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Enrichment of Rumen Solid-Phase Bacteria for Production of Volatile Fatty Acids by Long-Term Subculturing In Vitro

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Abstract: Rumen bacteria have the ability to efficiently degrade and acidify lignocellulosic biomass, among which rumen solid-phase bacteria are more dominant. However, the effectiveness of in vitro cultured ruminal solid-phase bacteria in producing volatile fatty acids (VFA) during lignocellulosic biomass degradation remains unclear. This study presents a feasibility analysis of the long-term subculture of rumen solid-phase bacteria in vitro for VFA production. The results indicated that VFA production could reach 0.20–0.30 g/g dry matter. After 40 generations (200 days) of subculturing, the bacterial community underwent alterations. The relative abundance of certain fiber-degrading, acid-producing bacteria, which were less abundant in rumen solids, such as *Oribacterium* and *Victivallis*, was significantly upregulated following subculturing in vitro. The success of this study in subculturing rumen solid-phase bacteria in vitro over an extended period and achieving efficient VFA production is of considerable importance for the practical application of rumen microorganisms in production settings.

Keywords: rumen solid-phase bacteria; volatile fatty acids production; long-term continuous subculture; lignocellulosic biomass degradation; microbial community alterations



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1. Introduction

Lignocellulosic biomass is one of the most abundant renewable resources on Earth, with an annual global production reaching approximately 20 billion tons [1]. However, a significant portion of lignocellulosic biomass is wasted or incinerated, leading to resource depletion and environmental pollution. Utilizing lignocellulosic biomass for the production of clean biofuels and high-value chemicals presents a crucial strategy for addressing energy shortages and promoting organic waste recycling. Notably, the conversion of lignocellulosic biomass into volatile fatty acids (VFA) represents a high-value biotransformation process that has garnered considerable attention in recent years [2,3]. Lignocellulosic biomass primarily consists of cellulose, hemicellulose, and lignin, and its complex, rigid structure poses challenges for degradation. Therefore, effective treatment methods must be implemented to overcome the biological barriers between the components of lignocellulosic biomass, thereby facilitating its degradation and biotransformation [4]. Microbial anaerobic fermentation is an efficient approach for the degradation of lignocellulosic biomass. Through the synergistic action of microorganisms, lignocellulosic biomass is converted into monosaccharides, which are subsequently transformed into high-value products such as VFA and methane [5].

The rumen of ruminants is a natural fermentation system for lignocellulose, efficiently converting it into VFA that can be digested and absorbed by ruminants [6]. The degradation

of lignocellulose and the subsequent production of acids in the rumen primarily depend on the synergistic actions of the vast and diverse microbial community present. Rumen bacteria account for more than 50% of rumen microorganisms and are the most abundant microbial group in the rumen. These bacteria are capable of secreting lignocellulose hydrolases, which effectively degrade lignocellulose [7]. The contents of the rumen consist of solid and liquid phases, with the solid phase containing roughly 75% of the total rumen flora [8]. Bacteria associated with fiber degradation, such as *Ruminococcus*, *Treponema*, and *Fibrobacter*, are more prevalent in the solid phase. Gene analysis of KO and CAZymes genes has revealed that genes related to fiber degradation are more abundantly expressed in the solid phase. Consequently, rumen solid-phase bacteria dominate lignocellulose degradation [9–11].

Previous studies have demonstrated that rumen microorganisms serve as effective anaerobic fermentation inocula for the efficient degradation of lignocellulosic biomass and the production of VFA. Zhang et al. reported that the anaerobic fermentation of rice straw using rumen microorganisms resulted in a VFA production of 0.36 g/g volatile solids (VSs), with degradation rates of cellulose, hemicellulose, and lignin at 47.8%, 58.9%, and 20.6%, respectively [12]. Wang et al. indicated that fermentation using rumen fluid significantly enhanced the hydrolysis and acidification efficiency of grass scraps, achieving a VFA production of 0.38 g/g VSs, which is 1.52 times greater than that of traditional fermentation [13]. Furthermore, it has been observed that rumen bacteria can regenerate into stable communities capable of effectively degrading corn stover and producing VFA through a long-term in vitro subculture method [14]. However, no in vitro rumen fermentation studies have reported the production of acid by rumen solid-phase bacteria through the anaerobic fermentation of lignocellulosic biomass. Furthermore, it remains unclear whether solid-phase bacteria can be enriched through subculturing to form a more efficient fiber-degrading and acid-producing bacterial community. Therefore, investigating the effects of in vitro subculture on the rumen solid-phase bacterial community and its acid-producing capacity is of significant importance in advancing the practical application of rumen microorganisms.

This study aims to evaluate the feasibility of long-term subculture in vitro of rumen solid-phase bacteria and its effect on acid production. Specifically, the objectives are: (1) to measure the VFA production, pH and gas production; (2) to comprehensively explore the bacterial diversity, abundance, and community; (3) to analyze the correlation of bacteria and VFA production. The successful long-term in vitro subculture of enriched rumen solid-phase bacteria for lignocellulosic biomass degradation and acid production presents a novel approach to enhance the efficiency of lignocellulosic biomass degradation and the generation of high-value intermediates, thereby contributing to the alleviation of environmental pollution and resource scarcity.

2. Materials and Methods

2.1. Materials

Rice straw was sourced from the experimental field at the College of Agriculture, Yangzhou University, located in Yangzhou City, Jiangsu Province, China (Geographic location: 32°23'21" N, 119°25'24" E). Following harvest, the straw was dried at 65 °C, subsequently ground using a mill, and passed through a 0.42 mm sieve. It was then sealed and stored to maintain its dryness. The total solids content of the rice straw was found to be 92.03%, with cellulose, hemicellulose, and lignin comprising 34.12%, 27.36%, and 6.23% of the total solids, respectively. The total solids content was determined according to the method outlined by APHA (2005) [15], while the cellulose, hemicellulose, and lignin contents were assessed following the methodology of Van Soest et al. [16].

Four Holstein cows, each weighing 700 ± 50 kg, were selected as donor animals at the Gaoyou Experimental Farm of Yangzhou University. These cows were in their dry period and equipped with permanent rumen fistulas. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yangzhou University (SYXK(Su)2016–0019). Two hours after morning feeding, rumen contents were collected via the rumen fistula, and the liquid phase components were filtered through four layers of sterile gauze. The solid phase contents of the rumen were promptly placed in a sealed autoclavable thermos flask and immediately transported to the laboratory.

2.2. Experimental Design

In vitro subculture of rumen mixed bacteria was conducted following the methodology outlined by Lin et al. [17]. All subculturing experiments were performed on a sterile ultra-clean bench. The experimental design included three replicates, with each replicate consisting of 1.2 g of fermentation substrate accurately weighed into a 128 mL fermentation flask. Subsequently, 18 mL of mineral salt buffer was rapidly added, and CO₂ was continuously poured in to exhaust the O₂ in the bottle, with the formulation and configuration of the mineral salt buffer detailed in Table 1. Following this, 0.15 mL of a 2.5% sodium sulfide solution was introduced, the flask was sealed with a rubber stopper, and it was exposed to light until the buffer lightened in color. An additional 0.8 g of rumen solid phase content was then added to each fermentation flask, which was subsequently sealed with a butyl rubber stopper and capped with an aluminum lid. After measuring the initial air pressure in the fermentation flasks, they were placed in a constant temperature incubator set to 39 °C for static incubation. A 2 mL aliquot of the mixed fermentation broth was transferred to a new generation every 5 days, and the specific experimental procedures were as described above, resulting in a cumulative total of 40 generations.

Table 1. Mineral salt culture buffer formulations and configuration methods [18].

Items	Composition
Liquid A	Mix and dissolve CaCl ₂ ·2H ₂ O 13.2 g, MnCl ₂ ·4H ₂ O 10.0 g, CoCl ₂ ·6H ₂ O 1.0 g, and FeCl ₃ ·6H ₂ O 8.0 g in distilled water at constant volume to 100 mL
Solution B	Dissolve 4.0 g NH ₄ HCO ₃ and 35 g NaHCO ₃ in 1000 mL distilled water
Liquid C	Dissolve 5.7 g NaH ₂ PO ₄ , 6.2 g KH ₂ PO ₄ , and 0.6 g MgSO ₄ ·7H ₂ O in distilled water at constant volume to 1000 mL
Azurazine solution	0.1% (w/v)
Mineral salt incubation buffer was configured from sterilized ultrapure water 400 mL + 0.1 mL solution A + 200 mL solution B + 200 mL solution C + 1 mL azurazine solution, and CO ₂ was passed continuously for 3 h.	

