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Microbial and Protease Fermentation of Mao-Tai Lees Alters Nutritional Composition and Promotes In Vitro Intestinal Proteolysis

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Abstract: Mao-tai lees (ML) is a by-product produced in the process of Mao-tai liquor production and contains high levels of crude protein, starch and fiber, and large yield. Thus, the ML has the potential to become feedstuffs alternatives in livestock production. The present study evaluated the nutritional values of ML and fermented ML (FML), including the first stage (FML I; microbial fermentation), the second stage (FMTL II; microbial fermentation), and the final stage (FFML; microbial fermentation with proteases), and explored their effects on in vitro intestinal fermentation. The results showed that the FFML had higher contents of acid detergent fiber, acid detergent lignin, crude fiber, crude protein, neutral detergent fiber, starch, Vitamin B_2, B_6, and B_{12}, whereas the FML II presented higher contents of calcium, copper, iron, manganese, magnesium, and Vitamin B_1 compared with the other groups. Compared with the ML, the total free amino acids (FAAs) and total bioamine contents were higher in the FML II and FFML and had lower total hydrolyzed amino acids and total other free organic acids contents, among which the FFML had higher total FAAs and total bioamine contents. The FMLs had lower *n*-6:*n*-3 PUFA ratio compared with the ML; however, the FFML had lower *n*-6:*n*-3 PUFA ratio than the other groups. Furthermore, the FFML had higher concentrations of 1,7-diaminoheptane, isobutyrate, isovalerate, putrescine, and spermidine in vitro fermentation, suggesting that the FFML had greater proteolysis than the other groups. Collectively, these findings suggest that microbial fermentation with proteases could alter the nutritional composition and promote in vitro intestinal proteolysis of ML, which may be an effective way for promoting the protein utilization of ML. The study provides an effective potential strategy to develop novel feedstuff alternatives.

Keywords: amino acid; bioamines; fatty acids; fermentation; in vitro; Mao-tai lees

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1. Introduction

The growing global population has raised the demand for foods of animal origin [1]. Feed resources play a crucial role in livestock production to determine the overall production cost, and they have received great attention from producers and consumers [2]. Corn and soybeans are conventional feedstuffs in livestock production due to their high nutritional value. However, there are shortages of high nutritional feedstuffs, and they are also expensive due to the increasing demand in recent years [3,4]. China is the largest pork producer in the world [3], and it is important to maintain the production cost with higher pork quality and establish a sustainable production system in China. Thus, it is urgent to find alternatives for conventional feedstuffs.

Agricultural industries in China produce a large quantity of agricultural by-products after production and processing, such as lees and okara [5,6]. However, these agricultural by-products usually have the disadvantages of poor palatability and more antinutritional

components, so they cannot be effectively utilized and are dumped or thrown away, resulting in the wastage of resources and environmental pollution [6,7]. Recent studies have found that fermentation can mitigate the disadvantages of agricultural by-products [8]. Microbial fermentation could significantly improve palatability and nutritional values and eliminate the antinutrients of animal feedstuffs from agricultural by-products [6,9]. *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Bacillus subtilis* are commonly used probiotic species in fermentation for improving feed utilization and intestinal health, and their combined fermentation with enzymes has been found to be more effective in improving the fermentation effect [8,10].

The nutritional quality of protein in feed depends on the composition and bioavailability of amino acids (AAs) [11], and the lower bioavailability of dietary protein can lead to nitrogen loss and environmental pollution. The protein can produce small peptides and free AAs (FAAs) under the fermentation process [12], which will promote its absorption and utilization by the intestine. Then, the AAs as protein components are usually decomposed into bioamines and short-chain fatty acids (SCFAs) by micro-organisms in the intestine [13,14]. Thus, microbial fermentation may alter the composition of AAs and FAAs in feed and lead to changes in the proteolysis degree in the intestine.

Mao-tai lees (ML) is one of the main by-products after the production and processing of sorghum with high protein (23%), fiber (17%), and starch (20%) contents and a variety of essential AAs, including leucine, lysine, valine, etc., and produces approximately 150,000 tons per year [15]. Our previous studies showed that the ML fermented by microorganisms was beneficial for the microbiota composition and intestinal health of pigs [5,13]. Thus, we hypothesized that microbial fermentation combined with proteases could promote the nutritional composition and fermentation characteristics of the ML, and thus influence gut health parameters by promoting intestinal proteolysis. Therefore, the present study evaluated the nutritional composition of ML and fermented MLs (FMLs) with or without proteases and determined in vitro intestinal fermentation characteristics, which may be an effective potential strategy for developing novel feedstuff alternatives.

2. Materials and Methods

2.1. Preparation of Fermented Mao-tai Lees

The FMLs were prepared by the Road Biological Environmental Co., Ltd., Sichuan, China. After sterilization, the ML (containing 55% water) was mixed with 5‰ *Saccharomyces cerevisiae*, 3‰ *Aspergillus niger*, and 0.6‰ *Bacillus subtilis*, and then, the FMLs were prepared by a continuous three-stage pure solid-state fermentation. The fermentation processes are as follows: the first stage (FML I), fermentation at 32 °C for 36 h under ventilation; the second stage (FMTL II), fermentation at 32 °C for 12 h under static and anaerobic conditions; and the final stage (FFML), addition of 0.5‰ acidic protease and 0.5‰ neutral protease and incubated at pH 5.0 and 60 °C for 12 h. The ML and the three FMLs were dried by oven drying at 65 °C (FD115; Binder GmbH Inc., Neckarsulm, Germany), pulverized, screened through a 0.45-mm mesh, and then packed in hermetical plastic bags for use. The types and concentrations of proteases were based on their stability in a lower acidic environment and the manufacturers' recommendation.

2.2. Analysis of Nutritional Composition

The contents of dry matter (DM; method 945.15), organic matter (OM; method 942.05), ether extract (EE; method 920.39), crude protein (CP, total N × 6.25; method 945.01), crude fiber (CF; method 994.13), and acid detergent lignin (ADL; 973.18) were analyzed according to the methods by AOAC [16]. Gross energy (GE) was measured using an isothermal automatic calorimeter (5EAC8018; Changsha Kaiyuan Instruments Co., Ltd., Changsha, China). The contents of a neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the methods described by Van Soest et al. [17] by adding a heat-stable α-amylase and sodium sulphite. The starch content was determined after pre-extraction with 80% ethanol (*v*/*v*), and glucose released from starch by enzyme

hydrolysis was measured using amyloglucosidase (Sigma Inc., St. Louis, MO, USA) according to Karthner and Theurer [18]. An Agilent 7900 ICP-MS system (Agilent Inc., Palo Alto, CA, USA) was used for the simultaneous determination of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), and phosphorus (P) contents according to a method by AOAC (method 984.27) [16], and vitamins B_1 (VB_1; method 942.23), VB_2 (method 940.33), VB_6 (method 961.15), and VB_{12} (method 961.15) contents according to a method by AOAC [16]. The nutritional composition of each sample was analyzed twice.

2.3. Analysis of Hydrolyzed Amino Acids

The ML and FMLs samples (1.0 g) were weighed in ampoules containing 10 mL of hydrochloric acid (6 mol/L) and were mixed vigorously. The mixtures were sealed with an alcohol burner, then digested at 110 °C for 24 h, diluted to 100 mL with distilled water, and the supernatants were filtered with 0.45-μm membranes for analysis [19]. An ion-exchange AA analyzer (L8800; Hitachi, Tokyo, Japan) was used to determine the contents of hydrolyzed AAs of ML and FMLs. The hydrolyzed AA content of each sample was analyzed twice.

2.4. Analysis of Long-Chain Fatty Acids

The long-chain fatty acids (LCFAs) composition of ML and FMLs was determined as previously described by Yin et al. [20] and Li et al. [21]. Briefly, methyl esters of LCFAs were prepared for gas chromatography (GC) analysis using KOH/methanol, which was determined using the Agilent 7890A GC (Agilent Inc., Palo Alto, CA, USA). The concentration of individual LCFA was expressed as a percentage of the total LCFAs. The LCFA composition of each sample was analyzed twice.

