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# Effect of soil contamination and additives on fermentative profile, microbial community and iron bioaccessibility of alfalfa silage

Tingting Jia[1*†], Ying Luo[2,3†], Lei Wang[2] and Zhu Yu[2,4*]

## Abstract

**Background** During alfalfa harvesting and preservation, it is important to minimize losses and preserve dry matter and nutrients. Soil contamination of alfalfa forage is a common issue that occurs during the ensiling process. Soil contamination can adversely influence the quality of silage, potentially altering the fermentation process, microbial composition, and iron content.

**Results** In this study, different levels of soil (0, 1.5% or 7.5% contamination on a wet basis; silt loam soil) and two types of additives (LP, *Lactobacillus plantarum* inoculant alone; MLP, combination addition of molasses and *Lactobacillus plantarum*) were added to alfalfa and subjected to anaerobic fermentation for 45 days to evaluate the iron content as well as the alpha diversity and relative abundance of bacterial and fungal communities. Soil-contaminated alfalfa contained lower levels of LA (14.2–41.8 g $kg^{-1}$ DM) and higher levels of AN (50.0–156.4 g $kg^{-1}$ DM) compared to uncontaminated alfalfa. Soil contamination of alfalfa forage increased the abundance of Clostridia, Actinobacteria, and Alphaproteobacteria in silage. The application of LP or MLP in soil-contaminated silage increased the abundance of *Lactobacillus* and inhibited the growth of *Enterococcus faecium*, *Pediococcus pentosaceus*, unclassified_f_Enterobacteriaceae, and *Weissella cibaria*. In addition, as the level of soil contamination increased, both the total and bioaccessible iron contents in alfalfa silage increased. The dominant bacteria *Lactobacillus plantarum* exhibited a positive relationship with LA and bioaccessible iron contents and a negative relationship with pH, AN and BA. The dominant fungi *Neocosmospora rubicola* showed a positive relationship with total iron, BA, AN and pH.

**Conclusions** Soil contamination of alfalfa increased the abundance of Clostridia, Actinobacteria, and Alphaproteobacteria and it also increased the total and bioaccessible iron content in silage. The addition of LP and MLP improved the fermentation quality of soil-contaminated silage by increasing LA production and reducing the relative abundance of *Enterococcus faecium*, *Pediococcus pentosaceus*, unclassified_f_Enterobacteriaceae, and *Weissella cibaria*.

**Keywords** Additives, Alfalfa silage, Iron content, Microbial community, Soil contamination

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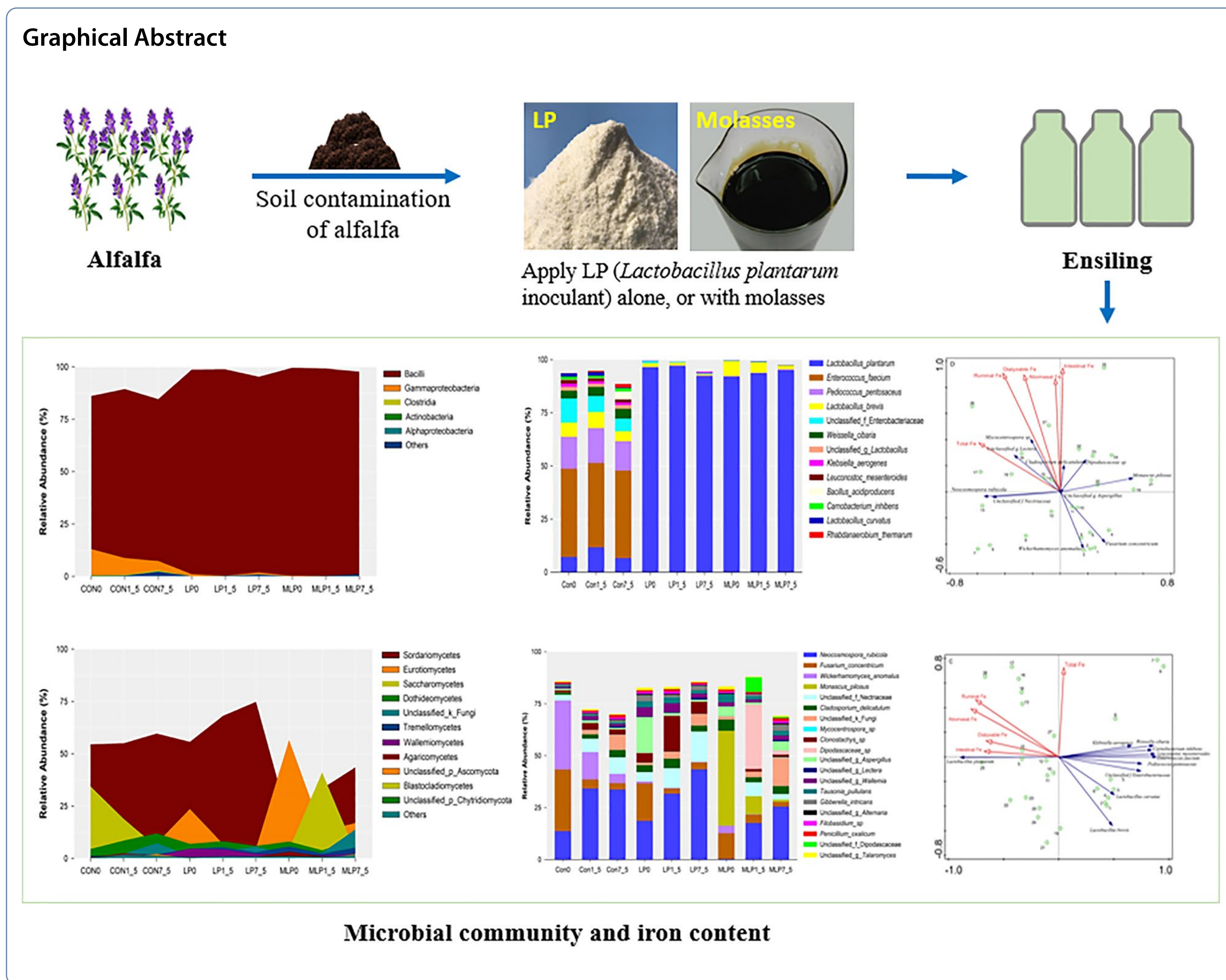


## Introduction

The preservation of alfalfa feed through anaerobic fermentation is a dependable strategy on livestock farms, as it is less susceptible to weather fluctuations and facilitating long-term storage. The essence of fermentation process lies the conversion of soluble carbohydrates into lactic acid by lactic acid bacteria (LAB) under anaerobic conditions, thereby reducing the pH level and inhibiting the growth of most aerobic spoilage bacteria [1]. Due to its high protein content, alfalfa has been extensively utilized in ruminant diets. During the anaerobic fermentation of alfalfa forage, it is crucial to minimize losses and ensure safety. However, factors such as soil contamination can influence the anaerobic fermentation process.

Soil is an important source of contamination during forage fermentation [2]. In China, soil contamination of forage is inevitable and frequent occurrence during ensiling. Factors such as harvesting, drying, picking, and shredding on unevenly planted land and mechanical processes that can disturb the soil surface, lead to an increase in soil contamination of alfalfa. Soil contamination can impair forage quality and decrease the digestibility of ingested organic matter [3, 4]. The soil contamination rate of fermented forage can reach as high as 8% (*wt/wt*) [2] and can easily increase the mineral content, especially iron in silage [5–7]. The impact of soil contamination on iron content is particularly significant because the iron content in soil is significantly higher than in plants [8, 9]. Healy [10] demonstrated that iron derived from soil in the gastrointestinal fluid of ruminants may be partially soluble, and the released ions could be absorbed or influence the mineral composition of the digestive fluid. Ruminants can tightly control iron metabolism through homeostasis mechanisms; excess iron can reduce cattle and sheep feed intake [6], lower feed utilization and daily gain, and increased competition for intestinal absorption with manganese and copper, negatively affecting the status of these essential trace

minerals in ruminants [11, 12]. But the effects of soil contamination on the iron content and composition of alfalfa silage remain unclear.

Microorganisms play a crucial role in the anaerobic fermentation of alfalfa forage. Aerobic microorganisms in silage remain active until oxygen is depleted, and acidification inhibits microbial metabolism [13]. In well-preserved forage, facultative anaerobic bacteria gradually lower the silage pH, promoting the growth of acid-tolerant lactic acid bacteria, which dominate the microbial community in properly fermented silage [14]. Among the many microbial species found in silage, some are considered undesirable as their presence can reduce dry matter (DM), leading to unfavorable fermentation, such as the production of butyric acid or toxins, posing adverse effects on the health of livestock and humans [15]. Former studies have explored the variations in bacterial flora among different silages [16, 17] and the spatiotemporal changes in microbial communities within silage [18]. Soil contamination can introduce additional microbial populations into the fermentation process, and some of these microbes might compete with or inhibit the growth of desirable anaerobic bacteria. A better understanding of the effects of soil contamination on microbial communities could provide further insights into improving methods for alfalfa silage preservation.

Therefore, in the present study, different levels of soil and two types of additives were added to alfalfa and subjected to anaerobic fermentation to evaluate the iron content as well as the alpha diversity and microbial communities. This study will provide guidance and suggestions for improving the quality and safety of alfalfa silage.

## Methods

### Experimental design and silage preparation

The experiment was conducted in Hujiachi Town, Hengshui City, Hebei Province. Alfalfa was harvested at the bud stage in May 2021 and wilted to 325.4 g kg$^{-1}$ dry matter. It was then chopped to a 1–2 cm theoretical length for silage preparation. A 3 × 3 factorial design with a control was employed to investigate the effects of soil contamination on the fermentation characteristics, iron content, and microbial composition of alfalfa silage. Factors included the level of soil contamination (0, 1.5%, and 7.5% on a wet basis; silt loam soil) and two additives: (i) *Lactobacillus plantarum*; (ii) a combination of molasses and *Lactobacillus plantarum*. We defined the soil contamination level in silage according to the study of Rafferty et al. [2], which found the soil contamination rate of fresh forage samples collected from the field before harvest was lower than 2% (*wt/wt*), while the soil contamination rate of samples collected after four months of harvest and storage was as high as 8% (*wt/wt*).

