud (https://www.ezbiocloud.net/identify) (accessed on 27 February 2023).

OTU assigned to lactic acid bacteria (LAB) was further grouped based on their fermentation manner, according to Bergey's Manual (3rd edition) [16]. Obligately homofermentative lactobacilli (homo-LAB): hexoses were fermented almost exclusively to lactic acid via the Embden–Meyerhof pathway; pentoses or gluconate were not fermented. Facultatively heterofermentative lactobacilli (Fc-Hetero-LAB): hexoses were fermented almost exclusively to lactic acid via the Embden–Meyerhof pathway, and pentoses were fermented to lactic acid and acetic acid via an inducible phosphoketolase. Obligately heterofermentative Lactobacilli (Ob-Hetero-LAB): hexoses were fermented into lactic acid, acetic acid, (ethanol), and CO_2 via the phosphogluconate pathway. Pentoses were fermented into lactic and acetic acids via the phosphoketolase pathway. Another LAB member was collectively grouped and designated as non-LAB.

3. Results

3.1. Effect of pH Control on the Batch Storage of Food Waste

The initial pH of the food waste after mincing and seed inoculation was 4.0 with 9.4 g/L lactic acid and 1.5 g/L acetic acid (Figure 2). In batch storage at 30 °C without pH neutralization, a gradual accumulation of lactic acid with a pH decrease of below 4 was observed (Figure 2A). In contrast, intermittent pH neutralization every 24 h resulted in a rapid increase in lactic acid concentration, reaching 53 g/L after 7 days (Figure 2B). When the stored food waste was neutralized after one day (day 0) (Figure 2D), approximately 20 g/L of lactic acid accumulated just one day after neutralization. However, after 2 days (day 0 and 1) (Figure 2C), the increase in lactic acid was terminated as the pH decreased to below 4.0. Under any control, a small amount of acetic acid (1–2 g/L) stably accumulated throughout the storage period, but no other type of volatile fatty acids, including formic acid, propionic acid, or butyric acid, was detected in any experimental run.

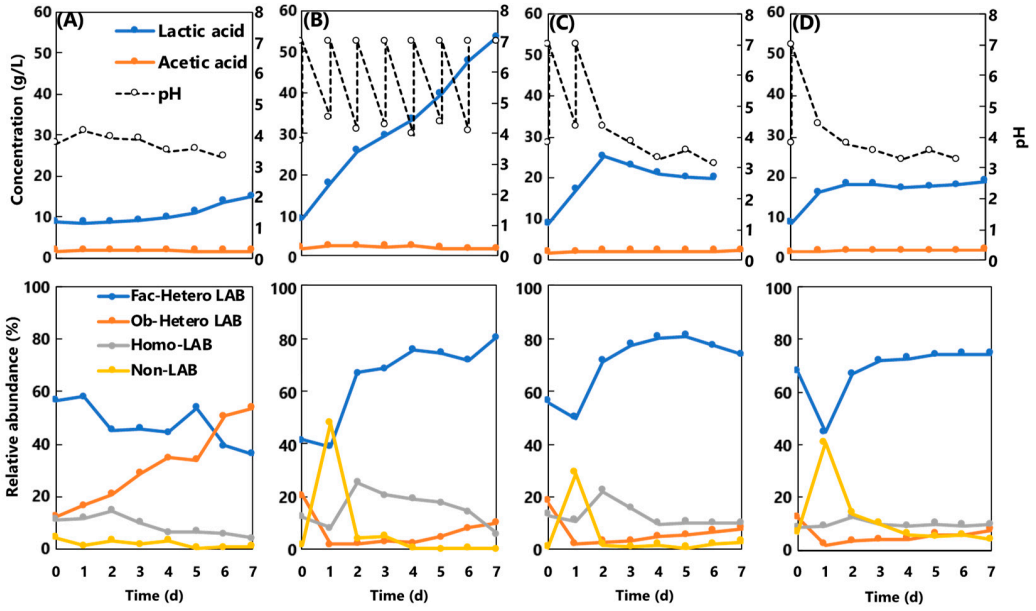

Figure 2. Effect of pH control on the primary storage of food waste. Run (**A**), no pH control; Run (**B**), pH neutralization every 24 h; Run (**C**), the pH was neutralized on days 0 and 1; Run (**D**), the pH was neutralized only on day 0.

The seed culture initially included Fc-Hetero-LAB (58–66%), Ob-Hetero-LAB (13–18%), Homo-LAB (14–15%), and Non-LAB (4–10%). During batch storage without pH neutralization (Figure 2A), the abundance of Fc-Hetero-LAB gradually decreased to 38%, whereas that of Ob-Hetero-LAB increased by 55% after 7 days. In contrast, in Runs (B)–(D), the occupancy of Fc-Hetero-LAB increased during the initial 2 days and maintained an abundance of 70–80% during incubation. Members belonging to non-LAB remained low, particularly in Run (D). The major OTUs in Fc-Hetero-LAB belonged to the genera *Lacticaseiobacillus* and *Lactiplantibacillus*.

3.2. Repetition of Fed-Batch Storage and Addition of Exogenous Contaminant

Considering the minimization of the burden of both manpower and the consumption of alkaline chemicals in primary storage, a simpler control seems better. Next, the effect of repetition on the fed-batch storage of food waste neutralized at 48 h was examined. As shown in Table 1, the analytical values of the first and second repetitions fluctuated. Storage stabilized after the third repetition, which was assessed based on the structure of the microbial community. During the 3rd–5th repetitions of fed-batch storage, 10.7% carbon was lost on average, with an accumulation of lactic acid (22.2 g/L) and acetic acid (1.9 g/L), resulting in a decrease in COD to 40,000 from the initial 43,000.

Table 1. Effect of the fed-batch storage repetition of kitchen refuse.

