


Article

Mixed Fermentation of *Lactiplantibacillus plantarum* and *Bacillus licheniformis* Changed the Chemical Composition, Bacterial Community, and Rumen Degradation Rate of Tea Residue

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Abstract: Tea residue, as a byproduct in tea processing, is highly nutritious and can be used as a good raw material for ruminant feed. This study aimed to investigate the effects of *Lactiplantibacillus plantarum* (*L. plantarum*) and *Bacillus licheniformis* (*B. licheniformis*) mixed fermentation of tea residue mixture (tea residue:wheat bran, 7:3) on chemical composition, bacterial community, and rumen degradation rate. Changes in chemical composition and bacterial community were evaluated after 0 (F0), 1 (F1), 3 (F3), and 5 (F5) days of fermentation. The rumen degradation rate was studied by the in situ nylon bag method. Compared with group F0, acid soluble protein in other groups increased while pH and neutral detergent fiber decreased ($p < 0.05$). The group F5 was the best. The diversity of bacterial communities in group F0 was significantly lower than those in the other groups ($p < 0.05$). The relative abundance of phylum Firmicutes and the genus *Lactobacillus* increased with increasing fermentation time. The rumen degradation rates of dry matter, crude protein, neutral detergent fiber, and acid detergent fiber were increased after fermentation. In conclusion, mixed fermentation of tea residue by *L. plantarum* and *B. licheniformis* can ameliorate chemical composition, reduce bacterial community diversity, and improve the rumen degradation rate.

Keywords: tea residue; *Lactiplantibacillus plantarum*; *Bacillus licheniformis*; fermentation; bacterial community; rumen degradation



Citation: Huang, X.; Xu, Y.; Wu, X.; Ding, Y.; Fan, C.; Xue, Y.; Zhuo, Z.; Cheng, J. Mixed Fermentation of *Lactiplantibacillus plantarum* and *Bacillus licheniformis* Changed the Chemical Composition, Bacterial Community, and Rumen Degradation Rate of Tea Residue. *Fermentation* **2022**, *8*, 380. <https://doi.org/10.3390/fermentation8080380>

Academic Editors: Mengzhi Wang and Michela Verni

Received: 24 June 2022

Accepted: 4 August 2022

Published: 9 August 2022

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1. Introduction

Tea is one of the most popular beverages in the world. It has a huge consumer group and market demand. During 2020, the global tea area harvested exceeded 5 million hectares, and its production exceeded 7 million tons [1]. China is one of the major tea producers. In 2020, the tea plantation area in China was 3.2 million hectares, and tea production was 2.9 million tons, ranking first in the world. The above two indicators increased by 3.6% and 5.6%, respectively, compared to 2019 [2]. Tea residues are produced from tea after drinking, extracting the active ingredients, or making tea beverages [3], and they increase along with the increase in the annual tea consumption and the development of processing technology in the tea industry. Along with having the largest tea consumption, China produces more than 5 million tons of tea waste each year [4]. Undesirably, most tea residues are discarded or burned as industrial waste, which not only causes waste of resources but also contributes to environmental pollution [5].

Ramdani [6] showed that black tea residue was nutrient-rich and contained 25.2% crude protein, 2.3% ether extract, 39.4% neutral detergent fiber, 28.5% acid detergent fiber, 13.0% total phenols, 10.5% total tannins, 0.9% calcium, and 0.22% phosphorus. Fortunately, people are increasingly seeking ways to reuse tea residue resources. At present, tea residues

are mainly used as fertilizer [7], fuel [8], adsorbent [4], and sources of active ingredients [9]. Kondo et al. [10] showed that tea residue has a high crude protein content and has potential as a source of feed protein. Nasehi et al. [11] also showed that replacing alfalfa hay in lamb diets with green tea residue improved its digestibility and nitrogen retention, and improved growth performance. However, fresh tea residue often has high fiber and moisture content, making it difficult to store and poorly utilized. Microbial fermentation of plant feed protein can degrade its constituent bio-logical macromolecules, such as polysaccharides and proteins, into easily absorbed small molecules, reducing anti-nutritional factors [12].

The most critical technology for solid-state fermentation is the selection of microbial strains and process parameters. *Lactobacillus* is a group of Gram-positive, anaerobic, or facultative aerobic cocci or rods, which can use carbohydrates to produce large amounts of lactic acid [13]. They can produce bacteriocins, which inhibit the growth of spoilage and pathogenic bacteria [14]. Therefore, *Lactobacillus* is often added to feeds in order to prolong their storage time. *Bacillus* is a group of Gram-positive, rod-shaped endospore-formers and are facultative anaerobes or aerobes [15]. They show strong tolerances to extremes in temperature, organic solvents, and pH [16]. *Bacillus* can secrete many enzymes, such as amylases, xylanases, chitinase, β -1, 3-glucanase, β -glucosidase, lipase, protease, cellulase, and others, which play important roles in the feed, food, pharmaceutical, and textiles industries [17].

Preliminary results (unpublished) from our laboratory showed that good fermentation can be achieved with a 1:1 ratio of *Bacillus licheniformis* (*B. licheniformis*) to *Lactiplantibacillus plantarum* (*L. plantarum*), a total inoculum of 5×10^7 CFU/g, a water content of 57%, and 5 day of fermentation at 34 °C. However, the fermentation process entails many types of microorganisms, produces various metabolites, and is extremely complex [18]. The rumen degradation rate of fermented tea residue is also unknown. Therefore, the aim of this study was to investigate the effects of *L. plantarum* and *B. licheniformis* mixed fermentation of tea residue mixture (tea residue: wheat bran, 7:3) on chemical composition, bacterial community, and rumen degradation rate.

