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THE INFLUENCE OF VARIOUS CONCENTRATIONS OF ESSENTIAL OILS ON THE GROWTH RATE OF FIBROLYTIC AND AMYLOLYTIC BACTERIA ISOLATED FROM RUMEN FLUID

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Abstract

The antibacterial capabilities of essential oils (EOs) have been shown to influence the rumen microbial population. On the basis of their antimicrobial properties, six EOs were selected: oleaster, orange peel, laurel, garlic, thyme and cinnamon. Scanning Electron Microscopy pollen morphotypes were identified to reliably identify the plant species from which EOs are extracted. The maximum specific growth rate of bacteria is one of the metrics that are used to quantify the growth rate of bacteria. Dose–response incubations were carried out so that their effect could be determined on the maximum specific growth rate (μmax) of bacteria. Ten different concentrations (0, 50, 100, 200, 300, 400, 600, 800, 1000, and 5000 ppm) were used for this purpose. In order to measure bacterial growth, the optical density at 650 nm was measured hourly until the reading for bacterial growth decreased. Using Micro Fit, the maximum bacterial growth rate before growth began was calculated (v 1.0). It was determined that the influence of essential oils, dosages, and the relationship between dose and oil had a statistically significant impact on the maximal specific growth rate (p<0001). The EO of thyme was found to have the most potent antibacterial effect on all bacteria examined in the study. Following thyme EO, laurel, oleaster, and cinnamon EOs displayed the strongest antibacterial effect. Based on this study, it could be concluded that the antibacterial effect was generally more pronounced at 400 ppm doses of essantial oils.

Key words: Essential oil, Dose effect, Rumen bacteria, Growth rate, SEM.

Introduction

The excessive formation of some metabolites such as methane, carbon dioxide and hydrogen sulfide due to digestive activity in the rumen causes nutrient loss and environmental problems (Sutton *et al*., 2021). In recent years, one of the highlighted issues of ruminant nutritionists and rumen microbiologists has been to manipulate the rumen microbial community in order to optimize the nutrient use efficiency of animals and hence lessen their environmental impact. (Calsamiglia *et al*., 2007; Patra & Yu, 2012; Smeti *et al*., 2015; Belanche *et al*., 2016; Joch *et al*., 2018; Sahan *et al*., 2018; Benchaar, 2021). It has been demonstrated that certain of the chemical feed additives, antibiotics, methane inhibitors, and plant extracts that are employed for this purpose boost rumen metabolism and the growth of animals. However, the presence of chemical residues in animal products, as well as the demonstration of bacterial resistance to antibiotics and concerns regarding the use of antibiotics in animal nutrition, led to an increase in the popularity of using plant EOs and their active compounds instead of antibiotics and chemical additives in studies for the manipulation of the rumen microbial ecosystem. This was done in an effort to better understand how to control the rumen's unique microbial community. Bacteria, which constitute the biggest proportion of the rumen's microbial community, can be classed as amylolytic, fibrolytic, or proteolytic depending on the substrates they degrade and use for growth. The balance of group of microorganism densities in rumen is important. Amylolytic bacteria can grow in low pH, but this is not the case for fibrolytic bacteria. The pH has an effect on the cellulolytic bacteria as well as the amylolytic bacteria group. In contrast to cellulolytic bacteria, amylolytic bacteria are more common when the diet contains a significant amount of grain and decrease when the pH of the rumen and its buffers increase. Cellulolytic populations diminish when grain intake is high, leading to a fall in pH and an increase in the acidity of the rumen. For this reason, it is considered important investigate the effects of EOs on fibrolytic and amylolytic bacteria individually to reach clearer information.

There are research demonstrating that EOs extracted by various ways from diverse plant parts possess antibacterial action against a variety of microbes, including gram (-) and gram (+) bacteria, protozoa, and fungi. (Helander *et al*., 1998; Saeed & Tariq, 2008; Belanche *et al*., 2016; Jahani-Azizabadi *et al*., 2019; Jan *et al*., 2019). Yet, what is known only to a limited extent is how the rumen microbial population responds to individual EOs. In addition, the EOs studied within the limited range of dose have failed to determine the optimum effect dose.

To the best of the author's knowledge, cinnamon, garlic, and thyme, which are known to have potent antibacterial properties, have been used in numerous studies; however, laurel, oleaster, and orange peel oil, which have been used to treat many diseases since ancient times, have not been used extensively in a study about ruminants, specifically how the rumen bacteria respond to individual EOs, until now. All these reasons mentioned above reveal the necessity of investigating the effects of doses of EOs on individual rumen bacteria. Therefore, this research was planned to investigate the influence of different doses (ranging between 50 and 5000 ppm) of EOs of oleaster, orange peel, laurel, garlic, thyme and cinnamon on the growth of rumen fibrolytic (*Butyrivibrio fibrisolvens, Ruminococcus albus, Clostrodium proteoclasticum*) and amylolytic (*Ruminobacter amylophilus, Selenomonas ruminantium*) bacteria *In vitro*.

