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Highlights

What are the main findings?

- Fe_3O_4 and ferrous precipitate (R-Fe_3O_4) effect on dark fermentation was evaluated.
- R-Fe_3O_4 enhanced hydrolysis of soluble compounds and VFAs production.
- R-Fe_3O_4 improved microbial diversity by promoting iron-reducing bacteria.

What are the implications of the main findings?

- Fenton-based by-products can serve as a valuable resource for sustainable recovery.

Abstract

Fenton-based processes are widely used advanced oxidation methods known for degrading persistent pollutants. However, these techniques often generate significant amounts of iron-containing sludge, which poses environmental disposal challenges due to its complex composition. Furthermore, the sludge produced by the Fenton process contains high content of Fe(III) compounds, which can serve as an iron source to stimulate dissimilatory iron reduction (DIR), enhancing the performance of anaerobic digestion. Based on the characterization results from a previous study, this work investigated the use of the ferrous precipitate generated by the electrochemical peroxidation process applied to tannery wastewater treatment as an additive to enhance volatile fatty acids (VFAs) production during dark fermentation. The performance of ferrous precipitate (R-Fe_3O_4) was compared to that of conventional magnetite (Fe_3O_4) during dark fermentation under high organic loading conditions, emphasizing their potential to enhance hydrolysis efficiency and VFAs production yields, while promoting sustainable resource recovery and reuse within a circular bioeconomy framework. The results showed that the addition of both Fe_3O_4 and R- Fe_3O_4 significantly increased VFAs yields, with a predominance of long-chain fatty acids. The presence of $CaCO_3$ in the ferrous precipitate contributed to maintaining a stable pH environment, supporting microbial activity and enhancing the hydrolysis of soluble compounds. Moreover, the availability of essential micronutrients within the ferrous precipitate favoured greater microbial diversity. Consequently, the addition of R-Fe_3O_4 promoted VFAs production even at higher organic loading rates, suggesting a promising application of Fenton-based by-products as functional additives to improve the economic and environmental performance of the dark fermentation process.

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1. Introduction

Today, the increasing consumption of environmental resources has led to a rise in greenhouse gas emissions, with a significant impact on global warming. At the same time, the depletion of fossil fuel resources has shifted the focus towards the production of biofuels from renewable sources [1].

Volatile fatty acids (VFAs) play a crucial role as a primary source of energy and carbon in the organic carbon cycle. These are low molecular weight organic acids, typically containing six or fewer carbon atoms, and are characterized by strong hydrophilic properties. The organic carbon derived from VFAs serves as a renewable resource and a key building block for the chemical industry, with several applications across the pharmaceutical sector, wastewater treatment, as well as in biodiesel production and bioplastic synthesis [2]. However, the petrochemical industry is typically the primary driver behind the commercial production of VFAs, significantly contributing to greenhouse gas emissions, as reported by Atasoy et al. [3]. Thus, extensive research has been focused on finding more environmentally sustainable solutions for producing materials, fuels, and chemicals.

Anaerobic digestion (AD) is an effective method for recovering valuable resources, including VFAs [4]. Generally, it consists of four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. However, the production of added-value products, such as VFAs, occurs during the first three stages of the AD process, also known as anaerobic or dark fermentation [5].

So far, different organic wastes have been utilized as substrates for VFA production, with significant efforts focused on optimizing the process to overcome challenges such as low yields and high costs, especially when compared to chemical synthesis routes [6]. During anaerobic fermentation, complex organic compounds are degraded into smaller molecules, which are subsequently converted into VFAs by acidogenic bacteria. Consequently, the efficiency of hydrolysis plays a critical role in maximizing VFA production [7]. Effective pretreatment techniques can significantly enhance the efficiency of hydrolysis.

Over time, a range of methods has been explored, including physical approaches (e.g., microwave and thermal pretreatments), chemical treatments (e.g., acid and alkaline hydrolysis), and biological methods (e.g., enzymatic hydrolysis) [8]. Among these, the use of additives, such as metal oxides, has also been explored [9,10].

In this context, iron-based oxides have been shown to enhance the effectiveness of hydrolysis and acidogenesis [11]. The addition of iron oxide in the anaerobic digestion of food waste was investigated in a two-stage continuous process by Yuan et al. [12], highlighting its promoting effect on hydrolysis stage. Meanwhile, several studies have found that Fe^{3+} may compete with methanogens for electrons as electron acceptor, potentially inhibiting methanogenesis. Thus, Fe_3O_4 could act as a valuable additive for promoting VFA production [13].

As reported by Wang [14], Fe_3O_4 effectively promoted VFA production in the anaerobic fermentation of food waste by stimulating the growth of Fe^{3+}-reducing bacteria and enhancing the decomposition and transformation of complex organic compounds. Furthermore, due to its magnetic properties, Fe_3O_4 can be easily recovered and reused, helping to prevent secondary pollution and reduce overall costs.

In this context, Fenton sludge produced from the Fenton process contains high content of Fe(III) compounds, which can serve as an iron source to stimulate dissimilatory iron reduction (DIR), thereby enhancing the performance of the anaerobic digestion process [15]. In our previous study, an electro-Fenton-based approach was applied for the tertiary treatment of tannery wastewater at neutral pH, where the characterization of the produced sludge showed that it was primarily composed of Fe_3O_4 and $CaCO_3$ [16]. Current methods for recovering and adding value to tannery sludge involve its conversion into biochar, catalyst production, and bioenergy generation [17].

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This study aimed to investigate the use of iron sludge, a by-product from tertiary treatment of tannery wastewater, as an additive to improve VFA production. Among the key challenges in the VFA production from dark fermentation process is the identification of cost-effective strategies to enhance hydrolysis and improve VFA yields, particularly when processing high-strength organic substrates. In this context, powdered milk, rich in readily biodegradable compounds, has been proposed as a suitable model substrate under high organic loading conditions.

The novelty of this work lies in the comparison between conventional magnetite (Fe_3O_4) and iron sludge in terms of their effectiveness in enhancing hydrolysis efficiency and VFA production yields, promoting resource recovery and reuse in a sustainable framework.

2. Materials and Methods

2.1. Experimental Materials Characterization

Powdered milk was selected as the organic substrate, and waste activated sludge (WAS) from the secondary clarifier of a municipal wastewater treatment plant (WWTP) located in Tuscany (Italy), was used as the inoculum for the dark fermentation tests.