2. Materials and Methods

2.1. Preparation of Tea Residue and Fermentation

20-kg fresh black tea residue was provided by Sanan Sino-Science Co., Ltd. (Luan, Anhui, China). It was mainly the residue from the production of black tea beverages by leaching. The chemical composition of the tea residue is shown in Table 1. A 10 kg amount of wheat bran was purchased from Hefei Huaren Agriculture and Animal Husbandry Group Co., Ltd. (Hefei, Anhui, China). It was used as subsidiary material to control the moisture content and to provide a carbon source. *B. licheniformis* was purchased from Jiangsu Lvkee Biotechnology Co., Ltd. (Yangzhou, Jiangsu, China). Its live bacteria content was $\geq 2 \times 10^{10}$ CFU/g. *L. plantarum* (ACCC 11016) was obtained from the Agriculture Culture Collection of China (Beijing, China). 70-g fresh black tea residue and 30-g wheat bran were mixed and used as fermentation substrates. The 100-g mixture was placed into polyethylene bags (18 mm \times 25 mm). Subsequently, *B. licheniformis* and *L. plantarum* were uniformly added to the tea residue mixture in a ratio of 1:1 and at a final concentration of 5×10^7 CFU/g. Finally, the tea residue mixture was fermented at 34 °C for 1 day (F1), 3 day (F3), and 5 day (F5). The tea residue mixture was fermented without bacteria for 0 d (F0) as a control group. Each treatment was performed in three replicates.

2.2. Chemical Composition Analysis

A fermentation sample (10 g) per bag was mixed with 90 mL of sterilized distilled water and stored at 4 °C for 24 h. The supernatant was filtered through four layers of gauze [19]. The pH values were determined using a pH meter (pHS-3C; SHENGCI Instrument Co., Ltd., Shanghai, China). Another 10 g fermentation sample per bag was stored in a -80 °C freezer for backup. The residual material was dried at 45 °C for 48 h then crushed in a grinder (QE-100; Zhejiang Yili Industry and Trade Co., Ltd., Jinhua, Zhejiang,

China) and passed through a 1.0 mm sieve to determine the chemical composition. The samples of tea residue mixture (F0, F1, F3, and F5) were analyzed for dry matter (DM), crude protein (CP), ether extract (EE), and ash (Ash) [20]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using the method reported by Van Soest et al. [21]. Protease activity (PA) and cellulase activity (CA) were determined according to GB/T 28715-2012 and NY/T 912-2020, respectively [22,23]. The acid-soluble protein (ASP) and tea tannin (TTN) contents were determined according to the protocols described by NY/T 3801-2020 and GB/T 27985-2011, respectively [24,25]. A standard curve was made using tannic acid to determine TTN. ($y = 0.0949x - 0.0357$ $R^2 = 0.9992$)

Table 1. Chemical composition of tea residue (% dry matter).

Items	Content (%)
Dry matter, DM	23.12
Crude protein, CP	22.81
Ether extract, EE	3.01
Acid detergent fiber, ADF	25.74
Neutral detergent fiber, NDF	39.12
Ash	3.44
Calcium, Ca	0.62
Phosphorous, P	0.21
Acid-soluble protein, ASP	2.35
Tea tannin, TTN	6.63

2.3. Bacterial Community Analysis

To assess the bacteria community by next-generation sequencing, we measured a 10 g sample of each treatment and stored it in a freezer at -80 °C. Samples were sent to Shanghai Majorbio Technology Co., Ltd. (Shanghai, China) for analysis.

2.3.1. DNA Extraction and Sequencing

Total microbial genomic DNA was extracted from tea residue mixture samples using the E.Z.N.A.[®] soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop[®] ND-2000 spectrophotometer (Thermo Scientific Inc., Wilmington, CA, USA) and kept at -80 °C prior to further use. Bacterial 16S rRNA genes spanning a hypervariable (V3–V4) region were amplified using the 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers [26]. For the V3–V4 region of bacterial 16S rRNA genes, the PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles at 95 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 10 min, ending at 4 °C. All samples were amplified in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus[™] Fluorometer (Promega, Madison, USA). The purified amplicons were pooled in equal amounts and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.3.2. Sequence Analysis

Raw FASTQ files were de-multiplexed using an in-house perl script, and then quality-filtered by FASTQ version 0.19.6 [27] and merged by FLASH version 1.2.7 [28]. Bioinformatic analysis of the tea residue mixture microbiota was implemented using the Majorbio Cloud platform (<https://cloud.majorbio.com>) (accessed on 30 January 2022). Based on the OTUs information, rarefaction curves and alpha diversity indices including observed

OTUs, Chao1 richness, Shannon index and Good's coverage were calculated with Mothur version 1.30.1 (Patrick Schloss, Ann Arbor, MI, USA) [29].

2.4. Animals and Experimental Design

2.4.1. Ethics Statement

The animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Anhui Agricultural University (No. SYDW-P20190600601). The experiments were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and the Standards for the Administration of Experiment Practices.

