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The effects of *Pinus brutia* bark extract on pure and mixed continuous cultures of rumen bacteria and archaea, and fermentation characteristics *in vitro*

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ABSTRACT

The aim of the study was to investigate the effects of *Pinus brutia* bark extract, which is rich in polyphenolic compounds of tannins, on both pure and mixed continuous cultures of rumen bacteria and archaea, as well as on rumen fermentation characteristics *in vitro*. Antimicrobial susceptibility assay with pure cultures was carried out in an anaerobic chamber. *Pinus brutia* bark extract exhibited a potential inhibitor activity ($P<0.05$) against pure cultures of *Ruminococcus flavefaciens*, *Eubacterium ruminantium*, and *Methanobacterium formicicum* while a growth stimulatory effect ($P<0.05$) was observed for *Ruminoccocus albus*, *Butyrivibrio fibrisolvens,* and *Streptococcus bovis. Pinus brutia* bark extract only had a potential inhibitor effect ($P<0.05$) on *R. albus* at the highest dose (1200 µg/mL). *Pinus brutia* bark extract also stimulated ($P<0.05$) the growth of pure cultures of *Fibrobacter succinogenes,* while it did not affect *Megasphaera elsdenii,* except at the highest dose. The effects of two doses (75 and 375 mg/L) of *P. brutia* bark extract on *in vitro* mixed cultures and rumen fermentation parameters were determined by the rumen simulation technique (Rusitec). Supplementation with *P. brutia* bark extract led to a quadratic decrease ($P<0.05$) in the cell numbers of *R. flavefaciens.* Production of total and individual short chain fatty acids (SCFA), acetate to propionate ratio (C2/C3), total protozoa, ruminal pH, and dry matter digestibility (DMD) did not change in the presence of *P. brutia* bark extract. Supplementation with both doses of *P. brutia* bark extract decreased ($P<0.05$) the ammonia-N concentrations. Ammonia-N concentration was lowest in the high-supplemented group ($P<0.05$). As a conclusion, inhibitory effects of *P. brutia* bark extract on some species in the pure cultures were in the same direction as with mixed ruminal cultures, while stimulatory effects disappeared. The lack of inhibitory effects on protozoa and on a large number of Gram-positive rumen bacteria in the mixed cultures suggests that its mechanism of action is not exactly similar to antibiotics. Although *P. brutia* bark extract did not alter ruminal SCFA, it could have potential to improve ruminal protein utilization without depressing rumen microbial fermentation.

Key words: *Pinus brutia* bark; rumen bacteria; anaerobic chamber; rumen fermentation; rusitec; tannins

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Introduction

Rumen harbors a diverse ecosystem, consisting of bacteria, protozoa, fungi, archaea, and viruses which have an important role in the physiology, performance characteristics and welfare level of the host organism (LOOR et al., 2016). Rumen microorganisms have the capability to break down indigestible feedstuffs and to convert them to short-chain fatty acids and microbial protein. Rumen microbial fermentation also has some disadvantages, such as excessive N excretion and methane emission, which reduce the efficiency of feed utilization, and also cause environmental pollution (CALSAMIGLIA et al., 2007). Antibiotic growth promotors have been used to alter inefficient microbial processes by selectively inhibiting certain groups of microbes, mainly Gram-positive rumen bacteria, protozoa (HOOK et al., 2009), and methanogenic archaea (KHORRAMI et al., 2015). However, antibiotic growth promotors have been banned in Europe since 2006 due to the concern about the appearance of resistant strains of human pathogenic bacteria (GOIRI et al., 2009). After the ban on antibiotic feed supplements, plant extracts and plant secondary metabolites have come forward as alternatives to antibiotics to improve animal productivity.

Pinus brutia Ten. (Turkish red pine*;* brutia pine) grows naturally in the Mediterranean, Aegean, and Black Sea regions of Turkey. This species covers 25% of the total Turkish forest area and therefore, its bark has major potential in the wood working industry as a waste material (UCAR et al., 2013). *Pinus brutia* bark is rich in antimicrobial phenolic compounds, *i.e.* flavonoids and phenolic acids which are particularly the constitutive units of tannins (KIVRAK et al., 2013; UCAR et al., 2013). Resin present in the bark of plants is used for medicinal purposes, for instance, to treat abscesses or relieve pain (SEZİK et al., 2001). Previous research has shown that extracts of *P. brutia* bark has antimicrobial activity against Gram-positive bacteria such as *Bacillus cereus, B. subtilis, B. megaterium, Staphylococcus aureus,* and *Mycobacterium smegmatis,* while it was ineffective against Gram-negative species, *Escherichia coli* and *Klebsiella pneumoniae* (DIĞRAK et al., 1999). However, the influences of *P. brutia* bark extract on rumen microorganisms and rumen fermentation have not been previously reported.

Determining the antimicrobial spectrum and the optimal dose are the major challenges in the rumen studies with plant extracts and plant secondary metabolites. Nonspecific and strong antimicrobial activity can depress ruminal fermentation overall, especially in high doses (BODAS et al., 2012). Therefore, this study aimed to investigate the effects of *P. brutia* bark extract on both pure and mixed continuous cultures of Gram-positive and Gram-negative rumen bacteria and rumen archaea, as well as on rumen fermentation characteristics *in vitro*.

