Effects of oregano essential oil, cobalt and synergistic of both of them on rumen degradation rate and fermentation characteristics for corn silage

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ABSTRACT

This study used oregano essential oil (EO), cobalt (Co) and synergistic of both of them additives instead of antibiotics to explore the effects of different additives on the corn silage fibre structure degradation, nutrient degradation rate and rumen fermentation characteristics of sheep. The additives used in the study as a product were provided by Ralco, Inc., USA. The Latin square experimental design was adopted with 5 treatments. The following treatments were fed through the rumen fistula: carrier (Car), carrier + EO, carrier + Co, carrier + EO + cobalt (EOC) and no additives as the control (CON). These treatments were fed to five sheep for five rounds at a dose of 80 mg/kg. At 48 h of degradation, the area of degraded corn leaf and husk reached 100%, SEM results showed that except for Car and CON treatment, there were obvious destroyed fibre structure differences under EO, Co and EOC treatment, showing total disappearance of leaf epidermal cells, palisade tissue and spongy tissue, especially under EOC. Under EOC treatment, DMD\textsubscript{48h}, CPD\textsubscript{48h}, NDFD\textsubscript{48h} and ADFD\textsubscript{48h} increased by 3.33%, 3.89%, 7.64% and 5.67%, respectively, compared with CON, while NDFD\textsubscript{48h} and ADFD\textsubscript{48h} increased by 4.70% and 6.05% under Co ($p>0.05$). The NH\textsubscript{3}-N concentration in rumen fluid decreased by 19.36%, 27.20% and 19.48%, while the microbial protein (MCP) content increased by 6.32%, 4.29% and 5.97% ($p>0.05$) under EO, Co and EOC treatments compared with CON at 48 h ($p<0.05$). The total VFA concentration under EO, Co and EOC treatments was lower than CON at 6 and 48 h ($p=0.016$, $p=0.022$) and the propionate and acetate concentrations were higher under EOC treatment than CON at 48 h ($p>0.05$). Feeding EOC increased the fibre structure degradation and nutrient degradation rate of corn silage and improved rumen fermentation in sheep, resulting in better effects than feeding EO or Co alone.

HIGHLIGHTS

- This article revealed the significance of oregano essential oil synergistic with cobalt (EOC) on rumen fermentation characteristics and nutrient degradation rate of sheep.
- In this study, the degradation of leaves in the rumen was observed by scanning electron microscopy, and the degradation area was calculated by electron microscopy for the first time.
- The results of this study will provide the theoretical basis for EOC as a substitute for antibiotics.

Introduction

Livestock production provides significant benefits to human life (Bar-On et al. 2018); however, the shortage of forage resources has become the main factor restricting the development of animal husbandry in China (Qin 2021). As a largely agricultural country, China produces a high amount of straw, especially corn straw but most of this straw is not utilised (Lu et al. 2018). Using these straw resources to feed animals would reduce not only environmental pollution but also the competition for food between humans and animals. However, corn straw contains cellulose, hemicellulose and lignin, which reduce its efficient utilisation by animals (Zhang 2014), so it needs to be processed to improve its utilisation, palatability and nutritional quality or the utilisation of straw needs to be improved by modifying the digestive function of the rumen in ruminants. Recently, it was
found that the addition of some feed additives in diets can improve rumen nutrient digestibility (Sheperd and Combs 1998; Liu 2008), and straw feed utilisation, which were all antibiotic additives. So anti-biotic-free animal products are favourable for human and animal health.

Therefore, this study used additives instead of antibiotics. Oregano essential oil (EO) is a natural plant-extracted EO and is composed of carvacrol, thymol, cymene and terpinene (Lambert et al. 2001; Rhayour et al. 2003). Due to their known antimicrobial and antifungal actions, such EOs are considered extensively for use in food, agriculture and livestock (Burt 2004; Bakkali et al. 2008). Recent research results have shown that EOs can improve the growth characteristics of animals (Tekippe et al. 2011; Baranauskaite et al. 2017), the rumen fermentation characteristics (Newbold et al. 2004; Ye 2013) and nutrient degradation rate of animals (Surface-Active Agents-Detergents 2018). Zhang et al. (2021) fed on a daily basal diet supplemented with EO in beef cattle and found that this increased the rumen digestive ability by modulating epithelial development and microbiota composition. Cobalt (Co) is a special trace element that is necessary for animal growth and development. It is involved in the synthesis of vitamin B12 and plays a very important role in ruminants. If Co requirements are not met, dietary supplementation with Co can enhance production performance, immune performance and haematopoietic function in ruminants (Mburu et al. 1993; Li et al. 2008), and Co has been shown to improve feed digestibility in both in vitro and in vivo studies (Poudel 2016; Pretz 2016).

