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Bacteriolytic activity of ruminal protozoa is affected by rate and type of common essential oils: Effect of thyme oil

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Abstract

Over the past two decades, research into the effects of essential oils (EOs) on rumen microorganisms as an alternative to antibiotics has intensified. Yet, there are few investigations on rumen protozoa. To evaluate if EOs have antiprotozoal efficacy against rumen protozoa, the present investigation was conducted. Using the potentiality of rumen protozoa to digest ^{14}C-labeled bacteria, the influence of EOs on *in vitro* protozoal activity was determined. For the study, orange peel, cinnamon, laurel, oleaster, garlic, and thyme essential oils were selected. Control (without essential oil), 500 mg/kg, 1000 mg/kg, and 5000 mg/kg dosages were used. Except for garlic oil, all essential oils showed a notable effect on antiprotozoal activity. The highest antiprotozoal impact was reported at 500 mg/kg and 1000 mg/kg doses of thyme oil, with respective reductions of 89.1% and 84.8% in bacteria degraded by protozoa. Cinnamon and oleaster oils had the strongest antiprotozoal efficacy at a concentration of 5000 mg/kg. At 5000 mg/kg doses of oleaster and cinnamon oils, the quantity of ^{14}C-labeled bacteria degraded by protozoa was reduced by 96% compared to the group that served as the control. In conclusion, orange peel, cinnamon, laurel, oleaster, and thyme oils demonstrated remarkable *in vitro* antiprotozoal activity; however, more comprehensive research is required to analyse the effects of the EOs used in this study on rumen living groups such as protozoa, bacteria, and yeast and on rumen dynamics.

Keywords: antiprotozoal agent, essential oil, feed additive, rumen protozoa, ^{14}C-labeled bacteria
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Introduction

Essential oils (EOs) extracted from plants using different methods have been used as feed additive alternatives to antibiotics for stabilizing rumen flora in recent years. Much research has described the effects of various EOs and their active components (secondary metabolites) on the microorganisms that live in the rumen, as well as the metabolic processes that take place in the rumen (McIntosh *et al.*, 2003; Benchaar *et al.*, 2007; Kekana *et al.*, 2021; Ünlü *et al.*, 2021). Most of these studies are regarding rumen bacteria and rumen fermentation. However, despite providing 50% of the biomass in the rumen, the importance of protozoa in the microbial ecosystem of the rumen is still not fully understood, i.e., there are insufficient studies on rumen protozoa.

Protozoa in the rumen are less in number than bacteria, but their biomass is comparable because they are larger in size. Since the metabolic activities of microorganisms are inversely related to their size, bacteria have a greater metabolic activity than protozoa (Newbold *et al.*, 2015). However, the activity of protozoa in breaking down amino acids is three times that of bacteria (Newbold *et al.*, 2015). Although protozoa may utilize cellulose and starch, their primary role is N metabolism (Jouany & Morgavi, 2007). In addition to the proteolytic and deaminase reactions that occur in the rumen, enormous quantities of bacteria are digested by protozoa. Therefore, protozoa also influence the reduction of bacterial protein transferred from the rumen to the duodenum (Dehority, 2003). According to Belanche *et al*. (2012), a commonly-occurring presence of protozoa is capable of degrading ~17%

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of the bacteria in the rumen every hour. Their observations illustrated the strong relationship between the protozoal population and the availability of bacterial protein in ruminants.

Since protozoa can negatively impact the ruminal and post-ruminal bacterial protein cycles (Castillo-Lopez & Domínguez-Ordóñez, 2019), limiting their presence in the rumen can reduce the demand for rumen-undegradable protein in the diet of animals (Newbold *et al.*, 2015). Additionally, Newbold *et al*. (2015) observed that defaunation can lower methane emissions. These authors also reported that saponins have strong inhibitory effects on ruminal protozoa. Unlike saponins, few studies have demonstrated antiprotozoal activities of tannins and plant-based essential oils.

This investigation was considered due to the possibility of antiprotozoal effects in the active chemicals found in essential oils, which exhibit a vast variation. Orange peel (*Citrus cinensis*), cinnamon (*Cinnamomum verum*), laurel (*Laurus nobilis*), oleaster (*Eleagnus angustifolia*), garlic (*Allium sativum*), and thyme (*Thymus vulgare*) contain a variety of secondary metabolites, including D-Limonen, cinnamalddehyde, 1-8 Cineol, eugenol, dially disulphide, and carvacrol. Several mechanisms have been reported for the antimicrobial effects of these active components. One mechanism operates through the hydrophobic properties of essential oils, as they affect the cell and mitochondrial membranes of microorganisms and disrupt their structures. This in turn affects the cell permeability and causes the intracellular ion balance to deteriorate (Swamy *et al.*, 2016). Other mechanisms include antibacterial activities, which affect the hydrophobic end of some proteins and render important enzymes, such as decarboxylase, dysfunctional (Calsamiglia *et al.*, 2007). It has been reported that this mechanism is primarily induced by the active terpene compounds found in essential oils. Carvacrol, one of the most abundant active ingredients of thyme, belongs to the group of monoterpenes. Due to the presence of these secondary metabolites, essential oils have the potential to modulate fermentation and microbial population dynamics of the rumen (via decreased methane synthesis, decreased ruminal protein degradation, defaunation, and pH stimulation).