Materials and methods

Plant extract. The extract of *P. brutia* bark was provided by the Kale Naturel Herbal Products Company, Ltd., Balikesir, Turkey. As specified by the manufacturer, bark samples were air dried, ground in a mill, and screened. *Pinus brutia* bark (powder) was extracted with distilled water (1/10, w/v) at 55 °C for 6 h and filtered to give a homogenous liquid. The extract was reduced to 1/5 of its volume using a rotary vacuum evaporator, and dried in a laboratory scale spray-dryer.

Table 1. Phenolic compounds of *P. brutia* bark extract

Phenolic compounds	μg/g
Gallic acid	2.2
Protocatechuic acid	1.4
Catechin	6.4
P-hydroxy benzoic acid	0.9
Caffeic acid	1.2
Epicatechin	5.8
Vanilin	0.4
P-coumaric acid	0.2
Ferulic acid	0.2
Quercetin	17.7
Luteolin	0.2
Kaempferol	0.2
Apigenin	0.3

Analyses of phenolic compounds of P. brutia bark extract. Phenolic compounds (Table 1)

of *P. brutia* bark extract were quantified using a high-performance liquid chromatography (HPLC) (Shimadzu, Japan) device equipped with a photodiode array detector. An Agilent Eclipse XDB-C18 (250 × 4.60 mm) 5 µm column at 30ºC and 0.8 mL/min flow speed was used.

Antimicrobial susceptibility assay with pure cultures.

Culture of bacterial and archaeal strains in anaerobic medium. Ruminococcus flavefaciens (ATCC 49949), *Ruminococcus albus* (ATCC 27210), *Eubacterium ruminantium* (ATCC 17233), *Butyrivibrio fibrisolvens* (ATCC 19171), and *Streptococcus bovis* (ATCC 33317) were the Gram-positive bacteria species used in the tests. A mesophilic, methanogenic archaeon, *Methanobacterium formicicum* (ATCC 33274), was used as a methane producer. *Megasphaera elsdenii* (ATCC 25940) and *Fibrobacter succinogenes* (ATCC 19169) were the Gram-negative bacterial species tested. The anaerobic medium for bacterial and archaeal cultures was prepared under CO_2 as previously reported (ORPIN, 1976). Bacterial and archaeal strains were grown at 37 °C for 24-72 h under strictly anaerobic conditions (80% nitrogen, 10% carbon dioxide, 10% hydrogen) in an anaerobic chamber (Whitley DG250, Don Whitley, West Yorkshire, UK).

Broth microdilution method. The antimicrobial activity assays of *P. brutia* bark extract were carried out using a broth microdilution method, following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016) in the anaerobic chamber. A stock solution of *P. brutia* bark extract (100 mg/mL) was prepared by dissolving the extract in 50% (v/v) ethanol. Serial 2-fold dilutions of the extract (1200, 600, 300, 150, 75, 37.5, 18.8, 9.4, 4.7, and 2.3 µg/mL) were made from the stock solution in the growth medium. For broth microdilution, 200 µL of each dilution was distributed over a 96-well plate (Corning 3599, Flat bottom, USA), and 20 µL of inoculum compromising 4×10^{10} cell/mL of the overnight culture, were added into each well. Each strain was tested in triplicate wells. At the same time, negative control wells without extract, and media control wells without microorganisms, were maintained for each set. The plates were incubated at 37 °C for 24 h in the anaerobic chamber. Microbial growth was detected with a microplate reader at 600 nm (Epoch, BioTek, USA). A significantly lower OD600 value compared to the control dose (0 µg/mL) was accepted as potential antimicrobial activity (KO et al., 2018) while a significantly higher value was accepted as a stimulatory effect (DAS et al., 2015).

In vitro testing with mixed continuous cultures

Experimental procedure. Rumen simulation technique (Rusitec) apparatus, a semi-continuous culture system, was used to simulate the rumen environment in the laboratory (CZERKAWSKI and BRECKENRIDGE, 1977). The system consisted of nine airtight vessels with 750 mL volume each. Vessels were immersed in a water bath maintained at 39 °C. Rumen content from a freshly slaughtered healthy two-year-old Brown Swiss bull with 500 kg mean body weight, was obtained from a commercial slaughter facility as the vessel inoculum. The rumen content was transported in warm (39 °C) thermos bottles to the Rusitec system within 30 min. On the first day of the experiment, each vessel was filled with 750 mL of strained rumen liquor. Solid ruminal content (80 g) was weighed into a nylon bag (150 µm pore size; 80 × 120 mm), which was then placed inside the feed container in each vessel, together with a bag of experimental feed (4 g barley straw and 6 g commercial concentrate). According to the information obtained from the owner, the animal had been fed a diet (12 kg DM/day) consisting of 40% barley straw and 60% commercial vitamin and mineral supplemented concentrate for growing cattle. The same diet was also used for *in vitro* incubation trials (Table 2).

