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Research Article

Sensitivity of pure cultures of some Gram-positive and Gram-negative rumen bacteria to sigla storax (*Liquidambar orientalis*)

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ABSTRACT

Extracted from the wounded bark of the *Liquidambar orientalis* tree, sigla storax is a semi-viscous, balsamic resin. This study aimed to evaluate the effects of sigla storax on the growth of pure cultures of select Gram-positive and Gram-negative rumen bacteria, thereby elucidating its potential mode of action on rumen metabolism as an alternative antibiotic feed additive. Under strictly anaerobic conditions, the antimicrobial activity of sigla storax was assessed using the broth microdilution method. With the exception of *Streptococcus bovis*, storax demonstrated potential antimicrobial activity on all bacteria at doses starting from 1-2 mg/ml ($P<0.05$). The most susceptible bacterium was *Ruminococcus flavefaciens*, which was completely inhibited at 4 mg/ml sigla, while the most resistant was *S. bovis*, which showed no complete inhibition. For other Gram-positive bacteria, the minimum inhibitory concentration (MIC) varied: 16 mg/ml for *Butyrivibrio fibrisolvens*, and 32 mg/ml for *Ruminococcus albus*, *Eubacterium ruminantium*, and *Methanobacterium formicicum*. Interestingly, at lower doses, sigla storax exhibited a growth-stimulating effect on *E. ruminantium* (0.06-0.125 mg/ml) and *S. bovis* (0.125-2 mg/ml) ($P<0.05$). The Gram-negative *Megasphaera elsdenii* also showed a slight stimulatory response to sigla storax at concentrations of 0.06-0.5 mg/ml ($P<0.05$). However, at 32 mg/ml, sigla storax completely inhibited both Gram-negative bacteria tested: *M. elsdenii* and *Fibrobacter succinogenes*. While Gram-positive bacteria generally exhibited higher sensitivity to sigla storax compared to Gram-negative bacteria, the study concluded that its mechanism of action differs from typical antibiotic feed additives. This distinction is due to sigla storax's antibacterial activity against Gram-negative bacteria and its stimulatory effects on certain Gram-positive bacteria.

INTRODUCTION

The rumen hosts one of the most diverse and densely populated microbial ecosystems known. A symbiotic relationship has evolved between ruminant animals and their ruminal microbes. In this mutually beneficial arrangement, the ruminant provides a hospitable environment within the rumen, while the microorganisms supply the animal with essential energy and nitrogen sources in the form of volatile fatty acids and microbial protein. Approximately half of the rumen's microbial population consists of bacteria (Genzebu and Tesfay, 2015).

Over recent decades, enhancing rumen fermentation efficiency through bacterial modification has been a primary research focus. This emphasis stems from the fact that Gram-positive rumen bacteria produce higher quantities of hydrogen, formate, lactate, and ammonia compared to their Gram-negative counterparts, resulting in energy and protein losses (Castillejos et al., 2007). Hydrogen released by acetate and butyrate-producing Gram-positive bacteria is used for methane synthesis by methanogenic archaea. On the other hand, propionate, which is produced by mostly Gram-negative bacteria, consumes reducing equivalents for methanogenesis (Bharanidharan et al., 2021; Knapp et al. 2014). Methane is ultimately eliminated by belching, which is widely reported to represent a loss of feed energy of up to 2-15% (Martin et al., 2021). Enteric methane is also a greenhouse gas responsible for 15-24% of global warming (Steinfeld et al., 2006). Ionophore antibiotics such as monensin specifically target Gram-positive hydrogen producers and alter the microbiota in favour of Gram-negative propionate producers (Callaway et al. 2003). In cattle treated with monensin, methane production is reduced by up to 30% as a result of a shift in fermentation towards propionate production (Callaway et al., 2003; Wedegaertner and Johnson, 1983). Monensin also limits lactate production by inhibiting lactate-producing bacteria such as Gram-positive *Streptococcus bovis*, which is often involved in the development of rumen acidosis. Accordingly, cattle fed monensin had lower lactate concentrations and higher ruminal pH (Russell and Strobel, 1989). Reducing nitrogen losses by restraining deamination and proteolysis by Gram-positive ammonia producers and protozoa is another favourable effect of monensin on rumen fermentation (Russell and Strobel, 1989). Thus, ionophoric antibiotics have been employed as feed additives for years to modify rumen microbiota and rumen fermentation to improve animal productivity (Thompson et al., 2021). However, following the prohibition of antibiotics as feed additives (OJEU 2003), researchers have intensively explored the use of natural, plant-derived antibacterial agents to modulate rumen microorganisms and fermentation processes (Stefańska et al., 2021). This shift in focus represents an ongoing effort to optimize rumen function while adhering to current regulatory