This study aimed to identify the antiprotozoal properties and optimal dosages of essential oils derived from orange peel, cinnamon, laurel, oleaster, and thyme.

Materials and Methods

The EOs used in this study were orange peel (*Citrus cinensis*), cinnamon (*Cinnamomum verum*), laurel (*Laurus nobilis*), oleaster (*Eleagnus angustifolia*), garlic (Allium sativum), and thyme (*Tymus vulgare*). These essential oils were all supplied by Doğa Bitki Ürünleri Gıda Limited (Antalya, Turkey). The hydro-distillation method was utilised to extract the essential oils from the plant material (Kumar Mahawer *et al.*, 2022). Prior to their use and examination by gas chromatography mass spectrometry (GC-MS), the EOs that were extracted were stored in opaque glass vials at a temperature of 4 °C. The principal secondary metabolites of these six essential oils (orange peel, cinnamon, laurel, oleaster, garlic, and thyme) have unique chemical structures and stereochemistry (Table 1).

A drop of material was dissolved in 1 mL of hexane before being prepared for gas chromatography analysis. The GC-MS was injected with 1 µL of material and examined. The analysis utilized a Perkin Elmer Clarus 500 GC/MS instrument and a ZB-5 MS column (30 m in length, 0.25 mm inner diameter, and 0.25 m film thickness). As the carrier gas, helium (He) gas with a flow rate of 10 mL per minute was utilized. In the analysis, the injector temperature was 240 ^{o}C, the GC temperature was 60 ^{o}C for 10 min; with increments of 4 ^{o}C, 220 ^{o}C was obtained and maintained for 10 min. It was maintained at 250 ^{o}C with 4 ^{o}C increases every 10 min and 20 ^{o}C/min. Using electronic libraries, active components in essential oils were characterized (WILEY, NIST, and NBS libraries). Table 1 lists the principal constituents of the EOs used in the study.

Table 1 Major components (secondary metabolites) of essential oils

Essential Oil	Major components	Major components (%)
Laurel	Alpha-Terpinenyl Acetate	43.5
	1,8 Cineol	23.9
	4-Terpineol	7.8
	Beta-Fenchyl Alcohol	7
	Trans-Caryophyllene	3.7
	Linalool	2.8
Oleaster	Cinnamaldehyde	61.22
	Benzylacetate	7.84
	Eugenol	6.74
	Diproplene Glycol	6.47
	2-Propanol 1-(1-Methyl-2-(2-Propenyloxy) Ethoxy-	5.45
	Amylcinnamic Aldehyde	2.69
Cinnamon	Benzyl Alcohol	22.72
	Linalool	1.21
	1,3 – Dioxolane 4-Methyl-2-Phenyl	0.69
	Eugenool	6.77
	Triacetin	1.93
	Cinnamaldehyde	66.68
Orange Peel	D-Limonene	34.2
	1,8-Cineole	14.02
	Trans-Carveol	11.89
	4-Terpineol	5.87
	Linalool	2.84
	Carvone	31.17
Thyme	Carvacrol	93.03
	Caryophyllene	3.4
	Linalool Oxide	1.86
	Caryophyllene Oxide	0.57
	Alpha-Humulene	0.33
	Trans Linalool Oxide	0.31
Garlic	Octadecanoic Acid Metyl Ester	39.84
	1-Dodecanol	22.16
	Hexadecanoic Acid Methyl Ester	14.05
	Diallyl Disulphide	10.63
	Allyl Trisulfide	4.88
	9-Octadecenoic Acid Methyl Ester	4.64

As described by Belanche *et al*. (2016) using *Streptococcus bovis* (ES1) as the labelled substrate, the influence of essential oils on *in vitro* protozoal activity was determined by observing the breakdown of ^{14}C-labelled bacteria by rumen protozoa in strained rumen fluid. The Institute of Biological, Environmental, and Rural Sciences (IBERS) at Aberystwyth University supplied the *S. bovis* used for the study. For preparing labelled *S. bovis*, a pure culture of *S. bovis* was grown in Wallace and McPherson (WM) media at 39 ^{o}C for three days. Daily, 1 mL of culture was transferred to 8 mL of new media. *Streptococcus bovis* was grown in WM media containing ^{14}C-leucine as the sole N source (1.44 Ci/8mL) at 39 ^{o}C for 24 h, the day before the experiment.

