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Investigation of the Mechanism for Removal of Typical Pathogenic Bacteria from Three-Compartment Septic Tanks under Low Temperature Conditions

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Abstract: Three-compartment septic tanks are a prominently advocated environmentally sustainable sanitation facility in rural China. However, the comprehensive elimination efficacy and underlying mechanisms of pathogenic bacteria within septic tanks remain incompletely understood. In particular, the operational performance in low-temperature conditions has received limited attention in the existing literature. In this work, a simulation of the three-compartment septic tank treatment system was conducted under low-temperature conditions (15 °C). The operational results exemplify the synergistic interplay of volatile fatty acids (VFAs), NH_3-N, and bacterial communities, culminating in a partial reduction in *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enteritidis*, within the three-compartment septic tank. Their respective population abundances were decreased by magnitudes of 2.2, 1.3, 0.03, and 1.46 logarithmic units (copies/mL), respectively. Through the utilization of qPCR and physicochemical indicators, it was observed that the bactericidal effect of VFA primarily occurred during the initial 0–21-day period, while NH_3-N consistently proved to be the most vital sterilizing agent throughout the operation of the three-compartment septic tank. Predominant bacterial communities within the septic tank, such as *Christensenellaceae_R-7_group*, *Brevundimonas*, *Acinetobacter*, and *Saccharimonadales*, exerted substantial inhibitory impacts on *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella enteritidis* through niche competition and suppression. In essence, this study elucidated the actual efficiency of elimination and the underlying mechanisms of typical pathogenic bacteria within three-compartment septic tanks in low-temperature conditions, thereby providing compelling evidence supporting the viability of environmentally sound treatment using such septic tanks. Concurrently, it also shed light on several limitations associated with this treatment approach, aiming to contribute valuable insights for the assessment of ecological risks and health hazards associated with the environmentally benign treatment of rural toilet waste.

Keywords: three-compartment septic tank; volatile fatty acids (VFAs); ammonia nitrogen; removal of typical pathogenic bacteria; anaerobic fermentation

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Citation: Cheng, S.; Yang, S.; Huang, J.; Liu, F.; Shen, F. Investigation of the Mechanism for Removal of Typical Pathogenic Bacteria from Three-Compartment Septic Tanks under Low Temperature Conditions. *Processes* **2024**, *12*, 87. https://doi.org/10.3390/pr12010087

Academic Editor: Antoni Sanchez

Received: 28 November 2023
Revised: 19 December 2023
Accepted: 21 December 2023
Published: 29 December 2023

1. Introduction

In the expansive rural regions of China, inhabited by a population of over 500 million people engaged in agricultural pursuits, the discharge of domestic wastewater from rural residents is of significant magnitude [1]. However, due to the intricate topography, dispersed settlements, and limited economic foundations, the implementation of centralized and large-scale sewage treatment networks is unsuitable for rural China [2,3]. Consequently, while the urban domestic wastewater treatment rate attains a commendable 94.50% [4], the treatment rate for rural domestic wastewater merely reaches 37.26% [5]. The insufficiently treated domestic wastewater, especially human waste from toilets, will potentially expose farmers in the region to significant safety risks and potential health hazards [6]. Pathogenic bacteria found in toilet waste can directly impact human well-being [7]. This is particularly relevant in developing countries, where pathogens responsible for ailments such as pneumonia, diarrhea, and malaria, transmitted through fecal waste, are prevalent causes of mortality among older children [8]. Untreated or inadequately treated toilet waste can further contaminate environmental mediums such as soil, surface water, and groundwater, engendering latent risks over the long term [9,10]. Effectively addressing toilet waste treatment and mitigating the adverse impact of pathogenic bacteria on human health have emerged as formidable challenges for rural China in recent years.

The notions of resource cycling and environmental health hold considerable significance in the discourse surrounding fecal waste treatment, as acknowledged by numerous scholars [11,12]. In recent times, the three-compartment septic tank has emerged as the principal technological model and the focal point of the "toilet revolution" in underdeveloped rural areas of China, dueto its inherent advantages of affordability, ease of operation and maintenance, and the potential for resource reutilization of fecal waste [13,14]. Functioning as a straightforward method of wastewater treatment, the septic tank primarily harnesses the forces of gravity and buoyancy within its first compartment (G1) to effectuate the separation of solids, liquids, and floating scum. The feces settle and partially decomposed in G1, facilitating their extended decomposition within the septic tank. The wastewater, following a preliminary phase of simple fermentation, proceeds to the second compartment (G2) for further anaerobic digestion. Within G2, the wastewater undergoes a retention period and maturation, leading to a partial elimination of pathogenic bacteria [15], before ultimately being stored in the third compartment (G3) [16,17]. Unlike the two-compartment septic tanks found in European and American countries, which are typically linked to sewage treatment networks or land treatment systems, the wastewater stored in the third compartment in China can be periodically extracted for agricultural irrigation and fertilization purposes [18].

Nonetheless, the mechanisms governing the control and complete elimination of typical pathogenic bacteria within septic tanks have not yet been fully comprehended. Recent findings increasingly indicate that the removal of pathogenic bacteria from the effluent of three-compartment septic tanks is notcomplete, and certain pathogens may even persist at relatively high levels [19,20]. This state of affairs is undeniably disconcerting.Recently, some researchers have made partial headway in exploring the mechanisms and effects of pathogenic bacteria treatment in mesophilic or thermophilic septic tanks [21]. Adequate anaerobic fermentation within septic tanks operating under high-temperature conditions during the summer season can yield favorable outcomesof *Escherichia coli* removal from fecal waste [21]. However, there is a lack of reports regarding the mechanismsof pathogenic bacteria removal under low-temperature conditions. Given that the average annual temperatures in most regions of northern China range from 10 to 12 °C [22,23], it becomes all the more imperative to investigate the mechanisms of pathogenic bacteria elimination within three-compartment septic tanks operating in low-temperature conditions, thereby providing practical guidance and valuable insights.

