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Optimal Fermentation of *Artemisia annua* Residues and Its Effects on Production Performance of Laying Hens

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Abstract: *Artemisia annua* residue (ARR) is a pharmaceutical by-product produced after the extraction of artemisinin; it is rich in protein, crude fat, vitamins, trace elements, and bioactive compounds and contains negligible anti-nutritional factors. The present study aimed to optimize the fermentation conditions of ARR, evaluate the compound and microbial compositions of fermented AAR, and explore its effects on the production performance of laying hens. A total of 288 Xinyang black-feather laying hens were randomly allocated into four treatments for 30 days, including a control group (basal diet) and a basal diet supplemented with 1%, 2%, and 4% fermented AAR, respectively. The results showed that the optimized fermentation conditions of AAR were 80% moisture content, 3% inoculation quantity, 34 °C fermentation for 6 days, initial pH at 8, and 60 mesh (sieving). The compounds of 2-furyl-5-methyl furan, deoxyartemisinin, phytol, n-hexadecanoic acid, aromandendrene, and calarene had higher contents (average 6.86%) in the fermented AAR. The bacteria of Proteobacteria and Firmicutes (average 45.18%) were the most abundant phyla, and *Acinetobacter*, *Bacillus*, and *Brevundimonas* (average 15.87%) were the most abundant genera in the fermented AAR. The fungi of Phragmoplastophyta, Vertebrata, and Ascomycota (average 30.13%) were the most abundant phyla, and *Magnoliophyta*, *Mammalia*, *Wickerhamomyces-Candida_clade*, and *Aspergillus* were the most abundant genera (average 21.12%) in the fermented AAR. Furthermore, dietary supplementation of fermented AAR increased the average daily feed intake (ADFI), egg weight, and albumen height. Dietary supplementation of 2% and 4% fermented AAR increased the laying rate, while 2% fermented AAR increased the Haugh unit and decreased the feed-to-egg ratio. Collectively, it is concluded that fermented AAR has the potential to become a phytogenic feed additive, and dietary supplementation of 2% fermented AAR had better effects on the production performance of laying hens.

Keywords: *Artemisia annua* residues; phytogenic feed additives; fermentation; production performance; laying hens

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1. Introduction

Phytogenic feed additives have been gaining more interest in animal feed in recent years [1]. Phytogenic feed additives are derived from natural plants, such as herbs, spices, fruits, and other plant parts [2]. These feed additives contain various bioactive components, such as polyphenols, alkaloids, and flavonoids, and have antimicrobial, antioxidant, growth promotion, and immune-regulatory functions [3,4]. However, with the increasing demand for phytogenic feed additives in animal husbandry and the limitation of existing resources in some countries, it is of great significance to develop novel phytogenic feed additives that are not fully utilized in the pharmaceutical industry.

Artemisia annua is an annual herb native to China that is rich in bioactive compounds, such as polyphenols and steroids, and it has long been used to treat many diseases, including plasmodium and various parasitic infections [5]. It also contains high levels of protein (16.11%), crude fat (5.89%), vitamins, trace elements, and negligible amounts of anti-nutritional factors (51.03 mg/100 g DM) such as phytate (43.05 mg/100 g DM) and tannin (0.24 mg/100 g DM) [5]. Previous studies have shown that *Artemisia annua* has the potential roles of improving growth performance, antioxidant, anti-heat stress, anti-inflammatory, and anti-coccidiosis in chickens [6–9]. Artemisinin is an effective antimalaria drug extracted from *Artemisia annua*, and its high demand has led to the rapid development of the extraction industry. However, after the production and processing of *Artemisia annua*, a large number of by-products, such as *Artemisia annua* residues (AAR), remain unused in many countries [10]. Moreover, the inadequate utilization of AAR leads to a great waste of the active ingredients of *Artemisia annua* [10], and the improper treatment of AAR may also cause environmental pollution, such as improper disposal. Therefore, the effective utilization of AAR would not only save resources but will also protect the environment. Furthermore, it would be a potential phytogenic feed additive for livestock and poultry production.

Fermentation is an effective way to mitigate the disadvantages of agricultural by-products [11]. Previous studies have also shown that microbial fermentation could significantly improve animal feed palatability, digestibility, and nutritional value [12,13]. In addition, the cell wall of *Artemisia annua* limits the dissolution of bioactive compounds [6]. Thus, the existence of a cell wall is not conducive to the utilization of AAR by livestock and poultry. To date, there has been a lack of research on the effects of AAR on poultry, and we hypothesized that fermented AAR might have positive effects on the laying performance and egg quality of laying hens. Thus, we fermented AAR with a lignin-degrading bacteria (*Bacillus amyloliquefaciens-c4*) to release the bioactive components and improve digestibility and palatability. In this study, we optimized the fermentation conditions, evaluated the compound and microbial compositions, and further supplemented the optimized fermented AAR to laying hens. This study will provide a theoretical basis for the application of AAR as a feed additive in laying hen diets.

2. Materials and Methods

2.1. Preparation of AAR and Microbial Inoculum

The AAR used in this study was provided by Fuyang Normal University, Fuyang, Anhui, China. The microbial inoculum (*Bacillus amyloliquefaciens-c4*; preservation number: CGMCC NO.15178) was provided and preserved by the Biological Inoculation Research and Development Center of Northeast Agricultural University, Harbin, Heilongjiang, China. The AAR was pulverized, and the microbial solid-state fermentation was carried out within 24 h.

