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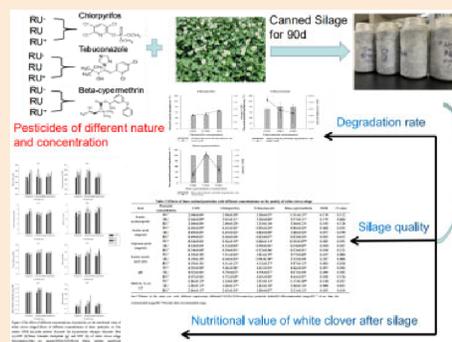
The degradation of residual pesticides and the quality of white clover silage are related to the types and initial concentrations of pesticides

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In order to understand the degradation of different residual pesticides of white clover silage and their influence on silage quality, three commonly used orchard pesticides with different concentrations were added to the white clover and fermented for 90 days. The results showed that the degradation rate of cypermethrin and its toxic degradation product 3-phenoxybenzoic acid (3-PBA) was the highest after silage, at different concentrations, both were 100%. The degradation rate of Tebuconazole and chloropyridine was 72.47–80.27% and 47.76–64.82%, of which 3,5,6-trichloro-2-pyridinol (TCP) content, poisonous toxic degradation product, increased 0.0525–0.253 mg·kg⁻¹. The residues of beta-cypermethrin and tebuconazole had reached safety standards after silage. As compared with the control, the contents of lactic acid, acetic acid, and propionic acid increased in the treated samples. The higher concentrations of three pesticides all significantly reduced the lactic acid content of silage ($p < 0.05$). Pesticides had different effects on the nutritional components of white clover silage. Conclusively, silage is a potential way to expand the utilization of covering plants in orchards.



Keywords: insecticide, fungicide, silage fermentation, pesticide degradation, white clover.

Introduction

Orchard cover plants are used as a mode of managing the ecology of orchards; herbaceous plants are grown between fruit trees or in the entire orchard so that the herbaceous plants and fruit trees can coexist harmoniously.¹⁾ This management model is widely used throughout the world and can produce a large number of cover plants every year. However, the current related research focuses on the effect on the soil and agroecosystems of returning mowed or composted plants to the orchard. In fact, there are drawbacks in the use of returning. For example, Celette

et al. reported that the mulching grass cover can only decompose in the soil and positively affect soil fertility and fruit tree growth for three years after returning to the orchard as a green manure.²⁾ Sholberg³⁾ and Bertilsson and Murphy⁴⁾ found that cover plants in the orchard will easily cause pests and diseases if they are not cut in time or if they are returned to the orchard in excess. Cover plants in the orchard are also an important feeding source, but they pose a great health risk due to the extensive use of pesticides in the management of fruit trees.⁵⁾ White clover (*Trifolium repens* L.) is one of the most important legume forages in the world. It is also the most ideal orchard-covering plant in the Loess Plateau, and it is widely planted in the apple orchard of 963,000 hm². Therefore, it is of great significance to the development of animal husbandry and sustainable agriculture.^{6,7)} For this reason, there is practical value in using scientific and effective methods to reduce pesticide residues on white clover as animal feed.

Chlorpyrifos, tebuconazole, and beta-cypermethrin are widely used in orchard management in many developing countries for insecticidal and bactericidal purposes.⁸⁾ However, due to the lack of proper supervision and management, the use of pesticides far

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exceeds the recommended level, and some studies have found that, after the phosphorus-oxygen bond on chlorpyrifos is broken, the formation of 3,5,6-TCP is riskier than the parent pesticide. Pyrethroid pesticides are widely used, and their degradation product, 3-PBA, can cause agricultural product secondary pollution.^{9,10)} Pesticide residues in feed are the main source of pesticides entering animals.¹¹⁾ If these orchard cover plants are directly fed, a large amount of pesticide residues will be enriched through the food chain, which will seriously threaten the health of livestock and humans, mainly through animal foods such as milk and meat. The excessive use of pesticides has become a source of many health and environmental problems.¹²⁾ Therefore, in order to prevent milk and meat from being contaminated, it is necessary to determine and reduce pesticide residues in feed.¹³⁾

Microorganisms play a key role in the biodegradation of chemical pesticides.¹⁴⁾ Silage is a method of feed preservation based on lactic acid bacterial (LAB) fermentation under anaerobic conditions.¹⁵⁾ One of the most important methods for reducing or removing pesticide residues is to utilize the biodegradation of microorganisms in the fermentation process.¹⁶⁾ The research on the degradation of pesticide residues by fermentation is mostly focused on liquid foods, such as wine, and there has been less research on animal feed.^{17,18)}

This study intends to detect pesticide residues, lactic acid, acetic acid, propionic acid, pH, ammonia nitrogen, dry matter (DM), crude protein (CP), crude fiber (CF), *etc.* of white clover silage over 90 days with different concentrations of chlorpyrifos, tebuconazole, and beta-cypermethrin. The purpose is: (1) to explore the pesticides' degradation of white clover silage, (2) to learn the effect of residual pesticides and their toxic metabolites on the quality of silage, and (3) to grasp the relationship between the degradation of different pesticides and the initial pesticide concentration. It is expected to provide a theoretical basis for the safe use of orchard plant as feed, a combination of planting and breeding, and the sustainable development of a "forest-grass-livestock" circular agriculture model.

Materials and methods

1. Materials

White clover was collected from the Apple Test Base of Modern Agricultural Innovation Park in Yangling District, Xianyang, Shaanxi Province (108°02'E, 34°18'N). The apple orchard in the experimental area was established in 2009. The fruit tree variety planted is "Changfu No. 2." The row spacing between fruit trees is 4 m, and the spacing between trees is 2 m. White clover is planted between rows.

