Running title: Antibacterial activity of (R)-(+)-Limonene

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

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# Novelty Declaration

Bacteria considered pathogenic can cause economic damage in the agricultural sector, and also have the potential to cause foodborne infections in individuals, as food throughout its processing stages can be contaminated. In this context, few studies have investigated (R)-(+)-Limonene against strains of Gram positiveand negativebacteriaisolated from foods. Our results demonstrate its promising potential for treatment of bacterial infections, recording significant antibacterial action, and varied effects when associated with differing classes of antimicrobials.

# Abstract

This study aimed to evaluate the potential antibacterial, anti-adherent activity and associations with synthetic antimicrobials of the monoterpene (R)-(+)-Limonene against strains of *Enterococcus faecalis* and *Enterobacter clocae.* The antibacterial character (R)-(+)-Limonene was verified: using broth microdilution technique to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), associations of the compound with antimicrobials infusion disc method, and Minimum Concentration of Adherence (MICA) in the presence of sucrose. It was observed that the compound presented an MIC of 1000 μg/mL for five of the six tested strains of *E. faecalis,* while for *E. clocae* the MIC was above 1000 μg/mL for all test strains. The MBC for *E. faecalis* was also 1000 μg/mL*.* As for the associations of the compound with antimicrobials, it presented an indifferent effect in the presence of Ampicillin, a synergistic effect in the presence of Gentamicin, synergistic and indifferent effects were observed in the presence of Ciprofloxacin, and indifferent and antagonistic effects were verified in the presence of Ceftriaxone. With respect to the MICA, it was observed that in the proportions tested, both (R)-(+)-Limonene and 0.12 % chlorhexidine digluconate failed to inhibit biofilm formation. Thus, it can be verified that the tested monoterpene does not present antibacterial effect against *E. cloacae* strains and presented moderate bactericidal effect against *E. faecalis* strains, besides being identified synergistic, antagonistic and indifferent effect when associated with different classes of antimicrobials. Thus, it shows promise for the treatment of bacterial infections, corroborating with conventional therapies.

**Key words:** Antibacterial activity, Antimicrobials, Phytotherapy, Monoterpene, Natural products.

# Introduction

Throughout its preparation, from collection to the moment it reaches the final consumer, food potentially suffers from contaminations which can cause intoxication in millions of people annually. Foodborne diseases are caused by many microorganisms, and most foods contain nutrients that support microbial growth (Flores and Melo 2015). Among these microorganisms there are bacteria, such as *Enterococcus faecalis* and *Enterobacter clocae* (Campos *et al.* 2013; Braga *et al.* 2020).

The genus Enterococcus belongs to the normal microbiota of the mammalian gastrointestinal tract, but it also colonizes other habitats such as waters, plants, soil, and fermented foods of both plant and animal origin (Lebreton *et al.* 2014; Chajecka-Wierzchowska *et al.* 2017). In addition, they can be found in raw foods, such as milk and meat, through environmental contamination or through the intestinal contents of the animals themselves (Sánchez Cabrera *et al.* 2021).

 *E. faecalis* is beneficially used in the food and pharmaceutical industry as an indicator of fecal contamination in food and water, helping to assess sanitary conditions (Werner *et al.* 2013). However, *E. faecalis,* as well as *Enterococcus faecium* are the most frequently found bacteria in animal and human intestines, and are described as responsible for causing nosocomial infections as well as antimicrobial resistance (Lebreton *et al.* 2014).

 Regarding the species *E. cloacae,* is considered ubiquitous in terrestrial and aquatic environments such as water, sewage, soil, and food. It occurs as an intestinal microbiota commensal in humans and animals, and is also a pathogen to plants and insects (Mezzatesta *et al.* 2012). *E. cloacae* has been isolated in food processing plants, in fresh vegetables, rice, meat, and meat products (Shaker *et al.,* 2007; Haryani *et al.* 2008; Nyenje *et al.* 2012). The *E. cloacae* species are causative of opportunistic infections, such as pneumonia, urinary tract infections, wound, skin and soft tissue infections, ophthalmic and bloodstream infections, particularly related to catheters (Storti *et al.* 2005; Mokracka *et al.* 2011).

Thus, the emergence and spread of antimicrobial resistance is a threat to global public health. The indiscriminate and inappropriate use of antibiotics to treat disease in animals allows antimicrobial resistant bacteria and antimicrobial resistance genes to be transmitted from animals to food and humans (Verraes *et al.* 2013).

 The One Health concept recognizes support for approaches to strengthen systems that help prevent, prepare, detect, respond, and recover from disease, especially infectious disease. One Health also considers related issues such as antimicrobial resistance that collectively threaten humans, animals, and the environment (Berthe *et al.* 2018).

In this context, the presence of alternative treatments is considered primitive and one of the options is phytotherapy, through the use of medicinal plants that show biological activities, having several applications for prevention, promotion and treatment of various existing pathologies (Almeida-Junior *et al.* 2020).

The use of herbal medicines as a medicinal resource owes much to the high cost of synthetic drugs, difficult access to medical care, and a tendency to use well known natural products rooted in society (Badke *et al.* 2012).

