# Nanoemulsion coatings and use of antimicrobial ginger essential oil on citrus

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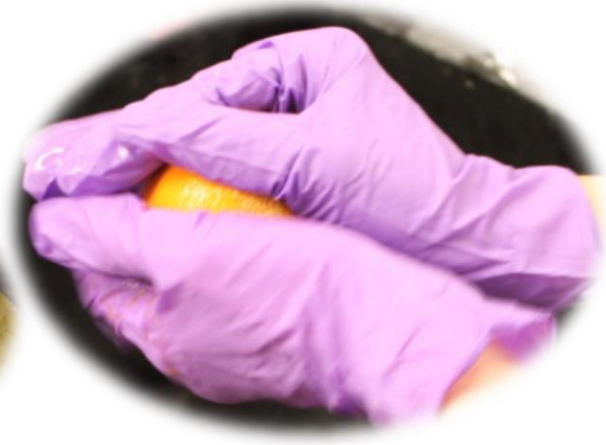


FAPESP CAPES unesp Embrapa Instrumentação

# Introduction

- ✓ Shellac and carnauba wax are commonly used on citrus, carnauba is more permeable to gases, less permeable to water, shellac is more shiny but can cause off-flavor
- ✓ Carnauba wax emulsion coatings are usually microemulsions, but the smaller the lipid mycelles, the more stable and shiny the coating, thus tested a nanoemulsion carnauba coating.
- ✓ Ginger (rhizome extracts) was found to reduce microorganisms in food
- ✓ Objective was to evaluate effects of (ginger) antimicrobial/nanoemulsion edible coatings to preserve fruit quality and decrease decay

# Antimicrobial activity: Ginger extracts

✓**Ethanolic extracts of essential oil**

**40 Kg 'Gigante' ginger**
**Piedade-SP, Brazil**

- ✓ Freeze-dried ginger
- ✓ Air oven dried ginger
- ✓ Fresh ginger

200 RPM at 25 °C 48h
+ rotary evaporator at 40 °C

EtOH 96%

**Ethanolic extracts**

EtOH 70%

**Hydroethanolic extracts**

Steam distillation
Clevenger apparatus
24h

**Ginger Essential oil**

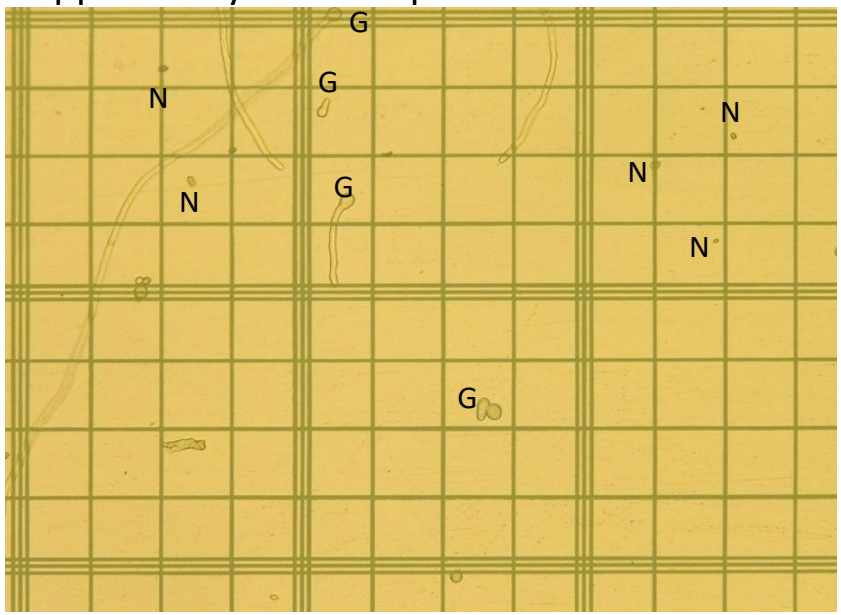
# Antimicrobial activity for ginger ethanolic extracts and essential oil

✓**Initial screening: concentration 1% and 3%**

## ✓Spore germination inhbition

***Penicillium digitatum***

- PD (potato dextrose) broth + Sample + spore suspension
- Final concentration of spores solution $10^6$
- Incubated at 25 °C by 120 RPM agitation
- Reading after 24 hours in Neubauer chamber supported by microscope with camera




500x magnification

N: not germinated; G: germinated

**Table 1. Percentage of germinated spores of P. digitatium, after 24 h incubation in ginger essential oil and alcoholic extracts.**

| | Samples | 1% | 3% |
|---|---|---|---|
| 1 | EtOH96 fresh | 18.1 bc | **2.9 a** |
| 2 | EtOH96 freeze-dried | 35.7 d | **1.2 a** |
| 3 | EtOH96 oven | 32.2 d | **0.6 a** |
| 4 | EtOH70 fresh | 24.6 c | 25.1 b |
| 5 | EtOH70 freeze-dried | 23.4 c | 19.9 b |
| 6 | EtOH 70oven | 14.6 b | **2.3 a** |
| 7 | GEO fresh | **0 a** | **0 a** |
| 8 | GEO freeze-dried | **0 a** | **0 a** |
| 9 | GEO air oven | **0 a** | **0 a** |
| 10 | GEO commercial | **5.3 a** | **0.6 a** |
| 11 | Negative control | 37.4 d | 37.4 c |
| 12 | Positive control | 0.6 a | 0.6 a |
| 13 | DMSO control | 35.7 d | 35.7 c |
| 14 | PVP control | 36.3 d | 35.7 c |

Columns with different letters are significantly different by Duncan test ($p<0.05$) applied after Anova.

➢**Ethanolic extracts at 3% and GEO at 1 and 3% showed the highest inhibition of spore germination. Commercial GEO had lowest activity compared to extracted GEO.**

# Antimicrobial activity

## ✓Poisoned food technique (test essential oils vs fungi)

***Penicillium digitatum***

- Samples into in solid medium (PDA)
- 10uL of spores suspension $10^5$
- Incubated at 25 °C

➢**Ethanolic and Hydroethanolic extracts did not exhibit antifungal activity at 1% and 3%**

➢ **GEO showed the highest mycelial zone inhibition**

- Oil was dissolved in Dimethyl sulfoxide (DMSO) and alcoholic extracts in Polivinilpirrolidone (PVP)

- Tween 80 (100 µL to 100mL of medium) to disperse the sample in the medium.