Material and Methods

Essential oils: The six EOs used in current research, that called SEOs, are orange peel (*Citrus cinensis*), cinnamon (*Cinnamomum verum*), laurel (*Laurus nobilis*), oleaster (*Eleagnus angustifolia*), garlic (*Allium sativum*) and thyme (*Tymus vulgare*). These SEOs were all supplied by Doğa Bitki Ürünleri Gıda Limited (Antalya, TURKEY). EOs were stored in dark glass vials at 4ºC prior to use.

These SEOs were selected due to the fact that the main bioactive components of each have distinct chemical and structural properties given in (Table 1). Shows the active compounds in EO with the first six highest percentage.

Strains of rumen bacteria: The following microbial species were obtained from Aberystwyth University UK; ATCC1917: -*Butyrivibrio fibrisolvens*, SY3 -*Rumınococcus albus*, B316-*Clostrodium proteoclasticum*, H18 *Ruminobacter amylophilus*, Z108 *Selenomonas ruminantium*. Bacteria were maintained in Hobson's M8 medium prior to use (Hobson, 1969).

Characterization of EO: Gas chromatography-mass spectrometry (GC-MS) analyses were conducted utilizing a Hewlett Packard 5973-6890 GC-MS system running in electron impact (EI) ionization mode (fitted with an HP 5MS 60 m x 0.25 mm x 0.25 m film thickness capillary column) with He (1.5 mL min-1) as the carrier gas. The column's initial temperature was 60°C, and it was gradually heated to 250°C at a rate of 4°C min–1. At 70 eV, mass spectra were collected. The mass range was from 35 to 425 m/z. By comparing the mass spectrum data and retention indices (RI) of EOs with spectra from the NIST/NBS Wiley libraries, EOs were discovered.

Table 1. Major components of EOs.

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
	Cinnamaldehyde	66.68
	Linalool	1.21
	Eugenol	6.77
	1,3-Dioxolane, 4-Methyl- 2 -Phenyl	0.69
	Triacetin	1.93
Orange Pell	D-Limonene	34.2
	1,8-Cineole	14.02
	Linalool	2.84
	4-Terpineol	5.87
	Trans-Carveol	11.89
	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

Table 2. Micro-structural characters via SEM of EOs yielding species.

Species	Family	Size	Type	Shape	Sculpturing	Polar diameter	Equatorial diameter	P/E Index
Orange Pell	Rutaceae	Medium	Tetracolporate	Prolate-spheroidal	Micro-reticulate	29.6 µm	26.4 µm	1.12
Cınnamon	Lauraceae	Small	Inaperturate	Spheroidal	Micro-echinate	23.4 µm	22.9 µm	1.02
Laurel	Lauraceae	Medium	Inaperturate	Spheroidal	Micro-echinate	36.4 µm	35.7 µm	1.01
Oleaster	Elaeagnaceae	Medium	Tricolporate	Prolate-spheroidal	Verrucate	34.5 µm	31.2 µm	1.1
Garlıc	Amaryllidaceae	Medium	Monosulcate	Oblate	Rugulate-perforate	27.3 µm	38.2 µm	0.71
Thyme	Lamiaceae	Medium	Hexacolpate	Oblate-spheroidal	Reticulate	26.5 µm	30.1 µm	0.88