2.4.2. Animal Management

Three 45-month-old healthy Holstein dairy cows (about 650 kg) with permanent rumen cannulas were used. The experimental diet was provided by Shanghai Xinghuo Dairy Farm (Shanghai, China). The composition and nutrient levels of the basic diet are shown in Table 2.

Table 2. Composition and nutrient levels of the basic diet.

Ingredients	Content (% of DM)	Nutrient Levels	Content (% of DM)
Alfalfa hay	21.0	NEL (Mcal/kg) ¹	1.73
Barley silage	17.5	Crude protein, CP	17.60
Corn	33.6	Neutral detergent fiber, NDF	29.17
Soybean meal	12.8	Acid detergent fiber, ADF	17.40
Cottonseed meal	4.3	Calcium, Ca	1.02
Rapeseed meal	2.5	Phosphorous, P	0.45
Cottonseed	4.5		
Beet pulp	2.1		
Dicalcium phosphate	0.52		
Salt	0.42		
Sodium bicarbonate	0.41		
Vitamin–mineral premix1	0.35		

¹ NEL is a calculated value, while the other nutrient levels were measured values.

2.4.3. In Situ Nutrient Degradability

In this study, the nylon bag method [30–32] was used to reanalyze the rumen degradation characteristics of DM, CP, ADF, and NDF. Each cow was regarded as a replicate, each forage three replicates, and each forage had two parallel replicates per cow ($n = 6$). A total of 54 nylon bags were prepared. The nylon bags (size: 10 cm × 15 cm; pore: 50 μm) were numbered, dried at 65 °C, then weighed. We weighed 10 g of unfermented tea residue (UTR) (F0) and fermented tea residue (FTR) (F5) into nylon bags. A maximum of six bags were attached to 50-cm-long plastic tubes that retained bags within the liquid phase of the ruminal content. The nylon bags were placed in the rumen of fistulated cattle at 08:00 a.m. and incubated for 2 h, 4 h, 8 h, 16 h, 24 h, 30 h, 36 h, and 48 h. The bags were then washed, dried at 65 °C, and weighed. A control group was set for 0 h (not incubated in the rumen). Once retrieved from rumen at each time point, the bags were rinsed in cool water, then dried to a constant weight at 65 °C for 48 h. The dried residues were crushed in a grinder and passed through a 1 mm sieve for further use.

2.4.4. Rumen Degradation Rate Analysis

We calculated the degradation rate using Equation (1):

$$A = \frac{(B - C)}{B} \times 100\% \quad (1)$$

where A is the rumen degradation rate of a nutrient in the material tested (%), B is the mass of a nutrient in the material tested (g), and C is the mass of a nutrient in the residue (g).

Ruminal degradation parameters and effective degradation rates of feeds were determined and calculated according to the mathematical index model of rumen kinetics proposed by Ørskov et al. [33].

$$Dp = a + b(1 - e^{-ct}) \tag{2}$$

$$ED = a + b \times c / (k + c) \tag{3}$$

where a is the rapidly degradable component (%), b is the potentially degradable component (%), c is the constant rate of degradation of b (%/h), $a + b$ is total degradable component (%), Dp is the nutrient disappearance rate in the rumen at time “ t ”. The effective degradability (ED , %) was determined using Equation (3), where k is the rumen outflow rate of the nutrient component (%/h), rendered as $k = 0.031\%/h$ [34].

2.5. Statistical Analysis

Statistical analyses were performed using the statistical program IBM SPSS 25.0 (IBM Co., Armonk, NY, USA). The chemical composition and alpha diversity of bacteria were analyzed using a one-way analysis of variance (ANOVA). The ruminal degradation kinetics (i.e., a , b , c , $a + b$, dp , and ED) of DM, CP, ADF, and NDF within various feeds were estimated using the NLIN procedure (Equations (2) and (3)). The data are expressed as mean \pm standard deviation. Significance was declared at $p < 0.05$.

3. Results

3.1. Chemical Composition of Tea Residue Mixture during Fermentation

As shown in Table 3, DM loss, ASP, CP, and EE increased with the extension of fermentation time from day 0 to day 5. Except for CP, the above indexes in group F5 were significantly higher than those in group F0 ($p < 0.05$). However, with the prolongation of fermentation time, the pH, CA, ADF, and NDF decreased ($p < 0.05$). PA first increased, then decreased, and reached a peak in group F3.

Table 3. Changes in the chemical composition of tea residue mixture at different times following fermentation by *L. plantarum* and *B. licheniformis*.