2.2. Optimization of Microbial Fermentation Conditions

The basic fermentation conditions were as follows: the AAR (not sieved, pH 5.7, and moisture content of 70%) was added with 5% brown sugar and inoculated with 5% microbial inoculum, and then the AAR was fermented at 28 °C for 6 days using a constant-temperature incubator (DRP-9162, Senxin Biotechnology Inc., Shanghai, China). The optimized fermentation conditions were as follows: (1) Moisture content: the moisture content was adjusted to 60%, 70%, 80%, 90%, and 100%, and the other fermentation conditions were unchanged. (2) Inoculation: the inoculation quantity of microbial inoculum was set to 1%, 3%, 5%, 7%, and 9%, and the other fermentation conditions were unchanged. (3) Temperature: the fermentation temperature was set to 28 °C, 30 °C, 32 °C, 34 °C, and 36 °C using a constant-temperature incubator (DRP-9162, Senxin Biotechnology Inc., Shanghai, China), and the other fermentation conditions were unchanged. (4) Fermentation time: the fermentation time was set to 0, 3, 6, 9, and 12 days, and the other fermentation conditions were unchanged. (5) Initial pH value: the initial pH was adjusted to 6, 7, 8,

9, and 10 using a pH meter (Gaozhi Precision Instrument Inc., Shanghai, China), and the other fermentation conditions were unchanged. (6) Particle size: the particle size was set to 20, 40, 60, 80, and 100 mesh using sieves (ZhenXing Inc., Guangzhou, China), and the other fermentation conditions were unchanged. Finally, the optimized fermentation conditions were selected for ARR fermentation to evaluate the surface morphology, chemical and microbial composition, and further feeding effects on laying hens. Each fermentation condition had three replicates.

2.3. Chemical Compound Composition Analysis

The artemisinin content was measured as previously described by Zhang [14]. Briefly, approximately 5.0 g of fermented AAR was accurately weighed into 100 mL of petroleum ether and transferred into an ultrasonic extractor (MAS-II PLUS; Xinyi Microwave Chemistry Technology Inc., Shanghai, China) for condensation reflux extraction (50 °C for 120 min). The extracted solution was filtered through filter paper and evaporated to dryness using a rotary evaporator (Hei-VAP Advantage ML/HB/G3; Schwabach, Germany) and redissolved with 10 mL of 95% (*w*/*v*) ethanol. Then, 2.5 mL of the redissolved solution was diluted to 10 mL with 95% (*w*/*v*) ethanol, adding 0.2% (*w*/*v*) NaOH solution to 50 mL, and the solution was then incubated with a water bath at 50 °C for 30 min. The absorbance value was measured at a wavelength of 292 nm with an ultraviolet spectrophotometer (T6 New Century; Puxi General Instrument Inc., Beijing, China), and 95% (*w*/*v*) ethanol was used as the blank control. The remaining extracted solution was concentrated 100 times using a rotary evaporator (Hei-VAP Advantage ML/HB/G3; Heidolph Inc., Schwabach, Germany) and filtered through 0.22-μm filter membranes to measure the chemical composition of the fermented AAR using a gas chromatography–mass spectrometer (Agilent 7890A/5975C; Agilent Inc., Santa Clara, CA, USA). The chemical composition of the fermented AAR was analyzed in triplicate.

2.4. Observation of Surface Morphology

The surface morphology of the fermented AAR was obtained by field emission scanning electron microscopy (FESEM; model SU8010; Hitachi, Tokyo, Japan). Briefly, fragments of the fermented AAR were fixed, rinsed, dehydrated, freeze-dried, and coated with gold before scanning. The images representing the average characteristics were screened with a magnification of 1000×.

2.5. Analysis of Microbiota Composition

The total microbial DNA of the fermented AAR was extracted with a TIANamp Soil DNA Kit (Tiangen Biochemical Technology Inc., Beijing, China) according to the manufacturer's instructions. The integrity of the DNA was measured by 1% agarose gel electrophoresis. The concentration and purity of the extracted DNA were measured using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Inc., Waltham, MA, USA), and the $OD_{260/280}$ ratio was 1.7–1.9. The polymerase chain reaction (PCR) amplification processes were carried out as previously described by Li et al. [15]. The universal primers 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3–V4 region of the bacterial 16S rRNA genes, and the universal primers F (5′-CCAGCASCYGCGGTAATTCC-3′) and R (5′-ACTTTCGTTCTTGATYRA-3′) were used to amplify the V4 region of the fungal 18S rRNA genes. AMPure XP Beads (Beckmann Inc., Bremen, Germany) were used to purify the DNA, and it was then dissolved in elution buffer. The sequencing library was constructed using a NGS™ dsDNA HS Assay Kit by Qubit® 3.0 Fluorometer (Life Technologies Inc., Carlsbad, CA, USA). Finally, the qualified libraries were sequenced on an Illumina Novaseq platform with the 250 bp mode (Illumina Inc., San Diego, CA, USA). The sequencing of the 16S bacteria and 18S fungi was carried out by the Boyuezhihe Biology Science and Technology Co., Ltd. (Wuhan, China). The relative abundances of bacteria and fungi at the phylum and genus levels were

included in the statistics. The microbiota composition of the fermented AAR was analyzed in triplicate.

2.6. Animal Experiment

A total of 288 healthy 50-week-old Xinyang black-feather laying hens with a similar body weight were selected from Duoduoli Agricultural Science and Technology Co., Ltd., Fuyang, Anhui, China. After 7 days of adaptation, the laying hens were divided into four groups (0% (control), 1%, 2%, and 4% of fermented AAR; DM basis) with a completely randomized block design. Each group contained nine replicates with eight hens per replicate. All laying hens were raised outside of the Ancient West Lake Modern Agricultural Science and Technology Demonstration Park, Fuyang, Anhui, China. The experimental hens were fed at 0600 and 1800 h and had free access to water at all times. The fermented AAR was mixed uniformly with the basal diet (Table 1) before feeding. The experiment lasted 30 days.