2.5. Analysis of Small Molecule Metabolites

The ML and FMLs samples (50 mg) were weighed into 2-mL Eppendorf (EP) tubes and homogenized with 600 μL of a MeOH/H_2O solution (2:1, *v*/*v*). The mixed samples were homogenized with a tissuelyser (Servicebio Inc., Wuhan, China) at 20 HZ for 90 s for polar metabolite extraction. The homogenized samples were sonicated for 60 s in an ice bath and stood for 60 s, and these procedures were performed in five cycles. After 2 min, samples were centrifuged at 12,000× *g* and 4 °C for 10 min, and the supernatants were transferred into 2-mL EP tubes. Then, 600 μL MeOH/H_2O solution was added to the supernatants for two extractions. The mixed supernatants were centrifugated at 16,000× *g* and 4 °C for 10 min, and then, the supernatants were transferred into 2-mL EP tubes to dry in a speed vacuum dryer for 24 h. After that, dried samples were transferred for freeze drying for 24 h. The dried powder was dissolved with Na/K buffer and kept at 4 °C for 2 min after vortexing, and approximately 550 μL supernatant of each sample was taken into 5-mm NMR tubes for analysis after centrifugation at 16,000× *g* and 4 °C for 10 min according to previous studies [22,23]. The content of small molecule metabolites of each sample was analyzed twice.

2.6. Analysis of Microbiota Composition

The total microbial DNA was extracted from ML and FMLs samples with QIAamp DNA stool Mini Kit (QIAGEN Company, Dusseldorf, Germany), following the manufacturers' protocol. The concentration (ng/μL) and purity (OD, 260/280) of the extracted DNA were determined by NanoDrop® ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DC, USA). The polymerase chain reaction (PCR) amplification, gel extraction, and sequencing library construction processes followed the methods described by Li et al. [24]. The universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3–V4 region of bacterial 16S rRNA gene [25], and the universal primers ITS3_KYO2 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the fungal ITS2 rRNA gene for pyrosequencing using Miseq sequencer [26]. The PCR products were

extracted using 1.0% agarose gels, purified using the SanPrep DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China), and quantified using the NanoDrop® ND1000 (NanoDrop Technologies Inc.). The equimolar purified amplicons were pooled and paired-end sequenced on Illumina MiSeq platform (MiSeq Reagent Kit v2; Illumina Inc., Santiago, MN, USA; 500 cycles). Sequencing of 16S bacteria and ITS2 fungi was carried out by the Chengdu Institute of Biology, Chinese Academy of Sciences (Chengdu, China).

2.7. In Vitro Batch Fermentation

The experiment contained four treatments, including ML, FML I, FMTL II, and FFML. The in vitro fermentation experiment was a completely randomized block design with three runs, and each sample had three fermentation bottles for each run (replicates).

The buffer solution was prepared following the method by Barry et al. [27]. The ileal and colonic chyme samples were obtained from six Ningxiang weaned pigs with an average body weight of 30 ± 1.50 kg. The pigs were euthanized by stunning electric shock (120 V, 200 Hz) to collect the ileal and colonic chyme samples. The samples were immediately placed in sterile thermos bottles prefilled with CO_2 upon collection. The ileal and colonic samples were filtered through four layers of cheesecloth and mixed with buffer medium (1:10, *w*/*v*). The CO_2 was continuously flushed into the culture medium solution for 30 min at 37 °C (pH 6.9–7.0), and then, 30 mL of culture medium solution was immediately placed into the fermentation bottles under the CO_2 stream. The fermentation bottles were placed in a constant temperature shaker (ZWY-200D; Zhicheng Instruments Co., Ltd., Shanghai, China) for 48 h at 37 °C and 55 r/min. The total gas production was recorded within 36 h.

2.8. Analysis of Short-Chain Fatty Acids, pH, Ammonia-N, and Bioamines

The concentrations of short-chain fatty acids (SCFAs; including acetate, butyrate, isobutyrate, isovalerate, propionate, and valerate) of fermented liquid were measured using GC (Agilent 7890 A, Agilent Inc., Palo Alto, CA, USA), according to the methods described by Wang et al. [28]. The pH value was measured using a pH meter (Delta 320, Mettler Company, Changsha, China). The ammonia-N (NH_3-N) concentration was measured using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan), according to the methods described by Mauricio et al. [29]. The concentrations of bioamines, including cadaverine, 1,7-diaminoheptane, phenethylamine, putrescine, spermidine, tyramine, and tryptamine, were measured in accordance with the procedure for small molecule metabolites above. The SCFAs, NH_3-N, and bioamines concentrations and pH values were measured in triplicates.

2.9. Calculations and Statistical Analysis

The dry matter degradation (DMD, g/kg of DM) was calculated as follows:

$$DMD = 1000\,[1 - W_1 \times (V_1/V_2)/W_2] \quad (1)$$

where W_1 is the DM weight of the residue after in vitro fermentation; W_2 is the DM weight of substrate before in vitro fermentation; V_1 is the volume of fermentation fluid in the bottle before sampling (i.e., 60 mL); V_2 is the volume of fermentation fluid in the bottle after sampling (i.e., 56 mL).

The logistic-exponential model [30] was used to analyze the kinetics of total gas production, and was expressed as follows:

$$GP_t = VF\,\frac{1 - exp(-kt)}{1 + exp(b - kt)} \quad (2)$$

where *GPt* is the accumulated gas production at time t (mL/g); *VF* is the final asymptotic gas production (mL/g); *k* is the fractional rate of gas production (/h); *b* is the shape parameter. Initial fractional rate of degradation at 0 h (FRD_0, /h) was calculated using the following equation:

$$FRD_0 = k/[1 + \exp\,(b)]. \quad (3)$$

The data from replicated bottles for each run were calculated and analyzed by one-way ANOVA, and the comparative analysis among the treatments was conducted using the Duncan multiple range test with SPSS 26.0 software (SPSS, Inc., Chicago, IL, USA). The data are presented as means ± SEM. Statistical significance value and trend toward difference were set at the levels of $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

3. Results

3.1. Nutritional Composition of Mao-tai Lees and Fermented Mao-tai Lees

The nutritional composition of ML and FMLs is shown in Table 1. Compared with the ML, the FML I, FML II, and FFML had lower OM (−2.91%, −4.35%, and −1.73%) content and higher CP (+1.06%, +4.63%, and +5.55%) content; the FML II and FFML had lower EE (−6.00% and −7.55%) content and higher GE (+1.42% and +3.09%) content. The EE (+15.27%) content was higher, and GE (−2.52%) content was lower in the FML I group compared with the ML group. The ADL (−13.62% and −14.24%), CF (−13.43% and −4.42%), and starch (−31.82% and −14.02%) contents were lower in the FML I and FML II groups, whereas those contents (ADL, +1.66%; CF, +4.06%; and starch, +8.38%) in FFML group were higher compared with the ML group. Moreover, the NDF (+7.05% and +9.22%) and ADF (+2.80% and +4.36%) contents were higher in the FML I and FFML groups, whereas those contents (ADF, −3.95% and NDF, −0.51%) were lower in the FML II group when compared with the ML group.

Table 1. Nutritional composition of Mao-tai lees and fermented Mao-tai lees.

Item	ML	FML I	FML II	FFML
Nutrient (g/kg of DM)				
Acid detergent fiber	364.70	374.90	350.30	380.60
Acid detergent lignin	174.80	151.00	149.90	177.70
Crude fiber	169.80	147.00	162.30	176.70
Crude protein	227.00	229.40	237.50	239.60
Dry matter	947.40	979.70	976.50	929.70
Ether extract	58.30	67.20	54.80	53.90
Gross energy (MJ/kg)	19.08	18.60	19.35	19.67
Neutral detergent fiber	432.90	463.40	430.70	472.80
Organic matter	917.70	891.00	877.80	901.80
Starch	200.50	136.70	172.40	217.30
Trace element (g/kg of DM)				
Ca	5.42	6.54	7.89	6.31
Cu	0.01	0.03	0.03	0.01
Fe	1.23	2.62	3.02	1.55
Mg	2.59	2.69	3.38	3.26
Mn	0.11	0.12	0.14	0.12
P	5.60	5.14	5.46	5.50
Vitamin B (mg/kg of DM)				
VB_1	186.95	143.05	220.04	96.93
VB_2	19.79	19.04	15.78	20.59
VB_6	40.54	29.07	44.48	47.10
VB_{12}	56.00	48.45	48.23	79.51

Data are presented as mean values of two determinations. Ca, calcium; Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; P, phosphorus; ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage.

Trace element composition showed that the FML I, FML II, and FFML had lower P (−8.21%, −2.50%, and −1.79%) content and higher Ca (+20.66%, +45.57%, and +16.42%), Cu (+200.00%, +200.00%, and +0.00%), Fe (+113.00%, +145.53%, and +26.02%), Mg (+3.86%, +30.50%, and +25.87%), and Mn (+9.09%, +27.27%, and +9.09%) contents compared with the ML.

