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Silica/Lignin Carrier as a Factor Increasing the Process Performance and Genetic Diversity of Microbial Communities in Laboratory-Scale Anaerobic Digesters

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Abstract: The article aims to present results of research on anaerobic digestion (AD) of waste wafers (WF-control) and co-substrate system–waste wafers and cheese (WFC-control), combined with digested sewage sludge, as inoculum. The purpose of this paper is to confirm the outcome of adding silica/lignin (S/L; 4:1) material, as a microbial carrier, on the process performance and genetic diversity of microbial communities. The experiment was conducted in a laboratory under mesophilic conditions, in a periodical operation mode of bioreactors. Selected physicochemical parameters of the tested carrier, along with the microstructure and thermal stability, were determined. Substrates, batches and fermenting slurries were subjected to standard parameter analysis. As part of the conducted analysis, samples of fermented food were also tested for total bacterial count, dehydrogenase activity. Additionally, DNA extraction and next-generation sequencing (NGS) were carried out. As a result of the conducted study, an increase in the volume of produced biogas was recorded for samples fermented with S/L carrier: in the case of WF + S/L by 18.18% to a cumulative biogas yield of 833.35 m^3 Mg^{-1} VS, and in the case of WFC + S/L by 17.49% to a yield of 950.64 m^3 Mg^{-1} VS. The largest total bacterial count, during the process of dehydrogenase activity, was maintained in the WFC + S/L system. The largest bacterial biodiversity was recorded in samples fermented with the addition of cheese, both in the case of the control variant and in the variant when the carrier was used. In contrast, three phyla of bacteria *Firmicutes*, *Proteobacteria* and *Actinobacteria* predominated in all experimental facilities.

Keywords: silica/lignin carrier; organic waste; anaerobic digestion; microbial genetic diversity; process performance

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1. Introduction

Currently, there is a strong trend towards the disposal of waste with the use of technologies that aim to reuse or recycle it. Anaerobic digestion of organic waste fits well with that concept because during that process organic waste is transformed into products, compost, and biogas that can be used in the economy. Anaerobic digestion as a process of disposal of organic substances was initially used for wastewater treatment and later on, for the stabilisation of sewage sludge in biological treatment plants with a total content

of solids of 3% to 10% [1–3]. The abovementioned process is successfully implemented to decompose solid waste of various origins in the form of suspension [4–6].

Anaerobic processes are performed in closed-loop systems, and the energy of produced biogas is used. The exhaust air from the ventilation of technological halls is cleaned with the use of biological filters. Therefore, there are no issues related to the emission of odor or bacterial aerosols. The best results are obtained by locating the fermentation installation near an industrial facility where waste heat from electricity production is used all year round [7].

Bioeconomy is a relatively new concept, which emerged as a response to the intensive development of the fossil fuel economy in a way that seriously threatens the efficient and sustainable use of natural resources. On the other hand, biorefineries are a key pillar in the development of a bioeconomy-based society. The idea of producing biofuels and bioproducts from a wide range of biomass sources in a flexible and integrated biorefinery opens the possibility of developing new and more sustainable processes and products that will eventually lead to a transition from the current scenario of a mainly oil-based economy to a true bioeconomy [8]. Today, biorefineries, built near large cities, are focused on the production of fuels and energy and the production of various organic substances of high market value. Lopes and Łukasik (2020) point out the significant economic benefits of wastewater utilisation under the biorefinery concept [9]. These researchers mention the reduction of additional investment costs in waste treatment facilities, reduction of operating costs (e.g., raw materials, utilities, maintenance), achieving high energy efficiency in the recovery and recycling of raw materials, reduction of logistics and supply chain costs by applying the biorefinery concept on a small scale, thus creating local synergies with suppliers and end-users. Additionally, the small-scale biorefinery concept, using wastewater and valorising biomass fractions, generates significantly lower greenhouse gas emissions and has a significant impact on fossil fuel depletion and eutrophication and water poisoning.

While discussing the issues of sustainable development and utilisation of by-products and waste products of agri-food industry processes, it should be mentioned that the waste products—confectionery, used in this research, are generated in large quantities and a continuous manner. The weekly waste generated in a typical production plant is estimated in tonnes, while the annual waste is in hundreds of tonnes [10]. Currently, the most common methods of waste utilisation are partial recycling or incineration. The latter method has always involved some problems, mainly due to the high level of pollution emitted by gases released in the process. It is also increasingly proposed to use confectionery waste in the production of animal feed. However, the direct application of food/confectionery waste as animal feed carries a high risk of increasing disease due to the shortening of the food chain. Biorefining, including anaerobic digestion, is in this case, the best alternative—the most cost-effective and environmentally friendly [2]. On the other hand, confectionery waste, due to its chemical composition and neutral pH (in most food waste, the pH is unfavourably acidic), has a high methanogenic potential.

Recently, Ximenes et al. (2021) proved that industrial waste and residue valorisation through biogas production is a feasible solution for a specific industrial scenario dealing with new socio-economic challenges [11]. This author presents the valorisation of wastes and residues from local fish, prawns and the vegetable-cultivation industry via biogas production forced to adapt to these new circumstances. It transpired that in a single year, as much as 189.74 tonnes of wastes and residues could be processed by the biogas production facility, producing as much as 94 GJ of cooling energy and 1 tonne of biofertiliser monthly.

A factor that guarantees high efficiency of biogas/methane production is the good condition of the bacterial flora as a catalyst for biochemical transformations. One of the methods of improving the conditions for the functioning of methanogens is their immobilisation with the use of a relevant carrier [12]. Interactions that take place between microorganisms and the material lead to biofilm formation, whose durability depends on, i.e., the type of a carrier and the individual characteristics of the environment. As a rule,

a good carrier should be insoluble, non-toxic, compatible, easily available, inexpensive, porous, and mechanically and thermally stable [13]. According to available reports on the conducted research, there were experiments performed related to methane fermentation with the use of i.e. zeolites, montmorillonite, bentonite, perlite, activated carbon, natural rubber, microcarriers, pine sawdust, and chitosan [14–17]. However, the aforementioned materials, in many cases, are characterised by functional imperfections such as low porosity and insufficient mechanical strength.

One of the substances with properties that are suitable for a microbial carrier is silica, i.e., a mesoporous material that has a well-formed surface area. Due to its specific physicochemical and electrochemical properties, silica is used in many industries such as pharmaceutical, cosmetic, chemical, paper, paint, and electrochemical [18]. When it comes to biomedical application, the fact that silica adsorbs proteins is well known [19]. Recently, the influence of microscopic silica particles on the organic matter decomposition in wastewater has been confirmed [20,21]. In turn, lignin, a natural polymer, is the primary wood component; it has a porous structure and shows hydrolytic enzyme resistance and thermal stability [22]. In recent years, the possibilities to use lignin as a potential microbe carrier have been explored in organic waste mono-digestion and co-digestion as described by Pilarska et al. (2018, 2019) [23,24]. The silica/lignin material was tested by Pilarska et al. (2020) as a way of anaerobic digestion of sewage sludge (SS) [25]. It should be mentioned that the favourable results of this experiment provided the rationale for the present study. The comparison of two carrier materials: pure kraft lignin and silica/lignin system (4:1) in SS fermentation, clearly showed the advantage of the material with SiO_2 (silica) addition. Incorporating the carrier into other types of substrate systems is necessary from a practical point of view. Previously, other researchers have also noted that the lignin and silica combination contributes to creating a material showing robust adsorption properties concerning, for instance, pigments, harmful organic substances and heavy metals [26,27]. Porous and biocompatible silica combined with lignin, characterised by strong adsorption properties and resistance to decomposition, is a good carrier and activator material for cells involved in anaerobic digestion.

The article aims to assess the effect of a microbial carrier in the form of silica/lignin system (4:1), not applied in the AD process so far, on the process performance and genetic diversity of microbial communities in laboratory-scale anaerobic digesters. The cumulative biogas/methane yield was determined for samples with waste wafers (WF) and co-substrates of waste wafers and cheese (WFC) as control variants and for analogous samples with the addition of a carrier. The addition of silica/lignin material resulted in increased activity of dehydrogenases, intensified proliferation of bacteria. As a consequence of the more effective operation of the biocatalysts, there was an increase in the rate of conversion of biomass and the volume of produced biogas, including methane. Furthermore, to determine the changes in bacterial metapopulations, next-generation sequencing was performed for the analysed experimental variants, which is the most innovative method applied to read the genetic sequence. 16S rRNA (the prokaryotic 16S ribosomal RNA gene) gene sequence analysis is widely used in the process of identification of microorganisms—bacteria, archaeons—and to examine the phylogenetic correlation between them [28]. The meta-taxonomic analysis conducted for this study showed the impact of a certain type of experimental facility on the structure of the bacterial microbiome.

The fact that the performed experiment was successful was because of individual properties of the components of the carrier, such as i.e., microstructure, chemical and quantitative composition, qualitative and quantitative selection of substrates for the AD process.

2. Materials and Methods

2.1. Substrates and Inoculum

Substrates in the experiment were waste wafers with filling and waste cheese (curd type), collected from production companies located in the area of Poznań. In addition, a local sewage treatment plant provided digested sewage sludge that was used as inoculum.