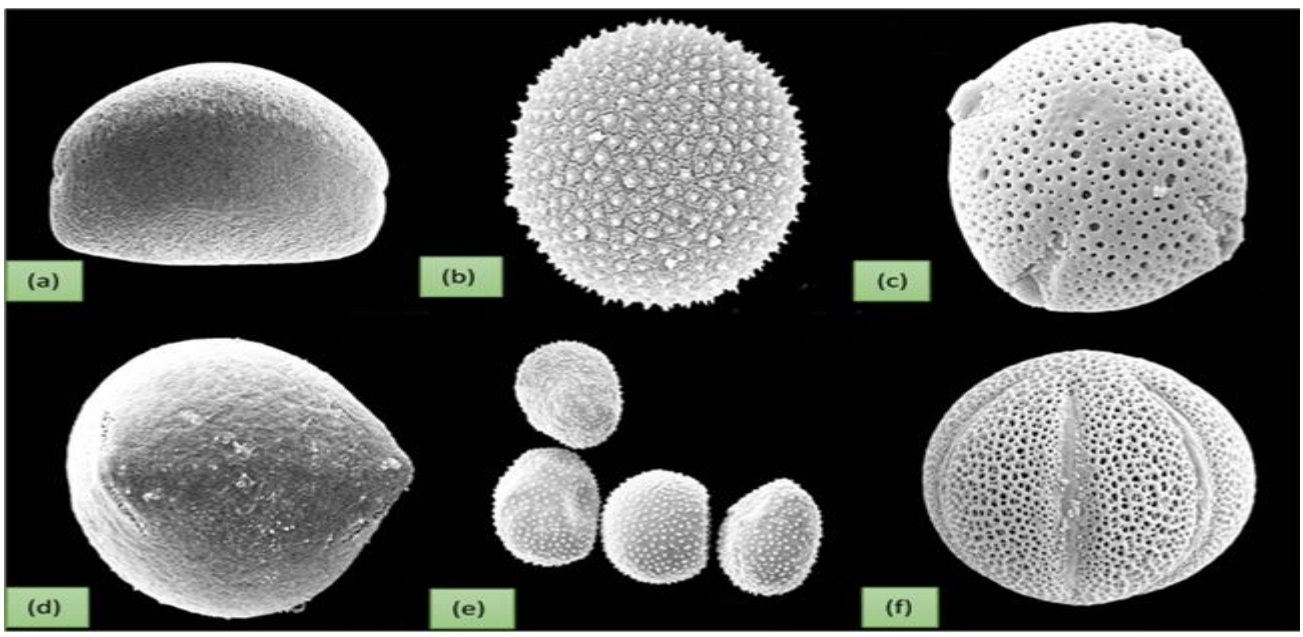

Fig. 1. Imaging analysis of pollen surface of EOs yielding species at 10 µm (a) Garlic (b) Orange peel (c) Cinnamon *(*d) Oleaster (e) Laurel (f) Thyme.

Pollen morphology: For scanning microscopy (SEM), pollen was also collected from six above mentioned essential oils yielding species. For SEM observations of pollen, anthers from flowers were separated by crushing the flowers in 45% acetic acid then pollen were suspended in 90% ethanol and mounted on metallic stubs. Before examination samples were sputter coated with gold palladium and micromorphological features of pollen was observed under SEM microscope (Majeed *et al*., 2022). All of the plant samples whose pollens studied are cultivated. These plant samples obtained from botany garden and from planted areas. Plants of this study identified with comparing floras of Turkey (Davis, 1965-1988).

Micro-morphology of EOs species via SEM: Before the oils were extracted, SEM pictures of the pollen of the selected plants were collected to identify the plant species (Fig. 1). In this section pollen visualization traits were quantified and measured showing diagnostic features of essential oil yielding species (Table 2 and Fig. 1).

Determination of the influence of essential oils on growing bacteria: The effect of essential oils on bacterial growth was investigated by inoculating Hobson's M8 medium-grown stock cultures with three-fold increases of EOs. After the medium was autoclaved, EO diluted in autoclaved water with 10% DMSO was added aseptically to give final concentrations of 50 to 5000 ppm (0.5 ml to each 6.5 ml of M8). The doses were used in the experiments 0, 50, 100, 200, 300 400, 600, 800, 1000 and 5000 ppm. The bacterial growth was assessed by taking hourly readings of the optical density at 650 nm until the growth readings dropped. The µmax [h^{-1}]) and the potential lag time (λ) before growth commenced were computed using the MicroFit v 1.0 (Institute of Foo Research, UK Ministry of Agriculture, Fisheries and Food (LINK Program). The concentration of EO required to decrease maximal growth (µmax) rates by 50% IC50 and to cause a doubling in the lag before growth commenced IC50 tlag was estimated after plotting µmax and tlag against EOs concentration using Curve Expert V1.4 (www.curveexpert.net) fitting a polynomial curve and using the analyze curve function to drive the required value. Every measurement was performed in triplicate.

Statistical Analyses

The 6 x 10 factorial arrangement was employed with SEOs and ten levels of dose (0, 50, 100 ,200, 300, 400, 600, 800, 1000, 5000 ppm) as main effects.

The data from the study were put through a two-way analysis of variance in the SPSS package program's General Linear Model The model was $Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$. where Y_{ij} = response variable, μ = overall mean, α_i = the effect of EO *i*th, β_j = the influence of dose *j*th, $(\alpha\beta)_{ij}$ = the interaction effect of EO and Dose and ε_{ij} =

error term. Duncan's New Multiple Range Test was used to compare the averages of different groups. The significant differences were declared at $p = 0.05$.

Results

On the μmax, the influence of EOs, their doses, and dose-oil interaction was statistically significant (p.0001). Although about all SEOs used in this study significantly reduced the *In vitro* growth of all bacteria species, they reacted differently to the EOs and their doses. The results of each bacterium are given in separate figures. The values depicted in the figures are the means, with the standard deviations indicated by vertical bars.

The effect of EO and dose interaction on the μmax of *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Clostrodium proteoclasticum*, which are among the rumen fibrolytic bacteria, are given in Figs. 2-4, respectively

The EOs and doses interaction effects on the μmax of *Butyrivibrio fibrisolvens* are shown in (Fig. 2). The μmax value of *Butyrivibrio fibrisolvens* was adversely affected by all oils and doses with the exception of 5000 ppm orange peel oil and 50 ppm thyme oil (p<0.0001). In comparison to the control, the 5000 ppm orange peel oil increased the μmax value by 3.33 percent (p<0.001). The lowest growth rate of *Butyrivibrio fibrisolvens* was seen at 600 and 800 ppm doses of oleaster oil and reduced the growth rate of *Butyrivibrio fibrisolvens* by 98.88% compared to the control.