Of the natural products with therapeutic potential, D-limonene or (R)-(+)-Limonene (4-isopropenyl-1-methylcyclohexene) a main constituent in several essential oils derived from citrus, such as orange, lemon, tangerine, lime, and grapefruit among others, has been generally recognized as safe (GRAS) for use as a food flavoring and preservative (Sun 2007). Yet due to its antibacterial, antifungal, and anti-inflammatory properties, the compound can also be used in medicine (Retajczyk and Wróblewska 2019).

#  In this context, according to the information regarding the therapeutic potential of natural products and the importance of combating infections caused by bacteria, this work aims to evaluate the possible antibacterial and anti-adherent activity and the study of association to synthetic antimicrobials

# of the monoterpene (R)-(+)-Limonene against strains of *E. faecalis* and *E. clocae*

# Materials and Methods

*In vitro* tests

**Test substance**

The monoterpene (R)-(+)-Limonene was purchased from Indústria Sigma - Aldrich ® (São Paulo-SP). To perform pharmacological tests, the substance was solubilized in DMSO (dimethylsulfoxide) and diluted in distilled water. The concentration of DMSO used was less than 0.1% v/v.

**Microorganisms**

*Enterococcus faecalis* (*ATCC 29212, Ef 46, Ef 47, Ef 48, Ef 49* and *Ef 50),* and *Enterobacter clocae* (*Ecl 41, Ecl 42, Ecl43, Ecl 44* and *Ecl 45)* strains were used. All were maintained on Mueller-Hinton Agar (MH) at 4°C. The inoculums were obtained from overnight culturesin MH at 35 ± 2ºC and diluted in sterile saline to obtain a final concentration of approximately 1.5 x 108 colony forming units per mL (CFU/mL), adjusted for McFarland scale turbidity at 0.5 (Bona *et al.* 2014).

**Culture Medium**

The culture media used in the assays to evaluate the antimicrobial activity was the liquid Mueller Hinton medium and the solid Muller Hinton agar medium, both purchased from Difco® and prepared according to 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 were added to each well of the plate. MIC determination was conducted with 10 µL of the microorganism in each well, approximately 1.5x108 CFU/mL. 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), 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; 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), 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 will be 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 R-(+)-Limonene - synthetic antimicrobials associations**

 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 disks. In 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 and Squires 1991). All assays were performed in duplicate.

**Determination of MICA**

The Minimum Adhesion Inhibitory Concentration (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

The Minimum Inhibitory Concentration (MIC) in liquid medium was established for (R)-(+)-Limonene at the different concentrations suggested in the methodology and determined by the lowest concentration capable of visibly inhibiting bacterial growth, as shown in Table 1. It was observed that the results for the monoterpene were 1000 μg/mL for five of the six tested strains of *E. faecalis.* Thus, revealing an MIC90 (lowest concentration capable of inhibiting growth by 90%) of 1000μg/mL. As for *E. clocae,* as shown in Table 2, it was found that the results for the monoterpene were above 1000 μg/mL in all strains tested, not indicating potential for antibacterial activity at the concentrations tested.

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Bacterial strain /Substance | *Atcc**29212* | *Ef* 46 | *Ef47* | *Ef 48* | *Ef 49* | *Ef 50* |
| 1000 μg/mL | **-** | **-** | **-** | **+** | **-** | **-** |
| 500 μg/mL | **+** | **+** | **+** | **+** | **+** | **+** |
| 250 μg/mL | **+** | **+** | **+** | **+** | **+** | **+** |
| 125 μg/mL | **+** | **+** | **+** | **+** | **+** | **+** |
| 62.5 μg/mL | **+** | **+** | **+** | **+** | **+** | **+** |
| 31.2μg/mL | **+** | **+** | **+** | **+** | **+** | **+** |
| Sterility control \_ | **-** | **-** | **-** | **-** | **-** | **-** |
| Growth control \_ | **+** | **+** | **+** | **+** | **+** | **+** |

 (**+)** with growth (**-)** without growth

**Table 2.** Minimum inhibitory concentration (MIC) of the monoterpene (R)-(+)-Limonene against different strains of *E. clocae.*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Bacterial strain /Substance | *Ecl 41* | *Ecl 42* | *Ecl 43* | *Ecl 44* | *Ecl 45* |
| 1000 μg/mL | **+** | **+** | **+** | **+** | **+** |
| 500 μg/mL | **+** | **+** | **+** | **+** | **+** |
| 250 μg/mL | **+** | **+** | **+** | **+** | **+** |
| 125 μg/mL | **+** | **+** | **+** | **+** | **+** |
| 62.5 μg/mL | **+** | **+** | **+** | **+** | **+** |
| 31.2μg/mL | **+** | **+** | **+** | **+** | **+** |
| Sterility control \_ | **-** | **-** | **-** | **-** | **-** |
| Growth control \_ | **+** | **+** | **+** | **+** | **+** |

(**+)** with growth (**-)** without growth

The Minimum Bactericidal Concentration (MBC) for *E. faecalis strains* was determined from the lowest concentration of the monoterpene that resulted in visible inhibition of the growth of the microorganism. According to Table 3, for the strains tested, it was observed that the values obtained were the same as the MIC (1000 μg/mL).