At the conclusion of each fermentation generation, the fermentation bottles were removed, and the air pressure within the bottles was measured using a barometer (Cecomp Electronics, Libertyville, IL, USA). Following uncapping, the pH of the fermentation broth was assessed with a pH meter (Sartorius, Göttingen, Germany) and 2 mL of the fermentation broth was collected and centrifuged at 12,000 rpm for 10 min. Subsequently, 1 mL of the supernatant was taken and mixed with 0.2 mL of a 25% meta-phosphoric acid solution containing 60 mmol/L of crotonic acid. The mixture was then thoroughly mixed and frozen overnight at −20 °C. After thawing, the sample was centrifuged again at 12,000 rpm for 10 min, filtered through a 0.22 µm membrane, and the VFA content was determined using gas chromatography. The gases contained in the bottles at the end of the 10th, 20th, 30th,

and 40th fermentation generations were collected using a vacuum collection bag, and the concentrations of carbon dioxide (CO₂), methane (CH₄), and hydrogen (H₂) were analyzed via gas chromatography.

2.3. Microbial Sequencing Analysis

The rumen solid phase and mixed fermentation broth at the end of the 1st, 10th, 20th, 30th, and 40th generations of fermentation were selected and stored in a freezing tube at −80 °C for 16S rDNA sequencing analysis. The samples were sent to Shanghai Lingen Biotechnology Co. (Shanghai, China) Microbial DNA was extracted using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocols. Primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3') were used for PCR amplification of 16S rDNA V3~V4 region and sequencing by PE250 mode of Novaseq 6000 (Illumina, San Diego, CA, USA). The PCR amplification system was 20 µL containing 4 µL of 5 × FastPfu buffer, 2 µL of dNTPs (2.5 mM), 0.8 µL each of the upper and lower primers (5 µM), 0.4 µL of FastPfu polymerase, and 10 ng of template DNA, and the volume was replenished with ddH₂O. The PCR amplification program was 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and finally extended at 72 °C for 10 min. The PCR products were detected by 2% agarose gel electrophoresis, and the PCR products were recovered by cutting the gel using the AxyPrep DNA Gel Recovery Kit (AXYGEN) (Axygen, New York, NY, USA) and eluted with Tris-HCl. Finally, the amplified products were subjected to online library construction and sequencing analysis using the PE250 mode of the Novaseq 6000.

2.4. Statistical Analysis

All statistical analyses were performed with SPSS 27.0 software. Chi-squared tests of variance were performed first, followed by one-way ANOVA and post hoc analyses, where 0.01 < *p*-value < 0.05 indicated a significant difference, while a *p*-value < 0.01 indicated a highly significant difference.

3. Results

3.1. pH and Gas Production During In Vitro Subculturing

Figure 1a shows the changes in the pH of the fermentation broth during subculture. The pH of the fermentation broth varied between 5.40 and 5.70 during in vitro subculturing up to the 40th generation, with pH values recorded as 5.38, 5.48, 5.70, 5.53, and 5.56 for the 1st, 10th, 20th, 30th, and 40th generations, respectively.

Figure 1b illustrates the changes in gas production during subculture. Gas production decreased significantly during the first three generations, declining from 97.76 mL/g dry matter (DM) in generation 1 to 78.73 mL/g DM in generation 3. Subsequently, gas production fluctuated but exhibited an overall significant upward trend, peaking at 101.21 mL/g DM in generation 36 (*p* < 0.01). The gas productions for the 10th, 20th, 30th, and 40th generations were 87.71 mL/g DM, 82.45 mL/g DM, 93.32 mL/g DM, and 90.58 mL/g DM, respectively. Table 2 shows the composition of the gas generated in the 10th, 20th, 30th, and 40th generations. The results indicated that CO₂ constituted the highest percentage of the total gas, with the percentage of CO₂ gas production in the 10th, 20th, 30th, and 40th generations showing an increasing trend (*p* < 0.01). The gas production ratios of CH₄ were 9.81%, 7.76%, 9.80%, and 4.00% in the 10th, 20th, 30th, and 40th generations, respectively, demonstrating a pattern of decrease, followed by an increase, and then a subsequent decrease (*p* < 0.01). H₂ represented the gas with the lowest proportion of the total gas, with the gas production ratio of H₂ in the 20th generation decreasing from 0.4832% in the 10th

generation to 0.0026%, followed by a significant increase. In generation 40, the percentage of H₂ gas production peaked at 1.7413% ($p < 0.05$).

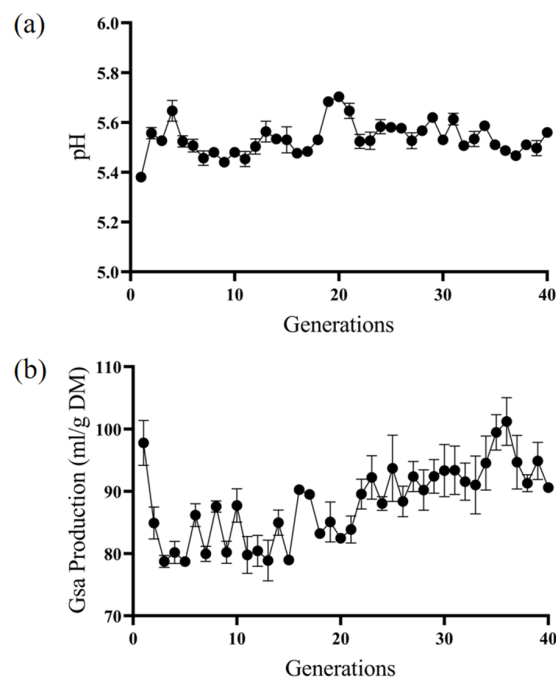


Figure 1. Curves of pH (a) and gas production (b) during in vitro subculturing.

Table 2. The 10th, 20th, 30th and 40th generation gas proportion and production.

Items	10	20	30	40	SEM	<i>p</i> -Value
Proportions (%)						
CO ₂	82.53 ^c	90.40 ^a	86.62 ^b	91.40 ^a	1.14	<0.01
CH ₄	9.81 ^a	7.76 ^b	9.80 ^a	4.00 ^c	0.73	<0.01
H ₂	0.4832 ^b	0.0026 ^b	0.8512 ^{ab}	1.7413 ^a	0.25	<0.05
Production (mL/g DM)						
CO ₂	72.35 ^b	74.54 ^b	80.84 ^a	82.79 ^a	1.33	<0.01
CH ₄	8.59 ^a	6.39 ^b	9.15 ^a	3.62 ^c	0.67	<0.01
H ₂	0.4322 ^b	0.0021 ^b	0.7851 ^{ab}	1.5762 ^a	0.22	<0.05

Note: In the provided data, distinct lowercase letters on the shoulder label signify a significant difference ($p < 0.05$). The same or no letters indicates no significant difference ($p > 0.05$).

3.2. VFA Production During In Vitro Subculturing

Figure 2 illustrates the changes in VFA production during the subculture. The production of VFA in vitro culture was 0.2138 g/g DM in the first generation, exhibiting a decreasing trend from generation to generation until the 20th generation. Notably, in the middle and late stages of long-term culture, VFA production increased significantly, peaking at 0.3015 g/g DM in the 32nd generation ($p < 0.01$). Acetic acid production was recorded at 0.1073 g/g DM in the first generation, with fluctuations observed prior to the 20th generation; however, the overall trend was downward. Following the 20th generation, acetic acid production rose significantly, reaching a peak of 0.1531 g/g DM in the 32nd generation ($p < 0.01$). Propionic acid production started at 0.0672 g/g DM in the first generation and exhibited a decline over 40 generations of subculture ($p < 0.01$). Butyric acid production, initially at 0.0278 g/g DM in the first generation, increased significantly after the 20th generation, peaking at 0.0641 g/g DM in the 36th generation ($p < 0.01$). Isobutyric and isovaleric acids were the two VFA with the lowest production levels during the

subculturing, with initial productions of 0.0015 g/g DM and 0.0044 g/g DM, respectively, in the first generation. The production of these two acids decreased steadily until the 21st generation but subsequently increased after continued culture ($p < 0.01$). Valeric acid production was 0.0041 g/g DM in the first generation, which significantly increased to 0.0148 g/g DM in the third generation, reaching a peak before experiencing a notable decline ($p < 0.01$). Hexanoic acid production began at only 0.013 g/g DM in the first generation, but significantly increased after subculture, peaking at 0.0178 g/g DM in the 29th generation. However, hexanoic acid production fluctuated and decreased after further incubation ($p < 0.01$).

