Universidade do Minho
Escola de Engenharia

CENTRO DE CIÊNCIA E
TECNOLOGIA TÊXTIL

# Eugenol-containing essential oils loaded onto chitosan/polyvinyl alcohol blended films and their ability to eradicate Staphylococcus aureus or Pseudomonas aeruginosa from infected microenvironments

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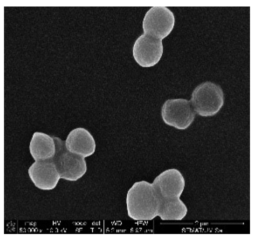
The 1st International Electronic Conference on Antibiotics:
The Equal Power of Antibiotics And Antimicrobial Resistance
**8-17th May 2021**

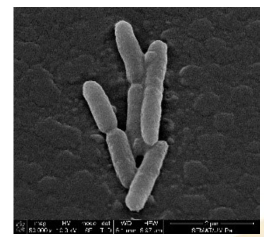
# Infected wounds

Bacteria are primarily responsible for diabetic foot ulcer (DFU)'s infections, being *S. aureus* the most common bacteria isolated (46.4%), followed by *P. aeruginosa* (22.8%)

*S. aureus* is a Gram-positive, commensal bacterium



*P. aeruginosa* is a Gram-negative, invasive bacterium



The increased resistance of bacteria against antibiotics

↓

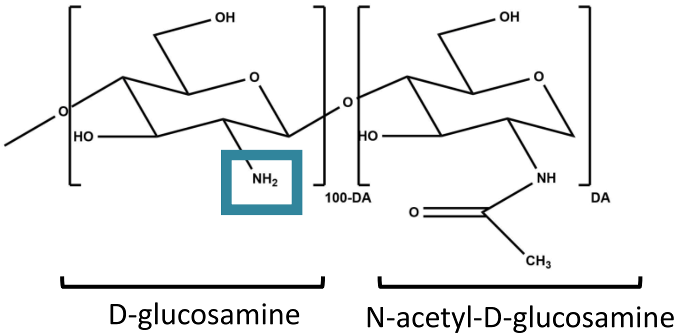
serious concerns about DFU therapeutic strategies

↓

Bio-based treatments with quick bactericidal action
and low tendency to induce resistance are greatly needed.

Tavares, TD, Antunes, JC *et al.*, *Antibiotics* **2020**, *9*(6), 314

# Antibacterial CS




It is suggested that the **antimicrobial activity of the** marine-derived polysaccharide **CS** results from **its cationic nature**

## Antimicrobial mechanisms

- ✓ **Electrostatic interaction** between positively charged R-$NH_3^+$ sites and negatively charged microbial outer **cellular components** and/or cellular membrane leads to cellular impermeability (inhibiting growth) or cellular lysis (killing bacteria). CS internalization and interaction with cytoplasmic constituents may also occur
- ✓ **Chelation of metals**, **suppression of spore elements** and **binding to essential nutrients** to microbial growth interfere with their growth and may contribute to their death

**CS's antimicrobial activity** is **influenced** by **various intrinsic and extrinsic factors**

CS itself (type, Mw, DA, viscosity, solvent and concentration)

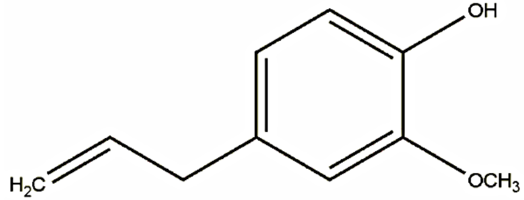
environmental conditions (test strain, its physiological state and the bacterial culture medium, pH, temperature, ionic strength, metal ions)

# Antibacterial CLO and CO

**Essential oils (EOs):**

- aromatic, volatile, lipophilic biomolecules, extracted from regions of plants (e.g. flowers, leaves, twigs, bark, wood, fruits, etc.)
- formed of complex mixtures of hydrophobic molecules, including thymol, carvacrol and eugenol (among others), which exhibit a broad spectrum of antimicrobial activity against bacteria, fungi, and viruses
- potential to replace antibiotics due to their inherent and strong anti-inflammatory, antiseptic, analgesic, spasmolytic, anesthetic, and antioxidative properties

**rich in eugenol** →

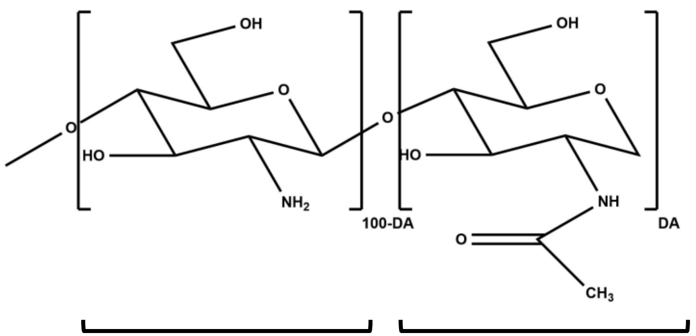



**strong Antibacterial activity**

Tavares, TD, Antunes, JC *et al.*, *Antibiotics* **2020**, *9*(6), 314

# Chitosan (CS) and Poly (vinyl alcohol) (PVA)

## CS

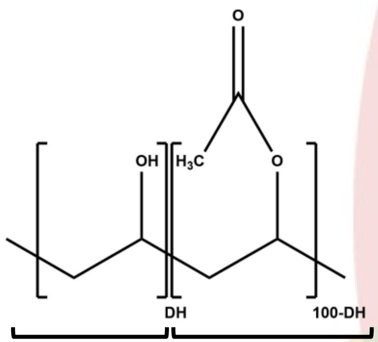



D-glucosamine N-acetyl-D-glucosamine

Natural and crystalline polymer
Biocompatible and biodegradable
Film-forming
High viscosity
Antibacterial and antifungal properties
Ability to absorb exudates