Bacteria from five Hungate tubes were pooled and harvested by centrifugation at 3000 × *g* for 15 min, washed once with Simplex-type Salt Solution (STS), and resuspended in STS containing 5 mmol/l of ^{12}C-leucine to prevent re-incorporation of released ^{14}C-leucine. After using the final *S. bovis* suspension as bacterial inoculum and collecting a sample to measure the level of radioactivity that was present, the experiment was carried out.

To a final concentration of 500, 1000, or 5000 ppm, EOs were added (diluted in autoclaved water containing 10% DMSO). *Streptococcus bovis* labelled with ^{14}C-luecine was incubated in triplicate for 3 h, with hourly samples obtained to detect bacterial decomposition resulting from the release of ^{14}C-

luecine. The total of 1 mL was removed into Eppendorf tubes for protein (triplicate and store at -20 °C) and 50 µL into Eppendorf tubes containing 0.25 mL 25% TCA before beginning additions (in triplicate). Using a 1 mL plastic syringe with a 23-G needle, 0.5 mL samples were collected at 0, 1, 2, and 3 h into Eppendorf tubes containing 0.125 mL of 25% TCA (w/v). For each EO and dose, triplicate measurements were performed. After that, samples of the supernatant fluid (200 µl) were added to 2 mL of scintillation fluid and counted by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). At each incubation time, the disintegration of ^{14}C-leucine-labeled bacteria was computed from the acid-soluble radioactive label and expressed as a percentage of the total disintegration per minute (dpm) contained in the labelled bacteria solution. The rate of degradation per hour was calculated as the difference from the linear portion of the degradation curve. The study procedure is summarized in Fig. 1.

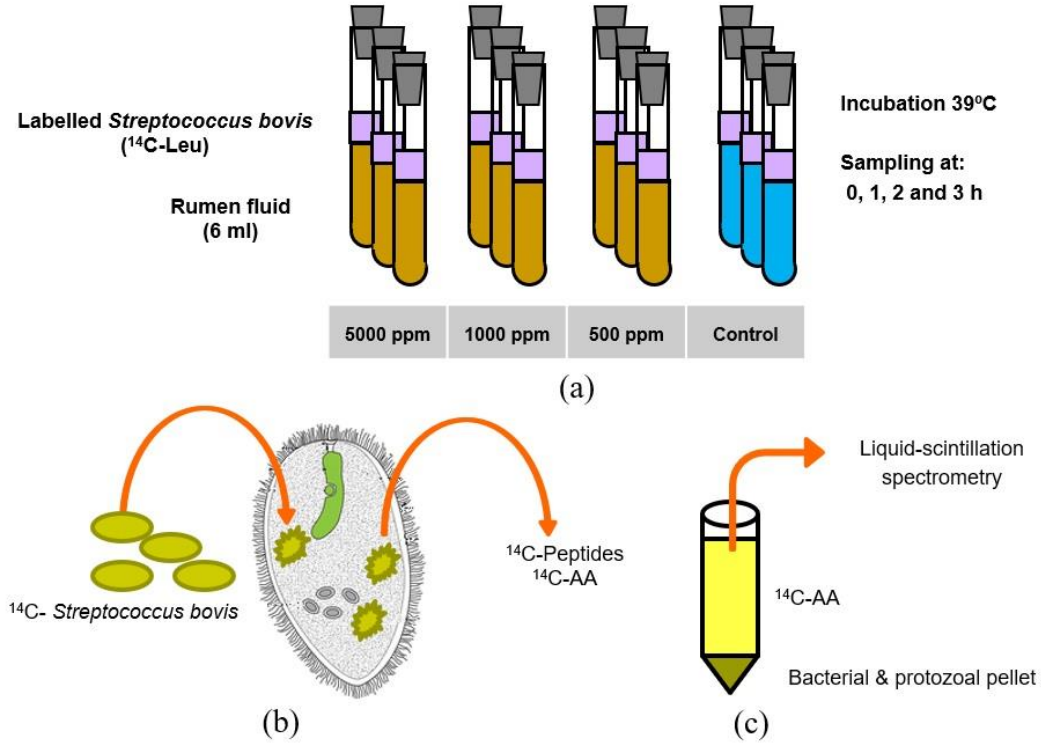

Figure 1 This applies to the study a) Incubation at 39 °C with labelled *Streptococcus bovis* and rumen fluid, b) labelled ^{14}C-*S. bovis* (ES1) degradation by protozoa, c) collection and quantification of labelled amino acid (AA = ^{14}C-leucine) released from *S. bovis* degraded by protozoa

All analysis was repeated at least three times, technically and biologically. Each variable was characterized by descriptive statistics. Using a one-way analysis of variance (ANOVA), the dose effect was determined. The model was

$$Y_{ij} = \mu + D_j + e_{ij} \quad (1)$$

where Y_{ij} represents the dependent variable, μ represents the overall mean, D_j represents the fixed effect of dose (j = 0, 500, 1000, and 5000 mg/kg), and e_{ij} represents the residual error. Any significant terms were compared using a Bonferroni adjustment when a statistically significant difference was found. In addition, the linear and quadratic effects of polynomial contrasts were analysed. In all analyses, P <0.05 was deemed significant. All data were analysed using Version 23.0 of IBM SPSS Statistics for Windows.