Studies have found that the combination of EO and Co (EOC) is a safe, economical and drug-free alternative to antibiotics. The primary components of EO are carvacrol and thymol, both phenolic compounds, which have a hydroxyl group that destroys bacterial cell membranes causing leakage of ions and molecules, thus exerting antibacterial activity (Lv et al. 2011). EO improves the microbial community structure of the intestinal tract and increases the ratio of beneficial to harmful intestinal microbiota, and Co is involved in the synthesis of vitamin B12 in the rumen of ruminants. Kuester (2016) added EOC to a full mixed ration fed to Holstein cows and found that EOC did increase the ratio of fat, protein and total intake in milk. A study at the University of Wisconsin, Platteville found that adding EOC to diets increased the performance of dairy cows by 4% in May and 8% in August and prevented the milk fat percentage from decreasing with temperature. Lei et al. (2018) added 0, 52 and 91 mg of EOC per head per day to the diet of velvet goats and found that EOC increased the average daily weight gain and improved apparent traits, and significantly increased the proportion of calcium and reduced the proportion of magnesium in the blood. Jiao et al. (2021) found that feeding Co, EO or EOC at 4 or 7 g/day enhanced ruminal nutrient digestion and fermentation parameters, which was visually confirmed by scanning electron microscopy (SEM) and stereoscopic microscopy (SM), but the dynamic changes in the rumen degradation rate and fermentation characteristics after additive treatment were not studied, and the degradation area of leaves in the rumen was not calculated via SEM. A unique and novel feature of this study is that the irregular leaf degradation area in the rumen was calculated using SM and the changing pattern in the rumen after additive treatment was investigated. The calculation of the leaf degradation area can more reliably confirm the results observed by microscopy. This study hypothesised that EOC treatment could affect fibre structure degradation of corn silage leaves and husks, and change rumen fermentation function of sheep. Therefore, the objective of this experiment is to investigate the effects of different additive treatments on fibre structure degradation, nutrient degradation rate and rumen fermentation parameters through rumen fistula technology and finally to provide a theoretical basis for the rational use of EOC in meat sheep production.

Materials and methods

Experimental treatments, design and animal

The experiment was conducted according to the Standard for the Care and Use of Research Animals (NRC, 2010) on farm 155, Tao Lin village, An Ning Fort, An Ning District, Gansu Province or at Gansu Agricultural University, Lanzhou, China.

The different additives were carrier (Car), carrier + oregano EO, carrier + Co and carrier + oregano EO + cobalt (EOC). The specific compositions are given in Table 1. The three additives and Car were manufactured, packaged, shipped and donated for the research project by the Animal Health Division of Ralco, Inc. (Marshall, MN, USA). The amount of additive was determined according to the average weight of the sheep. Studies have shown (Liang 2016) that 4 g per sheep per day is the most effective amount of additive, and we used sheep with an average weight of 50 kg, so the amount added was 80 mg/kg.
The experiment was conducted in a $5 \times 5$ Latin square design with five treatments corresponding to the same dose of the Car, EO, Co and EOC additives and control (CON). Five sheep with fistulas were numbered 1–5, corresponding to the five treatments, with five replicates per treatment and five rounds (Runs 1–5). The experiment had a prefeeding period of 7 days and a treatment period of 48 h. The first experimental replicate started at 7:00 am on the first day and ended at 7:00 am on the third day (48 h). After 10 days of letting, the rumen environment rest and stabilise, the second replicate was conducted. The specific experimental design is given in Table 2.

Five healthy rams (two generations of crossbred Small-Tail Han sheep $\times$ Lanzhou local sheep) with permanent rumen fistula installed at $\sim 10$ months of age, an average rectal temperature of $39 \, ^{\circ} C$ and an approximate weight of $45 \pm 5$ kg were used as experimental animals. The fistulated rams were blocked by body weight and age and randomly assigned to one of five Latin squares while being housed in shade-covered, open-sided, naturally ventilated pens. The total mixed ration (TMR) was formulated to meet the nutrient requirements of a ram weighing 50 kg that gained 50 g/day according to the mutton sheep breeding standards (NY/T816-2004) of the Agricultural Industry Standard of the People’s Republic of China. The TMR was mixed daily (Lanzhou Zhengda Co., Ltd., Lanzhou, China) and fed to the rams twice daily at 9:00 am and 5:00 pm, and the rams had ad libitum access to water and were fed at a daily intake of $3.06 \, kg/ \text{head/d}$. The ingredient and nutrient compositions of the TMR are given in Table 3. All procedures were approved by the Institutional Animal Care and Use Committee of Gansu Agricultural University (no.: GSAU-2th-AST-2021-138).