**Table 2. Percentage of mycelial zone inhibition compared to control after 5 days.**

| | Samples | 1% | 3% |
|---|---|---|---|
| 1 | EtOH96 fresh | * | * |
| 2 | EtOH96 freeze-dried | * | * |
| 3 | EtOH96 oven | * | * |
| 4 | EtOH70 fresh | * | * |
| 5 | EtOH70 freeze-dried | * | * |
| 6 | EtOH oven | * | * |
| 7 | GEO fresh | 11.58 | 12.24 |
| 8 | GEO freeze-dried | 23.82 | 27.59 |
| 9 | GEO air oven | 10.08 | 21.94 |
| 10 | GEO commercial | 2.35 | 3.10 |
| 11 | Negative control(water) | * | * |
| 12 | Positive control (Cyclohemide) | 100.00 | 100.00 |
| 13 | DMSO control | * | * |
| 14 | Tween 80 control | * | * |
| 15 | PVP control | * | * |

*No inhibition was observed for *Penicilium digitatium*.

# Antimicrobial activity

## ✓Inverted Petri dish test

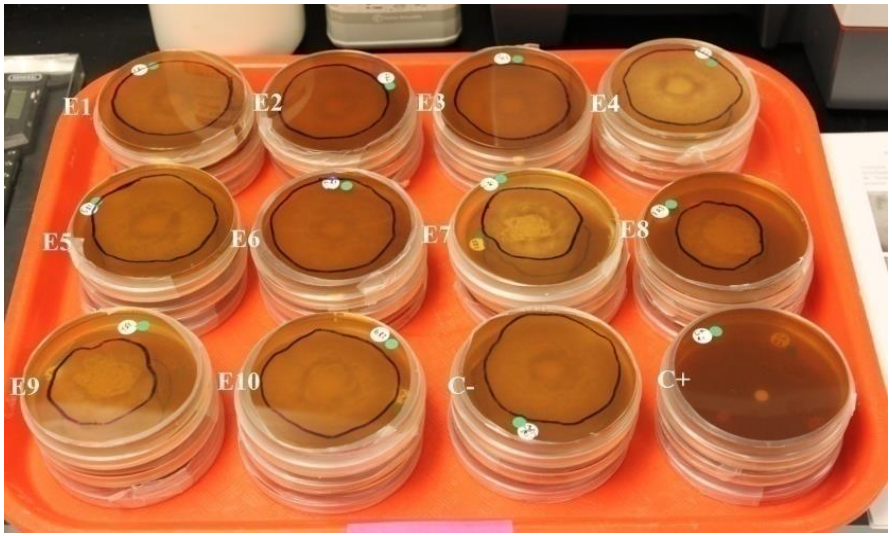



**Figure**: *Penicillium digitatum,* Neg. control: and Positive control: ammonia

- Samples placed on 6mm disc on the Petri dishes lid
- 10uL of spores suspension $10^5$ on PDA medium
- Incubated at 25 °C

**Table 3. Percentage of mycelial zone inhibition compared to control after 5 days.**

| | Samples | 0.5% | 1% | 3% |
|---|---|---|---|---|
| 7 | GEO fresh | 8.8 | 9.4 | **34.1** |
| 8 | GEO freeze-dried | 7.9 | 7.3 | **52.6** |
| 9 | GEO air oven | 0 | 1.3 | **36.1** |
| 10 | GEO commercial | 0 | 5.3 | **11.6** |

- **Ginger oil is rich in sesquiterpenes and monoterpenes**
  - **Zingiberene and geranial usually are the major constituents and theirs levels make the oil more potent**
  - **Investigation into the levels of these and other compounds is underway to explain the different antifungal activity between the samples in this study**

# Antimicrobial activity

✓**Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)**

- **MIC –** Macrodilution-NCCLS (2002)
- Samples in PD broth , spores suspension $10^6$
- Incubated at 25 °C by 125 RPM
- Reading after 7days

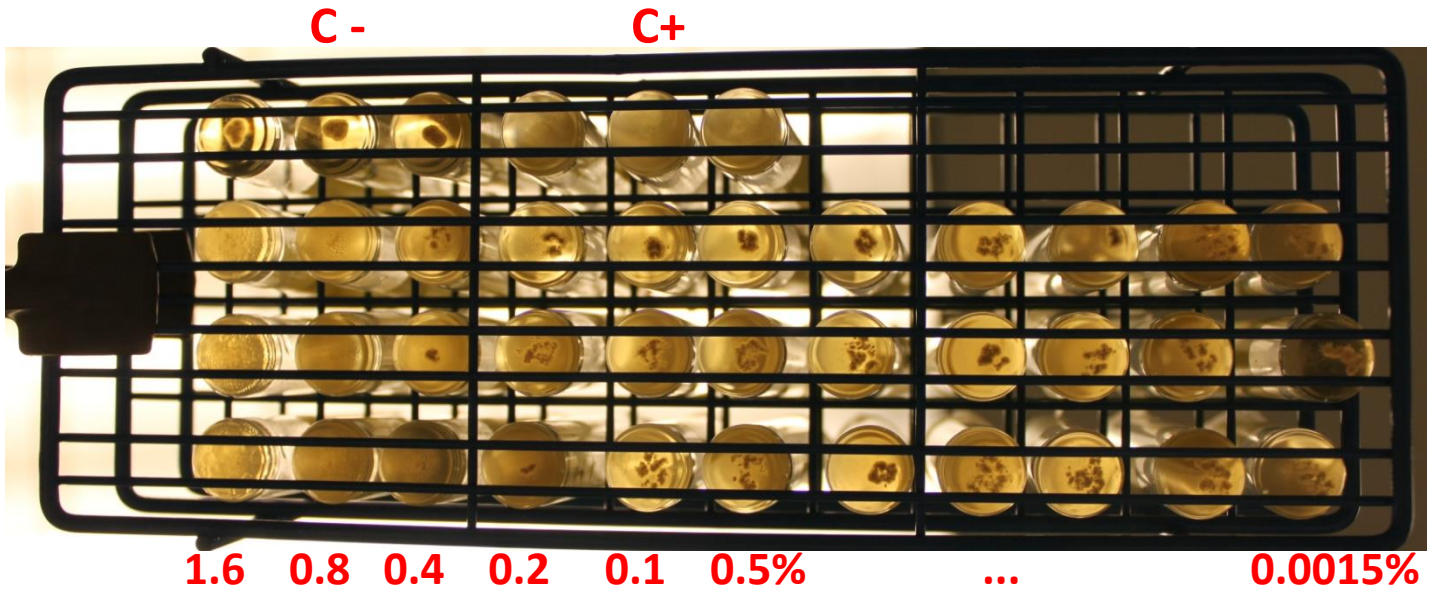
**Green mold**
***Penicillium digitatum***