The aimof this study was to reveal the mechanisms underlying the elimination of typical pathogenic bacteria in the three-compartment septic tank and evaluate the corresponding impacts. The absolute abundance of typical pathogenic bacteria, including *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enteritidis*,inwastewater under low-temperature conditions was investigatedwithin a simulated three-compartment septic tank system. Rigorous and continuous monitoring was implemented, accompanied by an integrated analysis of physicochemical indicators and microbial community composition within the system. The study aspires to furnish a scientifically grounded theoretical framework that can inform future enhancements in the treatment system of three-compartment septic tanks, leading to enhanced sterilization efficacy. The advancements will facilitate the safe and sustainable utilization of fecal waste in rural areas.

2. Material and Methods

2.1. Laboratory Small Three-Compartment Septic Tanks and the Overall Test Design

Acustom-designed small-scale three-compartment septic tank was used as the reactor, with a total effective volume of 16.8 L. This volume was achieved by scaling down a standard 1.5 m^3 septic tank by a factor of 89.3. The ratio of effective volumes among the three chambers was set as G1:G2:G3 = 2:1:3, with an effective depth of 20 cm. The wastewater was directed to G1 through an inlet and discharged via an outlet in G3. A peristaltic pump was employed to connect the inlet. All three chambers were equipped with inspection ports located at the top (Figure 1). The design of the septic tank adhered to the specifications outlined in the "Technical specification for construction of rural household latrine with three-compartment septic tank" [24]. The hydraulic and leakage tests were conducted to ensure the watertight integrity of each chamber and the absence of cross-flow between chambers. Three identical small-scale three-compartment septic tanks were arranged in a constant-temperature chamber, following a parallel design, with a consistent operating temperature maintained at 15 °C.

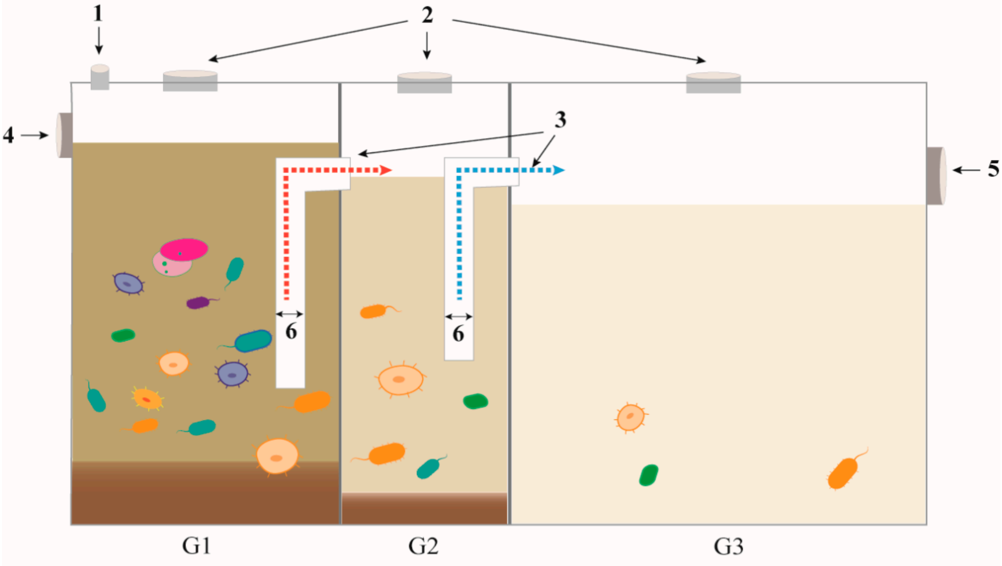

Figure 1. Three-compartment septic tank artificial simulation diagram. (Note: 1. exhaust port, inner diameter 3 mm, 2. access openings, diameter of diameter 40 mm, 3. the port through which feces pass, 20 mm × 20 mm, 4. water inlet, diameter of diameter 10 mm, 5. water outlet, diameter of diameter 20 mm, 6. the tube through which feces pass, 20 mm × 20 mm).

2.2. Inflow and Inoculum

The influent samplewas derived from the middle layer of the three-compartment septic tanks in Tianjin, China. The raw sewage underwent thorough mixing and subsequent filtration to eliminate large particles and insoluble matter, thereby preventing the peristaltic pump from clogging. The mixed sewage was promptly divided into 20 portions and frozen at a temperature of −80 °C. Before usage, each portion was thawed and activated at room temperature (15 °C). Approximately three days' volume of influent was extracted from the freezer during each cycle. The inoculum for the three-compartment septic tank was derived from the authentic rural three-compartment septic tanks.Thesewage was subjected to a comprehensive fermentation process, resulting in the acquisition of sludge for inoculation. On the initial day of influent introduction into G1, a one-time inoculation of 5.0 g of anaerobic sludge was added. To simulate wastewater input, wastewater inputs were introduced six times daily, with water being added every 4 h at a rate of 48 mL each time. The retention time was set at 20 days for G1, 10 days for G2, and 30 days for G3. On the 60th day, G3 reached its full capacity, and the wastewater was discharged through theoutlet.

2.3. Sample Collection and Physicochemical Parameter Analysis

In order to conduct physicochemical analysis and other measurements, sterile syringes were utilized to obtain a 20 mL water sample from each chamber through the sampling port. Sampling was collected at intervals of 7 days for test until day 63. Initially, all the compartments were empty, sampling for G2 began on day 28, while sampling for G3 commenced on day 35.

The physicochemical parameters, including pH, COD, and NH_3-N, were assessed by the established methodologies (referenced as [25]). The determination of volatile fatty acid (VFA)was conducted utilizing a Thermal Trace-1300 gas chromatograph outfitted with a TR-FFAP column [26]. Samples from three parallel septic tanks were collected for analysis, and the results were summarized as the mean value ± standard deviation. Statistical computations and data analysis were performed employing the SPSS 22.0 statistical software package (IBM, Armonk, NY, USA), while the data were graphically presented using Origin 2023.