Repeat	Carbon			Organic Acid (g/L)			LAB Relative Abundance (%)		
	Total (g/L)	Residual (%)	Loss (%)	Lactate	Acetate	Butyrate	Homo	Fac Hetero	Ob Hetero
0	198.2	100.0	-	7.5	1.8	0	12.4	31.2	34.1
1st	152.6	76.9	23.1	8.5	2.5	0	16.5	28.1	3.5
2nd	187.4	94.5	5.5	21.0	2.1	0	32.9	56.1	5.1
3rd	170.0	85.8	14.2	22.6	2.0	0	1.4	90.2	3.8
4th	180.5	91.0	9.0	21.9	1.8	0	1.6	87.0	4.5
5th	180.6	91.1	8.9	22.0	1.8	0	0.3	95.6	1.4
Av. (3rd–5th)	177.0	89.3	10.7	22.2	1.9	0	1.1	90.9	3.2

No 16S amplicon reads of pathogenic food bacteria were detected during primary fed-batch storage. When *Escherichia coli* HB101, a hygienic indicator bacterium, or *Clostridium acetobutyricum*, a representative unpleasant odor producer, was added to the storage, the population of both decreased quickly to their detection limit (<10^4 copies/g). These results suggest that the accumulation of lactic acid and the resulting decrease in pH during simple primary storage effectively suppress the proliferation of spoilage bacteria, including food pathogens.

3.3. Effect of Primary Strage of Food Waste on Second Stage of Anaerobic Digestion

The successive production of biogas from the primary storage of food waste via the TSMTAD was evaluated compared with that of the SSAD of untreated food waste (Figure 3). Samples obtained after 2 days of the primary storage of food waste were used as substrates. It is reasonable to conclude that primary storage was effective in accelerating the time course of biogas accumulation (Figure 3A). The highest productivity of biogas from non-treated and pre-treated food waste was observed after 16.5 days in the case of TSMTAD compared to 18.5 days for SSAD. Fifty percent of the total methane accumulated after 7.5 days in the case of TSMTAD compared to 12 d for SSAD. Although it ultimately reached almost the same percentage of methane generation (80%) at 26.3 days, the time required for the accumulation of half the amount of total methane was different (after 7.5 and 11.5 days, respectively) (Figure 3B), representing a change in the metabolic pathway in the AD process in TSMTAD compared to SSAD. The mean daily biogas yields of TSMTAD and SSAD were

compared using Welch's *t*-test. The results showed that the differences were significant on all days. The average methane concentrations of the biogas obtained by TSMTAD and SSAD were also tested using Welch's *t*-test; their differences were significant every day from the 1st to the 20th day and on the 31st day.

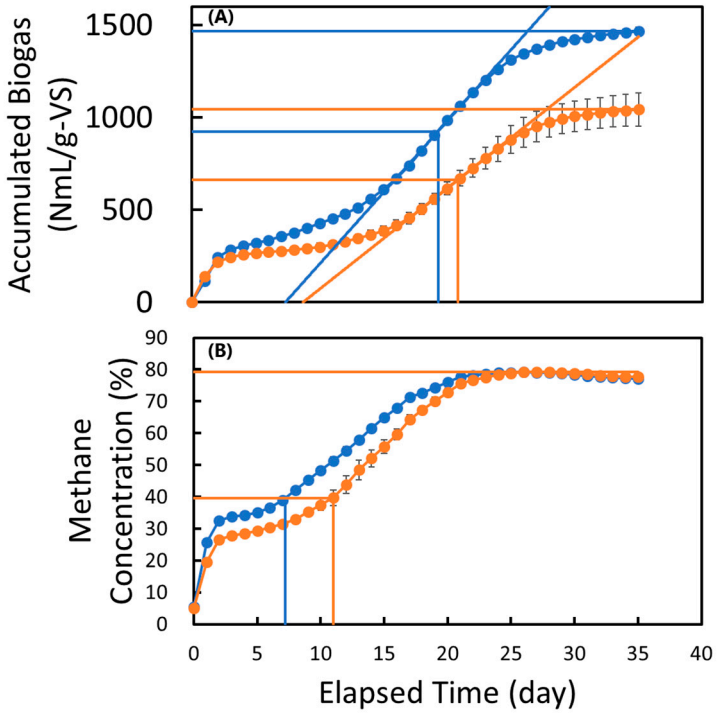

Figure 3. Time course of biogas production on TSMTAD (blue) and SSAD (orange). (**A**) Accumulated biogas; (**B**) Methane concentration.

As summarized in Table 2, the amount of biogas and methane accumulated in the second stage of TSMTAD was 1.4-fold higher than that from non-treated food waste on SSAD. Furthermore, the methane content was slightly higher in the second stage of TSMTAD (61.2%) than that on SSAD (58.0%). Thus, the average methane yield in the second stage of TSMTAD (897 NmL/g-VS) was 1.48-fold larger than that on SSAD. In contrast, the amount of residual organic acids decreased in TSMTAD compared with that in SSAD.

Table 2. Summary of biogas production by TSMTAD and SSAD.

Parameters		TSMTAD	SSAD	Ratio (TSMTAD/SSAD)
Substrate used (g)		888.7 ± 17.3	900.9 ± 10.6	-
Organic acid in digestate (g/L)	Formate	0	0	-
	Acetate	0.17 ± 0.03	0.51 ± 0.03	0.33
	Propionate	3.12 ± 0.11	4.37 ± 0.17	0.71
	Lactate	0	0	-
	Butyrate	0.65 ± 0.03	0.69 ± 0.02	0.94
Biogas (NmL/g-VS)		1465.8 ± 22.2	1043.5 ± 24.3	1.4
Methane (NmL/g-VS)		896.9 ± 89.0	604.9 ± 54.8	1.48
Methane Content (%)		61.2	58.0	1.06

The carbon yield (i.e., the fate of carbon atoms) in each experimental run was estimated based on that of the original food waste (Table 3). The carbon yield of methane from the original food waste was estimated to be 47.7% and 40.8% in the case of SSAD, respectively. It is worth noting that the methane yield was 6.9% higher in TSMTAD than in SSAD, despite the preceding carbon loss during the primary microaerobic storage of food waste.