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standards and addressing concerns about antibiotic resistance. Neverthless, the non-specific, broad-spectrum effect of most phytochemicals on rumen bacteria, which may depress rumen fermentation, is one of the major challenges in rumen studies (Bodas et al., 2012; Demirtas et al., 2021).

Commonly known as "sigla oil," sigla storax (Styrax liquidus) is a semi-liquid, balsam-like resin extracted from the wounded bark of the *Liquidambar orientalis* tree (Kılınç et al., 2020). This endemic member of the Hamamelidaceae family is native to Turkey's southwestern regions, particularly around Köyceğiz, Ula, Marmaris, and Fethiye. In Anatolian traditional medicine, sigla storax finds diverse applications. It is used to treat peptic ulcers, stomach aches, burns, wounds, cracked lips, bronchitis, and both parasitic and fungal infections (Atmaca et al., 2022). Recent research has explored its potential beyond traditional uses. Studies have investigated its cytotoxic effects on human cancer cells (Atmaca et al., 2022; Çetinkaya et al. 2022) and its neuroprotective properties (Zhang et al. 2019; Zhou et al. 2022). Sigla storax has demonstrated a broad spectrum of antimicrobial activity, particularly against various Gram-positive bacteria (Aşkun et al. 2021; Sağdıç et al. 2005; Keyvan and Savas 2021). Notably, surgical silk sutures coated with sigla storax exhibited the most potent anti-adhesion activity against the oral Gram-positive pathogen *S. aureus* (Kılınç et al. 2020). In addition, storax-loaded polymeric scaffolds demonstrated antibacterial and anti-biofilm properties against *S. aureus*, which is known to cause infections in chronic wounds (Demir et al., 2022). Given its pronounced antimicrobial effects, especially against Gram-positive bacteria, sigla storax shows promise as a potential antibiotic alternative for modifying rumen microorganism composition and activity. Supporting this potential, a recent study reported that sigla storax reduced rumen methane production without adversely affecting short-chain fatty acid (SCFA) production or feed digestibility. Importantly, it did not substantially alter the microbiota compared to monensin, a common antibiotic feed additive (Demirtas et al., 2023). However, to date, the literature lacks data on sigla storax's effects on pure cultures of rumen bacteria. An assessment of sigla storax's potential inhibitory or stimulatory impacts on specific rumen bacterial species would significantly contribute to elucidating its mechanism of action on rumen metabolism. Therefore, the present study aimed to investigate the effects of sigla storax on the growth of pure cultures of select Gram-positive and Gram-negative rumen bacteria.

MATERIALS and METHODS

Sigla storax

Sigla storax was sourced from a local supplier in Köyceğiz, Muğla, Turkey in August 2021, under the supervision of the Köyceğiz Forestry Management, which operates under the General Directorate of Forestry, Ministry of Environment and Forestry, Turkey. Until its use, the storax was stored at +4ºC. The traditional and producer-verified process for obtaining sigla storax is as follows: In early spring (around April), the trunks of *L. orientalis* trees are deliberately damaged, prompting the inner bark to absorb an exudate. By early July, the outer bark is shaved using specialized knives, and the balsam, along with the inner bark, is boiled in water for 10 to 30 minutes. As the mixture boils, the storax rises to the surface and is skimmed off. Afterward, the remaining bark is pressed to separate any remaining storax (Sağdıç et al., 2005). The resulting sigla storax in balsam form was used in the experiments.