Waste wafers were used as a stand-alone material and in combination with waste cheese. The sewage sludge used, due to its high alkalinity, achieves a significant buffer capacity, which, as noted in previous work by Pilarska et al., may be crucial for maintaining a stable pH value during biomass decomposition [1–3].

2.2. Experimental Procedure

2.2.1. Batch Preparation

The following control samples were analysed (with the addition of waste wafers and waste wafers along with cheese as co-substrates) and samples with the addition of silica/lignin carrier, marked accordingly: WF-control, WFC-control, WF + S/L, WFC + S/L. The proportion of substrates and inoculum in the samples was determined according to the German standard VDI 4630, and the proportions of the mixture were set, as per the norm, so that the content of dry matter in the batches did not exceed 10% [29]. The composition of the batches, along with their most important physicochemical parameters, is presented in Table 1.

Table 1. Composition and selected properties of the substrate/inoculum samples.

Samples	WF (g)	CE (g)	S/L (g)	Inoculum (g)	pH	TS (%)	VS (%)
WF-control	9.8	-	-	830.2	7.15	4.01	65.64
WF + S/L	9.8	-	20.0	830.2	7.08	4.00	64.62
WFC-control	5.5	2.9	-	832.6	6.96	3.76	65.57
WFC + S/L	5.5	2.9	20.0	832.6	6.75	3.76	64.55

WF = wafers, CE = cheese, WFC = WF + CE co-substrate system, TS = total solids, vs. = volatile solids, S/L = silica/lignin.

2.2.2. Carriers Preparation

Two types of material were used to prepare the carrier: silica, fumed (silicon dioxide, powder) and kraft lignin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

In the sample of silica/lignin carrier (4:1), there was 16 g of silica and 4 g of lignin, a dose per 1 L of a batch. The generation of the hybrid system was done in the process of mechanical grinding of the output components (and mixing of the components at the same time) with the use of a ball mill (PULVERISETTE 23, FRITSCH, Germany). Next, the carrier material was rinsed with PBS solution (phosphate buffered saline) and then treated with the use of sterile distilled water and subsequently dried to obtain a constant initial weight at 70 °C.

2.2.3. Bacillus Amyloliquefaciens Biomass

The bacterial cell biomass of the culture that included the examined carrier was determined by weighing. First, the PBS solution was used to rinse the carrier; subsequently, the carrier was rinsed again using sterile distilled water, after which it was dried to a constant initial weight at 70 °C. Later, in the amount calculated per 100 mL, the carrier was mixed in a flask that contained 0.6 g of glucose and 1.3 g of regular broth, to which distilled water was added to get a volume of 100 mL. Then, it was sterilised for 40 min at 110 °C in an autoclave. An autochthonous strain of Bacillus amyloliquefaciens was then placed on the obtained substrate, isolated from fermented sewage sludge. All inoculated samples (excluding controls) were subjected to incubation at the temperature of 24 °C for 5 days (Compact Shaker KS 15 B–Edmund Bühler GmbH) and shook (75 rpm). Following 5 days, the cultures, controls included, were further centrifuged (15,000 rpm, 15 min) with the use of a Hettich Universal 16 R centrifuge. The bacterial cell biomass that multiplied on the substrate with carriers was determined based on the difference in weight (g) between the uninoculated substrate and the substrate inoculated with an autochthonous strain of *Bacillus amyloliquefaciens* [25].

2.2.4. Anaerobic Digestion

Figure 1 presents the multi-chamber bioreactor that was used for the anaerobic digestion (AD). The total number of fermentation chambers in the experiment was 12 (each sample was analysed three times). The 1.0 L bioreactors (5) were filled in with a substance that was stirred one time per day. The reactors were placed in a container with water (4), coupled to a heater (1), which allowed to perform the process at a set range of temperatures (mesophilic conditions). The produced biogas was directed (7) into tanks (8) (with a scale), where it was stored.

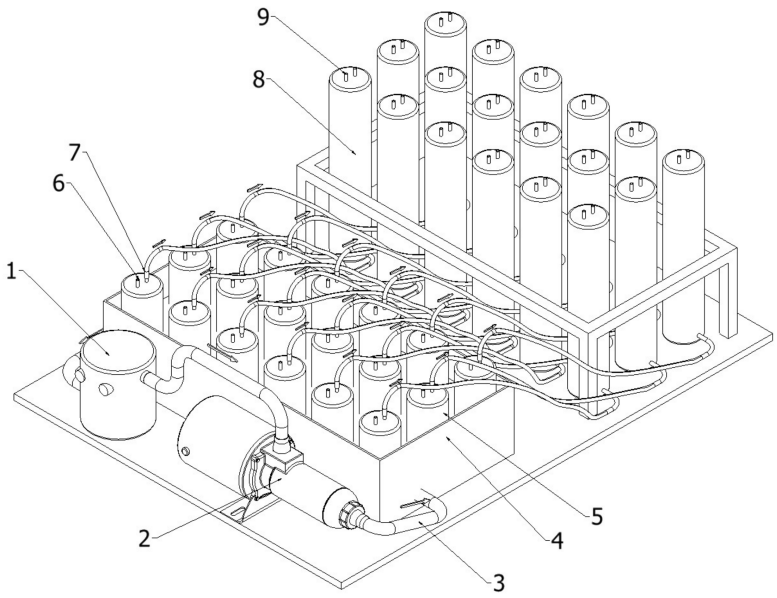

Figure 1. The (12-chamber) anaerobic bioreactor that was employed for the biogas production experiment: 1-water heater; 2-water pump; 3-insulated heating tubes; 4-water jacket (39 °C); 5-bioreactor (1.4 L); 6-slurry sampling valve; 7-biogas transport tube; 8-graduated biogas tank; 9-gas sampling valve.

As per the German standard DIN Guideline 38 414-S8 (DIN, Deutsches Institut für Normung Berlin, Germany) [30], the experiment was performed until daily production of biogas dropped below 1% of the total biogas produced in all biofermenters. The amount of produced biogas was checked every 24 h. Methane, carbon dioxide, hydrogen sulphide, ammonia and oxygen levels in biogas were established with the use of the Geotech GA5000 gas instrument. Assessment of biogas yield (in m^3 Mg^{-1}), in terms of total solids and volatile solids, was performed based on experimental data. In the case of bioreactors with batch control and with batch along with a carrier, the total amount of biogas was calculated with the use of formulas that had been described in the previous works of the authors [1,3,31].

2.3. Analysis Techniques

2.3.1. Physicochemical Analysis

Substrates and batches underwent pH analysis (potentiometric analysis). Properties such as electrolytic conductivity were measured with the use of the Elmetron CP-215 apparatus (ELMETRON, Zabrze, Poland). For the same material, total solids (TS) were determined by means of drying at 105 °C (Zalmed SML dryer, Zalmed, Łomianki, Poland). In contrast, volatile solids (VS) were determined using combustion at 550 °C (MS Spectrum PAF 110/6 furnace, MS Spectrum, Warsaw, Poland), known as gravimetric analysis.

In addition, the substrates and fermented samples were also tested in terms of carbon content using combustion at 900 °C followed by carbon dioxide determination (Infrared

Spectrometry, OI Analytical Aurora 1030 W TOC Analyzer, Picarro Inc., Santa Clara, CA, USA); the content of nitrogen–titration, Kjeldahl method with the use of 0.1 n HCl, with Tashiro's indicator; the content of ammonium nitrogen–distillation and titration, using of 0.1 n HCl, in the with Tashiro 's indicator.

To determine the content of volatile fatty acids (VFAs), total alkalinity (TA) and finally the volatile fatty acids-to-total alkalinity ratio (VFA/TA ratio) in the fermentation load, 5 mL of a given sample was collected (a sample of 5 mL of fermentation substrate) and titrated to 0.1 N of sulphuric acid solution (H_2SO_4) up to pH 5.0 to establish TA. The VFA level was established after a second titration between pH 5.0 and pH 4.4.

Images showing the morphology and microstructure of the silica/lignin system for examination and analysis were taken with an FEI Quanta 250 FEG scanning electron microscope (Thermo Fisher Scientific, Waltham, MA, USA), operating in a low vacuum mode at 70 Pa and an accelerating voltage of 10 kV. Prior to examination, the sample was covered with Au for 5 s using a Balzers PV205P coater (Oerlikon Balzers Coating SA, Balzers, Switzerland).

The silica/lignin carrier was also examined in a nitrous environment in terms of its thermal stability using a TGA 4000 thermogravimetric analyser (PerkinElmer, Waltham, MA, USA). Nitrogen was used to heat the samples from 25 to 995 °C at a flow rate of 20 mL $min^{-1,}$ and the temperature of 995 °C was maintained for 1 min before the samples were cooled [25].

2.3.2. Microbiological and Biochemical Analysis

The digested samples underwent biochemical analysis employing the spectrophotometric method. Their dehydrogenase activity was determined by applying the method developed by [32] after certain adjustments. Samples that were approximately 5 mL in volume were incubated at 30 °C, at a pH of 7.4 for 24 h with 2,3,5-triphenyltetrazolium chloride (TTC). Triphenylformazan (TPF) was obtained, extracted using 96% ethanol, and then quantified at 285 nm using a spectrophotometer. The dehydrogenase activity was given as μmol TPF mL^{-1} DM (dry matter) of waste 24 h^{-1}.