**Anthracnose**
***Colletotrichum. gloeosporioides***

**Assessed concentrations :**

Ethanolic extracts: 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 %

GEO: 1.6, 0.8, 0.4, 0.2, 0.1, 0.5, 0.025, 0.0125, 0.0062, 0.0031, 0.0015%




10uL → **MFC**

**Figure**. MIC_GEO-fresh_***P. digitatium***

- **MFC** – Placed 10 uL from MIC tube and subsequent higher concentration on PDA medium

# Antimicrobial activity

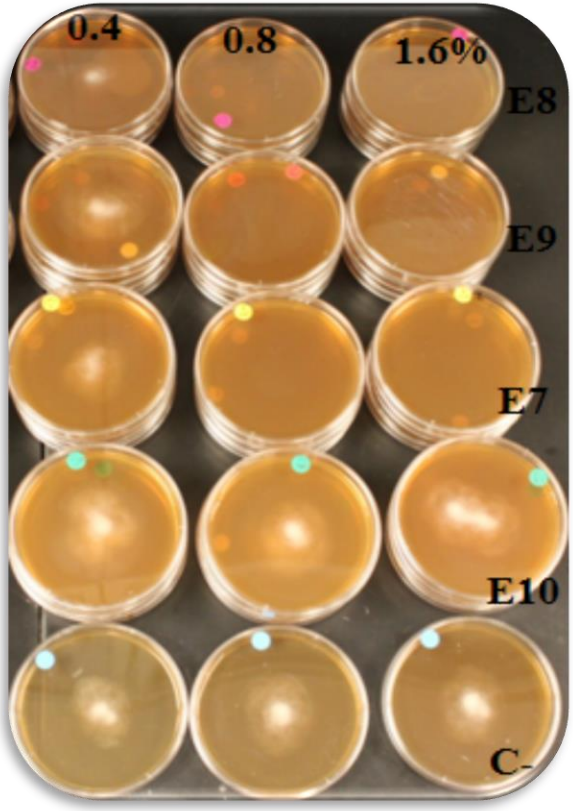



**Figure**. MFC_GEO- *P. digitatium*

**Table 4. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Penicilium digitatium* and *Colletotrichum gloeosporioides* after 7 days of incubation.**

| | | MIC | MFC | MIC | MFC |
|---|---|---|---|---|---|
| | **Samples** | ***Penicilium*** | | ***Colletotrichum*** | |
| E1 | Ethanolic extract-fresh ginger | 2.5% | * | 4.0% | 6.0% |
| E2 | Ethanolic extract-freeze-dried | 2.5% | * | 2.5% | 5.0% |
| E3 | Ethanolic extract- air oven | 2.5% | * | 2.5% | 5.0% |
| | Ethanol 96% control | 3.0% | * | 4.0% | 5.0% |
| E4 | HydroEthanolic extract-fresh ginger | 3.0% | * | 5.0% | * |
| E5 | HydroEthanolic extract-freeze-dried | 3.0% | * | 3.0% | 4.0% |
| E6 | HydroEthanolic extract- air oven | 2.5% | * | 2.5% | 4.0% |
| | Ethanol 70% control | 4.0% | * | 5.0% | 6.0% |
| E7 | GEO –fresh ginger | **0.4%** | 0.8% | **0.4%** | 0.4% |
| E8 | GEO - freeze-dried ginger | **0.4%** | 0.8% | **0.1%** | 0.2% |
| E9 | GEO - air oven | **0.4%** | 0.8% | **0.8%** | 1.6% |
| E10 | GEO commercial | 0.8% | * | 1.6% | * |

*No total inhibition at the highest concentration tested

# Antimicrobial activity

- Ethanolic and hydroethanolic extracts did not show fungicide activity against *P. digitatium*, however exhibited antifungal activity against C. *gloeosporioides* between 4 and 6%