Result and Discussion

In this study, the effect of various doses of essential oils on the activity of rumen protozoa was evaluated *in vitro* by measuring the amount of ^{14}C-labelled *S. bovis* that was degraded by rumen protozoa. The results are given in Table 2 and Figures 2 and 3. Protozoal activity was substantially affected by all EOs, except garlic oil (Table 2). Although garlic is known to have substantial effects on

microorganisms, in this study, it has not found to have a marked effect on protozoa. Yang *et al*. (2007) studied the effects of monensin, an antibiotic, and the essential oils of garlic and juniper berry on ruminal pH and protozoa numbers in lactating dairy cows. They observed that garlic oil had no effect on feed digestion, the characteristics of rumen fermentation, or protozoa populations. This result is consistent with the findings of the current study. In general, the antimicrobial action of garlic has been linked to organosulfur compounds, notably allicin (Ankri & Mirelman, 1999). The fact that the expected antimicrobial effect of the garlic oil utilized in our investigation was not detected may be attributable to the low amount of organosulfur compounds or the likelihood that the active structures of these compounds were compromised during the study. In this investigation, essential oils were dissolved with 10% DMSO. Organosulfites, which are also contained in garlic, are very reactive and can swiftly react with other compounds. Garlic failed to display a potent antimicrobial impact in our investigation. This circumstance was interpreted as the loss of garlic's antimicrobial characteristics as a result of a probable reaction between the utilized solvent and garlic's active components.

Table 2 Effect of different doses of essential oils on rumen protozoa activity

Essential oil	Dose				SEM	p-value	Contrasts	
	0	500	1000	5000			Linear	Quadratic
Orange	3.48 ^{a}	3.02 ^{b}	3.14 ^{ab}	0.21 ^{c}	0.12	***	***	***
Cinnamon	3.48 ^{a}	2.48 ^{b}	2.50 ^{b}	0.13 ^{c}	0.25	***	***	**
Laurel	3.48 ^{a}	1.98 ^{b}	2.78 ^{ab}	0.38 ^{c}	0.42	***	***	NS
Oleaster	3.48 ^{a}	1.81 ^{b}	1.32 ^{b}	0.13 ^{c}	0.18	***	***	NS
Garlic	3.48	3.31	3.56	3.28	0.20	NS	NS	NS
Thyme	3.48 ^{a}	0.38 ^{b}	0.53 ^{b}	0.61 ^{b}	0.17	***	***	***

a, b, c: Different letters on the same line are different from each other; SEM, standard error of mean; ***P <0.001; **P <0.01; NS: not significant

In studies on EOs, the dose to be employed is as crucial as the chosen plant. In this study, Fig. 2 demonstrates the influence of the dose on protozoal activity. Feitoza *et al.* (2022) exposed five cannulated adult Santa Ines sheep to *Prosopis juliflora* at five concentrations (0, 200, 400, 600, and 800 mg/mL) to assess the effects on ruminal protozoa populations. Protozoa react dose-dependently to *Prosopis juliflora*, according to Feitoza *et al.* (2022). However, larger concentrations (> 600 mg/mL) do not necessarily result in more protozoa being eliminated. According to their evaluation of these findings, protozoa are capable of developing mechanisms of resistance to phytogenic additives such as bacteria. The Laurel group clearly showed that protozoa can develop resistance mechanisms to phytogenic additives (Fig. 2).

When doses in the current study are evaluated, thyme oil at 500 mg/kg had the best anti-protozoal efficacy (Fig. 3 and Table 2). With thyme oil, the amount of bacteria degraded by protozoa at a 500 mg/kg dose was reduced by 89.08%. Little *in vitro* research directly examines the effect of thyme oil on rumen protozoa as in the current study (Teferedegne, 2000; Wang *et al.*, 2022). Patra *et al*. (2010) evaluated the effect of clove, eucalyptus, garlic, origanum, and peppermint EOs at three different doses (0.25, 0.50, and 1.0 g/L) on protozoa *in vitro* and observed all EOs displayed some level of antiprotozoal activity. The reduction in protozoa caused by origanum oil and peppermint oil was the greatest (by nearly 3 log units), followed by the reduction caused by clove oil (by 2 log units), followed by eucalyptus and garlic oils (by 1 log unit).