Analyses performed with the application of selective agar standard by Merck (Darmstadt, Germany) made it possible to measure colony-forming units (CFUs) of anaerobic bacteria (AnB). The count of the bacteria population was recorded after 24 h of incubation at 35 °C. Anaerobic conditions under which Petri dishes were incubated were maintained using the Anaerocult anaerobic incubation system (Merck).

2.3.3. DNA Extraction and Next-Generation Sequencing (NGS)

Total DNA was extracted from 500 mg of each sample using Genomic Mini AX Soil kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction. The extracted DNA was quantified using Quant-iT HS ds.-DNA assay kit (Invitrogen, Carlsbad, CA, USA) on Qubit2 fluorometer (Invitrogen); 2 μL of extracts were examined with the use of 0.8% agarose gel.

Metagenomic analysis was conducted based on the hypervariable region V3–V4 of the 16S rRNA gene. Specific primers 341F and 785R were used for amplification of this region and library preparation. PCR (polymerase chain reaction) was conducted with the use of Q5 Hot Start High-Fidelity DNA Polymerase kit (NEB Inc., Ipswich, MA, USA) at reaction conditions according to the manufacturer's specifications. Sequencing was conducted with the use of a MiSeq sequencer in 2 × 250 bp paired-end (PE) technology using the v2 Illumina chemistry kit. The reactions were carried out according to the Illumina V3–V4 16S RNA amplification protocol (Illumina, Inc., San Diego, CA, USA) and sequencing was performed according to the Illumina MiSeq PE300 (Genomed S.A., Warsaw, Poland). Automatic data analysis was performed on MiSeq and in Cloud environment BaseSpace by Illumina, using the 16S Metagenomics protocol (ver. 1.0.1). The libraries were prepared in an analogous way, according to the attached Illumina protocol.

2.3.4. Statistical and Bioinformatics Analyses

The *DADA2* (1.14) package [33] in R software (3.6.0) (R Core Team, 2016) was used to process demultiplexed fastq files [34]. Based on the quality plots, the last 20 and 70 bases were trimmed off forward and reverse reads accordingly. Primer sequences were excluded from all readings. Filter parameters were as follows: maxN = 0, maxEE for both reads = 2, truncQ = 2. The error rates were estimated by *learnErrors* using one million reads and exact sequence variants were resolved using dada. Next *removeBimeraDenovo* was used to remove chimeric sequences. After the filtration steps, 111,413–142,459 (mean = 131,475) of the reads were kept for further analysis. Taxonomy was assigned against the latest version of the modified RDP (Ribosomal Database Project) v18 database using IDTAXA taxonomic classification algorithm [35] on the sequences table, which was the outcome of the above *DADA2* workflow. Then, the results were transformed and imported into the *phyloseq* (1.22.3) package [36]. Chloroplast or mitochondrial DNA sequences were excluded. Next, the total number of individual taxa reads was transformed into a percentage for further analysis, assuming all taxa sum to be 100% for every sample. On average, 55% of all reads, which were correctly classified to the genus level, were aggregated and counted. Unclassified reads were grouped with *vsearch* [37] implemented in version 2.1 of seed software [38], at a 99% similarity level. Each of 71 clustered groups of unclassified reads was then marked as Unclassified_001 to Unclassified_071 and merged with the previous table (containing reads classified to the genus level). This approach enabled the statistical processing of the true alpha and beta diversity, regardless of whether a sequence exists in the reference database or not. As a result, 227 uncommon taxa (in terms of genus plus unclassified clusters) were found in every sample in total. The *phyloseq* package was used to calculate alpha diversity indices [39,40]. In addition, principal component analysis (PCA) was carried out and visualised using Past 3.25 software (Oslo, Norway).

Statistical analyses were performed with Statistica 13.3 software (StatSoft Inc. 2013, Tulsa, OK, USA). Two-way ANOVA was applied to determine the significance of the variation in the bacteria count groups and the enzymatic activity. Tukey's test was employed to calculate homogeneous mean subsets at a level of significance of $p < 0.05$. Lastly, stepwise regression was employed to find the set of optimal variables for specific bacteria and dehydrogenase activity characteristics.

3. Results and Discussion

3.1. Substrate Characteristics

Both waste wafers and waste cheese have a high proportion of volatile solids (VS), as shown in Table 2. However, it is the chemical structure of waste wafers and the presence of carbohydrates (high C concentration and high C/N ratio) that provide significant BMPs (biochemical methane potential) of the confectionery waste. Detailed results of the analysis of waste wafers were presented in previous publications of the authors [2,23].

Table 2. Substrate and inoculum physicochemical properties.

Waste	pH	Cond.	TS	VS	C/N Ratio	C	N	N-NH_4
		(mS cm^{-1})	(wt %)	(wt $\%_{TS}$)		(wt $\%_{TS}$)	(wt $\%_{TS}$)	(wt $\%_{TS}$)
Wafers	6.92	1.88	71.62	99.53	46.22	43.45	0.94	0.32
Cheese	4.59	74.36	32.15	94.86	3.48	49.64	14.25	0.49
Inoc.	7.03	32.16	3.21	65.24	3.30	26.37	7.99	3.97

Cond. = conductivity, TS = total solids, vs. = volatile solids, Inoc. = Inoculum.

Waste cheese is characterised by an extremely high conductivity value (Table 2), which indicates a large share of macronutrients in the composition of curd, including calcium and sodium. There are both positive and negative effects of increased concentration of Ca and Na on the operation of an anaerobic reactor [41,42]. Excessive levels of calcium may cause the formation of mill scale, however, regarding the amount of calcium in cheese waste [24]

and applied to the reactor, that macronutrient may contribute to the formation of cell aggregates. Salt (including sodium), which can be found in cheese waste in an anaerobic system, may negatively impact the microorganism activity and disrupt their metabolism. However, a low sodium level, i.e., 230–350 mg L^{-1}, is required for methanogens to form adenosine triphosphate and oxidize NADH [41]. Potential issues stemming from slightly elevated levels of sodium in cheese and its acidic pH (pH = 4.59) can be resolved through mandatory confectionery waste and buffering sewage sludge [3].

3.2. Properties and Efficiency of a Carrier

The dominant proportion of silica in the silica/lignin system (4:1) promoted the formation of aggregates and agglomeration of particles. SEM images (see Figure 2a,b) demonstrate the morphological diversity of that carrier as irregular shapes and porous microstructures are present in the particle clusters. Rough and porous surfaces promote the immobilisation of microorganisms and their multiplication [25,43,44]. In turn, the formation of irregular clusters positively affects the development of the BET surface (Brunauer–Emmett–Teller) [23,45]. As demonstrated by research, which has recently been published by Pilarska et al. (2020) [25], the significant proportion of silica resulted in the formation of the specific surface area of the analysed carrier, up to 151.5 m^2/g, with a pore volume of 0.35 m^3/g and diameter as large as 10.8 nm. From an economic point of view, a high value of the surface area of carriers is desirable.

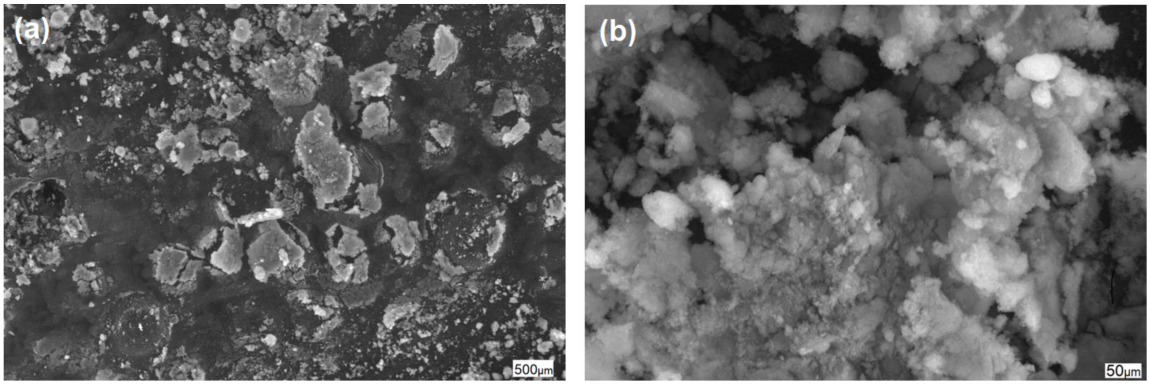

Figure 2. SEM images of the silica/lignin carrier at different magnifications: (**a**) 500 µm and (**b**) 50 µm diameter particles.