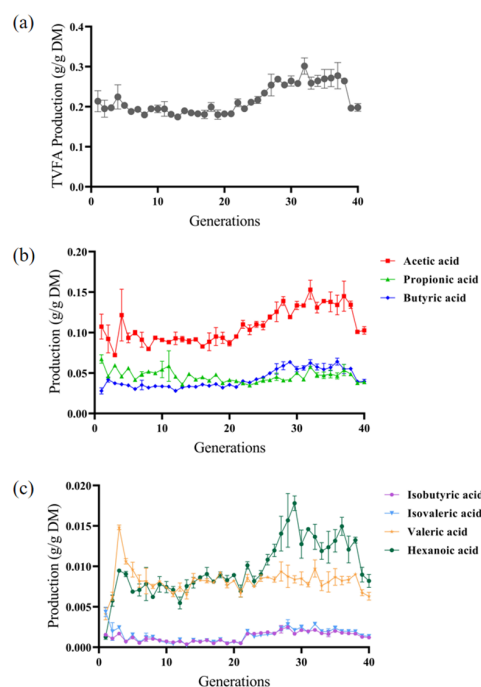


Figure 2. Variation curves of TVFA (a) as well as acetic, propionic, butyric (b), isobutyric, isovaleric, valeric, and hexanoic acid (c) production during in vitro subculturing.

3.3. Analysis of Bacterial Communities During In Vitro Subculturing

3.3.1. Analysis of Alpha and Beta Diversity

Table 3 presents the changes in the diversity and abundance of rumen solid-phase bacteria over 40 generations of subculture. The Chao 1 and ACE indices are indicative of bacterial diversity, whereas the Shannon and Simpson indices reflect bacterial abundance. The results indicate a significant decrease in both the Chao 1 and ACE indices. In contrast, the Shannon and Simpson indices exhibited a significant decline until the 10th generation, after which they increased progressively with each subsequent generation. These findings suggest that while bacterial diversity continued to diminish during subculture, bacterial abundance increased in the later stages of this process.

Table 3. Diversity and richness indices of bacteria during in vitro subculturing.

Index	0	1	10	20	30	40	SEM	p-Value
Chao 1	3305.68 ^a	3248.24 ^a	1974.42 ^b	2012.68 ^b	1394.19 ^c	999.01 ^d	212.50	<0.01
ACE	3310.05 ^a	3289.62 ^a	2079.14 ^b	2159.06 ^b	1472.94 ^c	1020.70 ^d	209.53	<0.01
Shannon	6.38 ^a	5.54 ^b	3.87 ^d	4.35 ^c	4.29 ^c	4.82 ^c	0.21	<0.01
Simpson	0.99 ^a	0.96 ^b	0.89 ^c	0.96 ^b	0.97 ^b	0.97 ^b	0.01	<0.01

Note: In the provided data, distinct lowercase letters on the shoulder label signify a significant difference ($p < 0.05$). The same or no letters indicates no significant difference ($p > 0.05$).

To visualize the differences between the rumen solid-phase bacteria and the bacterial communities of the 1st, 10th, 20th, 30th, and 40th generations, PCoA plots were generated using the Bray–Curtis distance matrix, with the percentage of variation explained by PC1 (47.8%) and PC2 (24.9%) (Figure 3a). The results indicated that the rumen solid-phase bacteria, including the 1st, 10th, 20th, and 30th generation flora, exhibited distinct distributions within their respective areas. This finding suggests significant differences in the bacterial community structure among the groups, highlighting that the bacterial community structure has undergone changes. Furthermore, the 30th and 40th generation bacterial communities were observed to be more closely aligned, indicating a stabilization of colony structure during the later stages of subculture; this finding was further supported by the NMDS analysis graphs (Figure 3b).

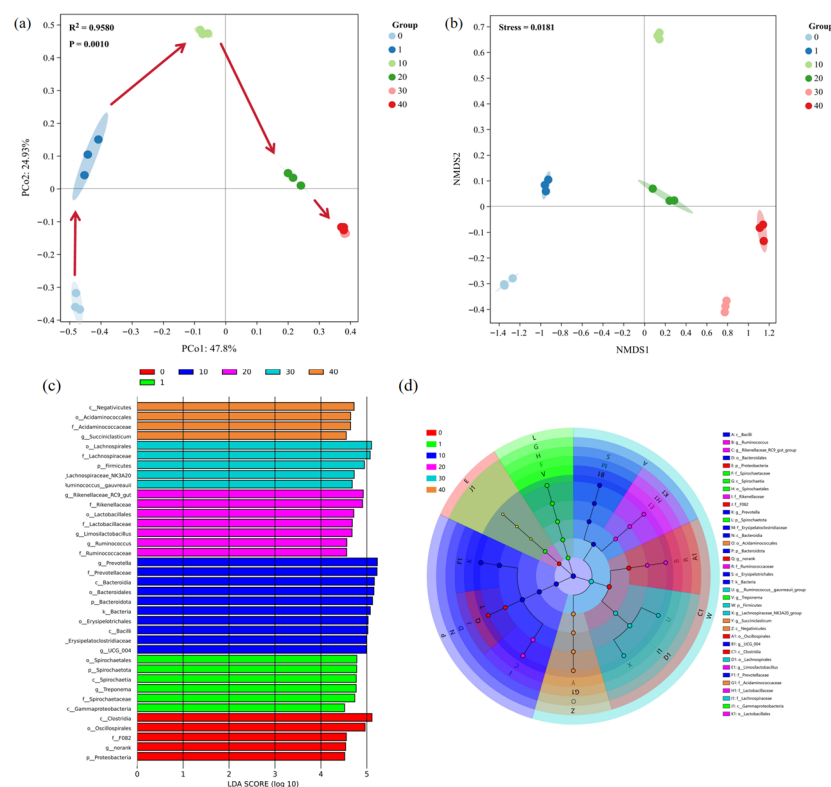


Figure 3. Beta-diversity analysis of bacteria during in vitro subculturing. (a) Principal coordinate analysis (PCoA); (b) non-metric multidimensional scaling analysis (NMDS); (c) calculation of histograms with LDA scores > 4.5 for each taxonomic unit from phylum to genus; (d) linear discriminant analysis (LeFSE).

LeFSe analyses were conducted on rumen solid-phase bacteria, as well as on bacteria from generations 1, 10, 20, 30, and 40, to evaluate the impact of intergroup differences in bacterial populations and evolutionary divergence. This analysis aimed to identify specific bacterial taxa within each group (Figure 3c,d). The results indicated that 37 bacterial taxa at various taxonomic levels exhibited LDA scores greater than 4. The bacteria showing the most significant differences in generation 1 were *Treponema*, while *Prevotella* and *UCG-004* were most notable in generation 10. In generation 20, significant differences were observed in *Rikenellaceae* RC9 gut group, *Limosilactobacillus*, and *Ruminococcus*. In the later stages of subculture, specifically in the 30th and 40th generations, the genera with the most significant differences included the *Lachnospiraceae* NK3A20 group, *Ruminococcus gawvreauui* group, and *Succiniclasticum*.