Table 2. Ingredients of the experimental diet used in the Rusitec as fed basis

Nutrients, %	Concentrate	Barley straw
Dry matter	93.41	93.40
Ash	8.96	11.48
Crude fiber	5.70	35.11
Crude protein	13.90	3.37
Ether extract	4.77	2.13
Organic matter	84.45	81.92
Nitrogen-free extract	60.08	41.31
Metabolizable energy (MJ/kg)	11.67	6.78

The commercial concentrate consisted of corn, wheat bran, corn gluten feed, molasses, sunflower seed meal, barley, corn dried distillers grains, soya bean meal, vinasse, vegetable oil, calcium carbonate, sodium chloride, and a vitamin-mineral premix. On subsequent days, the feed bag that had remained 48 hours in each vessel was replaced by a new bag of feed. Fermentation vessels received a continuous infusion of a buffer (pH 7.4) (DEMIRTAS et al., 2020) at a rate of 750 mL/day.

Experimental design. The incubation trial consisted of a 6-day adaptation period (Day 1-6) followed by a 6-day collection period (Day 7-12). The trial was conducted as a completely randomized design (CRD) with three treatments and three replicates per treatment. The extract was used at 75 mg/L (low dose) and 375 mg/L (high dose) according to the recommendations for *in vitro* screening studies (CALSAMIGLIA et al., 2007). (These doses correspond to 75 and 375 μg/mL in comparison with the doses used in the pure culture assays in the present study). The treatments consisted of no additives (control), 75 mg/L, and 375 mg/L of *P. brutia* bark extract. Dried extract of *P. brutia* bark was added directly to the fermentation vessels.

Sample collection and analyses. Analyses of the dry matter (DM), crude protein (CP), crude fiber (CF), ash, ether extract (EE) and organic matter contents of the experimental diets (Table 2) were performed according to the procedure of the AOAC (Association of Official Analytical Chemists) (2000). Nitrogen-free extract (NFE) was calculated as follows: NFE% = DM-(CP+CF+EE+Ash) (VAN SOEST, 1982). Metabolizable energy was determined according to the Turkish Standards Institute (TSE, 1991) method.

The pH values of ruminal fluids in each fermentation vessel were measured daily during feed bag exchange using an epoxy body pH electrode (WD-35801-00, Oakton, USA) connected to a pH-meter (Ion 6, Acorn series, Oakton, USA).

Samples for SCFA and ammonia-N analyses were taken from the overflow flasks of the Rusitec which were placed into ice to prevent microbial activity and to preserve the fermentation products. Before keeping at -20 °C, 90 μL of H_2SO_4 (12N) was added to 5 mL of samples for SCFA analyses. The SCFA were measured by HPLC (Dionex Summit P680, ASI100, USA) as described previously (DEMIRTAS et al., 2019). Daily production of SCFA was calculated by multiplying the concentrations by the volume of effluent accumulated. Ammonia-N concentration was detected colorimetrically using the indophenol blue method, and absorbance was measured at 546 nm with a spectrophotometer (UV-150-02, Shimadzu, Japan) (CHANEY and MARBACH, 1962).

For protozoa counting, 1 mL of rumen fluid sample taken from the fermentation vessel was mixed with 1 mL of a solution of 0.6 g methyl green, 8 g NaCl, and 100 mL formaldehyde (37%) filled up to 1000 mL with distilled water. Total numbers of protozoa were determined with a counting chamber (Fuchs-Rosenthal: 0.2 mm deep; 0.0625 mm^2; Marienfeld, Germany) using a light microscope (Leica CME, USA).

After the 48 hour fermentation, the feed bags removed from the fermentation vessels were washed by squeezing gently in nylon bags containing 50 mL of buffer solution. The residual buffer in the nylon bags was transferred back to the fermenter to ensure transfer of solid-phase-associated microorganisms. The feed bags were dried at 65 °C for 48 h. The dry matter digestibility at 48 h was calculated from the difference between the original dry matter sample weight and the dry matter residue weight, divided by the original sample weight. This value was then multiplied by 100 to calculate the percentage digestibility of the dry matter (DEMİRTAŞ and PİŞKİN, 2020).

DNA extraction and quantitative Real-Time PCR (qRT-PCR) assay. Samples for DNA extraction were collected from the fermentation vessels on the 5th day of the collection period for rumen bacterial and archaeal profiling. Samples were taken from the fermentation vessels at the time of the feed bag exchange, therefore containing both planktonic and solid-phase-associated microorganisms (WATANABE et al., 2010). The samples were immediately placed in liquid nitrogen and stored at -20 °C until processing. The samples were thawed and centrifuged at 10,000 rpm for 10 min at room temperature. Pellets were resuspended in 2×phosphate-buffered saline (KHAFIPOUR et al., 2009). Total DNA extraction from the pellets was performed with an E.Z.N.A.™ stool DNA extraction kit (Omega Bio-Tek, USA).