Although the abovementioned properties have already been analysed, the determination of the structure of the surface of silica is not easy. Both silica and lignin are described in detail in the works of Klapiszewski et al. (2015) [46]. The silicon dioxide surface (see Figure 3a) contains silanol (≡Si-OH) and siloxane (≡Si-O-Si≡) groups. What is more, it should be mentioned that silanol groups can have different forms, such as isolated (free), neighbouring and geminal-containing two hydroxyl groups connected by a common silicon atom. The share of lignin in the analysed silica/lignin carrier is much smaller, which is why that compound has an insignificant influence on the microstructure of the system and its chemical composition [25]. Lignin is the organic substance that binds the cells, fibres and vessels that make up wood and the woody parts of plants, as in straw. Lignin is one of the basic components of wood (along with cellulose and hemicelluloses), in which it is found in an amount of about 20%. After cellulose it is the most abundant renewable carbon source on earth. It is not possible to determine the exact structure of lignin as a chemical molecule. All lignins show some variability in their chemical composition. However, the definition common to all is a dendritic network polymer whose monomers are organic compounds derived from phenolic alcohols, namely, p-coumaryl, coniferyl and sinapyl alcohols (Figure 3b). Lignin is characterised by a unique three-dimensional chemical structure, cross-linked by ether and covalent carbon-carbon (C-C) bonds.

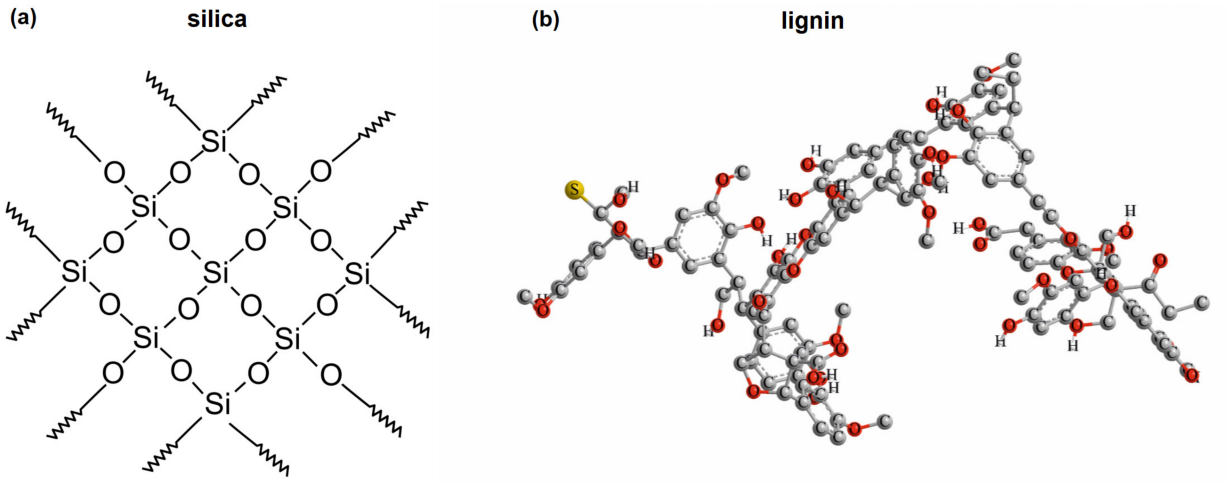

Figure 3. The structural formulas of (**a**) silica and (**b**) lignin.

The multiple functional groups found in the compound molecules, particularly the surface located carboxylic and phenolic groups, contribute to the biosorption properties of lignin [13,47].

Another advantage of the hybrid silica/lignin system as a microbial carrier is its high thermal stability. The silica/lignin carrier dominated by silica shows greater thermal stability when it comes to a broad scope of temperatures than pure lignin, as was demonstrated by the same research team in their previous work [25]. The TG (thermogravimetry) curve shape (black line) indicates a slight loss of mass in the temperatures applied during methane fermentation (0.5–1%), including fermentation conducted under thermophilic conditions (Figure 4). The greatest mass loss of heated material was noticed at very high temperatures (~745 °C), and it was 10%. Based on literature data, colloidal silica is characterised by high thermal resistance and mechanical strength, and due to those properties, it is often used in the production of composite materials, for example, made of carbon fibres [48].

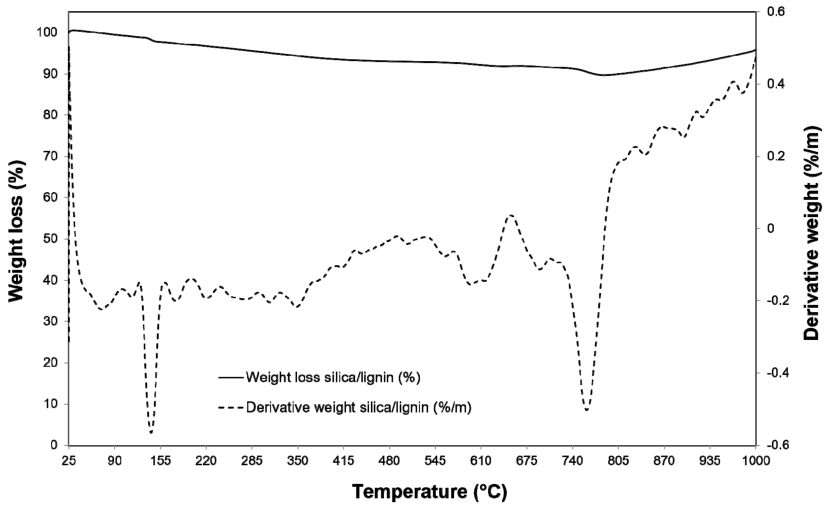

Figure 4. Thermograms of the silica/lignin carrier.

To verify the effect of the silica/lignin carrier, an autochthonous strain of Gram-positive sporulating *Bacillus amyloliquefaciens*, isolated from digested sewage sludge, was applied (Figure 5a,b). Those bacteria have a highly developed enzymatic apparatus, which contributes to the efficiency of substrate digestion and, at the same time, to the efficiency of

anaerobic digestion [49]. What is more, cells of *Bacillus amyloliquefaciens* grow rapidly and secrete large amounts of protein into the culture medium, so they can be used to produce heterogeneous proteins.

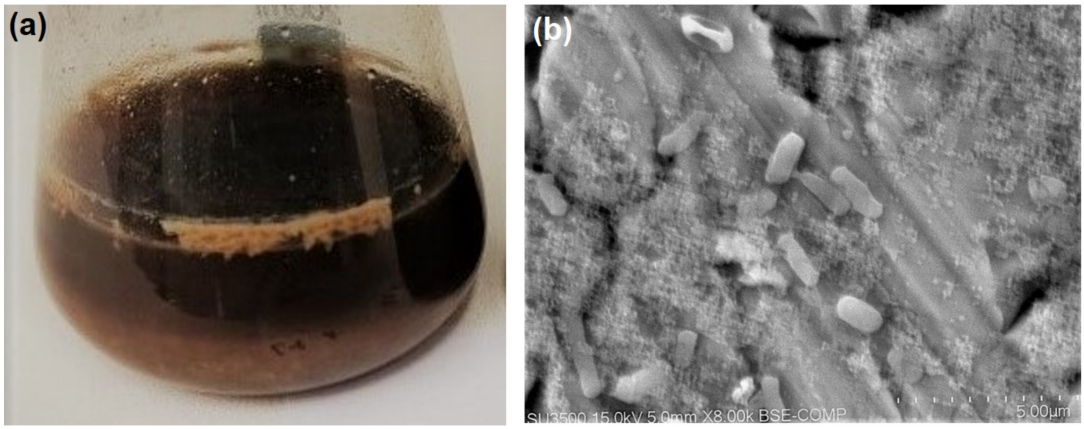

Figure 5. (**a**) A *Bacillus amyloliquefaciens* culture with added carrier, (**b**) SEM images of cell *B. amyloliquefaciens* colonisation.

As noted by Pilarska et al. (2020), in their previous article [25], a significant share of silica in the composition of the carrier material has a beneficial effect on the proliferation of bacteria. According to literature data, the addition of silica increases the production of intracellular proteins [50] and enhances the activity of methanogens in the AD process due to intensification of the decomposition of the macromolecular compounds, which can be found in, i.e., sewage sludge [21]. In the described experiment, the determined bacterial cell biomass in cultures containing silica/lignin carrier was 1.12 ± 0.05 g/100 mL and it is comparable to the results obtained in earlier studies [25].

3.3. Stability and Performance of Anaerobic Digestion

Basic indicators of stability of anaerobic digestion, such as pH, VFA to TA ratio (volatile fatty acids-to-total alkalinity ratio) and the N-NH_4^+ concentration, are each time tested during the process. Their values, determined by experiments, are known to be optimal for the development and activity of methanogens [31]. For pH, the range is from 6.5 to 7.2. Based on the obtained results, with regards to the fermented samples (Table 3), there is no acidification during the first stage of degradation–hydrolysis. The increase in pH to the value of 7.53 in the case of the system with the addition of cheese waste (WFC-control), was caused by, as noted by the authors in their earlier article [1], the breakdown of casein contained in milk. The produced ammonia, in the form of ammonia water, which makes the environment slightly alkaline, did not affect the stability of the process.

In the case of the VFA/TA ratio (Table 3), where the upper limit value for stable systems is 0.4, there was an increase in this parameter to 0.43 for the system with cheese (WFC-control), and then, there was a decline observed in the subsequent stages of the process. The rapid return to a state of full activity of methanogens did not adversely affect the results of biogas production. A decline in VFA/TA ratio indicates effective decomposition and removal of organic matter [13,24].