2.4. RNA Extraction and Real-Time PCR Analysis

Utilizing the aforementioned collected water (at identical time points as the physicochemical indicators), RNA extraction was carried out. Sample pretreatment encompassed centrifugation at 12,000 rpm for 2 min at 4 °C to harvest the bacterial cells (ensuring that the maximum quantity of collected cells did not surpass 1×10^9 cells/mL) while meticulously eliminating all supernatant. The bacterial total RNA extraction kit (Tiangen, Beijing, China) was employed to extract 50–70 µL of RNA from each sample. The extracted RNA was evaluated for concentration and purity through UV absorbance measurement, and its RNA integrity was further ascertained through denaturing agarose gel electrophoresis, both of which confirmed satisfactory outcomes. The RNA was applied for subsequent experiments. Reverse transcription and cDNA synthesis were performed employing the PrimeScript™ RT reagent Kit with gDNA Eraser (TakaRa, Tokyo, Japan). Finally, Real-Time PCR (Polymerase Chain Reaction) sample detection was conducted using the ABI 7500 fluorescent quantitative PCR instrument (Applied Biosystems, Waltham, MA, USA) [27]. Primers targeting the bacteria of interest (*Enterococcus faecalis* [28], *Escherichia coli* [29], *Staphylococcus aureus* [30], *Salmonella enteritidis* [31]) were selected to construct common target genes and synthesized using Invitrogen (Waltham, MA, USA). For details regarding primer information, please refer to Supplementary Table S1. Plasmid cloning was executed using competent DH5α cells to generate the standard for absolute quantitative PCR detection [32,33]. The standard curve equation for the target bacteria was established based on the amplification curve of the standard. The amplification efficiency of the target gene was satisfactory, demonstrating a robust linear relationship ($R^2 = 0.99$). The detection limits for the target genes of the four pathogenic bacteria were all below 10^2 copies/mL, thereby ensuring the accuracy of this experiment. The initial assessment of absolute abundance values for the four typical pathogenic bacteria presented relatively high levels (Table 1).

Table 1. Absolute abundance of typical pathogenic bacteria in influent/raw water (Lg (copies/mL)).

Enterococcus faecalis	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella enteritidis*
7.270 ± 0.620	7.656 ± 0.443	7.125 ± 0.702	6.608 ± 0.638

2.5. 16S rRNA Gene Sequencing

The samples D1G1, D14G1, D28G1, D28G2, D35G1, D35G2, D35G3, D63G1, D63G2, and D63G3 (D:reaction time, G1: first pool, G2: second pool, G3: third pool) were chosen as the samples for 16S rRNA detection. DNA extraction from fecal water samples, intended for 16S rRNA sequencing experiments, was carried out using the Fast DNA Spin Kit for Soil (MP, Santa Ana, CA, USA). Following the procedure outlined in Section 2.4, the evaluation of DNA purity and concentration was undertaken. Gel electrophoresis was employed to validate the appropriateness of PCR product sizes, ensuring the adequate quantity for genome library construction, thus satisfying the prerequisites for the preparation of two or more gene libraries. The amplification region selected for the 16S rRNA gene was V3-V4 (338-806) [34], with the primer sequences as follows: Forward primer: 5′-ACTCCTACGGGAGGCAGCAG-3′, Reverse primer: 5′-GGACTACCAGGGTATCTAAT-3′. The annealing temperature was set at 55 °C. High-throughput sequencing of the 16S rRNA gene was carried out using the Illumina HiSeq platform (Ollwegene Technologies, Nanjing, China). The raw sequencing data have been deposited in the SRA database at the NCBI data center. The acquired Fastq data underwent quality control processing to ensure a high-quality sequence distribution, with the majority of sequences falling within the range of 380–440 bp. The R language data statistics and graphics package was utilized to generate plots depicting the distribution of OTUs (Operational Taxonomic Units), species composition, redundancy analysis, and other visualizations [35,36].

3. Results and Discussion

3.1. The Role of VFA in the Early Killing of Pathogenic Bacteria in Septic Tanks

Figure 2 depicts the temporal fluctuations in pH and VFAs within the three-compartment septic tank. Clearly, the VFA levels displayed an initial surge followed by a subsequent decline during the 1–21-day period, ultimately reaching a lower overall concentration. Simultaneously, the pH values decreased from day 1 to day 7. The gradual pH increase observed between days 7 and 14, despite the continuous rise in VFA, can be attributed to the concomitant increase in NH_3-N within chamber G1, leading to pH fluctuations. During the initial 0–7-day period, the intricate organic compounds in the digester rapidly decomposed into simpler, soluble organic substances through microbial activity. This initial fermentation process, involving hydrogen- and acetate-producing bacteria, led to the accumulation of VFAs within chamber G1 [37]. VFAs, as volatile fatty acids with low molecular weight, can permeate microbial cell membranes and disrupt bacterial functions. Acting as a broad-spectrum substance capable of eliminating typical pathogenic bacteria, VFAs effectively function as a disinfectant during the early to mid-stages of the anaerobic digestion process. Within the 0–14-day period, the population of *Enterococcus faecalis* and *Salmonella enteritidis* within chamber G1 decreased by approximately one order of magnitude (Figure 3a,d), while *Escherichia coli* decreased by about 0.4 orders of magnitude (Figure 3b). However, *Staphylococcus aureus* only experienced a decline of 0.1 orders of magnitude (Figure 3c), owing to its inherent resistance to VFAs [38]. It should be noted that between 14–21-day, as the VFA concentration in the system decreased, the residual nutrients allowed the proliferation of *Staphylococcus aureus*, which resulted in a transient surge in the number of *Staphylococcus aureus*.

Previous studies have indicated that in order to enhance the bactericidal effect of VFAs within the system, it is necessary to promote the generation of more lipophilic, unbound VFAs in a neutral or acidic fermentation environment [39,40]. Therefore, a lower pH would be more conducive to the bactericidal efficacy of VFAs. In the three-compartment septic tank, which predominantly receives human excrement, the environment tends to be alkaline. By adjusting the pH of the influent or introducing more acid-producing bacteria during the initial stages [41], the bactericidal effect (VFA action) during the first cycle of the anaerobic digestion process can be significantly enhanced. However, excessive accumulation of VFAs or an excessively acidic environment can have adverse effects on anaerobic fermentation (acid inhibition) [42]. Hence, it is crucial to control the rate of acid production and duration of acid influence, and to cultivate and domesticate relevant acid-producing bacterial strains, to achieve simultaneous improvements in anaerobic fermentation and bactericidal efficacy.