Items	F0 (0 day)	F1 (1 day)	F3 (3 day)	F5 (5 day)	<i>p</i> -Value
Dry matter loss, DM Loss (%)	-	0.98 \pm 0.03 ^b	1.08 \pm 0.05 ^b	1.86 \pm 0.01 ^a	<0.01
pH	5.91 \pm 0.04 ^a	4.40 \pm 0.04 ^b	3.97 \pm 0.04 ^c	3.93 \pm 0.04 ^c	<0.01
Protease activity, PA (U/g)	0.15 \pm 0.02 ^d	328.96 \pm 0.98 ^b	523.18 \pm 2.79 ^a	143.41 \pm 13.10 ^c	<0.01
Cellulase activity, CA (U/g)	0.11 \pm 0.01 ^d	30.85 \pm 0.24 ^a	11.78 \pm 0.43 ^b	6.48 \pm 0.23 ^c	<0.01
Acid-soluble protein, ASP (% DM)	1.92 \pm 0.04 ^d	2.48 \pm 0.13 ^c	3.11 \pm 0.03 ^b	4.00 \pm 0.15 ^a	<0.01
Crude protein, CP (% DM)	18.09 \pm 0.50	18.32 \pm 0.15	18.32 \pm 0.15	18.52 \pm 0.40	0.682
Ether extract, EE (% DM)	3.59 \pm 0.03 ^c	3.63 \pm 0.13 ^c	3.93 \pm 0.03 ^b	4.38 \pm 0.12 ^a	<0.01
Acid detergent fiber, ADF (% DM)	23.16 \pm 0.54 ^a	22.18 \pm 0.17 ^{ab}	20.46 \pm 0.01 ^{bc}	19.94 \pm 1.29 ^c	0.030
Neutral detergent fiber, NDF (% DM)	49.94 \pm 1.11 ^a	46.53 \pm 1.00 ^b	45.57 \pm 0.79 ^b	43.90 \pm 0.30 ^c	<0.01

F0: fermentation without bacteria for 0 d; F1: fermentation with bacteria for 1 d; F3: fermentation with bacteria for 3 d; F5: fermentation with bacteria for 5 d. ^{a,b,c,d} Means \pm SD ($n = 3$) in rows and with different superscript letters indicate a significant difference at $p < 0.05$.

3.2. Bacterial Community of Tea Residue Mixture during Fermentation

A total of 285 OTUs were identified in the four groups from day 0 to day 5. Among these, 58 OTUs were found in all groups, accounting for 20.35% of the total OTUs (Figure 1a). Based on the PCoA graph (Figure 1b), the clouds of the four groups were divided into three main sections, with groups F3 and F5 close together. This indicated that the difference in

the bacterial community succession was evident between group F0 and other groups, but that between group F3 and group F5 was minor.

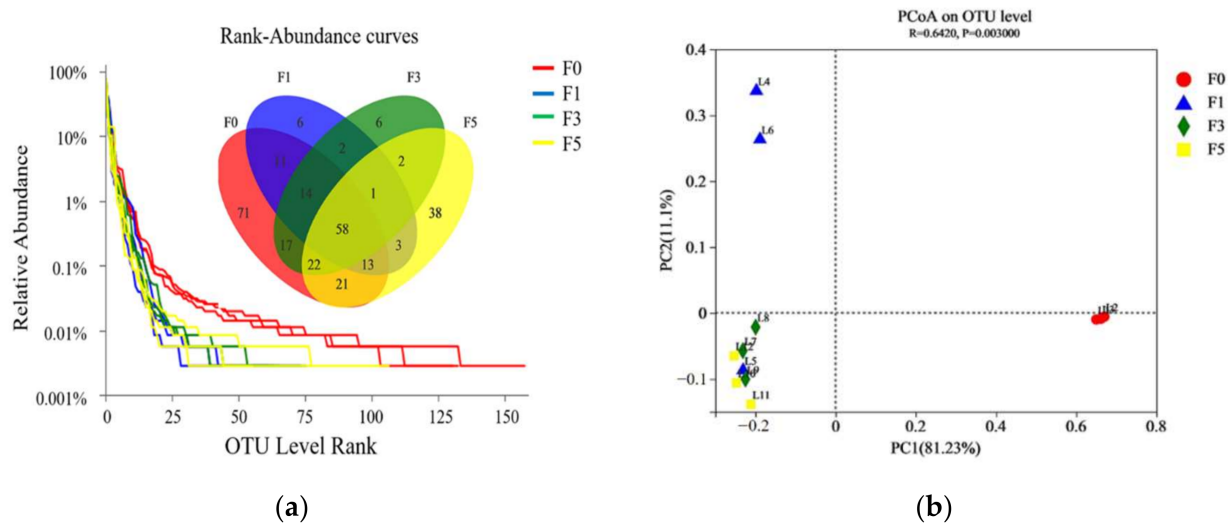


Figure 1. The rank-abundance curves were derived from the microbial OTU level. (a) The Venn graph illustrates the overlap of microbial OTUs among the treatment groups F0, F1, F3, and F5 at a 3% dissimilarity level; (b) PCoA analysis of taxonomical classifications in groups F0, F1, F3, and F5. Each group has three replicates. Group F0 contains L1, L2, and L3. Group F1 contains L4, L5, and L6. Group F3 contains L7, L8, and L9. Group F5 contains L10, L11, and L12.

As shown in Table 4, the coverage values of all groups were around 99.9%, indicating that the analysis of the bacterial community was credible. The Chao1, Ace, and Shannon indices were significantly higher in group F0 than in group F1, F3, and F5 ($p < 0.05$), indicating that the diversity of bacteria community contained in group F0 was significantly higher than in the other groups. The diversity of the bacteria community contained in groups F1, F3, and F5 were not significantly different ($p > 0.05$).

Table 4. Alpha diversity of tea residue mixture during *L. plantarum* and *B. licheniformis* fermentation.