Table 1. Ingredients and chemical compositions of the basal diet (DM basis).

Item	Value
Ingredient, g/kg	
Corn	645.08
Soybean meal	225.50
Limestone powder	88.90
DL-methionine	0.52
Premix [a]	40.00
Nutrient levels [b], g/kg	
Available phosphorus	3.30
Calcium	32.30
Crude protein	168.0
Metabolizable energy, MJ/kg	11.54
Lysine	6.70
Methionine	3.10
Total phosphorus	4.60

[a] Providing the following amounts of vitamins and minerals per kg of a complete diet (DM basis): vitamin A, 10,000 IU; vitamin D_3, 2500 IU; vitamin E, 18 IU; vitamin K_3, 1 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3.5 mg; vitamin B_{12}, 15 μg; nicotinic acid, 63 mg; pantothenic acid, 18 mg; folic acid, 0.4 mg; biotin 0.15 mg; 130 ferrum, 80 mg; cuprum, 9 mg; zinc, 70 mg; manganese, 80 mg; iodine, 0.6 mg; and selenium, 0.3 mg. [b] The data of calcium, crude protein, metabolizable energy, and total phosphorus were analyzed in triplicate according to the methods described by AOAC [16]. The data of available phosphorus, lysine, and methionine were calculated.

2.7. Determination of Laying Performance and Egg Quality

During the experimental period, eggs and feed were weighed daily by an electronic scale (PTY-B1200; Mettler Toledo Instrument Inc., Shanghai, China). The average egg weight, average daily feed intake (ADFI), feed-to-egg ratio, and laying rate (number of eggs laid daily/number of laying hens × 100) were calculated.

The longitudinal and transverse diameters of each egg were measured using a Vernier caliper (171-502; Sanliang Measuring Tool Inc., Dongguan, China) to calculate the egg shape index (transverse diameter/longitudinal diameter). The average value of the two ends and the middle part of the eggshell were measured using a micrometer screw (211–115; Sanliang Measuring Tool Inc., Dongguan, China) as the eggshell thickness without egg membranes. The albumen height, Haugh unit (calculated based on albumen height), and yolk color were determined using a multifunctional egg quality meter (EA-01; Tenovo International Inc., Beijing, China).

2.8. Statistical Analysis

The data of the optimized fermentation conditions and the production performance of the laying hens were analyzed by one-way ANOVA. The comparative analysis among the treatments was conducted using the Duncan multiple range test. All analyses were

performed using the SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). The data of the optimized fermentation conditions are presented as means and standard error; the data of the production performance of laying hens are presented as means and standard error of the mean (SEM). Statistical significance value was set at $p < 0.05$.

3. Results

3.1. Optimization of Microbial Fermentation Conditions

The results of optimization of the microbial fermentation conditions are shown in Figure 1. Compared with the AAR with an 80% moisture content, the AAR with 60%, 70%, 90%, and 100% moisture contents had a lower ($p < 0.05$) artemisinin content after fermentation. Moreover, the AAR with an 70% moisture content had a higher ($p < 0.05$) artemisinin content compared with the AAR with 60%, 90%, and 100% moisture contents (Figure 1A). The AAR with a 3% inoculation quantity had a higher ($p < 0.05$) artemisinin content after fermentation compared with the other (1%, 5%, 7%, and 9%) inoculation quantities. Compared with the AAR with a 1% inoculation quantity, the AAR with 5%, 7%, and 9% inoculation quantities had a higher ($p < 0.05$) artemisinin content after fermentation (Figure 1B). Among the different fermentation temperatures, the AAR fermented at 34 °C (0.54 mg/g of DM) and 36 °C (0.53 mg/g of DM) had a higher ($p < 0.05$) artemisinin content after fermentation compared with those with 28 °C and 30 °C fermentation temperatures (Figure 1C). Among the different fermentation times, the AAR with 6 days of fermentation had a higher ($p < 0.05$) artemisinin content than those with the other fermentation times (0, 3, 9, and 12 days). The AAR with 3 days of fermentation had a higher ($p < 0.05$) artemisinin content compared with those with 9 and 12 days of fermentation (Figure 1D). Among the different initial pH values, the AAR with pH 8 had a higher ($p < 0.05$) artemisinin content after fermentation than those with the other pH values (6, 7, 9, and 10). Additionally, the AAR with pH 7 had a higher ($p < 0.05$) artemisinin content after fermentation than those with a pH at 6, 9, and 10, while it was lower ($p < 0.05$) at pH 6 than those with a pH at 7, 8, and 9 (Figure 1E). There was no significant difference ($p > 0.05$) in the artemisinin content of the AAR with different particle sizes (20, 40, 60, 80, and 100 mesh) after fermentation (Figure 1F). The optimized fermentation conditions of the AAR were selected as an 80% moisture content, a 3% inoculation quantity, 34 °C fermentation for 6 days, initial pH at 8, and 60 mesh.