To simulate the impact of soil contamination on alfalfa silage, the top 5 cm soils from the alfalfa fields were air-dried and crushed before being mixed with the alfalfa materials according to the method of Hansen and Spears [5]. The soil used in present study is not polluted with heavy metals or organic matter, the soil texture was silt loam with a pH of 7.8, organic matter 14.4 g kg$^{-1}$, potassium of 11.8 mg g$^{-1}$, calcium of 29.0 mg g$^{-1}$, sodium of 0.1 mg g$^{-1}$, magnesium of 9.2 mg g$^{-1}$, copper of 13.2 μg g$^{-1}$, iron of 25,431.3 μg g$^{-1}$, zinc of 70.6 μg g$^{-1}$. The soil was thoroughly mixed with the alfalfa materials at the appropriate inclusion levels (0, 1.5%, and 7.5%) before fermentation. *Lactobacillus plantarum* was isolated by Li et al. [19] and applied at a concentration of $1.0 \times 10^6$ cfu g$^{-1}$ of fresh matter (FM) [19, 20]. The Molasses was purchased from Weifang Lvlong Biotechnology Co., LTD and was applied at a rate of 2.0 g kg$^{-1}$ of FM [21]. The two types of additives (LP, *Lactobacillus plantarum* inoculant alone; MLP, a combination of molasses and *Lactobacillus plantarum*) were dissolved in deionized water. Then, 10 mL of the solution per kg of alfalfa material was applied. The control was sprayed with an equal volume of deionized water. The 1 L plastic jars with screw-top lids were used as experimental silos. After treatment application, each silo was packed with alfalfa materials. Triplicate silos for each treatment were sealed and allowed to ferment at ambient temperature (25 ℃ ± 1 ℃). On day 45, the silos were opened, and the top 5 cm of oxygen-exposed fermented materials were removed.

### Fermentation characteristics and iron content analysis

The pH value, ammonia nitrogen (AN), and organic acid contents of each sample were determined. Samples (20 g) from each silo were added to 180 mL of deionized water and homogenized using a juice squeezer for 2 min. The slurry was then strained through four layers of cheesecloth and one layer of qualitative filter paper. The pH of the supernatant was measured using a pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd., Shanghai, China). The lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were determined using high-performance liquid chromatography (HPLC) (Liu et al., 2013). The analysis was performed using a Shodex RS pak KC-811 column from Showa Denko K.K. in Kawasaki, Japan. A diode array detector (DAD) with a wavelength of 210 nm (SPD-M10AVP, Shimadzu Co., Ltd., Kyoto, Japan) was used. The eluent consisted of 3 mmol/L $HClO_4$, and the flow rate was set at 1.0 mL min$^{-1}$. The column temperature was maintained at 50 ℃. AN was determined using the phenol and sodium hypochlorite method [22].

Approximately 100 g samples of each silo were dried at 65 °C for 48 h to measure the iron content. The dried samples (0.5 g) were wet digested for iron content analysis using a mixture of nitric, sulfuric, and perchloric acids (8:1:1, *v/v/v*), following the method described by Fischer and L'abbé [23]. The total iron content of all samples was determined by ICP-MS (PlasmaQuant-MS Elite, Germany).

#### Bioaccessible iron content analysis

The simulated in vitro digestion system was used to analyze the bioaccessible iron content with modifications based on the method described by Hansen and Spears [5]. A mixture of enzymes was used to simulate rumen digestion because rumen fluid is highly variable and contain high contents of trace minerals. Dried samples (0.5 g) were added to a 50 mL flask containing 30 mL of acetic acid buffer. Cellulase (EC 3.2.1.4) from *Aspergillus niger* was added at a concentration of 10 units/mL of buffer. Then, hemicellulase (EC 232–799-9) from *A. niger* was included at a level of 1.67 units/mL of buffer. Additionally, 50 μL of heat-stable amylase (EC 3.2.1.1) was added to each flask. The flasks were sealed with parafilm and placed in a 39 °C water bath. They were then oscillated at a rate of 30 times per minute for 24 h. After the 24 h of digestion period, a 3 mL aliquot of the fluid was centrifuged at 580×*g* for 5 min to separate solid components before conducting iron content analysis.

The abomasal stage follows ruminal digestion. Approximately 3 mL of a 5% pepsin solution (388 units/mg; EC 3.4.23.1) in 1 N HCl was added to each flask, resulting in a buffer pH below 2.5. The flasks were then sealed with parafilm and placed in a 37 °C water bath. They were oscillated at a rate of 30 times per minute for 1 h. After 1 h of digestion, a 3 mL aliquot of the fluid was extracted following the previously described method. Intestinal digestion followed the abomasal stage, and the pH was adjusted to 6.8 by adding 1 M NaOH. Subsequently, 0.4 mL of a 10% trypsin (EC 232–468-9) solution was added to each flask. The flasks were again oscillated in a 37 °C water bath, with a frequency of 30 oscillations per minute, for a duration of 2 h. The dialyzable iron content was measured using a dialysis tube with a molecular cut-off of 15,000 Da (Spectra/Por 7 Dialysis Membrane, Spectrum Laboratories, Rancho Dominguez, CA). After the process of intestinal digestion, the flasks were inverted and then transferred into a 50 mL polypropylene tube. Subsequently, they were centrifuged at 580×*g* for a duration of 10 min. Approximately 8 mL of the supernatant was transferred into a 50 mL dialysis tube segment, which was approximately 8 cm in length. The catheter was suspended in a 600 mL beaker filled with 500 mL of deionized water and covered with a large sealing film. The sample was placed in an oscillating water bath at 37 °C for a duration of 2 h to simulate the approximate retention time of digestive juices in the intestinal tract. The soluble mineral elements in the intestinal supernatant and dialysate were analyzed. The dialyzable iron content was determined as the amount of soluble iron that disappeared from the dialysis tubing during the 2 h time period (initial supernatant iron content—dialysis tubing iron content). The iron contents of ruminal, abomasal, intestinal, and digestion and dialyzable solutions were determined using ICP-MS (PlasmaQuant-MS Elite, Germany).

#### Microbial community analysis

##### *DNA extraction and PCR amplification*

The microbial total DNA extraction of all samples was performed using the method described by Zheng et al. [24]. The sample (20 g) was mixed with 80 mL of sterile water and stirred at 120 rpm for 2 h at 4 °C. The samples were filtered through two layers of sterile gauze and then centrifuged at 10,000×*g* for 15 min at 4 °C. The pellet was washed three times in sterile water and then used to extract the total genomic DNA. Microbial community genomic DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to the manufacturer's instructions. The DNA extract was checked on a 1% agarose gel, and the DNA concentration and purity were determined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA).

The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified using primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95 ℃ for 3 min, followed by 27 cycles of denaturing at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 45 s, and single extension at 72 ℃ for 10 min, followed by cooling to 4 ℃. The PCR mixtures consisted of 5×FastPfu buffer (4 μL), 2.5 mM dNTPs (2 μL), forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, FastPfu Polymerase 0.4 μL, BSA 0.2 μL, template DNA (10 ng), and $ddH_2O$ (up to 20 μL).

The PCR primers targeting the full-length ITS rRNA were ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′). The PCR amplification of the ITS rRNA gene was performed as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, along with a single extension at 72 °C for 10 min, and the

reaction was then cooled to 4 °C. The PCR mixtures contained 10 × buffer (2 μL), 2.5 mM dNTPs (2 μL), forward primer (5 μM, 0.8 μL), reverse primer (5 μM, 0.8 μL), rTaq Polymerase (0.2 μL), BSA (0.2 μL), template DNA (10 ng,) and $ddH_2O$ (up to 20 μL).

PCRs were performed in triplicate. The PCR product was extracted from a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), following the manufacturer's instructions. The purified product was then quantified using a Quantus™ Fluorometer (Promega, USA).

#### *Illumina MiSeq sequencing*

Purified amplicons were pooled in equimolar amounts and paired-end sequenced using an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) following the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database with the following accession Numbers: PRJNA907969 and PRJNA908062.

#### *Processing of sequencing data*

The raw 16S rRNA and ITS rRNA gene sequencing reads were demultiplexed and quality-filtered using fastp version 0.20.0 [25]. The reads were then merged using FLASH version 1.2.7 [26] according to the following criteria: (i) reads with an average quality score of less than 20 over a 50 bp sliding window were truncated at that site, and any resulting truncated reads shorter than 50 bp were discarded. Reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled based on their overlapped sequence. The maximum mismatch ratio of the overlapping region is 0.2. Reads that could not be assembled were discarded; and (iii) Samples were distinguished based on the barcode and primers, and the sequence direction was adjusted to ensure an exact barcode match, allowing for a 2-nucleotide mismatch allowed in primer matching.

Operational taxonomic units (OTUs) were clustered using UPARSE version 7.1 [27], with a 97% similarity cutoff. Chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed using RDP Classifier version 2.2 [28] against the 16S rRNA database (e.g., Silva v138) with a confidence threshold of 0.7.

### Statistical analysis

The fermentation characteristics and data on total and bioaccessible iron content were analyzed using analysis of variance (ANOVA) with the general linear model-univariate procedure of SPSS 19.0 software. ANOVAs were performed for soil contamination and additive treatments as the two main factors, as well as for the interaction between these two factors. The mean values were compared using Duncan's multiple range tests. Differences between means were considered significant when $P < 0.05$. We assessed the diversity of the community using the Shannon index. The Shannon indices were calculated using Mothur (v.1.30) and analyzed using the Wilcoxon rank-sum test for estimators at the OTU level. Bar plots depicting bacterial and fungal communities at the class and species levels were generated using Simplot 12.5 software. The Circos graph was constructed using Circos software [29]. Redundance analysis (RDA) was used to analyze the correlation of microbial community and silage characteristics by CANOCO 5.0.

## Results

### Fermentation characteristics

The fermentation characteristics of the alfalfa silage are shown in Table 1. Increasing level of soil contamination from 0 to 7.5% resulted in an increase in pH value, BA, and AN contents, while decreasing the LA, AA, and PA contents. Compared to the control group, the application of LP and MLP significantly decreased the pH, BA, and AN contents, while increased the LA content of alfalfa silage ($P < 0.05$). The PA content of MLP silage was significantly higher than that in the control and LP silage ($P < 0.05$).

### Total and bioaccessible iron content

The soil contamination significantly influenced the total and bioaccessible iron contents of alfalfa silage (Table 2). With the increasing level of soil contamination from 0 to 7.5%, the total iron content increased dramatically. Bioaccessible iron contents following simulated ruminal digestion were also affected by soil contamination levels and additives (Table 2). Increasing level of soil contamination from 0 to 7.5% resulted in an increase in the bioaccessible iron content in alfalfa silage. The ruminal, abomasal, intestinal, and dialyzable digestible iron contents of 7.5% soil level were higher than those in 0 and 1.5% soil contamination treatments. The ruminal, abomasal, and intestinal iron contents in LP and MLP silage were significantly higher than those in the control silage ($P < 0.05$).