Food and Drug Administration (FDA)-approved as a wound dressing material (topical intended use)

## PVA




Poly (vinyl alcohol) Poly (vinyl acetate)

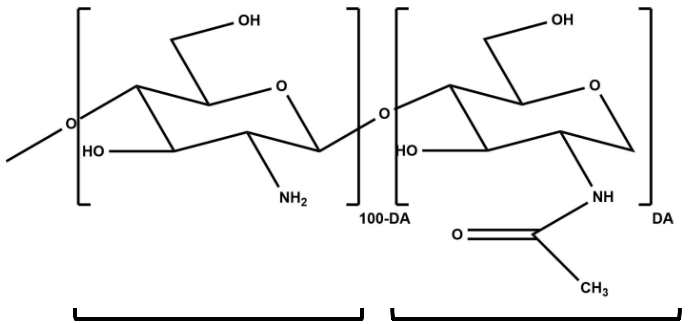
Synthetic and semi-crystalline polymer
Biocompatible and biodegradable
Film-forming
Good mechanical properties: flexibility and swelling capability in aqueous environments
Water-soluble

Multiple FDA-approved medical uses, in the form of transdermal patches, jellies, oral tablets, ophthalmic preparations, intradermal patches and sutures, among others

# Production of CS/PVA films

CS

Antimicrobial properties

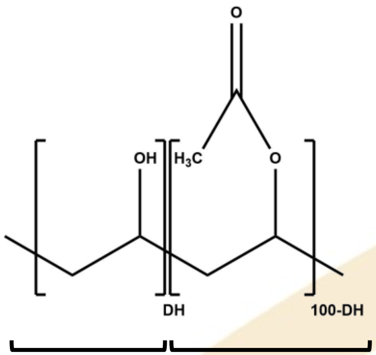



D-glucosamine N-acetyl-D-glucosamine

**Blend**

PVA

Flexibility and hydrophilicity




Poly (vinyl alcohol) Poly (vinyl acetate)

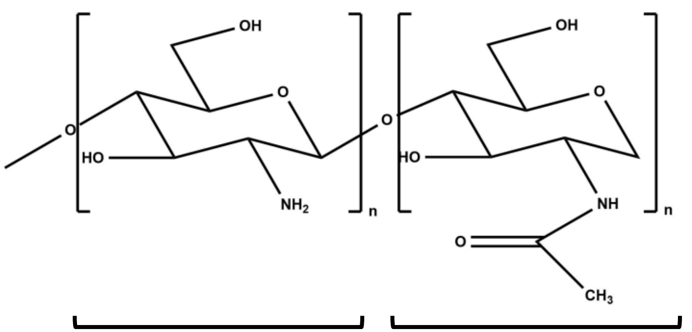
- good capacity to form intermolecular hydrogen bonds
- readily forms hydrogen bonds due to a large number of hydroxyl groups

✓ Increase hydrophilicity, improve mechanical properties

✓ Improve stability in aqueous environments

# Production of CS/PVA films

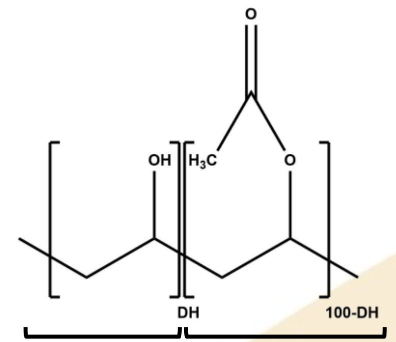
CS




D-glucosamine N-acetyl-D-glucosamine

**Blend**

PVA




Poly (vinyl alcohol) Poly (vinyl acetate)

**Main Applications:**

Food packaging, controlled release of biomolecules, wound dressing, tissue engineering, membrane bioreactors, pervaporation, reverse osmosis, dye removal, fuel cells