Items	F0 (0 day)	F1 (1 day)	F3 (3 day)	F5 (5 day)	p-Value
Chao1	153.6 ± 10.58 ^a	90.24 ± 22.52 ^b	92.73 ± 10.16 ^b	107.68 ± 18.32 ^b	<0.01
Ace	158.82 ± 12.28 ^a	124.04 ± 21.44 ^b	100.31 ± 9.69 ^b	118.00 ± 20.43 ^b	0.016
Shannon	1.65 ± 0.20 ^a	1.27 ± 0.24 ^b	1.24 ± 0.05 ^b	1.13 ± 0.17 ^b	0.034
Simpson	0.35 ± 0.08	0.42 ± 0.15	0.46 ± 0.04	0.50 ± 0.12	0.444
Coverage (%)	99.94 ± 0.01	99.94 ± 0.02	99.93 ± 0.02	99.93 ± 0.01	0.861

F0: fermentation without bacteria for 0 d; F1: fermentation with bacteria for 1 d; F3: fermentation with bacteria for 3 d; F5: fermentation with bacteria for 5 d. ^{a,b} Means ± SD ($n = 3$) in rows and without a common superscript are significantly different ($p < 0.05$). Alpha diversity is the diversity within a particular region or ecosystem and is a composite indicator of abundance and evenness.

The bacterial communities of the tea residue mixture at different fermentation times are displayed in Figure 2. Three bacterial phyla (Firmicutes, Proteobacteria, and Cyanobacteria) were identified in the tea residue with a relatively high abundance (>1%) (Figure 2a). The main bacteria in group F0 were Cyanobacteria (60.02%) and Proteobacteria (35.03%). As the fermentation time increased, Cyanobacteria and Proteobacteria gradually decreased, while Firmicutes gradually increased. The top ten dominant bacterial genera are shown in Figure 2b. The main microbiota in group F0 was *norank_f_norank_o_Chloroplast* (60.02%) and *Pseudomonas* (18.15%). The main bacteria in group F1 were *Lactobacillus* (39.07%), *Pantoea* (42.30%), and *Bacillus* (13.51%). However, *Lactobacillus*, *Bacillus*, and *Pediococcus* were the main bacteria in groups F3 and F5.

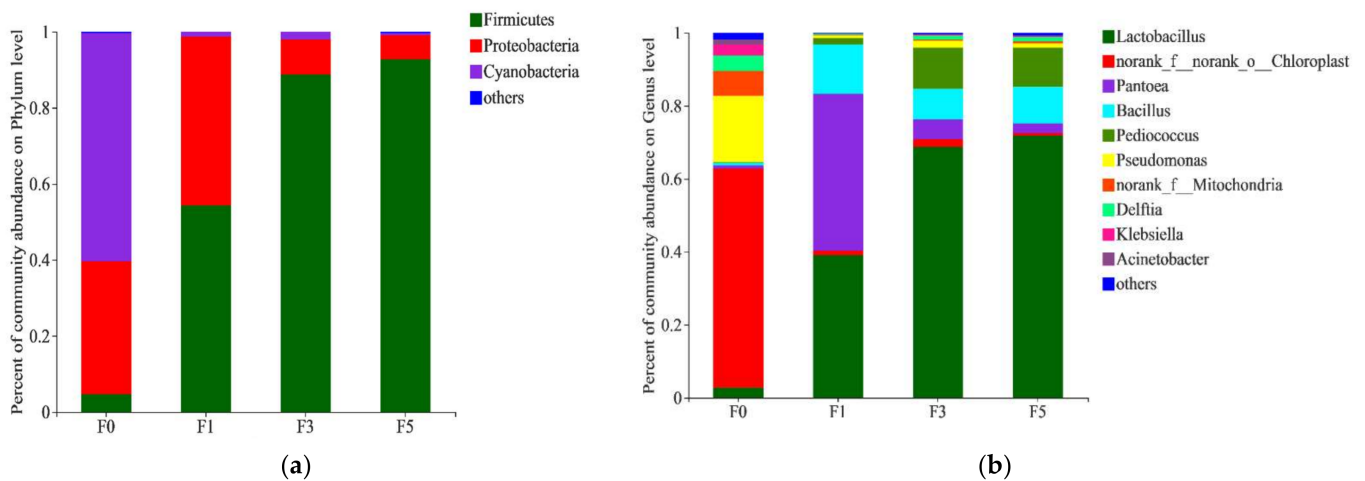


Figure 2. Relative abundance, following fermentation of the tea residue mixture treatment groups F0, F1, F3, and F5; bacterial communities at the phylum level (a) and genus level (b).

The bacterial communities were mainly correlated with pH and ASP, ADF, and NDF content (Figure 3). *Enterococcus*, *Pediococcus*, and *Lactobacillus* were the main organisms that affected these indicators. *Enterococcus* was positively correlated with pH, ADF, and NDF, and negatively correlated with ASP ($p < 0.05$). *Pediococcus* and *Lactobacillus* were positively correlated with ASP but negatively correlated with pH, ADF, and NDF. *Methylobacterium-Methylobacterium* and *Sphingomonas* were positively correlated with NDF. However, *Methylobacterium-Methylobacterium* had a negative effect on EE and *Sphingomonas* had a negative effect on ASP.