Table 3. Primers used in the qRT-PCR assay

Target species	Forward primer (5′-3′)	Reverse primer (5′-3′)	References
Total bacteria (16S rRNA)	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	Denman and McSweeney, (2006)
Methanogenic archaea (*mcrA*)	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	Denman et al., (2007)
HAP bacteria (16S rDNA)	GAGTTTGATCCTGGCTCAG	AAGGAGGTGATCCAGCC	Attwood et al., (1998)
R. albus (16S rDNA)	CAAAACCCTAAAAGCAGTCT-TAGTTCG	GACGGGCGGTGTGTACAAG	Li et al., (2014)
R. flavefaciens (16S rRNA)	C G A A C G G A G A T A A T T T-GAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGG-TATTACC	Denman and McSweeney, (2006)
B. fibrisolvens (16S rDNA)	ACACACCGCCCGTCACA	TCCTTACGGTTGGGTCACAGA	Klieve et al., (2003)
S. bovis (16S rDNA)	CTAATACCGCATAACAGCAT	AGAAACTTCCTATCTCTAGG	Tajima et al., (2001)
F. succinogenes (16S rRNA)	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	Denman and McSweeney, (2006)
M. elsdenii (16S rDNA)	GACCGAAACTGCGATGCTAGA	CGCCTCAGCGTCAGTTGTC	Ouwerkerk et al., (2002)
S. ruminantium (16S rDNA)	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	Tajima et al., (2001)

qRT-PCR: Quantitative Real-Time PCR; HAP bacteria: Hyper-ammonia producing bacteria

Purity and concentration of DNA were measured spectrophotometrically at A260/280 with the Take3 plate of a microplate reader (Epoch, BioTek, USA).

The quantification of representative rumen microorganisms was done with SYBRGreen based qRT-PCR assay using specific primers (Table 3) in a LightCycler 480II Real-Time PCR machine (Roche, Germany), as reported previously (DEMIRTAS et al., 2019). Melting curve analysis demonstrated that each of the primer pairs amplified a single product. The microbial numbers were calculated using the calibration curves, which were obtained according to the Cp values of known concentrations of the reference microorganisms for the respective target (JIAO et al., 2013).

Statistical analyses. A one-way ANOVA test was used for evaluation of the data from the antimicrobial assay, followed by Dunnet's test for comparison between the control and treatments. Each well of the 96-well plate was considered as an experimental unit.

A repeated analysis of variance (ANOVA) test was conducted using the SigmaStat Program (version 3.1, Systat Software, Erkrath, Germany) to analyze the data on the rumen fermentation characteristics. The individual fermenters were used as the experimental units. Treatments, time, and their interaction were considered as fixed effects, and fermenters as random effects. Post hoc multiple comparisons between means was conducted using the Duncan test.

Polynomial regression with sequential analysis of variance was used to assess the linear and quadratic effects of *P. brutia* bark extract dosage on rumen bacterial and archaeal populations in the Rusitec, determined by qRT-PCR.

Data on protozoa, bacteria and archaea were transformed by Log_{10} before variance analysis. The differences were considered significant at $P \leq 0.05$ for all data analyses.

Results

Antimicrobial susceptibility of pure cultures. Effects of *P. brutia* bark extract on pure cultures of bacterial and archaeal strains are presented in Fig. 1 and Fig. 2. *Pinus brutia* bark extract exhibited potential antimicrobial activity (P<0.05) on *R. flavefaciens* at all used doses. *Pinus brutia* bark extract also showed potential antimicrobial activity (P<0.05) on *E. ruminantium* and *M. formicicum* at doses starting from 18.8 and 37.5 µg/mL, respectively. *Pinus brutia* bark extract, on the other hand, showed a growth stimulatory effect (P<0.05) on *S. bovis* and *B. fibrisolvens* at doses starting from 2.3 and 9.4 µg/mL, respectively. The growth stimulatory activity of *P. brutia* bark extract on *S. bovis* was most obvious at the highest dose. The growth of *R. albus* was also promoted (P<0.05) by *P. brutia* bark extract at doses of 2.3-600 µg/mL, while potential antimicrobial activity was observed at 1200 µg/mL (P<0.05). *Pinus brutia* bark extract did not have any significant effect on *M. elsdenii* except at the highest dose, at which it had potential antimicrobial activity (P<0.05). On the other hand, it exhibited a growth stimulatory effect (P<0.05) on *F. succinogenes*, the other Gram-negative bacterium, at a dose starting from 4.7 µg/mL.