According to the critical values reported by Chen et al. (2008), the obtained concentrations of N-NH_4^+ for fermentable batches, as the inhibitor of the AD process, remained at an acceptable level [41]. Measurements of concentration of N-NH_4^+ are important for the AD process implemented with the use of protein substrates and sewage sludge as rich sources of organic nitrogen. During anaerobic decomposition of biological matter, organic nitrogen is transformed into ammonium nitrogen, and part of that organic nitrogen is bound in biomass [3]. Hence, during the experiment, a successive increase in the concentration of N-NH_4^+ was noticed (see Table 3), and there was a decline recorded in the last stage of the process due to the exhaustion of organic matter. The efficiency of the formation of

ammonium nitrogen hinges on the load of the fermentation chamber and the heat during the process [51,52].

Table 3. Changes in stability parameters during the anaerobic digestion.

Samples	Fermentation Time (Days)							
	1	**3**	**6**	**9**	**12**	**15**	**18**	**21**
					pH (-)			
WF-control	6.92	7.05	7.13	7.28	7.16	7.09	7.21	7.29
WF + S/L	6.89	6.98	7.05	7.17	7.23	7.14	7.17	7.25
WFC-control	7.05	7.12	7.31	7.44	7.38	7.50	7.46	7.53
WFC + S/L	6.85	6.94	7.10	718	7.32	7.29	7.14	7.16
					VFA/TA ratio (-)			
WF-control	0.36	0.43	0.38	0.31	0.29	0.25	0.26	0.24
WF + S/L	0.42	0.37	0.36	0.33	0.40	0.29	0.25	0.22
WFC-control	0.38	0.40	0.43	0.42	0.39	0.34	0.28	0.25
WFC + S/L	0.41	0.39	0.42	0.38	0.35	0.37	0.29	0.23
					$N–NH_4^+$ (mg L^{-1})			
WF-control	148	164	173	189	222	278	249	216
WF + S/L	165	169	214	225	238	296	264	183
WFC-control	805	812	875	892	936	998	917	881
WFC + S/L	871	939	986	964	905	893	815	792

The volume of biogas obtained from the waste wafers, converted into volatile solids (VS) was 705.16 m^3 Mg^{-1} vs. (see Figure 6) with a methane 356.11 m^3 Mg^{-1} VS. These obtained results are comparable to the biogas yield of wafers obtained in previous studies conducted by the authors [2,13,24,31], as well as to the yield of other food waste, including highly processed, post-production flour [4] and molasses [3].

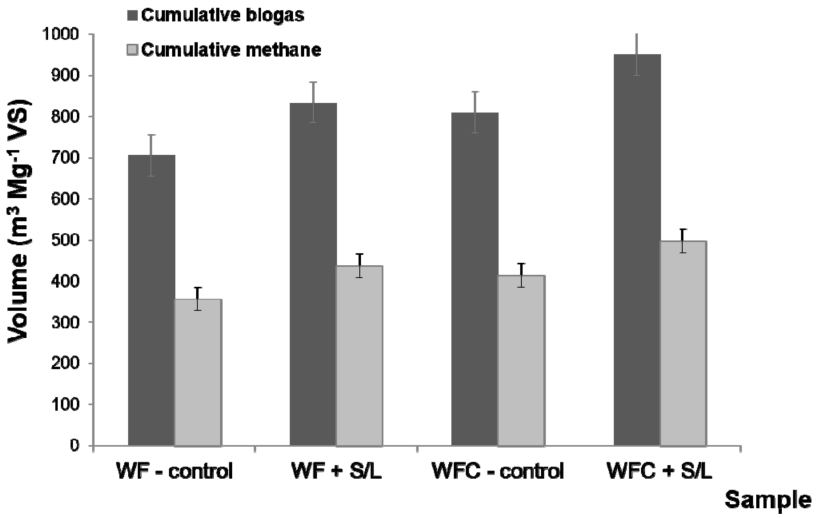

Figure 6. Cumulative biogas and methane yield from tested samples.

The addition of silica/lignin carrier to the WF system resulted in an increase in the volume of produced biogas, including methane, by 18.18% (833.35 m^3 Mg^{-1} VS; see Figure 6). Due to the functional properties of the S/L hybrid components, i.e., lignin sorptive properties and silica microstructural properties, this hybrid is an exceptional cell carrier for anaerobic digestion [25]. It has been proven that silica adsorbs proteins (differential protein adsorption concept) and that it also enhances protein proliferation [27]. In turn, the combination of co-substrates in the form of waste wafers and waste cheese (WFC-control),

similarly to one of the previous works of the author [24], increased the biogas yield, compared to the WF-control sample, to 809.11 m^3 Mg^{-1} vs. of biogas with 51.1% share of methane (Figure 6). The obtained results prove the synergy effect between carbohydrate confectionery waste and protein dairy waste, which is a great prognosis with regards to the implementation of the system on an industrial scale. The implementation of the S/L carrier into the WFC system resulted in a surge in the produced biogas, including methane, in that case by 17.49%. Thus, the best result was obtained when the co-substrate system with the prepared S/L carrier material (WFA + S/L) was used: biogas: 950.64 m^3 Mg^{-1} vs. methane: 497.19 m^3 Mg^{-1} VS.

3.4. Total Bacterial Count and Dehydrogenase Activity in Digested Samples

Bacterial count and their activity in the system of fermented food waste (waste wafers and cheese, WFC) were statistically significantly more varied with regards to the sampling date and the type of experimental variant (Figure 7, Table 4). Based on the study conducted by Pilarska et al. (2019) [24], the bacterial content in fermented waste wafers and cheese with the addition of lignin as cell carrier was different and depended on the chemical make-up of the ferment and the anaerobic digestion length.

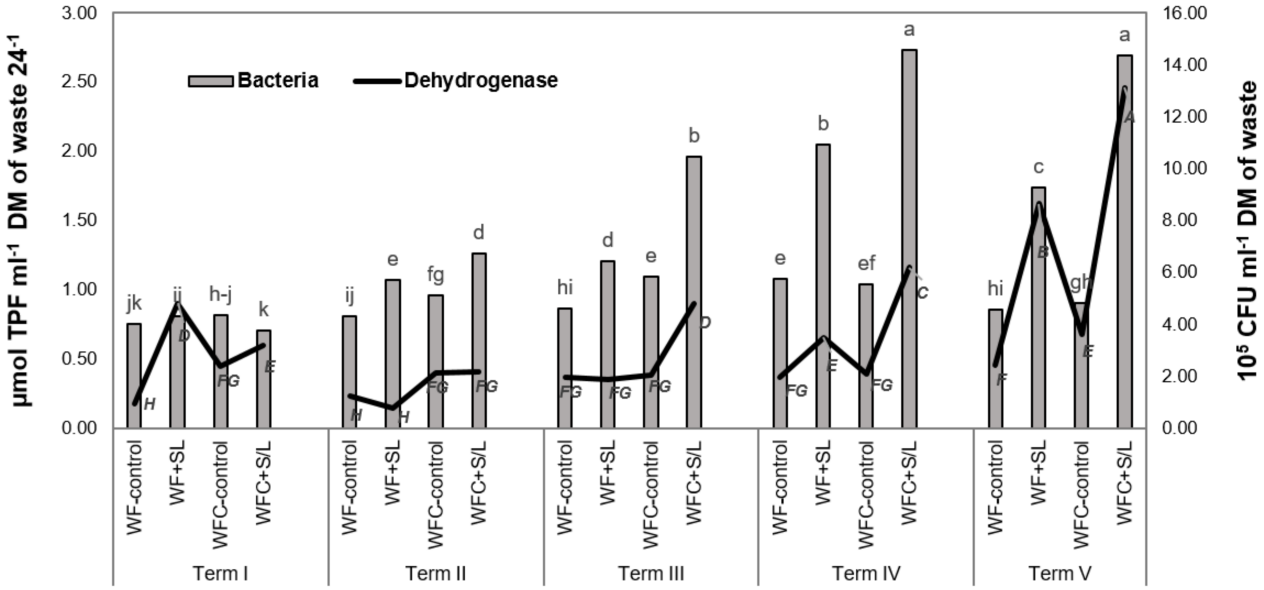

Figure 7. Bacteria total count and dehydrogenase activity changes found in the digested sampled material. Explanation: The same letter indicates a lack of significant differences ($p < 0.05$).

Table 4. Results of *F* test statistics and levels of significance of two-way ANOVA concerning dehydrogenase activity and the bacteria count associated with combination and fixed factor-related research terms (*** $p = 0.001$).

Parameter	Term	Combination	Interaction
Bacteria	2225.42 ***	3915.19 ***	600.59 ***
Dehydrogenase	1882.39 ***	1924.33 ***	452.00 ***

The results (see Figure 7) demonstrate that the multiplication of anaerobic bacteria increased in all the analysed experimental variants over time, except for the last term (V).

The highest bacterial abundance during the analyses was maintained in the co-substrates with the addition of a silica/lignin carrier (WFC + S/L). This finding is connected to both the presence of nutrients, carbohydrates and available forms of nitrogen (see Tables 1 and 3) and the carrier, which, thanks to its adsorption properties, provided an ideal habitat for the growth of the autochthonous microbiome [12,23]. The study by

Tapia-Olivares et al. (2019) demonstrates that lignin, as a non-toxic, efficient and above all, renewable organic resource, is an excellent carrier for bacteria [53], as is silica which increases the adhesion of microorganisms and their stability and dispersion in culture fluids [54].