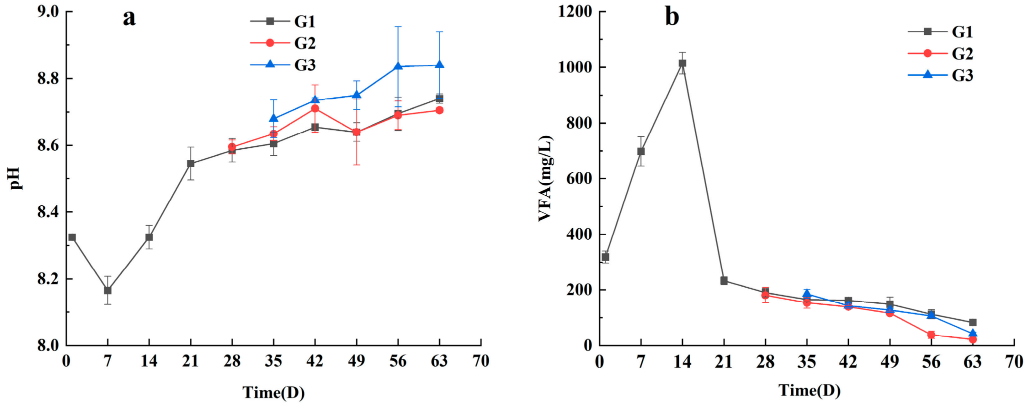

Figure 2. The monitoring diagram of pH and VFA in the whole operation process of three-compartment septic tank. (**a**). Line graph of pH changes, (**b**). Line graph of VFA changes.

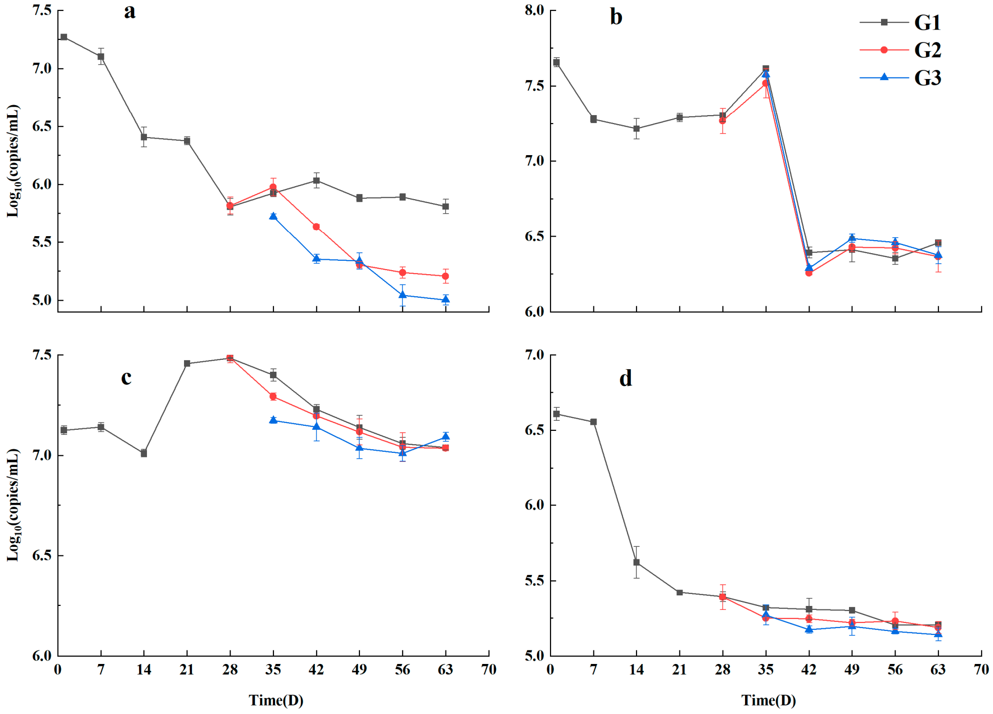

Figure 3. Absolute abundance of typical pathogenic bacteria in three-compartment septic tanks: (**a**) *Enterococcus faecalis*, (**b**) *Escherichia coli*, (**c**) *Staphylococcus aureus*, (**d**) *Salmonella enteritidis*.

3.2. NH_3-N Impact Strategies on Typical Pathogenic Bacteria

NH_3-N plays a pivotal role as an indicator in the anaerobic fermentation of toilet waste. Given that raw toilet waste inherently contained a certain quantity of NH_3-N, the initial concentration of NH_3-N was relatively elevated. As the reaction proceeded, the NH_3-N content gradually increased until reaching day 14 due to the degradation of organic matter (Figure 4), as evidenced by the declining COD values during this period. NH_3-N can exist in two forms: NH_4^+ and free ammonia nitrogen (FAN). Some researchers argue that FAN exerts bactericidal effects akin to VFAs by penetrating cell membranes and impairing cellular physiological activities, while others contend that NH_4^+ serves as the primary inhibitory factor [43,44]. Although there is ongoing debate concerning the precise mechanisms by which NH_3-N inhibits methanogenic bacteria within anaerobic fermentation systems, its capability to eradicate pathogenic bacteria is well established. Overall, from day 21 to day 42 of the treatment process, as the NH_3-N content within the system steadily rose, there was a notable reduction in the population of *Enterococcus faecalis* and *Escherichia coli* by approximately 0.5 orders of magnitude (Figure 3a) and around 1 order of magnitude (Figure 3b), respectively. Additionally, there was a decrease in *Staphylococcus aureus* within the anaerobic digester, particularly in chamber G1, by approximately 0.5 orders of magnitude (Figure 3c), although its overall abundance remained high. Regarding *Salmonella enteritidis*, its population was already diminished to a very low level by day 21; hence, the bactericidal effect of NH_3-N on *Salmonella* was not significant.

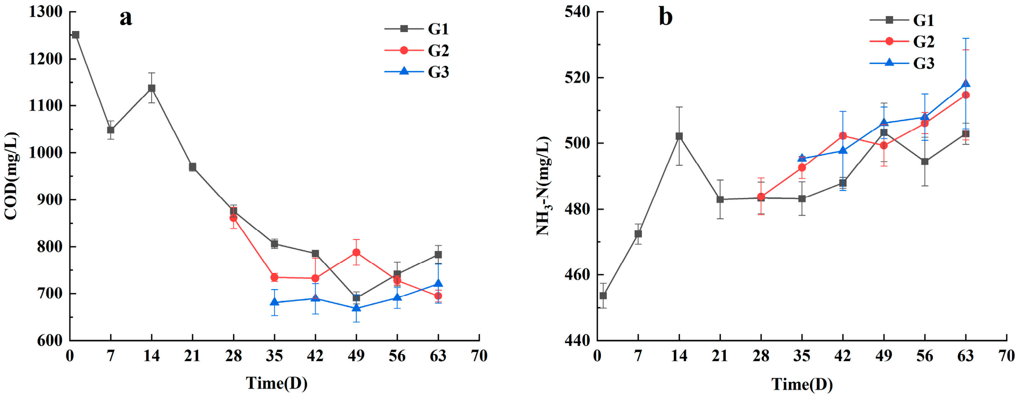

Figure 4. The monitoring diagram of COD and ammonia nitrogen in the whole operation process of the three-compartment septic tank. (**a**). Line graph of CODchanges, (**b**). Line graph of NH_3-N changes.