3.2. Chemical Compound Composition and Surface Morphology of Fermented AAR

The chemical composition of the fermented AAR is shown in Table 2. The compounds with less than 1.0% relative proportions and less than 50% qualitative values were excluded from further analysis. The artemisinin content of the fermented AAR was 0.88 mg/g of DM. Furthermore, 2-furfuryl-5-methylfuran had the highest proportion (12.13%) in the fermented AAR, followed by deoxyartemisinin (9.38%), phytol (7.21%), *n*-hexadecanoic acid (5.24%), aromandendrene (3.71%), calarene (3.51%), caryophyllene oxide (3.20%), octamethyl cyclotetrasiloxane (2.90%), 5,6,8,9,10,11-hexahydrobenz[a]anthracene (2.53%), 1,5,5-trimethyl-6-methylene-cyclohexene (2.14%), 4-isopropenyl-4,7-dimethyl-1-oxaspiro[2.5]octane 3a (1.92%), 9-dimethyldodecahydrocyclohepta[d]inden-3-one (1.84%), decamethylcyclopentasiloxane (1.79%), caparratriene (1.56%), alloaromadendrene (1.41%), dodecamethylcyclohexasiloxane (1.32%), octadecamethylcyclononasiloxan (1.31%), tetradecamethylcycloheptasiloxane (1.22%), 5-butyl-6-hexyloctahydro-1H-indene (1.05%), and cis-jasmone (1.01%).

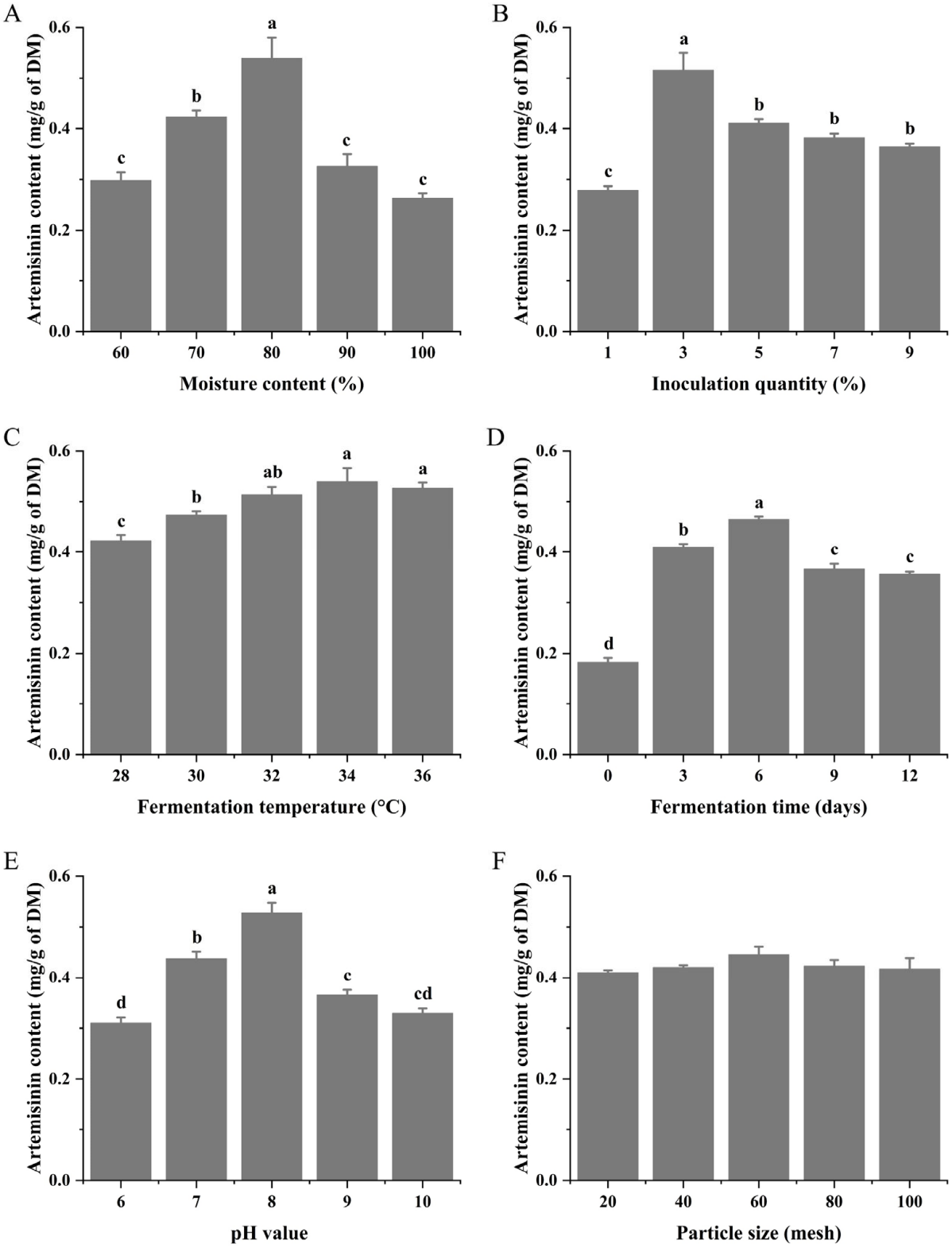

Figure 1. Effects of moisture content (**A**), inoculation quantity (**B**), temperature (**C**), time (**D**), initial pH (**E**), and particle size (**F**) on artemisinin content of *Artemisia annua* residues (AAR) during fermentation. Data are presented as means ± standard error (n = 3). $^{a–d}$ Different letters indicate significant differences ($p < 0.05$).

Table 2. Chemical compound composition of fermented *Artemisia annua* residues (AAR).