### Table 1. Compositions of additives.

| Additives | Component content |
|-----------|-------------------|
| Car       | $75\%$ zeolite + $12\%$ limestone + $10\%$ diatomaceous earth and a herbal package (small amounts of lactic acid, kelp, roughage products, chicory root, red pepper, fenugreek flavour extract, anise oil, cloves, saccharin sodium and guar gum) |
| EO        | $1.13\%$ essential oil (oregano oil particles $< 5 \, \mu m$ diameter + small amount of olive oil) + $97\%$ carrier + $1.87\%$ herbal package |
| Co        | $0.1425\%$ cobalt lactate + $97\%$ carrier + $2.8575\%$ herbal package |
| EOC       | $0.1425\%$ cobalt lactate + $1.13\%$ oregano essential oil + $97\%$ carrier + $1.7275\%$ herbal package |

### Table 2. Design of the experiment.

| Run | 1  | 2  | 3  | 4  | 5  |
|-----|----|----|----|----|----|
| 1   | CON | Car | EO | Co | EOC |
| 2   | EO  | CON | EOC| Car| Co  |
| 3   | EOC | Co  | CON| EO | Car |
| 4   | Co  | EOC | Car| CON| EO  |
| 5   | Car | EO  | Co | EOC| CON |

CON: control; Car: feeding carrier; EO: feeding essential oil; Co: feeding cobalt; EOC: feeding cobalt and essential combination.

### Table 3. Compositions and nutritional levels of the experimental diets (air drying basis).

| Formula composition | Proportion (%) | Nutritional level | Content |
|---------------------|---------------|-------------------|---------|
| Corn                | 38            | Dry matter (%)    | 36.23   |
| Corn germ meal      | 20            | Digestive energy (MJ kg$^{-1}$) | 14.23 |
| Corn cob flour      | 9             | Metabolisable energy (MJ kg$^{-1}$) | 11.67 |
| Rice husk powder    | 8             | Calcium (%)       | 0.76    |
| Sprayed corn husk   | 6             | Phosphorus (%)    | 0.65    |
| Cornhusk            | 5             | Crude protein (%) | 9.4     |
| Cotton meal         | 3             |                   |         |
| Rapeseed meal       | 2             |                   |         |
| Soybean meal        | 3.5           |                   |         |
| Bean curd           | 3.5           |                   |         |
| 1% Premixa$^a$      | 1             |                   |         |
| Salt                | 1             |                   |         |
| Total               | 100           |                   |         |

$^a$One kilogram of the premix contained the following: VA 650,000 IU, VDs 300,000 IU, VE 4,000,000 IU, Fe 500 mg, Cu 500 mg, Mn 1000 mg, Zn 500 mg, Co 15 mg and Se 40 mg.

Ruminal digestion of corn silage, leaf and husk tissue

Before the experiment began, fresh whole corn silage was taken at random from the silage pond, brought to the laboratory, dried in an oven at $65 \, ^{\circ} C$, crushed in a high-speed grinder for $\sim 1 \, min$ and then divided into two parts using the following quadratic method: one part was used for determining the dry matter (DM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents of the original sample and the other was used for packing nylon bags ($3 \times 4 \, cm$ sizes nylon bags made from $0.05 \, mm$ nylon cloth and tied with nylon cord) with a constant weight of $1.5 \, g$ each. To eliminate systematic error, three nylon bags were placed in the rumen of each fistulated sheep. At the beginning of the experiment, $4 \, g$ of each additive (Car, EO, Co and EOC) wrapped in paper was first put into the rumen, and then 12 nylon bags were put into the rumen at 4-time points (6, 12, 24 and 48 h), and 3 nylon bags were randomly removed at each time point. After 48 h, the first round
of the experiment was completed, and the sheep were allowed to rest for 10 days before the second round of the experiment. Three of the nylon bags that were attached via a nylon cord were removed at 6, 12, 24 and 48 h, placed in cold water to terminate the fermentation and washed gently with running water at 39 °C until the rinse water was clear. The rinsed bags were then placed in a 65 °C drying oven and dried to constant weight to determine the nutrient degradation rates of dry matter degradation rate (DMD), crude protein degradation rate (CPD), neutral detergent fibre degradation rate (NDFD) and acid detergent fibre degradation rate (ADFD). The calculation of the corn silage ruminal nutrient degradability (DMD, CPD, NDFD and ADFD) via the in situ nylon bag-based method was conducted as follows: nutrient degradation (%) = 100 × (sample nutrient concentration – residue nutrient concentration)/sample nutrient concentration.

Additionally, corn leaves and husks that were well preserved after silage fermentation and had sizes sufficient to be cut into 1 × 1 cm samples were selected. On the morning of the first day, i.e. at 0 h at the start of the experiment, the leaf and husk sample was placed in a separate nylon bag, sealed, each nylon bag containing 3 leaves and husks sample and placed through the ruminal fistula into the rumen at the same time as the corn silage sample for nutrient degradability determination. After 48 h, the sample was removed from the rumen, and the nylon bag was placed and fixed to a glass slide containing a pre-cooled 2.5% glutaraldehyde solution and then processed by the method of Jiao et al. (2016). Since each nylon bag contains 3 leaves and husks, which eliminates systematic errors, we used the leaf and husk samples from the fifth round by SEM and SM to observe the fibre structure and calculate the area of degradation.