3.3.2. Changes at the Phylum Level

Table 4 shows the changes in the microbial community at the phylum level in the rumen solid phase and the 1st, 10th, 20th, 30th, and 40th generations of the subculture. Firmicutes, Bacteroidota, and Proteobacteria were identified as the three dominant phyla of rumen solid-phase bacteria. The relative abundance of these dominant phyla changed significantly with continuous subculturing. Firmicutes exhibited the highest abundance among the rumen solid-phase bacterial phyla, accounting for 47.47%. Following in vitro subculture to the 10th generation, its abundance decreased to 39.91%; however, after subculturing, the abundance of Firmicutes significantly increased, reaching a peak of 57.57% in the 30th generation ($p < 0.01$). The relative abundance of Bacteroidota, another rumen solid-phase bacterium, was initially 32.51%, increasing to 52.72% in the 10th generation, before significantly decreasing to a minimum of 26.83% in the 30th generation ($p < 0.01$). After 40 generations of subculturing, the relative abundance of Proteobacteria decreased from 8.25% to 1.80%, indicating a significant difference ($p < 0.05$). Spirochaetota initially had a relative abundance of 1.19% in rumen solids, which increased to 13.60% after one generation of in vitro culture, followed by a subsequent decrease in relative abundance ($p < 0.01$). Additionally, Desulfobacterota, Actinobacteriota, Synergistota, and Fibrobacterota demonstrated a significant increase in relative abundance after long-term in vitro subculturing ($p < 0.01$).

Table 4. Changes in bacteria at the phylum level during in vitro subculturing.

Phylum	0	1	10	20	30	40	SEM	p-Value
Firmicutes	47.47 ^{bc}	36.46 ^d	39.91 ^{cd}	50.02 ^{ab}	57.57 ^a	55.71 ^{ab}	2.07	<0.01
Bacteroidota	32.51 ^{bc}	39.19 ^b	52.72 ^a	38.83 ^b	26.83 ^d	28.96 ^d	2.22	<0.01
Proteobacteria	8.25 ^a	8.00 ^a	1.00 ^b	1.06 ^b	2.23 ^b	1.80 ^b	0.98	<0.05
Spirochaetota	1.19 ^b	13.60 ^a	2.72 ^b	2.10 ^b	1.94 ^b	1.96 ^b	1.07	<0.01
Actinobacteriota	1.44 ^b	0.43 ^b	1.23 ^b	4.82 ^a	5.00 ^a	6.65 ^a	0.60	<0.01
Desulfobacterota	1.95 ^b	0.29 ^c	0.37 ^c	0.98 ^{bc}	3.77 ^a	3.55 ^a	0.36	<0.01
Verrucomicrobiota	4.07 ^a	1.15 ^{bc}	0.07 ^e	0.85 ^{cd}	1.63 ^b	0.54 ^{de}	0.32	<0.01
Synergistota	0.32 ^{bc}	0.12 ^c	0.67 ^b	1.21 ^a	0.76 ^b	0.38 ^{bc}	0.01	<0.01
Fibrobacterota	0.13 ^{bc}	0.25 ^{bc}	1.19 ^a	0.06 ^c	0.21 ^{bc}	0.31 ^b	0.10	<0.01
Planctomycetota	1.07 ^a	0.08 ^b	0.01 ^b	0.02 ^b	0.01 ^b	0.01 ^b	0.10	<0.01

Note: In the provided data, distinct lowercase letters on the shoulder label signify a significant difference ($p < 0.05$). The same or no letters indicates no significant difference ($p > 0.05$).

3.3.3. Changes at the Genus Level

Table 5 shows the changes in the microbial community at the genus level in the rumen solid phase and the 1st, 10th, 20th, 30th, and 40th generations of the subculture. At the genus level, the top five dominant genera of rumen solid-phase bacteria were the *Rikenellaceae* RC9 gut group, *Ruminococcus*, the *Christensenellaceae* R-7 group, *Prevotella*, and *Acinetobacter*, all of which exhibited relative abundance greater than 5%. The *Rikenellaceae* RC9 gut group was the most abundant genus in the rumen solid phase, with a relative abundance of 11.03%. This relative abundance declined to 5.13% by the 10th generation, increased significantly to 22.83% in the 20th generation, and subsequently decreased again to 6.94% in the 40th generation ($p < 0.01$). Similarly, the relative abundance of *Ruminococcus*, the *Christensenellaceae* R-7 group, and *Acinetobacter* followed the same trend as the *Rikenellaceae* RC9 gut group, demonstrating a pattern of decrease, followed by an increase, and then a decrease again ($p < 0.01$). The relative abundance of *Prevotella* among rumen solid-phase microorganisms was 5.60%, which rose to 39.55% after 20 generations of subculture, making it the most abundant genus in the 20th generation. However, with continued subculturing, its relative abundance significantly decreased, falling to

7.86% in the 40th generation ($p < 0.01$). Additionally, genera such as *Succiniclasticum*, *Acidaminococcus*, *Acetitomaculum*, the *Lachnospiraceae* NK3A20 group, *Oribacterium*, the *Ruminococcus gauvreauii* group, *Syntrophococcus*, UCG-004, the *Eubacterium nodatum* group, *Solobacterium*, *Megasphaera*, *Clostridium sensu stricto* 1, *Prevotella_7*, *Bacteroides*, *Victivallis*, *Desulfovibrio*, and *Olsenella* exhibited significantly higher relative abundance after long-term in vitro subculturing ($p < 0.01$).

Table 5. Changes in bacteria at genus level during in vitro subculturing.