Vitamin B composition results showed that the FML I and FFML had lower VB_1 (−23.48% and −48.15%) content, whereas the FML II had higher VB_1 (+17.70%) content

compared with the ML. The VB_2 (−3.79% and −20.26%) and VB_{12} (−13.48% and −13.88%) contents were lower in the FML I and FML II groups, whereas those contents (VB_2, +4.04% and VB_{12}, +41.98%) were higher in the FFML group compared with the ML group. Furthermore, the FML II and FFML had higher VB_6 (+9.72% and +16.18%) content, whereas the FML I had lower VB_6 (−28.29%) content compared with the ML.

3.2. Hydrolyzed Amino Acid Composition of Mao-tai Lees and Fermented Mao-tai Lees

The hydrolyzed AA composition of ML and FMLs is presented in Table 2. The FML I, FML II, and FFML had lower aspartic acid (−0.00%, −4.84%, and −7.26%), L-cysteine (−33.33%, −4.76%, and −0.00%), glutamic acid (−1.35%, −4.81%, and −1.35%), isoleucine (−2.35%, −5.88%, and −7.06%), leucine (−1.00%, −5.03%, and −7.04%), L-threonine (−1.52%, −4.55%, and −6.06%), total hydroxy AA (−0.00%, −4.03%, and −6.04%), total BCAA (−2.33%, −5.43%, and −6.98%), total acidic AA (−1.09%, −4.81%, and −2.48%), and valine (−4.85%, −5.83%, and −6.80%) contents, while had a higher arginine (+13.95, +2.33%, and +25.58%) content compared with the ML. The alanine (−4.70% and −2.01%), glycine (−3.70% and −7.41%), histidine (−3.13% and −3.13%), lysine (−1.47% and −4.41%), phenylalanine (−3.19% and −2.13%), proline (−4.12% and −4.12%), serine (−3.61% and −6.02%), total SCAA (−4.35% and −3.91%), total aromatic AA (−0.71% and −2.84%), and total AA (−4.14% and −3.36%) contents were lower in the FML II and FFML groups, whereas alanine (+34.90%), glycine (+8.64%), histidine (+9.38%), lysine (+2.94%), phenylalanine (+1.06%), proline (+0.59%), serine (+1.20%), total SCAA (+25.65%), total aromatic AA (+4.26%), and total AA (+2.88%) contents were higher in the FML I group compared with the ML group. The tyrosine (+10.64% and +4.26%) content was higher and total SAA (−13.95% and −4.65%) was lower in the FML I and FML II groups, whereas tyrosine (−4.26%) content was lower and total SAA (+2.33%) was higher in the FFML group compared with the ML group. Furthermore, the FML I and FFML had higher methionine (+4.55% and +4.55%) and total basic AA (+7.69% and +4.90%) contents, whereas the FML II had lower methionine (−4.55%) and total basic AA (−0.70%) contents compared with the ML.

Table 2. Hydrolyzed amino acid composition of Mao-tai lees and fermented Mao-tai lees (g/kg of DM).

Item		ML	FML I	FML II	FFML
Hydroxy AA	Thr	6.60	6.50	6.30	6.20
	Ser	8.30	8.40	8.00	7.80
SCAA	Gly	8.10	8.80	7.80	7.50
	Ala	14.90	20.10	14.20	14.60
BCAA	Val	10.30	9.80	9.70	9.60
	Ile	8.50	8.30	8.00	7.90
	Leu	19.90	19.70	18.90	18.50
Aromatic AA	Tyr	4.70	5.20	4.90	4.50
	Phe	9.40	9.50	9.10	9.20
Basic AA	Lys	6.80	7.00	6.70	6.50
	His	3.20	3.50	3.10	3.10
	Arg	4.30	4.90	4.40	5.40
Acidic AA	Asp	12.40	12.40	11.80	11.50
	Glu	52.00	51.30	49.50	51.30
SAA	Cys	2.10	1.40	2.00	2.10
	Met	2.20	2.30	2.10	2.30
Imino acid	Pro	17.00	17.10	16.30	16.30

Table 2. *Cont.*

Item	ML	FML I	FML II	FFML
Total Hydroxy AA	14.90	14.90	14.30	14.00
Total SCAA	23.00	28.90	22.00	22.10
Total BCAA	38.70	37.80	36.60	36.00
Total Aromatic AA	14.10	14.70	14.00	13.70
Total Basic AA	14.30	15.40	14.20	15.00
Total Acidic AA	64.40	63.70	61.30	62.80
Total SAA	4.30	3.70	4.10	4.40
Total AA	190.70	196.20	182.80	184.30

Data are presented as mean values of two determinations. AA, amino acid; Ala, alanine; Arg, arginine; Asp, aspartic acid; BCAA, branched-chain amino acid; Cys, L-cysteine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; SCAA, straight-chain amino acid; Ser, serine; SAA, sulfur-containing amino acid; Thr, L-threonine; Tyr, tyrosine; Val, valine. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage.

3.3. Long-Chain Fatty Acids Composition of Mao-tai Lees and Fermented Mao-tai Lees

The LCFA composition of ML and FMLs is shown in Table 3. Compared with the ML group, the C14:0 (+20.45% and +54.55%), C18:0 (+9.63% and +44.06%), and MUFA (+1.57% and +1.96%) percentages were higher in the FML I and FML II groups and had lower percentages of C14:0 (13.64%), C18:0 (17.42%), and MUFA (4.65%) in the FFML group, whereas the PUFA (−5.92% and −13.32%) percentage was lower in the FML I and FML II groups and higher (+2.12%) in the FFML group. The FFML had lower percentages of C14:0 (−13.64%), C18:0 (−17.42%), and MUFA (−4.65%) and had higher PUFA (+2.12%) percentages; the FML I, FML II, and FFML had higher percentages of C16:0 (+5.80%, +12.79%, and +1.30%), C16:1 (+19.54%, +75.86%, and +44.83%), C17:0 (+20.00%, +16.67%, and +273.33%), C18:3*n*6 (+127.27%, +63.64%, and +1445.46%), C20:1 (+11.18%, +18.43%, and +0.00%), C20:3*n*6 (+115.79%, +5.26%, and +442.11%), SFA (+7.74%, +19.77%, and +3.37%), and *n*-3 PUFA (+127.27%, +136.36%, and +1909.09%), whereas lower percentages of C18:1*n*9 (−0.00%, −1.84%, and −6.57%), C18:2*n*6 (−6.79%, −13.92%, and −4.89%), UFA (−2.41%, −6.17%, and −1.05%), PUFA/SFA (−12.87%, −28.07%, and −1.17%), *n*-6 PUFA (−6.83%, −13.79%, and −5.12%), and *n*-6/*n*-3 PUFA (−57.44%, −61.78%, and −95.07%) compared with the ML group. Furthermore, the FML I and FFML had higher C20:0 (+43.90% and +158.54%) and lower C20:4*n*6 (−12.50% and −62.50%) percentages, whereas the FML II had lower C20:0 (−2.44%) and higher C20:4*n*6 (+18.75%) percentages compared with the ML.

Table 3. Long-chain fatty acids (LCFAs) composition of Mao-tai lees and fermented Mao-tai lees (% of total LCFAs).

Item	ML	FML I	FML II	FFML
C14:0	0.44	0.53	0.68	0.38
C16:0	17.75	18.78	20.02	17.98
C16:1	0.87	1.04	1.53	1.26
C17:0	0.30	0.36	0.35	1.12
C18:0	4.88	5.35	7.03	4.03
C18:1*n*9	31.51	31.51	30.93	29.44
C18:2*n*6	40.08	37.36	34.50	38.12
C18:3*n*6	0.11	0.25	0.18	1.70
C20:0	0.41	0.59	0.40	1.06
C20:1	3.31	3.68	3.92	3.31
C20:3*n*6	0.19	0.41	0.20	1.03
C20:4*n*6	0.16	0.14	0.19	0.06
C22:6*n*3	0.00	0.00	0.08	0.50

Table 3. *Cont.*

Item	ML	FML I	FML II	FFML
MUFA	35.68	36.24	36.38	34.02
n-3 PUFA	0.11	0.25	0.26	2.21
n-6 PUFA	40.24	37.49	34.69	38.18
n-6/*n*-3 PUFA	350.53	149.17	133.97	17.29
PUFA	40.55	38.15	35.15	41.41
SFA	23.77	25.61	28.47	24.57
PUFA/SFA	1.71	1.49	1.23	1.69
UFA	76.23	74.39	71.53	75.43

Data are presented as mean values of two determinations. MUFA, monounsaturated fatty acid (C16:1 + C18:1*n*9 + C20:1); PUFA, polyunsaturated fatty acid (C18:2*n*6 + C18:3*n*6 + C20:3*n*6 + C20:4*n*6 + C22:6*n*3); *n*-3 PUFA, C18:3*n*6 + C22:6*n*3; *n*-6 PUFA, C18:2*n*6 + C20:4*n*6; SFA, saturated fatty acid (C14:0 + C16:0 + C17:0 + C18:0 + C20:0); UFA, unsaturated fatty acid (C16:1 + C18:1*n*9 + C18:2*n*6 + C18:3*n*6 + C20:1 + C20:3*n*6 + C20:4*n*6 + C22:6*n*3). ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage.