The three selected pesticides are all commonly used in orchards: 45% chlorpyrifos EC, produced by Shaanxi Shangge Road Biological Science Co., Ltd., is an organophosphate insecticide; tebuconazole (430 g/L) suspension, produced by Shaanxi Hengtian Biological Agriculture Co., Ltd., is a high-efficiency broad-spectrum fungicide; active ingredient 2.5% beta-cypermethrin water emulsion, produced by Shaanxi Hengtian Biological Agriculture Co., Ltd., is a pyrethroid insecticide.

Table 1. The design of the white clover silage experiment.

Test treatment		Concentration, g/L	Spray amount, mL
CON	water	—	15
RU ⁻	chlorpyrifos	0.50	15
	tebuconazole	0.30	15
	beta-cypermethrin	0.30	15
RU	chlorpyrifos	0.75	15
	tebuconazole	0.40	15
	beta-cypermethrin	0.40	15
RU ⁺	chlorpyrifos	1.00	15
	tebuconazole	0.50	15
	beta-cypermethrin	0.50	15

Note: The above concentrations are the active ingredients of each pesticide; CON=control, no pesticide added; RU=Recommended usage; RU⁻=Less than the recommended usage; RU⁺=Greater than recommended usage.

Pesticides were diluted with distilled water to three concentrations (lower than recommended concentration (RU⁻), the recommended concentration (RU), and higher than recommended concentration (RU⁺) before use (Table 1), and stored at 4°C. Chlorpyrifos, tebuconazole, and beta-cypermethrin standard products were purchased from the testing center.

2. Silage preparation

In late September 2018, white clover in the full blooming period was mowed and brought back to the laboratory. The white clover was spread and ventilated indoors until its water content was 49.2%. Then, it was cut into 1–2 cm sections and sprayed with pesticide according to Table 1. Sucrose (2%) was added to the processed raw materials and mixed evenly, and it was then put into a 1 L plastic silage bottle, about 800 g per bottle. After compaction, the bottle was sealed. All treatments were replicated three times and kept at room temperature for 90 days (Table 1).

3. Chemical analyses

3.1. Determination of nutritional components and quality of silage fermentation

The nutrient content of white clover before silage is shown in Table 2. Before white clover silage, the water content was controlled to 49.2%. The CP content was 19.42% DM. The ash content was 11.2% DM, and the CF was 18.6%.

After fermentation for 90 days, the silage was taken out of the bottle and mixed as a sample. Each 20 g sample was put into a conical flask, 180 mL of distilled water was added, it was stirred evenly, sealed with a sealing film, kept at 4°C for 24 hr, filtered with four layers of gauze, and filtered with a funnel. The liquid was filtered to determine the pH, organic acids, water-soluble carbohydrates (WSC), and ammonia nitrogen. A 150 g sample was dried in an oven at 65°C to a constant weight and then crushed to determine the DM, CP, CF, acid detergent fiber (ADF), neutral detergent fiber (NDF), ether extract (EE), and moisture content.

Table 2. Chemical composition of white clover before silage.

Test items	DM, %	CP, %DM	EE, %DM	CF, %DM	Ash, %DM	NDF, %DM	ADF, %DM
Content	50.82	19.42	9.68	18.60	11.20	27.10	22.40

Note: DM means dry matter; CP stands for crude protein; EE stands for ether extract; CF stands for crude fiber; NDF stands for neutral detergent fiber; ADF stands for acid detergent fiber.

Chemical composition analysis was determined according to the method of Zhang Q.¹⁹⁾ The pH was determined with a Mettler Lido DELTA 320 pH meter. The content of organic acids was determined by high-performance liquid chromatography. Ammonia nitrogen was determined by phenol-sodium hypochlorite colorimetry. WSC was determined by the anthrone-sulfuric acid colorimetric method. The Kjeldahl method was used to determine the crude protein content. The NDF and ADF were determined by the acid-base digestion method. The contents of dry matter and moisture were determined by the drying method. The EE content was determined by the residual method (SOX406 Fat Analyzer).

3.2. Pesticide residue determination

Extraction and purification of chlorpyrifos, β -cypermethrin pesticide residues, and their toxic metabolites

Twenty g of homogenized white clover was accurately weighed into a 250 mL Erlenmeyer flask, 80 mL of acetonitrile solution containing 0.1% acetic acid was added, it was homogenized for 3 min, shaken for 1 hr, filtered with suction, and the filtrate was poured into the container. Then, 5 g of sodium chloride was measured into a 100 mL cylinder with a stopper, it was shaken vigorously for 3 min and allowed to stand for 20 min. Then, 10 mL of the supernatant organic phase was placed in a flat-bottomed flask, and it was spun and concentrated to dryness, using 2 mL of *n*-hexane to make the volume constant and passing through a 0.22 μ m filter membrane for testing.

Extraction and purification of tebuconazole pesticide residue

A 5.00 g sample of homogenized white clover was weighed into a 50 mL centrifuge tube, 20 mL of acetonitrile was added, and it was homogenized for 3 min, shaken well, and centrifuged at 5000 r/min for 3 min. After standing for stratification, 2.00 mL of the acetonitrile layer was taken, 100 mg PSA was added, along with 50 mg C18 for purification. It was then vortexed and mixed for about 30 sec, and 0.20 mL of the acetonitrile layer was taken, to which 0.80 mL of acetonitrile was added to dilute, and it was passed through a 0.22 μ m organic membrane HPLC-MS/MS for detection. The samples in the super linear range were diluted with wheat straw blank matrix to the linear range for detection.