**Table 3.** Minimum bactericidal concentration (MBC) of the monoterpene (R)-(+)-Limonene against different strains of *E. faecalis.*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Bacterial strain /Substance | *Atcc**29212* | *Ef* 46 | *Ef47* | *Ef 49* | *Ef 50* |
| 1000 μg/mL | **-** | **-** | **-** | **-** | **-** |
| Sterility control \_ | **-** | **-** | **-** | **-** | **-** |
| Growth control \_ | **+** | **+** | **+** | **+** | **+** |

(**+)** with growth (**-)** without growth

For the (R)-(+)-Limonene/antimicrobials association tests, the Ampicillin (APM10), Gentamicin (GEN10), Ciprofloxacin (CIP), and Ceftriaxone (CRO) associations presented the following: an indifferent effect in the presence of Ampicillin, a synergistic effect in the presence of Gentamicin, a synergistic and indifferent effect in the presence of Ciprofloxacin, and an indifferent and antagonistic effect in the presence of Ceftriaxone. The results were obtained using comparative observations for inhibition halos in the presence of the isolated antimicrobials, against inhibition halos in the presence of the antimicrobial/monoterpene associations, as can be seen in Table 4.

**Table 4.** Study of the association of monoterpene (R)-(+)-Limonene with synthetic antimicrobials.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **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(↓) |

**HIATB:** inhibition zone in the presence of the antimicrobial. **RL:** (R)-(+)-Limonene. Synergistic effect (↑); antagonistic effect (↓); indifferent effect (\*).

 It was also observed that neither (R)-(+)-Limonene nor 0.12% chlorhexidine digluconate were able to inhibit biofilm formation at the tested concentrations; as shown in Table 5.

**Table 5.** Minimum 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** | **1:2** | **1:4** | **1:8** | **1:16** | **1:32** | **1:64** | **1:128** |  |
|  |  |  | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |  |
|  **Digluconate in 0.12% chlorhexidine** |
|  | μg/mL |  | **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.

# Discussion

Products from plants have been fomenting great interest in research for many years, as they are potentially useful in the control and reduction of microorganisms that cause losses in food industries (Souza *et al.* 2011). Thus, the antimicrobial activities of (R)-(+)-Limonene have already been proven with different species of food-related microorganisms, such as Staphylococcus aureus, Listeria monocytogenes, Salmonella enterica, Saccharomyces bayanus among others (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, moderate for an MIC of 600 to 1500µg/mL and weak for an MIC above 1500µg/mL. According to the results, the monoterpene (R)-(+)-Limonenedemonstrated moderate antibacterial activity against *E. faecalis strains*, as it obtained an MIC90 of 1000µg/mL. For *E. clocae,* classification was not possible, since the MIC was not found using the concentrations tested.

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,* whereas for *Ef48* the observed action was bacteriostatic.

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 256µg/mL as well as Gram-negative strains (*Pseudomonas aeruginosa*) with an MIC of 512µg/mL.

The (R)-(+)-Limonene/antimicrobial associations study resulted in synergistic, antagonistic, and indifferent effects against different strains of *E. faecalis.* Other studies corroborate this analysis, such as Costa *et al.* (2019) observing that (R)-(+)-Limonene presents synergistic effect when associated with

gentamicin against *S. aureus* and *Escherichia coli*,and 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 (Silva *et al.* 2021).

Studies with other natural products can also be highlighted, such as that of Santana *et al.* (2021) who observed the association of the essential oil of Lavandula Hybrida Grosso with the antimicrobial cephalothin, which showed a synergistic effect. Thus, confirming that this association of natural products natural with synthetic antimicrobials can be considered an important therapeutic option in combating bacterial infections.

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 Souza *et al.* (2021)however*,* has verified the biofilm inhibiting potential of *Lavandula hybrida "Grosso"* essential oilagainst *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.

# Conclusion

Based on these preliminary results, it can be verified that the tested monoterpene does not present effect antibacterial against *E. cloacae* strains and presented an moderate bactericidal effect against *E. faecalis* strains. When associated with different classes of antimicrobials (R)-(+)-Limonene presented synergistic, antagonistic and indifferent effects. This reveals (R)-(+)-Limonene promise for the treatment of bacterial infections, corroborating with conventional therapies. However, further studies are needed to confirm and elucidate its efficacy and mechanisms.

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# Authors' contributions

MSA conducted the experiment, wrote and revised the manuscript. MAAM conducted the experiment and reviewed the manuscript. AAOF supervised the entire process and reviewed the manuscript. BS, PSCC, LMMON, WSM, VRLS, VGSR and HLFP interpreted the results. All authors have read and approved the final manuscript.

**Conflict of interests**

The authors declare no conflicts of interest.

**Ethics Approval**

Not applicable in this document.

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