The main physico-chemical characteristics of both the substrate and the inoculum, including pH, total solids (TS), volatile solids (VS), total organic carbon (TOC), chemical oxygen demand (COD), ammonium (NH_4^+-N), total nitrogen (TN) and phosphate (PO^{3-}_4-P) are summarized in Table 1.

Prior to the dark fermentation experiment, the inoculum was incubated at 105 °C for 30 minutes to inhibit methanogenic microorganisms [18]. Fe_3O_4 particles, with sizes ranging from 50 to 100 nm and a purity of 97%, were purchased from Sigma-Aldrich. A ferrous precipitate obtained from the electrochemical peroxidation of tannery wastewater was employed in this study and previously characterized in our earlier work [16].

Given its composition, primarily iron oxides (e.g., Fe_3O_4) and calcium carbonate, the iron precipitate was used as an additive and compared with commercially available Fe_3O_4. Prior to the experiments, the collected precipitate was dehydrated in an oven at 105 °C for 24 hours.

Table 1. Physical-chemical properties of milk powdered and inoculum sludge.

	Unit	Milk powdered	Inoculum
pH	-	6.7	7.8
Total solids (TS)	% w/w	96.9	0.8
Volatile solids (VS)	%	91.3	0.6
Total organic carbon (TOC)	mg/L	7780	817
Chemical oxygen demand (COD)	mg/L	69850	1558
Ammonium (NH_4^+-N)	mg/L	73.3	17.5
Total nitrogen (TN)	mg/L	982	252
Phosphate (PO_4^{3-}-P)	mg/L	351	25

2.2. Dark Fermentation Tests

Batch dark fermentation assays were performed in triplicate using 1000 mL serum bottle reactors, each filled to a working volume of 350 mL.

Based on literature, higher substrate-to-inoculum (S/I) ratios are beneficial by inhibiting methanogenic consumption of VFAs, thus enhancing VFA accumulation [19]. However, excessive substrate loading may inhibit hydrolysis, thus reducing subsequent VFA production [20].

To assess the impact of organic overloading on VFA production, powdered milk and inoculum were mixed at elevated S/I ratios of 2, 3, and 4. The S/I ratio was calculated based on volatile solids (VS) content [21], using the standardized method proposed by Angelidaki et al. [22] and subsequently adapted by Pecorini et al. [23].

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Based on literature data, an optimal dosage of 10 g/L was added to reactors containing either commercial magnetite (Fe_3O_4) or iron precipitate recovered from the electrochemical process (R-Fe_3O_4) [14]. The reactors were labelled as S/I = 2, S/I = 3, and S/I = 4, respectively. Reactors with the addition of commercial Fe_3O_4 were labelled as S/I = 2 Fe_3O_4, S/I = 3 Fe_3O_4, and S/I = 4 Fe_3O_4, respectively. Reactors with the addition of recovered iron precipitate (R- Fe_3O_4) were labelled as S/I = 2 R- Fe_3O_4, S/I = 3 R-Fe_3O_4, and S/I = 4 R-F Fe_3O_4, respectively.

Following purging with high-purity nitrogen, the reactors were securely sealed with caps equipped with ball valves and incubated at 37 ± 1 °C for 7 days. Sampling and parameter measurements were conducted every 2 days for each experimental condition.

2.3. Analytical Methods

pH was measured with a PC 700 pH meter (Eutech Instruments).

Total solids (TS) and volatile solids (VS) in the organic substrate and inoculum of the fermented samples were quantified using standard analytical methods [24].

To determine the soluble components, all samples were first filtered through 0.45 µm membrane filters. All analyses were performed in triplicate.

The concentration of soluble Chemical Oxygen Demand (sCOD) was measured using the potassium dichromate oxidation method with a Hach DR3900 spectrophotometer.

The concentrations of ammonium nitrogen (NH_4^+-N) and orthophosphate phosphorus (PO_4^{3-}-P) were also determined using the Hach DR3900 spectrophotometer, based on the standard methods of the American Public Health Association (APHA) [24].

For the analysis of soluble carbohydrates, samples were further filtered through 0.22 µm cellulose acetate syringe filters to remove particulates and reduce sample coloration. Soluble carbohydrate concentrations were determined using the phenol–sulfuric acid colorimetric method according to Dubois et al. [25].

VFAs, namely acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids, were quantified using a gas chromatograph (model 7890B, Agilent Technologies, USA) equipped with a CP-FFAP column (0.25 mm ID, 0.5 mm film thickness, 30 m length) and a flame ionization detector operating at 250 °C. Hydrogen was used as the carrier gas. The column temperature was programmed to increase from 60 °C to 250 °C at a rate of 20 °C per minute.

Prior to analysis, samples were centrifuged at 13,500 rpm for 30 minutes and then filtered through a 0.45 µm membrane. An aliquot of 500 µL of the filtrate was mixed with isoamyl alcohol (1.00179, Merck KGaA, Germany) in a 1:1 volumetric ratio, followed by the addition of 200 µL of phosphate buffer solution (pH 2.1), sodium chloride, and 10 µL of hexanoic-D11 acid solution (10,000 ppm), which served as the internal standard. The resulting mixture was homogenized using a Mortexer Multi-Head vortex mixer (Z755613-1EA, Merck KGaA, Germany) for 10 minutes. The final liquid suspension was introduced into the gas chromatograph using an auto-sampler [26].

2.4. Microbial Community's Investigations

Samples were collected from each group after 7 days of anaerobic fermentation to investigate the relative abundance and diversity of microbial communities.

Total genomic DNA was extracted using the Qiagen DNA extraction kit (DNeasy 96 PowerSoil Pro QIAcube HT).

The V3-V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primer pair Pro341F (5′-CCTACGGGNBGCASCAG -3′) and Pro805R (Rev 5′-GACTACNVGGGTATCTAATCC -3′) for 25 cycles. The PCR products were enzymatically purified and subjected to a second PCR step to attach Illumina adapters and indices for sequencing and sample identification.

High-throughput sequencing was performed on an Illumina platform in paired-end mode (2 × 300 bp), generating approximately 50,000 reads per sample.

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Raw sequencing data quality was assessed prior to bioinformatic analysis. The analysis workflow included preprocessing steps such as adapter trimming, quality filtering, and paired-end reads merging. Amplicon Sequence Variants (ASVs) were identified, and taxonomic assignment was carried out using the SILVA 138 database targeting the V3-V4 region of the 16S rRNA gene [27].

