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## Effects of oregano (*Oreganum onites* L.) aromatic water on rumen microbial fermentation of Holstein calves

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**Abstract:** The study aimed to determine the effects of the addition of aromatic oregano water (AOW) on rumen fermentation of Holstein calves. For this purpose, 20 Holstein calves were divided into four groups (n = 5) and fed with three different doses of AOW (1, 1.5, and 2%). The experimental groups were formed as follows; the control group (CNT): milk + starter; G1: 1% AOW supplemented milk + starter; G2: 1.5% AOW supplemented milk + starter; G3: 2% AOW supplemented milk + starter. The rumen fluid was taken from the oesophagus at 60 days of age with the aid of a rumen probe. In acetic acid (AA), propionic acid (PA), and butyric acid (BA) except for total volatile fatty acid (TVFA) (P < 0.05), the numerical differences between the means of the groups were not found statistically significant. The rumen pH values of the groups were not affected by the addition of AOW. Likewise, the difference between the total bacteria and the numbers of protozoa, Entodinium, Diplodinium, Isotrichia, and Daystrichia were not statistically significant. The results of the study showed that the addition of AOW did not have a negative or positive effect on rumen fermentation of the calves.

**Key words:** Calves, aromatic oregano water, ruminal fermentation, bacteria, protozoa

### 1. Introduction

The population of rumen microorganism is the most important digestive system difference between ruminants and single stomachs. Rumen microorganisms consist of bacteria, protozoa, and fungi [1]. Digestion in the rumen is entirely based on microorganism activities. However, the variation of the microorganism population depends on the structure, quantity, and microbial interaction of the ration. The bacteria in the rumen are active in the digestion of hemicelluloses, cellulose, pectin, lignin, and lipids due to their different enzymes. The fungi complement the symbiotic cycle by converting the polysaccharides in the cell walls of plants to cellobiose and glucose. The majority of the protozoa in the rumen are composed of ciliates; their number varies rapidly according to the composition of the ration [2].

Today, 1/3 of 300 plant species grown in nature contain essential oils. Essential oils are found in many plants due to their protective role against bacterial or fungal infections or insecticides. They are mostly composed of cyclic hydrocarbons and their aldehyde or ester derivatives [3]. Although it is not known precisely how essential oils support nutrient digestion, it is reported that they increase

the synthesis of digestive enzymes [4]. In ruminant animals, the pH value of the rumen fluid and the changes in microorganism species such as bacteria and protozoa which decrease and increase by these pH values affect the yield and quality [5,6]. In order to improve the yield and quality of the products in ruminants, feed additives that are antibacterial-efficient and feed-enhancing are used [7]. For many years, ionophore antibiotics have been used to improve the quality and quantity of rumen fermentation events in ruminant rations and feed utilisation [8]. However, due to the possibility of bacterial resistance, the importance of natural and safe alternatives such as probiotics, prebiotics, or phytobiotics has been emphasised, and the use of antibiotics as growth promoters has been discussed [9–11]. Based on the idea that essential oils may be an alternative to antibiotics due to their antimicrobial properties, the essential oils used on their own have different effects on rumen bacteria, and have less harmful toxic effects compared to oxygenate compounds of monoterpene hydrocarbons, monoterpene alcohols, and aldehydes, and in some cases stimulates microbial activity [3,12]. Besides, it was stated that essential oils did not affect the total bacteria count in the rumen; however, the

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number of hyper-ammonia-producing bacteria decreased by 77% [13]. Ando et al. [14] observed a decrease in the number of rumen protozoa in steers fed with peppermint. In their study with herbally formulated AV/DAC-16 drug, Singh and Bhatia [15] reported that they did not observe the effect of AV/DAC-16 on the number of protozoa, but the bacteria count increased significantly compared to the control group. Khiaosa-ard and Zebeli [10] stated that low-dose essential oil bioactive compounds increase the butyrate concentration and the protozoa number while high-dose compounds cause a significant decrease in the protozoa number and the acetate-propionate ratios. A study carried out on clove, peppermint, and lemongrass stated that there was no difference in the total VFA concentrations, but the addition of cloves decreased the propionate concentration [16]. In the study by Akbarian-Tefaghi et al. [17] carried out with the addition of thyme, eucalyptus, and celery to the starter, while the addition of thyme reduced the butyrate concentration, eucalyptus and celery increased it; however, rumen pH, acetate, and propionate concentration was not affected by adding these herbs. Busquet et al. [18] observed that garlic and cinnamaldehyde increased the propionate and butyrate ratio and reduced the acetate ratio.