The EOs and doses interaction effects on the μmax of *Ruminococcus albus* are given in Fig. 3. The highest μmax value of *Ruminococcus albus* was observed at 100 ppm and 400 ppm doses of the cinnamon oil (Fig. 3) compared to the control. The strongest antibacterial effect for *Ruminococcus albus* was observed at 1000 ppm dose of oleaster oil, oleaster reduced the μmax value of *Ruminococcus albus* by 96% compared to the control. The μmax value of *Ruminococcus albus* decreased after at 100 ppm dose then it remains low. At 400 ppm dose of thyme oil, the μmax of *Ruminococcus albus* began to decrease, and the lowest value was observed at 600 ppm.

The EOs and doses interaction effects on the μmax of *Clostrodium proteoclasticum* are given in (Fig. 4). The μmax of *Clostrodium proteoclasticum* was affected differently from all EOs depending on the dose. The EOs showing strong antibacterial effect on the *Clostrodium proteoclasticum* was thyme. After a dose of 800 ppm, the antibacterial effect of the oils was replaced by a bacterial growth-enhancing effect. After the control, the μmax value of *Clostridium proteoclasticum* was highest at 50 ppm and 1000 ppm doses of oleaster oil.

When the effects of dose interaction with EOs on the μmax values of fibrolytic bacteria (Figs. 2-4) are examined, it is clear that oleaster oil has the strongest antibacterial effect for *Butyrivibrio fibrisolvens* and *Ruminococcus albus*, while thyme has the strongest antibacterial effect for *Clostrodium proteoclasticum* (p<0.0001).

The effect of EO and dose interaction on the μmax of *Ruminobacter amylophilus* and *Selenomonas ruminantium*, which are among the rumen amylolytic bacteria, are given in (Figs. 5 and 6), respectively.

In Fig. 5, where the interaction effect of EO and dose on the growth rate of Ruminobacter amylophilus is shown, it is seen that the 1000 ppm dose of thyme significantly reduces the growth rate compared to the control. The adverse effect seen in thyme was observed in almost all doses of laurel and oleaster as well.

When Fig. 6 is examined, the growth rate of *Selenomonas ruminantium* increased at 200, 300, 400 and 500 ppm doses of orange pell oil and 50 ppm of cinnamon oil compared to the control. The growth rate of *Selenomonas ruminantium* decreased compared to the control in all oils and doses other than these. Among these reductions, the most significant reduction was in the 1000 ppm dose of thyme oil. Surprisingly and intriguingly significant growth acceleration was seen following a 1000 ppm dose of orange peel oil.

To clarify the impacts of the available essential oils, the results for each oil were also displayed in a separate graph. Consequently, the influence of EOs and dose interaction on the μmax of bacteria (*Butyrivibrio fibrisolvens, Ruminococcus albus, Clostrodium proteoclasticum, Ruminobacter amylophilus,* and *Selenomonas ruminantium*) are illustrated in Fig. 7 for every SEOs. Examining (Fig. 7) reveals that thyme, oleaster, and laurel oils have an antibacterial impact on all microorganisms.

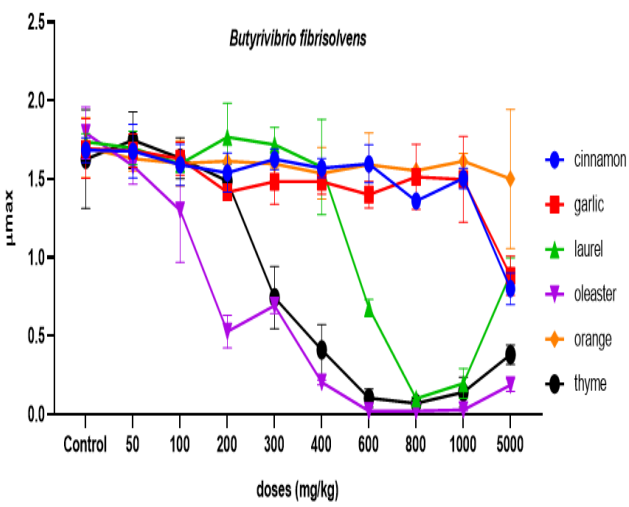

Fig. 2. The EO and doses interaction effects on the μmax of *Butyrivibrio fibrisolvens*.