2.5. Statistical Analysis

Significant differences in VFA concentration and microbial diversity indices in all reactors with R- Fe_3O_4 and Fe_3O_4 additions were compared to those in the control group at the same S/I ratio using one-way analysis of variance (ANOVA), followed by a Tamhane post hoc test to identify significant differences ($p < 0.05$) between the experimental conditions. The normality of the data and homogeneity of variances were previously verified using Shapiro-Wilk and Levene tests, respectively.

3. Results and Discussion

3.1. Changes in pH and Soluble Chemical Oxygen Demand (sCOD)

Carbohydrates are quickly converted into monomeric sugars, which can be efficiently fermented into VFAs. Several studies have shown that using substrates with higher concentrations of carbohydrates tends to enhance total acid production, particularly under neutral pH condition [28].

Thus, all experiments were conducted without adjusting the initial pH, in order to reflect realistic operating conditions and to promote VFAs production.

The changes in pH at different S/I ratios in the control reactor, as well as in reactors with the addition of Fe_3O_4 and R-Fe_3O_4, are shown in Figure 1. The pH decreased rapidly during the dark fermentation period. Notably, larger pH drops were observed at higher S/I ratios of 3 and 4. Higher substrate amounts led to increased acid production, resulting in a decrease in pH. However, when the pH is lower than the pKa value of VFAs dissociation, VFAs production in the anaerobic fermentation process can be inhibited.

Under these conditions, the acids remain mostly undissociated, which limits the activity of hydrolytic and acidogenic bacteria by increasing their energy requirements for cell maintenance [29]. On the other hand, the pH decrease observed in the reactors, as the S/I ratio increased, followed the order: R-Fe_3O_4 < Fe_3O_4 < control. Specifically, the R-Fe_3O_4 reactors showed a smaller pH drop compared to the Fe_3O_4 reactors and the control. This effect could be attributed to the carbonate presence in the R- Fe_3O_4 reactors, which acts as a buffering agent, mitigating acidification and maintaining a more stable pH environment. As a result, this contributes to enhanced hydrolysis of soluble components and increased VFA production as described in the following sections.

Moreover, similar findings were reported by Wang et al., who demonstrated that Fe_3O_4 supplementation in the anaerobic fermentation of food waste can prevent excessive pH drops, thereby preserving the activity of key hydrolytic and acidogenic bacteria [14]. During anaerobic fermentation, hydrolysis constitutes a crucial initial stage and is typically assessed by measuring the amount of sCOD released from the biomass [30].

Figure 2 shows the changes in sCOD concentration at different S/I ratios, as it reflects the concentration of dissolved organic compounds. In reactors operated at an S/I ratio of 2, the sCOD concentration exhibited an initial decline, followed by a slight increase. Conversely, at higher organic loading ratios (S/I = 3 and S/I = 4), the sCOD concentration showed a slight increase over time.

The sCOD concentration increased during the initial 4 days of the dark fermentation process, suggesting rapid hydrolysis of particulate organic matter into soluble compounds. Hydrolysis and acidogenesis proceed concurrently, with acidogenesis becoming dominant in the later stages. Consequently, sCOD levels stabilized around day 7, reflecting the metabolic shift towards enhanced production of VFAs. Moreover, reactors supplemented with R-Fe_3O_4 exhibited a moderate increase in sCOD levels during the later stages of the dark fermentation process. This trend suggests that R-

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Fe_3O_4 enhanced the hydrolysis step by promoting the conversion of particulate organic matter into soluble compounds.

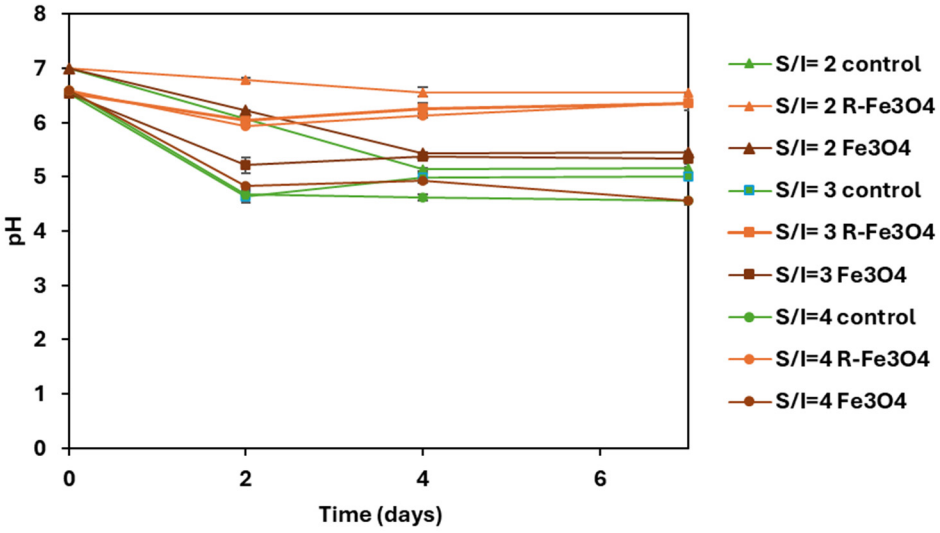

Figure 1. Changes in pH at different S/I ratios and experimental conditions.

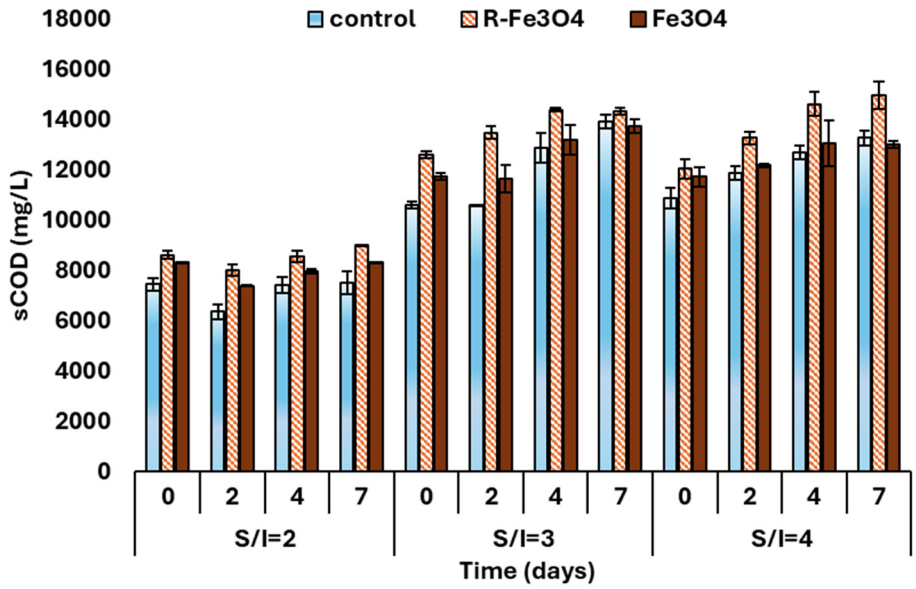

Figure 2. Changes in sCOD concentration at different S/I ratios and experimental conditions (error bars represent the standard deviation).