Bacterial strains and anaerobic medium

The study examined several Gram-positive bacterial strains. These included *Ruminococcus albus* (ATCC 27210) and *Ruminococcus flavefaciens* Sijpesteijn C97 (ATCC 49949), both known for producing hydrogen and formate. Butyrate producers *Butyri-*

Table 1. Composition of the anaerobic medium (for 100 ml) (Orpin 1976)

Component*	
Mineral solution 1**	15
Mineral solution 2***	15
Clarified rumen fluid****	15
$NaHCO_3$ (Sigma S5761)	0.6
Yeast extract (Sigma Y1625)	0.25
Trypticase peptone (BD 211921 Bacto™)	1
Resazurin (%0.1, v/v) (Sigma R7017)	1
Cysteine HCl (Sigma C7880)	0.1
Cellobiose (Sigma 22150)	0.5
Deionized water	55

*Units are ml for liquid components and g for solid components.
**Mineral solution 1: 3 g/l K_2HPO_4 (Sigma P3786)
***Mineral solution 2: 3 g/l KH_2PO_4 (Sigma P9791), 6 g/l $(NH_4)_2SO_4$ (Sigma A4915), 6 g/l NaCl (Sigma S7653), 0.6 g/l $MgSO_4 \cdot 7H_2O$ (Sigma 230391) and 0.6 g/l $CaCl_2$ (Sigma C1016)
****The ruminal fluid collected from the slaughterhouse was mixed and filtered through three layers of cheesecloth to seperate into liquid and solid (digesta) fractions. The liquid fraction was centrifuged at 15,000 rpm and, the clear supernatant was used as a component of the anaerobic medium.

vibrio fibrisolvens D1 (ATCC 19171) and *Eubacterium ruminantium* GA 195 (ATCC 17233) were also studied, along with the lactate producer *Streptococcus bovis* (ATCC 33317). *Methanobacterium formicicum* (ATCC 33274), a mesophilic methanogen, served as the methane producer. Additionally, two Gram-negative bacteria were included: *Megasphaera elsdenii* LC1 (ATCC 25940) and *Fibrobacter succinogenes* S85 (ATCC 19169), both known for producing succinate and propionate. To maintain anaerobiosis, anaerobic medium was prepared under CO_2 following Orpin's (1976) method. Table 1 details the chemical composition of the anaerobic media. The medium was heated to 60ºC in a hot water bath while being bubbled with CO_2 to eliminate oxygen. Resazurin (0.1%, v/v), an indicator of redox potential, was used to monitor oxygen elimination, with a color change from bluish-purple to dull yellow signifying successful oxygen removal. After preparation, the flask containing the medium was stoppered and autoclaved. Anaerobic bacteria were cultivated for 24 to 72 hours in an anaerobic cabinet (Whitley DG250, Don Whitley, West Yorkshire, UK) maintained at 37ºC with a N_2-CO_2-H_2 (80:10:10) atmosphere.

Antimicrobial susceptibility assay

To assess the impact of sigla storax on rumen bacterial growth, a broth dilution technique was employed in an anaerobic cabinet, adhering to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). The process began with the preparation of a sigla storax stock solution (350 mg/ml) by dissolving sigla in ethanol (99.8% purity, Sigma 32221). This stock was then serially diluted two-fold in anaerobic medium to achieve concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 mg/ml. Using a flat-bottom 96-well plate (Corning 3599), 200 µl of each dilution was pipetted into designated wells. Each well was then inoculated with 20 µL of bacterial suspension (4×10^{10} cells/ml). For reliability, triplicate wells were used for each concentration. The experimental design included negative control wells (pure medium without sigla storax solution) and medium control wells (without bacteria) for each set. Following preparation, the plates were incubated in an anaerobic chamber at 37ºC for 24 hours. Microbial growth was subsequently assessed using a microplate reader (Epoch, BioTek, USA) set at 600 nm. Interpretation of results followed established guidelines: The minimum inhibitory concentration (MIC) was defined as the lowest concentration yielding an OD600 ≤ 0.1 (Kang et al., 2008). OD600 values significantly lower than the control dose (0 mg/ml) were considered indicative of potential antimicrobial activity (Ko et al., 2018), while significantly higher OD600 values were interpreted as evidence of stimulatory activity (Das et al., 2015).