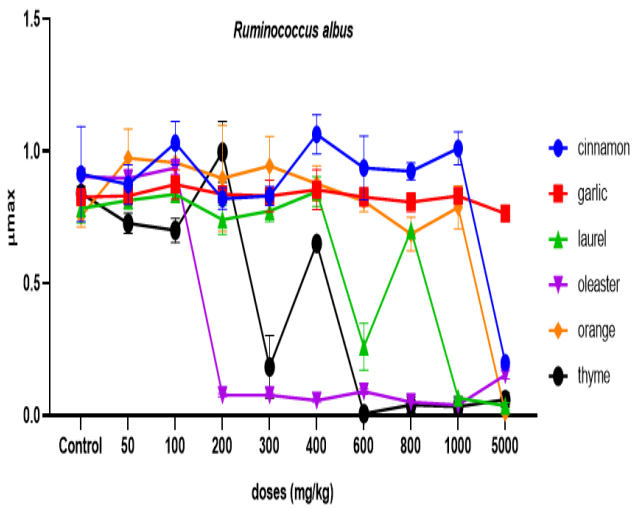

Fig. 3. The EO and doses interaction effects on the μmax of *Ruminococcus albus*.

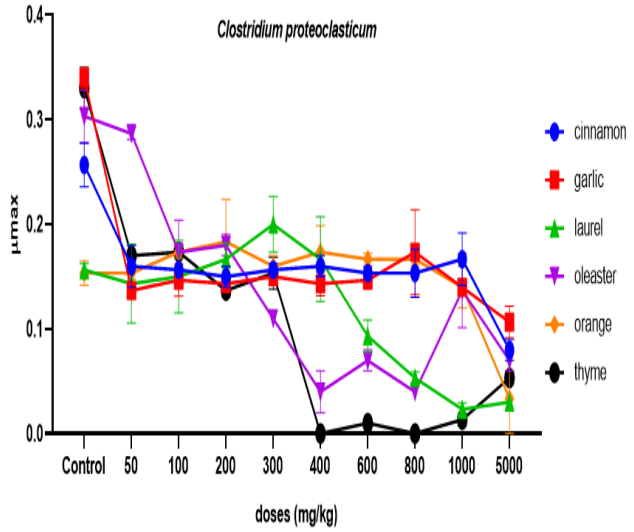

Fig. 4. The EO and doses interaction effects on the μmax of *Clostrodium proteoclasticum.*

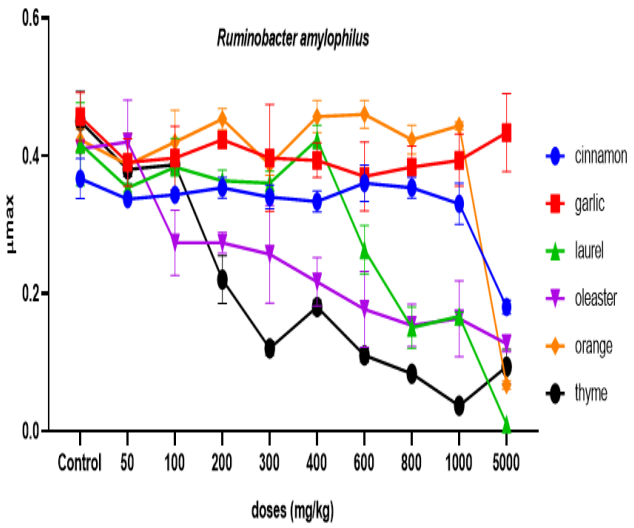

Fig. 5. The EO and doses interaction effects on the μmax of *Ruminobacter amylophilus*.

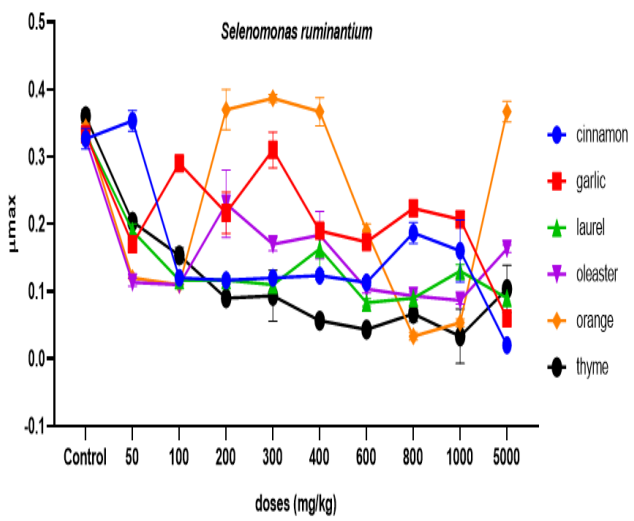

Fig. 6. The EO and doses interaction effects on the μmax of *Selenomonas ruminantium*.

The μmax value of *Ruminococcus albus* was suppressed almost entirely at a dose of 1000 ppm cinnamon oil. It is observed that a similar decrease occurs in *Butyrivibrio fibrisolvens*. (Fig. 7a). The strongest bactericidal action against *Butyrivibrio fibrisolvens* was observed at doses of 800 ppm laurel oil, at 400, 600, 800, and 1000 ppm oleaster oil and 600 ppm thyme oil as shown in the graphs clearly. (Fig. 7c, Fig. 7d and Fig. 7f).