Spearman Correlation Heatmap

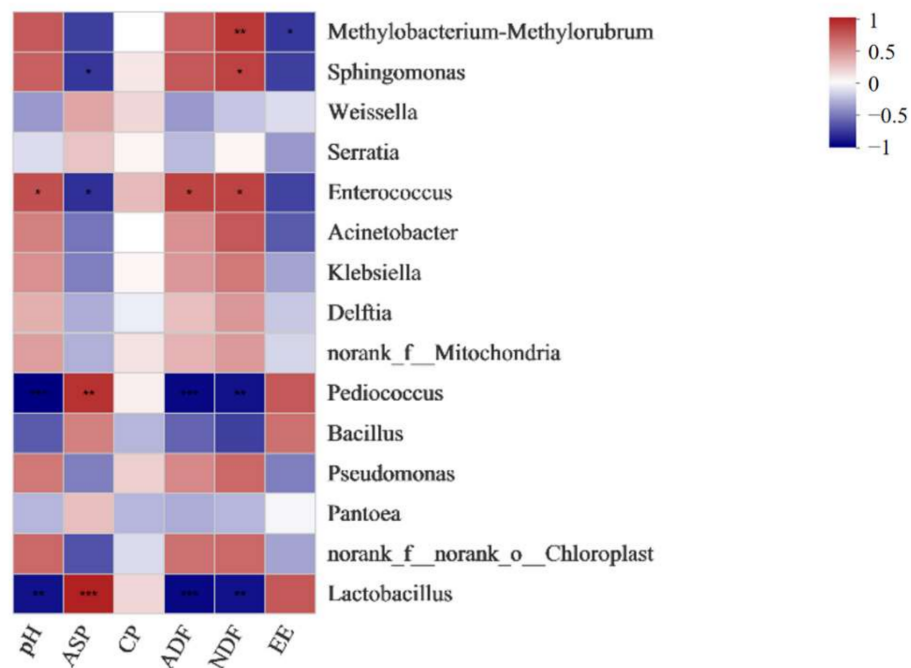


Figure 3. Correlation analyses between the chemical compositions and the top 15 tea residue mixture bacterial genera. The red squares show the positive correlations, while the blue squares show the negative correlations. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. ASP = acid-soluble protein, CP = crude protein, ADF = acid detergent fiber, NDF = neutral detergent fiber, and EE = ether extract.

3.3. Rumen Degradation Rates of Unfermented and Fermented Tea Residue Mixtures

As shown in Figure 4, the rumen degradation rates of DM, CP, ADF, and NDF increased with the extension of the incubation time from 0 to 48 h. All of the above indicators were higher in the FTR than in the UTR. The rumen degradation rate of DM was significantly higher in FTR than in the UTR for all incubation times except 24 h ($p < 0.05$). The rumen degradation rate of CP was significantly higher in the FTR than in the UTR at all incubation times ($p < 0.05$). The rumen degradation rate of ADF was significantly higher in the FTR than in the UTR for all incubation times except 2 h and 8 h ($p < 0.05$). The rumen degradation rate of ADF was significantly higher in the FTR than in the UTR when the incubation time was 2 h, 4 h, 16 h, or 48 h ($p < 0.05$).

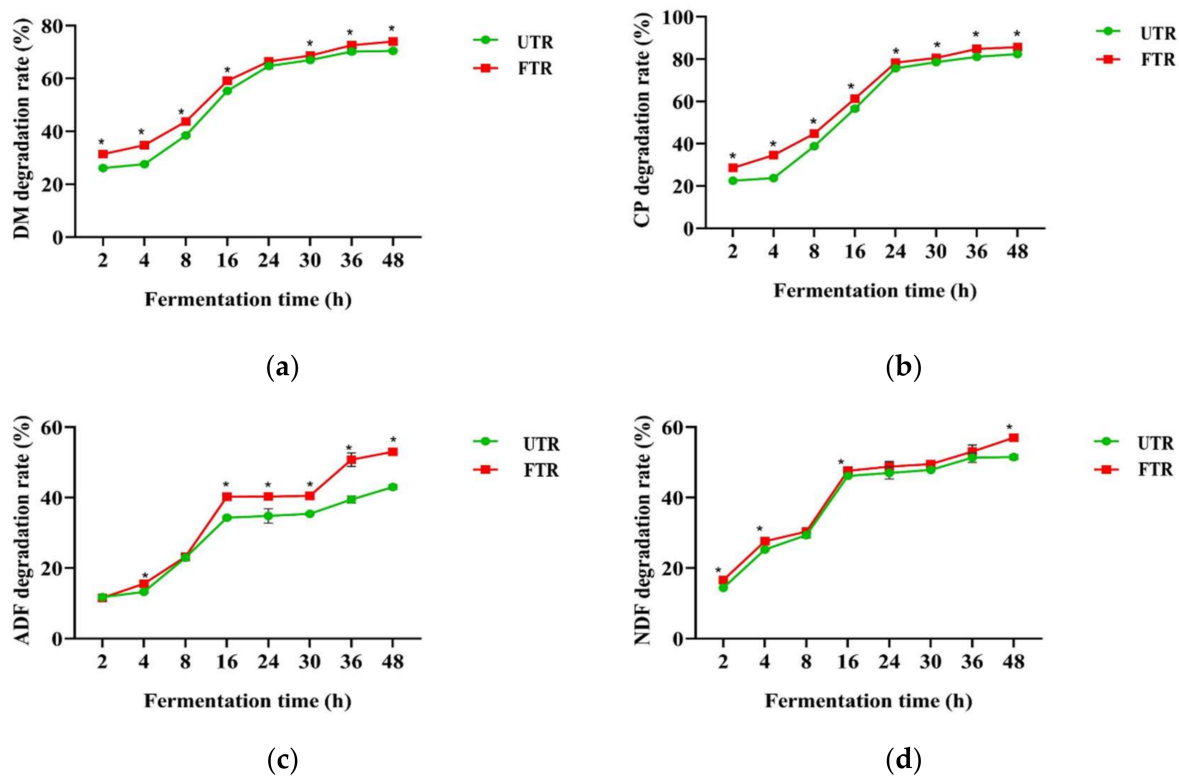


Figure 4. Plots of the cumulative rumen degradation rate of components dry matter (DM) (a), crude protein (CP) (b), acid detergent fiber (ADF) (c), and neutral detergent fiber (NDF) (d) in unfermented (UTR) and fermented (FTR) tea residue mixtures.