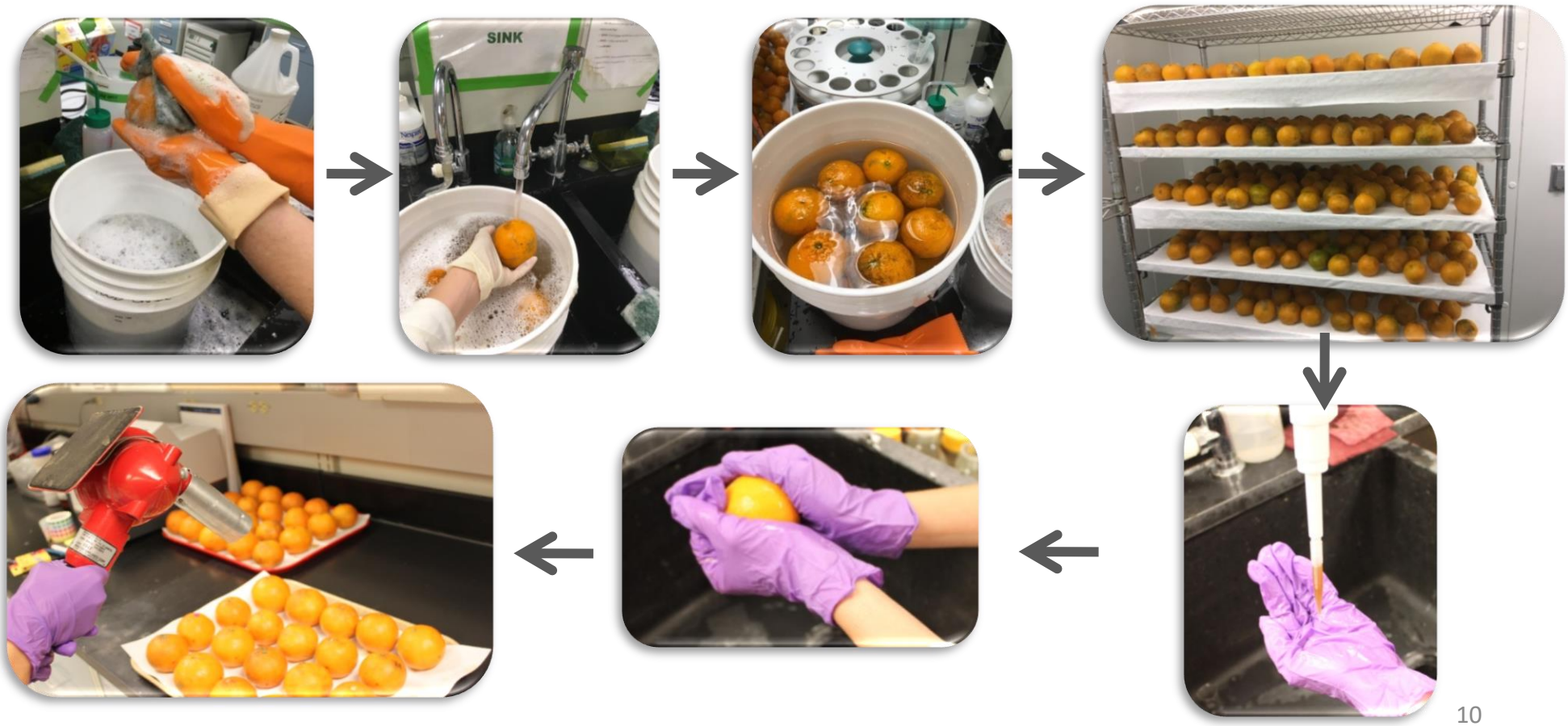
- Ethanolic and Hydroethanolic extracts did not exhibit antifungal activity in the 1% to 3% range in the poisoned food test, however they showed MIC at 2.5 and 3% in pd broth.
  - More studies are needed to study the diffusibility and evaporation of ethanol for extracts in solid systems to improve their performance and minimize ethanol activity .

- GEO fresh, freeze dried or air oven dried showed higher antimicrobial activity toward both microorganisms evaluated. Commercial GEO had lower activity compared to extracted GEO.

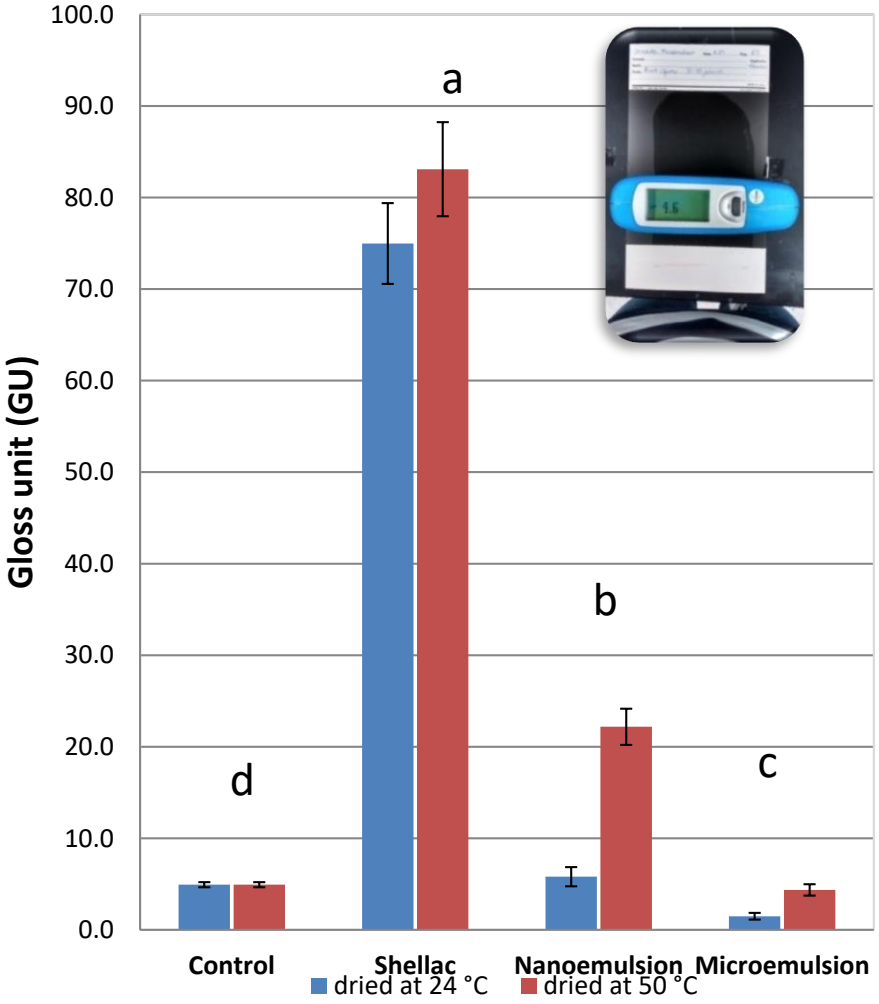
- GEO-air oven was selected for incorporation into a nanoemulsion coating because it had the highest yield (3.5 % w/w) compared to essential oil from fresh or freeze-dried ginger (0.2 % and 3.2%, respectively).