This statement is confirmed by the results of our original research concerning the evaluation of dehydrogenase activity levels, i.e., enzymes representing oxidoreductases, considered biological and chemical indicators of microorganism metabolic activity [55]. Analysis of dehydrogenase activity (DHA) in fermented food waste showed the stimulating effect of the applied carriers on the activity levels of the enzymes studied (Figure 6). Similar to the bacterial count, the highest dehydrogenase activity was observed in the variant, which, apart from the carrier, contained waste wafers and cheese (WFC + S/L). On the other hand, analysis of the dynamics of changes in DHA activity during the study showed that irrespective of the experimental variant, the activity of the examined enzymes gradually increased, reaching the highest level in term V.

To estimate the cause–effect relationships present between microbiological, enzymatic and chemical parameters studied (Figure 8), a principal component analysis (PCA) was used, which showed a positive relationship between the number of bacteria and the activity of dehydrogenases and the methane content formed during the fermentation of waste wafers.

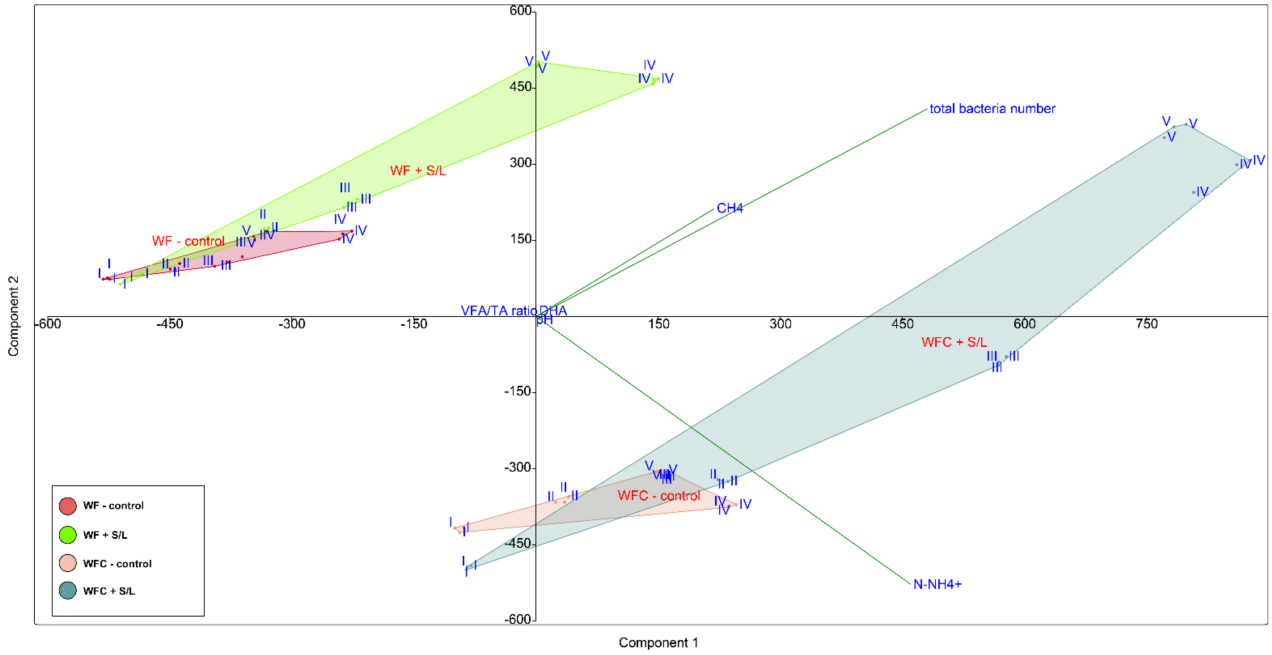

Figure 8. Distribution of microbiological and chemical properties in four variants, in two PCA axes.

This analysis also illustrated the lack of correlation between the number of microorganisms and dehydrogenase activity (DHA) and the pH value, as well as the significant effect of ammonium ion content on the growth and development of anaerobic bacteria in the tested experimental variants. In addition, the study by Yenigün and Demirel (2013) shows that the ammonium ion content during methane fermentation significantly shapes the multiplication of bacteria, inhibiting their growth when the value of 2.7 NH_4^+ g/L is exceeded [56].

The results further confirmed the positive relationship between pH value and methane emission. According to Bhatta et al. (2006), pH is one of the main factors affecting microbial production of volatile fatty acids (VFAs) and methane during the methane fermentation process [57]. The authors showed that lowering the pH value to 6 reduced the total production of VFAs, the ratio of acetate to propionate and the total methane production.

3.5. Bacterial Community Abundance and Composition

In addition to the traditional bacterial culture methods (see Figure 9), which were applied to indicate the dynamics of changes in their abundance during the fermentation of confectionery waste, a qualitative assessment of the present microbiome was also carried out using an increasingly popular, and at the same time extremely sensitive, method of determining differences and similarities of microorganisms in a given environment (Figures 8 and 9) [58,59]. The sets of bacterial DNA sequences obtained by next-generation sequencing (NGS) of the tested samples provided us with a large amount of information on the occurrence of given taxa in the bacterial taxonomic hierarchy. During testing, 18 phyla and between 137 and 167 genus of bacteria were found, depending on the experimental variant. Such a large number of operational taxonomic units (OTUs) meant that only the most common ones were presented graphically.

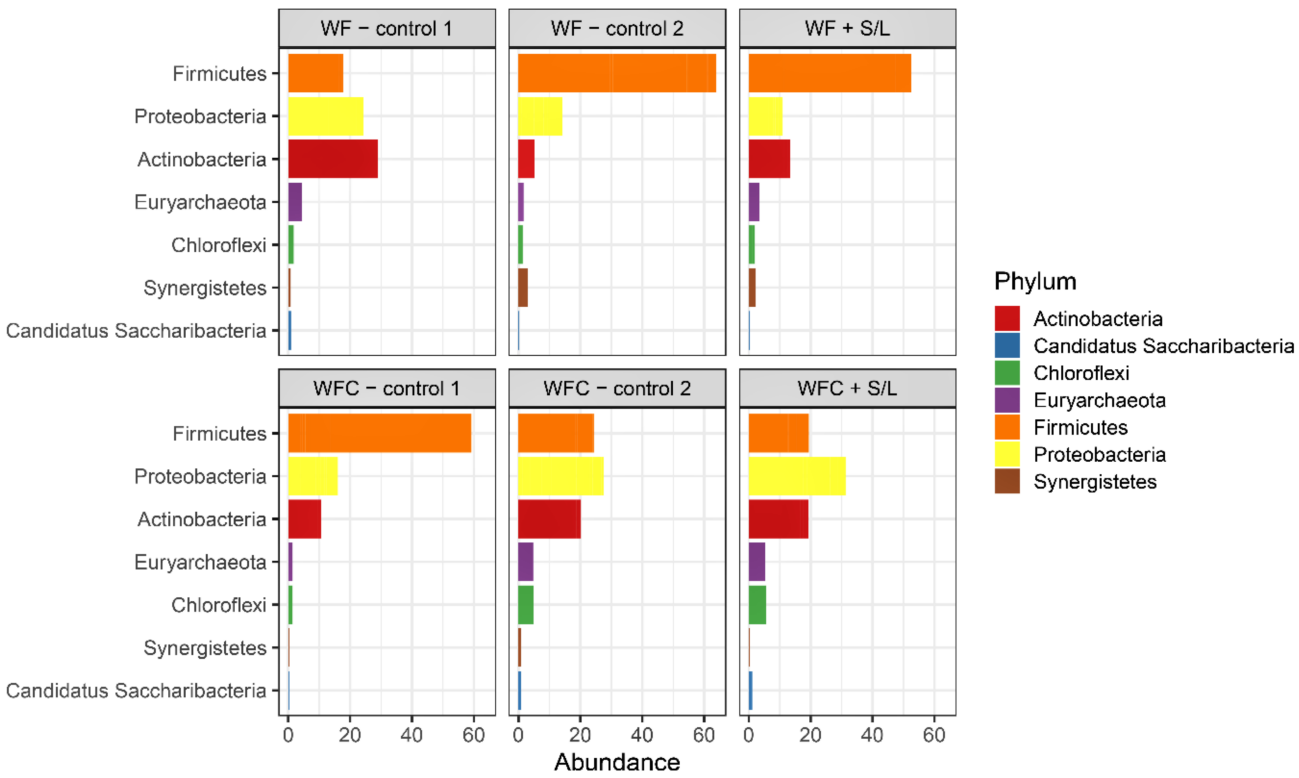

Figure 9. The percentage content of selected bacterial phyla in the experimental variants used.

A comparative analysis was carried out based on the following material samples: WF-control 1 and WFC-control 1, i.e., systems without carrier addition, sampled during the first phase of the process, as a reference to the following: WF-control 2 and WFC-control 2 (samples without carrier addition, sampled during the final stage of the process) and WF + S/L and WFC + S/L (samples with addition of carrier, taken at the final stage of the process).