3.2. Changes in Soluble Carbohydrates

Figure 3 illustrates the changes in total carbohydrate concentrations across all reactors during the dark fermentation process. Due to the significant decrease in concentration, the data were plotted on a logarithmic scale to improve visualization.

The concentration gradually decreased over time, suggesting efficient carbohydrate degradation. Notably, reactors supplemented with R-Fe_3O_4 and Fe_3O_4 showed lower carbohydrate levels at all S/I ratios. Previous research confirmed that carbohydrates were rapidly consumed within two days, and that Fe_3O_4 addition effectively accelerated the removal of soluble carbohydrates [31].

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Moreover, in this study, the addition of R-Fe_3O_4 at the highest organic loading ratio (S/I = 4) enhanced carbohydrate degradation.

This result aligns with a previous study demonstrating that Fe_3O_4 addition during the anaerobic fermentation of food waste enhances the hydrolysis of soluble carbohydrates, particularly at higher S/I ratios [14].

Thus, based on the discussion in Section 3.1, the results confirm that R-Fe_3O_4 not only promotes the breakdown of particulate organic matter into soluble compounds but also accelerates their consumption even under high organic loading conditions.

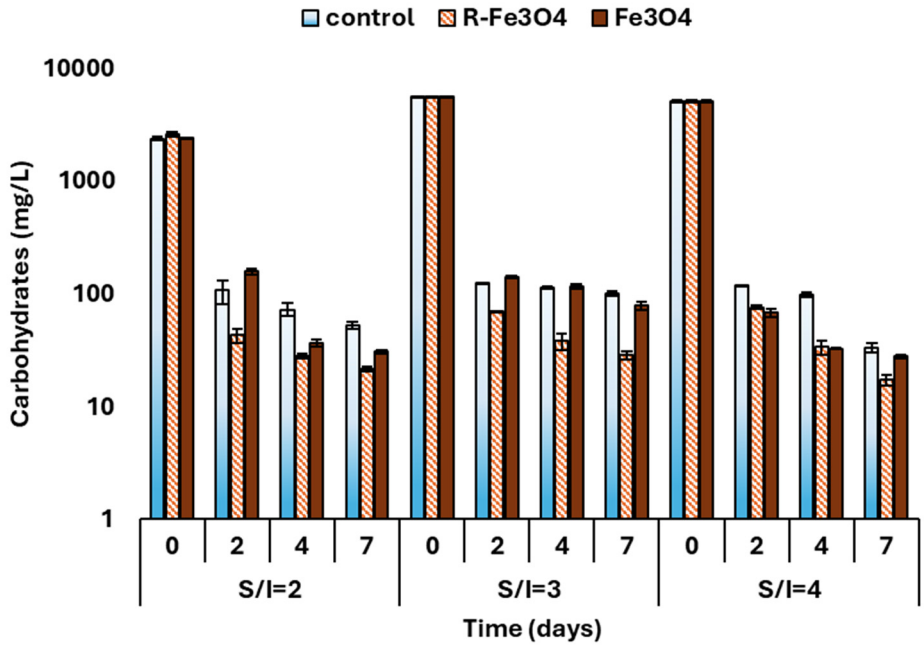

Figure 3. Changes in total carbohydrate concentrations at different S/I ratios and experimental conditions (error bars represent the standard deviation).

3.3. Changes in Dissolved Inorganic Salt (NH_4^+-N and PO_4^{3-}-P)

NH_4^+-N is formed as a result of protein hydrolysis [12]. High molecular weight organic compounds, such as proteins, are first broken down into amino acids by hydrolytic, acid-producing bacteria, and these amino acids are then further degraded by microorganisms, releasing NH_4^+-N [32]. Therefore, NH_4^+-N concentration was measured and used as an indicator of the hydrolysis of soluble proteins and the degradation of amino acids. Figure 4 presents the changes in NH_4^+-N concentrations at different S/I ratios.

Throughout the dark fermentation process, reactors supplemented with R-Fe_3O_4 consistently exhibited higher NH_4^+-N concentrations than those with Fe_3O_4 or without any additive, suggesting that R- Fe_3O_4 may promote protein hydrolysis more effectively.

Notably, the NH_4^+-N concentration slightly decreased on the last day of dark fermentation in the reactors with S/I ratios of 2 and 3.

According to Puga et al. [33], nitrogen loss is mainly due to the volatilization of NH_4^+-N, which may occur under alkaline conditions. Ma and Huang [34] also assessed the effect of acidic and alkaline treatments on VFAs production from sludge and chicken manure in six experimental groups with different pH levels. Their findings highlighted that pH had a strong influence on NH_4^+-N concentrations. Strongly acidic conditions inhibited the release of macromolecular organic compounds such as proteins, while alkaline treatments promoted hydrolysis and solubilization.

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Moreover, the trend observed by the authors was consistent with the results of our study, where NH_4^+-N concentrations under neutral and alkaline conditions initially increased, then decreased between the sixth and eighth day of fermentation and finally rose again.

In contrast, in our study, reactors with an S/I ratio of 4 showed a gradual increase in NH4+-N concentration throughout the dark fermentation process. Meanwhile, reactors with an S/I ratio of 3 exhibited relatively higher NH_4^+-N concentrations compared to other conditions, reaching a maximum of 569.5 ± 23.3 mg/L on the day 4 of dark fermentation following the addition of R-Fe_3O_4.

In addition to NH_4^+-N, the concentration of PO_4^{3-}-P was monitored across all experimental groups, with the results presented in Figure 5. Phosphate originated from the breakdown of phospholipid bilayers and polyphosphate particles.

However, alkaline conditions led to a reduction in PO_4^{3-}-P concentration because of phosphate precipitation. As shown in Figure 5, PO_4^{3-}-P concentration decreased over time in all reactors regardless of the S/I ratio. Specifically, PO_4^{3-}-P concentration initially increased and then declined in the control groups and reactors with Fe_3O_4, whereas in the presence of R-Fe_3O_4, PO_4^{3-}-P concentration decreased drastically throughout the dark fermentation process.