Statistical analyses

Each well of the 96-well plate served as an experimental unit. Each sample has three replicates. Data of observed optical density (OD600) were statistically analysed by one-way ANOVA followed by Dunnet's test. A *P*-value of ≤0.05 was considered statistically significant.

RESULTS

Table 2 summarizes the MIC values of sigla storax, while Figures 1 and 2 illustrate its effects on Gram-positive and Gram-negative rumen bacteria, respectively. Among the tested species, *R. flavefaciens* exhibited the highest sensitivity, with growth inhibition occurring at 4 mg/ml of sigla storax. *R. albus* showed complete inhibition at 32 mg/ml, but potential antimicrobial effects were observed at 2-16 mg/ml ($P<0.05$). For *B. fibrisolvens*, potential antimicrobial activity was noted from a concentration of 1 mg/ml ($P<0.05$), with complete inhibition at 16 mg/ml. Sigla storax showed potential antimicrobial activity against both *E. ruminantium* and *M. formicicum* at 1-16 mg/ml ($P<0.05$) and complete inhibition at 32 mg/ml. Interestingly, sigla storax stimulated growth of *E. ruminantium* and *S. bovis* at lower concentrations: 0.06-0.125 mg/ml and 0.125-2 mg/ml, respectively ($P<0.05$). *S. bovis* emerged as the most resistant species. While no complete inhibition was observed, potential antimicrobial effects were noted at 8-32 mg/ml ($P<0.05$). Regarding Gram-negative bacteria, sigla storax exhibited a slight stimulatory effect on *M. elsdenii* at 0.06-0.5 mg/ml concentrations ($P<0.05$). Sigla storax showed potential antimicrobial activity against both *M. elsdenii* and *F. succinogenes* starting from 2 mg/ml ($P<0.05$), with complete inhibition at 32 mg/ml.

Table 2. Minimum inhibitory concentration (MIC) values of sigla storax on rumen bacteria

Rumen bacteria	MIC values (mg/ml)
Gram-positives	
R. albus	32
R. flavefaciens	4
B. fibrisolvens	16
E. ruminantium	32
S. bovis	-
M. formicicum	32
Gram-negatives	
F. succinogenes	32
M. elsdenii	32

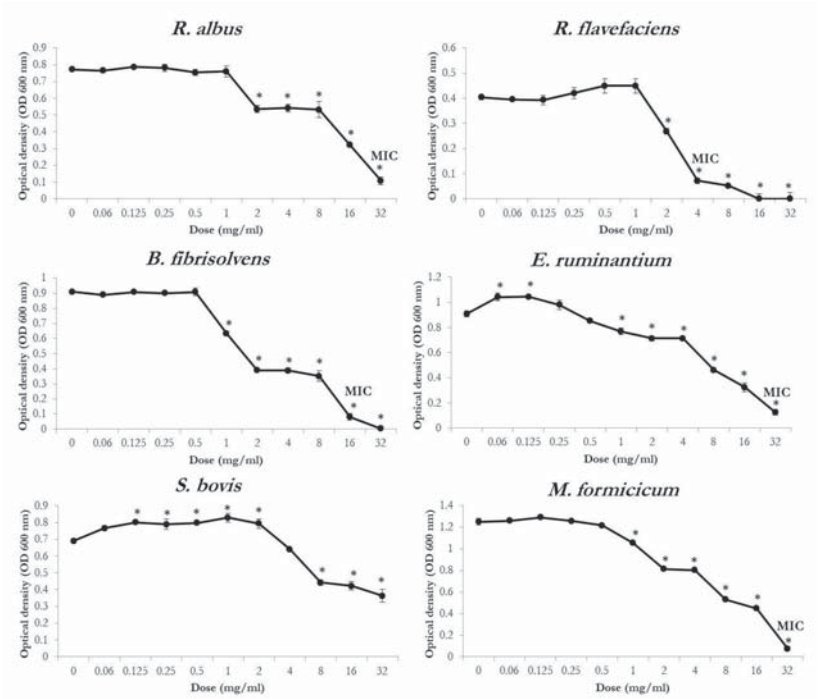

Figure 1. Effects of sigla storax on Gram-positive rumen bacteria by the broth microdilution method. The results represent the mean ± standard error. *P<0.05, difference of sigla storax-treated culture compared with the control. Control level was 0 mg/ml of the storax. MIC: Minimum inhibitory concentration.