3.4. Small Molecule Metabolite Composition of Mao-tai Lees and Fermented Mao-tai Lees

The small molecule metabolite composition of ML and FMLs is presented in Table 4. For free AA (FAA) content, the FML I, FML II, and FFML had higher alanine (+13.35%, +5.88%, and +70.91%), aspartic acid (+27.27%, +10.61%, and +22.73%), dimethylglycine (+0.00%, +7.89%, and +38.16%), glutamic acid (+0.56%, +4.71%, and +26.74%), glutamine (+1.88%, +20.38%, and +115.01%), isoleucine (+7.83%, +7.47%, and +43.77%), leucine (+2.13%, +7.45%, and +37.77%), lysine (+0.18%, +5.42%, and +31.46%), methionine (+8.47%, +16.13%, and +43.95%), 3-methylhistidine (+0.00%, +28.57%, and +38.10%), proline (+6.34%, +4.65%, and +31.83%), and valine (+8.50%, +6.80%, and +55.44%) contents compared with the ML group. The arginine (+3.38% and +21.41%), phenylalanine (+18.75% and +38.54%), tyrosine (+11.65% and +21.36%), and total FAA (+6.63% and +25.37%) contents were higher in the FML II and FFML groups, whereas the arginine (−3.38%), phenylalanine (−2.08%), tyrosine (−3.88%), and total FAA (−2.47%) contents were lower in the FML I group compared with the ML group. Moreover, the FML I and FFML had a lower glycine (−19.59% and −23.30%) content, whereas the FML II had a higher glycine (+3.62%) content compared with the ML.

Table 4. Small molecule metabolites composition of Mao-tai lees and fermented Mao-tai lees (mg/kg of DM).

Item	ML	FML I	FML II	FFML
Free amino acids				
Alanine	6.29	7.13	6.66	10.75
Arginine	3.55	3.43	3.67	4.31
Aspartic acid	0.66	0.84	0.73	0.81
Dimethylglycine	0.76	0.76	0.82	1.05
Glycine	19.91	16.01	20.63	15.27
Glutamic acid	5.31	5.34	5.56	6.73
Glutamine	3.73	3.80	4.49	8.02
Leucine	5.64	5.76	6.06	7.77
Isoleucine	2.81	3.03	3.02	4.04
Lysine	5.53	5.54	5.83	7.27
Methionine	2.48	2.69	2.88	3.57
3-Methylhistidine	0.21	0.21	0.27	0.29
Phenylalanine	0.96	0.94	1.14	1.33
Proline	7.10	7.55	7.43	9.36
Tyrosine	1.03	0.99	1.15	1.25
Valine	2.94	3.19	3.14	4.57
Total free amino acids	68.91	67.21	73.48	86.39

Table 4. *Cont.*

Item	ML	FML I	FML II	FFML
Other free organic acids				
Acetate	7.47	5.49	8.19	21.85
Butyrate	7.16	7.29	6.20	5.74
Formate	0.44	0.31	0.30	0.33
Fumarate	0.008	0.008	0.12	0.12
Isobutyrate	26.70	28.89	24.00	27.66
α-Ketoglutarate	1.11	1.16	1.31	1.96
Lactate	197.17	202.43	133.82	126.91
Pyruvate	3.00	3.28	3.14	4.10
Succinate	11.06	12.60	11.24	15.78
Total other free organic acids	254.12	261.46	188.32	204.45
Bioamines				
Dimethylamine	1.73	1.83	1.81	2.11
Trimethylamine	1.91	1.99	1.94	2.59
TMAO	8.50	8.16	9.29	9.75
Total bioamines	12.14	11.98	13.04	14.45
Other metabolites				
Choline	4.14	4.80	5.02	6.67
GPC	5.80	5.12	6.18	5.38
Myo-inositol	2.99	3.58	3.60	3.98
α-Glucose	1.28	1.74	2.88	2.05
β-Galactose	1.61	1.74	2.05	1.48

Data are presented as mean values of two determinations. TMAO, trimethylamine-N-oxide; GPC, glycerophosphoryl choline. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage.

For other free organic acid (FOAs) content, the FML I, FML II, and FFML had higher fumarate (+0.00%, +1400.00%, and +1400.00%), α-ketoglutarate (+4.50%, +18.02%, and +76.58%), pyruvate (+9.33%, +4.67%, and +36.67%), and succinate (+13.92%, +1.63%, and +42.68%) contents and had lower formate (−29.55%, −31.82%, and −25.00%) content compared with the ML group. The FML II and FFML had higher acetate (+9.64% and +192.50%) content and lower butyrate (−13.41% and −19.83%) and other FOAs (−25.89% and −19.55%) contents, whereas the FML I had lower acetate (−26.51%) content and higher butyrate (+1.82%) and other FOAs (+2.89%) contents compared with the ML group. Moreover, the isobutyrate (+8.20% and +3.60%) content was higher in the FML I and FFML groups and lower (−10.11%) in the FML II group compared with the ML group. The FML I had higher (+2.67%), and the FML II and FFML had lower (−32.13% and −35.63%) lactate content compared with the ML group.

For bioamines content, the FML I, FML II, and FFML had higher dimethylamine (+5.78%, +4.62%, and +21.97%) and trimethylamine (+4.19%, +1.57%, and +35.60%) contents compared with the ML group. Furthermore, the FML II and FFML had higher total bioamine (+7.41% and 19.03%) and TMAO (+9.29% and 14.71%) contents, whereas the FML I had lower total bioamine (−1.32%) and TMAO (−4.00%) contents compared with the ML group.

For others metabolite content, the FML I, FML II, and FFML had higher choline (+15.94%, +21.26%, and +61.11%), myo-inositol (+19.73%, +20.40%, and +33.11%), and α-glucose (+35.94%, +125.00%, and +60.16%) contents compared with the ML group. Furthermore, the FML II had a higher GPC (+6.55%) content; FML I and FML II had higher β-galactose (+8.07% and +27.33%) content; FML I and FFML had lower GPC (−11.72% and −7.24%) content; and FFML had lower β-galactose (−8.07%) content when compared with the ML group.

3.5. Bacteria Composition of Mao-tai Lees and Fermented Mao-tai Lees

The bacteria composition of ML and FMLs at phylum level is shown in Figure 1A. The *Proteobacteria* (26.26–95.95%) and *Firmicutes* (2.64–69.37%) were the most dominant

phyla (>1%) in all groups, and *Bacteroidetes* and *Actinobacteria* accounted for 13.73% and 3.35% in the ML, 1.06% and 3.17% in the FML II, and 3.35% and 1.41% in the FFML groups, respectively. The FML I had higher relative abundance of *Proteobacteria* (+86.01%) and lower *Firmicutes* (−91.23%), whereas the FML II and FFML had lower *Proteobacteria* (−49.15% and −15.70%) and higher *Firmicutes* (+130.41% and +69.59%) compared with the ML group. The FML I, FML II, and FFML had lower relative abundances of *Actinobacteria* (−94.74%, −5.26%, and −57.89%) and *Bacteroidetes* (−97.44%, −92.31%, and −75.64%) compared with the ML group. Furthermore, the FML I and FML II had lower relative abundance of *Tenericutes* (−66.67% and −100.00%), whereas FFML had higher *Tenericutes* (+33.33%) compared with the ML.