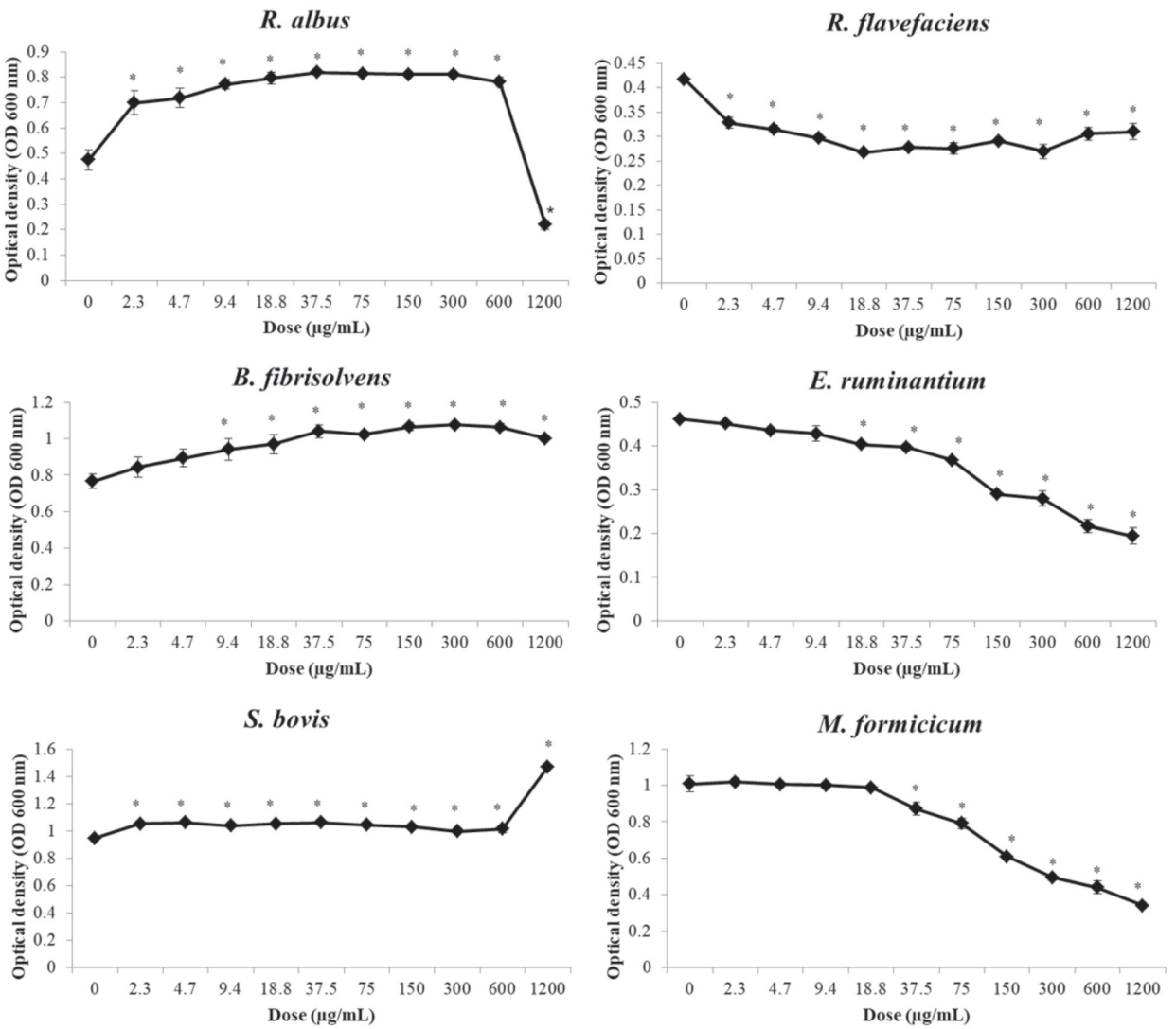

Fig. 1. Effects of *P. brutia* bark extract on pure cultures of Gram-positive rumen bacteria and methanogenic archaeon *(M. formicicum)* by the broth microdilution method. The results represent the mean ± standard error. *P<0.05, difference of *P. brutia* bark extract-treated culture compared with the control.

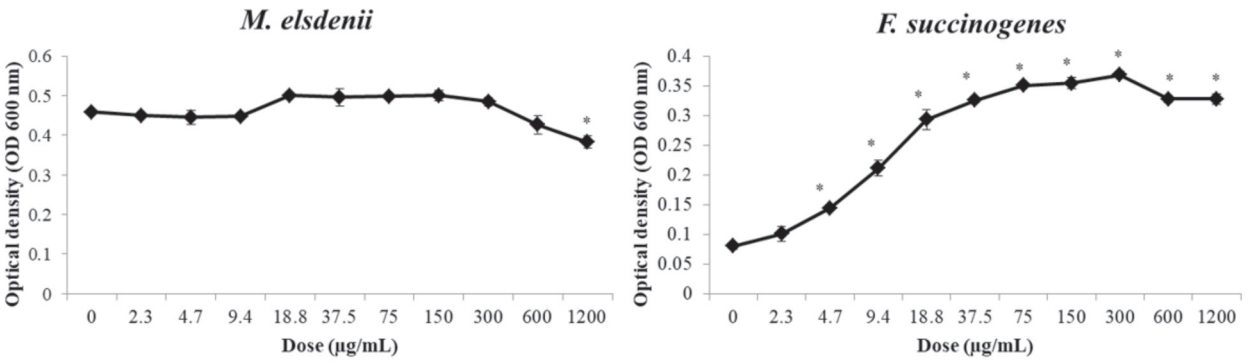

Fig. 2. Effects of *P. brutia* bark extract on pure cultures of Gram-negative rumen bacteria by the broth microdilution method. The results represent the mean ± standard error. *P<0.05, difference of *P. brutia* bark extract-treated culture compared with the control.

Table 4. Effects of two doses (mg/L) of *P. brutia* bark extract on rumen fermentation characteristics in the mixed continuous Rusitec cultures