Through a cross-comparison of the different chambers, it becomes evident from Figure 4 that as a result of ongoing anaerobic fermentation, G2 and G3 displayed higher concentrations of NH_3-N in comparison to G1. The elevated NH_3-N levels in G2 and G3 during the 42–63-day period exerted a more pronounced bactericidal effect on *Enterococcus faecalis*. Their absolute abundance decreased by approximately one order of magnitude compared to G1 (Figure 3a). However, for other pathogenic bacteria, the increase in NH_3-N concentration did not significantly enhance the bactericidal effect. Overall, when horizontally examining the three chambers of the three-compartment septic tank, there was no substantial disparity in the efficacy of pathogenic bacteria removal among G1, G2, and G3.

In terms of the overall effectiveness in removing pathogenic bacteria, the three-compartment septic tank achieved approximately 2.2 orders of magnitude reduction for *Enterococcus faecalis*, 1.3 orders of magnitude reduction for *Escherichia coli*, 0.03 orders of magnitude reduction for *Staphylococcus aureus*, and 1.46 orders of magnitude reduction for *Salmonella enteritidis* by day 63. Regarding the physicochemical properties of the water, despite a certain degree of reduction in the COD and NH_3-N content of the effluent after the completion of treatment in the three-compartment septic tank, the levels still remained high at 720.6 mg/L and 518.1 mg/L, respectively. The value of COD exceed the irrigation requirements for water and drought-resistant crops specified in the "Standard for irrigation water quality" [45]. Consequently, under low-temperature conditions, direct agricultural utilization of the effluent from the three-compartment septic tank presents significant risks of environmental pollution and adverse effects on human health.

Based on the experimental design, it was discovered that each 48 mL of water inflow at the inlet corresponded to an actual flushing volume of 1.1 L. However, the surveys have indicated that most water-saving toilets available in the Chinese market have a flushing volume of approximately 3–5 L, significantly exceeding the requirements of three-compartment septic tanks. As a result, the actual retention time of fecal wastewater in the three-compartment septic tank is only about 20 days. Consequently, in practical construction and usage, the three-compartment septic tank can only achieve partial effectiveness in removing pathogenic bacteria, as observed in the experiments. To enhance the treatment performance of septic tanks in rural areas, it is necessary to reduce the flushing amount of the water toilet, increase the effective volume of the three-compartment septic tank or incorporate additional post-treatment facilities.

NH_3-N plays a vital role in the elimination of typical pathogenic bacteria, but it also poses a detrimental factor to the continuous anaerobic digestion process [46]. Studies have shown that when NH_3-N concentration reaches 3000 mg/L, methanogenic acetate bacteria are inhibited, resulting in a slowdown of the fermentation process and a decrease in anaerobic methane production efficiency [47]. Fortunately, in rural septic tanks, the NH_3-N concentration has not yet reached this threshold, possibly due to factors such as increased flushing or low temperature conditions. Therefore, it may be possible to optimize the structure of septic tanks in a rational manner to enhance the NH_3-N concentration within the anaerobic digestion system, thereby achieving better removal effectiveness without compromising the ability of anaerobic fermentation. Furthermore, we can also cultivate and domesticate anaerobic fermentation microbial communities that exhibit strong tolerance to NH_3-N [48] to address the inhibitory effects of ammonia while eliminating harmful microbial communities within the system. We will continue to conduct further research in this area.

3.3. Effects of Microbial Communities

3.3.1. Effects of Changes in Microbial Species

The microbial community within the three-compartment septic tank is diverse and undergoes shifts in composition, which can potentially impact the fate of pathogenic bacteria [49]. Figure 5 provides an overview of the bacterial community composition at the phylum and genus levels. The top 10 taxa in terms of relative abundance were *Proteobacteria*, *Bacteroidota*, *Firmicutes*, *Patescibacteria*, *Actinobacteriota*, *Synergistota*, *Abditibacteriota*, *Desulfobacterota*, *Verrucomicrobiota*, and *Chloroflexi* (Figure 5a). In the influent sample (D1G1), the phylum *Firmicutes* exhibited the highest abundance (58.6%), followed by *Patescibacteria* (12.6%), *Desulfobacterota* (8.1%), and *Proteobacteria* (5.9%). *Firmicutes*, known as hydrolytic acidogenic microorganisms, play a crucial role in anaerobic fermentation by secreting extracellular enzymes to hydrolyze complex organic compounds present in the influent [50,51]. *Bacteroidota*, *Patescibacteria*, and *Desulfobacterota* also have similar functionalities, as supported by previous research findings [52–54]. These microbial taxa contributed to the decrease in pH and the significant production of VFA in the early stage of the three-compartment septic tank system. Several studies have confirmed the positive influence of these taxa on VFA production [55]. As fermentation progresses, the relative abundance of *Firmicutes* decreased significantly to 15.1% at 14 days and further dropped to only 6% of the initial value at 28 days. During this period, *Proteobacteria* surpassed *Firmicutes* and became the dominant phylum with the highest relative abundance in three-compartment septic tanks, continuing until the completion of the entire treatment process. This shift suggests that certain bacterial populations of *Firmicutes*, abundant in the early stage of the three-compartment septic tank's low-temperature anaerobic fermentation, may have lower tolerance to VFA accumulation compared to other bacterial phyla, leading to niche displacement by other bacterial groups. The significant decline in the abundance of *Enterococcus faecalis*, belonging to the *Firmicutes* phylum, further supported this observation. Some reports indicate that *β-Proteobacteria* within the *Proteobacteria* phylum are the predominant group in microbial communities that utilize propionate, butyrate, and acetate [56], which allows them to replace *Firmicutes* and occupy more available resources in high VFA concentrations. However, considering the overall treatment process, *Firmicutes* still maintain a sustained high abundance within the system (3.66%), indicating their persistence and significant role in both three-compartment septic tanks' treatment and subsequent fecal sludge application processes [57]. At the phylum level, the relative abundance of *Bacteroidota* continued to increase throughout the treatment process, reaching the second-highest position in the system after 28 days. *Bacteroidota*, as an anaerobic bacterium, employed a competitive strategy by lacking peptidoglycan and carbohydrates in its cell wall, making it insensitive to many antibiotics and metabolic byproducts of other bacteria. Additionally, *Bacteroidota* has evolved complex symbiotic relationships under anaerobic conditions, enabling efficient utilization of polysaccharide substances for growth and reproduction [58], which allows it to occupy a favorable position within the system. Similarly, *Patescibacteria*, also known as CPR superphylum, possesses ultra-small cells and simplified membrane structures. Researchers have confirmed that *Patescibacteria* lacks CRISPR virus defense but evades predation by bacteriophages through the deletion of common membrane phage receptors and other alternative strategies [59], providing *Patescibacteria* with a competitive advantage in microbial competition within three-compartment septic tanks. These findings highlight the dynamic nature of the microbial community in three-compartment septic tanks and the potential impact of specific microbial taxa on the overall functioning of the system.