Phylum	Family	Genus	0	1	10	20	30	40	SEM	p-Value
Firmicutes	Ruminococcaceae	<i>Ruminococcus</i>	6.55 ^b	2.17 ^c	0.16 ^d	8.27 ^a	0.08 ^d	0.01 ^d	0.82	<0.01
	Christensenellaceae	<i>Christensenellaceae</i> R-7 group	5.97 ^a	3.20 ^b	0.71 ^d	1.63 ^c	0.08 ^e	0.09 ^e	0.51	<0.01
	Oscillospiraceae	NK4A214 group	2.15 ^a	0.70 ^{bc}	1.48 ^{ab}	0.76 ^{bc}	0.37 ^c	0.58 ^c	0.18	<0.01
		UCG-002	1.79 ^a	0.27 ^b	0.04 ^c	0.06 ^c	0.00 ^c	0.00 ^c	0.16	<0.01
	Acidaminococcaceae	<i>Succiniclasticum</i>	2.14 ^b	2.06 ^b	1.43 ^b	7.08 ^a	7.15 ^a	9.75 ^a	0.83	<0.01
		<i>Acidaminococcus</i>	0.00 ^d	0.02 ^d	0.08 ^{cd}	0.41 ^c	1.47 ^b	2.43 ^a	0.26	<0.01
	Hungateiclostridiaceae	<i>Saccharofermentans</i>	1.21 ^b	1.52 ^{ab}	0.07 ^c	1.79 ^a	0.03 ^c	0.00 ^c	0.19	<0.01
		<i>Eubacterium</i> <i>ruminantium</i> group	1.12 ^b	2.35 ^a	0.06 ^c	0.26 ^c	0.03 ^c	0.00 ^c	0.22	<0.01
	Lachnospiraceae	<i>Acetitomaculum</i>	0.79 ^c	0.10 ^d	0.10 ^d	0.58 ^{cd}	3.18 ^b	3.82 ^a	0.37	<0.01
		<i>Lachnospiraceae</i> NK3A20 group	0.74 ^{cd}	0.47 ^d	0.43 ^d	1.74 ^c	12.22 ^a	9.40 ^b	0.17	<0.01
		<i>Butyrivibrio</i>	0.55 ^c	1.28 ^a	0.97 ^b	0.30 ^{cd}	0.04 ^d	0.00 ^d	0.12	<0.01
		<i>Lachnospiraceae</i> NK4A136 group	0.25 ^b	1.25 ^a	1.30 ^a	0.16 ^b	0.05 ^b	0.01 ^b	0.14	<0.01
		<i>Oribacterium</i>	0.24 ^c	1.70 ^b	0.34 ^c	0.17 ^c	3.33 ^a	2.06 ^b	0.29	<0.01
		probable genus 10	0.19 ^c	1.30 ^a	0.04 ^c	0.68 ^b	0.02 ^c	0.00 ^c	0.12	<0.01
		<i>Lachnospiraceae</i> FCS020 group	0.17 ^c	1.29 ^a	0.05 ^c	0.64 ^b	0.04 ^c	0.01 ^c	0.12	<0.01
		<i>Ruminococcus</i> <i>gauvreauii</i> group	0.13 ^c	0.11 ^c	0.30 ^c	0.62 ^c	10.20 ^a	7.53 ^b	1.00	<0.01
		<i>Syntrophococcus</i>	0.06 ^c	0.06 ^c	0.30 ^c	0.57 ^b	2.71 ^a	2.84 ^a	0.29	<0.01
	Streptococcaceae	<i>Streptococcus</i>	0.36 ^b	4.48 ^a	0.13 ^b	0.31 ^b	0.04 ^b	0.00 ^b	0.39	<0.01
		<i>Erysipelotrichaceae</i> UCG-009	0.15 ^b	0.08 ^b	1.03 ^a	1.50 ^a	0.10 ^b	0.11 ^b	0.15	<0.01
	Erysipelotrichaceae	UCG-004	0.12 ^c	0.29 ^c	20.67 ^a	0.85 ^c	3.86 ^b	3.60 ^b	1.77	<0.01
		<i>Eubacterium</i> <i>nodatum</i> group	0.09 ^d	0.04 ^d	0.12 ^d	0.51 ^c	0.92 ^b	1.27 ^a	0.11	<0.01
	Lactobacillaceae	<i>Limosilactobacillus</i>	0.08 ^b	0.05 ^b	0.73 ^b	10.40 ^a	0.10 ^b	0.04 ^b	0.93	<0.01
	Erysipelotrichaceae	<i>Solobacterium</i>	0.08 ^c	0.03 ^c	0.15 ^c	1.74 ^b	1.51 ^b	2.16 ^a	0.22	<0.01
	Veillonellaceae	<i>Megasphaera</i>	0.03 ^d	0.65 ^b	0.03 ^d	0.30 ^c	0.71 ^b	1.23 ^a	0.11	<0.01
	Clostridiaceae	<i>Clostridium sensu</i> <i>stricto</i> 1	0.03 ^b	0.07 ^b	1.26 ^b	1.59 ^b	5.32 ^a	4.83 ^a	0.54	<0.01
Bacteroidota	Rikenellaceae	<i>Rikenellaceae</i> RC9 gut group	11.03 ^b	6.00 ^d	5.13 ^d	22.83 ^a	8.77 ^{bc}	6.94 ^{cd}	1.48	<0.01
	Prevotellaceae	<i>Prevotella</i>	5.60 ^c	25.84 ^b	39.55 ^a	8.27 ^c	4.27 ^c	7.86 ^c	3.25	<0.01
		<i>Prevotella_7</i>	0.03 ^d	0.18 ^d	4.03 ^a	0.46 ^d	2.42 ^b	1.63 ^c	0.35	<0.01
	Bacteroidaceae	<i>Bacteroides</i>	0.05 ^c	0.12 ^c	1.39 ^b	1.27 ^b	4.55 ^a	4.59 ^a	0.47	<0.01
Proteobacteria	Moraxellaceae	<i>Acinetobacter</i>	5.18 ^a	0.14 ^b	0.00 ^b	0.04 ^b	0.00 ^b	0.01 ^b	0.48	<0.01
	Succinivibrionaceae	<i>Succinivibrio</i>	0.52 ^b	1.46 ^a	0.06 ^b	0.05 ^b	0.01 ^b	0.01 ^b	0.15	<0.01
		<i>Ruminobacter</i>	0.44 ^b	5.46 ^a	0.13 ^b	0.16 ^b	0.01 ^b	0.00 ^b	0.71	0.15
Verrucomicrobiota	Victivallaceae	<i>Victivallis</i>	0.04 ^b	0.02 ^b	0.02 ^b	0.02 ^b	1.55 ^a	0.27 ^b	0.14	<0.01
Desulfobacterota	Desulfovibrionaceae	<i>Desulfovibrio</i>	1.82 ^b	0.22 ^c	0.36 ^c	0.97 ^{bc}	3.77 ^a	3.55 ^a	0.37	<0.01
Actinobacteriota	Atopobiaceae	<i>Olsenella</i>	0.12 ^c	0.13 ^c	1.01 ^c	3.24 ^b	3.24 ^b	4.98 ^a	0.47	<0.01
Spirochaetota	Spirochaetaceae	<i>Treponema</i>	1.02 ^c	13.59 ^a	2.68 ^b	2.09 ^{bc}	1.94 ^{bc}	1.95 ^{bc}	1.07	<0.01
Synergistota	Synergistaceae	<i>Pyramidobacter</i>	0.08 ^c	0.11 ^{bc}	0.50 ^b	1.04 ^a	0.34 ^{bc}	0.18 ^{bc}	0.09	<0.01
Fibrobacterota	Fibrobacteraceae	<i>Fibrobacter</i>	0.13 ^{bc}	0.25 ^{bc}	1.19 ^a	0.06 ^c	0.21 ^{bc}	0.31 ^b	0.10	<0.01
Tenericutes	Anaeroplasmataceae	<i>Anaeroplasma</i>	0.04 ^c	0.76 ^b	1.91 ^a	0.20 ^c	0.18 ^c	0.30 ^c	0.16	<0.01

Note: In the provided data, distinct lowercase letters on the shoulder label signify a significant difference ($p < 0.05$). The same or no letters indicates no significant difference ($p > 0.05$).

3.3.4. Effect of Bacterial Communities on VFA Production

Figure 4 illustrates the relationship between 39 dominant bacteria and the production of VFA across generations 1, 10, 20, 30, and 40. The production of VFA exhibited a significant positive correlation with *Oribacterium* and *Victivallis*, while showing a negative correlation with *Erysipelotrichaceae* UCG_009, the NK4A214 group, and *Ruminococcus*. Notably, *Oribacterium*, *Victivallis*, and *Megasphaera* were significantly and positively correlated with the productions of acetic, isobutyric, and isovaleric acids. Additionally, propionic acid production was positively associated with *Butyrivibrio*, *Ruminobacter*, UCG_002, the *Eubacterium ruminantium* group, *Treponema*, *Streptococcus*, *Lachnospiraceae* NK4A136 group, the *Christensenellaceae* R_7 group, probable genus 10, and *Lachnospiraceae* FCS020 group. The production of butyric and hexanoic acids was similarly correlated with the relative abundance of *Succiniclasticum*, *Desulfovibrio*, *Acetitomaculum*, the *Lachnospiraceae* NK3A20 group, the *Ruminococcus gauvreauii* group, *Olsenella*, the *Eubacterium nodatum* group, *Solobacterium*, *Syntrophococcus*, *Bacteroides*, *Victivallis*, *Clostridium sensu stricto* 1, *Acidaminococcus*, and the *Rikenellaceae* RC9 gut group. Furthermore, valeric acid production was significantly and positively correlated with *Pyramidobacter*, the *Rikenellaceae* RC9 gut group, the *Ruminococcus gauvreauii* group, and *Limosilactobacillus*.

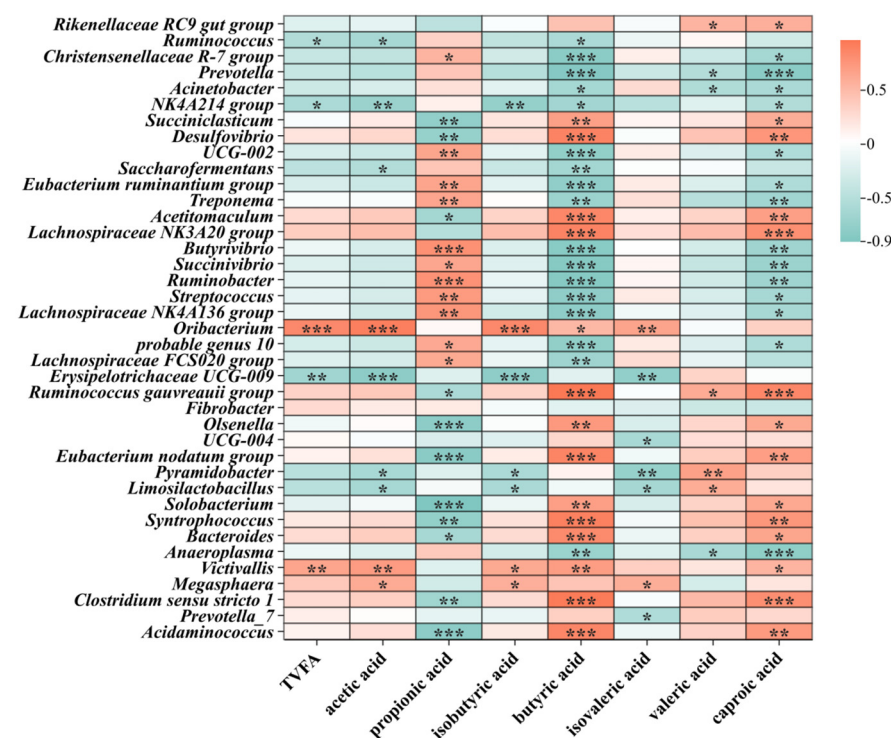


Figure 4. Heatmap of environmental factors and bacteria based on Spearman rank correlations at the genus level (red indicates positive correlations, blue indicates negative correlations; $0.01 < p < 0.05$, $0.001 < p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively).