	Treatments				P values		
Parameters	Control	*P. brutia*-75	*P. brutia*-375	SEM	Treatment	Time	T × T
Ruminal pH	6.65	6.63	6.61	0.01	0.102	0.002	0.381
DMD (%)	49.51	52.17	50.35	1.66	0.547	<0.001	0.272
Protozoa (log_{10}/mL)	3.52	3.81	3.77	0.078	0.076	0.001	0.711
Ammonia-N (mmol/L)	6.42a	5.79b	5.00c	0.13	<0.001	0.008	0.608
Total SCFA (mmol/d)	36.18	38.31	36.55	1.02	0.354	0.383	0.409
Individual SCFA (mmol/d)							
Acetate	18.34	19.80	19.27	0.47	0.167	0.412	0.757
Propionate	8.82	9.43	8.82	0.46	0.588	0.540	0.259
Butyrate	5.29	5.44	5.06	0.20	0.448	<0.001	0.068
Isobutyrate	0.30	0.31	0.28	0.03	0.829	0.155	0.635
Isovalerate	1.11	1.08	1.02	0.05	0.434	0.217	0.263
Valerate	2.33	2.26	2.11	0.10	0.328	0.040	0.123
C2/C3	2.10	2.10	2.28	0.10	0.375	0.170	0.513

a-c: Means in the same row followed by different superscripts differ significantly (P<0.05). SCFA: Short chain fatty acids; C2/C3: acetate to propionate ratio; DMD: Dry matter digestibility; T × T: Treatment × Time interaction.

Rumen fermentation characteristics. The effects of two doses (75 and 375 mg/L) of *P. brutia* bark extract on rumen fermentation characteristics in the mixed continuous Rusitec cultures are presented in Table 4. Supplementation with both doses of *P. brutia* bark extract decreased (P<0.05) the ammonia-N concentrations. Ammonia-N concentration was lowest in the high-supplemented group (P<0.05). Ruminal pH, DMD, total protozoa, production of total and individual SCFA, and C2/C3 did not change in the presence of *P. brutia* bark extract.

Rumen bacterial and archaeal populations assessed by qRT-PCR. The effects of two doses (75 and 375 mg/L) of *P. brutia* bark extract on rumen bacterial and archaeal populations in the Rusitec are shown in Table 5.

Supplementation with *P. brutia* bark extract led to a quadratic decrease (P<0.05) in the cell numbers of *R. flavefaciens. Pinus brutia* bark extract had no effect on the abundance of other microbial populations in the Rusitec.

Table 5. Effects of two doses (mg/L) of *P. brutia* bark extract on rumen bacterial and archaeal populations in the Rusitec determined by qRT-PCR[1]

	Treatments			P values	
Microorganism (log_{10}/mL)	Control	*P. brutia*-75	*P. brutia*-375	Linear	Quadratic
Methanogenic archaea	4.46 ± 0.15	4.41 ± 0.09	4.21 ± 0.07	0.086	0.979
Total bacteria	9.41 ± 0.07	9.34 ± 0.06	9.37 ± 0.03	0.872	0.369
Gram-positives					
HAP bacteria	5.05 ± 0.24	4.79 ± 0.23	4.69 ± 0.20	0.337	0.525
R. albus	6.70 ± 0.10	6.60 ± 0.07	6.65 ± 0.06	0.909	0.361
R. flavefaciens	0.28 ± 0.07a	0.07 ± 0.02b	0.09 ± 0.06b	0.159	0.030
B. fibrisolvens	5.02 ± 0.09	4.99 ± 0.13	5.07 ± 0.12	0.681	0.782
S. bovis	3.46 ± 0.43	3.11 ± 0.41	4.18 ± 0.11	0.074	0.289
Gram-negatives					
F. succinogenes	4.90 ± 0.26	4.74 ± 0.25	5.03 ± 0.22	0.541	0.561
M. elsdenii	7.47 ± 0.12	7.52 ± 0.07	7.43 ± 0.07	0.647	0.652
S. ruminantium	6.15 ± 0.76	5.97 ± 0.94	6.60 ± 0.43	0.574	0.783

[1] Values are means ± SEM. a, b: The different letters in the same row indicate statistical difference (P<0.05) between the means of treatments. qRT-PCR: Quantitative Real-Time PCR. HAP bacteria: Hyper-ammonia producing bacteria

Discussion

Manipulating rumen microbial populations by using antimicrobial feed additives has proved to be a favorable strategy to maximize production efficiency in ruminants. The selective activity of antimicrobial agents on microorganisms is critical in order not to suppress ruminal fermentation completely (BODAS et al., 2012). In the present study, the effects of *P. brutia* bark extract were investigated on both pure and mixed continuous cultures of some rumen microorganisms, to obtain a more detailed conclusion regarding the spectrum of antimicrobial action.

The *P. brutia* bark extract used in the present study contained several polyphenolic compounds, such as gallic acid, protocatechuic acid, cafeic acid, quercetin, catechin, and epicatechin (Table 1). The phenolic composition of *P. brutia* bark extract was in accordance with previous reports (KIVRAK et al., 2013; UCAR et al., 2013). Phenolic acids and flavonoids determined in the *P. brutia* bark extract were generally identified as constitutive units of hydrolysable and condensed tannins (MCSWEENEY et al., 2001). Tannins are plant phenolics synthesized in plant parts, such as the bark, wood, roots, seeds, buds, leaves, and floral parts (BECHTOLD and MUSSAK, 2009). An extract of Turkish red pine bark was reported to contain 34% tannin (BAYSAL et al., 2003).