This was caused in part by an increase in the biogas yield (5.51%) and a decrease in the CO_2 yield (−1.41%).

Table 3. Summary of carbon yield estimation.

Parameters	TSMTAD	SSAD
C (g in 1 kg kitchen refuse)	30.1 ± 0.58	30.1 ± 0.36
C (g in 1 kg each substrate)	23.6 ± 0.46	30.1 ± 0.36
CH_4-C (g from each reactor)	12.76 ± 0.50	11.07 ± 1.52
CH_4-C (g from 1 kg each substrate)	14.36 ± 0.42	12.28 ± 1.57
CO_2-C (g from each reactor)	7.81 ± 0.085	8.3 ± 1.0
CO_2-C (g from 1 kg each substrate)	8.79 ± 0.098	9.21 ± 1.02
Biogas-C (g-Biogas from 1 kg each substrate)	23.15 ± 0.45	21.49 ± 2.58
Residual organic acids-C (g in digestate from 1 kg kitchen refuse)	2.19 ± 0.11	3.11 ± 0.04
CH_4-C yield from each substrate (%)	60.85 ± 1.8	40.8 ± 5.2
CH_4-C yield from kitchen refuse (%)	47.7 ± 1.4	40.8 ± 5.2
C-CO_2 yield from each substrate (%)	37.23 ± 0.4	30.6 ± 3.4
C-CO_2 yield from kitchen refuse (%)	29.19 ± 0.3	30.6 ± 3.4
C-Biogas yield from each substrate (%)	98.08 ± 1.9	71.39 ± 8.6
C-Biogas yield from kitchen refuse (%)	76.9 ± 1.5	71.39 ± 8.6

3.4. Microbial Community Analysis of the Second Stage of Anaerobic Digestion

The microbial communities in both anaerobic digestion liquids were compared (Table 4). The cell concentrations estimated from the quantitative 16S rRNA gene copy number in each system were at the same level (2×10^8 copy/mL), while their alpha diversity differed. The Shannon value decreased in TSMTAD but was higher than that in SSAD. The values of the observed species in TSMTAD seeds were much higher than those in SSAD and AD seeds. This indicated that the microbial community in the second stage of TSMTAD was more complex than that in SSAD.

Table 4. Cell number and alpha diversity of each sludge.

Treatments	Cell Number ($\times 10^8$/mL)	Shannon	Observed Species
TSMTAD	2.40 ± 0.90	2.33 ± 0.33	43.3 ± 4.9
SSAD	2.45 ± 0.17	1.75 ± 0.16	25.7 ± 1.8
AD Seed	ND	2.89 ± 0.64	24.8 ± 9.9

Major OTUs showing a population abundance > 0.5% were deduced to be the closest microbial species [17–36] and compared (Table 5). Almost all OTUs were common to both runs, and many of them were attributed to possible thermophilic species and reasonable consortia acclimated from a reactor operating at high temperatures, except for OTU1, OTU2, OTU15, OTU16, OTU21, and OTU22. The first two OTUs were Fc-Hetero-LABs, *Levilactobacillus brevis* [17], and *Loigolactobacillus coryniformis* [18], which are known components of primary stored food waste. OTU3 to OTU15 were related species with higher abundances in TSMTAD than in SSAD. By contrast, OTU16 to OTU23 showed a lower abundance in TSMTAD. OTU14 was the only methane-producing archaea closely related to *Methanothermobacter thermautotrophicus* [28] that was found in both treatment systems. This species is a thermophilic hydrogen-assimilating methane bacterium that is frequently found during high-temperature anaerobic digestion [37]. The populations were similar for both TSMTAD and SSAD. OTU17 and -18 were collectively assigned to *Defulviltoga tunisiensis* [31] and were the most abundant in both systems. However, their abundance was found to decrease in the population of TSMTAD (from 77.1% to 71.4%).

Table 5. List of major OTUs in TSMTAD and SSAD.

OTU	Closest Species	Similarity (%)	Relative Abundance				Reference
			AD Seed (%)	TSMTAD (%)	SSAD (%)	TSMTAD /SSAD	
OTU 1	*Levilactobacillus brevis*	100	ND	1.07 ± 0.21	ND	-	[17]
OTU 2	*Loigolactobacillus coryniformis*	100	ND	0.57 ± 0.15	ND	-	[18]
OTU 3	*Acetomicrobium flavidum*	98.4	ND	0.58 ± 0.15	0.21 ± 0.36	2.81	[19]
OTU 4	*Hydrogenispora ethanolica*	89.4	2.2	1.99 ± 0.41	0.81 ± 0.73	2.47	[20]
OTU 5	*Clostridium isatidis*	100	ND	0.62 ± 0.18	0.27 ± 0.24	2.27	[21]
OTU 6	*Atribacter laminatus*	88.6	ND	0.74 ± 0.12	0.40 ± 0.35	1.85	[22]
OTU 7	*Thermoanaerobacter kivui*	89.8	ND	0.54 ± 0.11	0.30 ± 0.28	1.79	[23]
OTU8, 9, 10	*Capillibacterium thermochitinicola*	93.3, 95.7, 96.7	ND	0.52 ± 0.17	0.33 ± 0.09	1.58	[24]
OTU 11	*Thermanaerovibrio acidaminovorans*	87.4	2.04	2.31 ± 0.76	1.54 ± 0.22	1.50	[25]
OTU 12	*Thermoclostridium caenicola*	95.3	ND	0.62 ± 0.04	0.44 ± 0.40	1.41	[26]
OTU 13	*Thermacetogenium phaeum*	95.5	2.2	2.64 ± 0.81	2.00 ± 0.31	1.32	[27]
OTU 14	*Methanothermobacter thermautotrophicus*	100	0.52	0.91 ± 0.28	0.82 ± 0.18	1.11	[8]
OTU 15	*Syntrophaceticus schinkii*	97.2	ND	1.62 ± 0.37	1.60 ± 0.30	1.01	[29]
OTU 16	*Advenella faeciporci*	100	3.46	2.28 ± 0.19	2.39 ± 0.13	0.95	[30]
OTU 17, 18	*Defluviitoga tunisiensis*	99.6, 100	71	71.4 ± 5.7	77.1 ± 1.7	0.93	[31]
OTU 19	*Thermoanaerobacter sulfurigignens*	85.8	ND	1.13 ± 0.22	1.23 ± 0.13	0.91	[32]
OTU 20	*Acetomicrobium thermoterrenum*	100	1.28	0.86 ± 0.12	1.03 ± 0.36	0.84	[33]
OTU 21	*Halocella cellulosilytica*	92.5	ND	1.24 ± 0.16	1.53 ± 0.75	0.81	[34]
OTU 22	*Proteiniborus indolifex*	89.7	1.06	1.13 ± 0.04	1.41 ± 0.13	0.80	[35]
OTU 23	*Bacillus infernus*	98.8	2.4	2.07 ± 0.36	3.10 ± 0.24	0.67	[36]