Studies are generally conducted in vitro. However, it is usually related to the effects of essential oils on animals, and there are few studies on aromatic water. Özkaya et al. [19] reported that aromatic oregano water added to milk replacer tended to improve calves' growth and health and could be safely used in calves. In a previous study [20], they observed that aromatic oregano water applied at 40, 60, and 80 ml/L did not affect in vitro gas production, metabolic energy level, and organic matter digestion, but increased parameters other than methane production at a dose of 40 ml/L. Besides, they also reported that the total concentration of VFA, acetate, propionate, and butyrate in the rumen fluid increased with the addition of aromatic oregano water. There is a very limited number of studies on the effects of the addition of AOW, which is a by-product obtained when producing oregano oils, on rumen fermentation of calves. Therefore, the aim of this study was to determine the effects of this by-product added to the calves' milk on rumen fermentation.

## 2. Material and methods

### 2.1. Aromatic water production

Dried oregano leaves (500 g) were treated with 2.5 L of tap water and placed in a sor (5 L) connected to the condenser of the Clevenger hydrodistillation apparatus as specified by the European Pharmacopoeia. After hydrodistillation, the aromatic water was separated from oregano oil distillation and collected. The total phenolic amount and

the components of AOW were determined as described by Özkaya [19].

### 2.2. Feeding of the calves

A total of 20 (average  $40.01 \pm 0.82$  kg) calves were used in the study. The AOW was added to the calves' milk by 1, 1.5, and 2% [19]. The milk was given to the calves in two equal meals per day (4L/day). The calves were allowed to take the starter and water as *ad libitum*. The calves were fed with commercial starter (18% CP-2800 kcal/kg). The rumen fluid was taken from the calves approximately 3 h after drinking milk with rumen probe designed for the calves at 60 days of age. The experimental groups were designed as follows with five replicates; the control group (CNT): milk + starter; G1: 1% AOW added milk + starter, G2: 1.5% AOW added milk + starter, G3: 2% AOW added milk + starter.

### 2.3. Protozoa count and identification of the rumen fluid

The protozoa count and the identification of the rumen fluid were performed using a microscope using the method determined by Ogimoto and Imai [21].

Protozoa count: For this, 0.1 ml of the rumen fluid was removed, and 0.9 ml of the MFS solution (100 ml of formaldehyde solution (30%), 900 ml of distilled water, 0.6 g of methyl green, 8 g of NaCl) was placed and counted under the microscope.

The following equation was used to calculate the number of protozoa in the rumen fluid: the number of cells in  $\text{cm}^3$  (ml) :  $1000 \times \text{counted cells} / \text{counted total square} \times \text{dilution} \times \text{volume}$  [22].

Protozoa identification: The definition of ciliate, the shape of the protozoa and cilia location was made. For this purpose, the figures were used, which were indicated by Ogimoto and Imai [21]. In the rumen fluid taken on the lam, the protozoa species were counted up to 100, and the percentage of protozoa was calculated.

### 2.4. Bacterial count in the rumen fluid

In order to determine the total bacteria count, the rumen fluid was diluted in formaldehyde and measured spectrophotometrically at a wavelength of 600 nm (T80 + UV/VIS Spectrometer, PG Instrument Ltd. UK).

### 2.5. Determination of VFA in the rumen fluid

The samples taken from the rumen were centrifuged at  $400 \times g$  for 10 min, and volatile oils were determined using supernatant liquid gas chromatography. Acetic acid (AA), Propionic acid (PA), and Butyric acid (BA) were determined in the rumen.