In accordance with Zhao *et al.*'s method, liquid chromatography-tandem mass spectrometry was used to determine the pesticide residue of tebuconazole.²⁰⁾ According to the methods of Hunter *et al.*, chlorpyrifos and beta-cypermethrin pesticide residues were determined by gas chromatography.²¹⁾

Method accuracy and precision

The recovery rate of chlorpyrifos is 77.2–95.6%, the relative standard deviation (RSD) is 1.4–3.7%, and the minimum detection limit is 0.02 mg/kg; the recovery rate of beta-cypermethrin is

72.1–87.2%, the relative standard deviation (RSD) is 5.2–7.9%, and the lowest detection limit is 0.003 mg/kg; the recovery rate of tebuconazole is 87.2–101.3%, the relative standard deviation (RSD) is 3.1–7.9%, and the lowest detection limit is 0.00056 mg/kg; the TCP recovery rate of the toxic metabolite 3,5,6-trichloro-2-pyridinol of chlorpyrifos is 91–101%, the relative standard deviation (RSD) is 2.2–4.9%, and the minimum detection limit is 0.0005 mg/kg. For the toxic metabolite of beta-cypermethrin, 3-phenoxybenzoic acid, the recovery rate of 3-PBA is 59–77%, the relative standard deviation (RSD) is 3.2–4.5%, and the minimum detection limit is 0.2 mg/kg.

Results and discussion

1. Degradation of three pesticides with different concentrations after white clover silage

After white clover was fermented at ambient temperature for 90 days, beta-cypermethrin treatment had the highest degradation, reaching 100%, followed by tebuconazole treatment; the lowest was chlorpyrifos treatment, with which TCP content increased by 0.0153–0.172 mg·kg⁻¹ (Table 3). Silage fermentation is a complex reaction process involving multiple microorganisms and chemical substances.²²⁾ The degradation effect of microorganisms on pesticides during silage is mainly due to its certain adsorption effect on pesticides. Some bacteria can use pesticides as energy sources, such as carbon sources and phosphorus sources, to promote the production of organic acids.²³⁾ However, due to the different chemical properties and structures of the three pesticides, the absorption and utilization efficiency of microorganisms are different. The chemical properties and structures of pesticides are also important factors affecting their degradation by microorganisms.²⁴⁾ The degradation efficiency of some pesticides, such as beta-cypermethrin,²⁵⁾ is higher in anaerobic conditions than in aerobic conditions, while the degradation of beta-cypermethrin is mainly through microbial degradation. Regueiro *et al.* reported that the action of *Saccharomyces cerevisiae* completely degrades pyrethroid insecticides.²⁶⁾ Shi *et al.* showed that LAB can promote the degradation of theta-cypermethrin (CYP)²⁷⁾; this may also be why beta-cypermethrin had the best degradation effect in white clover and the lowest residual amount after silage. The degradation products of chlorpyrifos can reduce the activity of microorganisms. Studies have found that the degradation intermediate 3,5,6-trichloropyridinol (TCP) of chlorpyrifos is resistant to microorganisms in different media, which slows its degradation to chlorpyrifos.²⁸⁾ In this study, the TCP content after silage fermentation increased by 0.0525–0.2530 mg·kg⁻¹. This may be why chlorpyrifos of white clover silage had the slowest degradation. Wang *et al.* showed

Table 3. Degradation rate of three pesticides at different concentrations after 90 days of white trifoliolate silage fermentation.

Pesticide types	Pesticide concentration	The initial concentration, mg/kg	Residual concentration, mg/kg	Detection limit, mg/kg	Degradation rate, %	Recovery rates mean, %
chlorpyrifos	RU ⁻	9.38	4.90±0.09 ^{b)}	0.02	47.76±0.94 ^{b)}	77–95
	RU	14.06	7.00±0.22 ^{a)}		50.21±1.56 ^{b)}	
	RU ⁺	18.76	6.60±0.23 ^{c)}		64.82±1.21 ^{a)}	
tebuconazole	RU ⁻	5.63	1.55±0.13 ^{b)}	0.00056	72.47±2.35 ^{b)}	87–101
	RU	7.5	1.48±0.13 ^{b)}		80.27±1.73 ^{a)}	
	RU ⁺	9.38	1.99±0.12 ^{a)}		78.78±1.38 ^{a)}	
beta-cypermethrin	RU ⁻	5.63	undetected	0.003	>99%	72–87
	RU	7.5	undetected		>99%	
	RU ⁺	9.38	undetected		>99%	
TCP	RU ⁻	—	0.2530 ^{a)}	0.00056	—	91–101
	RU	—	0.1720 ^{b)}		—	
	RU ⁺	—	0.0525 ^{c)}		—	
3-PBA	RU ⁻	—	undetected	0.2	—	59–77
	RU	—	undetected		—	
	RU ⁺	—	undetected		—	

Note: The data are the mean±S.D. ($n=3$). There are differences in the mean values of ^{a)–b)} in the same column of the same pesticide with different superscripts ($p<0.05$). CON=control, no pesticide added; RU=Recommended usage; RU⁻=Less than the recommended usage; RU⁺=Greater than recommended usage; TCP=Chlorpyrifos toxic metabolite 3,5,6-trichloro-2-pyridinol; 3-PBA=Beta-cypermethrin toxic metabolite 3-phenoxybenzoic acid.

that the degradation rate of chlorpyrifos is 52.4% after the silage of chlorpyrifos straw for 40 days.²⁹⁾ Tebuconazole pesticide mainly inhibits the growth and metabolism of fungi, while bacteria and actinomycetes are the main microbial groups that transform and degrade pesticides. Sehnem *et al.* isolated and screened three bacterial groups with high activity for degrading tebuconazole from soil contaminated with tebuconazole and found that they are mainly *Pseudomonas*, *Serratia*, and *Enterobacter*.³⁰⁾ This can explain why most of the tebuconazole can be degraded during the fermentation of white clover.