4. Discussion

In this study, a long-term in vitro subculturing of rumen solid-phase bacteria was carried out to investigate the feasibility of anaerobic fermentation of rice straw for the production of volatile fatty acids (VFA), and long-term stable fermentation was successfully achieved for 40 generations (200 days). It was found that the VFA production was about 0.20 g/g DM in the early and middle stages of in vitro subculturing, which showed a slightly decreasing trend. However, with the continuation of the subculture, the VFA production increased significantly in the late stage of the experiment, and the peak value

reached 0.3015 g/g DM. It maintained the high VFA production for a longer period. The production of VFA in the present study was significantly higher than in previous studies using rumen bacteria as inoculum: Nguyen et al. used a rumen membrane bioreactor and produced VFA stably for only 35 days at 1% substrate loading; Jin et al. produced VFA stably for 100 days at 3% substrate loading, but their productions were much lower than the present study; Liang et al. conducted a long-term (120 days) *in vitro* fermentation experiment of corn stover using rumen bacteria and the VFA production was stable at 0.213–0.339 g/g DM, which was similar to the results of the present study [14,19,20]. The present study showed a decreasing trend of VFA production during the first and middle stages, and the studies of Jin et al., Liang et al., and Nguyen et al. also found a significant decrease in VFA production during the pre-anaerobic fermentation period [14,19,20]. Ozbayram et al. proposed that differences in microbial communities are the main cause of differences in fermentation products [21]. Therefore, we hypothesized that in the early stages of subculture, rumen solid-phase bacteria did not adapt quickly to the *in vitro* culture environment, and the bacterial community was altered, resulting in lower VFA production. As subculturing progressed, some fiber-degrading acid-producing bacteria gradually adapted to the *in vitro* culture environment and began to enrich, and VFA production increased significantly.

The VFA produced in this study were mainly composed of acetic acid, propionic acid, and butyric acid, which accounted for 36.57–53.71%, 16.38–31.68%, and 13.12–30.17% of the total VFA production, respectively. The production of acetic acid and butyric acid increased significantly after the 20th generation, similar to the trend of total VFA production. However, the production of propionic acid continued to decrease over the generations. Studies by Jin et al. and Nguyen et al. reported fluctuations in the production of acetic acid, propionic acid, and butyric acid during long-term fermentation, and a study by Liang et al. found significant increases in the production of acetic acid and butyric acid and fluctuations in the production of propionic acid during long-term *in vitro* incubation, which is similar to the results of this study [14,19,20]. In addition, in this study, we found that hexanoic acid production increased significantly after long-term *in vitro* culture, from the lowest value of 0.0013 g/g DM in the 1st generation to the highest value of 0.0178 g/g DM in the 29th generation, which was an increase of 1369.23%. Hexanoic acid is a high-value volatile fatty acid that serves as an important metabolic product in anaerobic fermentation and possesses significant energy and economic value. No study has reported the trend of hexanoic acid production during long-term *in vitro* culture, so we hypothesized that the acetic, butyric, and hexanoic acid-producing bacteria in the rumen solid-phase bacteria were better adapted to the *in vitro* culture environment compared with the other acid-producing bacteria.

Fiber-degrading acid-producing bacterial colonies are key drivers of VFA production by anaerobic fermentation of lignocellulose. Therefore, this study analyzed the changes in the colony structure of rumen solid-phase bacteria during long-term subculture *in vitro* to investigate the reasons for the increase in VFA production in mid- to late subculture. In this study, we found that the diversity index of solid-phase bacteria decreased from generation to generation, similar to the results of Jin et al. and Nguyen et al. They hypothesized that some rumen bacteria could not adapt to the *in vitro* culture environment and disappeared after long-term subculture *in vitro*, resulting in a decrease in bacterial diversity [19,20]. The enrichment index of the results of this study decreased until the 10th generation and then increased significantly, indicating that at the beginning of the transmission, most bacteria could not adapt to the *in vitro* culture environment and decreased in abundance. As the subculture continued, some bacteria gradually adapted to the *in vitro* culture environment and enrichment occurred. The community structure of fiber-degrading acid-producing

bacteria determines the effect of anaerobic fermentation [13]. Therefore, we hypothesized that although the solid-phase bacterial community structure was altered by long-term subculture in vitro, some fiber-degrading acid-producing bacteria (especially those producing acetic, butyric, and hexanoic acids) gradually adapted to the in vitro culture environment and enrichment occurred, resulting in a significant increase in the production of VFA.

To demonstrate whether the enrichment of some fiber-degrading acid-producing bacteria occurred through subcultures, the present study analyzed the succession of solid-phase bacterial community structure at the phylum level. At the phylum level, Firmicutes and Bacteroidota were the two phyla with the highest abundance during long-term subcultures in vitro, accounting for about 75.65% to 88.85% of the total bacteria. Firmicutes and Bacteroidota contained a variety of bacteria capable of degrading lignocellulose and producing acids, such as *Prevotella*, *Ruminococcus*, *Butyrivibrio*, and *Bacteroides*. These bacteria can be rapidly enriched in environments with high levels of soluble organic matter, degrading cellulose and hemicellulose into sugars, which are then converted into VFA [22]. Analysis of rumen solids and colony structure of the 1st, 10th, 20th, 30th, and 40th generations revealed that Firmicutes, Actinobacteria, Desulfobacterota, Synergistota, and Fibrobacterota in the dominant phylum significantly increased in abundance during the middle to late stages of subculture, and this trend was similar to the change in VFA production. We speculate that some fiber-degrading bacteria within these phyla gradually adapted to the in vitro culture environment and were enriched by subculturing.

Therefore, the present study further analyzed the succession of solid-phase bacterial community structure at the genus level. It was found that the *Rikenellaceae* RC9 gut group, *Ruminococcus*, the *Christensenellaceae* R-7 group, *Prevotella*, *Acinetobacter*, the NK4A214 group, *Succinivibrionaceae*, *Desulfovibrio*, UCG-002, and *Saccharofermentans* were the top ten dominant bacteria in terms of abundance. Most of the above genera are core genera in the rumen of ruminants and have an important impact on lignocellulose degradation and VFA production [23]. Most of the dominant genera in Firmicutes and Bacteroidota were subjected to subcultures and their abundance showed varying degrees of increase. These genera mainly belong to the family Acidaminococcaceae, Lachnospiraceae, Prevotellaceae, Bacteroidaceae, and Clostridiaceae, all of which have been repeatedly reported to be associated with lignocellulose degradation and capable of producing VFA such as acetic acid and propionic acid [24–28]. The genera *Victivallis*, *Desulfovibrio*, *Olsenella*, *Treponema*, *Pyramidobacter*, and *Fibrobacter* exhibited a significant increase in abundance during the later stages of subculture, which corresponds closely to the phyla that were found to be up-regulated in abundance. Based on the research findings, it is speculated that rumen solid-phase bacteria associated with lignocellulose degradation functions struggle to adapt rapidly to in vitro culture environments. This struggle leads to a decrease in their relative abundance, subsequently causing a reduction in VFA production. However, through long-term culturing, some bacteria gradually adapt to the in vitro environment, ultimately forming a bacterial community capable of degrading cellulose and efficiently producing VFA.