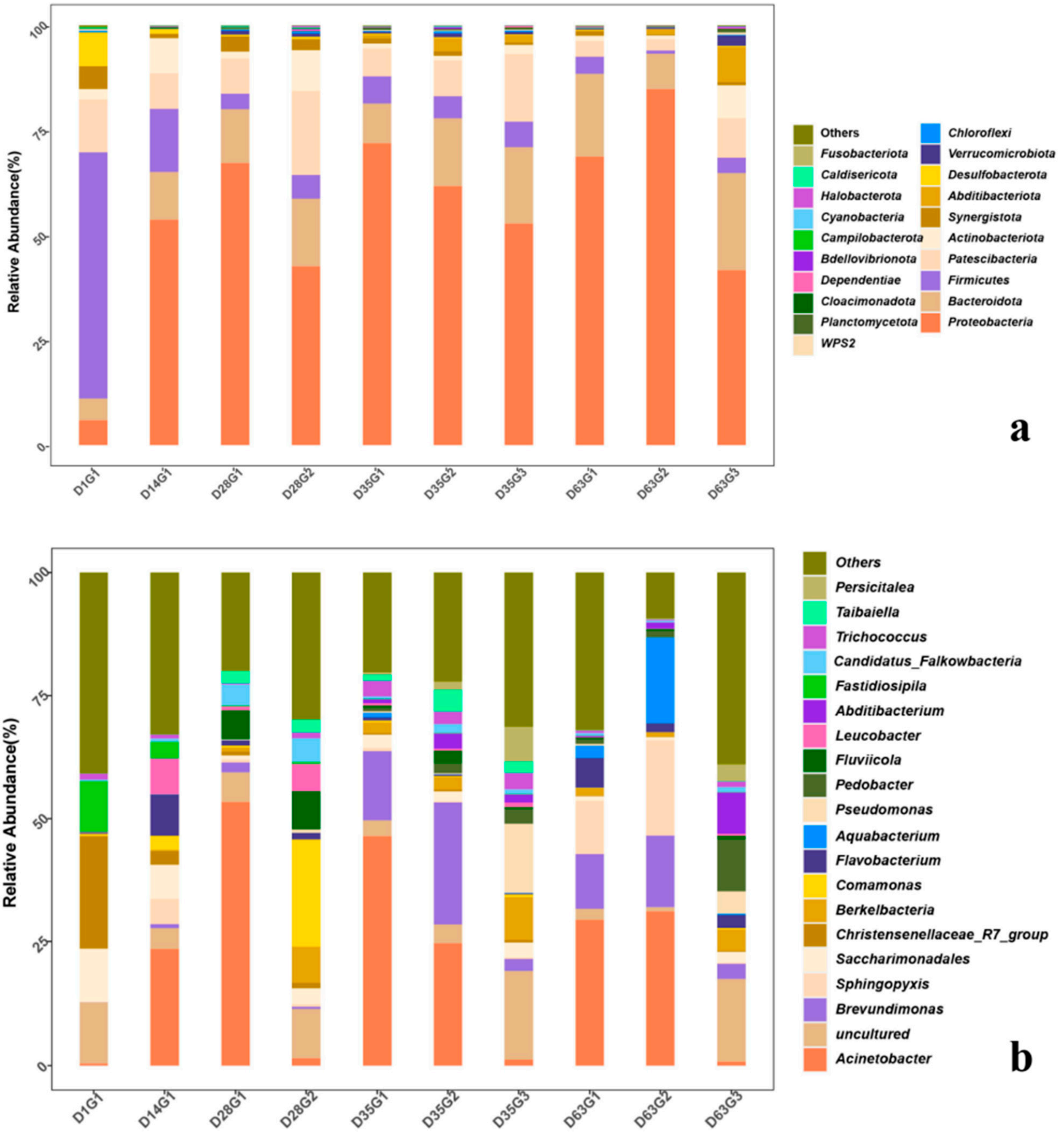

Figure 5. Bar plots for bacteria at the phylum level (**a**) and genus level (**b**).