# Production of CS/EO/PVA films

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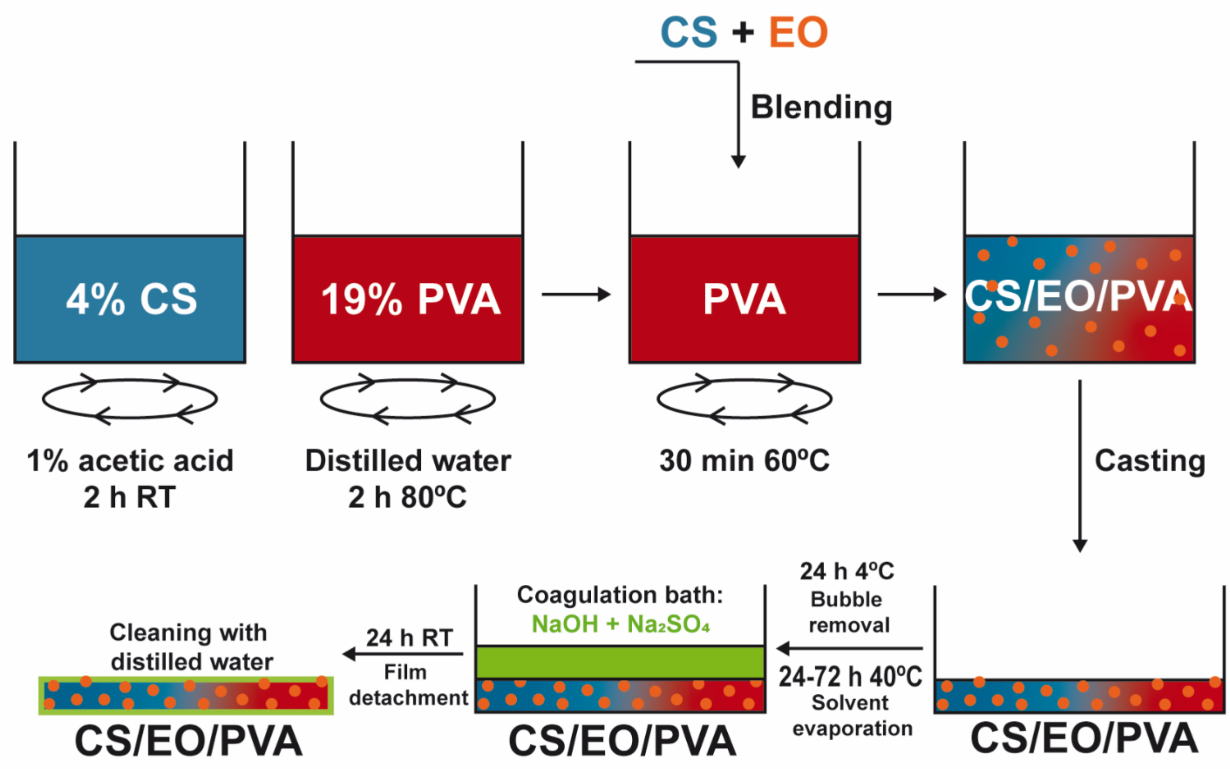
**Solvent Casting + Phase Inversion**

**CS:** 100-300 kDa and 9.6±1.4% DA **PVA:** 72 kDa and 88% DH




JC Antunes *et al.*, Pharmaceutics (2021) doi:10.3390/pharmaceutics13020195

# Production of CS/EO/PVA films

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## Solvent Casting + Phase Inversion

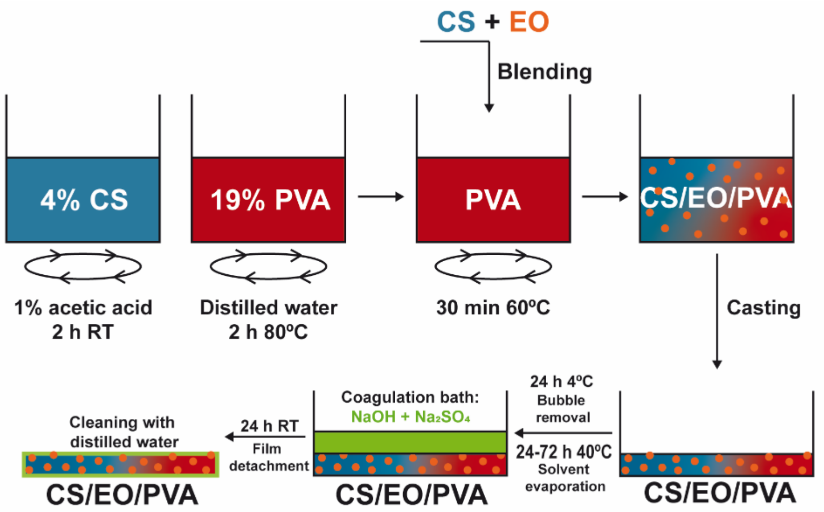


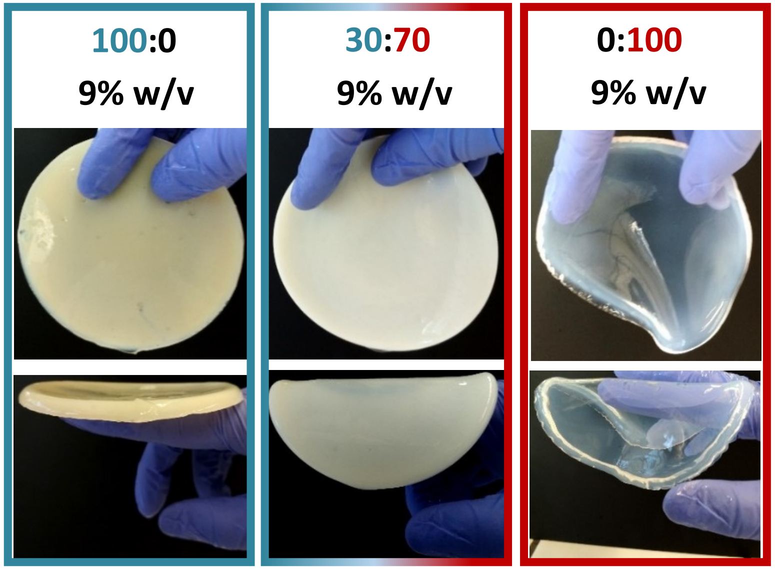

**Table 2.** Data required to build tested CS/EO/PVA blended films, specifically EO loading amount (in µL), mass (g), and volume (mL) of polymer solutions for each case, total mass percent (%*w*/*v*), total volume (mL), and selected CS/PVA mass ratios.