In the present study, *R. flavefaciens* was the most susceptible bacterium to *P. brutia* bark extract, in both pure and mixed culture assays. Furthermore, *P. brutia* bark extract had potential to inhibit pure cultures of *E. ruminantium* among Gram-positive rumen bacteria and *M. formicicum* as a methanogenic archaeon. To our knowledge,

there is no literature to date on the effects of *P. brutia* bark extract on rumen microorganisms. However, similar to our results, pure cultures of *R. flavefaciens* and *E. ruminantium* were also included in sensitive groups to Ginkgo extract, which is rich in polyphenols (OH et al., 2017). *Ruminococcus flavefaciens* was reported to be sensitive to cashew nut shell liquid, another phenolic-rich compound, both on pure cultures and in Rusitec experiments (WATANABE et al., 2010), as in the present study. Total populations of methanogenic archaea in Rusitec fermenters supplemented with *P. brutia* bark extract also tended to decrease linearly (P = 0.086) in accordance with the pure culture experiments in the present study. The main flavonoid present in the *P. brutia* bark extract was quercetin (Table 1). OSKOUEIAN et al. (2013) reported that quercetin significantly suppressed *in vitro* populations of total methanogens in the rumen. The flavonoids generally act against microorganisms by inhibiting cytoplasmic membrane function, inhibiting microbial cell wall synthesis, or inhibiting nucleic acid synthesis (CUSHNIE and LAMB, 2005).

On the other hand, *P. brutia* bark extract stimulated the growth of some acetate, formate, butyrate, and lactate producing Gram-positive rumen bacteria, such as *B. fibrisolvens, R. albus,* and *S. bovis* in the pure culture experiments. A decrease in *R. albus* density was observed at the highest dose, while the increase in *S. bovis* was more prominent at the same dose. *Streptococcus bovis* was also one of the most resistant species among pure cultures of rumen bacteria to aldehydes from green leaf volatiles in a previous study (DEMIRTAS et al., 2019). Many studies have revealed that phenolic compounds can interact with microorganisms in a positive, as well as in a negative way, especially in anaerobic environments (DEMİRTAŞ and PİŞKİN, 2020; BROUDISCOU and LASSALAS, 2000). Rumen bacteria can degrade many polyphenols, particularly constituents of tannins such as gallic acid, pyrogallol, phloroglucinol, and quercetin, to overcome the inhibitory effects of these compounds, and use the end products as carbon and energy sources (BHAT et al., 1998). CHESSON et al. (1982) reported that cellulolytic strains of rumen bacteria showed a considerable ability to hydrogenate *trans-p*-coumaric acid and *trans*-ferulic acids, with *Ruminococcus* spp. proving the most effective. The authors suggested that hydrogenation may serve to protect cellulolytic strains against the toxic effects of these phenolic compounds. TZOUNIS et al. (2008) indicated that the dietary polyphenols (+)-catechin and (-)-epicatechin can be utilized by beneficial fecal bacteria even in the presence of favorable carbon sources, such as sucrose and fructo-oligosaccharides. Quercetin, epicatechin, catechin, gallic acid, caffeic acid, and tannic acid did not inhibit probiotic *L. acidophilus*. Furthermore quercetin tended to stimulate the growth of *L. acidophilus* (HERVERT-HERNÁNDEZ et al., 2009).

Succinate and propionate producing Gram-negative rumen bacteria, *M. elsdenii* and *F. succinogenes,* were generally resistant to *P. brutia* bark extract in both pure cultures and mixed continuous cultures. Pure cultures of *M. elsdenii* were also previously reported to be insensitive to the phenolic-rich extracts (OH et al., 2017; WATANABE et al., 2010). The growth of *F. succinogenes,* on the other hand, was stimulated at all doses in the pure cultures. This effect, however, disappeared in the mixed continuous cultures (Rusitec). The same conflicting results between pure cultures and mixed cultures were also obtained for *R. albus* and *B. fibrisolvens* in the present study. The mechanism responsible for this effect could mainly be the competition for substrate utilization in the mixed ruminal cultures. Hence, the amount of end products from the degradation of the phenolic compounds may be insufficient to stimulate bacterial growth in the mixed cultures, in contrast to the pure cultures.

Pinus brutia bark extract supplementation did not affect the production of total and individual SCFA in the Rusitec. The abundance of bacterial species which are related to the production of SCFA also did not change, except for acetate and formate producing *R. flavevaciens.* Although the abundance of *R. flavevaciens* was depressed by *P. brutia* bark extract supplementation, acetate, valerate, and isovalerate production and DMD were not affected. This suggests that some other species which contribute to fiber digestion, such as *Prevotella* (STEVENSON and WEIMER, 2007)

and/or uncharacterized fibrolytic bacteria replaced the suppressed bacteria, and potentially occupied this niche. The abundance of total bacteria also did not change in the Rusitec. The fact that the *P. brutia* bark extract does not affect total SCFA production and DMD indicated that it did not suppress ruminal fermentation. This is one of the expected properties of feed additives to be used for modification of ruminal fermentation (COBELLIS et al., 2016; BODAS et al., 2012).