To investigate the extent to which bacteria enriched by subculturing contribute to the production of VFA, the present study was conducted to correlate the succession of colony structure with changes in the production of VFA in subculture. The results showed that the production of VFA was significantly and positively correlated with *Oribacterium* and *Victivallis*. *Oribacterium* belongs to the Lachnospiraceae of the Firmicutes. Previous studies have shown that *Oribacterium* can ferment glucose, galactose, and sucrose to produce acetic and lactic acids, but cannot be maintained in vitro for more than 2 weeks [29]. *Victivallis* belongs to the Victivallaceae of the Verrucomicrobiota. It was found that *Victivallis* can be cultured anaerobically in vitro in the liquid medium, with optimal growth conditions of a temperature of 37 °C and pH of 5.0–7.5. *Victivallis* is able to ferment a variety of sugars to

produce acetic acid, ethanol, and H₂ [30]. In the present study, long-term in vitro culture of *Oribacterium* and *Victivallis* was achieved and significantly contributed to the upregulation of the productions of acetic, isobutyric, butyric, isovaleric, and hexanoic acids. Therefore, it is reasonable to hypothesize that *Oribacterium* and *Victivallis* are better adapted to long-term in vitro subcultures than other solid-phase bacteria and contribute significantly to the increase in the production of VFA.

The pH is a crucial factor influencing anaerobic fermentation. Extremely high or low pH levels can inhibit bacterial activity and the secretion of fiber-degrading enzymes, which subsequently affects the hydrolysis of lignocellulose and acid production. Therefore, maintaining an appropriate pH is beneficial for stable and efficient anaerobic fermentation [31]. In this study, the pH varied between 5.40 and 5.70. It was found that the suitable pH range for rumen bacteria is 6.0–6.8, which helps to maintain efficient microbial homeostasis in vivo. However, there are some differences between in vivo and in vitro anaerobic fermentation. VFA produced by in vivo fermentation can be taken up and transported by rumen epithelial cells, whereas VFA produced by in vitro fermentation accumulate, leading to a decrease in pH [32]. Therefore, the low pH of the in vitro fermentation in this study may be due to the accumulation of VFA. It was found that the optimum pH of most lignocellulose-degrading enzymes was around 5.0–6.5, so the pH in this study provided a suitable environment for fiber-degrading bacteria, which was conducive to achieving lignocellulose degradation and acid production [33].

During the anaerobic fermentation process, H₂ production is closely linked to the composition of the rumen microbial community. Rumen bacteria generate H₂ while degrading complex carbohydrates, such as lignocellulose, and the metabolic activities of certain fiber-degrading bacteria, such as *Ruminococcus*, also contribute to H₂ production. Consequently, an increase in H₂ production may indicate a rise in the relative abundance or enhanced activity of these fiber-degrading bacteria [34]. In this study, H₂ production significantly increased during the middle and late stages of subculture, suggesting that certain fiber-degrading bacteria were enriched, the fiber degradation process became more active, and fermentation efficiency improved. The production of CH₄ from the gas components is typically considered the final stage of anaerobic fermentation. In fermentation systems, methanogenic bacteria utilize intermediates such as VFA, hydrogen, etc. to produce CH₄ [35]. In this study, it was found that none of the methanogenic bacteria such as *Methanobacterium*, *Methanospirillum*, and *Methanosarcina* reached the relative peak of the dominant bacteria during long-term subculture, which indicates that methanogenic bacteria maybe cannot adapt to the environment of in vitro cultivation. Maximizing the production of VFA requires not only optimizing the production method but also minimizing its consumption. The results of this study suggest that long-term in vitro subculture of rumen solid-phase bacteria not only enriches some key fiber-degrading acid-producing bacteria but also reduces the abundance of methanogenic bacteria, thus ensuring high production of VFA during anaerobic fermentation.

5. Conclusions

The conversion of lignocellulosic biomass into renewable energy utilizing rumen microorganisms exhibits significant performance and promising application prospects. In this study, rumen solid-phase bacteria were subjected to 40 generations (200 days) of continuous long-term subculturing in an in vitro environment. Initially, the production of volatile fatty acids (VFA) decreased; however, as the experiment progressed, VFA production significantly increased during the middle and late stages of culturing, resulting in efficient acid production from lignocellulose degradation. Given that the in vitro environment differs from the native in vivo habitat of these bacteria, the adaptation of solid-phase

bacteria to in vitro conditions resulted in alterations to their colony structure. Nevertheless, after extended subculturing, the bacteria re-established a relatively stable and efficient community structure. Within this structure, fiber-degrading, acid-producing bacteria such as *Oribacterium* and *Victivallis* demonstrated enhanced adaptation to the in vitro culture environment, significantly contributing to the increased production of VFA. The findings of this study confirm the feasibility of the long-term subculture of rumen solid-phase bacteria in vitro, while also providing a novel approach to enhance the production of economically valuable intermediate products through the anaerobic fermentation of straw.

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References

1. Nguyen, L.N.; Nguyen, A.Q.; Johir, M.A.H.; Guo, W.; Ngo, H.H.; Chaves, A.V.; Nghiem, L.D. Application of Rumen and Anaerobic Sludge Microbes for Bio Harvesting from Lignocellulosic Biomass. *Chemosphere* **2019**, *228*, 702–708. [[CrossRef](#)] [[PubMed](#)]
2. Dahiya, S.; Rapoport, A.; Singh, B. Biotechnological Potential of Lignocellulosic Biomass as Substrates for Fungal Xylanases and Its Bioconversion into Useful Products: A Review. *Fermentation* **2024**, *10*, 82. [[CrossRef](#)]
3. Rashmi, R.; Tripathi, T.; Pandey, S.; Kumar, S. Transforming Lignocellulosic Biomass into Biofuels: Recent Innovations in Pretreatment and Bioconversion Techniques. *Int. J. Res. Appl. Sci. Eng. Technol.* **2024**, *12*, 1079–1089. [[CrossRef](#)]
4. Yang, X.; Zhang, Y.; Sun, P.; Peng, C. A Review on Renewable Energy: Conversion and Utilization of Biomass. *Smart Mol.* **2024**, *2*, e20240019. [[CrossRef](#)]
5. Dong, W.; Yang, Y.; Liu, C.; Zhang, J.; Pan, J.; Luo, L.; Wu, G.; Awasthi, M.K.; Yan, B. Caproic Acid Production from Anaerobic Fermentation of Organic Waste—Pathways and Microbial Perspective. *Renew. Sustain. Energy Rev.* **2023**, *175*, 113181. [[CrossRef](#)]
6. Bhujbal, S.K.; Ghosh, P.; Vijay, V.K.; Rathour, R.; Kumar, M.; Singh, L.; Kapley, A. Biotechnological Potential of Rumen Microbiota for Sustainable Bioconversion of Lignocellulosic Waste to Biofuels and Value-Added Products. *Sci. Total Environ.* **2022**, *814*, 152773. [[CrossRef](#)]
7. Liang, J.; Zhang, P.; Zhang, R.; Chang, J.; Chen, L.; Wang, G.; Tian, Y.; Zhang, G. Response of Rumen Microorganisms to pH during Anaerobic Hydrolysis and Acidogenesis of Lignocellulose Biomass. *Waste Manag.* **2024**, *174*, 476–486. [[CrossRef](#)]
8. Zhao, W.; Abdelsattar, M.M.; Wang, X.; Zhang, N.; Chai, J. In Vitro Modulation of Rumen Fermentation by Microbiota from the Recombination of Rumen Fluid and Solid Phases. *Microbiol. Spectr.* **2023**, *11*, e03387-22. [[CrossRef](#)]
9. Bowen, J.M.; McCabe, M.S.; Lister, S.J.; Cormican, P.; Dewhurst, R.J. Evaluation of Microbial Communities Associated with the Liquid and Solid Phases of the Rumen of Cattle Offered a Diet of Perennial Ryegrass or White Clover. *Front. Microbiol.* **2018**, *9*, 2389. [[CrossRef](#)]
10. Su, M.; Hao, Z.; Shi, H.; Li, T.; Wang, H.; Li, Q.; Zhang, Y.; Ma, Y. Metagenomic Analysis Revealed Differences in Composition and Function Between Liquid-Associated and Solid-Associated Microorganisms of Sheep Rumen. *Front. Microbiol.* **2022**, *13*, 851567. [[CrossRef](#)]
11. Wang, P.; Qi, M.; Barboza, P.; Leigh, M.B.; Ungerfeld, E.; Selinger, L.B.; McAllister, T.A.; Forster, R.J. Isolation of High-Quality Total RNA from Rumen Anaerobic Bacteria and Fungi, and Subsequent Detection of Glycoside Hydrolases. *Can. J. Microbiol.* **2011**, *57*, 590–598. [[CrossRef](#)] [[PubMed](#)]