**Ruminal fluid**

After removing the nylon bags that contained corn silage leaves and husks, 30 mL of rumen fluid was subsequently extracted from each ram with a spoon through the rumen fistula for 0, 6, 12, 24 and 48 h and was filtered through four layers of cheesecloth into a clean sampling container. The rumen fluid pH was immediately recorded using a pH metre (P611, Shanghai, China) with a glass electrode. The rumen fluid was then preserved by freezing in a cryopreservation fridge (–20 °C). The samples were thawed and analysed for ammonia nitrogen (NH₃-N), microbial protein (MCP) and volatile fatty acid (VFA) contents.

**Laboratory analysis**

Raw corn silage samples and rumen degradation residues were analysed using standard procedures for DM (Method No. 967.03) and CP (Method No. 990.03) (AOAC 2000). The NDF content was determined as described by Van Soest et al. (1991) using heat-stable α-amylase (FAA, Ankom Technology, Macedon, NY) and sodium sulphite. The ADF content was determined according to AOAC method 973.18 (AOAC 1997).

The VFA content was determined by gas chromatography (GC-2010 Plus; Shimadzu, Kyoto, Japan) according to the method of Zhou et al. (2011), Using internal standards, internally labelled 2-ethylbutyric acid (2 EB) was used. The chromatographic column was an AT-FFAP (30 m × 0.32 mm × 0.25 μm) capillary column. The chromatographic column temperature was programmed as follows: 60 °C maintained for 1 min, followed by an increase to 115 °C at 5 °C/min, no hold and an increase to 180 °C at 15 °C/min. The detector temperature was 260 °C, and the injection port temperature was 250 °C.

The ruminal NH₃-N concentrations were measured following colorimetric methods as described by Feng and Gao (2010) using a 721-type spectrophotometer (TU-1901). The ruminal NH₃-N concentration was calculated from the standard curve with R² = 0.9992 and y = 0.5758x – 0.0062, where y is the absorbance and x is the NH₃-N concentration at 700 nm. The rumen fluid sample was diluted with distilled water 20-fold before the chemical reaction. MCP was determined by the purine method according to Liu (Liu 2014). The MCP concentration was calculated from the standard curve with R² = 0.9984 and y = 0.0016x + 3.5135, where y is the absorbance and x is the RNA determination value at 260 nm (mg/mL).

**SEM for silage tissue**

After 48 h of ruminal fermentation and digestion in the fifth round of the experiment, the nylon bags containing the corn silage tissues leaf and husk were removed, the bags were opened and the residues were collected, which was followed by washing with distilled water repeatedly until the tissue surface was cleaned. Then, the whole leaf or husk samples were examined by SM (Discovery. V 20, Jena, Germany) at 10 × magnification to view the fibre structure and
calculate the degradation area. After completing the SM observation, the samples were subsequently fixed, rinsed and dehydrated via freeze-drying (freeze dryer VFD-21S, Yamato Scientific Co., Ltd., Koto-Ku, Japan), after which a film coating was applied (Sputter coater, MSP-1S, Hitachi High-Technologies, Minto-Ku, Japan). The prepared leaf and husk samples were viewed using SEM (S 3400 N, Hitachi Science and Technology, Minto-Ku, Japan) to visually observe and record the sample cell fibre microstructure at 50 \( \times \) magnification (Jiao et al. 2021) and take photographs of the samples under each additive treatment.

**Statistical analyses**

The irregular area of corn silage leaf and husk tissues after rumen degradation was calculated by SM. Data were carried out in Statistical Analysis Systems (SPSS) version 24 (Windows, SPSS Inc., Chicago, IL). Before any statistical analyses were conducted, all data were subjected to Levene’s test of equality of variance using one-way ANOVA, and data that did not pass this test were tested by the Kruskal–Wallis nonparametric test. The effect of the different treatments was assessed by the general linear model for a Latin square design. All sources of variation were considered fixed, except for ram, which was considered random. Duncan’s multiple range test was used to identify differences between specific treatments; \( p < 0.05 \) was considered statistically significant.

**Results**

**SEM and SM scans of corn silage husk and leaf fibre structures after ruminal digestion**

**Corn silage leaf fibre structure**

The differences for corn silage leaf fibre structures of five treatments after degradation were not obvious, epidermal cells were all destroyed, the whole leaf blade showed thin and the degraded area \( a_{48h} \) under five treatments was 0.80, 0.75, 0.82, 0.85 and 0.92 cm\(^2\), respectively (Figure 1). SEM results showed that except for Car and CON treatment, there were obvious destroyed fibre structure differences under EO, Co and EOC treatment, showing the total disappearance of leaf epidermal cells, palisade tissue and spongy tissue, only left the netted veins (Figure 2C–E), especially under EOC.