4. Discussion

To accelerate the treatment of food waste created by individual households on a daily basis and facilitate the recovery of energy, two-stage meso- and thermophilic anaerobic digestion (TSMTAD) was examined in this study. The conditions of the first stage comprised primary storage for the efficient collection and treatment of food waste (Figure 1). For comparison with existing methods, the total methane productivity of successive high-temperature anaerobic digestion was compared to that of single-stage anaerobic digestion (SSAD). In conclusion, a simple but stable primary storage was demonstrated, and higher methane productivity was observed in the successive stages of TSMTAD compared with SSAD. The microbial characteristics underlying these results are discussed below.

4.1. Simple Control of the First Stage in TSMTAD

In the first stage, the fed-batch storage of food waste resulting in lactic acid accumulation was evaluated not only for its ability to store organic carbon but also to prevent non-LAB growth. Initial pH neutralization and inoculation of LAB seeds under mesophilic condition were sufficient for the domination of Fc-Hetero-LAB and the suppression of non-LAB (Figure 2). During several repetitions of the fed and drawn-out tests, the bacterial community structure became stable, and exogenously inoculated non-LAB were eliminated

(Table 1). Approximately 2% lactic acid and 0.2% acetic acid selectively accumulated, while an average of 89.3% of the carbon atoms were retained. This could be achieved by establishing non-strict anaerobic and acidic conditions via initial pH neutralization and intermittent mixing [36] for Fc-Hetero-LAB, but not for non-LAB, including methanogens. LAB are generally known as oxygen-tolerant mesophilic anaerobes [38] and show an adaptive response to acidic conditions [39]. Fc-Hetero-LAB is a LAB group that converts hexoses almost exclusively to lactic acid via the Embden–Meyerhof pathway, while pentoses are fermented to lactic acid and acetic acid [16], which would contribute to the storage of organic carbon in the sludge.

The hydrolysis of macromolecules is considered a limiting step in anaerobic digestion [9]. To address this issue, various types of pretreatments have been reported, including ozone treatment [40] and alkaline treatment [41]. In addition, the TSAD of food waste has been investigated as an advantageous system for controlling the hydrolysis/acidogenesis process in AD separately from acetogenesis/methanogenesis [42].

However, to our knowledge, TSMTAD has not yet been reported [9,42]. In this context, although the effect of pretreatment on lactic acid fermentation in TSAD has also been studied, the methane yield in this case was similar to that in SSAD [43]. Another study examined the effect of pH-controlled lactic acid fermentation on TSAD, but methane production at the second stage was not stimulated [44]. One potential explanation for the lack of precise microbial communities reported in previous studies is the mesophilic conditions of the second AD stage, as discussed below. In another study, the aerobic pretreatment of food waste was found to increase the methane yield rate but not the methane yield potential [45]. In this case, the first stage proceeded at a weak alkaline pH and accumulated various volatile fatty acids (VFAs), including acetic acid, propionic acid, and n-butyric acid, and a successive mesothermal AD distinguishable microbial process would be proceeded. Thus, as shown in the present study, mesophilic incubation with LAB seeds and the initial pH neutralization could contribute to the formation of a stable consortium of Fc-Hetero-LAB, accelerating hydrolysis and selective acidogenesis. Further investigation of the incubation temperature to minimize energy input while maintaining the microbial community structure and suppressing food pathogens, as well as examining sensitivity to feedstock variability, is required for large-scale application.

4.2. Microbial Community Structure Resulting from the Second Stage of TSMTAD

Although a certain amount of carbon loss was observed in this study, the mesophilic and LAB-rich primary storage of food waste stimulated high levels of methane production during the thermophilic anaerobic digestion of the latter, indicating that much more carbon was recovered as methane in TSMTAD compared to SSAD (Table 3). In addition, methane productivity (Table 2) was higher than that of a previously reported AD process including two-stage AD [6,9,13,14]. These results suggest that both the effective digestion of macromolecules in lactic acid fermentation during the first stage and an efficient conversion to methane via lactic acid during the second anaerobic digestion stage occurred in TSMTAD (Figure 4). However, further comparative studies on carbon yield using various food wastes should be conducted. As deduced from the microbial community structure, one possible change in the bioconversion pathway is the production of acetic acid from lactic acid. Although the microbial community structures in the anaerobic digestion stage in both TSMTAD and SSAD were similar, including the methanogen *Methanothermobacter thermotrphicus* (OTU14) [28], the microbial community in TSMTAD showed several distinct structural features (Table 5). OTUs closely related to *Defluviitoga tunisiensis* (OTU17 and -18) were highly abundant in both AD systems and decreased in abundance in TSMTAD. This species is popular in the thermophilic AD process [46], harboring a large set of genes that utilize a variety of complex polysaccharides, including cellulose, pectin polysaccharide, galactan, and hemicellulose, where ethanol, acetate, H_2, and CO_2 are the end products [31]. Notably, *Defulviltoga tunisiensis* does not utilize lactate as a substrate, whereas sugars in food waste are already degraded to lactate in the first stage of TSMTAD. In addition, some OTUs that