This could be attributed to the presence of $CaCO_3$ in the R- Fe_3O_4 sample and the consequent formation of calcium phosphate, which precipitated under neutral conditions.

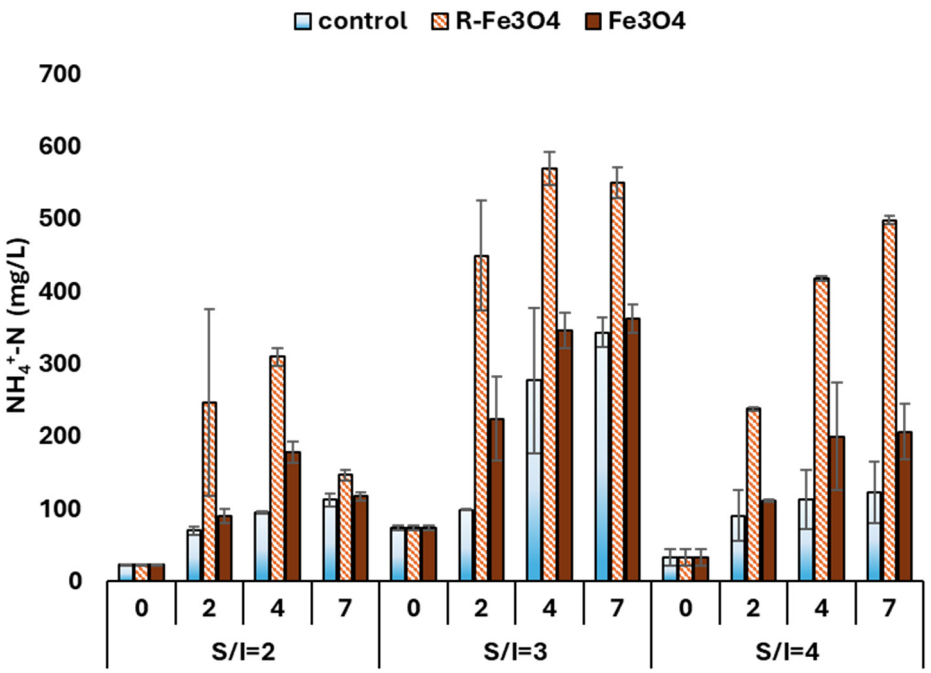

Figure 4. Changes in NH_4^+-N concentration at different S/I ratios and experimental conditions (error bars represent the standard deviation).

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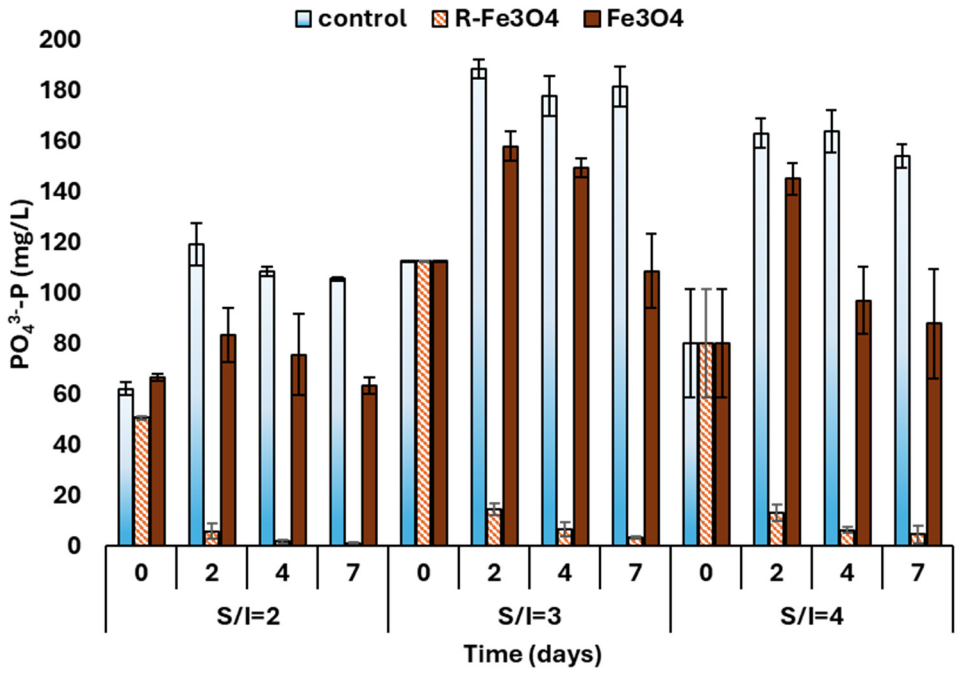

Figure 5. Changes in PO_4^{3-}-P concentration at different S/I ratios and experimental conditions (error bars represent the standard deviation).

3.4. Changes in VFAs Production and Composition

The production of VFAs was monitored to evaluate the effect of Fe_3O_4 and R-Fe_3O_4 additions at different S/I ratios. The VFA production, expressed as g C VFA/kg VS over time, is shown in Figure 6.

The statistical analysis showed a significant difference for the data obtained at S/I = 4 ($p = 0.001$). Furthermore, a significant difference was observed between the reactors with the addition of R-Fe_3O_4 and Fe_3O_4 ($p = 0.005$). An increase in VFAs concentration was observed with increasing dark fermentation time. Regardless of the S/I ratio, the addition of R-Fe_3O_4 and Fe_3O_4 enhanced VFA production. This was consistent with the findings by Wang et al. [35], who demonstrated that the addition of Fe_3O_4 nanoparticles further promotes the degradation of low molecular weight soluble organics by facilitating the conversion of higher molecular weight VFAs into acetic acid, CO2, and trace amounts of CH4.

In all cases, the highest VFA production was achieved in the presence of R-Fe_3O_4 reaching 191.7, 320.9, and 367.5 g C VFA/kg VS at S/I ratios of 2, 3, and 4, respectively. In comparison, VFA concentrations recorded without iron addition were 158.9, 275.1, and 267.0 g C VFA/kg VS, while the addition of Fe_3O_4 resulted in 165.6, 300.3, and 294.3 g C VFA/kg VS at the same S/I ratios. Notably, the highest VFA yield was recorded at the end of the dark fermentation process in the presence of R-Fe_3O_4 at an S/I ratio of 4. By contrast, no significant differences were observed between the control group and the reactors with Fe_3O_4 at the same S/I ratio, suggesting that R-Fe_3O_4 can promote VFA synthesis even under high organic loading conditions. Furthermore, VFA production with Fe_3O_4 at S/I = 4 was lower than the VFA concentrations achieved at S/I = 3 in the presence of Fe_3O_4.