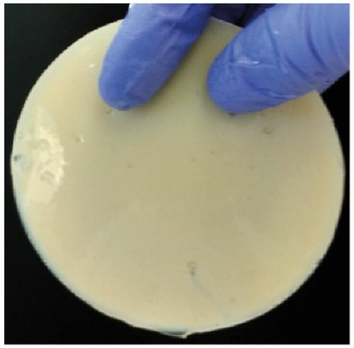
| | EO | | CS Solution | | PVA Solution | | Total % *w*/*v* | $V_{Total}$ (mL) | CS/PVA Mass Ratios |
|---|---|---|---|---|---|---|---|---|---|
| | m (mg) | V (µL) | $m_{CS}$ (g) | V (mL) | $m_{PVA}$ (g) | V (mL) | | | |
| CS | - | - | 3.51 | 39 | - | - | 9 | 39 | 100/0 |
| PVA | - | - | - | - | 3.51 | 39 | 9 | 39 | 0/100 |
| CS/PVA | - | - | 1.05 | 26 | 2.46 | 13 | 9 | 39 | 30/70 |
| CS/PVA/CLO 1% | 35.1 | 39.2 | 1.05 | 26 | 2.46 | 13 | 9 | 39 | 30/70 |
| CS/PVA/CLO 10% | 351.0 | 392.0 | 1.05 | 26 | 2.46 | 13 | 9 | 39 | 30/70 |
| CS/PVA/CO 1% | 35.1 | 33.2 | 1.05 | 26 | 2.46 | 13 | 9 | 39 | 30/70 |
| CS/PVA/CO 10% | 351.0 | 332.0 | 1.05 | 26 | 2.46 | 13 | 9 | 39 | 30/70 |

4% CS and 19% PVA solutions were used.

# Characterization of CS/EO/PVA films

CS | CS/PVA | PVA



| | CS | PVA | CS/PVA | CS/PVA/CLO 1% | CS/PVA/CLO 10% | CS/PVA/CO 1% | CS/PVA/CO 10% |
|---|---|---|---|---|---|---|---|
| Thickness (mm) | 1.73 ± 0.11** | 0.47 ± 0.06 | 0.72 ± 0.02 | 0.95 ± 0.03 | 1.31 ± 0.07* | 0.83 ± 0.04 | 1.08 ± 0.14 |
| DS (%) | 87.45 ± 6.04 | 72.01 ± 6.68 | 85.22 ± 2.93 | 85.33 ± 2.70 | 92.49 ± 0.25* | 86.73 ± 2.10 | 90.96 ± 0.96 |

Statistical significance (**p < 0.005) found through the Kruskal-Wallis test, followed by the Dunn's multiple comparisons test, to compare each unpaired group (n=4).

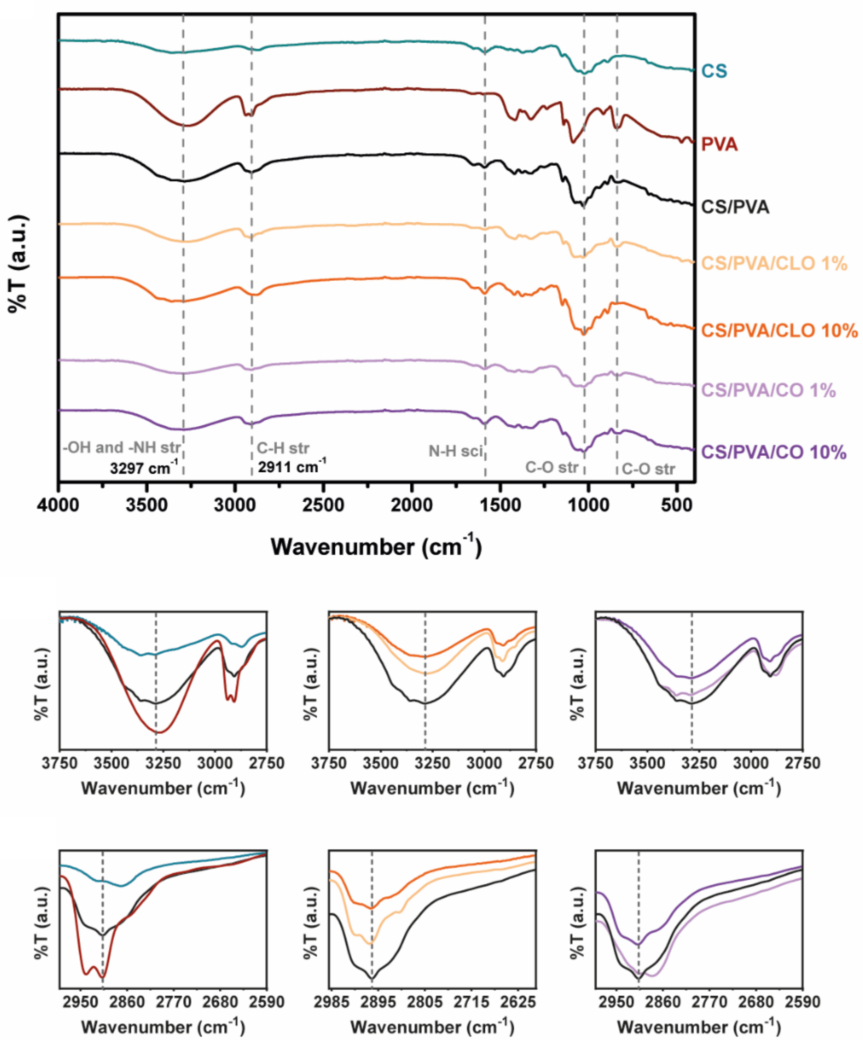
Hydrophobic **EO** loading —**resulted in**→ increased film thickness up to 182 (10% CLO) and overall water retention capacity