**Corn silage husk fiber structure**

The husk structure was destroyed, only leaving parallel veins, especially under Co and EOC treatment (Figure...
The degraded areas under five treatments were 0.80, 0.75, 0.92, 1.00 and 1.00 cm² by SM. It was shown the same results that the palisade tissue and spongy tissue appeared and husk flesh was degraded more than that in CON (Figure 4C–E) by SEM, especially EOC was fed.
Corn silage nutrient digestibilities
Changes in nutritional quality in corn silage
The CP content of corn silage after 48 h degradation decreased compared with the raw material, especially digested at 6 and 12 h under EO, Co and EOC treatment, while NDF and ADF increased, it increased by 3.48% and 5.20%, respectively, under EOC degraded at 6 h (p < 0.05) (Table 4).

The rumen degradation rate of corn silage
The DMD, CPD, NDFD and ADFD of corn silage under each group increased as the degradation time extended and peaked at 48 h (Figure 5). Compared with CON, the DMD$_{48\text{h}}$ and CPD$_{48\text{h}}$ under EOC increased (p > 0.05) by 3.33% and 3.89%, while CPD$_{48\text{h}}$ under EO increased (p < 0.05) by 5.21%. There were no significant differences between the Car group and CON at any time (p > 0.05) (Figure 5A and B); the NDFD$_{48\text{h}}$ and ADFD$_{48\text{h}}$ under EOC increased by 7.64% and 5.67%, respectively, while under Co increased by 4.70% and 6.05% (p > 0.05) (Figure 5C and D).

Ruminal fermentation
Changes in pH, NH$_3$-N and MCP
There were no significant differences in pH between treatments (p > 0.05) (Table 5). Compared with CON, the MCP under EO, Co and EOC treatment increased (p > 0.05) by 6.32%, 4.29% and 5.97% at 48 h, respectively. The NH$_3$-N concentration in the EO, Co and EOC groups was significantly decreased at 6, 12 and 48 h (p < 0.05), and at 48 h, it was decreased (p = 0.031) by 19.36%, 27.20% and 19.48%, respectively.

Changes in VFA
Compared with CON, the Total VFA concentration under EO, Co and EOC were lower at 6 and 48 h (p = 0.016, p = 0.022), while higher in EO at 12 h (p = 0.022) (Table 6), and the acetate (p = 0.012), propionate (p > 0.05) concentration and were higher under EOC (p > 0.05) at 48 h. The acetate/propionate (A/P) ratio was higher under EO than that in CON at 0 and 48 h (p = 0.022, p = 0.044). All of the indexes showed no significant differences between Car treatment and CON at any time (p > 0.05).

Discussion
Effects of different additives on the fibre structure of corn silage leaf and husk
Plant cell walls and fibrous components of cellulose, hemicellulose and lignin are usually the most difficult material for ruminant animals to degrade (Gao 2004). Thus, rupturing the plant cell wall will expose cell contents, which is critical for feed degradation, nutrient digestion and feed additive effectiveness. In this study,
when sheep were fed EO, Co and EOC, the epidermal cells, palisade tissue and spongy tissue of corn silage leaves were completely degraded, only left the netted veins, especially under EOC. From a macroscopic perspective, it was found that additive treatment significantly increased the degree of straw fibre degradation. This is the active ingredient Co in EOC increased the cellulolytic bacterial activity and abundance in the rumen and promoted fibre digestion (Singh and Chhabra 1995; Kadim et al. 2003; Jiang 2005; González-Montaña et al. 2020) and also may have occurred because EOC is a microbial catalyst that

### Table 4. Cornstalk silage residue nutrients or treated with different additives (%).

| Parameter | Time (h) | CON  | Car  | EO   | CO   | EOC |
|-----------|---------|------|------|------|------|-----|
|           | 6       | 4.59 ± 0.16 | 4.41 ± 0.10 | 4.42 ± 0.13 | 4.51 ± 0.10 | 4.49 ± 0.15 | 5.38 |
|           | 12      | 4.65 ± 0.19 | 4.46 ± 0.09 | 4.39 ± 0.18 | 4.52 ± 0.10 | 4.62 ± 0.07 | 4.62 |
|           | 24      | 4.67 ± 0.16<sup>b</sup> | 4.22 ± 0.26<sup>b</sup> | 5.14 ± 0.47<sup>a</sup> | 4.62 ± 0.37<sup>ab</sup> | 4.92 ± 0.29<sup>a</sup> |
|           | 48      | 4.76 ± 0.18<sup>abc</sup> | 4.34 ± 0.33<sup>a</sup> | 4.56 ± 0.24<sup>abc</sup> | 4.62 ± 0.30<sup>a</sup> | 4.62 ± 0.14<sup>a</sup> |
| CP        | 6       | 55.54 ± 3.50<sup>*</sup> | 63.24 ± 1.81<sup>*</sup> | 57.23 ± 2.96<sup>ab</sup> | 57.29 ± 4.43<sup>ab</sup> | 52.57 ± 4.25<sup>b</sup> | 46.11 |
| NDF       | 12      | 60.06 ± 2.77 | 64.94 ± 1.37 | 57.85 ± 3.93 | 62.80 ± 3.46 | 57.31 ± 4.24 |
|           | 24      | 59.19 ± 5.51 | 62.89 ± 4.01 | 57.25 ± 3.43 | 62.26 ± 5.11 | 59.18 ± 4.85 |
|           | 48      | 69.88 ± 0.74 | 69.08 ± 0.60 | 69.86 ± 1.25 | 69.63 ± 1.43 | 72.31 ± 3.28 |
| ADF       | 6       | 33.25 ± 2.19<sup>a</sup> | 39.53 ± 2.09<sup>a</sup> | 34.17 ± 1.64<sup>ab</sup> | 34.59 ± 2.61<sup>ab</sup> | 30.83 ± 2.53<sup>b</sup> | 24.15 |
|           | 12      | 36.47 ± 1.87<sup>ab</sup> | 40.17 ± 0.64<sup>a</sup> | 34.90 ± 2.25<sup>ab</sup> | 37.21 ± 2.10<sup>ab</sup> | 34.44 ± 2.21<sup>b</sup> |
|           | 24      | 41.79 ± 1.17 | 38.35 ± 2.63 | 35.23 ± 2.27 | 38.45 ± 2.76 | 39.05 ± 3.01 |
|           | 48      | 43.04 ± 0.86<sup>ab</sup> | 42.83 ± 0.55<sup>b</sup> | 42.66 ± 0.59<sup>b</sup> | 43.22 ± 0.39<sup>ab</sup> | 45.28 ± 1.49<sup>a</sup> |