Item	Value
Artemisinin (mg/g of DM)	0.88
Chemical compound composition (% of total compounds)	
Alloaromadendrene	1.41
Aromandendrene	3.71
5-butyl-6-hexyloctahydro-1H-indene	1.05
Calarene	3.51
Caparratriene	1.56
Caryophyllene oxide	3.20
Decamethylcyclopentasiloxane	1.79
Deoxyartemisinin	9.38
3a9-dimethyldodecahydrocyclohepta[d]inden-3-one	1.84
Dodecamethylcyclohexasiloxane	1.32
2-furfuryl-5-methylfuran	12.13
n-hexadecanoic acid	5.24
5,6,8,9,10,11-hexahydrobenz[a]anthracene	2.53
4-isopropenyl-4,7-dimethyl-1-oxaspiro[2.5]octane	1.92
cis-jasmone	1.01
Octadecamethylcyclononasiloxan	1.31
Octamethyl cyclotetrasiloxane	2.90
Phytol	7.21
Tetradecamethylcycloheptasiloxane	1.22
1,5,5-trimethyl-6-methylene-cyclohexene	2.14
Others	33.61

The surface morphology of the fermented AAR is shown in Figure 2. The scanning electron microscopy analyses showed that the fermented AAR had more pores destroyed by microorganisms than the AAR.

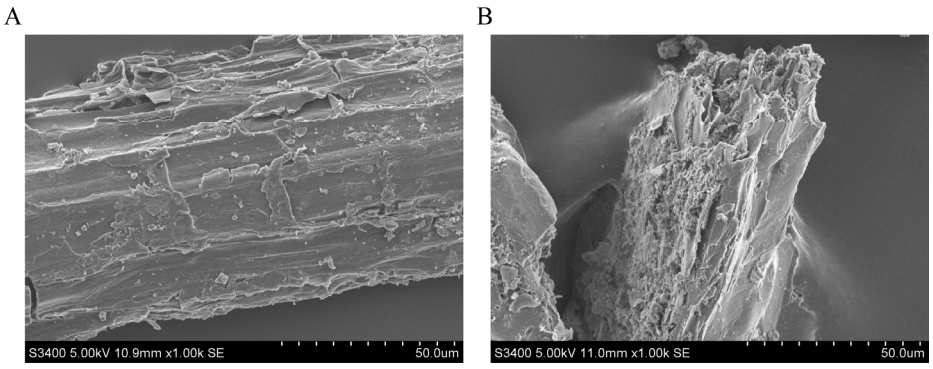

Figure 2. Scanning electron microscopy images of *Artemisia annua* residues (AAR) before (**A**) and after (**B**) fermentation.

3.3. Microbiota Composition of Fermented AAR

The bacterial composition of the fermented AAR is shown in Figure 3. The phyla with less than 1.0% relative abundances were excluded from further analysis. Proteobacteria (47.01%) and Firmicutes (43.34%) were the most dominant phyla in the fermented AAR, followed by Bacteroidota (3.09%), Actinobacteriota (2.29%), Spirochaetota (1.45%), and Deinococcota (1.27%) (Figure 3A). The genera with less than 1.0% relative abundances and those that were unassigned were excluded from further analysis. *Acinetobacter* (22.20%) and *Bacillus* (17.19%) were the most dominant genera in the fermented AAR, followed by *Brevundimonas* (8.22%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (4.53%), *Subdoligranulum* (3.01%), *Oceanobacillus* (2.66%), *Clostridia_UCG-014* (1.94%), *Porphyrobacter* (1.76%), *Enhydrobacter* (1.71%), *Faecalibacterium* (1.55%), *Treponema* (1.43%), *Ruminococcus gauvreauiiand* (1.28%), *Deinococcus* (1.27%), and *Eubacterium fissicatena* (1.27%) (Figure 3B).