Different initial pesticide concentrations also had an impact on the degradation of pesticides after silage. The degradation rate of different concentrations of beta-cypermethrin was 100%. With increased pesticide concentration in the treatment of chlorpyrifos, the degradation rate increased from 47.76 to 64.82%, while the TCP content was the opposite. The degradation rates of tebuconazole at RU⁻, RU, and RU⁺ concentrations were 72.47, 80.27, and 78.78%, respectively; the degradation rate at RU⁻ concentration was significantly lower than that at RU⁺ concentration ($p<0.05$) (Table 3). This result may be attributed to the fact that the degradation of pesticides by microorganisms is a complicated process, during which different concentrations of pesticides can decrease some microorganisms, and in the absence of competition, specific pesticide-degrading microorganisms may change the biological activity of microorganisms for pesticide degradation. For example, Dordevic and Durovic found that, as compared with low concentrations of chlorpyrifos, high concentrations of chlorpyrifos can better promote the metabolism of *Lactobacillus casei*, thereby increasing the degradation rate of chlorpyrifos.³¹⁾ However, some studies have shown that TCP, a degradation intermediate product of chlorpyrifos,

can slow down the degradation of chlorpyrifos, which is inconsistent with our results. This may be because the high concentration of chlorpyrifos or TCP changed the biological life of some microorganisms, thus accelerating the degradation of TCP, resulting in the more effective degradation of chlorpyrifos when treated with a high initial concentration of chlorpyrifos, as the TCP is degraded by microorganisms, and the content is reduced. Li *et al.* found that strain T6 can degrade TCP due to the action of a certain concentration of pesticides.³²⁾ Therefore, the reason the degradation of chlorpyrifos increased with increasing concentration needs further study. This result may also be because the pesticide concentration had an impact on the metabolic process of some microorganisms that degrade pesticides, which, in turn, led to changes in the pesticide degradation rate. Youness *et al.* showed that when the initial concentration of tebuconazole reaches a certain value, the degradation rate of tebuconazole by *Bacillus* sp. 3B6 remains at about 50%.³³⁾

Our study shows that after silage, beta-cypermethrin was completely degraded, tebuconazole residue was 1.55–1.99 mg/kg, and chlorpyrifos was 4.90–7.00 mg/kg. Additionally, the degradation rate of chlorpyrifos and tebuconazole increased as the initial concentration increased. According to reports, the 96-hour lethal concentrations of 50% (LC⁵⁰) chlorpyrifos to Nile tilapia and tebuconazole to zebrafish are 98.67–154.01 µg/L³⁴⁾ and 26.8 mg/L, respectively.³⁵⁾ As a high-efficiency isomer of cypermethrin, high-biologically active beta-cypermethrin has contact-killing and stomach-toxicity effects; its 24-hour LC⁵⁰ value for Nile tilapia fingerlings is 21.4 µg/L.³⁶⁾ The three pesticides studied are widely used in various crops, and their excessive use will cause environmental pollution and harm the health of livestock and humans. According to the European Food Safety Authority, the maximum resi-

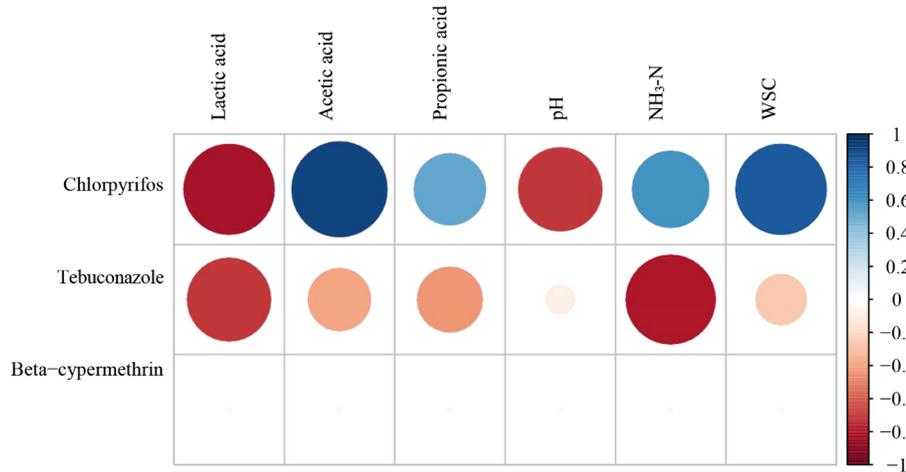


Fig. 1. Correlation between the degradation of pesticides with different properties and chemical substances in silage. The shape in the box is the correlation coefficient “r” between the row variable and the column variable. The larger the absolute value of the correlation coefficient “r”, the larger the area of the circle, and the darker the color (red means negative correlation, blue means positive correlation; 0.8–1.0 very strong correlation, 0.6–0.8 strong correlation, 0.4–0.6 moderate correlation, 0.2–0.4 weak correlation, 0.0–0.2 very weak correlation or no correlation).

dues of chlorpyrifos, tebuconazole, and beta-cypermethrin in crop animal feed are 4.44 mg/kg,³⁷⁾ 6.00 mg/kg,³⁸⁾ and 2.01 mg/kg,³⁹⁾ respectively. After silage fermentation, the pesticide residues of white clover with different concentrations of tebuconazole and beta-cypermethrin were far below the safety standards. Although chlorpyrifos was significantly reduced, the residue was still higher than the safety standards.