For a 500 mg/kg dose, the reduction in bacteria broken down by protozoa was statistically significant for oleaster, laurel, cinnamon, and orange peel oil (P <0.001). There reductions were in the order of 48.0%, 43.1%, 28.7%, and 13.2%, respectively. When 1000 mg/kg doses of essential oils were tested for antiprotozoal effect, thyme was again found to have the strongest effect in comparison with the control. Bacteria broken down by protozoa was decreased 84.8% with thyme oil at a dose of 1000 mg/kg. This effect of thyme EO was significant in both linear and quadratic aspects (P <0.001).

At a 1000 mg/kg dose, oleaster oil exhibited the strongest antiprotozoal activity following thyme oil, with a 62% increase in protozoal impact. At 5000 mg/kg, oleaster and cinnamon oils exhibited the strongest efficacy. The breakdown of bacteria by protozoa was suppressed by 96% at 5000 mg/kg oleaster oil and cinnamon oil containing cinnamaldehyde compared to the control group (Table 2). Conversely, Benchar *et al*. (2008) showed that adding cinnamaldehyde to the diets of four lactating Holstein cows fitted with ruminal cannulas had no effects on rumen protozoal activity. As has been stated numerous times in the literature, the results obtained in *in vitro* studies with EO do not always

translate to *in vivo* and *in sacco* studies. The amount of EO utilized is one of the most influential aspects on these findings. To be able to ensure that *in vitro* results can be repeated *in vivo*, it is necessary to keep the dose range as broad as feasible in EO investigations.

Of all the oils used in the study, only oleaster oil showed that the antiprotozoal effect increased linearly with increasing doses. According to Han *et al*. (2020), the reason could be the presence of terpengustifol, a triterpenoid saponin. Many studies have shown that herbal saponins have potent antiprotozoal properties and can be used as a defaunating agent (Wallace, 2004; Guo *et al.*, 2008; Hanim *et al.*, 2009; Ramos-Morales *et al.*, 2019; Tan *et al.*, 2020). Saponins' antiprotozoal activity may be due to their ability to form irreversible complexes with cholesterol in protozoal cell membranes, resulting in membrane breakdown, cell lysis, and death (Francis *et al.*, 2002). Another mechanism of saponin may be due to saponin binding with sterol in protozoa cell membranes, altering cell wall permeability (Newbold *et al.*, 1997).

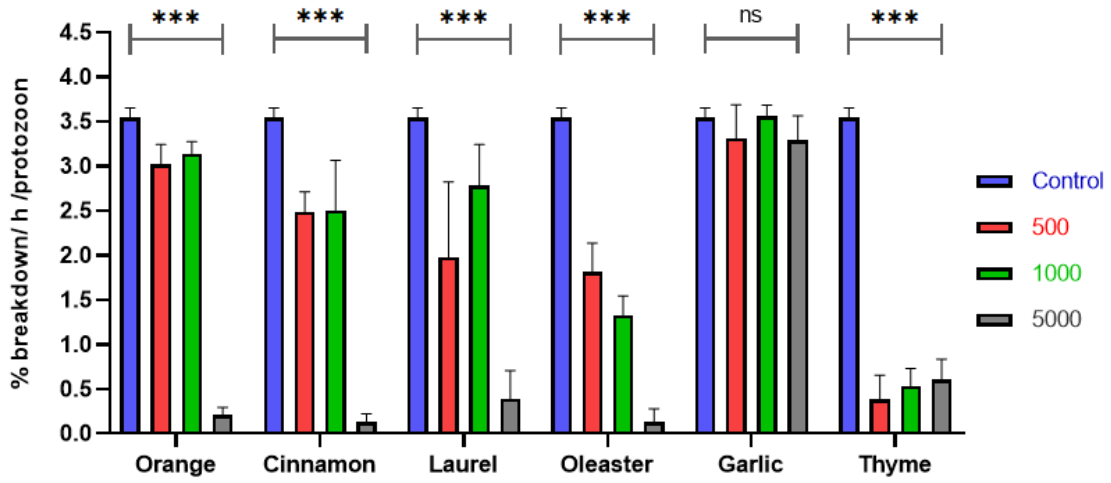

Figure 2 Effect of different doses of essential oils on rumen protozoal activity. *** indicates *P* <0.001, NS: not significant