showed lower abundance in TSMTAD had macromolecule-degrading abilities, including OTU17 and -18, *Defluviltoga tunisiensis*; OTU21, *Halocella cellulosilytica* [34], compared to those in SSAD. On the contrary, many of the OTUs that showed higher abundances in TSMTAD were possible hydrogen and acetate producers, including OTU3, *Acetomicrobium flavidum* [19]; OTU4, *Hydrogenispora ethanolica* [20]; OTU5, *Clostridium isatidis* [21]; OTU8, -9, and -10, *Capillibacterium thermochitinicola* [24], or syntrophic acetate-oxidizing bacteria (SAOB), including OTU6, *Atribacter laminatus* [22]; OTU7, *Thermoanaerobacter kivui* [23]; OTU11, *Thermanaerovibrio acidaminovorans* [25]; and OTU15, *Syntrophaceticus schinkii* [26]. Many of these possible SAOB were undetectable in the AD seeds (Table 5) but could convert acetic acid to H_2 and CO_2 via symbiotic cooperation with *Methanothermobacter thermautotrophicus* [28]. Of the two methanogenic pathways, acetoclastic conversion via acetate was observed only in the presence of *Methanosaetaceae*, whereas acetate oxidation and successive hydrogenotrophic conversion were the dominant pathways when *Methanosaetaceae* were not present [47]. As such, the AD system shown here harbored the latter pathway, in which *Methanothermobacter thermotrphicus* used CO_2 and H_2 as substrates for methanogenesis symbiotically with unique SAOB (Figure 4) [48,49]. Considering that the ratio of CH_4-C/CO_2-C in the biogas was higher in TSMTAD than in SSAD (Table 2), a restructuring of the members of the microbial community could result in an increase in methane productivity in TSMTAD. Because the results were obtained using limited food waste, the effects of food waste from various sources on TSMTAD should be investigated. In addition, the long-term stability of the TSMTAD for large-scale applications must be clarified in future studies.

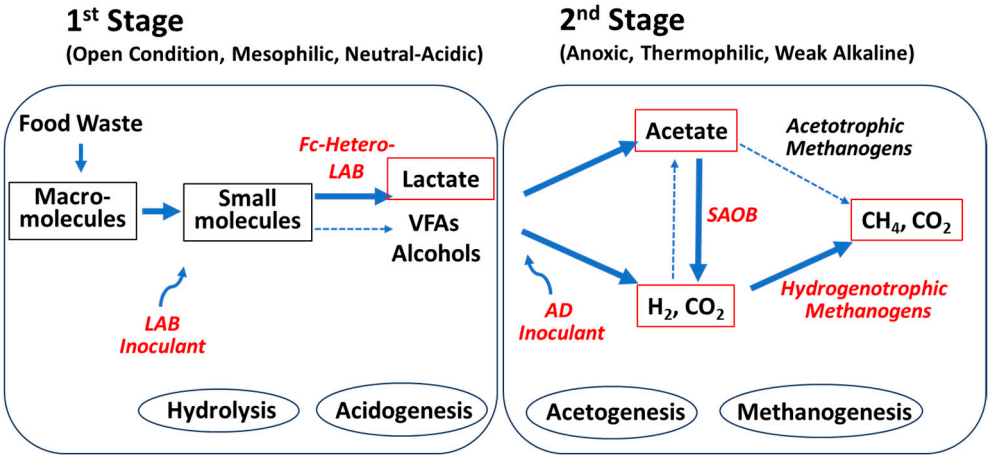

Figure 4. Characteristic pathway in TSMTAD. Stimulated pathways (solid arrow) and metabolites (red square) by specific microorganisms (red italics) at each stage of TSMTAD are shown. SAOB: syntrophic acetate-oxidizing bacteria.

4.3. Applicability of TSMATAD to "Hub-and-Spoke" Collection Model

In addition to the advantages represented by the independent control of the former hydrolysis/acidogenesis stage and the latter acetogenesis/methanogenesis stages in the TSAD system, the simple control of the first stage of TSMTAD also facilitates food waste treatment. The initial neutralization and inoculation of LAB seeds enable the stabilization of food waste via repeated fed-batch storage (Table 1). Furthermore, successive thermophilic AD resulted in high methane productivity (Figure 3, Table 4). Thus, TSMTAD shows potential in its applicability to a "hub-and-spoke" collection model [12] of food waste. The model has been discussed in terms of its merits and demerits for applications in industries such as the airline industry, shipping, overnight express delivery, and other transportation activities [50]. This system would have several logistically advantageous points, comprising a first stage of primary storage at multiple regional places (hub nodes) and a second stage of biogas production at a centralized location (demand node, Figure 1). The energy and manpower required for the collection and transportation of household

food waste could be reduced to larger quantities, and the concentration of the second stage at the centralized biogas production location would benefit from inventory management. The effective recycling of major by-products, sludge, and digestate, is also of interest in the system. Our initial estimates highlight a potential reduction in energy, manpower, and capital investment required for biogas production in a certain local city by the application of TSMTAD. A precise study of the efficiency and cost–benefit analysis of this type of logistics system with TSMTAD will need to be evaluated in a future study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10120607/s1, Table S1: Effect of seed source on organic acid accumulation and LAB community structure.