<sup>a-c</sup>Different superscripts within a row indicate a significant difference (<i>p</i> < 0.05).

CON: control; Car: feeding carrier; EO: feeding essential oil; Co: feeding cobalt; EOC: feeding cobalt and essential combination; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein.

### Figure 5. Effect of adding different additives at various degradation times and ruminal nutrient digestibility of corn silage (%).

<sup>a-b</sup>Different lowercase letters indicate significant differences between different additive treatments at the same time (<i>p</i> < 0.05).

DMD, dry matter degradability; NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility; CPD, crude protein digestibility. CON: control; Car: feeding carrier; EO: feeding essential oil; Co: feeding cobalt; EOC: feeding cobalt and essential combination.
increases the area of microbial action; therefore, the leaves were more completely degraded.

The percent area of husk degradation at 48 h was 100% upon adding Co and EOC; the epidermal cells, palisade tissue and spongy tissue were all destroyed and the arrangement was disordered, which was probably because Co changed the microbial flora structure, increased the abundance of cellulolytic bacteria and destroyed the plant cell walls (Lopez-Guisa and Satter 1992). The effect of adding Co and EOC on corn silage husk was more obvious than that of EO, which is consistent with the results of Jiao et al. (2021). EO is known to exhibit bacteriostatic efficacy that could maintain the ruminal microbial balance to ensure a healthy rumen with normal fermentation (Zhou et al. 2019), and Liu et al. (2020) found that EO could

| Parameter                  | Time (h) | CON   | Car   | EO    | Co    | EOC   | SEM   | p-Value |
|----------------------------|----------|-------|-------|-------|-------|-------|-------|---------|
| pH                         | 0        | 6.15  | 6.23  | 6.20  | 6.20  | 6.21  | 0.017 | 0.255   |
|                            | 6        | 6.13  | 6.41  | 6.34  | 6.25  | 6.29  | 0.027 | 0.206   |
|                            | 12       | 6.25  | 6.38  | 6.26  | 6.45  | 6.42  | 0.022 | 0.459   |
|                            | 24       | 6.23  | 6.34  | 6.25  | 6.25  | 6.43  | 0.011 | 0.140   |
|                            | 48       | 6.42  | 6.43  | 6.66  | 6.55  | 6.56  | 0.010 | 0.054   |
| Ammonia nitrogen (NH₃-N)   | 0        | 0.00  | 15.08 | 14.71 | 11.39 | 11.42 | 12.11 | 0.706   |
|                            | 6        | 14.61 | 13.03 | 13.08 | 9.67  | 9.87  | 12.87 | 0.580   |
|                            | 12       | 14.61 | 14.89 | 12.94 | 9.16  | 11.23 | 0.292 | <0.001  |
|                            | 24       | 14.06 | 12.14 | 11.54 | 11.32 | 12.97 | 0.510 | 0.157   |
|                            | 48       | 15.55 | 14.60 | 12.54 | 11.32 | 12.52 | 0.458 | 0.031   |
| Microbial protein (MCP) mg/mL | 0        | 58.33 | 57.90 | 57.02 | 62.83 | 61.96 | 0.320 | 0.896   |
|                            | 6        | 54.70 | 61.45 | 59.81 | 65.15 | 61.53 | 2.210 | 0.277   |
|                            | 12       | 62.34 | 59.53 | 65.73 | 65.38 | 61.20 | 2.449 | 0.449   |
|                            | 24       | 58.51 | 58.55 | 63.99 | 64.64 | 67.26 | 2.348 | 0.769   |
|                            | 48       | 64.28 | 63.41 | 68.34 | 67.04 | 68.12 | 3.044 | 0.680   |

*a–dDifferent superscripts within a row indicate a significant difference (p < 0.05).