2. Effects of three pesticides on the fermentation quality of white clover

The pH value is an important indicator reflecting the activity of microorganisms and the fermentation quality of silage. Us-

ually, the lower the pH, the better the fermentation quality is. The pH reduction is achieved by some strains producing acid through fermentation.⁴⁰⁾ In this study, with the exception of RU⁻ chlorpyrifos, the pH value of each pesticide-spraying treatment was significantly lower than that of the control ($p < 0.05$). The degradation rate of chlorpyrifos was negatively correlated with pH ($r = -0.7$), and the degradation rate of tebuconazole had no correlation with pH ($r = -0.09$, Fig. 1). After spraying pesticides, the pH value of white clover silage was as follows: tebuconazole < beta-cypermethrin < chlorpyrifos (Table 4). This result was consistent with the lactic acid, acetic acid, and propionic acid content of silage. This may be because the meta-

Table 4. Effects of three orchard pesticides with different properties on the quality of white clover silage.

Item	Pesticide concentration	CON	chlorpyrifos	tebuconazole	beta-cypermethrin	SEM	p-value
Lactic acid, mg/mL	RU ⁻	2.04 ± 0.09 ^{c)}	2.98 ± 0.30 ^{b)}	3.50 ± 0.07 ^{a)}	3.31 ± 0.13 ^{a,b)}	0.174	0.112
	RU	2.04 ± 0.09 ^{d)}	3.01 ± 0.11 ^{c)}	3.30 ± 0.06 ^{b)}	3.57 ± 0.11 ^{a)}	0.175	0.604
	RU ⁺	2.04 ± 0.09 ^{c)}	2.40 ± 0.20 ^{b)}	3.35 ± 0.10 ^{a)}	3.56 ± 0.23 ^{a)}	0.195	0.128
Acetic acid, mg/mL	RU ⁻	0.49 ± 0.05 ^{b)}	0.42 ± 0.02 ^{c)}	0.92 ± 0.02 ^{a)}	0.88 ± 0.03 ^{a)}	0.068	0.423
	RU	0.49 ± 0.05 ^{c)}	0.45 ± 0.02 ^{c)}	0.84 ± 0.06 ^{b)}	1.06 ± 0.02 ^{a)}	0.077	0.199
	RU ⁺	0.49 ± 0.05 ^{c)}	0.56 ± 0.04 ^{c)}	0.82 ± 0.07 ^{b)}	0.92 ± 0.03 ^{a)}	0.055	0.613
Propionic acid, mg/mL	RU ⁻	0.24 ± 0.04 ^{c)}	0.36 ± 0.12 ^{b)}	0.60 ± 0.14 ^{a)}	0.32 ± 0.07 ^{b,c)}	0.043	0.221
	RU	0.24 ± 0.04 ^{c)}	0.32 ± 0.04 ^{b)}	0.50 ± 0.01 ^{a)}	0.34 ± 0.05 ^{b)}	0.030	0.367
	RU ⁺	0.24 ± 0.04 ^{b)}	0.39 ± 0.01 ^{a)}	0.32 ± 0.06 ^{a)}	0.33 ± 0.01 ^{a)}	0.018	0.121
Lactic:acetic acid ratio	RU ⁻	4.19 ± 0.56 ^{b)}	7.21 ± 0.03 ^{a)}	3.81 ± 0.15 ^{b)}	3.77 ± 0.04 ^{b)}	0.437	0.004
	RU	4.19 ± 0.56 ^{b)}	6.64 ± 0.03 ^{a)}	3.94 ± 0.34 ^{b,c)}	3.35 ± 0.04 ^{c)}	0.387	0.006
	RU ⁺	4.19 ± 0.56 ^{a)}	4.31 ± 0.12 ^{a)}	4.12 ± 0.27 ^{a)}	3.87 ± 0.12 ^{a)}	0.093	0.018
pH	RU ⁻	4.92 ± 0.06 ^{b)}	5.06 ± 0.06 ^{a)}	4.61 ± 0.03 ^{c)}	4.66 ± 0.02 ^{c)}	0.057	0.302
	RU	4.92 ± 0.06 ^{a)}	4.79 ± 0.03 ^{b)}	4.54 ± 0.07 ^{d)}	4.67 ± 0.04 ^{c)}	0.044	0.585
	RU ⁺	4.92 ± 0.06 ^{a)}	4.72 ± 0.08 ^{b)}	4.62 ± 0.06 ^{b)}	4.64 ± 0.01 ^{b)}	0.038	0.126

Note: Data are means ± S.D. ($n = 3$). ^{a)-d)} Means in the same row with different superscripts differed ($p < 0.05$). CON = control, no pesticide added; RU = Recommended usage; RU⁻ = Less than the recommended usage; RU⁺ = Greater than recommended usage.

bolic processes of some microorganisms in the silage fermentation were changed by the pesticides. For example, Ayana *et al.* showed that pesticides have a negative effect on the acid production of LAB strains (*Streptococcus salivarius subsp. thermophilus* H, *Lactobacillus acidophilus* (Type 145), *Lactobacillus casei* ssp. *casei*, *Lactobacillus delbrueckii* ssp. *Bulgaricus*, and *Bifidobacterium* spp. 420),⁴¹⁾ while the study by Doignon and Rozes showed that triazoles fungicides can promote the metabolism of *Saccharomyces cerevisiae* to produce unsaturated free fatty acids, while inhibiting the production of short free fatty acids.⁴²⁾