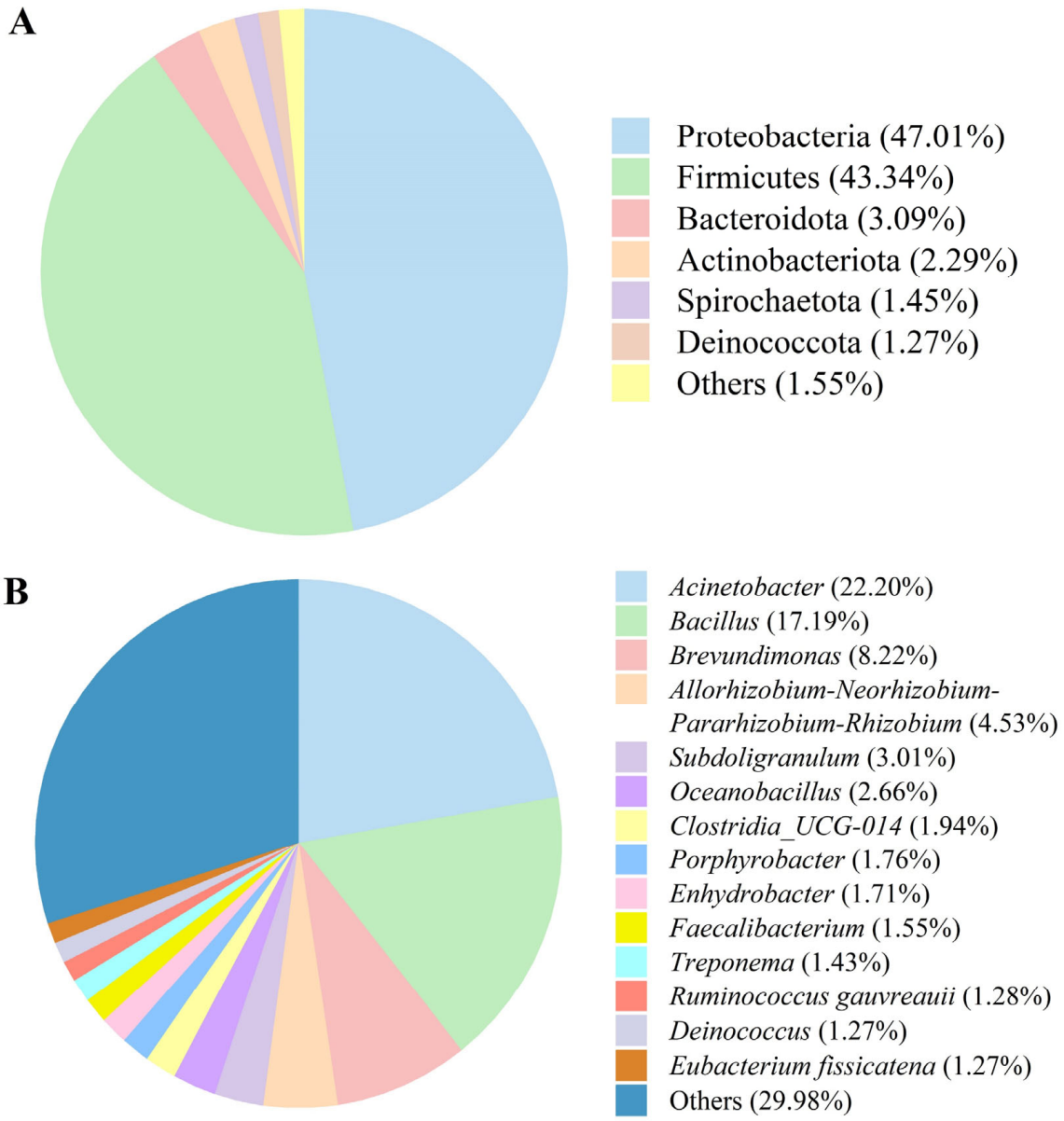

Figure 3. The bacterial composition of fermented *Artemisia annua* residues (AAR) at the phylum (**A**) and genus (**B**) levels.

The fungal composition of the fermented AAR is shown in Figure 4. The phyla with less than 1.0% relative abundances and those that were unassigned were excluded from further analysis. Phragmoplastophyta (32.37%), Vertebrata (31.32%), and Ascomycota (26.70%) were the most dominant phyla in the fermented AAR, followed by Mucoromycota (2.95%), Basidiomycota (2.56%), and Ciliophora (1.67%) (Figure 4A). The genera with less than 1.0% relative abundances and those that were unassigned were excluded from further analysis. *Magnoliophyta* (31.51%) and *Mammalia* (30.86%) were the most dominant genera in the fermented AAR, followed by *Wickerhamomyces-Candida_clade* (13.59%), *Aspergillus* (8.51%), *Beauveria* (3.75%), *Mortierella* (2.39%), and *Malassezia* (1.17%) (Figure 4B).