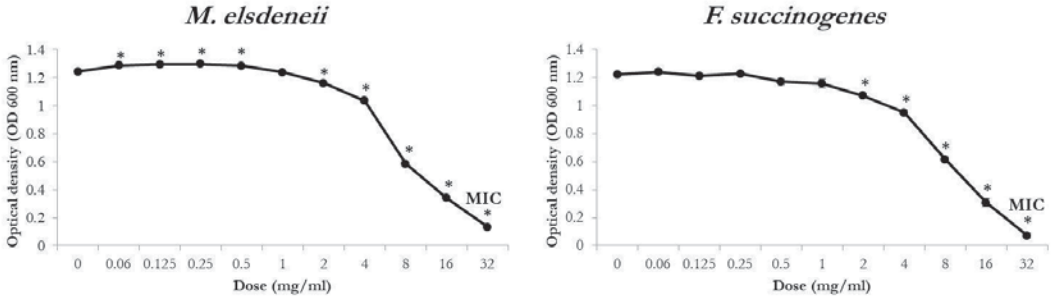

Figure 2. Effects of sigla storax on Gram-negative rumen bacteria by the broth microdilution method. The results represent the mean ± standard error. *P<0.05, difference of sigla storax-treated culture compared with the control. Control level was 0 mg/ml of the storax. MIC: Minimum inhibitory concentration.

DISCUSSION

Phytochemicals have been extensively studied as potential alternatives to antibiotics for modifying rumen microorganisms and fermentation (Kholif and Olafadehan, 2021). The desired effect of these agents is antibacterial activity, particularly against Gram-positive rumen bacteria that produce acetate and butyrate. These bacteria release more hydrogen, which is subsequently utilized in methane synthesis (Demirtas et al., 2018). In this study, sigla storax demonstrated inhibitory effects on representative species of acetate and butyrate-producing bacteria. Specifically, *R. flavefaciens* and *B. fibrisolvens* were inhibited at doses of 4 and 16 mg/ml, respectively. For other members of these groups, namely *R. albus* and *E. ruminantium*, the MIC was 32 mg/ml. A previous study using the same storax (Demirtas et al., 2023) employed an *in vitro* semi-continuous rumen microbial culture system (rumen simulation technique; Rusitec). With 0.5 mg/ml sigla storax supplementation, this study observed a reduction in the abundance of acetate-producing bacteria from the Ruminococcaceae family, including genera *Ruminococcus* 2 and *Ruminococcaceae* UCG-013, and uncultured *Ruminococcaceae* NK4A214 species. Additionally, some butyrate producers such as *Lachnospiraceae* UCG-002 and *Lachnospiraceae* UCG-006 were also suppressed. Notably, the same study reported suppression of the methane-producing genus *Candidatus Methanomethylophilus* and an uncultured genus from Methanobacteriaceae. These findings align with the observed mitigating effect of sigla on methane production. Likewise, in this study, sigla exhibited potential antimicrobial activity at concentrations ranging from 1 to 16 mg/ml, while demonstrating inhibitory effects on the methane-producing *M. formicicum* at 32 mg/ml. The discrepancies in effective sigla dosages across studies may be attributed to variations in bacterial species and the possible transformation of sigla within

the Rusitec's mixed culture environment, potentially yielding derivatives with enhanced antibacterial properties. The antimicrobial impact of sigla storax on methanogenic archaea and certain Gram-positive bacteria has been linked to cinnamic acid esters. Demirtas et al. (2023) identified two primary components in the essential oil composition of storax: *E*-cinnamyl cinnamate (38.8%) and 3-phenylpropanyl cinnamate (38.1%). As secondary plant metabolites, essential oils are renowned for their wide-ranging antimicrobial effects (Seow et al., 2014). In line with this, cinnamic acid derivatives have been reported to possess antibacterial, antiviral, and antifungal properties (Sova, 2012; Tawata et al., 1996).