Little *in vivo* research has been conducted on the effects of EOs on rumen protozoa, with conflicting results. According to Newbold *et al.* (2004) and Benchaar *et al.* (2007), ruminal protozoan counts were unaffected when sheep and dairy cows were fed 110 mg/day and 750 mg/day, respectively, of a mixture of essential oils. McIntosh *et al.* (2003) found that the consumption of a blend of EOs at a dosage of 1000 mg/d in dairy cattle had no influence on the protozoan activity. Clove EOs, on the other hand, reduced total protozoa (Patra *et al.*, 2010). Similarly, Ando *et al.* (2003) discovered that feeding 200 g/d of peppermint to steers lowered the number of protozoa by nearly 50%. In contrast, when beef heifers were fed a mixture of cinnamaldehyde and eugenol, Cardozo *et al*. (2006) noticed an increase in protozoa. Supplementing with juniper oil had no effect on individual protozoal population concentrations, only on the total protozoa population, according to Yesilbag *et al*. (2017). The differences in the results are due to many variables, such as the origin of EOs and concentration of the principal bioactive dosage, the type of studied animal, the type of protozoa, and methods employed. The elimination of those differences can be achieved by increasing the number of studies both *in vivo* and *in vitro* on the effect of EOs upon rumen protozoa. In addition, the effects of EOs on particular species of rumen protozoa rather than the total numbers of rumen protozoa should be the primary focus of research that is conducted in the years to come.

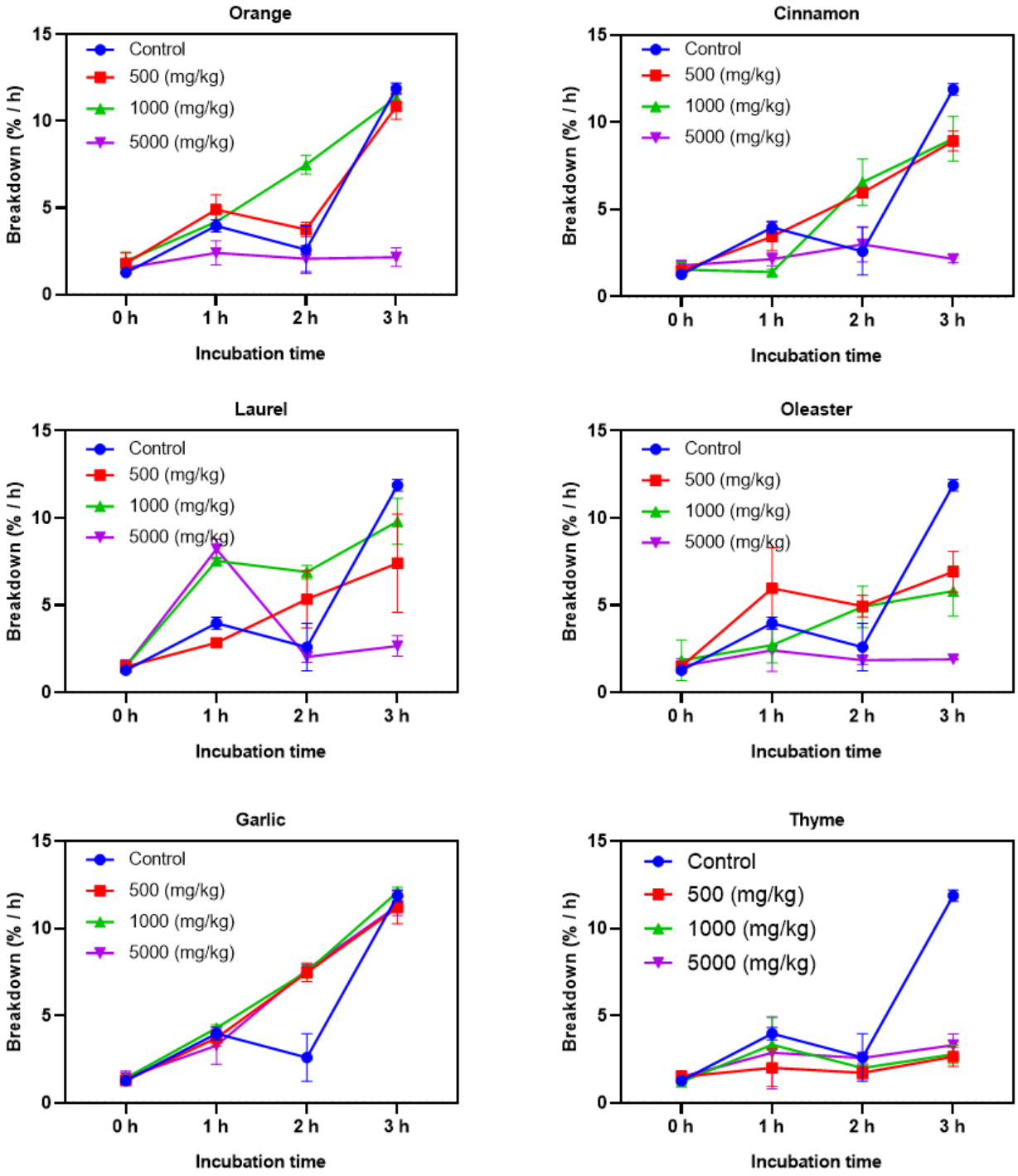

Figure 3 Effect of different dosages of essential oils (orange peel, cinnamon, laurel, oleaster, garlic, thyme) on the activity of rumen protozoa at various sampling times

Conclusions

The antiprotozoal efficacy of orange peel, cinnamon, laurel, oleaster, and thyme EOs varied depending on the type of EO and application dose. In particular, thyme oil has shown strong activity against ruminal protozoa in a dose-dependent manner. However, our study has several limitations. The most important limitation is the lack of surface analysis for the optimum EO inclusion. Therefore, further studies are needed to determine optimum testing conditions. Analysis of the active components of tested EOs is of great interest to determine which active components of the oils are more effective for antiprotozoal activity. In conclusion, more comprehensive studies are needed to fully understand the activity of these EOs.