Of all the bacterial phyla obtained, only seven in all experimental variants were marked by the content above 1.5% (*Actinobacteria*, *Candidatus Saccharibacteria*, *Chloroflexi*, *Euryarchaeota*, *Proteobacteria*, *Synergistota*) (Figure 10). It was shown that three bacterial phyla dominated the facilities used, namely *Firmicutes* accounting for 20.63% to 66.83% of all isolated types, *Proteobacteria* (12.64% to 31.33%) and *Actinobacteria* (8.01% to 29.17%), respectively.

The study by Banach et al. (2019) also shows that *Firmicutes* and *Proteobacteria* are the dominant bacterial phyla in the methane fermentation process, in this case, wastewater [60]. According to the aforementioned authors, many of the known syntrophic acetate-oxidising bacteria indeed are *Firmicutes*. In addition, this phylum includes a large group of mi-

croorganisms playing a significant role in the degradation of volatile fatty acids and the digestion of polysaccharides, oligosaccharides and proteins, including the *Clostridium* genus, which in our study accounted for more than 5% of all readings, depending on the experimental variant. According to Walter et al. (2012), the *Clostridia* class to which the abovementioned bacterial genus belongs constitutes the typical microbiome of fermented wastes and appears to be responsible for the first step in the syntrophic oxidation of acetate to CH_4 [61].

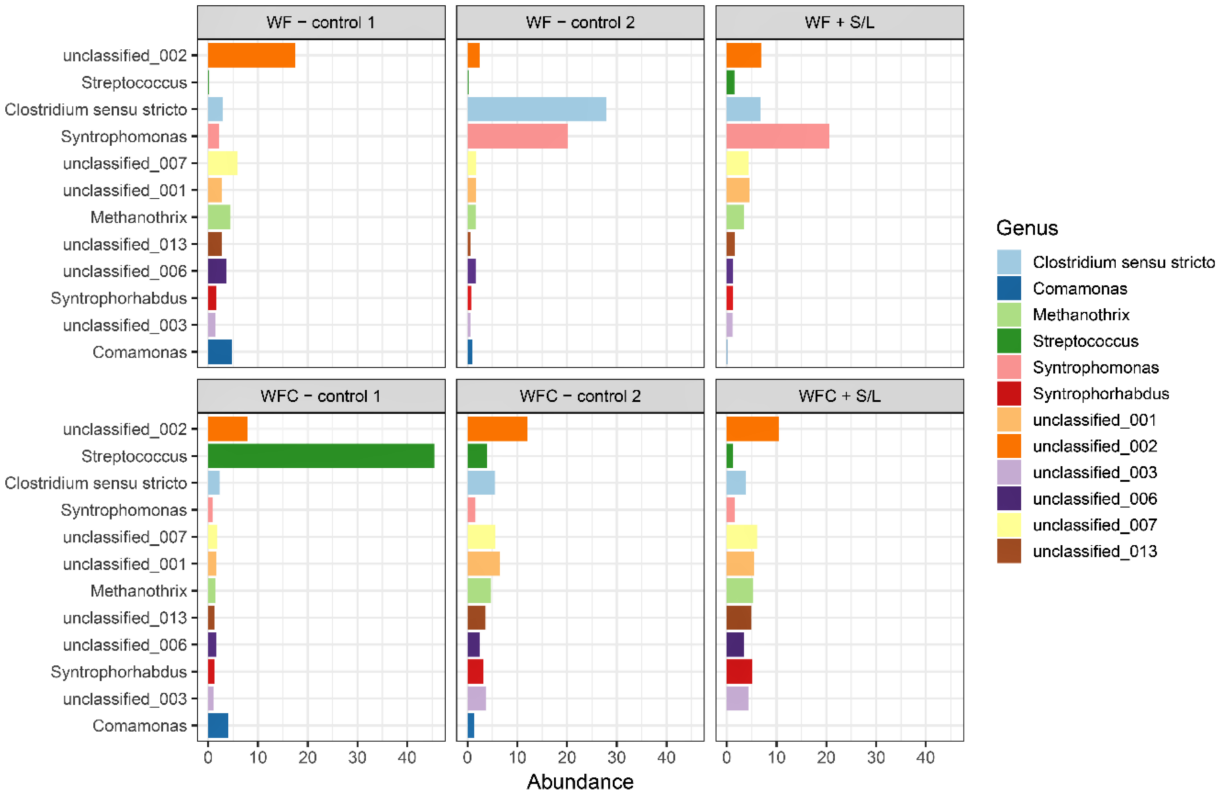

Figure 10. The percentage content of selected bacterial genus in the experimental variants used.

The metataxonomic analysis of fermented confectionery waste, irrespective of the experimental variant applied, also revealed the dominance of the *Syntrophomonas*, *Streptococcus*, *Methanotrix*, *Syntrophorhabdus* genus, as well as several genera designated as unclassified (Table 5).

Table 5. The available information about selected, most abundant unclassified sequences, based on the NCBI database.

Unclassified Symbol (in This Research)	NCBI Accession Numbers (% of Sequence Identity)	Source/Environment	References (If Available)	Closest Relative
unclassified_001	EF059533 (97.2%)	PCB-dechlorinating enrichment culture	Bedard et al. (2007) [62]	*Sedimentibacter* sp.
	AY766467 (96.5%)	Anaerobic coculture enriched with a hexachlorocyclohexane (HCH) polluted soil.	Wim van Doesburg et al. (2005) [63]	*Sedimentibacter* sp.
unclassified_002	MK143173 (98.8%)	Algae (Iceland)	Costa et al. (2019) [64]	*Knoellia* sp.
	KX256211 (98.8%)	Eastern Mediterranean Sea Sediment	Gärtner et al. (2016) [65]	*Intrasporangium* sp.

Table 5. *Cont.*

Unclassified Symbol (in This Research)	NCBI Accession Numbers (% of Sequence Identity)	Source/Environment	References (If Available)	Closest Relative
unclassified_003	NR_041354 (97%)	Thermophilic digester sludge, methanogenic propionate-degrading consortia	Yamada et al. (2007) [66]	*Bellilinea caldifistulae*
	KX261406 (93.8%)	Sludge and beet sugar industrial wastewater	-	*Levilinea saccharolytica*
unclassified_006	KC252871 (100%)	Activated sludge	-	*Comamonadaceae bacterium*
	KF751647 (99.8%)	Wastewater treatment system	-	*Diaphorobacter* sp.
	NR_125656 (99.3%)	Coking wastewater	Geng et al. (2014) [67]	*Ottowia* sp.
unclassified_007	AB021325 (98%)	Activated sludge with phenol as the sole carbon source	Watanebe et al. (1999) [68]	Uncultured/unclassified
	JQ899231 (97.5%)	Marine soil sediment	-	*Streptomyces aomiensis*
unclassified_013	AB529706 (98%)	Rhizoplane	Tanaka et al. (2012) [69]	Uncultured/unclassified
	HM124367 (96.8%)	Lake sediment	-	*Hyphomicrobium* sp.

In the case of confectionery waste with added cheese, it was observed that the fermentation process contributed to an increase in % sequences belonging to the *Syntrophorhabdus* genus involved in the degradation of compounds such as phenols, p-cresol, isophthalate and benzoate in syntrophy with hydrogenotrophic methanogenic archaea [70]. Research by Yuan et al. (2020) indicates that the aforementioned type of bacteria constitutes the natural microbiome of fermented food waste and plays an important role in the methane fermentation process [71]. In addition, the addition of zero-valent iron contributes to increasing the multiplication of these bacteria.

Based on an original study, it was shown that the Syntrophomonas *genus,* known for beta-oxidation of saturated fatty acids to acetate or acetate and propionate, growing syntrophically with hydrogenotrophic methanogens, was also dominant in the fermentation of waste wafers [72]. An increase in the multiplication of bacteria belonging to this genus was mainly recorded in the WF variants without cheese addition. In the above experimental facilities at the end of the experiment (WF-control 2), especially in the fermentation additionally enriched with lignin and silica (WF + S/L), an additional higher percentage of sequences belonging to the *Streptococcus genus* was observed. A different observation was made in the case of wastes additionally enriched with cheese, in which the fermentation process contributed to a lower multiplication of these bacteria. The study by Laothanachareon et al. (2014) shows that bacteria belonging to the *Streptococcus* genus play an important role in hydrogen and acetate production [73]. Their initial dominance in fermented wastes related to the high amount of readily available organic matter is then displaced by the *Clostridia* class, including the *Clostridium* genus, which was supported by the findings of the original research on the dynamics of changes in the microbiome of waste wafers enriched with cheese (see Figure 9).

The meta-taxonomic analysis showed that the type of experimental facility influenced the structure of the bacterial microbiome, which is confirmed by the results presented in a Venn diagram (Figure 11). Considering all taxa within the genus, 86 common taxa were identified, including such genus as *Acinetobacter, Aminivibrio, Acetoanaerobium, Clostridium, Defluviimonas, Devosia, Streptococcus, Syntrophobacter, Syntrophomonas* and *Syntrophorhabdus*.

