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Evaluation of the antibacterial effect of (R)-(+)-Limonene against *Enterococcus faecalis* and *Enterobacter cloacae* strains isolated from food

Avaliação do efeito antibacteriano do (R)-(+)-Limoneno contra cepas de *Enterococcus faecalis* e *Enterobacter cloacae* isoladas de alimentos

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Highlights

(R)-(+)-Limonene exhibited moderate bactericidal effect against *E. faecalis.*
(R)-(+)-Limonene had no antibacterial effect against *E. cloacae.*
The compound showed different modulating effects when associated with antimicrobials.
(R)-(+)-Limonene had no anti-adherent effect against the strains tested.

Abstract

The aim of this study was to evaluate the potential antibacterial and anti-adherent activities of the monoterpene (R)-(+)-limonene, as well as its synergistic potential with synthetic antimicrobials against strains of *Enterococcus faecalis* and *Enterobacter cloacae.* The antibacterial properties of (R)-(+)-limonene were assessed using the broth microdilution technique to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Additionally, the infusion disc method was employed to explore the association of the compound with antimicrobials, and the test tube method was used to determine the minimum inhibitory concentration of adherence (MICA). It was

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observed that the MIC for (R)-(+)-limonene was 1000 µg mL^{-1} for five of the six *E. faecalis* strains tested, while for *E. cloacae*, the MIC exceeded 1000 µg mL^{-1} for all strains tested. Identical values were recorded for the MBC in *E. faecalis.* In terms of its combination with synthetic antimicrobials, (R)-(+)-limonene demonstrated a synergistic effect with gentamicin and ciprofloxacin for most strains. Regarding the MICA, both (R)-(+)-limonene and 0.12% chlorhexidine digluconate failed to inhibit biofilm formation at the tested concentrations. Given the need for new therapeutic alternatives for treating bacterial infections, this study revealed that the tested monoterpene exhibited moderate bactericidal effects against *E. faecalis* strains and no antibacterial effect against *E. cloacae* strains. However, when combined with various classes of antimicrobials, (R)-(+)-limonene showed synergistic effects with gentamicin and ciprofloxacin for most strains. This suggests that (R)-(+)-limonene holds promise for enhancing the treatment of bacterial infections and could support conventional therapies. Nonetheless, further *in vitro*, *ex vivo*, and *in vivo* studies are necessary to confirm and elucidate its efficacy and mechanisms.
Key words: Antibacterial activity. Antimicrobials. Phytotherapy. Monoterpene. Natural Products.

Resumo

Esse estudo teve como objetivo avaliar a possível atividade antibacteriana, antiaderente e o estudo de associação a antimicrobianos sintéticos do monoterpeno (R)-(+)-Limoneno contra cepas de *Enterococcus faecalis* e *Enterobacter cloacae.* O caráter antibacteriano do monoterpeno (R)-(+)-Limoneno foi verificado pela técnica de microdiluição em caldo para determinação da Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM), como também através do método de disco de infusão foi realizado a associação do composto a antimicrobianos e pelo método com tubos de ensaio a Concentração Inibitória Mínima de Aderência (CIMA). Foi observado que o composto apresentou uma CIM de 1000 µg mL^{-1} para cinco, das seis cepas testadas de *E. faecalis*, já para *E. cloacae* a CIM foi acima de 1000 µg mL^{-1} para todas as cepas teste, em relação à CBM foi registrado os mesmos valores que a CIM para a *E. faecalis.* Quanto á associação do (R)-(+)-Limoneno com antimicrobianos sintéticos, verificou-se que o composto combinado com diferentes antimicrobianos, apresentou efeito sinérgico com Gentamicina e Ciprofloxacino para a maioria das cepas. A respeito da CIMA observou-se que tanto o (R)-(+)-Limoneno, quanto digluconato de clorexidina 0,12% não conseguiram inibir a formação de biofilme nas proporções testadas. Tendo em conta a necessidade de uma nova alternativa terapêutica para o tratamento de infecções bacterianas, este estudo demonstrou que o monoterpeno testado teve um efeito bactericida moderado contra estirpes de *E. faecalis* e nenhum efeito antibacteriano contra estirpes de *E. cloacae.* Quando associado a diferentes classes de antimicrobianos, o (R)-(+)-Limoneno apresentou efeitos sinérgicos em relação à Gentamicina e à Ciprofloxacino para a maioria das estirpes testadas. Isto mostra que o (R)-(+)-Limonene é promissor para o tratamento de infecções bacterianas, corroborando as terapias convencionais. No entanto, são necessários mais estudos *in vitro*, *ex vivo* e *in vivo* para confirmar e elucidar a sua eficácia e mecanismos.
Palavras-chave: Atividade antibacteriana. Antimicrobianos. Fitoterapia. Monoterpeno. Produtos Naturais.