# Evaluation of coatings on citrus fruit

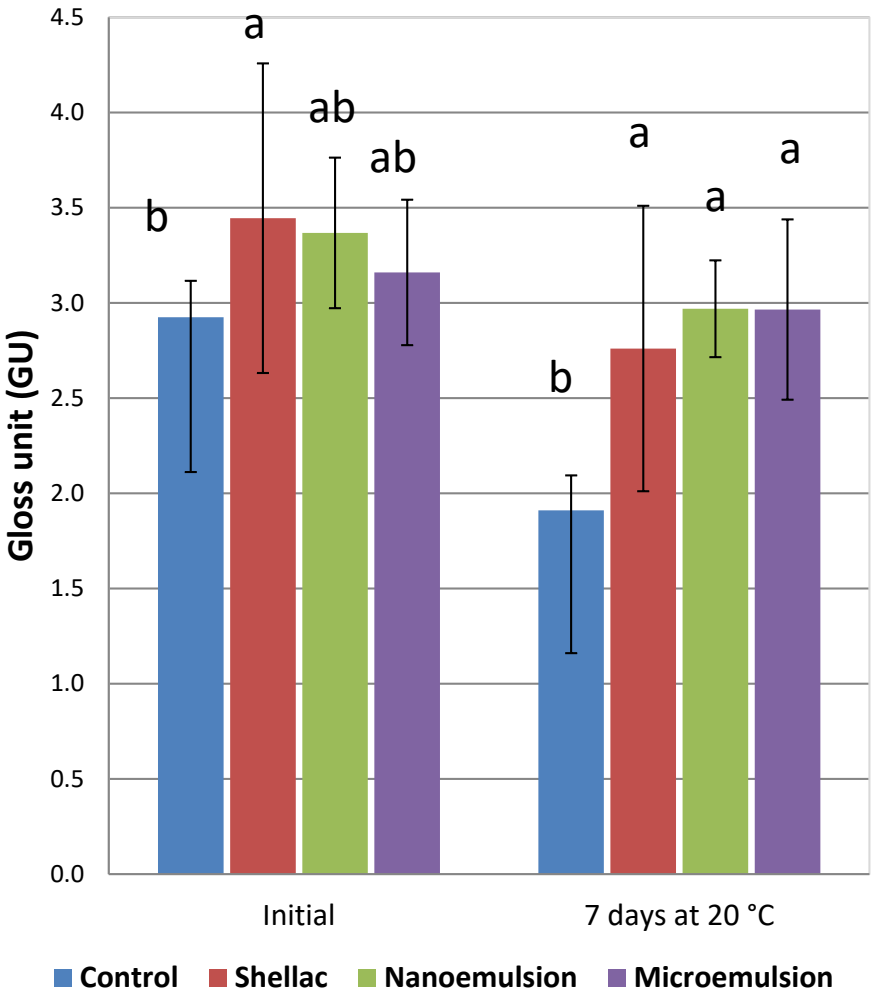
- **Experiment 1- ‘Nova’ mandarin**
  - Commercial carnauba and shellac microemulsions and an experimental carnauba nanoemulsion coating were compared to an uncoated control for fruit stored 7 d at 20 °C
  - Quality analyses were done for initial (0d) and at the end of storage (7d)



# Exp.1: Quality results - gloss

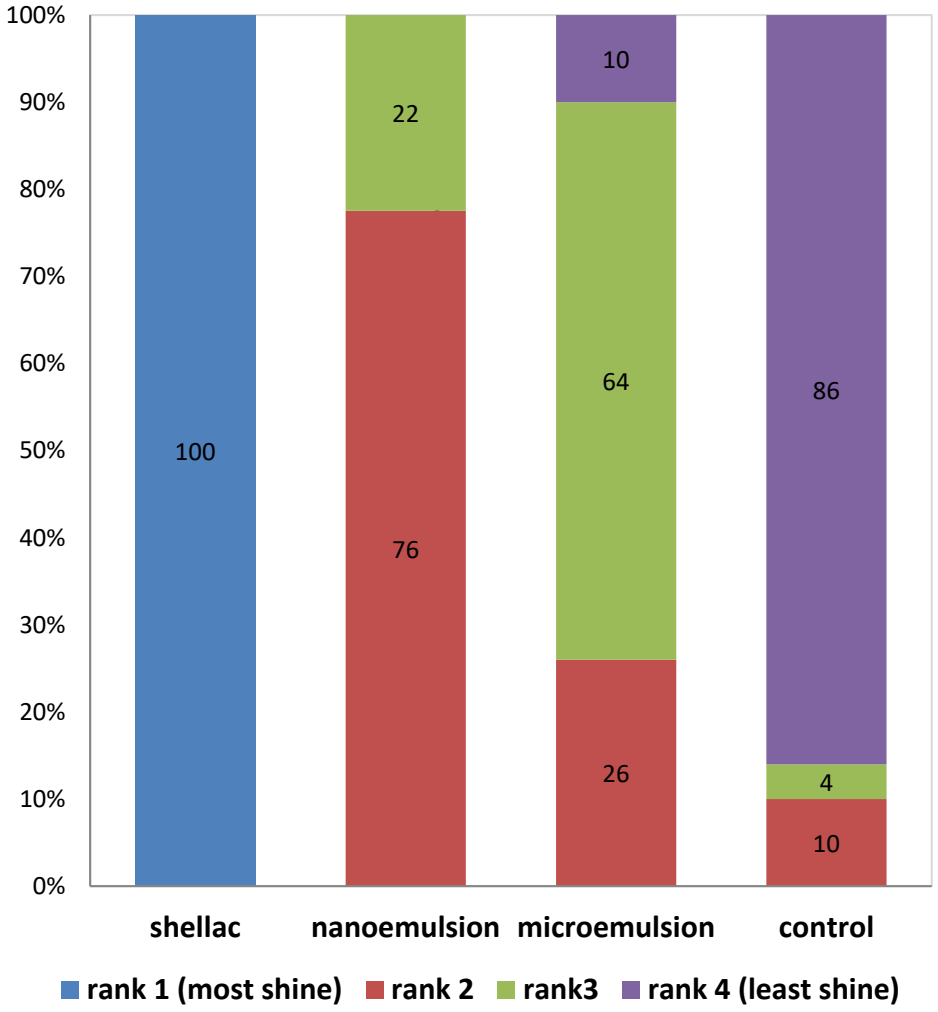



Columns with different letters are significantly different by Duncan test ($p<0.05$) applied after Anova.