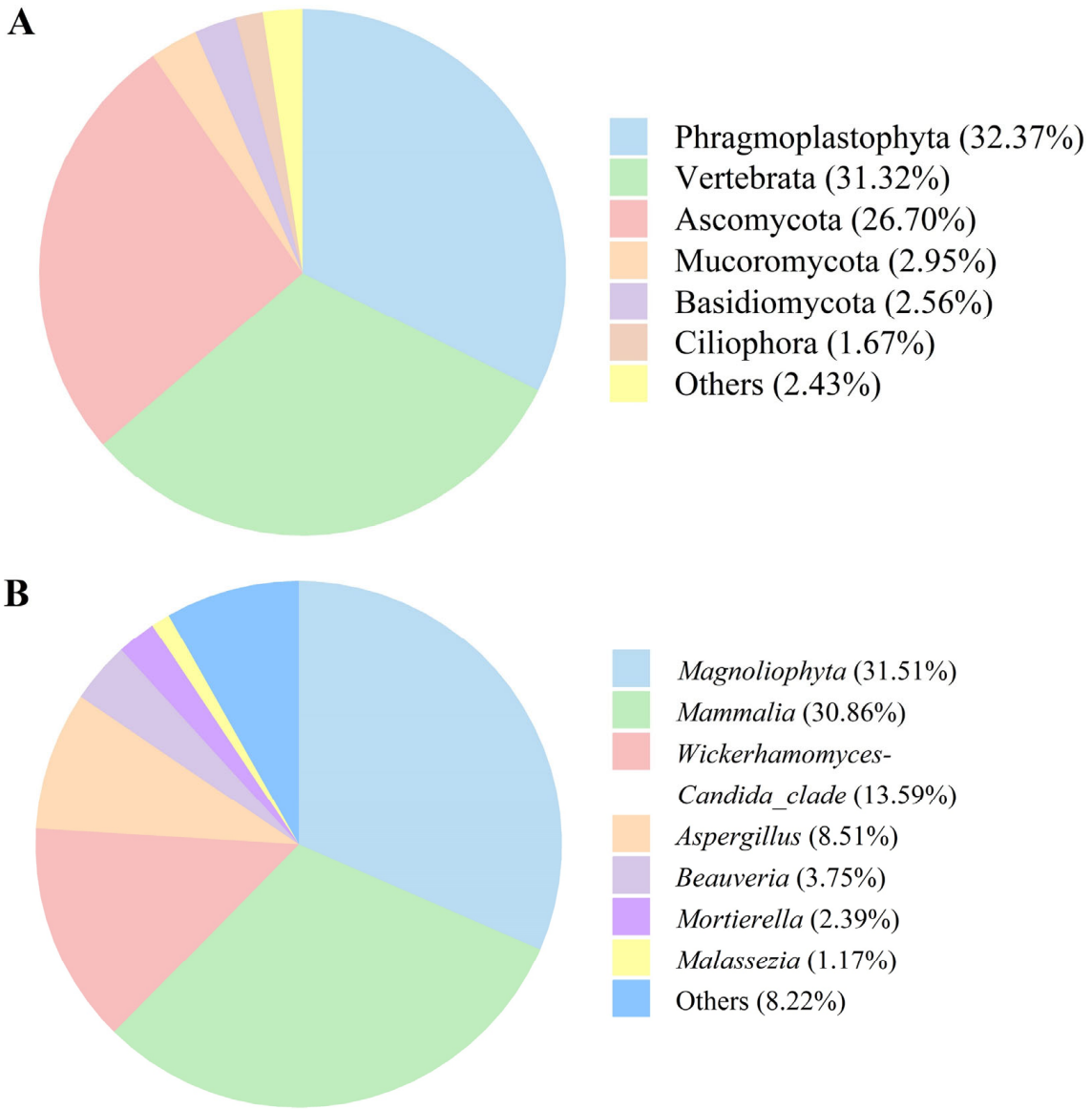

Figure 4. The fungal composition of fermented *Artemisia annua* residues (AAR) at the phylum (**A**) and genus (**B**) levels.

3.4. Laying Performance and Egg Quality

The effects of the fermented AAR on the laying performance and egg quality of laying hens are presented in Table 3. The hens fed with 1%, 2%, and 4% fermented AAR had a higher ($p < 0.05$) ADFI and egg weight, while the hens fed with 2% fermented AAR had a lower ($p < 0.05$) feed-to-egg ratio compared with the control group. The hens fed with 2% and 4% fermented AAR had higher ($p < 0.05$) laying rates compared with the control and 1% fermented AAR groups. Furthermore, the hens fed with 2% and 4% fermented AAR had a higher ($p < 0.05$) ADFI, and the hens fed with 2% fermented AAR had a higher ($p < 0.05$) egg weight when compared with the 1% fermented AAR groups.

Table 3. Effects of fermented *Artemisia annua* residues (AAR) on laying performance and egg quality of laying hens.

Item	Fermented AAR Levels in Diet, DM Basis				SEM	*p*-Values
	0%	1%	2%	4%		
Laying performance						
ADFI (g/d)	91.61 ^{c}	103.70 ^{b}	112.58 ^{a}	110.31 ^{a}	1.642	<0.001
Egg weight (g)	50.50 ^{c}	53.68 ^{b}	57.45 ^{a}	55.58 ^{ab}	0.599	<0.001
Feed-to-egg ratio	2.05 ^{a}	1.93 ^{ab}	1.81 ^{b}	1.92 ^{ab}	0.026	0.009
Laying rate (%)	71.63 ^{b}	74.41 ^{b}	84.22 ^{a}	81.85 ^{a}	1.512	0.004
Egg quality						
Albumen height (mm)	4.29 ^{b}	4.87 ^{a}	5.11 ^{a}	4.95 ^{a}	0.081	<0.001
Egg shape index	1.35	1.35	1.33	1.34	0.006	0.626
Eggshell thickness (mm)	0.36	0.37	0.38	0.38	0.003	0.051
Haugh unit	69.06 ^{b}	72.12 ^{ab}	75.88 ^{a}	72.85 ^{ab}	0.838	0.031
Yolk color	4.17	4.56	4.81	4.53	0.118	0.291

Data are presented as means with their SEM (n = 9). $^{a–c}$ Different letters indicate significant differences ($p < 0.05$). ADFI: average daily feed intake.

Regarding the egg quality traits, the 1%, 2%, and 4% fermented AAR groups had a higher ($p < 0.05$) albumen height, and the 2% fermented AAR group had a higher ($p < 0.05$) Haugh unit compared to the control group. However, there was no significant difference ($p > 0.05$) in the egg shape index, eggshell thickness, and yolk color among the different treatment group.

4. Discussion

Artemisia annua is a traditional Chinese medicinal herb with potential anti-malaria, anti-coccidiosis, anti-inflammatory, analgesic, antioxidant, anticancer, etc., effects [9,17,18]. *Artemisia annua* and its extracts have been widely used in medicine [19]; however, residues of *Artemisia annua* after extracting the effective components have not been effectively utilized. Furthermore, fermentation can effectively enrich the nutritional value and efficacy of animal feed additives. Thus, the present study optimized the fermentation conditions of AAR and evaluated the effects of the fermented AAR as a phytogenic feed additive fed to laying hens. The results demonstrated that fermented AAR had positive effects on the production performance of the laying hens. As a phytogenic feed additive, *Artemisia annua* was found to be safe for chickens at a dosage below 5% in previous studies [6,20]; thus, 1%, 2%, and 4% of fermented AAR additions (below 5%) were used in the present study.

Artemisinin is an important component in *Artemisia annua*. It has various biological effects such as anti-malaria, anti-coccidiosis, anti-tumor, and immunomodulatory effects [17,18,21]. Thus, we used the artemisinin content as a representative index to optimize the fermentation conditions of the AAR. In the present study, there was no significant difference in the artemisinin content of AAR with 34 °C and 36 °C fermentation temperatures and different particle sizes. Considering the measured value and cost, the fermentation temperature of 34 °C was more preferable due to the potential for electricity saving than the fermentation temperature of 36 °C. The particle size of 60 mesh had a numerically higher artemisinin content than other particle sizes. Thus, the optimized fermentation conditions of the AAR were considered with an 80% moisture content, a 3% inoculation quantity, 34 °C fermentation for 6 days, initial pH at 8, and 60 mesh.