The content of organic acids produced during fermentation is an important factor in evaluating the quality of silage fermentation.⁴³⁾ Lactic acid, acetic acid, propionic acid, and butyric acid are the most important organic acids produced by silage fermentation. Additionally, butyric acid is produced by *Clostridium butyricum*, which gives the feed a sour smell and reduces the quality of silage. In this study, butyric acid was not detected. As compared with the control, the content of lactic acid, acetic acid, and propionic acid of white clover silage increased with each treatment. At RU⁻ concentration, the contents of lactic acid and acetic acid with tebuconazole treatment were significantly higher than those treated with chlorpyrifos; however, there was no significant difference from silage treated with beta-cypermethrin. The content of propionic acid with tebuconazole treatment was significantly higher than that of chlorpyrifos and beta-cypermethrin ($p < 0.05$). The content of lactic acid and acetic acid with beta-cypermethrin treatment was significantly higher than that with other treatments at a concentration of RU. Additionally, the content of lactic acid and acetic acid in the treatment of chlorpyrifos was the lowest. Tebuconazole treatment caused the highest concentration of propionic acid ($p < 0.05$), and there was no significant difference in pro-

ponic acid content between chlorpyrifos and beta-cypermethrin treatments. At concentrations of RU⁺, the content of lactic acid treatment with beta-cypermethrin and tebuconazole was significantly higher than that with chlorpyrifos treatment, and acetic acid with beta-cypermethrin treatment was significantly higher than those with other treatments ($p < 0.05$). There was no significant difference in the propionic acid content between pesticide treatments (Table 4). The low lactic acid content of chlorpyrifos may be due to the physicochemical properties of chlorpyrifos or the influence of chlorpyrifos intermediates on microbial activity, which inhibited the metabolism of lactic acid. For example, studies have shown that dicofol has a great inhibitory effect on malolactic fermentation, while the bactericide chlorothalonil has little effect on it.⁴³⁾ The concentration of propionic acid in tebuconazole seems to be about three times higher than that in the control, so it can be explained that the fungicide can promote the increase of propionic acid. Simultaneously, the fungicide, tebuconazole may reduce the activity of yeast and promote the metabolism of acid-producing microorganisms. For example, He *et al.* showed that the fungicide chlorothalonil can effectively inhibit yeast fermentation of alcohol.¹⁷⁾ Beta-cypermethrin treatment may promote fermentation. Research by Antwi *et al.* has shown that pyrethroid pesticides can promote the fermentation of rumen microorganisms,⁴⁴⁾ and in the process of heterosexual fermentation of lactic acid bacteria, lactic acid is produced as well as a large amount of acetic acid.⁴⁵⁾ Therefore, this treatment can finally obtain a large amount of lactic acid and acetic acid.

The degradation rate of chlorpyrifos was negatively correlated with the content of lactic acid ($r = -0.83$) and positively correlated with the content of acetic acid ($r = 0.92$) and propionic acid ($r = 0.52$). The lactic acid content decreased significantly

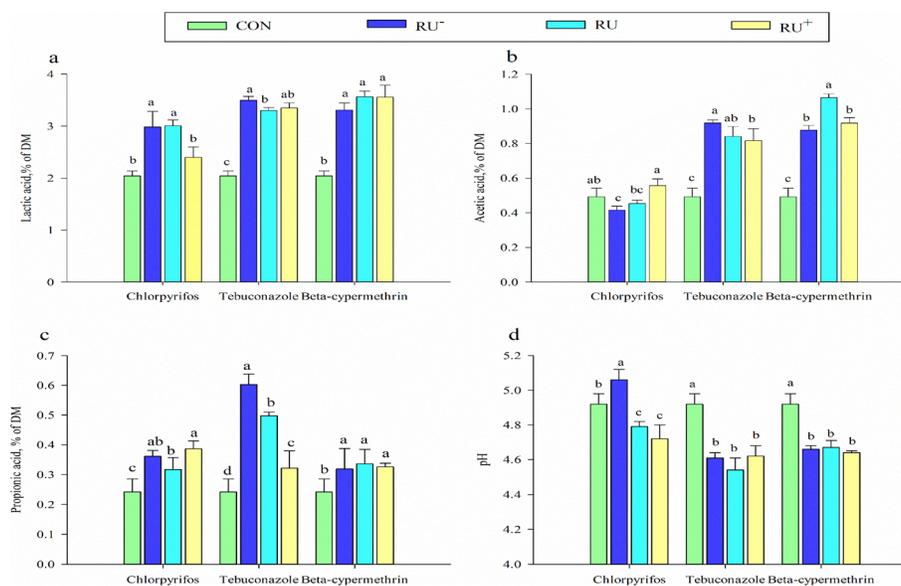


Fig. 2. Effects of different pesticide concentrations on the quality of white clover silage. Effects of different concentrations of three pesticides on lactic acid (a), acetic acid (b), propionic acid (c), and pH (d) of white clover silage fermentation. Data are the means \pm S.D. ($n = 3$). Different letters indicate significant differences at $p < 0.05$, according to the least significant difference (LSD).

as the concentration of chlorpyrifos increased ($p < 0.05$), and the content of acetic acid and propionic acid increased significantly ($p < 0.05$, Figs. 1, 2). This may be because the degradation of high concentrations of chlorpyrifos led to the accumulation of chlorpyrifos degradation intermediates, which inhibited the activity of acid-producing microorganisms such as lactic acid bacteria, thereby reducing the content of lactic acid and acetic acid, but had little effect on the microorganisms that produce propionic acid.²⁸⁾ The degradation rate with tebuconazole treatment was negatively correlated with the contents of lactic acid ($r = -0.7$), acetic acid ($r = -0.4$), and propionic acid ($r = -0.43$). The contents of lactic acid, acetic acid, and propionic acid decreased significantly with increased concentrations ($p < 0.05$, Figs. 1, 2). This may be because different concentrations of the fungicide tebuconazole inhibited the metabolic activity of some microorganisms, while the microorganisms that were not inhibited by tebuconazole continued to carry out metabolic activities. Studies such as that of Doignon and Rozes showed that as the concentration of triazole pesticides increases, yeast metabolism is inhibited.⁴²⁾ Because all degradation rates of beta-cypermethrin were greater than 99%, Fig. 1 cannot show its correlation with various indicators. However, it can be seen from Fig. 2 that the concentration of beta-cypermethrin had no significant effect on the contents of lactic acid and propionic acid. The highest acetic acid content was obtained at RU, which was significantly higher than that with RU⁻ and RU⁺ treatments ($p < 0.05$). Beta-cypermethrin may be greatly affected by microbial degradation. Additionally, it was degraded in the early stage of fermentation, and the degradation rate was greater than 99% under different treatment concentrations. Therefore, the effect of different concentrations of beta-cypermethrin on silage organic acids was not significant. Tang *et al.* found that the degradation rate of beta-cypermethrin by strain *Brevibacillus parabravis* BCP-09 was 75.87% within 3 days (pH 7.41, 38.9°C, 30.9 mg/L Beta-CP).²³⁾ In summary, the degradation of pesticides had different effects on the quality of white clover silage due to the different properties of pesticides. After silage, the content of organic acids of silage increased with all treatments. With the exception of RU⁻ chlorpyrifos, the pH value of the other treatments was significantly reduced ($p < 0.05$).