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Conflict of interest declaration

The author declares that no competing interests.

Author contribution

The article was prepared by a single author.

References

Ando, S., Nishida, T., Ishida, M., Hosoda, K., & Bayaru, E., 2003. Effect of peppermint feeding on the digestibility, ruminal fermentation, and protozoa. Livest. Prod. Sci. 82, 245–248. https://doi.org/10.1016/S0301-6226(03)00012-5.

Ankri, S., Mirelman, D. 1999. Antimicrobial properties of allicin from garlic. Microbes Infect, 1, 125–129. https://doi.org/10.1016/S1286-4579(99)80003-3.

Belanche, A., De la Fuente, G., Moorby, J. M., Newbold, C. J., 2012. Bacterial protein degradation by different rumen protozoal groups. J. Anim. Sci. 90. https://doi.org/10.2527/jas.2012-5118.

Belanche, A., Ramos-Morales, E., Newbold, C. J., 2016. *In vitro* screening of natural feed additives from crustaceans, diatoms, seaweeds, and plant extracts to manipulate rumen fermentation. J. Sci. Food. Agric. 96, 3069–3078. https://doi.org/10.1002/jsfa.7481.

Benchaar, C., McAllister, T. A., & Chouinard, P. Y., 2008. Digestion, ruminal fermentation, ciliate protozoal populations, and milk production from dairy cows fed cinnamaldehyde, quebracho condensed tannin, or *Yucca schidigera* saponin extracts. J. Dairy. Sci. 91, 4765–4777. https://doi.org/10.3168/JDS.2008-1338.

Benchaar, C., Petit, H. V., Berthiaume, R., Ouellet, D. R., Chiquette, J., Chouinardt, P. Y., 2007. Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. J. Dairy Sci. 90, 886–897. https://doi.org/10.3168/JDS.S0022-0302(07)71572-2.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., & Ferret, A., 2007. Invited review: Essential oils as modifiers of rumen microbial fermentation. J. Dairy Sci. 90, 2580–2595. https://doi.org/10.3168/jds.2006-644.

Cardozo, P. W., Calsamiglia, S., Ferret, A., Kamel, C., 2006. Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. J. Anim. Sci. 84, 2801–2808. https://doi.org/10.2527/jas.2005-593.

Castillo-Lopez, E., Domínguez-Ordóñez, M. G., 2019. Factors affecting the ruminal microbial composition and methods to determine microbial protein yield. Rev. Mex. Cienc. Pecu. 10, 120–148. https://doi.org/10.22319/rmcp.v10i1.4547.

Dehority, B. A., 2003. Rumen microbiology. Nottingham University Press, Nottingham.

Feitoza, E. R. F., Alves Júnior, R. T., da Rocha Costa, G. R., da Silva, C. S., Torres, T. R., de Lima, J. S., Pereira, K. P., de Souza, E. J. O., 2022. Phytogenic additive from *Prosopis juliflora* on populations of rumen ciliate protozoa and its correlation with nutrition of sheep. Arch. Microbiol. 204, 425. https://doi.org/10.1007/s00203-022-03041-2.

Francis, G., Kerem, Z., Makkar, H. P. S., Becker, K., 2002. The biological action of saponins in animal systems: A review. Br. J. Nutr. 88, 587–605. https://doi.org/10.1079/bjn2002725.

Guo, Y. Q., Liu, J. X., Lu, Y., Zhu, W. Y., Denman, S. E., McSweeney, C. S., 2008. Effect of tea saponin on methanogenesis, microbial community structure, and expression of *mcrA* gene in cultures of rumen micro-organisms. Lett. Appl. Microbiol. 47, 421–426. https://doi.org/10.1111/J.1472-765X.2008.02459.X.

Han, J., Chen, X., Liu, W., Cui, H., Yuan, T., 2020. Triterpenoid saponin and lignan glycosides from the traditional medicine *Elaeagnus angustifolia* flowers and their cytotoxic activities. Molecules. 25, 462. https://doi.org/10.3390/MOLECULES25030462.