**suggesting** ↓

Polymer chain rearrangements and EO entrapment inside the matrix

# Characterization of CS/EO/PVA films

## CS/EO/PVA film:




Peaks of both polymers are present
No new peaks are formed

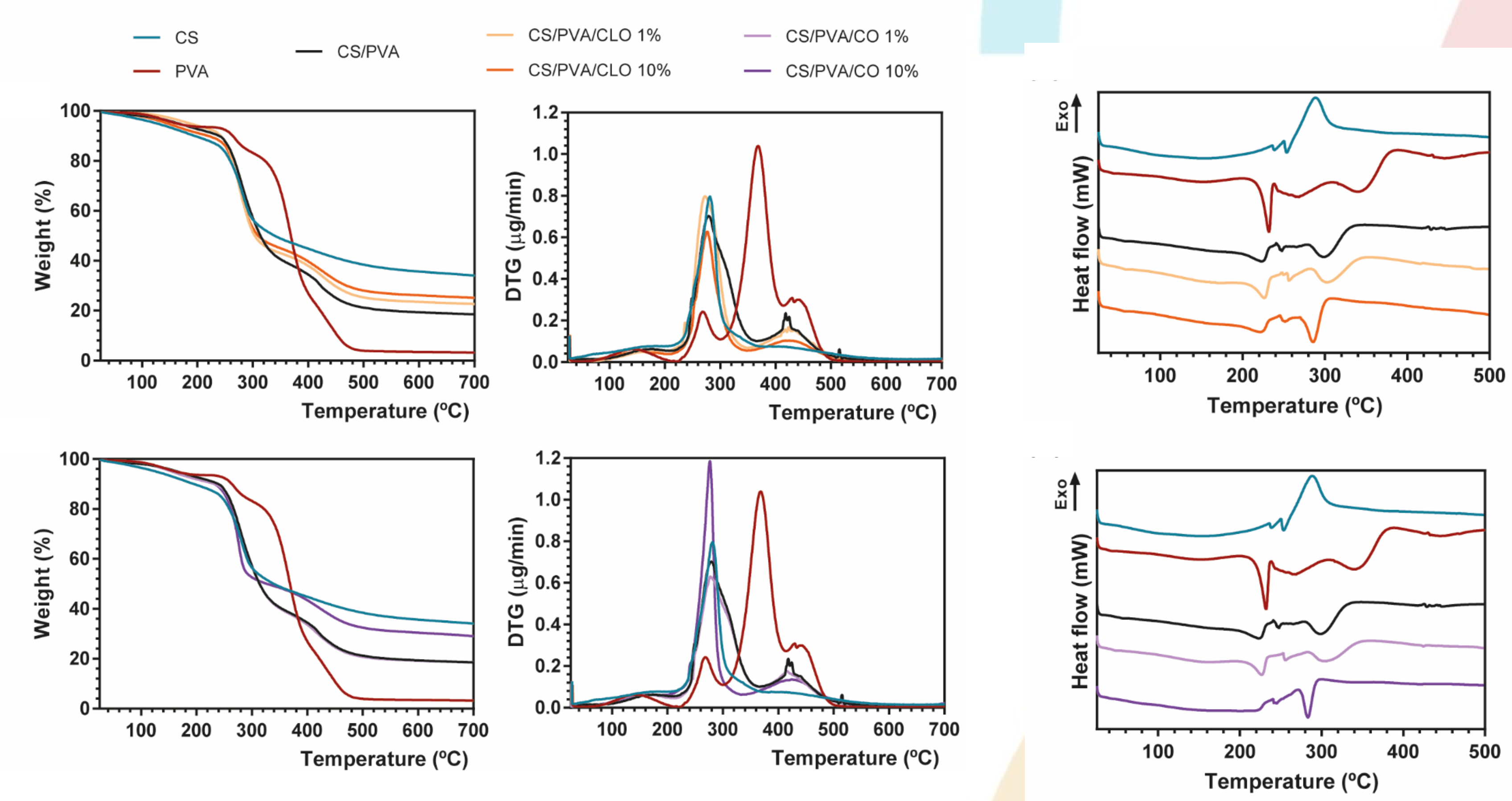
**suggesting**

Polymers blend
Hydrogen bond formation

**suggesting**

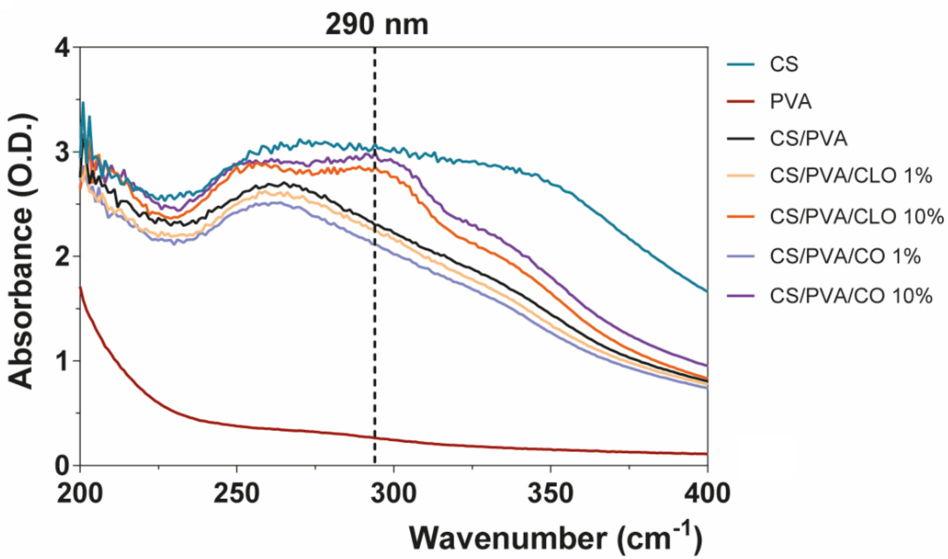
Commitment of free -OH groups with increasing EO amount is noticeable with both EOs