Introduction

Throughout its preparation and distribution, from collection to final consumption, food can become contaminated, potentially leading to intoxication in millions of people annually. Foodborne diseases are commonly caused by several microorganisms, and most foods contain nutrients that support microbial growth (Flores & Melo, 2015). Notable among these microorganisms are bacteria such as *Enterococcus faecalis* and *Enterobacter cloacae* (Siqueira et al., 2022; Braga et al., 2020).

The genus Enterococcus is part of the normal microbiota of mammalian gastrointestinal tracts but also colonizes other environments such as water, plants, soil, and both plant- and animal-origin fermented foods (Lebreton et al., 2014; Chajęcka-Wierzchowska et al., 2017). Additionally, they are present in raw foods like milk and meat due to environmental contamination or through the intestinal contents of animals themselves (Sánchez Cabrera et al., 2021).

Enterococcus faecalis is beneficially used in the food and pharmaceutical industries as an indicator of fecal contamination in food and water, aiding in the assessment of sanitary conditions (Werner et al., 2013). However, *E. faecalis,* along with *Enterococcus faecium,* are frequently found in animal and human intestines and are known for causing nosocomial infections and contributing to antimicrobial resistance (Lebreton et al., 2014).

E. cloacae, a significant Enterobacteriaceae member found in the environment, is part of the intestinal microbiota of humans and animals and can cause infections, especially in immunocompromised individuals (Bennett et al., 2019; Davin-Regli et al., 2019). This species is responsible for various diseases, e.g., pneumonia, urinary tract infections (UTIs), skin and soft tissue infections (SSTIs), and intravascular infections (Weiner et al., 2016). The emergence and spread of antimicrobial resistance pose a global public health threat, exacerbated by the indiscriminate and inappropriate use of antibiotics in animals, allowing antimicrobial-resistant bacteria and genes to be transmitted from animals to food and humans (M. O. Silva & Aquino, 2018).

The One Health concept supports approaches that strengthen systems to prevent, prepare for, detect, respond to, and recover from diseases, particularly infectious diseases. It also addresses related issues such as antimicrobial resistance that collectively threaten humans, animals, and the environment (Berthe et al., 2018).

Given this context, the exploration of alternative treatments such as phytotherapy is essential. Medicinal plants exhibit biological activities that provide preventive, promotive, and therapeutic options for various pathologies (Almeida et al., 2020).

The utilization of herbal medicines is often driven by the high costs of synthetic drugs, limited access to medical care, and societal preferences for well-known natural products (Badke et al., 2012). Among natural products, Limonene, a common cyclic monoterpene with the molecular formula $C_{10}H_{16}$, occurs naturally in optical forms like R-(+)-limonene (d-limonene) and S-(-)-limonene (l-limonene), or as a racemic mixture (Farmer et al., 2018).

The study of chemical isomers is relevant, as natural products like limonene consist of isomers that confer distinct characteristics to the compound. The isomer selected for this study, D-limonene or (R)-(+)-limonene (4-isopropenyl-1-methylcyclohexene), is a primary constituent of several essential oils derived from citrus fruits, including orange, lemon, tangerine, lime, and grapefruit. It has been generally recognized as safe (GRAS) for use as a flavoring agent and food preservative (Sun, 2007). Additionally, due to its antibacterial, antifungal, and anti-inflammatory properties, D-limonene can also be utilized in medicine and is considered an economically viable natural product (Retajczyk & Wróblewska, 2019).

In light of the therapeutic potential of natural products and the crucial role they play in combating bacterial infections, this study aimed to evaluate the antibacterial and anti-adherent activities of the monoterpene (R)-(+)-limonene, as well as its potential synergistic effects with synthetic antimicrobials against strains of *Enterococcus faecalis* and *Enterobacter cloacae.*

Material and Methods

Test substance

The monoterpene (R)-(+)-limonene was procured from Indústria Sigma - Aldrich® (São Paulo-SP, Brazil). For pharmacological testing, the substance was dissolved in DMSO (dimethyl sulfoxide) and further diluted in distilled water, with the final DMSO concentration kept below 0.1% v v^{-1}.