The highest number of unique taxa (14) in the experimental facilities used was found in WF-control 1. They included such a genus as *Sphingopyxis*, which comprises strains

that show strong bioremediation properties [74], *Sphingobacterium* representing the natural microbiome of methane-fermented waste [75] and *Chryseomicrobium* represented by haloalkalitolerant bacterial species [76]. In contrast, the lowest number of unique taxa was found in WF-control 2, including the *Paracandidimonas* genus, representing the natural microbiome of wastewater and sewage sludge [71,77].

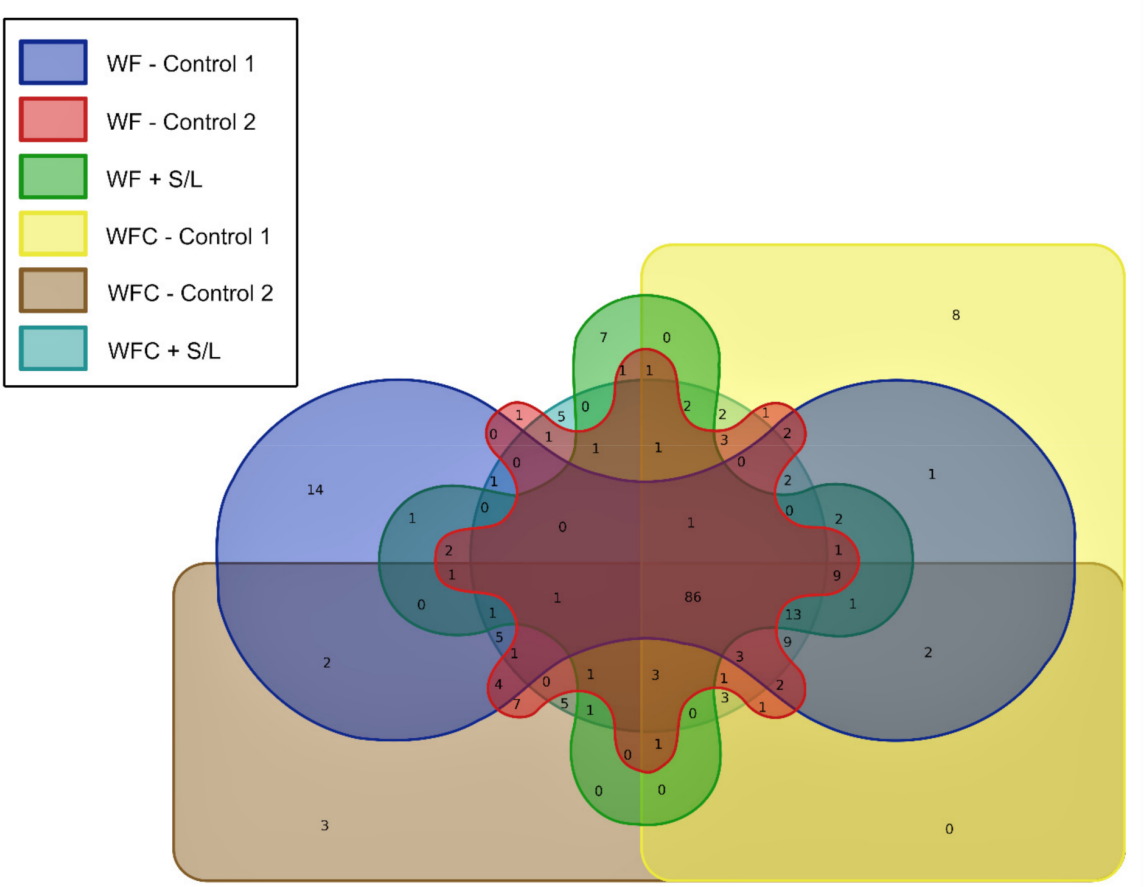

Figure 11. Venn diagram of overlapping bacterial communities from the six variants.

The NGS analysis further showed that the addition of cheese to methane-fermented waste wafers contributed to the appearance of bacteria of the *Brachymonas*, *Brooklawnia* and *Paraclostridium* genus, whose presence was not found in the other experimental variants. On the other hand, in those including lignin and silica carriers, a total of 12 unique bacteria were found, e.g., from such genus as *Bacillus, Ligilactobacillus, Micromonospora, Microbacterium, Staphylococcus, Nitratireductor* or *Dechloromonas.*

The fermentation process of waste wafers with added cheese (WFC-control 2 and WFC + S/L) also contributed to the highest values of Simpson's and Shannon's biodiversity indices (see Table 6). However, the lowest values of the analysed indices were recorded in the WFC-control 1 variant. The values of the abovementioned indices were slightly different in waste wafers subjected to the fermentation process without cheese addition. This is because the highest value of Simpson's and Shannon's indices was characteristic for WF-control 1.

Table 6. Biodiversity indices of bacterial communities based on the genus level (including unclassified).

Sample	Observed	Shannon (H')	Simpson (1/D)
WF-control 1	167	3.869	0.9513
WF-control 2	137	3.089	0.8741
WF + S/L	138	3.541	0.9345
WFC-control 1	161	2.779	0.7803
WFC-control 2	166	3.896	0.9620
WFC + S/L	152	3.835	0.9635

Table 7 presents the most important abbreviations and their explanations.

Table 7. Explanation of abbreviations and symbols used.

Abbreviation	Meaning
AD	anaerobic digestion
NGS	next-generation sequencing
16S rRNA	prokaryotic 16S ribosomal RNA gene
TS	total solids
VS	volatile solids
WF	wafer waste
CE	cheese waste
WFC	wafer and cheese co-substrates
S/L	silica/lignin carrier
PBS	phosphate buffered saline
DIN	Deutsches Institut für Normung
VDI	Verein Deutscher Ingenieure
HRT	hydraulic retention time
VFA	volatile fatty acids
VFA/TA ratio	volatile fatty acids-to-total alkalinity ratio
C/N ratio	carbon/nitrogen ratio
TTC	2,3,5-triphenyltetrazolium chloride
TPF	triphenylformazan
DHA	dehydrogenase activity
CFU	colony-forming units
AnB	anaerobic bacteria
DM	dry matter
PE	paired-end
PCR	polymerase chain reaction
RDP	ribosomal database project
IDTAXA	taxonomic classification algorithm
DADA2	software package that models and corrects Illumina-sequenced amplicon errors
PCA	principal component analysis
SEM	scanning electron microscope
BET surface	Brunauer-Emmett-Teller
TG	thermogravimetry
OTUs	operational taxonomic units

4. Conclusions

The silica/lignin hybrid material, with a significant weight proportion of silica (4:1), as proven by the results of the analyses conducted in this experiment, shows high porosity, significantly developed specific surface area, irregular microstructure and thermal stability over a wide temperature range, making the system of such two compounds an effective microbial carrier for methane fermentation. Furthermore, this material is environmentally friendly, resistant to hydrolytic enzymes and biocompatible. The positive effect of this carrier on the intensity of bacterial multiplication was also confirmed using an indigenous strain of *Bacillus amyloliquefaciens*. It was mainly attributed to the action of silica, which increases the productivity of intracellular proteins.

Bacterial counts and activity in waste wafers and cheese (WFC) varied more significantly in response to the date of sampling and the experimental variant. Among the samples tested, the largest total bacterial count and dehydrogenase activity was maintained in the WFC + S/L system. In general, the addition of silica/lignin carrier intensified the multiplication and activity of bacteria, both in the variant with wafers and the system of both food wastes. This relationship translated into biogas/methane yields from these samples. For WF + S/L, there was an increase in cumulative biogas yield of 18.18% to a value of 833.35 m^3 Mg^{-1} VS, while for WFC + S/L, there was an increase of 17.49% to a volume of 950.64 m^3 Mg^{-1} VS. Monitoring process stability control parameters, including pH and VFA/TA, proved no inhibition periods for all samples. Furthermore, the principal component analysis showed a positive correlation between methane emission, the pH value and the number of anaerobic bacteria. The influence of the type of experimental facility on the structure of the bacterial microbiome was also demonstrated using meta-taxonomic analysis. The largest bacterial biodiversity was recorded in samples fermented with the addition of cheese, both in the case of the control variant and in the variant when the carrier was used. In contrast, three phyla of bacteria *Firmicutes*, *Proteobacteria* and *Actinobacteria* predominated in all experimental facilities.

The application and effective action of the silica/lignin carrier as a biocatalyst significantly contributed to increasing the biomass conversion rate, shortening the retention time and improving the efficiency of the AD process. The result of the implemented research activity is a preliminary, empirical verification of the effect of the microbial carrier dedicated to AD.

Author Contributions: Conceptualisation, A.A.P.; methodology, A.A.P., A.W.-M., A.N. and A.G. (Aleksandra Grzyb); software, K.P., J.G. and A.G. (Anna Gałązka); validation, A.A.P. and A.W.-M.; formal analysis, A.A.P. and A.N.; investigation, A.A.P., A.W.-M., K.P., M.A., A.G. (Aleksandra Grzyb); resources, A.A.P., A.W.-M. and A.N.; data curation, A.A.P., J.G. and A.G. (Anna Gałązka); writing—original draft preparation, A.A.P.; writing—review and editing, A.A.P., A.W.-M. and J.G.; visualisation, K.P. and J.G.; supervision, K.P., A.N. and M.A.; project administration, A.A.P.; funding acquisition, A.A.P. All authors have read and agreed to the published version of the manuscript.

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