Pinus brutia bark extract had remarkable effects on ruminal ammonia-N concentration in the present study. Ammonia-N concentrations declined by 9.8% and 22.1% in the presence of 75 and 375 mg/L *P. brutia* bark extract, respectively. A decrease in ruminal ammonia is preferred to improve feed N economy if the ammonia level is higher than the critical level for maintaining microbial protein synthesis (5 mg of N/dL) (GRISWOLD et al., 2003). The ammonia-N levels were 8.1 mg/dL and 7 mg/dL in the fermentation vessels supplemented with low and high doses of *P. brutia* bark extract, respectively. These levels were sufficient for microbial growth in the Rusitec. Inhibition of HAP bacteria and/or protozoa which contribute significantly to protein degradation in the rumen generally lowers the ruminal ammonia-N level (DEMIRTAS et al., 2019). However, the abundance of HAP bacteria and protozoa was not decreased by the *P. brutia* bark extract. The *P. brutia* bark extract used in this study contained several polyphenolics which are constitutive units of hydrolysable and condensed tannins, as mentioned before. It is well established that multiple phenolic hydroxyl groups of tannins bind proteins under the rumen pH conditions, and prevent the excessive degradation of proteins by microorganisms (BHATTA et al., 2015). This will increase protein availability/absorption in the small intestine (MCSWEENEY et al., 2001). WISCHER et al. (2013) reported that tannin-rich extracts from several plants and four tannin monomers reduced the ammonia-N accumulation in the Rusitec, as observed in this study. However, degradation of organic matter and dry matter was also reduced by tannin supplementation in that study. Tannins may reduce fibre digestion by complexing with lignocellulose and preventing microbial digestion, or by directly inhibiting cellulolytic microorganisms, or both (MCSWEENEY et al., 2001). The decrease in ammonia-N concentration without an adverse effect on DMD in the present study can be considered as positive in terms of the efficiency of rumen fermentation.

Conclusions

Pinus brutia bark extract exhibited potential inhibitor activity against pure cultures of some acetate- and formate-producing Gram-positive bacteria and methane producing archaeon. This effect was similar for some species in the mixed cultures. A growth stimulatory effect was also observed for some Gram-positive and Gram-negative bacteria. However, this effect disappeared in the mixed continuous cultures, probably because of the competition for substrate. *Pinus brutia* bark extract was also not effective enough to change SCFA profile in the Rusitec. Nevertheless, it decreased ruminal ammonia without depressing rumen microbial fermentation at the doses received. Therefore, *P. brutia* bark extract could have the potential to improve ruminal protein utilization. On the other hand, the fact that the extract had no inhibitory effect on protozoa and on many of Gram-positive rumen bacteria in the mixed cultures suggests that its mechanism of action is not exactly similar to antibiotics. The effects of higher doses of *P. brutia* bark extract can be investigated in future studies, and *in vivo* trials are required to validate its efficiency as a feed additive.

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SAŽETAK

Cilj ovog rada bio je istražiti učinak ekstrakta kore brucijskog bora (*Pinus brutia*), koji je bogat polifenolnim sastojcima tanina, na čiste i mješovite kulture buražnih bakterija i arheja kao i na *in vitro* fermentacijske značajke buraga. Proveden je test antimikrobne osjetljivosti s čistim kulturama u anaerobnim uvjetima. Ekstrakt kore brucijskog bora pokazao je potencijalnu inhibitornu aktivnost ($P < 0,05$) protiv čistih kultura bakterija *Ruminococcus flavefaciens, Eubacterium ruminantium* i *Methanobacterium formicicum*, a stimulacijski učinak na rast ($P < 0,05$) opažen je za bakterije *Ruminoccocus albus, Butyrivibrio fibrisolvens* i *Streptococcus bovis*. Ekstrakt kore brucijskog bora imao je potencijalan inhibitorni učinak ($P < 0,05$) na *R. albus* samo u najvećoj dozi (1200 µg/mL). Također je imao stimulacijski učinak ($P < 0,05$) na čiste kulture *Fibrobacter succinogenes*, a nije utjecao na *Megasphaera elsdenii* osim u najvećoj dozi. Učinak dviju doza (75 i 375 mg/L) ekstrakta kore brucijskog bora na *in vitro* mješovite culture i pokazatelje fermentacije u buragu određen je simulacijskom tehnikom (Rusitec). Dodatak ekstrakta kore brucijskog bora doveo je do kvadratnog smanjenja ($P < 0,05$) broja stanica *R. flavefaciens*. Nije bilo promjena u proizvodnji ukupnih i pojedinačnih kratkolančanih masnih kiselina (SCFA), omjeru acetata i propionata (C2/C3), ukupnom broju protozoa, buražnom pH i probavljivosti suhe tvari (DMD). Suplementacija objema dozama ekstrakta kore brucijskog bora smanjila je ($P < 0,05$) koncentracije amonijaka-N. Koncentracija amonijaka-N bila je najniža u skupini s najvećom dozom suplementa ($P < 0,05$). Zaključujemo da je inhibitorni učinak ekstrakta kore brucijskog bora na neke vrste u čistim kulturama bio jednak onomu u mješovitim buražnim kulturama, a nije bilo stimulacijskog efekta. Manjak inhibitornih učinaka na protozoe i na mnoge Gram-pozitivne buražne bakterije u mješovitim kulturama upućuje na to da njihov mehanizam djelovanja nije jednak onomu kod antibiotika. Premda ekstrakt kore brucijskog bora nije promijenio buražni SCFA, mogao bi poboljšati iskorištavanje proteina u buragu a da pritom ne suprimira mikrobnu fermentaciju.

Ključne riječi: kora brucijskog bora; buražne bakterije; anaerobni uvjeti; buražna fermentacija; rusitec; tanini