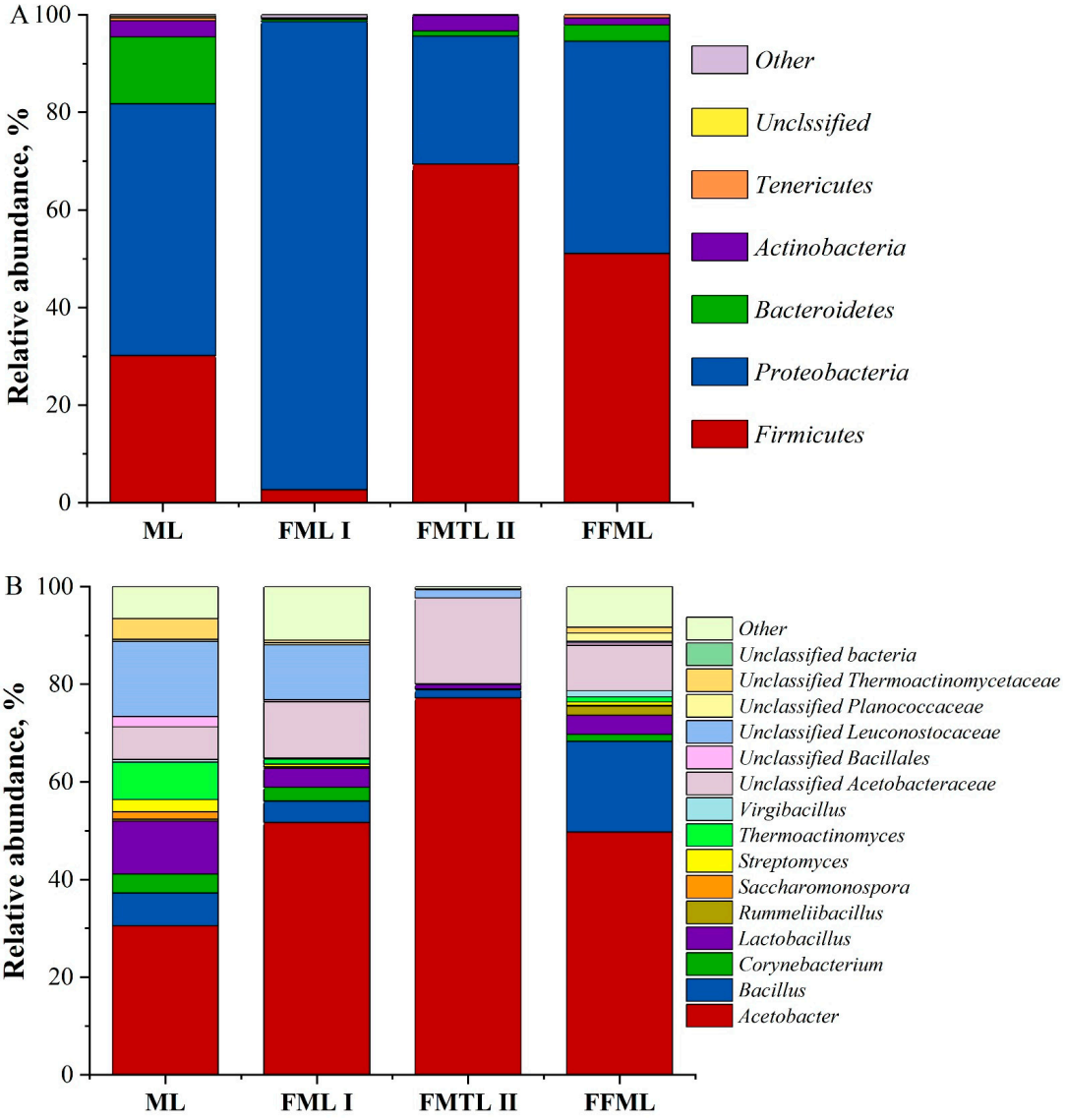

Figure 1. The bacterial composition of Mao-tai lees and fermented Mao-tai lees at the phylum (**A**) and genus (**B**) levels. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage.

The bacterial composition of ML and FMLs at the genus level is shown in Figure 1B. The bacterial genera with less than 1.0% relative abundance were excluded from further analysis. *Acetobacter* (30.53–77.20%) and *unclassified_Acetobacteraceae* (6.69–17.56%) were the most dominant genera in all groups. The FML I, FML II, and FFML had higher relative abundances of *Acetobacter* (+69.21%, +152.87%, and +62.73%) and *unclassified_Acetobacteraceae* (+73.09%, +162.48%, and +38.86%) while had lower relative abundance of *Corynebacterium*

(−27.62%, −94.63%, and −61.89%), *Lactobacillus* (−64.11%, −91.79%, and −64.02%), *Saccharomonospora* (−100.00%, −100.00%, and −86.90%), *Streptomyces* (−76.59%, −100.00%, and −73.02%), *Thermoactinomyces* (−87.08%, −98.96%, and −86.29%), *unclassified_Bacillales* (−83.41%, −100.00%, and −76.78%), *unclassified_Leuconostocaceae* (−26.83%, −88.98%, and −97.67%), and *unclassified_Thermoactinomycetaceae* (−88.44%, −99.53%, and −72.41%), when compared with the ML group. Furthermore, the relative abundance of *Bacillus* (34.83% and 75.49%), *Rummeliibacillus* (30.95% and 85.71%), and *Virgibacillus* (58.93% and 98.21%) in the FML I and FML II groups and *unclassified_Planococcaceae* (79.49%) in the FML II group were lower, whereas the relative abundances of *Bacillus* (+177.73%), *Rummeliibacillus* (+338.10%), and *Virgibacillus* (+123.21%) in the FFML group and *unclassified_Planococcaceae* (+10.26% and +335.90%) in the FML I and FFML groups were higher compared with the ML group.

3.6. Fungi Composition of Mao-tai Lees and Fermented Mao-tai Lees

The fungal composition of ML and FMLs at the phylum level is shown in Figure 2A. *Ascomycota* (57.14–99.50%) and *Basidiomycota* (excluded FML II; 1.93–13.86%) were the most dominant phyla in all groups, and *Zygomycota* accounted for 1.71% and 5.41% in the ML and FFML, respectively. Compared with the ML group, the relative abundance of *Ascomycota* (+3.31%) was higher and *Basidiomycota* (−83.81%) was lower in the FML II group; whereas the relative abundance of *Ascomycota* (−0.20% and −40.67%) was lower, and *Basidiomycota* (+91.13% and +619.07%) was higher in the FML I and FFML groups. Furthermore, the relative abundance of *Zygomycota* (+216.50%) was higher in the FFML group, whereas *Zygomycota* (−97.50% and −99.50%) was lower in the FML I and FML II groups compared with the ML group.

The fungal composition of ML and FMLs at the genus level is shown in Figure 2B. The fungal genera with less than 1.0% relative abundance were excluded from further analysis. *Aspergillus* (74.61%, 80.14%, and 62.50%) was the dominant genus in the ML, FML I, and FML II groups, whereas *Saccharomyces* (52.25%) was the dominant genus in the FFML group. Compared with the ML group, the relative abundances of *Saccharomyces* (+210.00%, +338.00%, and +10350.00%) and *unidentified_Fungi* (+366.67%, +1100.00%, and +36966.67%) were higher, whereas *Mucor* (−97.08%, −99.58%, and −2.50%), *Penicillium* (−31.12%, −80.36%, and −93.967%), *unidentified Sordariales* (−100.00%, −100.00%, and −40.42%), and *Wallemia* (−76.08%, −83.14%, and −98.43%) were lower in the FML I, FML II, and FFML groups. The relative abundance of *Aspergillus* (+7.41%) was higher in the FML I group, whereas *Aspergillus* (−16.23% and −95.39%) was lower in the FML II and FFML groups compared with the ML group. Furthermore, compared with the ML group, the relative abundance of *Pichia* (+489.64%) was higher, and *Rhodotorula* (−87.50%) and *Trichosporon* (−50%) were lower in the FML II group, whereas *Pichia* (−9.09% and −29.09%) was lower, and *Rhodotorula* (+4662.50% and +1362.50%) and *Trichosporon* (+2375.00% and +10,625.00%) were higher in the FML I and FFML groups.