The (Tables 3 and 4) display the doses corresponding to the highest and lowest μmax values of fibrolytic and amylolytic bacteria. Examining Tables 3 and 4, it can be seen that μmax values typically decrease with increasing dose. There are some exceptions, as was previously mentioned. In particular, high μmax values were seen at high doses for orange peel oil.

Discussion

Under *In vitro* conditions, the EOs utilized in this study were effective at promoting or inhibiting the growth rate of rumen amylolytic and fibrolytic bacteria. I would mention that mostly in such studies the rumen fluid has been used (Busquet *et al*., 2006; Benchaar *et al*., 2007; Calsamiglia *et al*., 2007; Chaves *et al*., 2008; Belanche *et al*., 2016; Yu *et al*., 2020), but in this study the pure bacteria strains were used.

Thyme, oleaster and laurel EOs were the most prominent antimicrobial effective EOs on the growth rates of fibrolytic bacteria were. When the studies investigating the antibacterial effects of those EOs on rumen bacteria are surveyed, it can be seen that there are scarcely any studies examining the individual effects of orange pell, oleaster and laurel oils on the rumen bacteria and rumen fermentation (Chaves *et al*., 2008; Kouazounde *et al*., 2015; Sahan *et al*., 2018). On the other hand, studies investigating the antimicrobial effect of thyme EOs on rumen bacteria are frequent (Evans & Martin, 2000; Busquet *et al*., 2006; Benchaar *et al*., 2007; Patra & Yu, 2012; Yu *et al*., 2020). The results of most of these studies are in line with the findings of the current research. A dose of 100 ppm and above of thyme oil, which was used in our study and the main component of which is carvacrol, displayed a marked antimicrobial effect against fibrolytic bacteria, especially *Butyrivibrio fibrisolvens*. In accordance with the findings of a research, McIntosh *et al*., (2003) noted that *Butyrivibrio fibrisolvens*, an important gram (-) bacterium, is very sensitive to EOs. Indeed, there are works indicating that EOs have stronger antimicrobial effects against gram (+) bacteria than gram (-) (Dorman & Deans, 2000; Burt, 2004). They interpreted this distinction by the absence of a protective outer membrane surrounding the cell wall of gram (+) bacteria. However, in their study investigating the effects of different doses (250, 500 and 1000 mg/liter) of five EO (peppermint oil, origanum oil, eucalyptus oil, clove oil, and garlic oil) on rumen fibrolytic bacteria measured by real-time PCR. Patra & Yu, (2012) stated that the decline observed in the population of *F. succinogenes*, a gram (-) species, did not differ significantly from that of *R. flavefaciens* or *R. albus*, both of which are gram (+) bacteria. This contradiction brings to mind the question of whether EO are effective on other cellular structures. It has also been proposed that EOs with antibacterial activity versus gram (-) bacteria include secondary metabolites tiny enough to pass through porin proteins in the outer membrane and enter the plasma membrane, thereby exerting antimicrobial activity (Dorman

& Deans, 2000). Some aromatic compounds, such as carvacrol and thymol, exhibit this characteristic. It can be stated that the strong antimicrobial effect against *Butyrivibrio fibrisolvens* one of the gram (-) bacteria-of the thyme plant used in the present study is due to its active ingredient carvacrol at an amount of 93%. Helander *et al*., (1998) revealed that carvacrol and thymol increase the release of membrane lipopolysaccharides of gram (-) bacteria and the level of cytoplasmic membrane permeability. Hence, the low molecular weights of these chemicals might make it possible for them to exert their effects on both gram (+) and gram (-) bacteria. Numerous studies also indicate that various phenolic and non-phenolic EO chemicals can interact with other physiologically active molecules, such as chemical groups of proteins and enzymes (Juven *et al*., 1994).

An examination of the active substance compounds of oleaster and cinnamon EOs used in the present study (Table 1) reveals that the most abundant active compound in both EOs is cinnamaldehyde. However, the influence of these two EOs on the growth rates of bacteria were not found to be similar, which indicates that the synergy between the active compounds contained in a certain type of EOs plays an important role in the antimicrobial effect. Indeed, this confirms the hypothesis of Burt, (2004) that the composition of active compounds in the EOs may cause additional or synergistic effects which may increase the productivity of the rumen bacterial population. In their study testing the antimicrobial activity of 5 active compounds related to cinnamaldehyde which is the main active component of cinnamon, Chang *et al*., (2001) reported that the aldehyde groups and the side chain lengths of the groups increased the antimicrobial activity.