1Treatments: CON: control; Car: feeding carrier; EO: feeding essential oil; Co: feeding cobalt; EOC: feeding cobalt and essential combination.

2SEM: standard error of the mean.

| Parameter                  | Time (h) | CON   | Car   | EO    | Co    | EOC   | SEM   | p-Value |
|----------------------------|----------|-------|-------|-------|-------|-------|-------|---------|
| Acetate (mmol/L)           | 0        | 162.00| 150.55| 137.51| 145.05| 152.54| 3.062 | 0.294   |
|                            | 6        | 158.35| 165.49| 125.62| 148.53| 151.46| 2.718 | 0.016   |
|                            | 12       | 160.83| 163.93| 164.51| 150.55| 120.09| 5.807 | 0.022   |
|                            | 24       | 161.38| 163.19| 163.43| 168.21| 157.72| 8.050 | 0.467   |
|                            | 48       | 167.34| 158.24| 142.19| 161.12| 146.29| 4.173 | 0.022   |
| Propionate (mmol/L)        | 0        | 58.49 | 59.06 | 67.68 | 61.05 | 67.71 | 2.430 | 0.706   |
|                            | 6        | 62.04 | 61.14 | 54.47 | 68.36 | 74.84 | 3.710 | 0.271   |
|                            | 12       | 74.27 | 75.98 | 66.53 | 72.08 | 71.16 | 4.040 | 0.314   |
|                            | 24       | 83.47 | 81.46 | 73.67 | 72.19 | 79.91 | 4.843 | 0.520   |
|                            | 48       | 61.20 | 60.24 | 79.64 | 80.83 | 76.18 | 3.720 | 0.012   |
| Butyrate (mmol/L)          | 0        | 17.56 | 14.64 | 16.79 | 20.63 | 16.18 | 1.110 | 0.463   |
|                            | 6        | 23.03 | 22.80 | 18.19 | 19.33 | 19.74 | 1.205 | 0.215   |
|                            | 12       | 16.00 | 15.96 | 13.71 | 18.42 | 15.96 | 0.615 | 0.076   |
|                            | 24       | 19.66 | 19.93 | 18.46 | 20.24 | 20.24 | 0.860 | 0.693   |
|                            | 48       | 17.14 | 16.56 | 18.77 | 17.22 | 15.91 | 1.264 | 0.698   |
| Acetate/propionate ratio    | 0        | 1.00  | 1.15  | 1.47  | 1.07  | 1.16  | 0.030 | 0.022   |
|                            | 6        | 1.21  | 1.35  | 1.28  | 1.42  | 1.17  | 0.060 | 0.026   |
|                            | 12       | 1.51  | 1.44  | 1.40  | 1.33  | 1.16  | 0.070 | 0.188   |
|                            | 24       | 1.20  | 1.15  | 1.29  | 1.29  | 1.26  | 0.205 | 0.694   |
|                            | 48       | 1.22  | 1.22  | 1.47  | 1.50  | 1.36  | 0.071 | 0.044   |

*a–dDifferent superscripts within a row indicate a significant difference (p < 0.05). SEM: standard error of the mean; VFA: volatile fatty acid.

1Treatments: CON: control; Car: feeding carrier; EO: feeding essential oil; Co: feeding cobalt; EOC: feeding cobalt and essential combination.

2SEM: standard error of the mean; VFA: volatile fatty acid.
improve the roughage degradation rate, regulate the rumen pH and promote rumen peristalsis. The activity of fibrinolytic bacteria was copromoted, thus facilitating the digestion of fibre (Wang et al. 2006). With each component demonstrating slightly different mechanisms, a combination of Co and EO would ensure greater degradation of straw and improve feed nutrient and fibre digestibility.

**Effect of different additives on the rumen degradation rate**

The rumen degradation rate of DM (DMD) and CP (CPD) are important indicators for feed digestibility. DMD and CPD increased continuously with the rumen fermentation time increasing and peaked at 48 h, which was consistent with the results of Mchrez and Frskove (1977). Compared with CON, DMD and CPD under EOC were higher than those when EO and Co were fed alone. A study conducted at South Dakota State University found that compared with CON, EOC treatment increased DMD by 2.30% and CPD by 3.50% for roughage (Kuester 2016) while the CPD was increased by 5.21% under EO in our study, and it was increased by 6.90% for broiler (Hu et al. 2004). The complex and diverse active chemical composition of plant EOs results in different functional properties and effects on nutrient digestibility. This suggests that EO and Co in EOC can produce synergistic effects in improving nutrient degradation rates. The effect of Co on feed DMD and CPD in this study was not significant (p > 0.05), maybe because of sheep breed and age and the position of the nylon bag placed in the rumen (Qiu et al. 2017). But the feed DMD and CPD were significantly higher under EOC than those under other treatments, suggesting that EOC had a significant effect on roughage degradation. It has also been shown that EOC can improve the palatability and nutritional quality of roughage (Mohamed et al. 2009) because it contains both EO and Co, both of which act synergistically to functionally improve the roughage digestibility by altering the rumen environment in sheep.