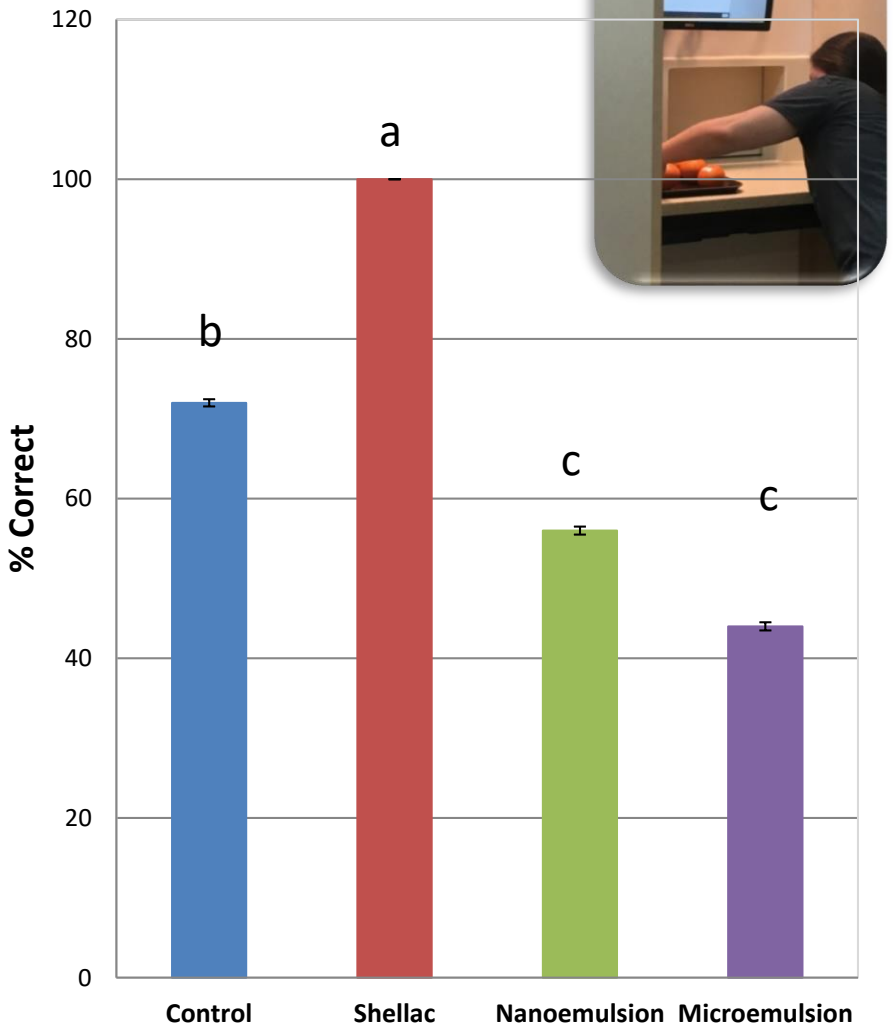



For each storage period, columns with different letters are significantly different by Duncan test ($p<0.05$) applied after Anova.

# Exp.1: Sensory Ranking for shine and tetrad test

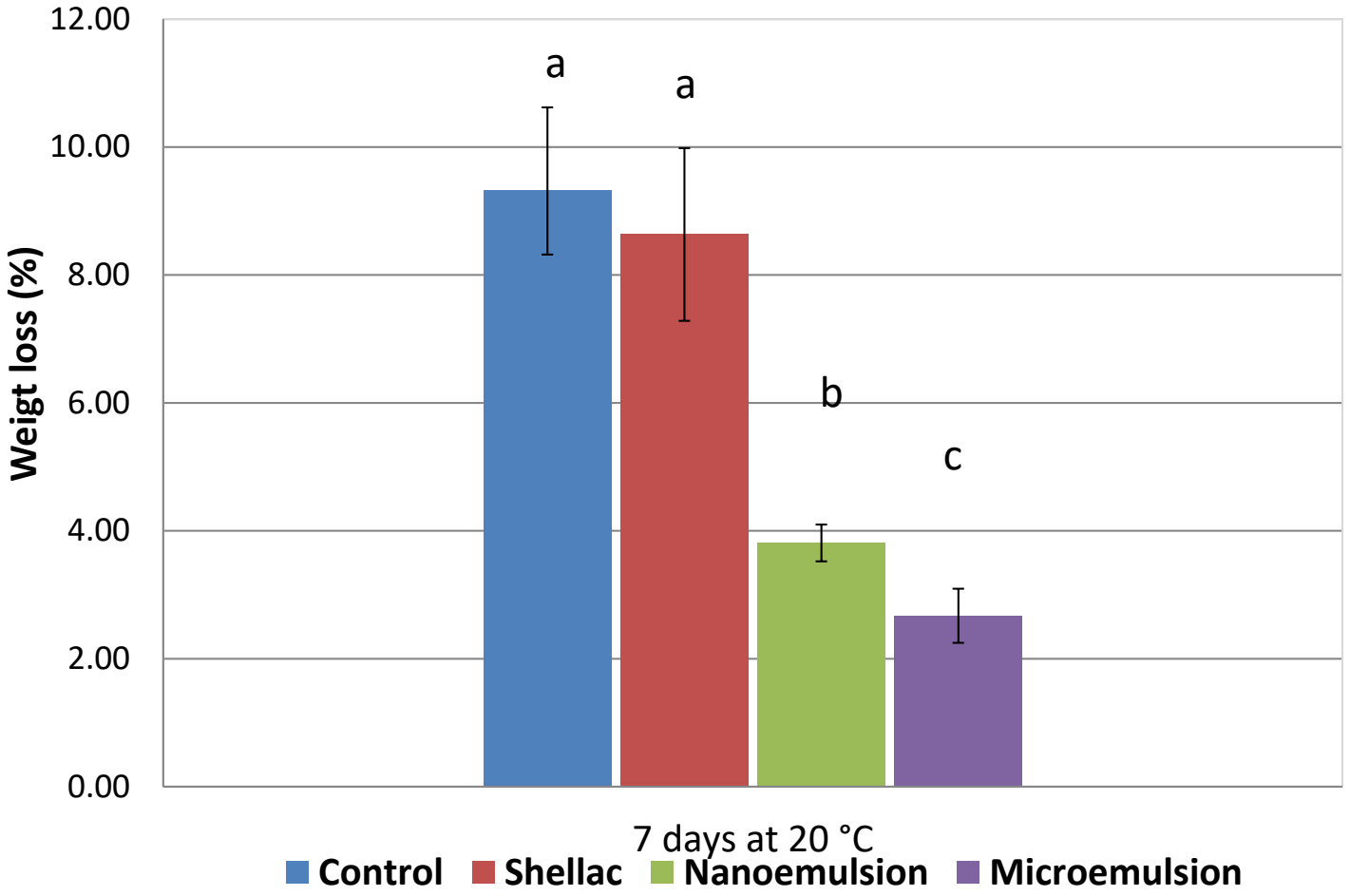



Columns with different letters are significantly diferent by critical absolute rank sum differences table at p<0.05 (Newell and MacFarlane, 1987),