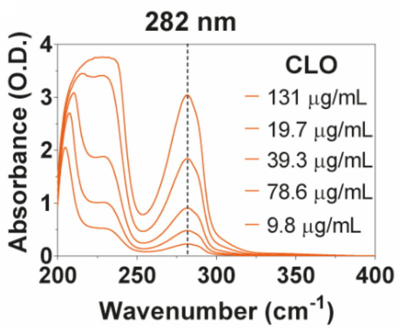
# Characterization of CS/EO/PVA films

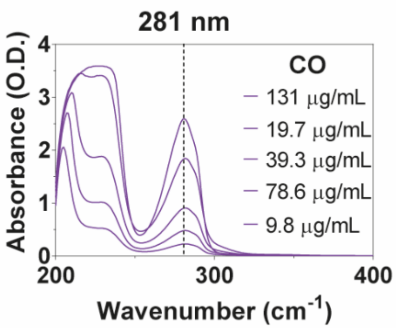



Film's thermal-induced behaviour → **reinforcing** → Polymer blending and EO entrapment

# Characterization of CS/EO/PVA films

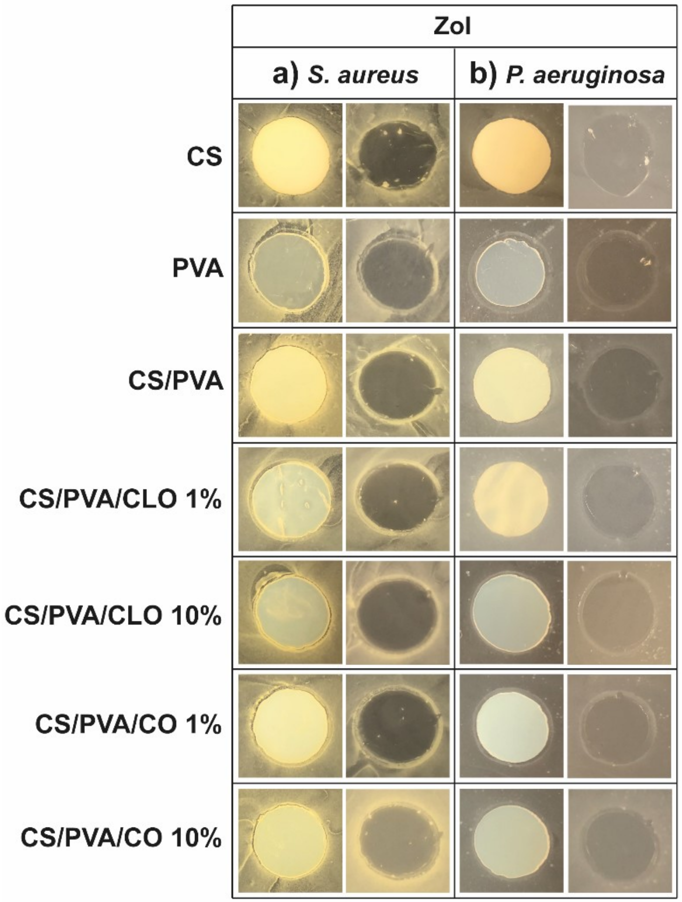



| | EO mass (µg) |
|---|---|
| CS/PVA/CLO 1% | **0.050** |
| CS/PVA/CLO 10% | **0.265** |
| CS/PVA/CO 1% | **0.038** |
| CS/PVA/CO 10% | **0.202** |