### 2.6. Statistical analyses

The results obtained were analysed using the Analysis of Variance (ANOVA) technique by using Minitab 17 (Minitab, Ltd. Coventry, UK). The differences between the averages of the groups were examined with the Tukey's test.

### 3. Results

As a result of the GC-MS analysis, 99.4% carvacrol and 0.6% thymol were found in the OW content.

Table 1 presents the parameters related to the rumen fermentation of the calves. The rumen pH values of the calves were not affected by AOW, but it was observed that G1 and G3 were lower than the other groups numerically. There was a significant difference between the total VFA of the groups ( $P < 0.05$ ). The total VFA increased in parallel with the AOW dose increase. The highest total VFA value was obtained in G3, while the lowest was obtained in GNT. However, numerical differences between the AA, PA, BA, and AA/PA averages of the groups were not found to be statistically significant. However, the addition of AOW tended to increase both the AA and PA values, while it tended to decrease the BA value.

The numerical differences between the pH values of the groups were not statistically significant (Table 1). The pH values in GNT and G2 were close to each other, while the pH values in G1 and G3 tended to decrease compared to the other groups.

Numerical differences between the total bacteria and protozoa counts of the groups were not found statistically significant (Table 2). In addition, the differences between the averages of protozoa species of the groups were also not statistically significant. Although the total bacteria count in G1 and G2 tends to increase compared to GNT and G3, a decrease tendency was observed in G3 compared to the other groups. The addition of AOW tended to decrease in the total protozoa count of the groups. Similarly, while a decreasing tendency was observed in the species of Entodinium and Diplodinium,

**Table 1.** Effects of aromatic oregano water addition on rumen fermentation.

	Treatments				P value
	CNT	G1	G2	G3	
Parameters	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	
TVFA	97.16 ± 4.42 <sup>B</sup>	102.10 ± 081 <sup>AB</sup>	105.22 ± 1.19 <sup>AB</sup>	110.84 ± 4.05 <sup>A</sup>	0.04
AA	51.54 ± 3.69	55.91 ± 1.13	57.90 ± 1.02	61.17 ± 3.18	0.10
PA	39.32 ± 2.38	40.49 ± 1.91	41.64 ± 0.36	44.17 ± 5.25	0.71
BA	6.30 ± 0.43	5.71 ± 0.48	5.68 ± 0.73	5.50 ± 0.41	0.73
AA/PA	1.33 ± 0.13	1.40 ± 0.08	1.39 ± 0.02	1.46 ± 0.17	0.89
pH	5.81 ± 0.40	5.46 ± 0.15	5.84 ± 0.20	5.56 ± 0.05	0.60

A,B: Means in the same row followed by the different letter are significantly different at  $P < 0.05$ .

TVFA: Total volatile fatty acid, AA: Acetic acid, PA: Propionic acid, BA: Butyric acid, AA/PA: Acetic acid/Propionic acid; CNT: milk + starter (Control group); G1: 1% AOW added milk + starter, G2: 1.5% AOW added milk + starter, G3: 2% AOW added milk + starter.

**Table 2.** Effect of aromatic oregano water addition on rumen microorganisms.

	Treatments				P value
	CNT	G1	G2	G3	
Parameters	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	
Total Bacteria ( $\times 10^8$ / mL)	14.47 ± 4.04	15.50 ± 3.01	15.26 ± 4.61	12.86 ± 3.98	0.96
Total Protozoa ( $\times 10^5$ / mL)	3.46 ± 0.28	3.38 ± 0.13	3.34 ± 0.14	2.99 ± 0.25	0.43
Protozoa species					
Entodinium ( $\times 10^4$ / mL)	15.68 ± 0.69	14.59 ± 1.15	13.76 ± 0.94	12.80 ± 0.92	0.21
Diplodinium ( $\times 10^4$ / mL)	10.18 ± 1.16	8.77 ± 0.44	8.83 ± 0.72	7.62 ± 0.96	0.26
Isotrichia+Dasytrichia ( $\times 10^4$ / mL)	8.76 ± 1.57	9.47 ± 0.86	10.18 ± 0.82	10.50 ± 0.59	0.65

CNT: milk + starter (Control group); G1: 1% AOW added milk + starter, G2: 1.5% AOW added milk + starter, G3: 2% AOW added milk + starter.

an increasing tendency was observed in *Isotrichia* + *Daystrichia* species.