Microorganisms

Strains of Enterococcus faecalis (*ATCC 29212, Ef 46, Ef 47, Ef 48, Ef 49,* and *Ef 50*) and *Enterobacter cloacae* (*Ecl 41, Ecl 42, Ecl 43, Ecl 44,* and *Ecl 45*) were used. All strains were maintained on Mueller-Hinton Agar (MH) at 4 °C. Inocula were prepared from overnight cultures in MH broth at 35 ± 2 °C, diluted in sterile saline to achieve a final concentration of approximately 1.5 x 10^8 CFU mL^{-1}, adjusted to a 0.5 McFarland standard turbidity (Bona et al., 2014).

Culture medium

The assays to evaluate antimicrobial activity employed both liquid and solid Mueller Hinton media from Difco®, prepared following the manufacturer's instructions.

Determination of the minimum inhibitory concentration (MIC)

The MIC was determined using microdilution technique in a 96 well U bottom plate. 100 µL of doubly concentrated Mueller Hinton broth and 100 µL of the product ((R)-(+)-Limonene) under study at concentrations of 1000, 500, 250, 125, 62.5, and 31.2 µL mL^{-1} were added to each well of the plate. MIC determination was conducted with 10 µL of the microorganism in each well, approximately 1.5x10^8 CFU mL^{-1}. A sterility control was also prepared in the penultimate well, with only 100 µL of broth, and a growth control was performed in the last well, containing 100 µL of doubly concentrated Muller Hinton broth, and the microorganism suspension. The entire assay was performed

in duplicate. The plates were incubated at 35 ± 2ºC for 24 hours, and afterwards, the first reading of the results was performed. 20 µL of sodium resazurin solution (SIGMA), recognized as a colorimetric oxide-reduction indicator for bacteria, previously solubilized in sterile distilled water 0.01% (w v^{-1}), was added to the plates, for incubation again at 35 ± 2ºC. The reading was visually performed using the absence or presence of microorganism growth by the formation of cell agglomerates (buttons), and also by observation of change in the color of the solution, from blue to pink, indicating growth. The MIC was determined as the lowest concentration of the product inhibiting visible growth of the microorganism, as verified by the unchanging color of the dye (Palomino et al., 2002; Ostrosky et al., 2008; Clinical and Laboratory Standards Institute [CLSI] (2012); Bona et al., 2014).

Determination of the minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) of the monoterpene was also determined for the bacterial strains. After reading the MIC, inoculates (10 µL) of the three dilutions prior to the MIC value were made in Mueller-Hinton broth medium (100 µL $well^{-1}$), in a sterilized microdilution plate, followed by incubation at 37ºC for 24 hours, after this, 20 µL of resazurin was added and then a new incubation at 35 ± 2ºC was performed. The reading was performed to confirm the concentration capable of inhibiting the total growth of the bacterial species, verified by a non-change in the coloration of the indicator dye (Ncube et al., 2008; Guerra et al., 2012).

Study of the association of R-(+)-Limonene with synthetic antimicrobials

The association of the product with the antimicrobials (Ampicillin, Gentamicin, Ciprofloxacin, and Ceftriaxone) was performed using disk diffusion technique in solid medium and filter paper discs (Bauer et al., 1966; Oliveira et al., 2006). In smooth sterile Petri plates containing Muller Hinton agar medium previously inoculated with the bacterial suspension, discs containing antimicrobials were introduced together with 20 µL of the MIC, a negative control was also performed containing only the antimicrobial disks with the bacterial suspension of the test product were added, then the plates were incubated at 35 ± 2ºC for 24-48h, and readings were taken. A synergistic effect was considered if the microbial growth inhibition halo formed by the association (product + antimicrobial) presented a diameter ≥ 2mm. When the inhibition halo resulting from the association was of a smaller diameter than that developed by the isolated action of the antimicrobial, it was considered an antagonistic effect. When the inhibition halo resulting from the association obtained a diameter equal to that resulting from the isolated application of the antimicrobial anti-microbial it was considered an indifferent effect (Cleeland & Squires, 1991). All assays were performed in duplicate.

Determination of the Minimum Inhibitory Concentration for Adherence (MICA)

The Minimum Inhibitory Concentration for Adherence (MICA) of the compound was determined in the presence of 5% sucrose, in accordance with Albuquerque et al. (2010)

with modifications, using concentrations of the pure compound to a dilution of 1:128. After bacterial growth, the bacterial strain was grown at 35 ± 2ºC in Mueller Hinton broth (DIFCO, Michigan, United States), then 0.9mL of the subculture was distributed in test tubes, and 0.1mL of the solution corresponding to the dilutions of the compound was then added. Incubation was performed at 35 ± 2ºC for 24 hours with the tubes inclined to 30º. The reading was performed by visually observing the adherence of the bacteria to the tube walls after shaking the tube. The assay was performed in duplicate. The same procedure was performed for the positive control 0.12 % chlorhexidine digluconate (Periogard®, Colgate-Palmolive Company, New York, USA). The MICA was considered as the lowest concentration of the agent in contact with sucrose that prevented adherence to the glass tube.