# Antibacterial testing



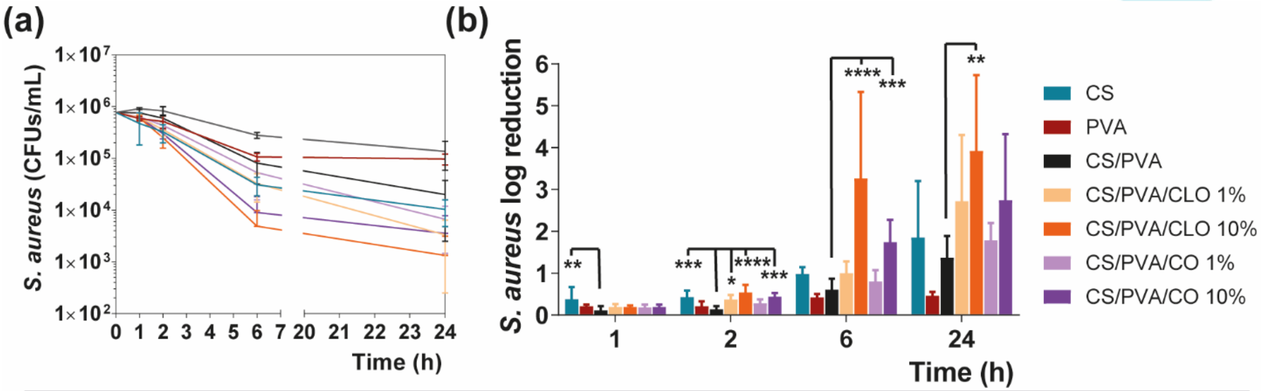

For each bacterium, left images depict films at their original location at the beginning of the assay, along with the bacteria that grew over the incubation period; while on the right, cultured films were carefully removed from the agar so that contact-kill could be visualized.

24h of incubation

↓

Slight antibacterial features

# Antibacterial testing

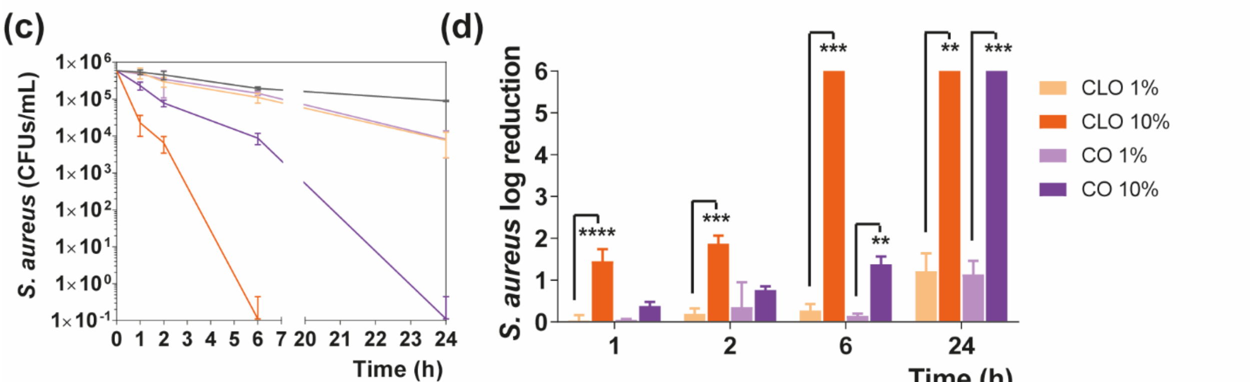



| CS/PVACLO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | | **** |
| 2 | | | | |
| 6 | | | | |
| 24 | | | | |

| CS/PVA/CLO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | *** | |
| 2 | | | | |
| 6 | | | | |
| 24 | | | | |

| CS/PVA/CO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | | *** |
| 2 | | | | * |
| 6 | | | | |
| 24 | | | | |

| CS/PVA/CO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | ** | **** |
| 2 | | | | |
| 6 | | | | |
| 24 | | | | |




| CLO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | | **** |
| 2 | | | | ** |
| 6 | | | | |
| 24 | | | | |

| CLO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | **** | ** |
| 2 | | | *** | |
| 6 | | | | |
| 24 | | | | |

| CO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | | *** |
| 2 | | | | ** |
| 6 | | | | |
| 24 | | | | |

| CO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|
| 1 | | | ** | **** |
| 2 | | | | ** |
| 6 | | | | |
| 24 | | | | |