3. Effects of three pesticides on nutritional components of white clover silage

DM, CP, EE, CF, ADF, NDF, and WSC contents of white clover silage after spraying pesticides for 90 days are shown in Figs. 3 and 4. Before starting the silage, the water content of white clover was 49.2%, and the DM content was 50.8%. With three different pesticide treatments, the loss of DM content of the raw materials was between 3 and 14.5%, following the order of chlorpyrifos > beta-cypermethrin > CON > tebuconazole treatment. Additionally, the difference was significant ($p < 0.05$, Fig. 3a), much lower than the DM loss of white clover as reported in the study of Johansson *et al.*⁴⁶⁾ Because the loss of DM of silage fermentation is mainly due to the aerobic activity of aerobic

microorganisms in the early stage, the abnormal fermentation produces volatiles that affect the quality of silage. For example, the metabolism of clostridium or yeast promotes the degradation of DM.^{40,47)} The addition of pesticides, such as chlorpyrifos and beta-cypermethrin, may inhibit this metabolism and prevent the degradation of DM. Tijana and Rada found that chlorpyrifos-methyl can effectively inhibit the formation of yeast communities, resulting in a 50% reduction in the number of yeast cells (CFU).⁴⁸⁾ With the increased pesticide concentrations in chlorpyrifos treatment, the DM content of white clover silage increased significantly ($p < 0.05$), but that with tebuconazole treatment was just the opposite. As for beta-cypermethrin treatment, the DM content of white clover first increased and then decreased, and the DM loss was the largest under the RU concentration treatment (Fig. 3a). This may be because low-concentration fungicide tebuconazole and high-concentration chlorpyrifos can better inhibit aerobic microorganisms, as well as anaerobic microorganisms that are not conducive to silage fermentation, reducing their extensive fermentation.⁴⁹⁾ Tijana and Rada found that high concentrations of chlorpyrifos-methyl severely affect yeast metabolism,⁴⁸⁾ thereby maximizing the retention of the dry matter of raw materials.

The degradation of CP is closely related to the production of ammonia nitrogen, and the deamination of CP produces ammonia nitrogen.⁵⁰⁾ The CP content of white clover treated with chlorpyrifos and tebuconazole was significantly higher than that of the control ($p < 0.05$), while there was no significant difference between that treated with beta-cypermethrin and the control. This was consistent with the changing trend of ammonia nitrogen (Fig. 3b, d). With increased pesticide concentration, the CP content with chlorpyrifos and beta-cypermethrin treatments showed a trend of first decreasing and then increasing. Contrary to the change trend of tebuconazole cp, the change trend of ammonia nitrogen at other concentrations is consistent with the change trend of CP. At the RU concentration, the CP content of chlorpyrifos was lowest, while the ammonia nitrogen content was highest at the RU⁺ concentration. At the RU concentration, the CP content of beta-cypermethrin is lowest, and the corresponding ammonia nitrogen content is highest. However, it was the opposite for tebuconazole treatment (Fig. 3b, d). Part of this result can be attributed to the fact that the spraying of chlorpyrifos and tebuconazole inhibited the growth of some obligate anaerobes of the genus clostridium or the activity of proteolytic enzymes,⁵¹⁾ resulting in the reduction of CP degradation and the preservation of CP in the raw materials.

In this experiment, with the degradation of pesticides, the content of NDF and CF in tebuconazole treatment were the highest, and those of chlorpyrifos treatments were the lowest. At the same concentration, the content of ADF with tebuconazole and beta-cypermethrin treatment was significantly higher than that of the control ($p < 0.05$), and that of the chlorpyrifos treatment was significantly lower than that of the control ($p < 0.05$, Fig. 4a, b, d). This may be because the degradation of NDF and CF was caused by a large number of aerobic and anaerobic mi-