Results and Discussion

The determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in liquid medium was established for (R)-(+)-Limonene at the different concentrations suggested in the methodologies against the strains of *E. faecalis* and *E. cloacae,* as shown in tables 1 and 2. The results for the monoterpene were 1000µg mL^{-1} for five of the six *E. faecalis* strains tested. Thus, considering an MIC90 (lowest concentration capable of inhibiting growth by 90%) of 1000µg mL^{-1}, in relation to the Minimum Bactericidal Concentration (MBC) for the same strains, the compound was found to have a MBC of 1000 µg mL^{-1}. On the other hand, for the *E. cloacae* strains, it was observed that the MIC results for the monoterpene were above 1000µg mL^{-1} for all the strains tested, thus indicating no potential antibacterial activity at the concentrations tested. As a result, the other methodologies (MBC, MICA and association with synthetic antimicrobials) were not carried out for this bacterium in this study.

Plant-derived products have been of interest in research for many years due to their potential utility in controlling and reducing microorganisms detrimental to the food industry (S. P. Souza et al., 2011). Thus, the antimicrobial activities of R-(+)-limonene have been confirmed against various food-related microorganisms, e.g. including *Staphylococcus aureus, Listeria monocytogenes, Salmonella enterica,* and *Saccharomyces bayanus* (Settanni et al., 2012; Chikhoune et al., 2013).

According Sartoratto et al. (2004), the classification of antimicrobial activity is as follows: strong for an MIC of up to 500µg mL^{-1}, moderate for an MIC of 600 to 1500 µg mL^{-1} and weak for an MIC above 1500µgmL^{-1}. According to the results, the monoterpene (R)-(+)-Limonene demonstrated moderate antibacterial activity against *E. faecalis* strains, as it obtained an MIC_{90} of 1000 µg mL^{-1}. For *E. cloacae,* classification was not possible, since the MIC was not found using the concentrations tested.

Table 1
Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the monoterpene (R)-(+)-Limonene against different strains of *E. faecalis*

Bacterial strain	*Atcc 29212*	*Ef* **46**	*Ef47*	*Ef 48*	*Ef 49*	*Ef 50*	*Ef* **46**
MIC (µg mL^{-1})	1000	1000	1000	>1000	1000	1000	1000
MBC (µg mL^{-1})	1000	1000	1000	-	1000	1000	1000

Legend: (-) not evaluated.

Table e
Minimum inhibitory concentration (MIC) of the monoterpene (R)-(+)-Limonene against different strains of *E. cloacae*

Bacterial strain	*Ecl 41*	*Ecl 42*	*Ecl 43*	*Ecl 44*	*Ecl 45*	*Ecl 41*	*Ecl 42*
MIC (µg mL^{-1})	>1000	>1000	>1000	>1000	>1000	>1000	>1000

Compounds can be evaluated as either bactericidal or bacteriostatic based on the MBC/MIC ratio. When the ratio is between 1: 1 to 2: 1, the compound is considered bactericidal, and when this ratio is greater than 2:1 the compound is considered bacteriostatic (Hafidh et al., 2011). Analyzing the results suggests that (R)-(+)-Limonene presents bactericidal activity for *E. faecalis* strains, such as *Atcc 29212, Ef 46, Ef47, Ef 49,* and *Ef50.*

Corroborating our study, according to Costa et al. (2019), (R)-(+)-Limonene demonstrates strong antibacterial activity against Gram-positive strains (*Staphylococcus aureus*) with an MIC of 256 µg mL^{-1} as well as Gram-negative strains (*Pseudomonas aeruginosa*) with an MIC of 512 µg mL^{-1}.

The combination of (R)-(+)-Limonene with the antimicrobials tested showed a synergistic effect with Gentamicin and Ciprofloxacin for most of the strains. This result was obtained by comparing the halo of inhibition in the presence of the antimicrobial alone with the halo of inhibition in the presence of the combination of antimicrobials with the monoterpene tested, as can be seen in Table 3.

Due to the lack of new antimicrobials, the combination of antibiotics has become a viable alternative for significantly reducing the treatment dose, while at the same time reducing the toxicity associated with the therapeutic dose of certain antibiotics, such as polymyxin B and aminoglycosides. One possible solution to this problem is the use of natural products as adjuvants to antibiotics, with the aim of achieving synergistic interactions (Langeveld et al., 2014; Milenković et al., 2018). This synergistic effect could be a clue for future research into the development of combinations of natural products and synthetic compounds in the form of technically elaborated pharmaceutical preparations, such as capsules, tablets, oils and oral suspensions, for example (Nacu & Hoerr, 2016).