The rumen degradation parameters are presented in Table 5. Compared to the UTR, the rapidly degradable components (a) of DM, CP, and NDF were all significantly higher in the FTR ($p < 0.05$). Furthermore, the total degradable component (a + b) and effective degradability (ED) of DM, CP, ADF, and NDF were also higher in the FTR ($p < 0.05$). However, the potentially degradable components (b) of DM and CP were significantly lower in the FTR ($p < 0.05$). The constant rate of degradation of b (c) of ADF and NDF of FTR were also significantly decreased ($p < 0.05$).

Table 5. The rumen degradation parameters of components in tea residue mixtures.

Items ²		a ¹ (%)	b (%)	c (%)	a + b (%)	ED (%)
DM	UTR	16.05 ± 0.32 ^b	58.45 ± 0.68 ^a	0.07 ± 0.002	74.50 ± 0.60 ^b	56.13 ± 0.16 ^b
	FTR	23.61 ± 0.32 ^a	53.50 ± 0.45 ^b	0.06 ± 0.003	77.11 ± 0.55 ^a	59.80 ± 0.39 ^a

Table 5. Cont.

Items ²		a ¹ (%)	b (%)	c (%)	a + b (%)	ED (%)
CP	UTR	9.61 ± 0.29 ^b	80.05 ± 0.78 ^a	0.06 ± 0.002	89.66 ± 0.80 ^b	62.67 ± 0.05 ^b
	FTR	19.13 ± 0.52 ^a	73.26 ± 1.00 ^b	0.06 ± 0.001	92.40 ± 0.48 ^a	67.16 ± 0.20 ^a
ADF	UTR	5.60 ± 0.73	36.30 ± 0.28 ^b	0.08 ± 0.010 ^a	41.90 ± 1.01 ^b	31.52 ± 0.14 ^b
	FTR	6.31 ± 0.63	48.45 ± 1.81 ^a	0.06 ± 0.003 ^b	54.76 ± 1.33 ^a	37.67 ± 0.23 ^a
NDF	UTR	6.90 ± 1.24 ^b	44.53 ± 0.47	0.11 ± 0.010 ^a	51.42 ± 1.05 ^b	41.41 ± 0.35 ^b
	FTR	11.36 ± 1.28 ^a	44.27 ± 1.78	0.09 ± 0.009 ^b	55.63 ± 1.37 ^a	43.83 ± 0.46 ^a

¹ a: Rapidly degradable component; b: the potentially degradable component; c: the constant rate of degradation of b; a + b: total degradable component; ED: effective degradability. ² Dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF), unfermented (UTR), and fermented (FTR). ^{a,b} Means ± SD ($n = 3$) in columns, with the same component, and without a common superscript are significantly different ($p < 0.05$).

4. Discussion

Tea residue is a byproduct of tea processing and is mostly discarded as industrial waste. Tea residue still contains many nutrients, and its rational processing can be expected to produce excellent animal feed. This study investigated the effect of the fermentation of tea residue mixed with wheat bran by *B. licheniformis* and *L. plantarum* on its chemical composition, bacterial community, and ruminal degradation rate.

In our study, ASP, CP, and EE of tea residue mixture were increased after fermentation, while pH, ADF, and NDF were decreased. Microbial fermentation of sugars and organic acids in crops releases gases such as carbon dioxide and hydrogen, which can cause dry matter loss [35]. As shown in this study, DM loss increased cumulatively, and the contents of ASP, CP, and EE in the tea residue wheat bran mixture also increased with the increase of DM loss. *L. plantarum* can produce lactic acid, acetic acid, propionic acid, phenyllactic acid, formic acid, and succinic acid, and then reduce the pH value [36]. In our study, the *L. plantarum* population increased with the duration of fermentation, which in turn caused a constant decrease in pH. In addition, Inca-Torres et al. [37] showed that *B. licheniformis* can inherently produce protease, cellulase, and lipase. Proteases decompose proteins by hydrolyzing them into amino acids and peptides [16], thus increasing the acid-soluble protein content [38]. In this study, protease activity increased and then decreased, reaching a maximum at 3 d of fermentation. Hao et al. [39] used *B. licheniformis* to anaerobically ferment litchi pomace. Their results, similar to the results of our experiment, showed that protease activity first increased and then decreased, and reached a maximum at 2.5 d of fermentation. Cellulase activity showed a decreasing trend. The results of our previous trials showed that both *B. licheniformis* and *L. plantarum* used in the current experiment reached their peak enzyme activity at 2 d of fermentation. The addition of *L. plantarum* and *B. licheniformis* to the tea residue mixture produced cellulase, which degraded cellulose to monosaccharides or oligosaccharides, thereby reducing the ADF and NDF content. Low fiber content can promote the utilization of the feed by rumen microorganisms, resulting in higher fermentation rates [40]. Shi et al. [41] fermented wheat bran with a complex bacterial solution containing *Bacillus subtilis*, which increased the EE and ASP content of the wheat bran and reduced the NDF content, similar to the results of our current study.