### Alpha diversity of the microbial community

Good's coverage estimation revealed that > 99% of the species were obtained in all alfalfa silage samples (Additional file 1: Table S1), indicating that all samples reached sufficient coverage. Alpha diversity of the microbial community in alfalfa silage is shown in Figs. 1 and 2. The Shannon index of bacteria in the control

**Table 1** The fermentation characteristics of alfalfa silage

| Item | Soil level (%) | Additives | | | SEM | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | | Con | LP | MLP | | S | A | S × A |
| pH values | 0 | $5.93^{a} \pm 0.05$ | $4.95^{bB} \pm 0.04$ | $4.71^{bB} \pm 0.19$ | 0.018 | < 0.001 | < 0.001 | 0.071 |
| | 1.5 | $5.91^{a} \pm 0.08$ | $4.94^{bB} \pm 0.01$ | $4.82^{cAB} \pm 0.02$ | | | | |
| | 7.5 | $5.95^{a} \pm 0.01$ | $5.18^{bA} \pm 0.04$ | $5.06^{bA} \pm 0.07$ | | | | |
| LA ($g\ kg^{-1}$ DM) | 0 | $17.3^{bAB} \pm 1.47$ | $39.4^{abA} \pm 1.29$ | $46.8^{a} \pm 9.75$ | 1.641 | 0.548 | < 0.001 | 0.952 |
| | 1.5 | $20.1^{bA} \pm 0.60$ | $37.3^{aAB} \pm 0.16$ | $41.8^{a} \pm 5.95$ | | | | |
| | 7.5 | $14.2^{bB} \pm 0.25$ | $35.6^{aB} \pm 0.63$ | $40.6^{a} \pm 3.18$ | | | | |
| AA ($g\ kg^{-1}$ DM) | 0 | $14.1^{a} \pm 0.58$ | $10.4^{bA} \pm 0.08$ | $12.9^{a} \pm 0.24$ | 0.433 | 0.094 | < 0.001 | 0.485 |
| | 1.5 | $14.6^{a} \pm 1.18$ | $7.2^{bB} \pm 0.80$ | $14.2^{ab} \pm 2.52$ | | | | |
| | 7.5 | $12.2^{a} \pm 0.93$ | $6.5^{bB} \pm 0.42$ | $11.7^{a} \pm 0.58$ | | | | |
| PA ($g\ kg^{-1}$ DM) | 0 | $8.3^{bA} \pm 0.41$ | $8.6^{bA} \pm 0.15$ | $16.4^{aA} \pm 0.46$ | 0.183 | < 0.001 | < 0.001 | 0.045 |
| | 1.5 | $5.7^{cB} \pm 0.13$ | $6.7^{bB} \pm 0.27$ | $15.8^{aA} \pm 0.01$ | | | | |
| | 7.5 | $4.6^{bB} \pm 0.30$ | $4.5^{bC} \pm 0.58$ | $11.0^{aB} \pm 0.94$ | | | | |
| BA ($g\ kg^{-1}$ DM) | 0 | $1.0^{aB} \pm 0.07$ | $0.3^{bC} \pm 0.05$ | $ND^{c} \pm 0.01$ | 0.057 | < 0.001 | < 0.001 | 0.004 |
| | 1.5 | $0.6^{aB} \pm 0.01$ | $0.4^{bB} \pm 0.07$ | $ND^{c} \pm 0.01$ | | | | |
| | 7.5 | $2.2^{aA} \pm 0.39$ | $0.9^{bA} \pm 0.03$ | $0.1^{b} \pm 0.08$ | | | | |
| AN ($g\ kg^{-1}$ TN) | 0 | $111.3^{aB} \pm 2.35$ | $51.4^{cC} \pm 0.40$ | $59.4^{bB} \pm 0.19$ | 0.724 | < 0.001 | < 0.001 | < 0.001 |
| | 1.5 | $118.0^{aB} \pm 3.64$ | $50.0^{bB} \pm 1.67$ | $55.6^{bC} \pm 0.23$ | | | | |
| | 7.5 | $156.4^{aA} \pm 1.05$ | $65.6^{cA} \pm 1.83$ | $74.9^{bA} \pm 1.45$ | | | | |

DM, dry matter; TN, total nitrogen

LA, lactic acid; AA, acetic acid; PA, propionic acid, BA, butyric acid; AN, ammonia nitrogen

Con, control; LP, *Lactobacillus plantarum* inoculant alone; MLP, combination addition of molasses and *Lactobacillus plantarum* inoculant

SEM, error of the means; S, soil contamination levels; A, additives; S × A, interaction between soil contamination levels and additives. Means within the same row (a, b) or within the same column (A, B) with different superscript differ significantly from each other ($P < 0.05$)

**Table 2** The total and bioaccessible iron contents of alfalfa silage

| Item | Soil levels (%) | Additives | | | SEM | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | | Con | LP | MLP | | S | A | S × A |
| Total iron ($\mu g\ g^{-1}$ DM) | 0 | $629.0^{B} \pm 101.7$ | $570.0^{B} \pm 54.2$ | $606.0^{C} \pm 79.5$ | 99.733 | < 0.001 | 0.033 | 0.079 |
| | 1.5 | $1180.5^{B} \pm 101.3$ | $1150.4^{B} \pm 130.0$ | $1512.9^{B} \pm 83.2$ | | | | |
| | 7.5 | $4154.6^{A} \pm 580.3$ | $2811.4^{A} \pm 323.6$ | $4476.2^{A} \pm 205.0$ | | | | |
| Ruminal iron ($\mu g\ g^{-1}$ DM) | 0 | $37.3^{bC} \pm 1.1$ | $117.7^{aC} \pm 3.6$ | $117.8^{aC} \pm 8.9$ | 3.768 | < 0.001 | < 0.001 | < 0.001 |
| | 1.5 | $59.3^{bB} \pm 1.7$ | $194.4^{aB} \pm 3.5$ | $209.3^{aB} \pm 10.2$ | | | | |
| | 7.5 | $121.7^{cA} \pm 4.6$ | $238.6^{bA} \pm 10.4$ | $344.6^{aA} \pm 20.6$ | | | | |
| Abomasal iron ($\mu g\ g^{-1}$ DM) | 0 | $4.7^{bC} \pm 0.1$ | $112.2^{aB} \pm 4.9$ | $115.6^{aB} \pm 4.0$ | 5.746 | < 0.001 | < 0.001 | 0.017 |
| | 1.5 | $44.8^{bB} \pm 3.9$ | $128.4^{aB} \pm 2.2$ | $134.0^{aB} \pm 6.6$ | | | | |
| | 7.5 | $63.0^{bA} \pm 0.7$ | $249.2^{aA} \pm 7.4$ | $272.6^{aA} \pm 40.3$ | | | | |
| Intestinal iron ($\mu g\ g^{-1}$ DM) | 0 | $11.5^{cB} \pm 0.1$ | $50.1^{bB} \pm 0.3$ | $101.4^{aC} \pm 4.5$ | 2.160 | < 0.001 | < 0.001 | < 0.001 |
| | 1.5 | $37.3^{cA} \pm 2.6$ | $76.6^{bA} \pm 8.7$ | $164.4^{aB} \pm 3.6$ | | | | |
| | 7.5 | $42.3^{cA} \pm 2.0$ | $89.4^{bA} \pm 0.7$ | $253.6^{aA} \pm 11.5$ | | | | |
| Dialyzable iron ($\mu g\ g^{-1}$ DM) | 0 | $6.4 \pm 0.1$ | $10.0 \pm 2.3$ | $19.8^{C} \pm 4.2$ | 2.097 | 0.007 | < 0.001 | 0.288 |
| | 1.5 | $7.6^{b} \pm 1.7$ | $28.3^{ab} \pm 8.4$ | $35.8^{aB} \pm 2.8$ | | | | |
| | 7.5 | $10.5^{b} \pm 1.7$ | $30.6^{ab} \pm 11.4$ | $50.9^{aA} \pm 1.0$ | | | | |

DM, dry matter

Con, control; LP, *Lactobacillus plantarum* inoculant alone; MLP, combination addition of molasses and *Lactobacillus plantarum* inoculant

SEM, error of the means; S, soil contamination levels; A, additives; S × A, interaction between soil contamination levels and additives; Means within the same row (a,b) or within the same column (A, B) with different superscript differ significantly from each other

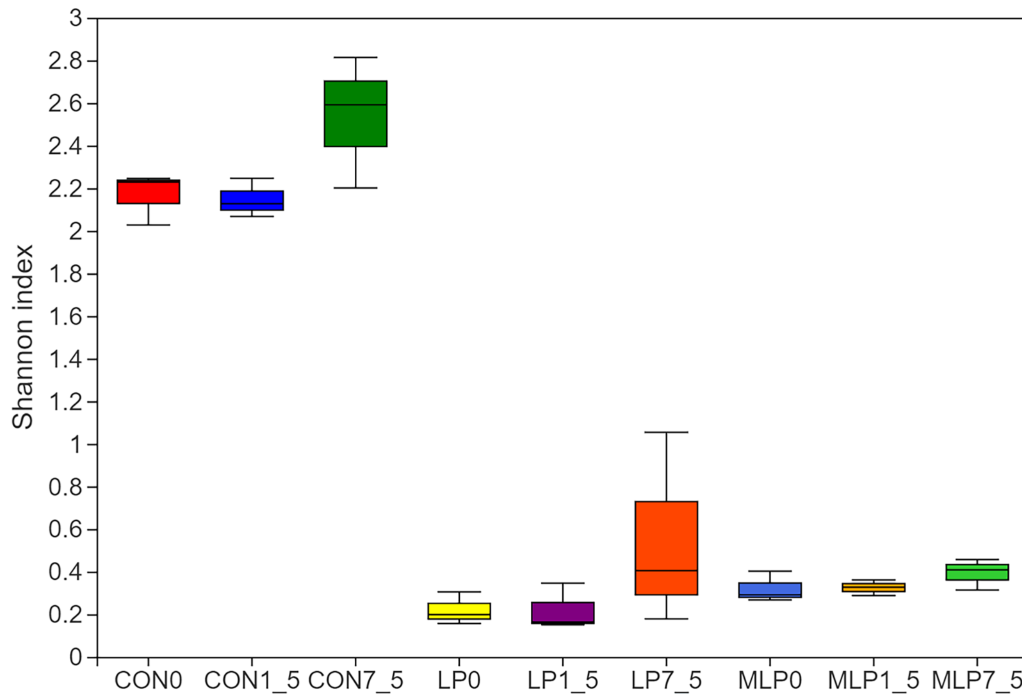


**Fig. 1** The Shannon indices of bacterial community in alfalfa silage. Con0, control without soil contamination; Con1.5, control with 1.5% soil contamination; Con7.5, control with 7.5% soil contamination; LP0, LP treatment without soil contamination; LP1.5, LP treatment with 1.5% soil contamination; LP7.5, LP treatment with 7.5% soil contamination; MLP0, MLP treatment with 1.5% soil contamination; LP7.5, MLP treatment with 7.5% soil contamination