#### 4. Discussion and Conclusion

AOW added to the milk of the calves had no significant effect on rumen parameters. However, the findings in this study showed that the addition of AOW tended to increase AA and PA and decrease BA. An in vitro study showed that garlic and cinnamaldehyde increased the percentage of propionate and butyrate but reduced the percentage of acetate [18]. However, the total VFA, AA, PA, BA, and AA/PA ratios decreased with the addition of thyme oil in vitro [23]. It has been indicated that the amount of acetate, propionate, butyrate, and the total VFA decreases in the rumens of Holstein steers [16]. In their study with buffalo calves, Singh and Bhatia [15] reported that the addition of the herbal formula AV/DAC-16 (a herbal medicine containing *Phyllanthus emblica*, *Terminalia chebula*, *Zingiber officinale*, and *Terminalia bellerica*) brought about a significant increase in the amount of the total VFA. Khiaosa-ard and Zebeli [10] stated that low-dose essential oil bioactive compounds increase the butyrate concentration and cause high reductions in the acetate-propionate ratio at high doses. In their study with dairy calves, Akbarian-Tefaghi et al. [17] observed that the addition of herbal plants and commercial essential oils did not affect acetate, propionate, and AA/PA at 70 days of age, but the addition of essential oils significantly increased butyrate. In a study conducted with the Ramie (*Boehmeria nivea*) plant with goats, the total VFA and individual volatile fatty acids were not affected [24].

The rumen pH varies between 5.5 and 7.0. This value depends on the type of feed given to the animal. Similarly, the antimicrobial effects of the bioactive components in plants also vary according to the type, dose, and pH of the feed given to the animals. The antimicrobial effect increases when the rumen pH falls from 6.5 to 5.5. The rumen pH in young calves is usually low, so it is suggested that changes in the structure of the active components of essential oils increase the susceptibility of bacteria to these molecules and thus increase the antimicrobial effect [25–27]. The pH values obtained in our study were within normal limits and were not affected by AOW addition. In studies with plants, many researchers reported that the rumen pH was not affected by plants [15–17].

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Rumen microorganisms provide nutrients needed by ruminants. These microorganisms can break up cellulose and pentosanes to feed organic acids (AA, PA, and BA). Rumen microorganisms increase the resistance of ruminants to toxins, and the microbial flora in the gastrointestinal tract has the effect of providing resistance to diseases. These microorganisms help to break up and digest the fibrous substance through the enzymes they secrete [28,29]. Rumen microorganisms consist of bacteria, protozoa, and fungi [27]. Protozoa constitute 50% of the total microbial mass. The presence of protozoa in the rumen, especially *Entodinium* species, reduces the transition of microbial nitrogen to intestines due to bacterial growth in the rumen and decreases methanogenesis by 30–40% [30]. Essential oils do not affect the total number of bacteria in the rumen but are known to reduce the amount of hyper-ammonia-producing bacteria by 77% [13]. Similarly, it was observed that essential oils did not cause a decrease in the rumen of protozoa [14]. Singh and Bhatia [15] reported that herbal medicines did not affect the number of protozoa but increased the number of bacteria. On the contrary, Khiaosa-ard and Zebeli [10] reported that essential oils applied at low doses increased the number of protozoa and at high doses they led to a decrease. Although the results obtained in our study showed that the difference between the total bacteria and protozoa count was not significant, the total bacteria and protozoa count in G3 decreased compared to the other groups (Table 2).

No studies were found on the microbial fermentation of the rumen due to the addition of AOW. Therefore, a comparison with previous studies was not possible.

In conclusion, AOW as a by-product has the potential to manipulate rumen fermentation and microorganisms of calves. There are not enough studies on the use of aromatic oregano water in ruminant feeding. There is a pressing need for more studies that will be carried out with different doses than those used in the present study in order to understand the mechanism of the action of aromatic oregano water.

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#### Conflict of interest

No potential conflict of interest was reported by the authors.

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