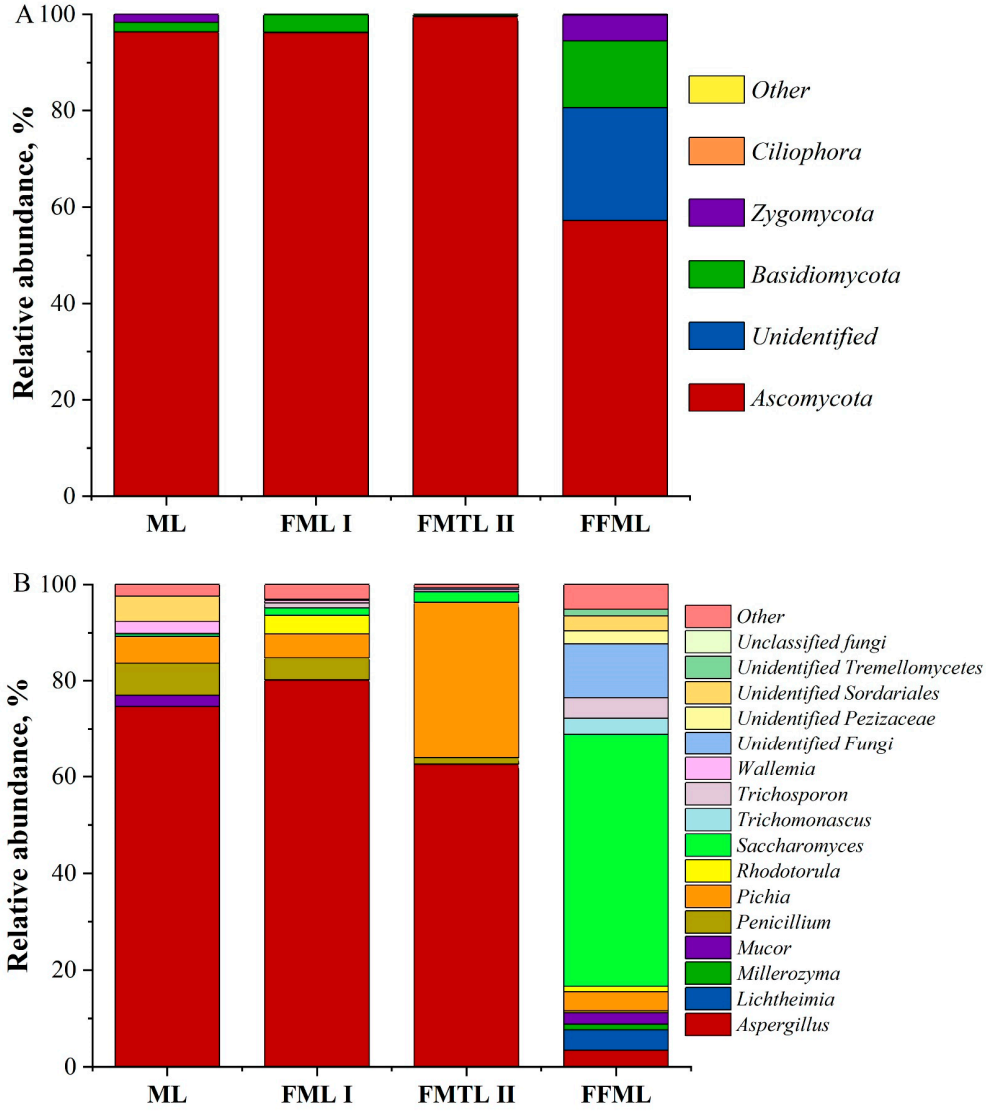

Figure 2. The fungi composition of Mao-tai lees and fermented Mao-tai lees at the phylum (**A**) and genus (**B**) levels. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage.

3.7. In Vitro Dry Matter Degradation and Gas Production Parameters of Mao-tai Lees and Fermented Mao-tai Lees

The DMD and gas production parameters of ML and FMLs in vitro fermentation are presented in Table 5. In the ileal fermentation, the DMD was lower ($p < 0.05$) in the FML I and FFML groups compared with the ML and FML II groups. The total gas production (GP) and the final asymptotic volume of total gas production (VF) were lower in the FML groups (FML I, FML II, and FFML) compared with the ML group, but the GP and VF were higher compared with the blank control group ($p < 0.05$). The FRD_0 of the FML I, FML II, and FFML groups was higher ($p < 0.05$) than the ML group in the ileum.

Table 5. In vitro dry matter degradation (DMD) and gas production parameters of Mao-tai lees and fermented Mao-tai lees in the ileum and colon.

Item	Blank	Glucose	ML	FML I	FML II	FFML	SEM	*p*-Values
Ileal fermentation								
DMD (g/kg)	-	992.40 ^{a}	401.90 ^{b}	340.20 ^{d}	399.10 ^{b}	355.90 ^{c}	66.40	<0.01
GP (mL)	38.23 ^{e}	132.65 ^{a}	122.68 ^{b}	104.75 ^{d}	111.36 ^{c}	106.77 ^{cd}	7.40	<0.01
VF (mL)	36.16 ^{e}	131.34 ^{a}	118.64 ^{b}	101.86 ^{d}	108.02 ^{c}	103.44 ^{cd}	7.36	<0.01
FRD_0 (/h)	0.20 ^{a}	0.09 ^{d}	0.15 ^{c}	0.18 ^{ab}	0.18 ^{ab}	0.19 ^{ab}	0.01	<0.01
Colonic fermentation								
DMD (g/kg)	-	976.10 ^{a}	441.60 ^{c}	413.80 ^{c}	498.60 ^{b}	345.30 ^{d}	55.60	<0.01
GP (mL)	21.68 ^{d}	136.23 ^{a}	106.53 ^{b}	89.59 ^{c}	103.45 ^{b}	91.45 ^{c}	8.48	<0.01
VF (mL)	21.72 ^{d}	133.01 ^{a}	106.11 ^{b}	87.32 ^{c}	104.19 ^{b}	90.30 ^{c}	8.31	<0.01
FRD_0 (/h)	0.25 ^{a}	0.16 ^{b}	0.09 ^{b}	0.10 ^{b}	0.08 ^{b}	0.09 ^{b}	0.02	0.02

Data are presented as mean values with their pooled SEM and *p*-values. DMD, dry matter degradation; GP, total gas production; VF, the final asymptotic volume of total gas production; FRD_0, initial fractional rate of degradation at 0 h. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage; Different letters indicate significant differences ($p < 0.05$).

In colonic fermentation, the DMD was higher ($p < 0.05$) in the FML II group compared with the ML, FML I, and FFML groups. The GP and VF were lower in the FML I and FFML groups compared with the ML and FML II groups; however, those contents were higher in the ML and FML groups compared with the blank control group ($p < 0.05$). Moreover, the FRD_0 was lower ($p < 0.05$) in the ML and FMLs groups than in the blank control group.

3.8. In Vitro Fermentation Parameters of Mao-tai Lees and Fermented Mao-tai Lees

The pH, NH_3-N, and SCFAs of ML and FMLs in vitro ileal and colonic fermentation are presented in Table 6. Compared with the ML group, ileal and colonic pH were higher ($p < 0.05$) in the FML I and FFML groups, whereas colonic NH_3-N concentration was higher ($p < 0.05$) in the FML I and FFML groups and lower ($p < 0.05$) in the FML II group. Colonic total SCFA concentration was lower ($p < 0.05$) in the FML I, FML II, and FFML groups than in the ML group. There was no difference in acetate concentration among the four groups in ileal and colonic fermentation ($p > 0.10$). Ileal and colonic propionate concentration was lower ($p < 0.05$) in the FML I, FML II, and FFML groups than in the ML group. Compared with the ML group, colonic butyrate concentration was higher ($p < 0.05$) in the FFML group, whereas it was lower ($p < 0.05$) in the FML I and FML II groups. Isobutyrate and isovalerate concentrations were lower ($p < 0.05$) in the ML, FML I, and FML II groups than in the FFML group in ileal and colonic fermentation.

Table 6. In vitro fermentation parameters of Mao-tai lees and fermented Mao-tai lees in the ileum and colon.

Item	Blank	Glucose	ML	FML I	FML II	FFML	SEM	*p*-Values
Ileal fermentation								
pH	7.24 ^{a}	5.90 ^{e}	6.24 ^{d}	6.40 ^{c}	6.31 ^{d}	6.51 ^{b}	0.10	<0.01
NH_3-N (mg/dL)	30.44 ^{a}	2.58 ^{b}	28.91 ^{a}	29.16 ^{a}	27.06 ^{a}	30.55 ^{a}	2.20	<0.01
SCFAs (mg/mL)								
Acetate	2.38 ^{b}	2.22 ^{b}	3.44 ^{a}	3.34 ^{a}	3.21 ^{a}	3.44 ^{a}	0.13	<0.01
Butyrate	0.48 ^{b}	1.16 ^{a}	1.31 ^{a}	1.13 ^{a}	1.16 ^{a}	1.10 ^{a}	0.07	<0.01
Isobutyrate	0.08 ^{ab}	0.03 ^{c}	0.08 ^{ab}	0.08 ^{ab}	0.07 ^{b}	0.09 ^{a}	0.00	<0.01
Isovalerate	0.17 ^{a}	0.05 ^{c}	0.168 ^{ab}	0.16 ^{ab}	0.15 ^{b}	0.18 ^{a}	0.01	<0.01
Propionate	2.52 ^{c}	4.26 ^{a}	3.86 ^{ab}	3.43 ^{b}	3.24 ^{b}	3.39 ^{b}	0.15	<0.01
Valerate	0.43 ^{c}	1.26 ^{ab}	1.48 ^{a}	1.21 ^{ab}	1.26 ^{ab}	1.10 ^{b}	0.09	<0.01
Total SCFAs	6.06 ^{b}	8.98 ^{a}	10.35 ^{a}	9.36 ^{a}	9.10 ^{a}	9.28 ^{a}	0.38	<0.01