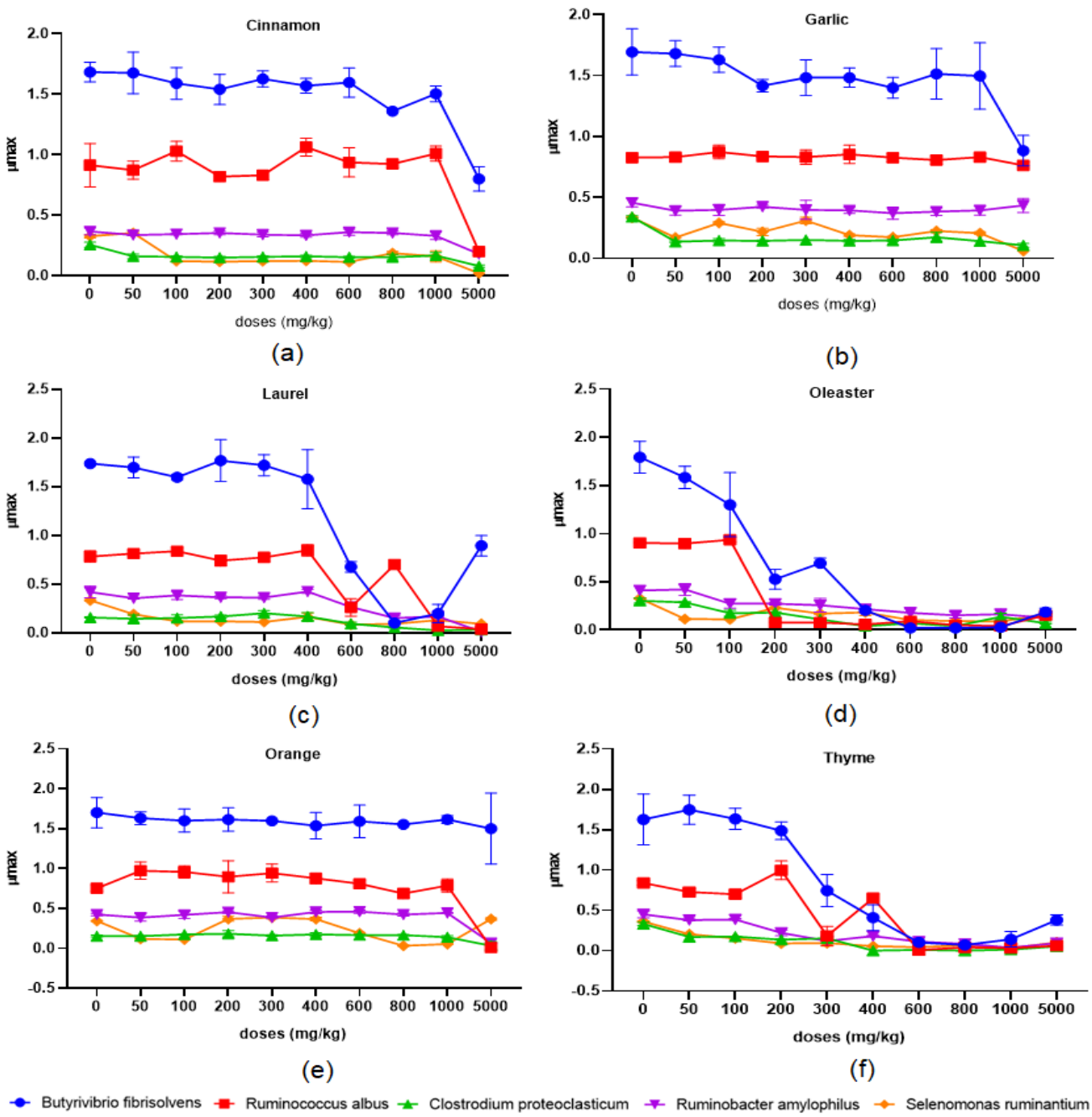

Fig. 7. EO and doses interaction effects on the µmax of bacteria. (a) cinnamon, (b) garlic, (c) oleaster, (d) laurel, (e) orange peel and (f) thyme.

Table 3. Doses corresponding to the highest and lowest μmax values of fibrolytic bacteria.

EO	*Butyrivibrio fibrisolvens*		Dose (ppm) *Ruminococcus albus*		*Clostrodium proteoclasticum*	
	Highest	Lowest	Highest	Lowest	Highest	Lowest
Cinnamon	0	5000	100	5000	0	5000
Garlic	0	5000	100	5000	0	5000
Laurel	200	800	400	5000	300	1000
Oleaster	0	600	100	1000	0	400
Orange pell	5000	400	50	5000	100	5000
Thyme	50	600	200	600	0	400

Table 4. Doses corresponding to the highest and lowest μmax values of amylolytic bacteria.