Analyzing the genus level of microbial composition provides additional insights into the dynamics of three-compartment septic tank systems. The abundance of *Acinetobacter*, *Christensenellaceae_R-7_group*, and *Brevundimonas* correlated with changes in the microbial community structure (Figure 5b). *Acinetobacter*, known for its high tolerance to temperature, pH, and antibiotics, exhibits exceptional resilience and maintains a high abundance within the system [60]. However, its relative abundance decreased in the G2 and G3 pools, and dropped to 0.8% in the final effluent. This decline may be associated with the sustained high levels of NH_3-N during the later stages of anaerobic fermentation. It is important to note that certain bacteria within the *Acinetobacter* genus are potential pathogens, which pose a risk to human health [61]. Thus, careful attention and monitoring of these bacterial groups are necessary. The *Christensenellaceae_R-7_group* is a group of effective sugar-fermenting bacteria that can break down glucose into short-chain fatty acids such as acetate and butyrate [62]. They also have the ability to degrade cellulose, making them important contributors to hydrolytic acidogenesis in the initial fermentation stage. The relative abundance of *Christensenellaceae_R-7_group* decreased during fermentation, from 22.8% to 2.8% at 14 days, and further declined to 0.2% in the effluent. The reduction in *Christensenellaceae_R-7_group* may be due to the scarcity of sugar-based organic compounds within three-compartment septic tanks. *Brevundimonas*, a member of *β-Proteobacteria*, has wide applications in environmental engineering, including wastewater treatment. It is considered a symbiont of animal gut bacteria and plays an important role in the removal of traditional pollutants within three-compartment septic tanks. Throughout the treatment process, no dominant species of typical pathogenic bacteria, accounting for more than 1% of the population, were found. This suggests that the bacterial species composition within the entire three-compartment septic tank system effectively inhibited the proliferation of the four typical pathogenic bacteria [63]. Overall, analyzing the genus-level composition provides a more detailed understanding of specific bacterial groups and their contributions to the functioning of the three-compartment septic tank system.

3.3.2. Microbial Diversity Change

The α-diversity of the samples was assessed using OTU-based sequencing, and the results are presented in Table 2. The sequencing coverage for all 10 sample groups exceeded 99%, indicating reliable and comprehensive sequencing results. Comparing the D1–D35 period to the D35–D63 period, there was a significant decrease in the Observed_species, Shannon, Simpson, and Chao1 indices. This suggests a substantial reduction in the distribution and abundance of microbial communities in three-compartment septic tanks after undergoing treatment. VFA and NH_3-N are identified as important environmental factors that influence the microbial community structure in the three-compartment septic tank treatment system, exerting a broad-spectrum effect on reducing the microbial population. In the D63G2 sample, the Observed_species, Shannon, Simpson, and Chao1 indices are reported as 3.55, 1471.00, 0.83, and 688.35, respectively. These values indicate that the second pool (G2) represents the location with the lowest species abundance and diversity within the entire anaerobic fermentation system at that particular time point.

Figure 6 provides an analysis of microbial community β-diversity, showcasing the differences in bacterial diversity among different stages of the three-compartment septic tank treatment process. The petal Venn diagram illustrates the shared and unique species among different systems. The diagram shows that there are 120 OTUs shared across all systems, indicating a core set of bacterial species present throughout the treatment process. Each system also has unique OTUs specific to that particular stage. Among the unique OTUs, D28G2 has the highest count with 886 OTUs. This suggests that during the early stages of acidification and other processes in the system, there is an establishment of a suitable anaerobic fermentation microbial system, leading to a slight increase in diversity. In terms of species diversity, G1 initially exhibited an increasing pattern followed by a decrease, indicating a fluctuation in microbial community composition. G2, inheriting the microbial community from G1, continued to decrease in diversity from D28 onwards. G3 showed an upward trend at D63, suggesting insufficient anaerobic fermentation substrates and the occurrence of anaerobic digestion inhibition, leading to changes in dominant bacterial genera and a slight increase in diversity. Overall, there were minimal species differences among G1, G2, and G3 during the same period, indicating a similarity in microbial community composition and suggesting consistent treatment performance among the three chambers.

Table 2. Diversity and richness of bacteria communities in the whole process of three-compartment septic tanks.

Treatment	Observed_Species	Shannon	Simpson	Chao1	Goods_Coverage
D1G1	1034.00	6.91	0.97	1160.73	1.00
D14G1	877.00	5.73	0.95	1212.25	0.99
D28G1	938.00	5.32	0.91	1207.00	0.99
D28G2	1006.00	6.17	0.94	1168.35	1.00
D35G1	771.00	4.76	0.85	1120.96	0.99
D35G2	743.00	5.28	0.91	942.30	1.00
D35G3	567.00	5.67	0.94	692.53	1.00
D63G1	611.90	5.00	0.93	760.76	1.00
D63G2	471.00	3.55	0.83	688.35	1.00
D63G3	691.00	6.09	0.96	839.35	1.00

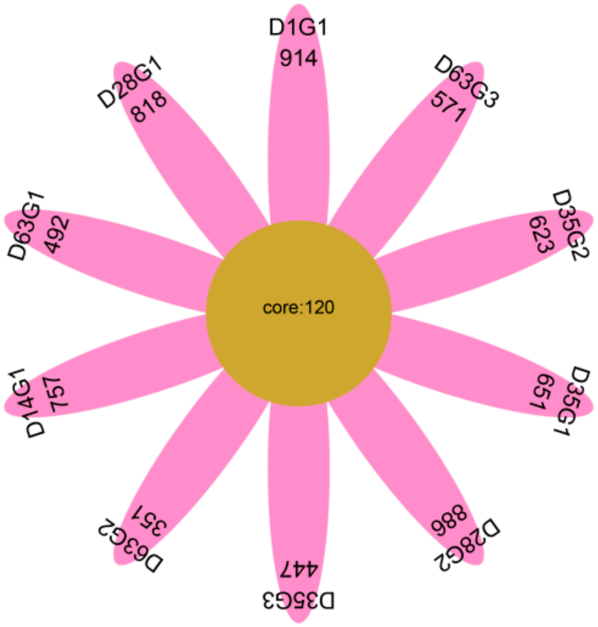

Figure 6. A Venn diagram depicting OTUs for bacterial communities in the three-compartment septic tank at various treatment durations and locations.