Table 6. *Cont.*

Item	Blank	Glucose	ML	FML I	FML II	FFML	SEM	*p*-Values
Colonic fermentation								
pH	6.94 ^{a}	5.31 ^{e}	6.02 ^{d}	6.09 ^{c}	6.08 ^{c}	6.13 ^{b}	0.11	<0.01
NH_3-N (mg/dL)	34.96 ^{c}	22.12 ^{d}	39.95 ^{abc}	43.16 ^{ab}	37.40 ^{bc}	46.61 ^{a}	2.02	<0.01
SCFAs (mg/mL)								
Acetate	0.92 ^{b}	3.48 ^{a}	3.68 ^{a}	3.49 ^{a}	3.56 ^{a}	3.66 ^{a}	0.24	<0.01
Butyrate	0.28 ^{d}	1.13 ^{a}	1.04 ^{b}	0.98 ^{bc}	0.94 ^{c}	1.06 ^{ab}	0.07	<0.01
Isobutyrate	0.07 ^{c}	0.10 ^{b}	0.10 ^{b}	0.10 ^{b}	0.09 ^{b}	0.12 ^{a}	0.00	<0.01
Isovalerate	0.14 ^{b}	0.19 ^{a}	0.15 ^{b}	0.16 ^{b}	0.14 ^{b}	0.21 ^{a}	0.01	<0.01
Propionate	0.56 ^{d}	3.48 ^{a}	3.39 ^{a}	3.20 ^{b}	3.04 ^{bc}	2.87 ^{c}	0.24	<0.01
Valerate	0.11 ^{d}	0.40 ^{a}	0.40 ^{a}	0.38 ^{b}	0.34 ^{c}	0.40 ^{a}	0.03	<0.01
Total SCFAs	2.09 ^{d}	8.79 ^{a}	8.76 ^{ab}	8.3 ^{bc}	8.10 ^{c}	8.3 ^{bc}	0.58	<0.01

Data are presented as mean values with their pooled SEM and *p*-values. NH_3-N, ammonia-N; SCFAs, short-chain fatty acids. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage. Different letters indicate significant differences ($p < 0.05$).

3.9. In Vitro Bioamine Concentrations of Mao-tai Lees and Fermented Mao-tai Lees

The bioamine concentrations of ML and FMLs in vitro ileal and colonic fermentation are presented in Table 7. In ileal fermentation, the concentrations of cadaverine, putrescine, and tryptamine in the FML II group and phenethylamine in the FML I, FML II, and FFML groups were lower ($p < 0.05$), whereas 1,7-diaminoheptane in the FML groups, putrescine in the FFML group, and tyramine in the FML I and FFML groups were higher than in the ML group ($p < 0.05$). In colonic fermentation, the concentrations of cadaverine, 1,7-diaminoheptane, and phenethylamine in the FML I group were lower, whereas 1,7-diaminoheptane and tyramine in the FFML group were higher compared with the ML group ($p < 0.05$).

Table 7. In vitro bioamine concentrations of Mao-tai lees and fermented Mao-tai lees in the ileum and colon (μg/mL).

Item	Glucose	ML	FML I	FML II	FFML	SEM	*p*-Values
Ileal fermentation							
Cadaverine	37.62 ^{a}	28.38 ^{b}	28.60 ^{b}	21.07 ^{c}	28.30 ^{b}	1.49	<0.01
1,7-Diaminoheptane	0.02 ^{d}	0.05 ^{c}	0.09 ^{a}	0.07 ^{ab}	0.08 ^{ab}	0.01	<0.01
Phenethylamine	0.14 ^{c}	0.26 ^{a}	0.21 ^{b}	0.18 ^{b}	0.20 ^{b}	0.01	<0.01
Putrescine	12.58 ^{d}	24.80 ^{b}	27.08 ^{ab}	20.54 ^{c}	29.20 ^{a}	1.74	<0.01
Spermidine	0.91 ^{a}	0.64 ^{bc}	0.67 ^{bc}	0.50 ^{c}	0.79 ^{ab}	0.04	<0.01
Tryptamine	1.35 ^{a}	1.39 ^{a}	1.29 ^{a}	0.90 ^{b}	1.51 ^{a}	0.07	0.047
Tyramine	2.56 ^{e}	9.17 ^{c}	12.16 ^{a}	8.26 ^{d}	11.24 ^{b}	0.97	<0.01
Colonic fermentation							
Cadaverine	1.85 ^{a}	0.70 ^{bc}	0.41 ^{d}	0.63 ^{bc}	0.88 ^{b}	0.16	<0.01
1,7-Diaminoheptane	-	0.094 ^{b}	0.065 ^{c}	0.085 ^{bc}	3.00 ^{a}	0.29	<0.01
Phenethylamine	0.21 ^{a}	0.10 ^{b}	0.07 ^{c}	0.08 ^{bc}	0.08 ^{bc}	0.02	<0.01
Putrescine	0.02 ^{c}	0.31 ^{ab}	0.22 ^{b}	0.28 ^{ab}	0.39 ^{a}	0.04	<0.01
Spermidine	-	-	-	-	-	-	-
Tryptamine	-	-	-	-	-	-	-
Tyramine	-	0.57 ^{b}	0.56 ^{b}	0.42 ^{b}	3.04 ^{a}	0.25	<0.01

Data are presented as mean values with their pooled SEM and *p*-values. ML, Mao-tai lees; FML, fermented Mao-tai lees; FML I, FML in the first stage; FML II, FML in the second stage; FFML, FML in the final stage. Different letters indicate significant differences ($p < 0.05$).

4. Discussion

Corn and soybeans are in short supply and expensive due to the increasing demand in recent years; thus, it is urgent to find alternatives for conventional feedstuffs. ML is the main by-product after the production and processing of sorghum, has high pro-

tein and fiber contents, a large yield, and the potential to become a feedstuff alternative. The present study evaluated the nutritional values of ML and FMLs and explored their effects on in vitro intestinal fermentation characteristics. Our results indicate that microbial fermentation could increase the nutritional composition of the ML. Furthermore, an in vitro study revealed the intestinal proteolysis characteristics of FMLs. These findings provide an effective potential strategy for ML and FMLs for further development of novel feedstuff alternatives.

We found that *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the most abundant phyla after fermentation, which is consistent with the bacterial phyla composition of many fermented foods [31,32]. However, FML I had a lower relative abundance of *Firmicutes*, while FML II and FFML had a higher relative abundance of *Firmicutes* compared with the ML group. The lower *Firmicutes* abundance in the FML I may be caused by the growth inhibition of the obligate anaerobic bacteria of the *Firmicutes* in the ventilated environment compared with the other groups. This result is consistent with a previous study, which found that *Firmicutes* abundance significantly decreased in an aerobic environment and was accompanied by an increase in *Proteobacteria* abundance [33]. In the present study, the FML I, FML II, and FFML had higher *Acetobacter* and lower *Lactobacillus* abundances than the ML group. Moreover, the FFML had a higher relative abundance of *Bacillus* than the other groups. *Bacillus* mainly decomposes macromolecular substances to produce flavor compounds [34]. Thus, the higher relative abundance of *Bacillus* in FFML might be associated with a better fermentation effect. The *Acetobacter* can produce acetate and is more competitive under acidic conditions, while the proliferation of *Lactobacillus* increases the acidity [34], which indicates that the lower *Acetobacter* and higher *Lactobacillus* abundances in FFML than in the FML II may be due to acidity-controlling ability of these bacteria species.

Furthermore, the present study showed that *Ascomycota*, *Basidiomycota*, and *Zygomycota* were the most abundant fungi in the four groups, which is consistent with the fungal phyla composition in previous studies [35,36]. The FFML had higher relative abundances of *Mucor*, *Saccharomyces*, and *Trichosporon* while had lower *Penicillium* and *Aspergillus*. *Aspergillus* and *Penicillium* can produce glucoamylase to hydrolyze starch [34], and the lower relative abundances of *Aspergillus* and *Penicillium* in FFML indicate lower starch hydrolysis. Moreover, *Mucor* has protease activity [34], while *Saccharomyces* and *Trichosporon* can produce flavor compounds [35,36]. Therefore, the higher relative abundances of *Mucor*, *Saccharomyces*, and *Trichosporon* in FFML represent a stronger fermentation effect.