The NDFD and ADFD increased with the extension of rumen degradation time, consistent with many previous reports (Ji et al. 2015). In this experiment, the NDFD$_{48h}$ and ADFD$_{48h}$ were higher under Co than those under EO, which verifies the previous described important role of Co in crude fibre (CF) degradation; thus, the effect of Co on CFD is better than that on DMD and CPD, which is consistent with the experimental results observed by microscopy, as described in section 2. Both NDFD and ADFD were higher at each degradation time under EOC than those under CON, indicating that EOC played a significant role in promoting the roughage CFD. EO and Co were present in EOC and played coordinated roles in improving the rumen environment, enhancing the activity of cellulolytic bacteria to promote rumen fermentation functions and forage utilisation. There was no significant difference for NDFD and ADFD under CON and Car treatment in this study (p > 0.05), indicating that the Car did not have any effect on the ruminal degradation of corn silage, thus eliminating the possibility of an error in the results caused by Car.

**Effect of different additives on rumen fermentation**

Ruminal pH is an important indicator for evaluating rumen microbial populations and homeostasis of the rumen internal environment, ranging from 5.5 to 7.0 for normal microbial activity in the rumen of sheep (Membrive CMB 2016). In this study, the rumen fluid pH in each group ranged from 6.13 to 6.66, within the normal range. Hristov et al. (2013) found that oregano leaves had no significant effect on the rumen pH in Holstein cows fed different amounts of oregano leaves (containing 1.58%/DM EO), with the same as the results of this study.

NH$_3$-N is not only the final product for nitrogenous substances degradation but also a raw material for MCP synthesis (Salter et al. 1979) and VFAs are produced from dietary carbohydrates by rumen fermentation. Studies have shown that adding EO to the diet significantly affects rumen fermentation and changes the VFAs concentration (Roy et al. 2014; Napoli et al. 2016). EO can reduce VFA production by inhibiting starch-hydrolysing and protein-hydrolysing bacteria (Patra 2011), EO can reduce protozoa population and decrease acetic acid and total VFA (Khiaosa and Zebeli 2013). The NH$_3$-N and total VFA were significantly lower at each time under EO and EOC than they were in CON in our study which may be due to EO promoting the rumen microbes’ growth and proliferation (Wang et al. 2007). NH$_3$-N concentration was closely related to MCP production and a suitable NH$_3$-N was beneficial to MCP synthesis (Zhang et al. 2014). The suitable range of NH$_3$-N concentration for rumen microbes growth is 6–30 mg/100 mL. In this study, the NH$_3$-N concentration was 9.16–15.55 mg/100 mL, which could provide a normal internal environment for MCP synthesis by rumen microorganisms. The additive treatment did not lead to significant differences in the
MCP content in this experiment, but the MCP yield was consistent with the results of Hua et al. (2018), indicating that the additive treatment had no side effects on MCP synthesis. Castillejos et al. (2006) reported that the in vitro addition of 500 mg/L thymol significantly increased the propionic acid concentration, while the acetate concentration under EO, Co and EOC treatment was higher than CON at 48 h in our study, indicating that the effect of EO, Co and EOC on acetate varies with time. The propionate concentration under EOC treatment was higher than that in CON at 48 h, which is consistent with the results of Wang et al. (2022), showing that EOC was beneficial for promoting propionate production and lipid metabolism in sheep, while showing that some EO components positively affected VFAs by decreasing acetic acid production and increasing propionate production patterns (Poudel et al. 2019). Higher propionic acid levels were generally considered beneficial for ruminant production. The VFA concentration increased first and then decreased because the additives stimulated the rumen microorganisms activity and reproduction, and the organic acids were absorbed in large quantities by the rumen wall or flowed into the back end of the digestive tract, and the VFA concentration gradually decreased again. We believe that EO and Co acted synergistically to alter the VFA levels to reduce feed energy loss in a way that reduced the Total VFA concentration and increased the propionate concentration. Overall, oregano EO and Co exerted synergistic effects in changing rumen fermentation parameters such as VFA structure and NH₃-N production, while the particular synergistic mechanisms to be studied in our subsequent studies.

Conclusions
We conclude that feeding EOC increased the DMD, CPD, NDFD and ADFD at all time points, which was visually verified by SEM, and decreased the rumen fluid NH₃-N and Total VFA and increased the propionate concentration. At 48 h of degradation, the area of degraded corn leaf and husk reached 100% under EOC treatment. In conclusion, EOC treatment can increase the fibre structure degradation and nutrient degradation rate of corn silage and improve rumen fermentation function in sheep and was more effective than feeding EO or Co alone.

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No potential conflict of interest was reported by the author(s).

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Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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