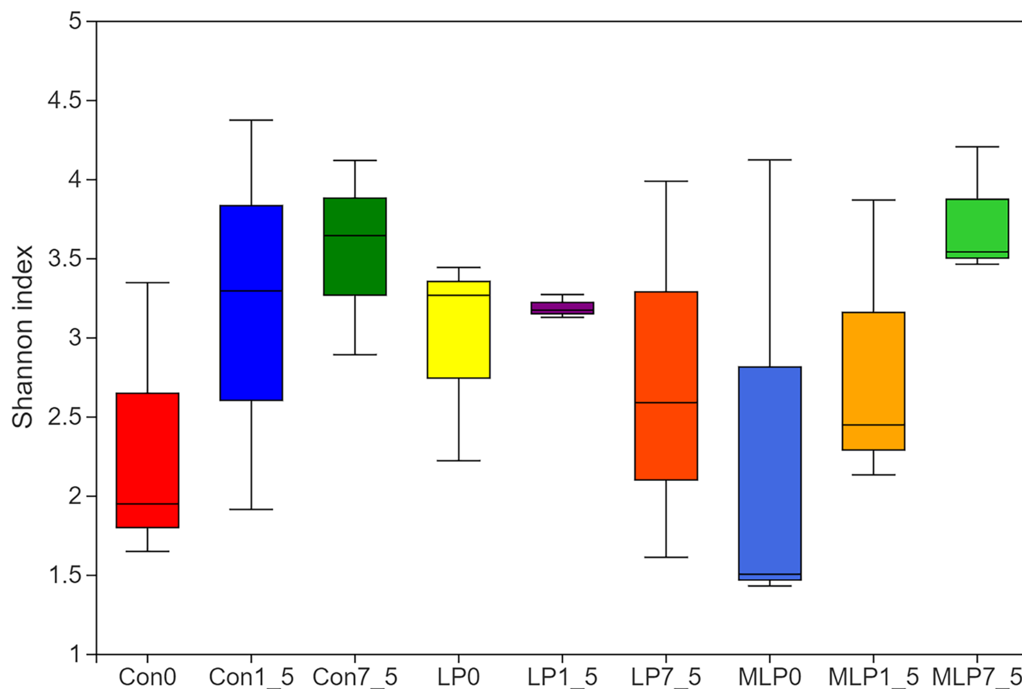


**Fig. 2** The Shannon indices of fungal community in alfalfa silage. Con0, control without soil contamination; Con1.5, control with 1.5% soil contamination; Con7.5, control with 7.5% soil contamination; LP0, LP treatment without soil contamination; LP1.5, LP treatment with 1.5% soil contamination; LP7.5, LP treatment with 7.5% soil contamination; MLP0, MLP treatment with 1.5% soil contamination; LP7.5, MLP treatment with 7.5% soil contamination

silage was significantly higher than in the LP and MLP silage (Fig. 1). Without additives, the Shannon indices of fungi in the 1.5% and 7.5% soil contamination treatments were higher than those in the control treatment (Fig. 2).

### Bacterial communities of alfalfa silage

The relative abundance of bacterial communities at class level is shown in Fig. 3. The identified five main classes were Bacilli, Gammaproteobacteria, Clostridia, Actinobacteria, and Alphaproteobacteria, the average abundances were 94.4 ± 1.3%, 3.7 ± 1.0%, 0.5 ± 0.2%, 0.5 ± 0.1%s and 0.3 ± 0.1%, respectively. Bacilli was most abundant in all alfalfa silage. The increasing level of soil contamination increased the abundance of Clostridia, Actinobacteria and Alphaproteobacteria. Additives significantly affected the abundance of Bacilli, Gammaproteobacteria, Clostridia, Actinobacteria, and Alphaproteobacteria (Additional file 1: Table S2, $P < 0.05$). The application of LP and MLP decreased the abundance of Gammaproteobacteria, Clostridia, Actinobacteria, and Alphaproteobacteria, while increased the abundance of Bacilli.

The relative abundance of bacterial community at species level in alfalfa silage is shown in Fig. 4. Increasing level of soil contamination led to an increase in the abundance of *Bacillus acidiproducens* in the control silage. The application of LP and MLP decreased the relative abundance of *Enterococcus faecium*, *Pediococcus pentosaceus*, unclassified_f_Enterobacteriaceae, and *Weissella cibaria* and increased the relative abundance of *Lactobacillus plantarum* (Additional file 1: Tables S3).

### Fungal communities of alfalfa silage

The relative abundance of fungal community at class level in alfalfa silage is shown in Fig. 5. The Sordariomycetes, Eurotiomycetes, Saccharomycetes, and Dothideomycete were predominant in all alfalfa silage. With the increasing level of soil contamination, the relative abundance of Eurotiomycetes, unclassified_p_Ascomycota, Blastocladiomycetes, and unclassified_p_Chytridiomycota increased in control silage. In LP and MLP silage, the relative abundance of Eurotiomycetes decreased as the soil contamination level increased (Additional file 1: Table S4).

The relative abundance of the fungal community at species level in alfalfa silage is shown in Fig. 6. The fungal community involved in the control silage consisted predominantly of the genera *Neocosmospora rubicola*, *Fusarium concentricum*, *Wickerhamomyces anomalus*, *Monascus pilosus*, and unclassified_f_Nectriaceae. With increasing level of soil contamination from 0% to 7.5%, the relative abundance of *Neocosmospora rubicola* increased, while the relative abundance of *Fusarium concentricum* decreased. The relative abundance of *Wickerhamomyces anomalus* in the control silage was higher than in the LP and MLP silage (Additional file 1: Table S5).

### Distribution of microbial communities in soil and alfalfa silage

The distribution of bacterial and fungal communities in soil and alfalfa silage samples at the genus level is shown in Figs. 7 and 8, respectively. The composition of

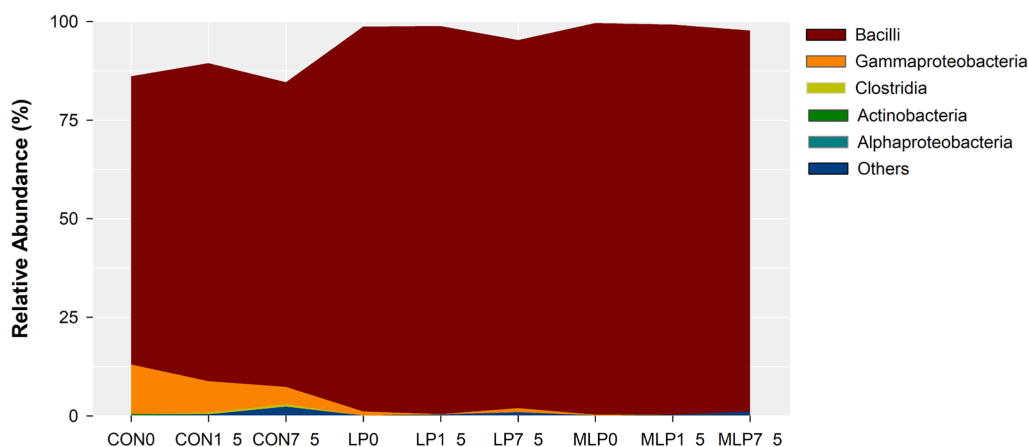


**Fig. 3** The relative abundance of bacterial community at class level in alfalfa silage. Con0, control without soil contamination; Con1.5, control with 1.5% soil contamination; Con7.5, control with 7.5% soil contamination; LP0, LP treatment without soil contamination; LP1.5, LP treatment with 1.5% soil contamination; LP7.5, LP treatment with 7.5% soil contamination; MLP0, MLP treatment with 1.5% soil contamination; LP7.5, MLP treatment with 7.5% soil contamination

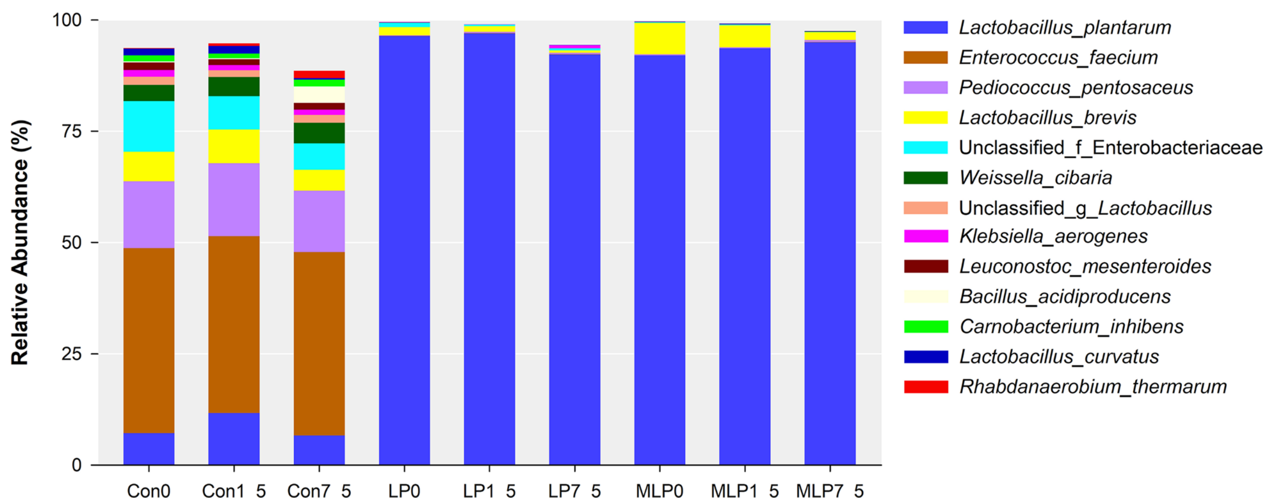


**Fig. 4** The relative abundance of bacterial community at species level in alfalfa silage. Con0, control without soil contamination; Con1.5, control with 1.5% soil contamination; Con7.5, control with 7.5% soil contamination; LP0, LP treatment without soil contamination; LP1.5, LP treatment with 1.5% soil contamination; LP7.5, LP treatment with 7.5% soil contamination; MLP0, MLP treatment with 1.5% soil contamination; LP7.5, MLP treatment with 7.5% soil contamination