Hanim, C., Yusiati, L. M., Alim, S., 2009. Effect of saponin as defaunating agent on *in vitro* ruminal fermentation of forage and concentrate. J. Indones. Trop. Anim. Agric. 34, 231–235. https://doi.org/10.14710/JITAA.34.4.231-235.

Jouany, J. P., Morgavi, D. P., 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. Animal. 1, 1443–1466. https://doi.org/10.1017/S1751731107000742.

Kekana, M. R., Luseba, D., Muyu, M. C., 2021. Effects of garlic supplementation on *in vitro* nutrient digestibility, rumen fermentation, and gas production. S. Afr. J. Anim. Sci. 51, 271–279. https://doi.org/10.4314/sajas.v51i2.15.

Kumar M., Himani, S., Arya, S., Kumar, R., Prakash, O., 2022. Extractions Methods and Biological Applications of Essential Oils. in Essential Oils - Advances in Extractions and Biological Applications. Santana de Oliveira, M., Helena de Aguiar Andrade, E., eds. IntechOpen, London.

McIntosh, F. M., Williams, P., Losa, R., Wallace, R. J., Beever, D. A., Newbold, C. J., 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. Appl. Environ. Microbiol. 69, 5011–5014. https://doi.org/10.1128/AEM.69.8.5011-5014.2003.

Newbold, C. J., El Hassan, S. M., Wang, J., Ortega, M. E., Wallace, R. J., 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. Br. J. Nutr. 78, 237–249. https://doi.org/10.1079/BJN19970143.

Newbold, C. J., De la Fuente, G., Belanche, A., Ramos-Morales, E., McEwan, N. R., 2015. The role of ciliate protozoa in the rumen. Front. Microbiol. 6, 1313. https://doi.org/10.3389/FMICB.2015.01313.

Newbold, C. J., McIntosh, F. M., Williams, P., Losa, R., Wallace, R. J., 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Anim. Feed Sci. Technol. 114, 105–112. https://doi.org/10.1016/j.anifeedsci.2003.12.006.

Patra, A. K., Kamra, D. N., Agarwal, N., 2010. Effects of extracts of spices on rumen methanogenesis, enzyme activities, and fermentation of feeds *in vitro*. J. Sci. Food Agric. 90, 511–520. https://doi.org/10.1002/jsfa.3849.

Ramos-Morales, E., Lyons, L., De La Fuente, G., Braganca, R., Newbold, C. J., 2019. Not all saponins have a greater antiprotozoal activity than their related sapogenins. FEMS Microbiol. Lett. 366, fnz144. https://doi.org/10.1093/FEMSLE/FNZ144.

Swamy, M. K., Akhtar, M. S., Sinniah, U. R., 2016. Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. Evidence-based Complementary and Alternative Medicine. 2016, 1–21. https://doi.org/10.1155/2016/3012462.

Tan, C., Ramírez-Restrepo, C. A., Shah, A. M., Hu, R., Bell, M., Wang, Z., McSweeney, C., 2020. The community structure and microbial linkage of rumen protozoa and methanogens in response to the addition of tea seed saponins in the diet of beef cattle. J. Anim. Sci. Biotechnol. 11, 1–10. https://doi.org/10.1186/S40104-020-00491-W.

Teferedegne, B., 2000. New perspectives on the use of tropical plants to improve ruminant nutrition. Proc. Nutr. Soc. 59, 209–214. https://doi.org/10.1017/S0029665100000239.

Ünlü, H. B., İpçak, H. H., Kandemir, Ç., Özdoğan, M., Canbolat, Ö., 2021. Effects of oregano essential oil and capsicum extract on fattening, serum constituents, and rumen fermentation of lambs. S. Afr. J. Anim. Sci. 51, 172–179. https://doi.org/10.4314/sajas.v51i2.4.

Wallace, R. J., 2004. Antimicrobial properties of plant secondary metabolites. Proc. Nutr. Soc. 63, 621–629. https://doi.org/10.1079/PNS2004393.

Wang, Z., Li, X., Zhang, L., Wu, J., Zhao, S., Jiao, T., 2022. Effect of oregano oil and cobalt lactate on sheep in vitro digestibility, fermentation characteristics and rumen microbial community. Animals. 12, 1–14. https://doi.org/10.3390/ANI12010118.

Yang, W. Z., Benchaar, C., Ametaj, B. N., Chaves, A. v., He, M. L., McAllister, T. A., 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. J. Dairy Sci. 90, 5671–5681. https://doi.org/10.3168/JDS.2007-0369.

Yesilbag, D., Biricik, H., Cetin, I., Kara, C., Meral, Y., Cengiz, S. S., Orman, A., Udum, D., 2017. Effects of juniper essential oil on growth performance, some rumen protozoa, rumen fermentation, and antioxidant blood enzyme parameters of growing Saanen kids. J. Anim. Physiol. Anim. Nutr. (Berl) 101, e67–e76. https://doi.org/10.1111/jpn.12560.