As previously reported, Fe_3O_4 promotes the enrichment of hydrolytic and acidogenic bacteria and enhances key enzymatic activities, thereby improving the breakdown of carbohydrates and proteins and subsequently boosting VFA production [36]. However, in this study, while pure Fe_3O_4 negatively influenced VFA production under high organic loading conditions, R-Fe_3O_4 did not exhibit such inhibitory effects. This may be attributed to the presence of carbonate in reactors supplemented with R-Fe_3O_4, which helps maintain a stable environment by acting as a buffering agent, thereby supporting microbial activity.

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On the other hand, the potential uses of VFAs are influenced not only by their overall yield but also by the specific composition of the individual VFAs [2]. Thus, the proportion of individual VFAs in the presence of Fe_3O_4 and R-Fe_3O_4 was examined at different S/I ratios.

As shown in Figure 7, butyric acid was the main component of dark fermentation in all reactors, meaning that substrate was preferentially converted into longer-chain VFAs at high organic loading. At an S/I ratio of 2, no significant differences were observed in the VFA composition between the control and the Fe_3O_4 supplemented reactors. However, the presence of Fe_3O_4 resulted in a higher relative abundance of caproic acid and isovaleric acid, and partially isobutyric acid, particularly on days 4 and 7 of the dark fermentation process. In contrast, while butyric acid remained the predominant VFA in reactors supplemented with R-Fe_3O_4, the relative abundance of acetic and propionic acid was notably higher compared to both the control and Fe_3O_4 supplemented reactors.

Several studies have suggested that increased acetic acid production may result from the degradation of longer-chain VFAs [37]. In this case, the observed decrease in butyric acid and the corresponding increase in acetic acid in the presence of R-Fe_3O_4 could suggest that R-Fe_3O_4 promoted the conversion of butyrate into acetate.

Different from the S/I ratio of 2, reactors operated at S/I ratios of 3 and 4 with Fe_3O_4 and R-Fe_3O_4 supplementation showed a gradual shift in VFA composition from butyric acid to caproic acid over time. This confirmed the tendency towards the production of longer-chain fatty acids in the presence of Fe_3O_4 and R-Fe_3O_4 under high organic loading conditions.

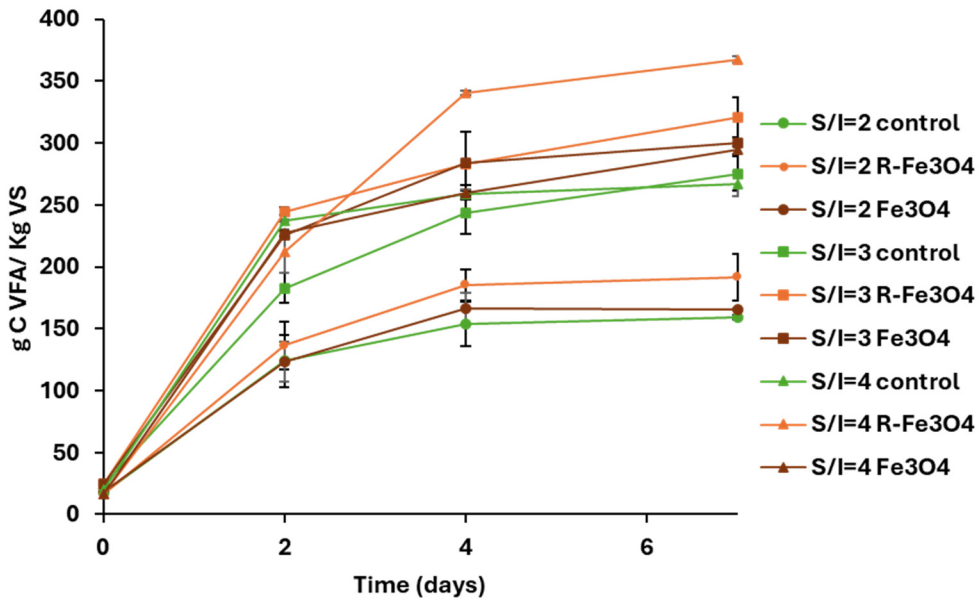

Figure 6. Total VFAs production at different S/I ratios and experimental conditions.

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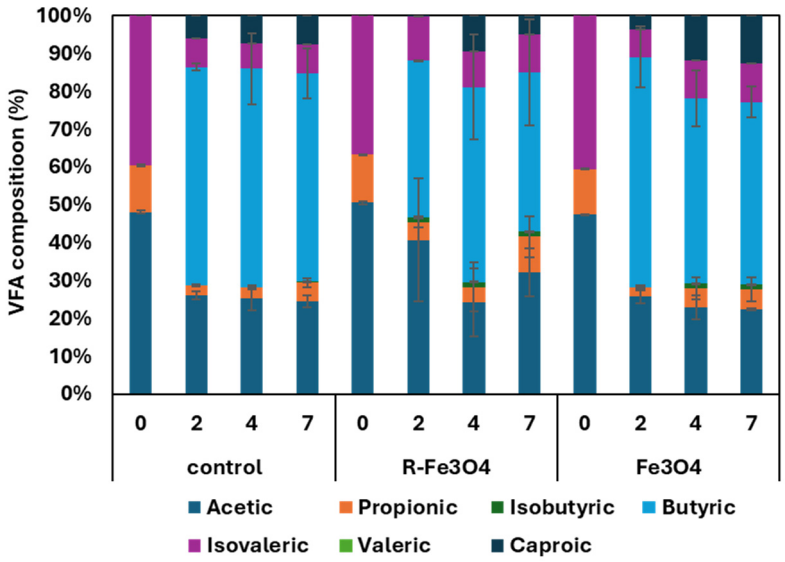
a)