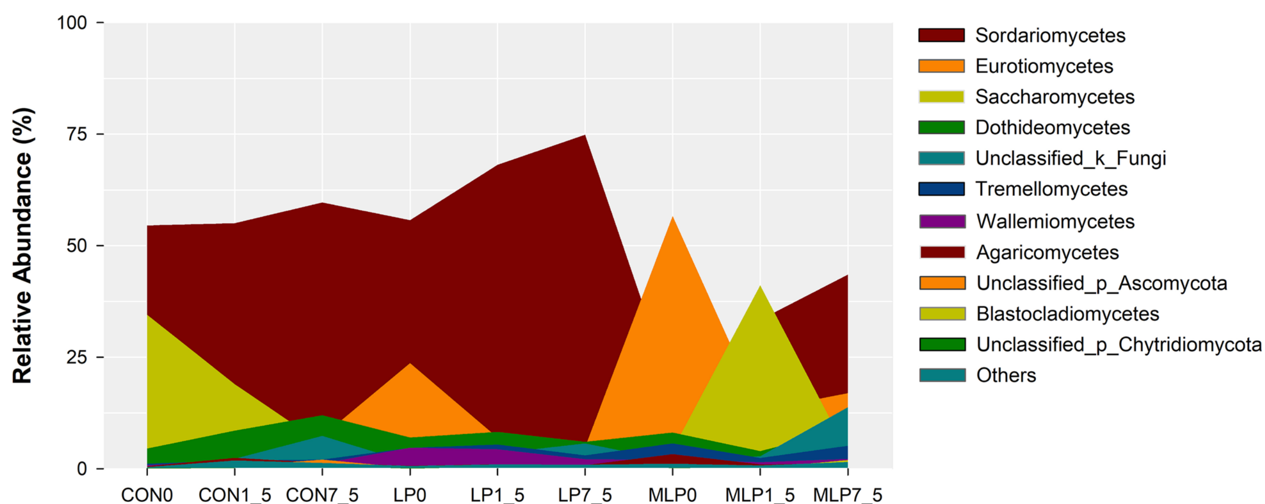


**Fig. 5** The relative abundance of fungal community at class level in alfalfa silage. Con0, control without soil contamination; Con1.5, control with 1.5% soil contamination; Con7.5, control with 7.5% soil contamination; LP0, LP treatment without soil contamination; LP1.5, LP treatment with 1.5% soil contamination; LP7.5, LP treatment with 7.5% soil contamination; MLP0, MLP treatment with 1.5% soil contamination; LP7.5, MLP treatment with 7.5% soil contamination

dominant bacteria in the LP and MLP groups of alfalfa silage is similar, in which *Lactobacillus* is the predominant bacteria with a relative abundance of over 90% (Fig. 7A). The dominant bacteria of Con0, Con1.5, and Con7.5 were similar, while *Enterococcus*, *Lactobacillus*, and *Pediococcus* were the dominant bacteria, with relative abundances above 40%, 10% and 10%, respectively. The distribution of communities in soil was found to be more diverse, with *Blastococcus* having the highest relative abundance at approximately 4.4%. The composition of dominant fungi in different silage samples was similar, with *Neocosmospora* and *Fusarium* being the dominant bacteria (Fig. 7B). Different from alfalfa silage, the dominant fungi in the soil were *Cladosporium*, *Mycocentrospora*, and *Lectera*.

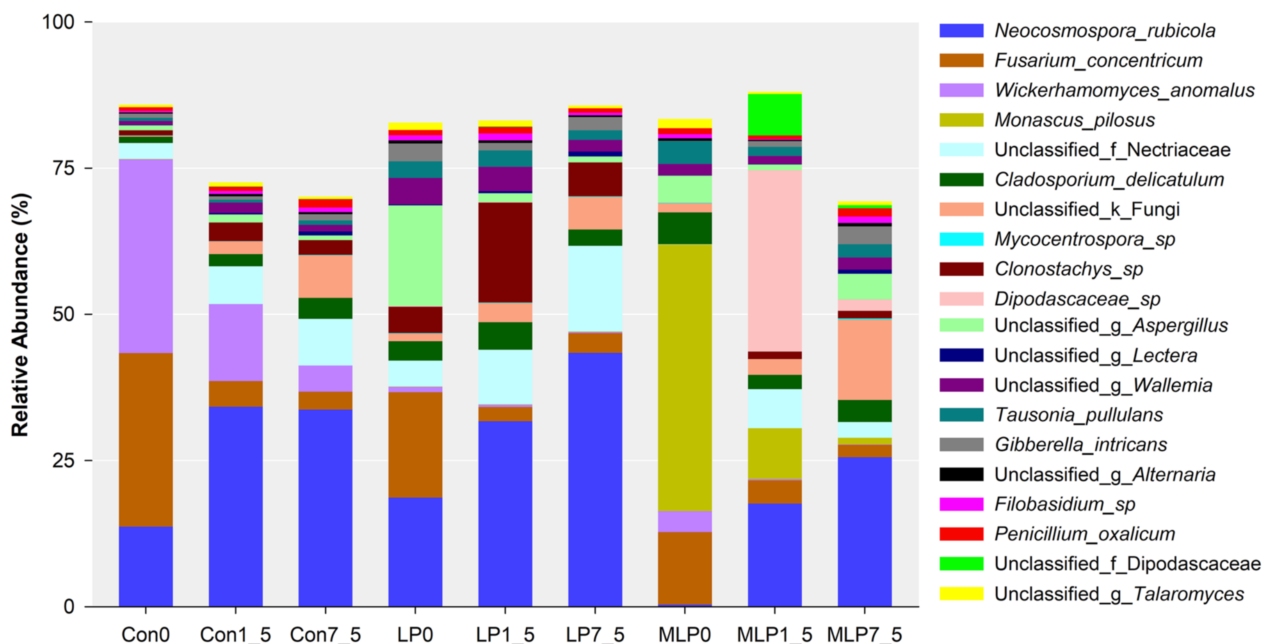


**Fig. 6** The relative abundance of fungal community at species level in alfalfa silage. Con0, control without soil contamination; Con1.5, control with 1.5% soil contamination; Con7.5, control with 7.5% soil contamination; LP0, LP treatment without soil contamination; LP1.5, LP treatment with 1.5% soil contamination; LP7.5, LP treatment with 7.5% soil contamination; MLP0, MLP treatment with 1.5% soil contamination; LP7.5, MLP treatment with 7.5% soil contamination

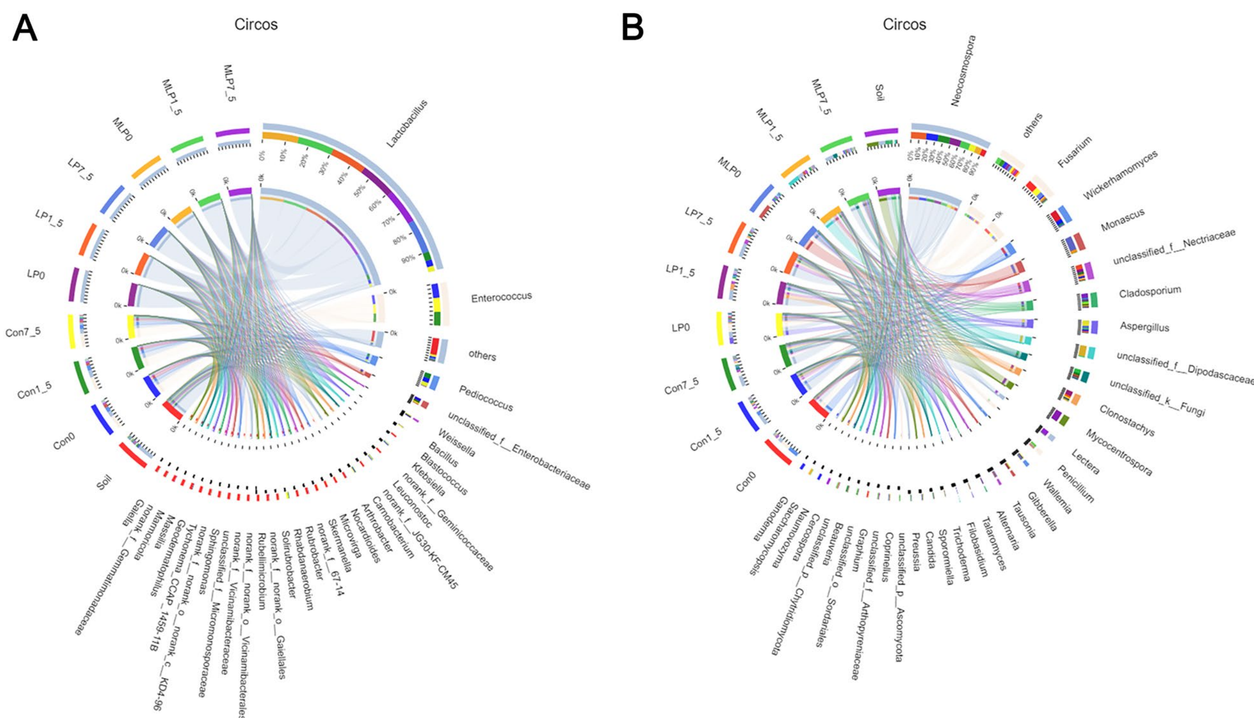


**Fig. 7** Distribution of bacterial (**A**) and fungal (**B**) communities in soil and alfalfa silage samples at the genus level