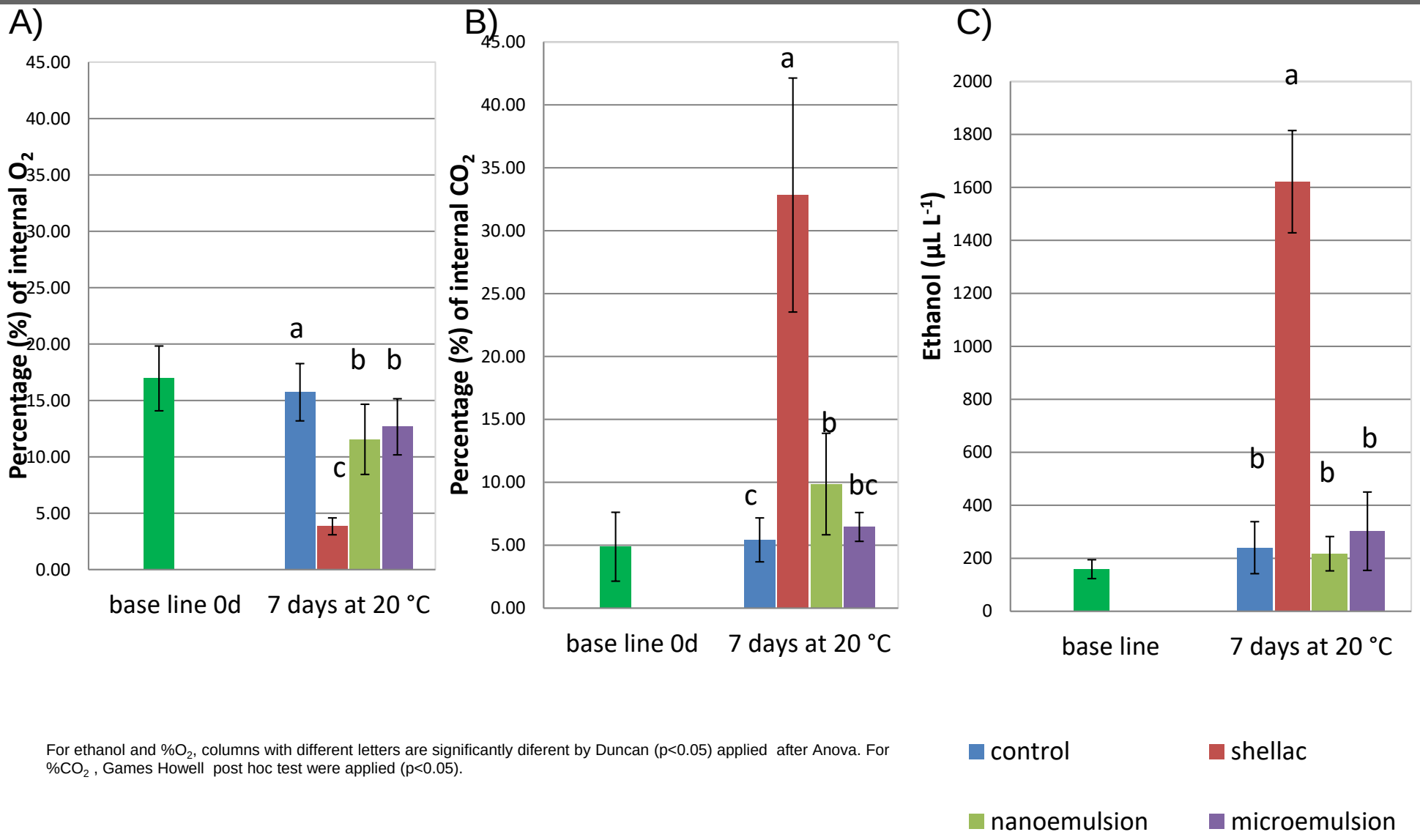

Columns with different letters are significantly diferent by approximation equation for tetrad (Z-test) at p=0.05,

# Exp.1: Weight loss




Columns with different letters are significantly diferent by Games Howell ($p<0.05$) applied after Anova.

# Exp.1: Internal $CO_2$ , $O_2$ and ethanol




For ethanol and $\%O_2$, columns with different letters are significantly diferent by Duncan ($p<0.05$) applied after Anova. For $\%CO_2$ , Games Howell post hoc test were applied ($p<0.05$).