We determined the compound composition of the fermented AAR and found that 2-furyl-5-methyl furan was the most abundant compound in the present study. The efficacy of furan includes anti-cancer, antidepressant, anti-anxiolytic, anti-inflammatory, antimicrobial, etc., effects [22], which may reduce the anxiety symptoms of laying hens in a closed environment and increase the production performance due to the higher content of 2-furyl-5-methyl furan in the fermented AAR. In addition, deoxyartemisinin, *n*-hexadecanoic acid, and aromandendrene have antimicrobial effects [23–25]. Phytol has antimicrobial,

anxiolytic, anticonvulsant, anti-inflammatory, and immunomodulatory effects [26], while calarene also has anxiolytic and anticonvulsant effects. Therefore, fermented AAR may effectively reduce depression, anxiety, stress, and other diseases in the production process and improve the laying rate and egg quality of laying hens. However, our findings were inconsistent with those of Mojarab-Mahboubkar and Sendi [27], who found that *Artemisia annua* contains high contents of artemisinin and artemisinic acid, which may be caused by the extraction of the bioactive components of AAR and the oxidation of artemisinin during fermentation. Previous studies have also found that *Artemisia annua* can effectively reduce the impairments of heat stress and inflammatory reaction of broilers [8,28]. Thus, our findings suggest that the fermented AAR has similar effects to *Artemisia annua*.

Proteobacteria and Firmicutes were the most abundant phyla in the fermented AAR, which was consistent with the bacterial phyla composition of *Artemisia annua* and other fermented feeds [29,30]. The genera *Acinetobacter,* with a relative abundance greater than 5%, plays an important role in umami peptide production because it can produce various proteases [31], and *Bacillus* mainly decomposes macromolecular substances to produce flavor compounds [32]. Thus, the higher abundances of *Acinetobacter* and *Bacillus* in the fermented AAR may increase the feed intake of poultry. Our results were similar to the findings of Husseiny et al. [29], who found that *Artemisia annua* also has higher relative abundances of *Acinetobacter* and *Bacillus*. *Bacillus amyloliquefaciens-c4* as a microbial inoculum in the present study may be one of the reasons for the higher relative abundance of *Bacillus*, which was also supported by the results that there were many pores that were destroyed by microorganisms in the surface morphology of the fermented AAR. In addition, *Brevundimonas* may inhibit inorganic sulfide and nitrogen oxide production [33], which may improve the quality of AAR fermentation and reduce the disease incidence in livestock and poultry production.

Meanwhile, in the present study, the higher abundances of Phragmoplastophyta and Ascomycota were consistent with other fermented feeds in a previous study by An et al. [34]. The genera *Magnoliophyta,* with a relative abundance greater than 5%, belongs to Phragmoplastophyta and widely exists in the air and environment [34], which may lead to its higher abundance in the fermented AAR. *Mammalia* belongs to the Vertebrata and is a filamentous alga [35], which may be parasitic in fermented AAR and proliferate during fermentation. *Wickerhamomyces* and *Aspergillus* can synthesize volatile components, and *Candida* produces flavor components during fermentation [36,37], which may also improve the taste and increase the feed intake of livestock and poultry. Furthermore, *Aspergillus* has been shown to have cellulase, xylanase, and antimicrobial activities [38,39], which are beneficial for destroying the cell wall of AAR and releasing active ingredients and may improve the feed digestibility of poultry. The higher relative abundance of *Aspergillus* might be another reason for more pores being destroyed by microorganisms in the surface morphology of the fermented AAR. These results were similar to the findings of Zhang et al. [38], who found that the relative abundance of endophytic *Aspergillus* was higher in *Artemisia annua*.

In order to evaluate the feeding effects, we fed the fermented AAR to laying hens. The results showed that supplementation of fermented AAR increased the laying performance of the laying hens, which might have been caused by the antimicrobial activity of the fermented AAR and the reduction of some harmful microorganisms in the digestive tract. The increased flavor and digestibility of AAR during fermentation may be another reason for the improved laying performance of the laying hens. Similarly, Brisibe et al. [40] found that supplementation of *Artemisia annua* increased the feed intake, weight gain, and laying rates in poultry. Therefore, our findings suggest that fermented AAR has similar effects to *Artemisia annua* for laying hens.

The Haugh unit and albumen height are important indexes for measuring the internal quality of eggs. The higher Haugh unit in the 2% fermented AAR group of the present study was consistent with the findings of Lee et al. [41], who found that the supplementation of *Artemisia annua* increased the Haugh unit of eggs (3 weeks), which may have been caused by

the active substances in the fermented AAR that improve the oxidative stability and prolong the freshness period of eggs. However, we found that there was no significant difference in yolk color, which was not consistent with Baghban-Kanani et al. [20], who found that supplementation of *Artemisia annua* led to an increase in yolk color, which might be due to the degradation of pigments caused by fermentation or the low content of pigments in the AAR in the present study. Overall, our findings indicated that supplementing fermented AAR had beneficial effects on the production performance of laying hens, and the 2% fermented AAR had better effects, which may be related to the optimization of the compounds and microbial compositions of AAR after fermentation, and it also enhanced the immunity, feed intake, and digestive performance of the laying hens.

5. Conclusions

The optimized fermentation conditions of the AAR in this study were considered as an 80% moisture content, 3% inoculation quantity, 34 °C fermentation for 6 days, initial pH at 8, and 60 mesh. Supplementing with fermented AAR had positive effects on the production performance of laying hens, including an increased ADFI, egg weight, laying rate, albumen height, and Haugh unit, whereas a decreased feed-to-egg ratio was also observed. These findings indicate that fermented AAR is an effective phytogenic feed additive and has similar effects to *Artemisia annua* for laying hens. However, future in-depth studies of AAR on poultry production are necessary to explore the safety of fermented AAR on laying hens and consumers.

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