In the study by Costa et al. (2019), (R)-(+)-Limonene was found to have a synergistic effect when associated with gentamicin against *S. aureus* and *Escherichia coli,* and an antagonistic effect when associated with norfloxacin and imipenem.

Yet the monoterpene (R)-(+)-Limonene presented an additive effect when combined with florfenicol, and an antagonistic effect when combined with oxytetracycline against *Aeromonas hydrophila* strains, strains and showed no synergism with the antimicrobials tested (E. G. Silva et al., 2021).

Table 3
Study of the association of the monoterpene (R)-(+)-Limonene with synthetic antimicrobials against strains of *E. faecalis*

Microorganism	Association	AMP 10	GEN 10	CIP	CRO
Atcc29212	HIATB	24mm	14mm	24mm	10mm
	HIATB + RL	24mm(*)	14mm(*)	24mm(*)	10mm(*)
Ef 46	HIATB	24mm	12mm	26mm	12mm
	HIATB + RL	24mm(*)	14mm(↑)	26mm(*)	5mm (↓)
Ef 47	HIATB	22mm	12mm	22mm	12mm
	HIATB + RL	28mm(↑)	12mm(*)	24mm(↑)	18mm(↑)
Ef 48	HIATB	10mm	12mm	24mm	14mm
	HIATB + RL	10mm(*)	14mm(↑)	24mm(*)	10mm(↓)
Ef 49	HIATB	26mm	10mm	20mm	10mm
	HIATB + RL	34mm(↑)	14mm(↑)	24mm(↑)	10mm(*)
Ef 50	HIATB	28mm	10mm	26mm	16mm
	HIATB + RL	28mm(*)	12mm(↑)	28mm(↑)	14mm(↓)

Legend: **HIATB:** halo of inhibition in the presence of the antibiotic. **RL:** (R)-(+)-Limonene. **APM10:** Ampicillin. **GEN10:** Gentamicin. **CIP:** Ciprofloxacin. **CRO:** Ceftriaxone. **(↑):** Synergistic effect. **(↓):** Antagonistic effect. **(*):** Indifferent effect.

Studies with other natural products can also be highlighted, such as that by Santana et al. (2021) who observed the association of *Lavandula hybrida Grosso* essential oil with the antimicrobial cephalothin against strains of *Staphylococcus aureus,* which showed a synergistic effect. This confirms that the combination of natural products with synthetic antimicrobials can be considered an important therapeutic option in the fight against bacterial infections.

Regarding the anti-adherent activity of the monoterpene under study against *E. faecalis* strains, it was observed that both (R)-(+)-Limonene and 0.12% chlorhexidine digluconate failed to inhibit biofilm formation in the proportions tested, as shown in Table 4.

In analyzing the anti-adherent activity of the compound, it was observed that none of the proportions tested was capable of inhibiting biofilm formation. Yet a study by

E. R. L. Souza et al. (2021) however, has verified the biofilm inhibiting potential of *Lavandula hybrida "Grosso"* essential oil against *Klebsiella pneumoniae,* and found that it is four times more potent than 0.12 % chlorhexidine digluconate, being thus considered a promising alternative to inhibiting biofilm formation.

Table 3
Adherence Inhibitory Concentration in µg/mL of monoterpene and 0.12% chlorhexidine digluconate against the *E. faecalis* strain *(Ef 49)*

R- (+)- Limonene								
µg mL^{-1}	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	+	+	+	+	+	+	+	+
Digluconate in 0.12% chlorhexidine								
µg mL^{-1}	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	+	+	+	+	+	+	+	+

(-) Without adhesion to the tube wall (+). With adhesion to the tube wall.

Conclusion

Given the need for a new therapeutic alternative for the treatment of bacterial infections, this study showed that the monoterpene tested had a moderate bactericidal effect against strains of *E. faecalis* and no antibacterial effect against strains of *E. cloacae.* When associated with different classes of antimicrobials, (R)-(+)-Limonene showed synergistic effects in relation to Gentamicin and Ciprofloxacin for most of the strains tested. This shows that (R)-(+)-Limonene is promising for the treatment of bacterial infections, corroborating conventional therapies. However, further *in vitro, ex vivo* and *in vivo* studies are needed to confirm and elucidate its efficacy and mechanisms.

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