3.3.3. The Interaction between Environmental Factors, Microbial Community, and Typical Pathogen Content

The redundancy analysis (RDA) methodology was employed to unveil the influence of various factors throughout the entirety of the three-compartment septic tank treatment process on the abundance of typical pathogenic bacteria. Figure 7 serves as an illustrative depiction, with RDA1 and RDA2 accounting for approximately 64.86% and 14.76%, respectively, of the overall variance pertaining to typical pathogenic bacteria. Notably, the collective explanatory power of NH_3-N and VFA encompasses approximately 70% of the total variance, thereby fortifying the findings expounded upon in Sections 3.1 and 3.2. Despite the rapid decline observed in VFA levels during the preliminary stages of septic tank treatment, which mitigates the influence of the entire process, its substantial role in the sterilization process should not be disregarded. Although VFA and NH_3-N exhibit an inverse correlation, the overarching contribution of NH_3-N surpasses that of VFA by more than twofold. This phenomenon suggests that during the 0–21-day interval of G1, as VFA levels decreased, the simultaneous increase in NH_3-N concentration concurrently facilitated the eradication of typical pathogenic bacteria. The bactericidal impact of these two factors deviates from a sequential "relay race" paradigm, instead resembling a "parallel run" with divergent endpoints. Hence, it can be inferred that VFA and NH_3-N synergistically processed the potential to eliminate typical pathogenic bacteria present within fecal matter. Consequently, in future enhancements and refinements of three-compartment septic tank processes, it becomes imperative to duly acknowledge the momentous influence stemming from this collaborative synergy.

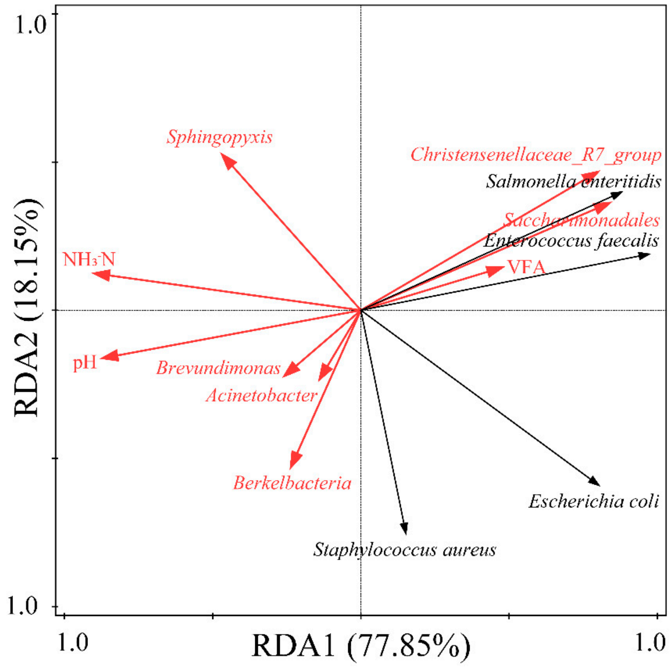

Figure 7. Redundancy analysis (RDA) of the relationship among environmental factors, microbial communities, and typical pathogenic bacteria content during three-compartment septic tank treatment.

The bacterial taxa *Christensenellaceae_R-7_group*, *Brevundimonas*, and *Saccharimonadales* significantly contributed to the variability observed in typical pathogenic bacteria, accounting for approximately 16.2%, 2.3%, and 2.0% of the total variance, respectively. *Christensenellaceae_R-7_group*, a vital group of fermentative bacteria within the intestinal microbiota of animals [64], played a pivotal role during the initial stages of hydrolysis and acidification in the three-compartment septic tank fermentation process. As the availability of organic nutrients within the system diminishes, the competition for resources intensified between *Christensenellaceae_R-7_group* and other bacterial groups, particularly in their contest with typical pathogenic bacteria. This heightened competition led to a significant reduction ($p < 0.05$) in the absolute abundance of *Enterococcus faecalis*, *Salmonella enteritidis*, and *Escherichia coli* during the period of 1–14 days. The generation of VFA and other compounds by *Christensenellaceae_R-7_group* within the system progressively deteriorated the growth environment for typical pathogenic bacteria within the three-compartment septic tank, thereby likely contributing to their substantial decline. *Brevundimonas*, a member of the *Caulobacteraceae* family, has increasingly been detected in various environmental matrices in recent years [65] and has exhibited efficacy in the removal of conventional pollutants [66]. It also demonstrates relevance in the elimination of *Enterococcus faecalis* and *Salmonella enteritidis*. *Saccharimonadales*, belonging to the phylum *Patescibacteria*, possesses a compact genome and exhibits a symbiotic lifestyle [67]. In recent investigations, *Saccharimonadales* has emerged as a significant bacterium in organic-rich sludge, exhibiting a synergistic association with genes involved in nitrogen cycling [68]. Consequently, *Saccharimonadales* may be associated with the bactericidal effects exerted by NH_3-N within the system. As an integral constituent of the three-compartment septic tank, the bacterial community assumes a critical role in the eradication of typical pathogenic bacteria. However, it is worth contemplating which dominant bacterial species can effectively achieve desirable treatment outcomes in terms of physicochemical parameters such as COD and NH_3-N while simultaneously eliminating typical pathogenic bacteria.

4. Conclusions

In this work, the rural three-compartment septic tank exhibited a certain degree of reduction in *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enteritidis*, with notable decreases in absolute abundance of approximately 2.2, 1.3, 0.03, and 1.46 orders of magnitude (copies/mL), respectively, over a 63-day period of stable operation at 15 °C. The septic tank displayed a moderate level of bactericidal efficacy against typical pathogenic bacteria. In the effluentof three-compartment septic tanks, there was a reduction of approximately 44.0% in COD, while the NH_3-N content increased by approximately 14.4%. However, overall, there was no substantial disparity in the efficacy of pathogenic bacteria removal among the three chambers of the three-compartment septic tank and there remain certain health risks associated with the effluent of septic tanks. VFA exhibited effective bactericidal properties during the initial stages of septic tank operation, while NH_3-N functioned as a bactericidal agent throughout the entire three-compartment septic tank treatment process. The decline in absolute abundance of pathogenic bacteria, such as *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella enteritidis*, as a result of ecological competition within microbial communities dominated by high-abundance groups such as *Christensenellaceae_R-7_group* and *Brevundimonas*, also played a significant role. The combined effects of VFA, NH_3-N, and microbial communities synergistically contributed to the elimination of typical pathogenic bacteria in the three-compartment septic tank.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12010087/s1, Table S1: PCR (qPCR) primers and the PCR parameters used in this study.

Author Contributions: S.C. Formal analysis, Methodology, Data curation, Visualization, Writing—original draft; S.Y. Investigation, Formal analysis; J.H. Writing—review and editing; F.L. and F.S. Conceptualization, Methodology, Resources, Investigation, Data curation, Funding acquisition, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Fundamental Research Funds for Central Public Welfare Research Institutes (2022-jcqyrw-lf).

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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