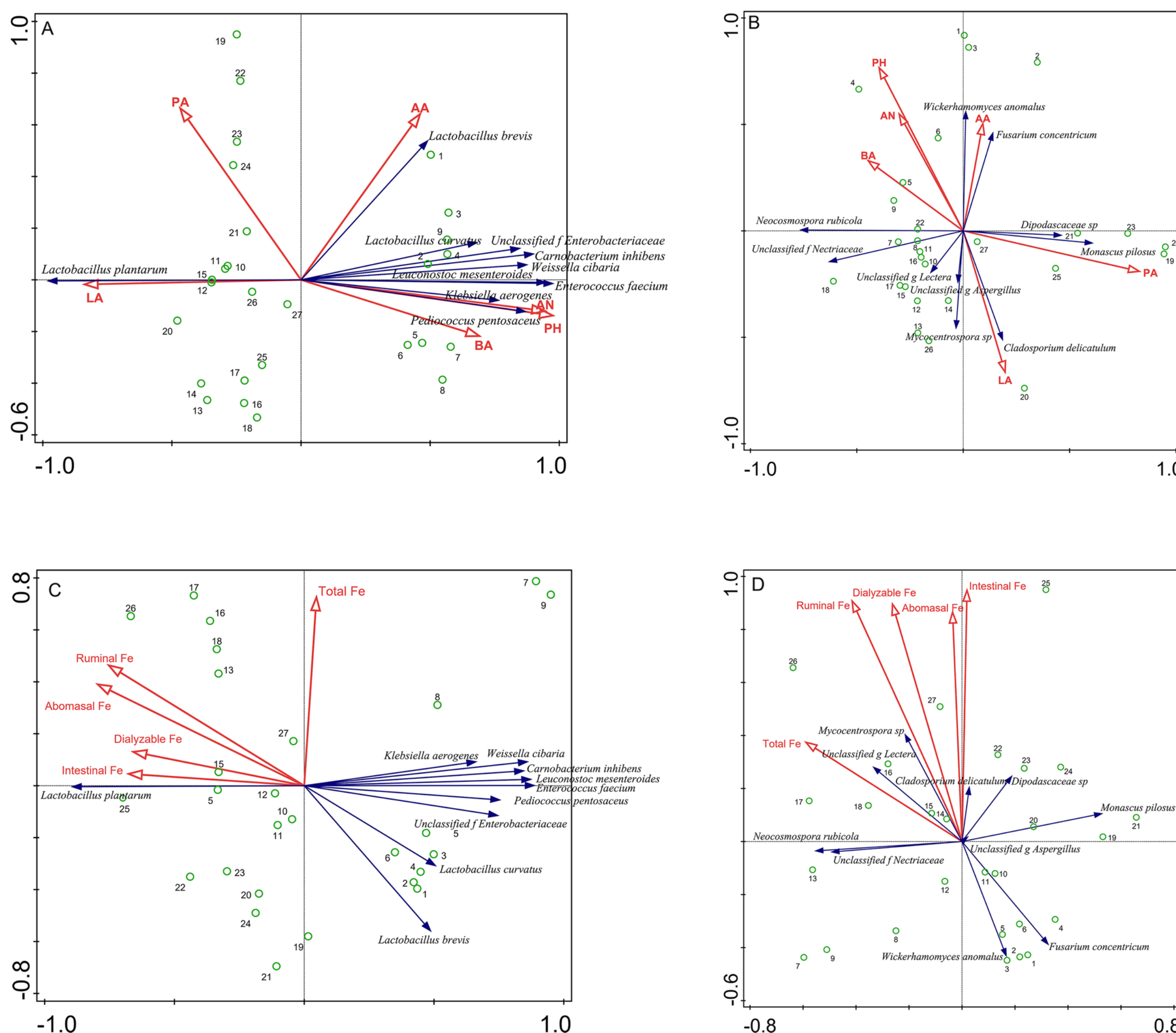


**Fig. 8** Redundancy analysis (RDA) of the relationship between bacterial community and fermentation characteristic (**A**), fungal community and fermentation characteristic (**B**), bacterial community and iron content (**C**), fungal community and iron content (**D**). LA, lactic acid; AA, acetic acid; PA, propionic acid, BA, butyric acid; AN, ammonia nitrogen

### Relationship between microbial communities and silage characteristics

The RDA showed that the bacterial and fungal communities were strongly correlated with fermentation characteristics and iron content (Fig. 8). For bacteria, the *Lactobacillus plantarum* exhibited a positive relationship with LA and bioaccessible iron; the *Lactobacillus brevis* showed a positive relation with AA; the *Lactobacillus curvatus*, Unclassified_f_Enterobacteriaceae, *Carnobacterium inhibens*, *Weissella cibaria*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Klebsiella aerogenes* and *Pediococcus pentosaceus* were positively correlated with the pH, BA and AN. For fungi, the *Neocosmospora rubicola* exhibited a positive relationship with total iron, BA, AN and pH; the *Wickerhamomyces anomalus* and *Fusarium concentricum* were positively correlated with AA; the *Dipodascaceae sp* and *Monascus pilosus* showed positive relationship with PA; the *Cladosporium delicatulum* were positively correlated with LA.

## Discussion

### Fermentation characteristics and iron content

The increasing level of soil contamination from 0 to 7.5% increased the pH value, BA, and AN contents, but decreased the LA, AA and PA contents. Silage pH is an important indicator for assessing fermentation quality, a

lower pH ensures better anaerobic fermentation and further inhibits microorganism. The increase of pH value in soil contaminated silage may be due to the high levels of basic nitrogenous substance such as AN and BA. Protein degradation is a significant issue in alfalfa fermentation, in which forage proteins are frequently broken down into AN and BA through plant protease and microorganism activity [30]. Higher levels of AN and BA were observed in soil-contaminated samples, indicating that soil contamination negatively affects the fermentation quality and leads to increased proteolysis in alfalfa silage. It is interesting to note that 1.5% soil level contamination without additives seem to promote lactic acid and acetic acid production and reduce pH value. This is similar with the result of Franco et al. [31], and the reason may be that a small amount of soil addition changes the competition among microorganisms in the silage. In addition, the LP and MLP additives significantly reduced the AN and BA contents, which may help to minimize protein loss and inhibit the growth of proteolytic bacteria. The pH values were also reduced in the LP and MLP samples compared to the control samples, indicating that the two additives helped convert water-soluble carbohydrates into organic acids. The reduction in pH value was mainly due to the accumulation of organic acids, especially LA [30]. The LA content in the LP and MLP samples was significantly higher than that in the control samples, indicating that the addition of LP and MLP can improve the fermentation quality of alfalfa silage.

With the increasing level of soil contamination from 0 to 7.5%, the total iron content increased dramatically. This suggests that soil contamination is the primary factor contributing to the increase in iron content. A similar pattern was observed in the study by Hansen and Spears [6], in which soil contamination had a significant impact on microelement contents, particularly iron. Iron is often found in high contents in ruminant diets, and one of the most likely explanations is that iron comes from soil contamination of feed [5, 6]. In the present study, the fermented alfalfa contaminated by soil (7.5%) contained high levels of iron (> 2000 mg/kg), which exceeded the recommended values of beef cattle (50 mg/kg) and dairy cattle (15–30 mg/kg) feeding standards in the NRC [32, 33]. In general, the iron in soils is thought to be tightly bound with chelating agents that ruminants cannot absorb. However, in acidic environments (such as during the ensiling of forage), the bioaccessibility of iron can be improved by reducing ferric iron to ferrous iron [34]. To evaluate the bioaccessibility of iron in alfalfa silage, we simulated the digestive system of ruminants to determine the effect of soil contamination on iron bioaccessibility. Microelement bioaccessibility is defined as the proportion of microelements that are soluble in the gastrointestinal tract and can be absorbed by animals [35]. In this study, the bioaccessibility of iron (ruminal iron, abomasal iron, intestinal iron, and dialyzable iron) in alfalfa silage was found to increase with increasing soil contamination level, suggesting that the exposure of soil to alfalfa silage may alter the composition of iron bound in soil. This result was similar to that of Hansen and Spears [5], when 1% and 5% level of soil were added to corn forage, the bioaccessible iron content increased after 90 days of fermentation. Iron is a well-known copper antagonist in ruminants, and a high-iron diet in ruminants may affect the absorption of essential trace minerals in the intestine. Soil contamination may be a major overlooked source of bioaccessible iron in ruminant feed. Minimizing soil contamination of harvested forage can improve the fermentation quality of alfalfa silage and reduce the risk of excessive iron levels.

#### Alpha diversity of the microbial community

Good's coverage estimation revealed that > 99% of the species were detected in all alfalfa silage samples, indicating that all samples reached sufficient coverage. The Shannon index of bacteria in the control group was significantly higher than that in the LP and MLP groups. During fermentation, the bacterial communities in the LP and MLP groups were dominated by *Lactobacillus*, leading to a decrease in bacterial diversity. The *Lactobacillus* did not dominate in the control group, resulting in a greater diversity of bacteria types in the control group. The use of additives in anaerobic fermented forage generally reduces bacterial diversity in the later stages of fermentation, such as corn fermented for 120 days [36], alfalfa fermented for 35 days [37], and oat fermented for 217 days [38]. Polley et al. [39] showed that the diversity of microbial communities was low when the abundance of dominant bacteria was high. Similarly, Ogunade et al. [36] also found that bacterial diversity in forage inoculated with *Lactobacillus plantarum* or *Lactobacillus buchneri* decreased bacterial diversity due to the increased relative abundance of the predominant genus (*Lactobacillus*). Without additives, the Shannon indices of fungi in the 1.5% and 7.5% soil-contaminated samples were higher than those in the control group. The soil-contaminated forage produced relatively higher Shannon indices of bacteria and fungi after fermentation, indicating that soil contamination increased the bacterial and fungal diversity after ensiling and may decelerate the growth of desirable bacteria such as *Lactobacillus* in this study.

#### Microbial communities of alfalfa silage

The microflora in the control group differed significantly from that in the LP and MLP groups in terms of

composition and proportion. After 45 days of fermentation, the bacterial community in fermented alfalfa was primarily composed of the classes Bacilli and Gammaproteobacteria, and a low abundance of Clostridia, Actinobacteria, and Alphaproteobacteria. In the control group, when the soil contamination level was increased to 7.5%, the relative abundances of Actinobacteria, Alphaproteobacteria, and Clostridia increased to 1.67%, 1.16%, and 2.97%, respectively, which were significantly higher than those observed in the LP and MLP groups. Among these bacteria, Actinobacteria are typical pollutants in the soil environment and have the ability to utilize a wide range of organic compounds [40]. Some genera of Alphaproteobacteria are known to be responsible for the hydrolysis of soluble proteins [41]. Clostridia in fermented forage will consume carbohydrates, proteins, and LA as energy sources and secrete BA [42]. The process of fermentation involves competition between lactic acid bacteria and other microorganisms. Most of the bacteria involved in lactic acid fermentation include genera such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella* [43]. In this study, the genera *Enterococcus*, *Pediococcus*, *Weissella*, and *Leuconstoc* were predominantly abundant in the control group. The study by Cai et al. [44] showed that coccal lactic acid bacteria, mainly including *Enterococcus*, *Pediococcus*, *Weissella*, and *Leuconstoc*, thrive only in the early stages of natural fermentation. These results suggest that incomplete fermentation occurred in the control group.

Lactic acid bacteria are prevalent in nature and are frequently used as inoculants on alfalfa to facilitate anaerobic fermentation. In this study, the addition of LP and MLP increased the abundance of *Lactobacillus plantarum* and decreased the abundance of *Enterococcus faecium*, *Pediococcus pentosaceus*, *Weissella cibaria*, and *Leuconstoc mesenteroides* compared to the control after 45 d of fermentation. Similar results were observed by Bai et al. [45] and Yan et al. [46], the abundance of *Lactobacillus* in lactic acid bacterium-inoculated forage was found to be increased compared to the control, indicating that inoculation with LP could significantly increase *Lactobacillus* abundance, thereby producing more LA. The lower abundances of *Lactobacillus plantarum* and the higher abundances of unclassified_f_Enterobacteriaceae in the control groups may explain their poor quality. The presence of gram-negative bacteria, such as Enterobacteriaceae, may decrease fermentation quality. These bacteria compete with LAB for water-soluble carbohydrates, resulting in slow acidification. Although the alfalfa silage was contaminated with soil (at levels of 1.5% or 7.5%), the bacterial species *Lactobacillus plantarum* still dominated the bacterial population when LP and MLP additives were applied, accounting for over 80% of the total. The main reason is that the addition of LP and MLP increases the abundance of favorable microorganisms such as *Lactobacillus plantarum* and generates antibacterial substances (including organic acids) in anaerobic conditions, thus inhibiting the growth of undesirable bacteria and improving the fermentation quality of soil-contaminated alfalfa. Increasing levels of soil contamination led to an increase in the abundance of *Bacillus acidiproducens* and *Rhabdanaerobium thermarum* in control samples. *Rhabdanaerobium thermarum* belongs to the family Eubacteriaceae in the order Clostridiales [47]. Bacteria belonging to the Clostridiales order typically result from soil contamination [48] and can break down protein [1], which may explain the poor fermentation quality of control samples contaminated by soil.