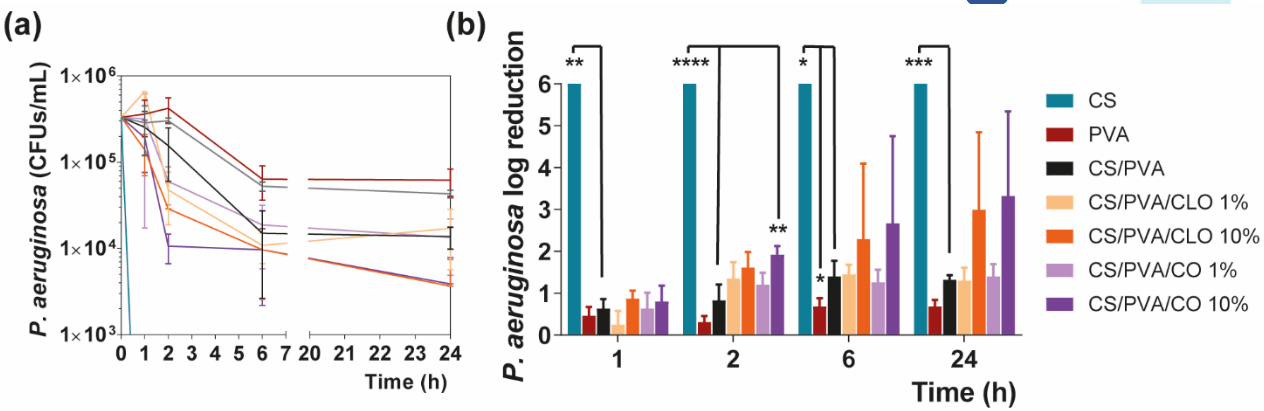
**CS/CLO 10%/PVA film:**

the most effective,
right after 6h with 10% EO

**CS film:**

quickest AM action
within 1h of incubation

# Antibacterial testing




| | CS/PVA/CLO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CS/PVA/CLO 1% | 1 | | | * | |
| | 2 | | | | |
| | 6 | | | | |
| | 24 | | | | |

| | CS/PVA/CLO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CS/PVA/CLO 10% | 1 | | | | *** |
| | 2 | | | | |
| | 6 | | | | |
| | 24 | | | | |

| | CS/PVA/CO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CS/PVA/CO 1% | 1 | | | | |
| | 2 | | | | |
| | 6 | | | | |
| | 24 | | | | |

| | CS/PVA/CO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CS/PVA/CO 10% | 1 | | *** | | ** |
| | 2 | | | | |
| | 6 | | | | |
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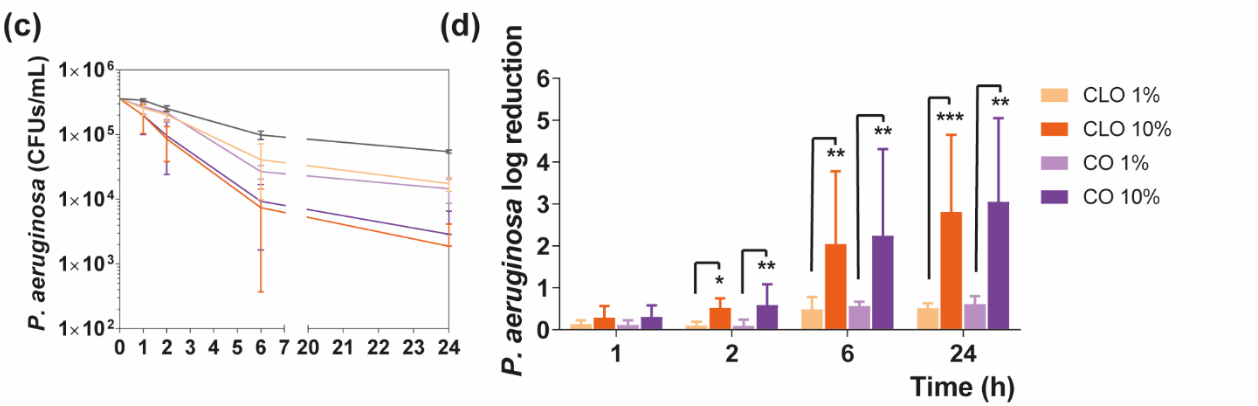


| | CLO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CLO 1% | 1 | | | | * |
| | 2 | | | * | ** |
| | 6 | | | | |
| | 24 | | | | |

| | CLO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CLO 10% | 1 | | | ** | **** |
| | 2 | | | * | ** |
| | 6 | | | | |
| | 24 | | | | |

| | CO 1% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CO 1% | 1 | | | ** | ** |
| | 2 | | | *** | *** |
| | 6 | | | | |
| | 24 | | | | |

| | CO 10% | 1 | 2 | 6 | 24 |
|---|---|---|---|---|---|
| CO 10% | 1 | | | ** | *** |
| | 2 | | | | * |
| | 6 | | | | |
| | 24 | | | | |

## CS/EO/PVA film:

10% CO led to a clear bactericidal trend after 2h of contact

## CS film:

Complete bacterial elimination in 1h, effect that endured until tested 24h

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# Conclusions and Future Work

- ✓ CS/PVA blended films were successfully built;
- ✓ CS and both EOS, the CLO and CO, show antibacterial activity against *S. aureus* and *P. aeruginosa;*
- ✓ The EOs were successfully incorporated in the CS/PVA films at 1 and 10%wt;
- ✓ CLO-loaded CS/PVA films showed evidently bactericidal effects right after 2h of direct contact with the bacteria, being significantly more efficient than unloaded films until the tested 24h.
- ✓ Films with 100% CS were particularly more effective than 10% EOO-loaded films against *P. aeruginosa*, by completely eradicating it during the first hour of incubation.

Future work will be directed towards a balance between AM action of CS and its mechanical hindrance after processing, together with the combination with the EOs to an intensified antimicrobial profile against both bacteria.

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Co-P.I. Professor M. Teresa P. Amorim
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