12. Zhang, H.; Zhang, P.; Ye, J.; Wu, Y.; Fang, W.; Gou, X.; Zeng, G. Improvement of Methane Production from Rice Straw with Rumen Fluid Pretreatment: A Feasibility Study. *Int. Biodeterior. Biodegrad.* **2016**, *113*, 9–16. [[CrossRef](#)]
13. Wang, S.; Zhang, G.; Zhang, P.; Ma, X.; Li, F.; Zhang, H.; Tao, X.; Ye, J.; Nabi, M. Rumen Fluid Fermentation for Enhancement of Hydrolysis and Acidification of Grass Clipping. *J. Environ. Manag.* **2018**, *220*, 142–148. [[CrossRef](#)] [[PubMed](#)]
14. Liang, J.; Fang, W.; Chang, J.; Zhang, G.; Ma, W.; Nabi, M.; Zubair, M.; Zhang, R.; Chen, L.; Huang, J.; et al. Long-Term Rumen Microorganism Fermentation of Corn Stover In Vitro for Volatile Fatty Acid Production. *Bioresour. Technol.* **2022**, *358*, 127447. [[CrossRef](#)]
15. American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, USA, 2005; 1220p.
16. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy. Sci.* **1991**, *74*, 3583–3597. [[CrossRef](#)]
17. Lin, M.; Dai, X.; Weimer, P.J. Shifts in Fermentation End Products and Bacterial Community Composition in Long-Term, Sequentially Transferred In Vitro Ruminant Enrichment Cultures Fed Switchgrass with and without Ethanol as a Co-Substrate. *Bioresour. Technol.* **2019**, *285*, 121324. [[CrossRef](#)]
18. Menke, K.H.; Raab, L.; Salewski, A.; Steingass, H.; Fritz, D.; Schneider, W. The Estimation of the Digestibility and Metabolizable Energy Content of Ruminant Feedingstuffs from the Gas Production When They Are Incubated with Rumen Liquor In Vitro. *J. Agric. Sci.* **1979**, *93*, 217–222. [[CrossRef](#)]
19. Nguyen, A.Q.; Nguyen, L.N.; Johir, M.A.H.; Ngo, H.-H.; Chaves, A.V.; Nghiem, L.D. Derivation of Volatile Fatty Acid from Crop Residues Digestion Using a Rumen Membrane Bioreactor: A Feasibility Study. *Bioresour. Technol.* **2020**, *312*, 123571. [[CrossRef](#)]
20. Jin, W.; Xu, X.; Yang, F. Application of Rumen Microorganisms for Enhancing Biogas Production of Corn Straw and Livestock Manure in a Pilot-Scale Anaerobic Digestion System: Performance and Microbial Community Analysis. *Energies* **2018**, *11*, 920. [[CrossRef](#)]
21. Ozbayram, E.G.; Kleinstuber, S.; Nikolausz, M.; Ince, B.; Ince, O. Effect of Bioaugmentation by Cellulolytic Bacteria Enriched from Sheep Rumen on Methane Production from Wheat Straw. *Anaerobe* **2017**, *46*, 122–130. [[CrossRef](#)]
22. Moraïs, S.; Mizrahi, I. Islands in the Stream: From Individual to Communal Fiber Degradation in the Rumen Ecosystem. *FEMS Microbiol. Rev.* **2019**, *43*, 362–379. [[CrossRef](#)] [[PubMed](#)]
23. Huws, S.A.; Edwards, J.E.; Lin, W.; Rubino, F.; Alston, M.; Swarbreck, D.; Caim, S.; Stevens, P.R.; Pachebat, J.; Won, M.-Y.; et al. Microbiomes Attached to Fresh Perennial Ryegrass Are Temporally Resilient and Adapt to Changing Ecological Niches. *Microbiome* **2021**, *9*, 143. [[CrossRef](#)]
24. Accetto, T.; Avguštin, G. Non-Oral Prevotella Stepping into the Spotlight. *Anaerobe* **2021**, *68*, 102321. [[CrossRef](#)] [[PubMed](#)]
25. Ahmad, A.A.; Yang, C.; Zhang, J.; Kalwar, Q.; Liang, Z.; Li, C.; Du, M.; Yan, P.; Long, R.; Han, J.; et al. Effects of Dietary Energy Levels on Rumen Fermentation, Microbial Diversity, and Feed Efficiency of Yaks (*Bos grunniens*). *Front. Microbiol.* **2020**, *11*, 625. [[CrossRef](#)]
26. Gharechahi, J.; Vahidi, M.F.; Bahram, M.; Han, J.-L.; Ding, X.-Z.; Salekdeh, G.H. Metagenomic Analysis Reveals a Dynamic Microbiome with Diversified Adaptive Functions to Utilize High Lignocellulosic Forages in the Cattle Rumen. *ISME J.* **2021**, *15*, 1108–1120. [[CrossRef](#)]
27. Ozbayram, E.G.; Kleinstuber, S.; Nikolausz, M. Biotechnological Utilization of Animal Gut Microbiota for Valorization of Lignocellulosic Biomass. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 489–508. [[CrossRef](#)]
28. Won, M.-Y.; Oyama, L.B.; Courtney, S.J.; Creevey, C.J.; Huws, S.A. Can Rumen Bacteria Communicate to Each Other? *Microbiome* **2020**, *8*, 23. [[CrossRef](#)]
29. Sizova, M.V.; Muller, P.A.; Stancyk, D.; Panikov, N.S.; Mandalakis, M.; Hazen, A.; Hohmann, T.; Doerfert, S.N.; Fowle, W.; Earl, A.M.; et al. *Oribacterium parvum* sp. Nov. and *Oribacterium asaccharolyticum* sp. Nov., Obligately Anaerobic Bacteria from the Human Oral Cavity, and Emended Description of the Genus *Oribacterium*. *Int. J. Syst. Evol. Microbiol.* **2014**, *64* Pt 8, 2642–2649. [[CrossRef](#)]
30. Zoetendal, E.G.; Plugge, C.M.; Akkermans, A.D.L.; De Vos, W.M. *Victivallis vadensis* Gen. Nov., sp. Nov., a Sugar-Fermenting Anaerobe from Human Faeces. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 211–215. [[CrossRef](#)]
31. Zhang, L.; Chung, J.; Jiang, Q.; Sun, R.; Zhang, J.; Zhong, Y.; Ren, N. Characteristics of Rumen Microorganisms Involved in Anaerobic Degradation of Cellulose at Various pH Values. *RSC Adv.* **2017**, *7*, 40303–40310. [[CrossRef](#)]
32. Liang, J.; Nabi, M.; Zhang, P.; Zhang, G.; Cai, Y.; Wang, Q.; Zhou, Z.; Ding, Y. Promising Biological Conversion of Lignocellulosic Biomass to Renewable Energy with Rumen Microorganisms: A Comprehensive Review. *Renew. Sustain. Energy Rev.* **2020**, *134*, 110335. [[CrossRef](#)]
33. Meng, Y.; Mumme, J.; Xu, H.; Wang, K. A Biologically Inspired Variable-pH Strategy for Enhancing Short-Chain Fatty Acids (SCFAs) Accumulation in Maize Straw Fermentation. *Bioresour. Technol.* **2016**, *201*, 329–336. [[CrossRef](#)] [[PubMed](#)]

34. Ma, H.; Dong, A.; Xu, Y.; Wu, Q.; Lambo, M.T.; Zhang, Y.; Dou, X.; Li, Y. Regulatory Effects of High Concentrate Diet Synergistically Fermented with Cellulase and Lactic Acid Bacteria: In Vitro Ruminal Fermentation, Methane Production, and Rumen Microbiome. *Anim. Feed. Sci. Technol.* **2025**, *319*, 116194. [[CrossRef](#)]
35. Wainaina, S.; Lukitawesa; Kumar Awasthi, M.; Taherzadeh, M.J. Bioengineering of Anaerobic Digestion for Volatile Fatty Acids, Hydrogen or Methane Production: A Critical Review. *Bioengineered* **2019**, *10*, 437–458. [[CrossRef](#)]

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