Interestingly, at low concentrations, sigla storax exhibited a mild growth-promoting effect on certain bacteria. This included Gram-positive species like *E. ruminantium* and *S. bovis*, as well as the Gram-negative *M. elsdenii*. Demirtas et al. (2023) reported that supplementing Rusitec with 0.5 mg/ml sigla increased the abundance of some butyrate-producing Gram-positive bacteria and propionate-producing Gram-negative bacteria. The used storax has been found to have high phenolic content, measuring 79.2 mg/g in catechin equivalents or 57.9 mg/g in gallic acid equivalents (Demirtas et al., 2023). Phenolic substances, including phenolic acids, flavonoids, and tannins, are phytochemicals or secondary plant metabolites that can interact both positively and negatively with microorganisms (Broudiscou et al., 2000). Many polyphenols, such as tannic acid, gallic acid, catechol, and catechin, can be hydrolyzed by rumen bacteria and used as carbon and energy sources for growth (Bhat et al., 1998). Thus, the phenolic compounds in sigla storax might be responsible for stimulating the growth of some of the studied bacteria. However, at higher doses, sigla demonstrated antibacterial activity against both Gram-negative bacteria. This dual effect is reminiscent of saponins, another type of secondary plant metabolite, which have been shown to promote the growth of certain rumen bacteria *in vitro* at low doses while potentially inhibiting bacterial growth at high doses (Patra et al., 2012).

One of the phytochemicals contained in sigla storax used in our study was the essential oil, dominated by *E*-cinnamyl cinnamate and 3-phenylpropanyl cinnamate. Essential oils, due to their hydrophobic nature, have a high affinity for the lipids of bacterial cell membranes. Their antibacterial properties appear to be linked to their lipophilic nature (Benchaar et al., 2008). The interaction with the cell membrane induces conformational alterations, leading to ion leakage across the membrane. This disruption causes a loss of the transmembrane ionic gradient, which results in energy depletion and ultimately leads to microbial cell death (Griffin et al., 1999). This mode of action enhances the effectiveness of essential oils against Gram-positive bacteria, as their cell membranes can directly interact with the hydrophobic components of the oils. In contrast, Gram-negative bacteria have a hydrophilic outer layer around their cell membrane that serves as a barrier to the penetration of hydrophobic compounds (Patterson et al., 2019). However, essential oils with small molecular weight such as thymol and carvacrol can penetrate the outer hydrophilic cell wall of Gram-negative bacteria and act on them (Calsamiglia et al., 2007). It has also been reported that this mechanism reduces the selectivity of essential oils against certain bacterial populations in the rumen (Calsamiglia et al., 2007). The efficacy of sigla storax against Gram-negative as well as Gram-positive bacteria in this study may be attributed to a similar mechanism of action of sigla storax essential oil. Thus, considering the antibacterial activity of sigla storax against Gram-negative bacteria along with stimulatory effect on certain Gram-positive bacteria, the impact of sigla on rumen bacteria appears to be less selective than that of antibiotic feed additives.

CONCLUSION

In this study, sigla storax demonstrated inhibitory effects against Gram-positive rumen bacteria at concentrations ranging from 4 to 32 mg/ml, while Gram-negative bacteria were inhibited at 32 mg/ml. Although Gram-positive bacteria generally showed higher sensitivity to sigla storax compared to Gram-negative bacteria, the findings suggest that its mechanism of action differs from that of typical antibiotic feed additives. This conclusion is based on two key observations: first, sigla storax's antibacterial activity extended to Gram-negative bacteria, and second, it exhibited growth-promoting effects on certain Gram-positive bacteria. These dual actions indicate a more complex interaction with rumen microbiota than traditional antibiotics. To fully elucidate sigla storax's mode of action and its potential for modifying rumen metabolism, further research is needed. Specifically, investigating its effects on a broader range of bacterial species within the rumen ecosystem would provide valuable insights.

DECLARATIONS

Ethics Approval

Not applicable.

Conflict of Interest

None declare.

Consent for Publication

Publication is appropriate.

Author contribution

Idea, concept and design: AD

Data collection and analysis: AD

Drafting of the manuscript: AD

Critical review: AD

Data Availability

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

Acknoledgements

Not applicable.

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