There are many studies focusing on mycotoxins in fermented forage, but there is limited information available on fungal communities in fermented alfalfa. The fungi Sordariomycetes, Eurotiomycetes, Saccharomycetes, and Dothideomycetes were predominant at the class level in all fermented alfalfa samples. With the increasing level of soil contamination, the relative abundance of Eurotiomycetes, unclassified_p_Ascomycota, Blastocladiomycetes, and unclassified_p_Chytridiomycota increased in the control group. In the LP and MLP groups, the relative abundance of Eurotiomycetes decreased as the soil contamination level increased. The fungi present in this study mainly belong to the genera *Neocosmospora*, *Fusarium*, *Wicherhamomyces*, *Monascus*, unclassified_f_Nectriaceae, *Aspergillus*, unclassified_f_Dipodascacea, and *Clonostachys*. In soil-contaminated alfalfa, the species *Neocosmospora rubicola* was found to be enriched. *Neocosmospora rubicola* is a filamentous fungus in the Nectriaceae family, and the genus *Neocosmospora* is widely found in various environments such as soil, plant debris, living plant material, air, water, and as opportunistic animal pathogens in the form of saprobes, endophytes, and pathogens [49]. *Neocosmospora* has also been reported to be related to mycotoxin poisoning in humans and animals [50]. In contrast to the species *Neocosmospora rubicola*, *Fusarium concentricum* was more abundant in the uncontaminated group than in the soil-contaminated group. The genus *Fusarium* is an important pathogen that can cause significant yield losses in crops [51]. Some species of *Fusarium* have been found in fermented forage [52], but *Fusarium concentricum* has rarely been reported in silage. This difference explains the lower abundance of *Fusarium concentricum* in soil-contaminated alfalfa. In addition, the species *Wickerhamomyces anomalus* was found to be relatively abundant in Con0. *Wickerhamomyces anomalus* is an ascomycete heteromycete yeast from the family Wicherhamomycetaceae. This species exists in many types of environments and has

been isolated from fermented corn [53]. It can grow over a wide pH range [54] and is a strong producer of AA in pure cultures [55]. In addition, *Monascus* was found to be relatively abundant in MLP0, which may be attributed to the high molasses and organic acid contents in the MLP treatment. The synergy among silage microbes involves the food web, where a metabolite produced by one type of bacteria, such as lactic acid, serves as an important nutrient for other lactic acid-consuming bacteria. LA can selectively promote the growth of microorganisms involved in these food chains*.* *Monascus* belongs to the family Monascaceae, and Zheng et al. [56] found that *Monascus* has a high capacity for polysaccharide utilization capacity and can tolerate a high content of organic acids (specifically LA to AA ratio of 3:1).

### Distribution of microbial communities in soil and alfalfa silage

The distribution of bacterial communities in soil was more diverse when compared to fermented alfalfa, and the most abundant bacteria was *Blastococcus*, accounting for approximately 4.4% of the total. There are hundreds of bacteria in fermented alfalfa, and dozens of common bacteria make up the majority of microorganisms in fermented alfalfa. The composition of dominant bacteria in the LP and MLP groups was similar, in which *Lactobacillus* was the predominant bacteria with a relative abundance of over 90%. The dominant bacteria of the control groups were similar, while *Enterococcus* was the dominant bacteria with a relative abundance above 40%. The composition of dominant fungi in different fermented alfalfa samples was similar, with *Neocosmospora* and *Fusarium* being the most prevalent species. In contrast to the fermented alfalfa samples, the dominant fungi in the soil were *Cladosporium*, *Mycocentrospora*, and *Lectera*. The detection rate and abundance of many bacteria and fungi in the soil were lower than those in fermented alfalfa, indicating that the microbial community composition of the fermented alfalfa differed from that of the soil.

### Relationship between microbial communities and silage characteristics

There are complex interactions between microbial communities and silage fermentation characteristics. Silage chemical factors and nutrients play a decisive role in microbial community, while the composition and diversity of microbial community are crucial for silage quality. The RDA showed that the dominant *Lactobacillus plantarum* exhibited a positive relationship with LA and a negative relationship with pH, AN and BA. This result was consistent with previous results, *Lactobacillus plantarum* can encourage the formation of lactic acid and thus reduce the acidity of silage, thereby inhibiting undesirable bacteria [57]. The *Lactobacillus brevis* showed a positive relationship with AA. *Lactobacillus brevis* is a heterofermentative lactic acid bacterium mainly produce AA [48], higher levels of AA were found in control groups may due to the property of *Lactobacillus brevis*. The bacteria *Lactobacillus curvatus*, Unclassified_f_Enterobacteriaceae, *Carnobacterium inhibens*, *Weissella cibaria*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Klebsiella aerogenes*, *Pediococcus pentosaceus* and fungi *Neocosmospora rubicola* were positively correlated with the pH, BA and AN, indicating that these microbial may promote the protein degradation in soil-contaminated alfalfa silage. The *Lactobacillus plantarum* was positively correlated with bioaccessible iron, this may be related to the acidic environment formed by its fermentation. The acidic environment enhances the reducibility of iron in soil and increases the dissociation of some iron from insoluble complexes [40]. Rooke et al. [58] observed increased solubility of minerals in grass silage compared to unfermented forage, suggesting that the acidic environment of silage promotes increased mineral release of naturally occurring elements in silage. Therefore, feed with a low pH value that may come into contact with soil should be considered as a potential source of bioaccessible iron.

## Conclusions

In summary, this study has identified microbial communities and investigated the iron content in alfalfa silage contaminated with soil. Soil contamination of alfalfa silage increased the abundance of Clostridia, Actinobacteria, and Alphaproteobacteria, which can have potential implications for silage quality. The addition of LP and MLP improved fermentation quality of soil-contaminated silage by increasing LA production and reducing the abundance of *Enterococcus faecium*, *Pediococcus pentosaceus*, unclassified_f_Enterobacteriaceae, and *Weissella cibaria*. The exposure of soil to alfalfa silage makes the iron more bioaccessible, this may affect the absorption of essential trace minerals in ruminant. Therefore, it is important to minimize soil contamination in feed and mitigate the risk of elevated iron content. This study provides guidance for high quality of alfalfa silage production.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00578-w.

**Additional file 1: Table S1.** The coverage of bacteria and fungi of alfalfa silage. **Table S2.** The list of relative abundance of bacterial community at class level. **Table S3.** The list of relative abundance of bacterial community

at species level. **Table S4.** The list of relative abundance of fungal community at class level. **Table S5.** The list of relative abundance of fungal community at species level.

**Author contributions**

JT, LY, and YZ designed the project. JT, LY, and WL developed the methods. JT and LY completed the data analysis. JT, LY, and YZ wrote the manuscript. JT and YZ provided the funding for the acquisition. All authors have read and approved the final manuscript.

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**Availability of data and materials**

The raw reads have been deposited at NCBI Sequence Read Archive (SRA) database (Accession Numbers: PRJNA907969 and PRJNA908062). The current study did not generate new code, all codes used to analysis in this study can be found according to corresponding reference.

## Declarations

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

This research has been confirmed for publication in the journal.

**Competing interests**

The authors have no competing interests.

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**References**

1. McDonald P, Henderson A, Heron S. The biochemistry of silage. Marlow: Chalcombe Publications; 1991.
2. Rafferty B, Dawson DE, Colgan PA. Soil and radiocaesium contamination of winter fodders. Sci Total Environ. 1994;153(1–2):69–76. https://doi.org/10.1016/0048-9697(94)90103-1.
3. Sun J, Zhao J, Xue Y, Liu H, Li W, Yu Z, Bai C. Effect of soil contamination and lactic acid bacteria inoculation on alfalfa silage quality. Chin J Grassl. 2021;43:114–20. https://doi.org/10.16742/j.zgcdxb.20200361.
4. Resch R. Challenges in silage production with regard to soil contamination. Viehwirtschaftliche Fachtagung: Milchmarkt und Zuchtung; Konservierungsverfahren Heu; Rindermast und Fleischqualitat; Milchviehfutterung; Tierische Produktion und Klima gemass Fortbildungsplan des Bundes. 25. und 26. Grimmingsaal HBLFA Raumberg-Gumpenstein, Austria. 2015:127–37.
5. Hansen SL, Spears JW. Bioaccessibility of iron from soil is increased by silage fermentation. J Dairy Sci. 2009;92:2896–905. https://doi.org/10.3168/jds.2008-1933.
6. Martens SD, Majewska-Pinda A, Benkmann A, Zentek J, Spolders M, Simon A, Schafft H, Steinhöfel O. Influence of soil contamination before and after ensiling on mineral composition of grass silages, feed intake and carry-over to body tissue of goats. J Anim Feed Sci. 2018;27:307–16. https://doi.org/10.22358/jafs/99863/2018.
7. Schlegel P, Wyss U, Arrigo Y, Hess HD. Changes in macro- and micromineral concentrations in herbage during the harvesting and conservation process. Grass Forage Sci. 2018;73:918–25. https://doi.org/10.1111/gfs.12382.
8. Bargagli R. The elemental composition of vegetation and the possible incidence of soil contamination of samples. Sci Total Environ. 1995;176:121–8. https://doi.org/10.1016/0048-9697(95)04838-3.
9. Wyttenbach A, Tobler L. Soil contamination in plant samples and in botanical reference materials: signature, quantification and consequences. J Radioanal Nucl Chem. 2002;254:165–74. https://doi.org/10.1023/A:1020818320501.
10. Healy WB. In vitro studies on the effects of soil on elements in ruminal, "duodenal", and ileal liquors from sheep. NZ J Agric Res. 1972;15:289–305. https://doi.org/10.1080/00288233.1972.10421257.
11. Grün M, Anke M, Hennig A, Seffner W, Partschefeld M, Flachowsky G, Groppel B. Excessive iron administration to sheep. 2. The effect on the iron, copper, zinc and manganese level in different organs (in German). Archiv für Tierernaehr. 1978;28:341–7. https://doi.org/10.1080/17450397809426811.
12. Suttle NF. Mineral nutrition of livestock. 4th ed. Oxfordshire: CAB International; 2010.
13. Okoye CO, Wang Y, Gao L, Wu Y, Li X, Sun J, Jiang J. The performance of lactic acid bacteria in silage production: a review of modern biotechnology for silage improvement. Microbiol Res. 2023;266:127212. https://doi.org/10.1016/j.micres.2022.127212.
14. Ávila CLS, Carvalho BF. Silage fermentation-updates focusing on the performance of micro-organisms. J Appl Microbiol. 2020;128(4):966–84. https://doi.org/10.1111/jam.14450.
15. Dunière L, Sindou J, Chaucheyras-Durand F, Chevallier I, Thévenot-Sergentet D. Silage processing and strategies to prevent persistence of undesirable microorganisms. Anim Feed Sci Technol. 2013;182(1–4):1–15. https://doi.org/10.1016/j.anifeedsci.2013.04.006.
16. Mogodiniyai Kasmaei K, Dicksved J, Spörndly R, Udén P. Separating the effects of forage source and field microbiota on silage fermentation quality and aerobic stability. Grass Forage Sci. 2017;72:281–9. https://doi.org/10.1111/GFS.12238.
17. Ni K, Minh T, Minh TuT, Tsuruta T, Pang H, Nishino N. Comparative microbiota assessment of wilted Italian ryegrass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. Appl Microbiol Biotechnol. 2017;101:1385–94. https://doi.org/10.1007/s00253-016-7900-2.
18. Kraut-Cohen J, Tripathi V, Chen Y, Gatica J, Volchinski V, Sela S, Weinberg Z, Cytryn E. Temporal and spatial assessment of microbial communities in commercial silages from bunker silos. Appl Microbiol Biotechnol. 2016;100:6827–35. https://doi.org/10.1007/s00253-016-7512-x.
19. Li D, Ni K, Zhang Y, Lin Y, Yang F. Influence of lactic acid bacteria, cellulase, cellulase-producing *Bacillus pumilus* and their combinations on alfalfa silage quality. J Integr Agric. 2018;17:2768–82. https://doi.org/10.1016/S2095-3119(18)62060-X.
20. Guo X, Ke W, Ding W, Ding L, Xu D, Wang W, Zhang P, Yang F. Profiling of metabolome and bacterial community dynamics in ensiled *Medicago sativa* inoculated without or with *Lactobacillus plantarum* or *Lactobacillus buchneri*. Sci Rep. 2018;8:357. https://doi.org/10.1038/s41598-017-18348-0.
21. Liu B, Yang Z, Huan H, Gu H, Xu N, Ding C. Impact of molasses and microbial inoculants on fermentation quality, aerobic stability, and bacterial and fungal microbiomes of barley silage. Sci Rep. 2020;10:5342. https://doi.org/10.1038/s41598-020-62290-7.
22. Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acid in ruminal fluid and in vitro media. J Dairy Sci. 1980;63:64–75. https://doi.org/10.3168/jds.S0022-0302(80)82888-8.
23. Fischer PWF, L'abbé MR. Acid digestion determination of iodine in foods. JAOAC. 1981;64:71–4. https://doi.org/10.1093/JAOAC/64.1.71.
24. Zheng Y, Zhang T, Lu Y, Wang L. *Monascus pilosus* YX-1125: an efficient digester for directly treating ultra-high-strength liquor wastewater and producing short-chain fatty acids under multiple-stress conditions. Bioresour Technol. 2021;331: 125050. https://doi.org/10.1016/j.biortech.2021.125050.

25. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–90. https://doi.org/10.1093/bioinformatics/bty560.
26. Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27:2957–63. https://doi.org/10.1093/bioinformatics/btr507.
27. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10:996–8. https://doi.org/10.1038/nmeth.2604.
28. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261–7. https://doi.org/10.1128/AEM.00062-07.
29. Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. Circos: an information aesthetic for comparative genomics. Genome Res. 2009;19:1639–45. https://doi.org/10.1101/gr.092759.109.
30. Kung LM Jr, Shaver RD, Grant RJ, Schmidt RJ. Silage review: interpretation of chemical, microbial, and organoleptic components of silages. J Dairy Sci. 2018;101:4020–33. https://doi.org/10.3168/jds.2017-13909.
31. Franco M, Tapio I, Pirttiniemi J, Stefański T, Jalava T, Huuskonen A, Rinne M. Fermentation quality and bacterial ecology of grass silage modulated by additive treatments, extent of compaction and soil contamination. Fermentation. 2022;8(4):156. https://doi.org/10.3390/fermentation8040156.
32. NRC. Nutrient requirements of beef cattle. 7th ed. Washington, DC: National Academy Press; 1996.
33. NRC. Nutrient requirements of dairy cattle. 7th ed. Washington, DC: National Academy Press; 2001.
34. Whitehead DC. Micronutrient cations: iron, manganese, zinc, copper and cobalt. In: Whitehead DC, editor. Nutrient elements in grassland: soil-plant-animal relationships. Wallingford: CABI; 2000.
35. Ruby MV, Davis A, Schoof R, Eberle S, Sellstone CM. Estimation of lead and arsenic bioavailability using a physiologically based extraction test. Environ Sci Technol. 1996;30:422–40. https://doi.org/10.1021/es950057z.
36. Ogunade IM, Jiang Y, Pech Cervantes AA, Kim DH, Oliveira AS, Vyas D, Weinberg ZG, Jeong KC, Adesogan AT. Bacterial diversity and composition of alfalfa silage as analyzed by Illumina Miseq sequencing: effects of *Escherichia coli* O157:H7 and silage additives. J Dairy Sci. 2018;101:2048–59. https://doi.org/10.3168/jds.2017-12876.
37. Bao W, Mi Z, Xu H, Zheng Y, Kwok L, Zhang H, Zhang W. Assessing quality of *Medicago sativa* silage by monitoring bacterial composition with single molecule, real-time sequencing technology and various physiological parameters. Sci Rep. 2016;24:28358. https://doi.org/10.1038/srep28358.
38. Romero JJ, Zhao Y, Balseca-Paredes MA, Tiezzi F, Gutierrez-Rodriguez E, Castillo MS. Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage. J Dairy Sci. 2017;100:1812–28. https://doi.org/10.3168/jds.2016-11642.
39. Polley HW, Wilsey BJ, Derner JD. Dominant species constrain effects of species diversity on temporal variability in biomass production of tallgrass prairie. Oikos. 2007;116:2044–52. https://doi.org/10.1111/j.2007.0030-1299.16080.x.
40. Krakat N, Schmidt S, Scherer P. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. Bioresour Technol. 2011;102:5692–701. https://doi.org/10.1016/j.biortech.2011.02.108.
41. Zhou Y, Chen Y, Guo J, Shen Y, Yang J. The correlations and spatial characteristics of microbiome and silage quality by reusing of citrus waste in a family-scale bunker silo. J Clean Prod. 2019;226:407–18. https://doi.org/10.1016/j.jclepro.2019.04.075.
42. Elferink SO, Driehuis F, Gottschal JC, Spoelstra SF. Silage fermentation processes and their manipulation. In Silage Making in the Tropics with Particular Emphasis on Smallholders; Mannetje, L., Ed.; FAO Plant Prod Prot Pap. 2000;161:17-30.
43. Ávila CLS, Carvalho BF. Silage fermentation-updates focusing on the performance of micro-organisms. J Appl Microbiol. 2020;128:966–84. https://doi.org/10.1111/jam.14450.
44. Cai Y, Benno Y, Ogawa M, Ohmomo S, Kumai S, Nakase T. Influence of *Lactobacillus spp.* from an inoculant and of *Weissella* and *Leuconostoc spp.* from forage crops on silage fermentation. Appl Environ Microbiol. 1998;64:2982–7. https://doi.org/10.1128/AEM.64.8.2982-2987.1998.
45. Bai J, Xu D, Xie D, Wang M, Li Z, Guo X. Effects of antibacterial peptide-producing *Bacillus subtilis* and *Lactobacillus buchneri* on fermentation, aerobic stability, and microbial community of alfalfa silage. Bioresour Technol. 2020;315:123881. https://doi.org/10.1016/j.biortech.2020.123881.
46. Yan Y, Li X, Guan H, Huang L, Ma X, Peng Y, Li Z, Nie G, Zhou J, Yang W, Cai Y, Zhang X. Microbial community and fermentation characteristic of Italian ryegrass silage prepared with corn stover and lactic acid bacteria. Bioresour Technol. 2019;279:166–73. https://doi.org/10.1016/j.biortech.2019.01.107.
47. Liu L, Jiao J, Salam N, Zhou E, Fang B, Xian W, Li M, Ding Y, Li W. *Rhabdanaerobium thermarum* gen. nov., sp. nov., a novel anaerobic bacterium isolated from a hot spring. Int J Syst Evol Microbiol. 2017;67:4584–8. https://doi.org/10.1099/ijsem.0.002335.
48. Pahlow G, Muck RE, Driehuis F, Elferink SJ, Spoelstra SF. Microbiology of ensiling. In: Buxton DR, Muck RE, Harrison JH, editors. Silage science and technology. Madison: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America; 2003. p. 31–94.
49. Sandoval-Denis M, Lombard L, Crous PW. Back to the roots: a reappraisal of *Neocosmospora*. Persoonia. 2019;43:90–185. https://doi.org/10.3767/persoonia.2019.43.04.
50. Antonissen G, Martel A, Pasmans F, Ducatelle R, Verbrugghe E, Vandenbroucke V, Li S, Haesebrouck F, Immerseel FV, Croubels S. The impact of Fusarium mycotoxins on human and animal host susceptibility to infectious diseases. Toxins (Basel). 2014;6:430–52. https://doi.org/10.3390/toxins6020430.
51. Bottalico A, Perrone G. Toxigenic Fusarium species and mycotoxins associated with head blight in small-grain cereals in Europe. Eur J Plant Pathol. 2002;108:611–24. https://doi.org/10.1023/A:1020635214971.
52. Eckard S, Wettstein FE, Forrer HR, Vogelgsang S. Incidence of Fusarium species and mycotoxins in silage maize. Toxins (Basel). 2011;3:949–67. https://doi.org/10.3390/toxins3080949.
53. Kurtzman CP, Fell JW. The yeast, a taxonomical study. 4th ed. Amsterdam: Elsevier Science; 1998.
54. Passoth V, Fredlund E, Druvefors UA, Schnürer J. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 2006;6:3–13. https://doi.org/10.1111/j.1567-1364.2005.00004.x.
55. Suárez-Lepe JA, Morata A. New trends in yeast selection for winemaking. Trends Food Sci Technol. 2012;23:39–50. https://doi.org/10.1016/j.tifs.2011.08.005.
56. Zheng M, Niu D, Jiang D, Zuo S, Xu C. Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. J Appl Microbiol. 2017;122:1456–70. https://doi.org/10.1111/jam.13456.
57. Fu Z, Sun L, Wang Z, Liu Y, Hao J, Gao C, Ge G. Effect of different regions on fermentation profiles, microbial communities, and their metabolomic pathways and properties in Italian ryegrass silage. Front Microbiol. 2023;13:1076499. https://doi.org/10.3389/fmicb.2022.1076499.
58. Rooke JA, Akinsoyinu AO, Armstrong DG. The release of mineral elements from grass silages incubated in sacco in the rumens of Jersey cattle. Grass Forage Sci. 1983;38:311–6. https://doi.org/10.1111/j.1365-2494.1983.tb01654.x.


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