Microbial fermentation of carbohydrates and proteins produces several metabolites, such as organic acids, FAAs, and volatile compounds [37]. Thus, the starch content was decreased and the EE content was increased after the microbial fermentation of FML I in the present study. However, the FFML had higher contents of ADF, ADL, CF, CP, NDF, and starch while lower DM content with the extension of fermentation time. These results were consistent with the findings by Gungor et al. [10], who reported that microbial solid-state fermentation increased the CP and CF contents and decreased the EE content of grape pomace, which was associated with enzymes produced by microorganisms and the presence of mycelia in cellulose. The lower DM content may cause a higher nutritional composition in FFML due to increased nutrient density. Moreover, the lower relative abundances of *Aspergillus* and *Penicillium* might be another reason for the higher starch content in FFML. The element compounds, such as phytate in the feed, can be decomposed into available trace elements via microbial fermentation [38], which might result in higher trace element contents in the FML I, FML II, and FFML than in the ML. In addition, we also found that microbial fermentation increased the VB_6 and VB_{12} contents in FFML, as VB_6 and VB_{12} are mainly involved in AA metabolism [39]. Thus, microbial and protease fermentation changed the nutritional composition of Mao-tai lees, which could alter intestinal fermentation characteristics.

Proteolysis is one of the main ways of protein degradation during feed fermentation, and the peptides produced in this process can be further decomposed into small peptides

and FAAs [12]. In the present study, the FML II and FFML had lower total hydrolyzed AA content and higher total FAAs content compared with the FML I and ML groups, whereas the FFML had higher total FAAs content compared with the other groups. These results were consistent with previous studies, which found that microbial fermentation increased FAA content in feed [37,40]. Among these FAAs, arginine, isoleucine, leucine, lysine, phenylalanine, and valine are essential AAs [21], and the higher contents of essential FAAs in FFML indicate that the proteolysis in ML was enhanced due to the microbial fermentation with proteases. Furthermore, AAs play an important role in food flavor. For example, alanine, aspartic acid, and glutamic acid are important for taste, and leucine and valine are important precursors of flavor compounds [21,41]. Therefore, the higher total FAAs, free alanine, free aspartic acid, and free glutamic acid contents in FFML may also increase the taste and intake of feed.

The LCFAs are the main energy sources of animals and humans and mainly synthesize structural lipids [21]. Polyunsaturated fatty acid (PUFA) is composed of *n*-3 and *n*-6 PUFA, and the PUFA status has been associated with cancer, inflammation, cardiovascular disease, and autoimmune diseases [42]. Previous studies showed that the lower *n*-6:*n*-3 PUFA ratio can promote protein metabolism in muscle [21]. In the present study, the FFML had a higher *n*-3 PUFA percentage and a lower *n*-6:*n*-3 PUFA ratio, suggesting that the FFML may have better intestinal protein fermentation characteristics and could be beneficial to intestinal health.

Microorganisms can use fermentable sugars to produce large amounts of organic acids through carbohydrate metabolism during fermentation [37]. In the present study, the FFML had higher contents of acetate, fumarate, α-ketoglutarate, pyruvate, and succinate, whereas the FML II and FFML had lower total other FOAs, butyrate, formate, and lactate contents compared with the ML group. Pyruvate is a key intermediate metabolite that is mainly produced by glucose and succinate via glycolysis and the citrate cycle [43], and it can be converted into lactate by lactate-dehydrogenase or metabolized into ethanol and acetate by pyruvate-formate lyase and pyruvate oxidase [37]; moreover, the lactate and acetate are mainly produced by *Lactobacillus* and *Acetobacter*, respectively [44,45]. The higher acetate content in FFML may be related to the higher pyruvate, succinate, and fumarate contents. However, the decrease in lactate synthesis in FFML may be due to the limitation of lactate-dehydrogenase, which is also supported by the lower relative abundance of *Lactobacillus* and higher level of *Acetobacter* in the present study. Additionally, the *Bacillus* can convert lactate to pyruvate via lactate-dehydrogenase and produce acetate [46]. The higher *Bacillus* abundance in FFML may be another possible reason for the decreased lactate content. Furthermore, α-ketoglutarate from the citrate cycle can be converted into glutamic acid by glutamate-dehydrogenase [47], and the higher α-ketoglutarate content in FFML may be associated with the higher free glutamic acid.

Trimethylamine-N-oxide (TMAO) has two degradation pathways: one is degradation to dimethylamine (DMA) and formaldehyde by TMAO demethylase, and the other is degradation to trimethylamine (TMA) by a microbial spoilage reaction [48]. Microbial enzymes can also convert choline into TMA [49]. In the present study, the higher TMAO and choline contents in FFML may lead to an increase in DMA and TMA contents. The TMA is an off-flavor compound [50]; however, the contents of TMAO, DMA, and TMA in the present study had very low levels compared to the higher TMAO level (1000 mg/kg) commonly found in aquatic products [48]. Therefore, we speculate that the lower TMA content in ML will not affect the quality of the feed. Myo-inositol is one of the most abundant forms of inositol, and it usually exists in the form of free or combined phospholipids or inositol phosphate derivatives [51]. In the present study, the FML I, FML II, and FFML had higher myo-inositol and lower P contents than the ML group, which might be a result of the existence of more myo-inositol in the form of phospholipids or inositol phosphate derivatives. Furthermore, myo-inositol has the effects of resisting oxidation and regulating carbohydrate metabolism [52], which may alter the fermentation pattern of ML in the intestine.

Compared with fiber, non-fiber carbohydrates, protein, and EE are more easily degraded by micro-organisms [53]. Carbohydrates are the main gas-producing nutrient, and the gas-producing efficiency of protein is only approximately 30% of carbohydrates [54]. In the present study, the ML and FML II had higher DMD, total gas production, and VF compared with the FFML group in ileal and colonic fermentation, whereas the ML had lower FRD_0 compared with other groups in ileal fermentation. The lower NDF and ADF contents in ML and FML II indicate that these substrates had fewer components that were difficult to degrade by micro-organisms, which may lead to higher DMD, whereas the lower CP and higher starch contents in ML may lead to a higher gas production efficiency. Moreover, the higher EE or CP content in FML I, FML II, and FFML may lead to a higher initial fermentation rate, whereas the lower degradation of CP by microbial fermentation in the colon may lead to the consistency of FRD_0, which is also proved by the lower bioamine concentrations in the colon in the present study. Overall, the FML II showed better substrate fermentation efficiency compared with the other groups because of its higher in vitro ileal and colonic degradation.

The SCFAs are the final metabolite products of carbohydrates and proteins produced by bacterial fermentation in mammalian guts [14]. In the present study, the FFML had higher concentrations of isobutyrate and isovalerate, whereas the ML had higher propionate concentrations than the other three groups in ileal and colonic fermentation. The straight-chain fatty acids (including acetate, butyrate, propionate, and valerate) are mainly produced by carbohydrates, and the branched-chain fatty acids (such as isobutyrate and isovalerate) are exclusively produced by the protein fermentation of intestinal bacteria, which come from L-leucine and L-valine, respectively. Therefore, the concentrations of isobutyrate and isovalerate in the intestine can be regarded as indicators of proteolysis [55]. The increased proteolysis of FFML may be related to its higher free leucine, valine, and total FAA concentrations. These results were consistent with the findings by Li et al. [5], who reported that dietary supplementation of microbial and protease-fermented lees increased protein utilization in weaned piglets. Thus, microbial fermentation with proteases may promote proteolysis rather than carbohydrate hydrolysis in ML.

Bioamines, including cadaverine, putrescine, and spermidine, have several physiological functions, such as regulating translation and transcription, regulating metabolism, promoting intestinal physiology, protecting the intestine and cardiovascular system, and resisting oxidation [56,57]. In the present study, the FFML had higher putrescine and spermidine concentrations in ileal fermentation and higher cadaverine, 1,7-diaminoheptane, putrescine, and tyramine concentrations in colonic fermentation compared with the other groups. Putrescine and cadaverine are the products of ornithine and lysine decarboxylation, respectively [58]. In addition, arginine can also promote putrescine production in the intestine [56]. The higher putrescine and cadaverine concentrations of FFML in colonic fermentation may reflect the increase in AA utilization, which is consistent with the increased free arginine and lysine contents. Spermidine is synthesized from putrescine and aminopropyl groups [58]. The higher ileal putrescine concentration in FFML also leads to a higher spermidine concentration. Thus, the FFML enhanced proteolysis in the intestine, which was reflected by its higher cadaverine, 1,7-diaminoheptane, putrescine, spermidine, and tyramine concentrations. However, the mechanisms of nutrients involved in intestinal proteolysis need to be further explored.

5. Conclusions

Microbial fermentation with/without proteases altered the nutritional composition of Mao-tai lees. Microbial fermentation with/without protease addition altered the in vitro intestinal proteolysis of Mao-tai lees, and microbial fermentation with protease promoted proteolysis. Thus, microbial fermentation with proteases showed a better utilization of Mao-tai lees than microbial fermentation. These findings provide an effective potential strategy for developing novel feedstuff alternatives for corn and soybean in livestock

production. Future studies are necessary to explore the association between nutritional composition changes and intestinal proteolysis.

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