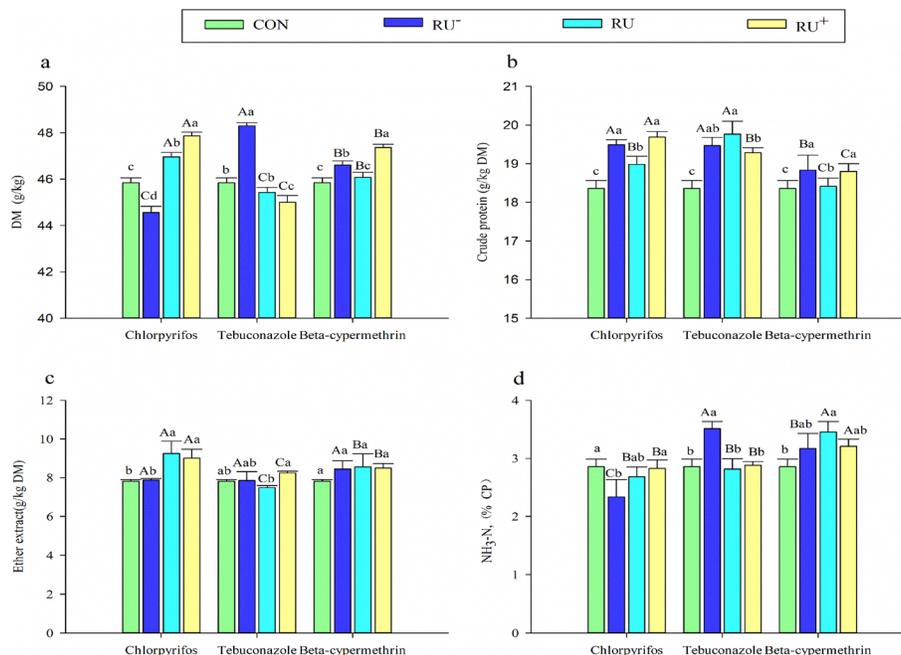


Fig. 3. The effect of three pesticides at different concentrations on the nutritional value of white clover silage. Dry matter (DM) (a), crude protein (b), crude fat (c), and ammonia nitrogen (d). Data are the mean \pm S.D. ($n=3$). Different letters a–c indicate that different color histograms with the same X value have significant differences (LSD) at $p<0.05$, and different letters A–C indicate that the same color histograms with different X values have significant differences (LSD) at $p<0.05$.

croorganisms. The spraying of fungicides may significantly affect the activities of these microorganisms, while pesticides had little effect on the activities of these microorganisms. Zhang *et al.* showed that tebuconazole can significantly inhibit soil microbial activity.⁵² Kato *et al.* showed that five kinds of microorganisms have been found (*Clostridium straminisolvans* CSK1, *Clostridium*

sp. strain FG4, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6). They can effectively degrade fiber, but a large amount of acetic acid inhibits this degradation.⁵³ This was consistent with the fact that the acetic acid content of tebuconazole treatment was significantly higher than that of chlorpyrifos treatment in this study. Howev-

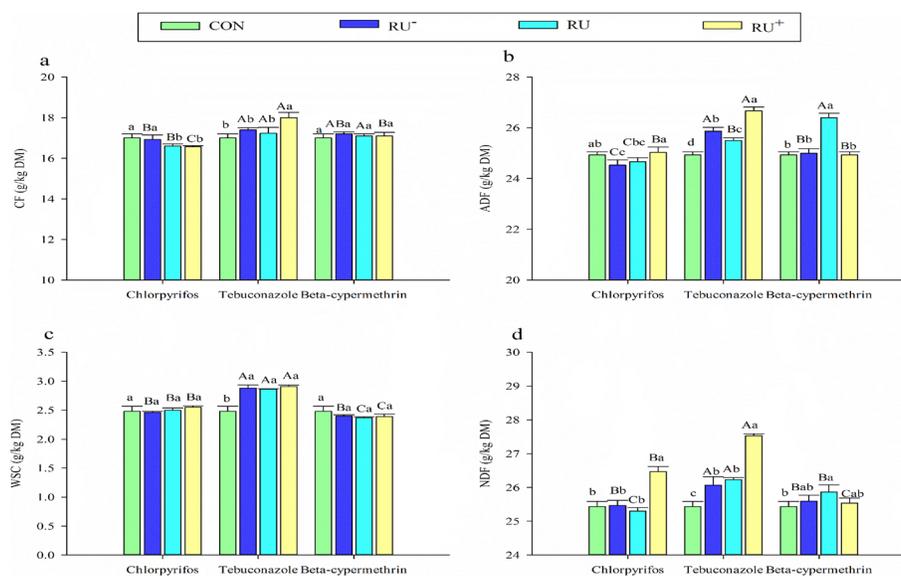


Fig. 4. The effect of three pesticides at different concentrations on the nutritional value of white clover silage. Crude fiber (a), ADF (b), water-soluble sugar (c), and NDF (d) fermented from clover silage. Data are the mean \pm S.D. ($n=3$). Different letters a–c indicate that different color histograms with the same X value have significant differences (LSD) at $p<0.05$, and different letters A–C indicate that the same color histograms with different X values have significant differences (LSD) at $p<0.05$.

er, further study is needed to discover the specific reason. Contrary to tebuconazole treatment, the CF content with chlorpyrifos treatment decreased as the pesticide concentration increased, and that of the beta-cypermethrin treatment was not affected by the pesticide concentration (Fig. 4a).

WSC can provide fermentation substrates for the proliferation of lactic acid bacteria during silage. The WSC content of the raw material is one of the most important factors determining the quality of fermentation. The content of WSC was in the order of tebuconazole>chlorpyrifos>CON>beta-cypermethrin ($p<0.05$). The content of WSC of all treatments was not affected by a pesticide's concentration and degradation (Fig. 4c). This may be because chlorpyrifos and tebuconazole pesticides had a negative impact on the metabolic activity of microorganisms, which weakened the metabolic activity of microorganisms and reduced the consumption of WSC.⁵⁴ However, due to its poor stability in anaerobic conditions and acidic environments, beta-cypermethrin decomposes quickly in the early stage and has no significant impact on microbial activity. Consequently, WSC consumption was relatively greater.⁵⁵

In conclusion, the residues of beta-cypermethrin and tebuconazole of white clover silage meet the safe feeding standards. Three orchard pesticides with different properties have no negative effects on the nutritional value of silage. Tebuconazole treatment is more conducive to the retention of nutrients as compared with beta-cypermethrin and chlorpyrifos treatments. The contents of DM, CP, and ammonia nitrogen of silage are related to the concentration of pesticides. The results can provide a theoretical basis for the development of orchard cover plant resources and the feed safety of white clover silage with pesticides before silage.

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Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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