This current study showed that the diversity of the bacterial community in the tea residue mixture was lower after fermentation (F1, F3, and F5) than before (F0), which was similar to the results reported by Xu et al. [42]. Prior to fermentation, there was a considerable abundance of aerobic and facultative anaerobic bacteria, such as *norank_f_norank_o_Chloroplast*, *Pseudomonas*, *norank_f_Mitochondria*, *Delftia*, and *Klebsiella*, but limited abundance of *Lactobacillus*. However, anaerobic bacteria became the dominant bacterial community due to the constant depletion of oxygen during fermentation [43]. Li et al. [44] also showed that the absolute dominant bacteria of the raw Pu-erh tea sample was *norank_o_Chloroplast*. *Bacillus*, *Pantoea*, *Pediococcus*, *Pseudomonas*, and other genera were also identified in raw Pu-erh tea and its fermented

variety. Same as our results, Sun et al. [45] reported that after anaerobic fermentation of paper mulberry silage, the relative bacterial abundance of *Lactobacillus* was enhanced.

Firmicutes, Proteobacteria, and Cyanobacteria were shown to be the dominant phyla in the tea residue mixture. Bacteria of the bacterial phyla Firmicutes are associated with fiber degradation and polysaccharide degradation [46]. In addition, Proteobacteria are involved in the formation of biofilms and the digestion of soluble carbohydrates [47]. Tong et al. [48] identified the main bacterial phyla from black and green tea leaves as Proteobacteria, Firmicutes, and Cyanobacteria along with Actinobacteria and Bacteroidetes, somewhat consistent with the results of our study. In our study, Firmicutes increased after fermentation. The reason for this result may be the addition of *B. licheniformis* and *L. plantarum* to groups F1, F3, and F5. Spearman correlation analysis demonstrated that *Pediococcus* and *Lactobacillus* were positively correlated with ASP but negatively correlated with pH, ADF, and NDF. However, *Enterococcus* had the opposite effect.

The in situ nylon bag method is an approach used to evaluate the difficulty of feed utilization and the degradation and utilization efficiency of feed in the rumen [49]. Ruminal microbes degrade dietary nutrients and convert them into ammonia nitrogen, microbial crude protein, and volatile fatty acids to provide nutrition for ruminants [46]. Dry matter degradability is an important index that can reflect the degradation extent of feed in the rumen [50]. In our study, the rumen degradation rates of DM, CP, ADF, and NDF for both FTR and UTR increased with the extension of the incubation time. Furthermore, the rumen degradation rate of DM, CP, ADF, and NDF for FTR were higher than those of UTR. It is possible that the *B. licheniformis* and *L. plantarum* used in fermentation in this study disrupted the cell wall structure of the tea residue, releasing more plant proteins and cellulose. Microorganisms use these plant proteins to synthesize microbial proteins that are better digested and absorbed by the animals, thereby increasing the rumen degradation rates of CP [51]. Furthermore, *Lactobacillus* can degrade part of lignin and release hemicellulose and cellulose. These sugars are broken down by rumen microorganisms into monosaccharides that are more easily absorbed by ruminants, accelerating the rumen degradation rates of ADF and NDF [52]. Li et al. [53] used *L. plantarum* for cassava silage and significantly improved its DM and CP degradation rate in the rumen of Hainan black goats. Zhao et al. [54] improved the rumen degradation rates of DM, CP, and NDF in dairy cows using lactic acid bacteria mixed with silage soybean residue and corn stover. In addition, the use of *L. plantarum* (Chikuso-1) for silage of stylo also increased the rumen degradation rates of DM, CP, ADF, and NDF in Hainan black goats by Li et al. [55], which was similar to the results of this experiment.

5. Conclusions

The mixed fermentation of tea residue mixture using *L. plantarum* and *B. licheniformis* can produce proteases and cellulase that degrade large molecules of protein and fiber, thereby improving the chemical composition and rumen degradation rate of the tea residue mixture. In addition, pH of the tea residue mixture decreased after fermentation which inhibits the growth of some harmful bacteria and reduces the diversity of the bacterial community. As such, *L. plantarum* and *B. licheniformis* can be used to develop tea residue fermented feed.

Author Contributions: Conceptualization, X.H.; methodology, X.H., Y.X. (Yinying Xu) and X.W.; software, X.H., Y.X. (Yinying Xu), X.W. and Y.D.; validation, Y.X. (Yinying Xu) and X.W.; investigation, X.H., Y.X. (Yinying Xu), X.W. and Y.D.; resources, X.H., Y.D. and X.W.; data curation, X.H., Y.X. (Yinying Xu) and X.W.; writing—original draft preparation, X.H. and Y.X. (Yinying Xu); writing—review and editing, J.C.; supervision, J.C., C.F., Z.Z. and Y.X. (Yanfeng Xue); funding acquisition, J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Key Research and Development Program of Anhui Province (202004a06020006; 202004a06020012) and the Shanghai Science and Technology Promotion Project for Agriculture (Shanghai Agriculture Science Promotion Project (2019) No. 1-2).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the first author. The data are not publicly available due to restrictions by the research group.

Conflicts of Interest: The authors declare no conflict of interest.

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