Author Contributions: Conceptualization, K.O., T.M., H.M., Y.T. and K.S.; methodology, Y.T. and K.O.; software, K.O. and T.K.; validation, K.O., Y.T. and K.S.; formal analysis, K.O., Y.O., H.T. and T.M.; investigation, K.O., Y.T., T.M. and H.M.; data curation, K.O., Y.O., T.K., T.M. and H.M.; writing—original draft preparation, K.S. and Y.O.; writing K.S.; review and editing, M.O., Y.T., K.O., T.M. and H.M.; visualization, T.K. and K.O.; supervision, K.O., Y.T. and K.S.; project administration, T.M. and H.M.; funding acquisition, T.M. and H.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded in part by NEDO, Phase B, Grant Number 2021-1015005, and Phase C, Grant Number 2024-514003. This work was also supported partly by JSPS KAKENHI Grant-in-Aid for Scientific Research (B) Grant Number JP 21H03663 and 23H03595. The authors would like to acknowledge the Center for Advanced Instrumental and Educational Supports and the Faculty of Agriculture, Kyushu University, for their support with MiSeq.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Conflicts of Interest: Authors Toshihito Morimitsu and Hideki Muraoka were employed by the company Momoyama Recycling Center R&D Division, Vioce Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Hamish, F.; Tom, Q.; Clementine, O. *Food Waste Index Report 2021*; United Nations Environment Programme: Nairobi, Kenya, 2021; ISBN 9789280738681.
2. Sakai, K.; Poudel, P.; Shirai, Y. Total Recycle System of Food Waste for Poly-L-Lactic Acid Output. In *Advances in Applied Biotechnology*; Petre, M., Ed.; IntechOpen Limited: London, UK, 2012; pp. 29–40. [CrossRef]
3. Sánchez, A. Decentralized Composting of Food Waste: A Perspective on Scientific Knowledge. *Front. Chem. Eng.* **2022**, *4*, 850308. [CrossRef]
4. Jeon, D.; Chung, K.; Shin, J.; Min Park, C.; Gu Shin, S.; Mo Kim, Y. Reducing Food Waste in Residential Complexes Using a Pilot-Scale on-Site System. *Bioresour. Technol.* **2020**, *311*, 123497. [CrossRef] [PubMed]
5. Adekunle, K.F.; Okolie, J.A. A Review of Biochemical Process of Anaerobic Digestion. *Adv. Biosci. Biotechnol.* **2015**, *6*, 205–212. [CrossRef]
6. Pilarska, A.A.; Kulupa, T.; Kubiak, A.; Wolna-Maruwka, A.; Pilarski, K.; Niewiadomska, A. Anaerobic Digestion of Food Waste-A Short Review. *Energies* **2023**, *16*, 5742. [CrossRef]
7. Harirchi, S.; Wainaina, S.; Sar, T.; Nojoumi, S.A.; Parchami, M.; Parchami, M.; Varjani, S.; Khanal, S.K.; Wong, J.; Awasthi, M.K.; et al. Microbiological Insights into Anaerobic Digestion for Biogas, Hydrogen or Volatile Fatty Acids (VFAs): A Review. *Bioengineered* **2022**, *13*, 6521–6557. [CrossRef]
8. Ishida, N.; Kawano, Y.; Fukui, R.; Zhang, M.; Tashiro, Y.; Sakai, K. Clarification of the Dynamic Autothermal Thermophilic Aerobic Digestion Process Using Metagenomic Analysis. *Microbiol. Spectr.* **2022**, *10*, 2. [CrossRef] [PubMed]
9. Holl, E.; Steinbrenner, J.; Merkle, W.; Krümpel, J.; Lansing, S.; Baier, U.; Oechsner, H.; Lemmer, A. Two-Stage Anaerobic Digestion: State of Technology and Perspective Roles in Future Energy Systems. *Bioresour. Technol.* **2022**, *360*, 127633. [CrossRef] [PubMed]
10. Chu, C.F.; Xu, K.Q.; Li, Y.Y.; Inamori, Y. Hydrogen and Methane Potential Based on the Nature of Food Waste Materials in a Two-Stage Thermophilic Fermentation Process. *Int. J. Hydrogen Energy* **2012**, *37*, 10611–10618. [CrossRef]