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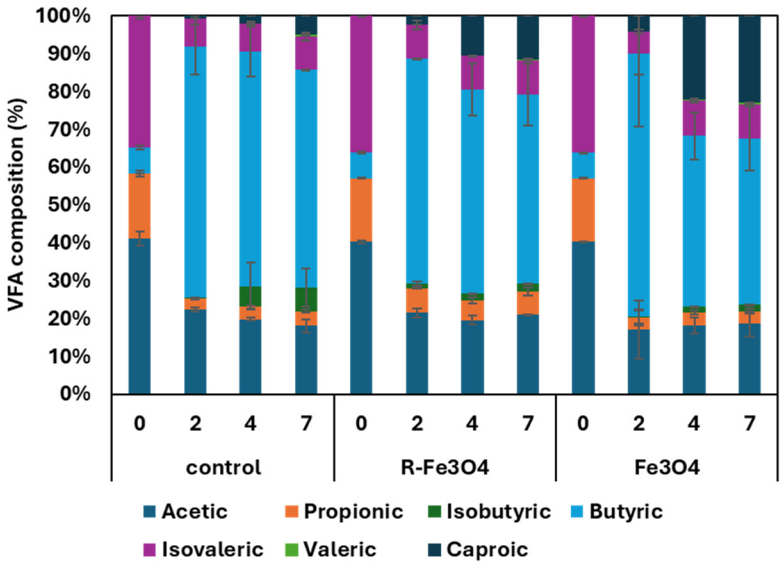

c)

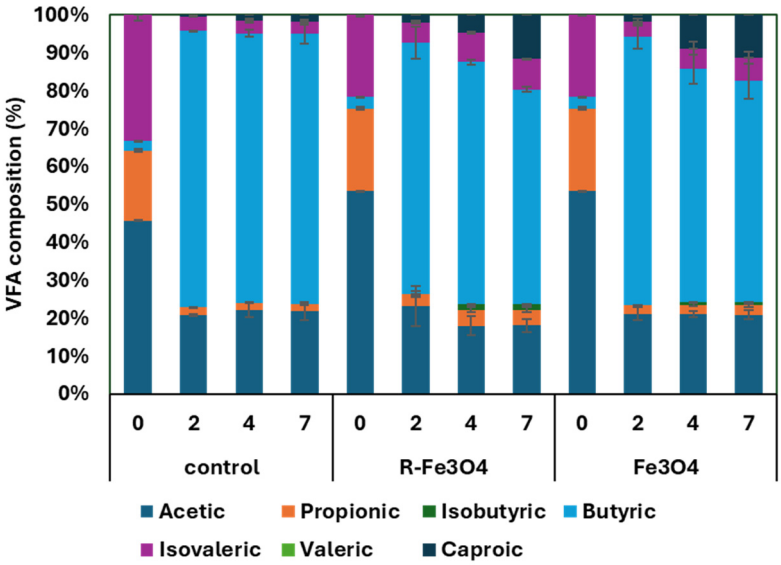

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Figure 7. Changes in VFAs composition at different S/I ratios and experimental conditions: S/I=2(a), S/I=3(b) and S/I=4(c).

3.5. Changes in Microbial Community

Microbial community profiles, including relative abundance and diversity, were assessed after 7 days of anaerobic fermentation under all experimental conditions (Figure 8a and 8b).

As shown in Figure 8a, the relative abundance of bacterial communities at the phylum level did not significantly change with Fe_3O_4 addition compared to the control groups. The predominant phylum across all samples was Firmicutes, accounting for approximately 90.5%, 89.7%, and 92.0% in the control samples at S/I ratios of 2, 3, and 4, respectively. In reactors with Fe_3O_4 addition, Firmicutes accounted for about 91.5%, 91.3%, and 92.6% at S/I = 2, 3, and 4, respectively. Firmicutes are well known for their key role in hydrolysis and acidogenesis during anaerobic fermentation, contributing significantly to the degradation of complex organic substrates.

In contrast, the addition of R-Fe_3O_4 induced significant changes in the bacterial community composition at the phylum level. The relative abundance of Firmicutes decreased substantially to 54.2%, 52.8%, and 82.8% at S/I ratios of 2, 3, and 4, respectively, while Proteobacteria significantly increased to 40.5%, 40.7%, and 14.6%, respectively.

It is well established that Fe_3O_4, due to its conductive properties, can act as a bridge facilitating electron transfer between iron-reducing bacteria and other microbial populations, thereby enhancing the degradation of organic matter [14].

For instance, Clostridium is capable of using Fe(III) as a terminal electron acceptor to oxidize organic compounds, reducing Fe(III) to Fe(II) in the process. In reactors supplemented with R-Fe_3O_4 fermentative iron-reducing bacteria such as Clostridium likely compete with other iron-reducing groups, including Proteobacteria, for available substrates and electron acceptors. Proteobacteria are also key iron-reducing and acidogenic bacteria, whose relative abundances were notably higher in reactors supplemented with R-Fe_3O_4, thus influencing microbial community dynamics and enhancing volatile fatty acid production.

Table 2 shows the different microbial diversity values for all reactors after 7 days of dark fermentation. The Chao1, Shannon, and Pielou indices were significantly higher in reactors supplemented with R-Fe_3O_4 compared to both the control groups and those with Fe_3O_4 addition. This could be attributed to a greater availability of micronutrients in the R-Fe_3O_4, as well as the presence of $CaCO_3$, which helps maintain a stable pH environment, thereby promoting more diverse microbial metabolic pathways. Thus, these results demonstrated that R-Fe_3O_4 enhanced microbial diversity and community evolution, promoting the growth of iron-reducing, acid-producing bacteria such as Proteobacteria and Acidobacteria, which contributed to the increased production of VFAs.

A one-way ANOVA was performed to assess significant differences in microbial diversity indices (Chao1, Shannon, and Pielou) for each S/I ratio. Statistically significant differences were observed only at S/I = 4, with p-values of 0.000 for Chao1, 0.002 for Shannon, and 0.004 for Pielou. Groups labeled with different letters indicate statistically significant differences according to the post hoc test.