EO	Dose (ppm) *Ruminobacter amylophilus*		*Selenomonas ruminantium*	
	Highest	Lowest	Highest	Lowest
Cinnamon	0	5000	50	5000
Garlic	0	800	0	5000
Laurel	400	5000	0	600
Oleaster	50	5000	0	1000
Orange pell	600	5000	300	800
Thyme	0	1000	0	1000

Unexpectedly, there was not an evident antibacterial effect of garlic oil in our study, whereas there are studies reporting that garlic oil has a strong antimicrobial effect on rumen bacteria (Yang *et al*., 2007; Patra & Yu, 2012). Garlic's antibacterial effect has been related to organosulfur compounds, specifically allicin, in general (Ankri & Mirelman, 1999). The fact that the antimicrobial effect expected from the garlic oil used in our study was not observed may be due to the low organosulfur compounds content or the possibility that their active structures were impaired during study process. In this study, 10% DMSO was used to dissolve the essential oils. Organosulfites, which are also present in garlic, are extremely reactive and can react rapidly with other chemicals. In our study, garlic was unable to demonstrate a potent antibacterial effect. This situation was evaluated as the loss of garlic's antibacterial properties due to a possible reaction between the used solvent and garlic's active compounds. However, Kongmun *et al*., (2010) found that garlic oil and coconut oil increased the *In vitro* digestion of organic matter. They detected an increase in the number of *Ruminococus albus*, a type of fibrolytic bacteria, which is consistent with our research.

The μmax of *Butyrivibrio fibrisolvens, Ruminococcus albus and Clostrodium proteoclasticum* was affected differently from all EOs depending on the dose. In the current study, the growth rates of fibrolytic bacteria displayed a tendency to decrease as of 400 ppm dose of EOs. However, the increase in the growth rate of *Ruminococcus albus* bacteria at a dose of 1000 ppm of orange pell and cinnamon oils suggested that the 1000 ppm of these essential oils may be optimal dose for the growth of bacteria. According to Busquet *et al*., (2006) EOs can have a dose-dependent effect on the activity of mixed rumen microbial communities.

Among the EOs used in this investigation, the strongest antimicrobial effect against the rumen amylolytic bacteria of *Ruminobacter amylophilus* and *Selenomonas ruminantium* was observed in thyme oil. On the other hand, after a 1000 ppm treatment of orange peel oil, development accelerated in an unexpected and intriguing manner for *Selenomonas ruminantium*. The mechanism of the strong antimicrobial effect of carvacrol, found in the structure of thyme oil, versus gram (-) bacteria was mentioned while discussing the results of fibrolytic bacteria. In addition, the results of the study showed *Selenomonas ruminantium* bacteria to be more sensitive to EOs than *Ruminobacter amylophilus*. Contrary to our results, McIntosh *et al*., (2003) stated in their study that *Ruminobacter amylophilus* bacteria are more sensitive to EOs than *Selenomonas ruminantium* bacteria. Yet, they also observed *Ruminobacter amylophilus* to be stronger in terms of adaptability, which demonstrates the fact that the antimicrobial effects of EOs are shaped by the type of bacteria and the type of EOs. Other EOs that stood out in terms of antimicrobial effect after thyme oil were oleaster and laurel oils. Studies on the effects of these two oils on rumen bacteria are scarce, but there are studies investigating their antibacterial effect against pathogenic microorganisms (Khan *et al*., 2016; Tehranizadeh *et al*., 2016). Okmen & Turkcan, (2014) indicated in their study that oleaster extract is effective against pathogens which cause mastitis.

Along with all the discussions, Cobellis *et al*., (2016) claimed that the effect of plant extracts on rumen microorganisms and therefore on rumen fermentation is highly variable. Considering that the growth stage of plants, harvesting and storage conditions may change the structure of bioactive molecules in plants, similar comparisons of studies are questionable.

As a result, it is crucial to do additional research in order to establish the possibility for employing plant essential oils, which are safer than antibiotics, in ruminant animals.

Conclusion

This study shows that six EOs (garlic, cinnamon, laurel, thyme, oleaster and orange peel) have *In vitro* antimicrobial action versus rumen fibrolytic and amylolytic bacteria. Furthermore, the wide range of doses in the study showed more clearly that the dose of EOs significantly affects antimicrobial effect. The EO showing strong antibacterial effect on the fibrolytic bacteria *Clostrodium proteoclasticum* and two of the amylolytic bacteria (*Ruminobacter amylophilus* and *Selenomonas ruminantium*) was thyme. For the fibrolytic bacteria, *Butyrivibrio fibrisolvens* and *Ruminococcus albus,* oleaster was the EOs with the strongest antibacterial effect. The antibacterial effect of EOs was generally observed at 400 ppm and doses higher than 400 ppm. The EO that

positively affects the growth rates of both fibrolytic and amylolytic bacteria is orange peel; but this effect has varied after 1000 ppm except on *Selenomonas ruminantium* and *Butyrivibrio fibrisolvens*. In conclusion, our study revealed that the effects of EOs are shaped depending on the dose. In addition, the antibacterial effects of EOs vary greatly according to the type of bacteria. In the light of the results of current study, the oils and mixtures used could be tested on the rumen microbial population to determine the using potential in optimizing rumen fermentation. Further *In vitro* and subsequently in vivo research is needed to confirm our results, to determine the best doses of EOs, and to use the determined EOs and doses in ruminant nutrition.

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