11. Tashiro, Y.; Inokuchi, S.; Poudel, P.; Okugawa, Y.; Miyamoto, H.; Miayamoto, H.; Sakai, K. Novel PH Control Strategy for Efficient Production of Optically Active L-Lactic Acid from Kitchen Refuse Using a Mixed Culture System. *Bioresour. Technol.* **2016**, *216*, 52–59. [CrossRef]
12. Thore, S. The Hub-and-Spoke Model—A Tutorial, SSRN Electronic Journal Aug. 2005. Available online: https://www.researchgate.net/publication/256069613 (accessed on 27 February 2023).
13. Wu, C.; Huang, Q.; Yu, M.; Ren, Y.; Wang, Q.; Sakai, K. Effects of Digestate Recirculation on a Two-Stage Anaerobic Digestion System, Particularly Focusing on Metabolite Correlation Analysis. *Bioresour. Technol.* **2018**, *251*, 40–48. [CrossRef]
14. Wang, P.; Wang, H.; Qiu, Y.; Ren, L.; Jiang, B. Microbial characteristics in anaerobic digestion process of food waste for methane production—A review. *Bioresour. Technol.* **2018**, *248*, 29–36. [CrossRef]
15. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Pēa, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
16. De Vos, P.; Garrity, G.M.; Jones, D.; Krieg, N.R.; Ludwig, W.; Rainey, F.A.; Schleifer, K.-H.; Whitman, W.B. *Bergey's Manual of Systematic Bacteriology*, 2nd ed.; The Low G + C Gram-Positive Bacteria (Bergey's Manual of Systematic Bacteriology); Springer: New York, NY, USA, 2009; Volume 3. Available online: http://hdl.handle.net/1854/LU-799073 (accessed on 27 February 2023).
17. Chen, Y.S.; Wu, H.C.; Lo, H.Y.; Lin, W.C.; Hsu, W.H.; Lin, C.W.; Lin, P.Y.; Yanagida, F. Isolation and Characterisation of Lactic Acid Bacteria from Jiang-Gua (Fermented Cucumbers), a Traditional Fermented Food in Taiwan. *J. Sci. Food Agric.* **2012**, *92*, 2069–2075. [CrossRef] [PubMed]
18. Gumustop, I.; Ortakci, F. Comparative Genomics of *Loigolactobacillus Coryniformis* with an Emphasis on *L. Coryniformis* Strain FOL-19 Isolated from Cheese. *Comput. Struct. Biotechnol. J.* **2023**, *21*, 5111–5124. [CrossRef] [PubMed]
19. Ben Hania, W.; Bouanane-Darenfed, A.; Cayol, J.L.; Ollivier, B.; Fardeau, M.L. Reclassification of *Anaerobaculum mobile*, *Anaerobaculum thermoterrenum*, *Anaerobaculum hydrogeniformans* as *Acetomicrobium mobile* comb. vov., *Acetomicrobium thermoterrenum* comb. nov. and *Acetomicrobium hydrogeniformans* Comb. nov., Respectively, and Emendation of the Genus *Acetomicrobium*. *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 1506–1509. [CrossRef]
20. Liu, Y.; Qiao, J.T.; Yuan, X.Z.; Guo, R.B.; Qiu, Y.L. *Hydrogenispora ethanolica* gen. nov., sp. nov., an Anaerobic Carbohydrate-Fermenting Bacterium from Anaerobic Sludge. *Int. J. Syst. Evol. Microbiol.* **2014**, *64*, 1756–1762. [CrossRef]
21. Padden, A.N.; Dillon, V.M.; Edmonds, J.; Collins, M.D.; Alvarez, N.; John, P. An Indigo-Reducing Moderate Thermophile from a woad vat, *Clostridium Isatidis* sp. nov. *Int. J. Syst. Bacteriol.* **1999**, *49*, 1025–1031. [CrossRef]
22. Katayama, T.; Nobu, M.K.; Kusada, H.; Meng, X.Y.; Hosogi, N.; Uematsu, K.; Yoshioka, H.; Kamagata, Y.; Tamaki, H. Isolation of a Member of the Candidate Phylum 'Atribacteria' Reveals a Unique Cell Membrane Structure. *Nat. Commun.* **2020**, *11*, 6381. [CrossRef]
23. Liu, F.; Conrad, R. Thermoanaerobacteriaceae Oxidize Acetate in Methanogenic Rice Field Soil at 50 °C. *Environ. Microbiol.* **2010**, *12*, 2341–2354. [CrossRef] [PubMed]
24. Ungkulpasvich, U.; Baramee, S.; Uke, A.; Kosugi, A. *Capillibacterium thermochitinicola* gen. nov., sp. nov., a Novel Anaerobic Thermophilic Chitinolytic Bacterium from Compost. *Int. J. Syst. Evol. Microbiol.* **2021**, *71*, 004693. [CrossRef]
25. Guangsheng, C.; Plugge, C.M.; Roelofsen, W.; Houwen, F.P.; Stams, A.J.M. *Selenomonas Acidaminovorans sp. nov., a Versatile Thermophilic Proton-Reducing Anaerobe Able to Grow by Decarboxylation of Succinate to Propionate*; Spinger: Berlin/Heidelberg, Germany, 1992; Volume 157, pp. 169–175.
26. Shiratori, H.; Sasaya, K.; Ohiwa, H.; Ikeno, H.; Ayame, S.; Kataoka, N.; Miya, A.; Beppu, T.; Ueda, K. *Clostridium Clariflavum* sp. nov. and *Clostridium Caenicola* sp. nov., Moderately Thermophilic, Cellulose-/Cellobiose-Digesting Bacteria Isolated from Methanogenic Sludge. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1764–1770. [CrossRef] [PubMed]
27. Hattori, S.; Kamagata, Y.; Hanada, S.; Shoun, H. *Thermacetogenium phaeum* gen. nov., sp. nov., a Strictly Anaerobic, Thermophilic, Syntrophic Acetate-Oxidizing Bacterium. *Int. J. Syst. Evol. Microbiol.* **2000**, *50*, 1601–1609. [CrossRef] [PubMed]
28. Smith, D.R.; Doucette-Stamm, L.A.; Deloughery, C.; Lee, H.; Dubois, J.; Aldredge, T.; Bashirzadeh, R.; Blakely, D.; Cook, R.; Gilbert, K.; et al. Complete Genome Sequence of *Methanobacterium thermoautotrophicum* ΔH: Functional Analysis and Comparative Genomics. *Int. J. Syst. Evol. Microbiol.* **1997**, *179*, 7135–7155.
29. Westerholm, M.; Roos, S.; Schnürer, A. *Syntrophaceticus schinkiigen*. nov., sp. nov., an Anaerobic, Syntrophic Acetate-Oxidizing Bacterium Isolated from a Mesophilic Anaerobic Filter. *FEMS. Microbiol. Lett.* **2010**, *309*, 100–104. [CrossRef] [PubMed]
30. Matsuoka, M.; Park, S.; An, S.Y.; Miyahara, M.; Kim, S.W.; Kamino, K.; Fushinobu, S.; Yokota, A.; Wakagi, T.; Shoun, H. *Advenella faeciporci* sp. nov., a Nitrite-Denitrifying Bacterium Isolated from Nitrifying-Denitrifying Activated Sludge Collected from a Laboratory-Scale Bioreactor Treating Piggery Wastewater. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 2986–2990. [CrossRef] [PubMed]
31. Maus, I.; Cibis, K.G.; Bremges, A.; Stolze, Y.; Wibberg, D.; Tomazetto, G.; Blom, J.; Sczyrba, A.; König, H.; Pühler, A.; et al. Genomic Characterization of *Defluviitoga tunisiensis* L3, a Key Hydrolytic Bacterium in a Thermophilic Biogas Plant and Its Abundance as Determined by Metagenome Fragment Recruitment. *J. Biotechnol.* **2016**, *232*, 50–60. [CrossRef]
32. Lee, Y.J.; Dashti, M.; Prange, A.; Rainey, F.A.; Rohde, M.; Whitman, W.B.; Wiegel, J. *Thermoanaerobacter Sulfurigignens* sp. nov., an Anaerobic Thermophilic Bacterium That Reduces 1 M Thiosulfate to Elemental Sulfur and Tolerates 90 MM Sulfite. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 1429–1434. [CrossRef] [PubMed]
33. Rees, G.N.; Patel, B.K.C.; Grassia, S.; Sheehy, A.J. *Anaerobaculum themoterrenum* gen. nov., sp. Nov., a Novel Thermophilic Bacterium Which Ferments Citrate. *Int. J. Syst. Bacteriol.* **1997**, *47*, 150–154. [CrossRef] [PubMed]