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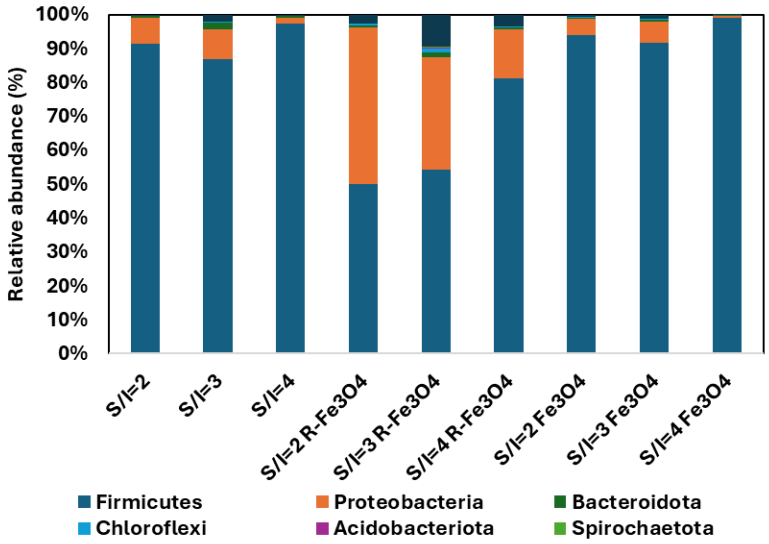
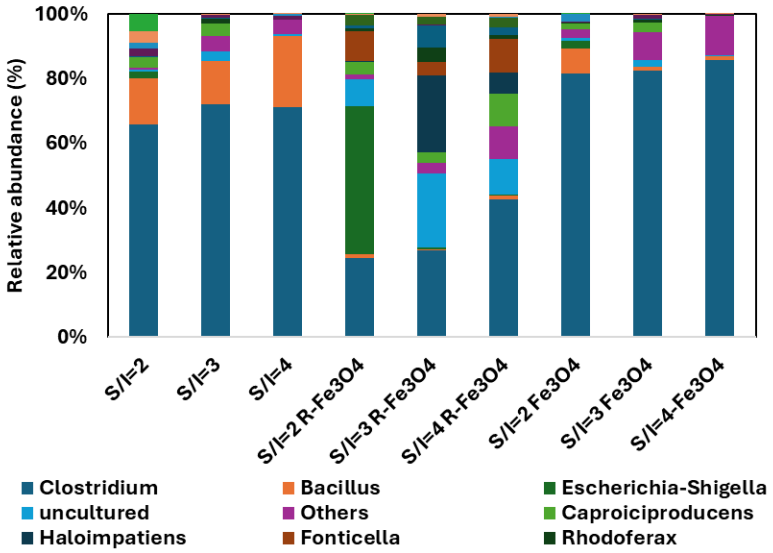
a)



b)



c)

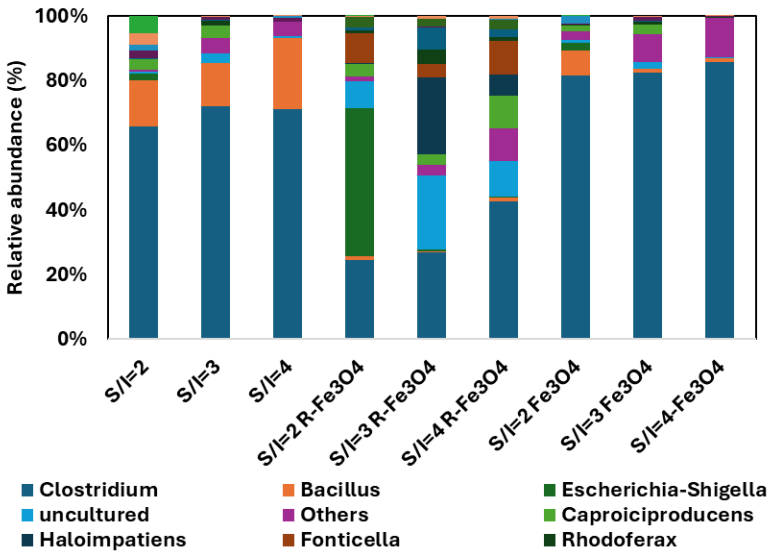


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Figure 8. Microbial community analysis at phylum (a) and genus (b) levels after 7 days of dark fermentation at different S/I ratios and experimental conditions.

Table 2. Alpha-diversity indices of microbial communities in different reactors after 7 days of dark fermentation.

	Chao1	Shannon	Pielou
S/I=2	188	2.89	0.55
S/I=2 R-Fe_3O_4	435.5	2.83	0.58
S/I=2 Fe_3O_4	187	2.50	0.41
S/I=3	292.5	3.54	0.50
S/I=3 R-Fe_3O_4	652.5	4.73	0.73
S/I=3 Fe_3O_4	232.5	4.03	0.47
S/I=4	168.5 [a]	2.13 [a]	0.49 [a]
S/I=4 R-Fe_3O_4	461 [b]	2.56 [b,c]	0.66 [b,c]
S/I=4 Fe_3O_4	86.5 [a]	1.38 [a,c]	0.31 [a,c]

4. Conclusions

In this study, a ferrous precipitate (R-Fe_3O_4) generated from the electrochemical peroxidation process for tannery wastewater treatment was used as an additive in the dark fermentation of powdered milk at high substrate-to-inoculum (S/I) ratios.

The comparison with commercial magnetite (Fe_3O_4) showed that the addition of iron, both as Fe_3O_4 and R-Fe_3O_4, significantly enhanced VFA production, with a notable increase in long-chain fatty acids.

The CaCO3 content in the ferrous precipitate played a crucial role in maintaining pH stability, which supported microbial activity and facilitated the hydrolysis of soluble components.

Thus, the addition of R- Fe_3O_4 promoted the enrichment of hydrolytic and acidogenic bacteria and enhanced key enzymatic activities, thereby improving the breakdown of carbohydrates and proteins and subsequently boosting VFA production. Furthermore, the highest VFA yield was recorded at the end of the dark fermentation process in the presence of R- Fe_3O_4 at an S/I ratio of 4, while pure Fe_3O_4 negatively influenced VFA production under high organic loading conditions. The results demonstrated that R- Fe_3O_4 enhanced microbial diversity and community evolution, promoting the growth of iron-reducing, acid-producing bacteria such as Proteobacteria and Acidobacteria, which contributed to the increased production of VFAs. This highlights the potential of electrochemical peroxidation by-products as valuable resources within circular bioeconomy frameworks, offering an environmentally sustainable and economically beneficial approach to improving dark fermentation processes.

Future research should focus on scaling up this approach, investigating the fundamental microbial mechanisms, and assessing the potential toxicity of the ferrous precipitate to ensure its safe application in full-scale systems.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, I..P. and E.P.; methodology, I.P. and E.P.; software, E.P. and S.C.; validation, I.P., R.I., G.P. and E.T.; formal analysis, I.P., E.P., F.P., S.C., T.L. and R.M.Z.; investigation, I.P., E.P. F.P., S.C., T.L. and R.M.Z.; resources, I.P. and R.I; data curation, , I.P., E.P., F.P., S.C., T.L. and R.M.Z.; writing—original draft preparation, E.P.; writing—review and editing, I.P., F.P. and S.C.; visualization, , I.P., E.P., F.P., S.C., T.L. and R.M.Z.; supervision, I.P., R.I., G.P. and E.T; project

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administration, I.P., R.I., G.P. and E.T.; funding acquisition, I.P. and R.I. All authors have read and agreed to the published version of the manuscript."

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