34. Simankova, M.V.; Chernych, N.A.; Osipov, G.A.; Zavarzin, G.A. *Halocella cellulolyticagen.* nov., sp. nov., a New Obligately Anaerobic, Halophilic, Cellulolytic Bacterium. *Syst. Appl. Microbiol.* **1993**, *16*, 385–389. [CrossRef]
35. Hahnke, S.; Langer, T.; Klocke, M. *Proteiniborus indolifex* sp. nov., Isolated from a Thermophilic Industrial-Scale Biogas Plant. *Int. J. Syst. Evol. Microbiol.* **2018**, *68*, 824–828. [CrossRef]
36. Boone, D.R.; Liu, Y.; Zhao, Z.; Balkwill, D.L.; Drake, G.R.; Aldrich, H.C. *Bacillus infernus* sp. nov., an Fe(III)-and Mn(IV)-Reducing Anaerobe from the Deep Terrestrial Subsurface. *Int. J. Syst. Bacteriol.* **1995**, *45*, 441–448. [CrossRef] [PubMed]
37. Sakai, K.; Murata, Y.; Yamazumi, H.; Tau, Y.; Mori, M.; Morjguchi, M.; Shirai, Y. Selective Proliferation of Lactic Acid Bacteria and Accumulation of Lactic Acid during Open Fermentation of Kitchen Refuse with Intermittent PH Adjustment. *Food Sci. Technol. Res.* **2000**, *6*, 140–145. [CrossRef]
38. Bryukhanov, A.L.; Klimko, A.I.; Netrusov, A.I. Antioxidant properties of lactic acid bacteria. *Microbiology* **2022**, *91*, 463–478. [CrossRef]
39. Papadimitriou, K.; Alegría, Á.; Bron, P.A.; de Angelis, M.; Gobbetti, M.; Kleerebezem, M.; Lemos, J.A.; Linares, D.M.; Ross, P.; Stanton, C.; et al. Stress Physiology of Lactic Acid Bacteria. *Microbiol. Mol. Biol. Rev.* **2016**, *80*, 837–890. [CrossRef]
40. Yue, L.; Cheng, J.; Hua, J.; Dong, H.; Zhou, J.; Li, Y.Y. Improving Fermentative Methane Production of Glycerol Trioleate and Food Waste Pretreated with Ozone through Two-Stage Dark Hydrogen Fermentation and Anaerobic Digestion. *Energy Convers. Manag.* **2020**, *203*, 112225. [CrossRef]
41. Grűbel, K.; Machnicka, A.; Wacławek, S. Alkalization of Surplus Activated Sludge on Biogas Production. *Ecol. Chem.Eng. S* **2013**, *20*, 343–351. [CrossRef]
42. Van, D.P.; Fujiwara, T.; Tho, B.L.; Toan, P.P.S.; Minh, G.H. A Review of Anaerobic Digestion Systems for Biodegradable Waste: Configurations, Operating Parameters, and Current Trends. *Environ. Eng. Res.* **2020**, *25*, 1–17. [CrossRef]
43. Feng, K.; Li, H.; Deng, Z.; Wang, Q.; Zhang, Y.; Zheng, C. Effect of Pre-Fermentation Types on the Potential of Methane Production and Energy Recovery from Food Waste. *Renew. Energy* **2020**, *146*, 1588–1595. [CrossRef]
44. Feng, K.; Li, H.; Zheng, C. Shifting Product Spectrum by PH Adjustment during Long-Term Continuous Anaerobic Fermentation of Food Waste. *Bioresour. Technol.* **2018**, *270*, 180–188. [CrossRef]
45. Wu, C.; Yu, M.; Huang, Q.; Ma, H.; Gao, M.; Wang, Q.; Sakai, K. Stimulation of Methane Yield Rate from Food Waste by Aerobic Pre-Treatment. *Bioresour. Technol.* **2018**, *261*, 279–287. [CrossRef]
46. Hania, W.B.; Godbane, R.; Postec, A.; Hamdi, M.; Ollivier, B.; Fardeau, M.L. *Defluviitoga tunisiensis* gen. nov., sp. nov., a *Thermophilic bacterium* Isolated from a Mesothermic and Anaerobic Whey Digester. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 1377–1382. [CrossRef] [PubMed]
47. Karakashev, D.; Batstone, D.J.; Trably, E.; Angelidaki, I. Acetate Oxidation Is the Dominant Methanogenic Pathway from Acetate in the Absence of Methanosaetaceae. *Appl. Environ. Microbiol.* **2006**, *72*, 5138–5141. [CrossRef] [PubMed]
48. Dyksma, S.; Jansen, L.; Gallert, C. Syntrophic Acetate Oxidation Replaces Acetoclastic Methanogenesis during Thermophilic Digestion of Biowaste. *Microbiome* **2020**, *8*, 105. [CrossRef]
49. Zheng, D.; Wang, H.Z.; Gou, M.; Nobu, M.K.; Narihiro, T.; Hu, B.; Nie, Y.; Tang, Y.Q. Identification of Novel Potential Acetate-Oxidizing Bacteria in Thermophilic Methanogenic Chemostats by DNA Stable Isotope Probing. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 8631–8645. [CrossRef]
50. Pels, E. Optimality of the Hub-Spoke System: A Review of the Literature, and Directions for Future Research. *Transp. Policy* **2021**, *104*, A1–A10. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.