# Aroma Volatiles for Nova mandarins

| Class | Compound | Base line | Control | Shellac | Nano carnauba | Micro carnauba |
|---|---|---|---|---|---|---|
| | | 0 day | 7 days at 20 °C | | | |
| Aldehydes | Acetaldehyde | 0.427 | 0.475* a | 0.584 a | 0.466 a | 0.489 a |
| | Hexanal | 0.013 | 0.009 bc | 0.007 c | 0.015 a | 0.013 ab |
| | Octanal | 0.012 | 0.015 a | 0.014 a | 0.018 a | 0.012 a |
| | Decanal | 0.343 | 0.344 b | 0.398 b | 0.569 a | 0.352 b |
| Alcohols | Methanol | 26.266 | 26.560 b | 67.170 a | 26.852 b | 44.283 b |
| | Ethanol | 158.705 | 240.010 b | 1621.475 a | 217.180 b | 302.219 b |
| | 2-MethyPropanol | 0.168 | 0.289 b | 1.753 a | 0.217 b | 0.350 b |
| | Hexanol | 0.110 | 0.087 b | 0.114 b | 0.156 a | 0.092 b |
| | cis-3-Hexenol | 0.173 | 0.240 b | 2.671 a | 0.298 b | 0.323 b |
| | trans-2-Hexenol | 0.003 | 0.026 a | 0.006 b | 0.002 b | 0.009 b |
| | Linalool | 0.132 | 0.136 a | 0.148 a | 0.128 a | 0.117 a |
| | Octanol | 0.414 | 0.870 a | 0.738 a | 1.010 a | 0.652 a |
| | Terpinen-4-ol | 0.083 | 0.079 a | 0.047 c | 0.063 b | 0.060 bc |
| | α-Terpineol | 0.117 | 0.278 a | 0.171 b | 0.108 c | 0.200 b |
| Terpenes | α-Pinene | 0.026 | 0.059 a | 0.049 a | 0.056 a | 0.046 a |
| | Sabinene | 0.004 | 0.005 b | 0.005 b | 0.013 a | 0.005 b |
| | Myrcene | 0.170 | 0.297 a | 0.297 a | 0.358 a | 0.225 a |
| | Limonene | 3.509 | 5.300 a | 5.119 a | 6.732 a | 3.909 a |
| | γ-Terpinene | 0.002 | 0.003 a | 0.004 a | 0.003 a | 0.003 a |
| | Valencene | 0.461 | 0.273 a | 0.284 a | 0.298 a | 0.254 a |
| Esters | Methyl Butanoate | 0.003 | 0.005 b | 0.038 a | 0.004 b | 0.005 b |
| | Ethyl Butanoate | 0.287 | 0.186 a | 0.679 a | 0.530 a | 7.258 a |
| | Ethyl Hexanoate | 0.019 | 0.080 a | 0.080 a | 0.112 a | 0.059 a |
| | Ethyl 3-hydroxyhexanoate | 2.792 | 2.414 b | 2.965 b | 3.936 a | 2.765 b |
| | Ethyl Acetate | 0.812 | 0.697 ab | 0.503 b | 0.957 a | 0.782 a |
| Ketones | Acetone | 68.129 | 87.555 b | 715.801 a | 101.312 b | 134.433 b |

*Values followed by the same letter within rows are not significantly different by Duncan´s test at the 0.05 level.

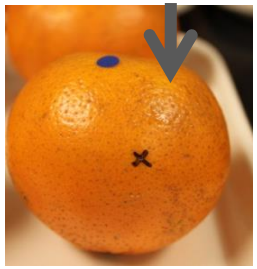
# Incorporation of ginger oil into coatings for effect on citrus quality and disease development

- **Experiment 2- ´Unique' ortanique tangor ('Valencia' orange/tangerine) quality**
  - Repetition of exp.1 with an **additional treatment of nanoemulsion containing 0.8% ginger oil** stored at 10 °C for 14 d followed by a simulated marketing period (7 d at 20 °C)

**Exp.3 - Natural decay and *P. digitatum*-inoculated 'Unique'** tangors stored at 20 °C for 31 days and 21 days, respectively, coated with:

1) carnauba nanoemulsion coating; 2) carnauba nanoemulsion coating + 0.8% GEO; 3) carnauba nanoemulsion coating + 0.8% commercial GEO; 4) 0.8% GEO; 5) 0.8% commercial GEO and 6) uncoated/untreated control

Sanitized fruit



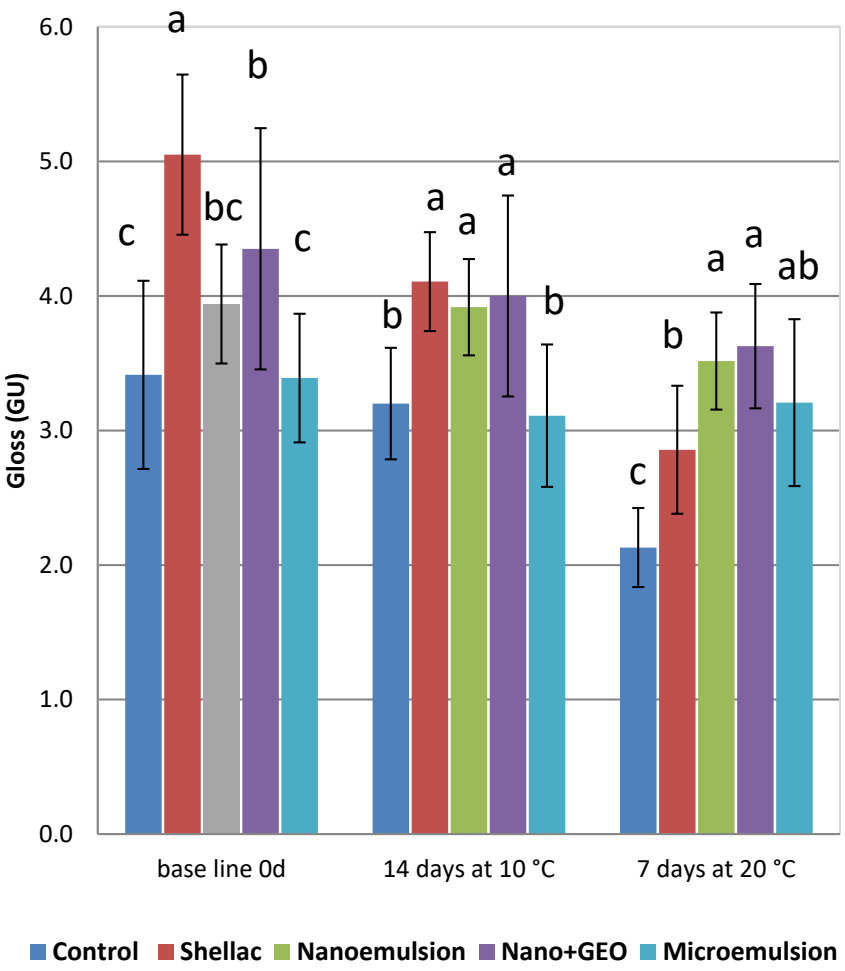
Wound : 1 mmm

7uL spores suspension $10^6$

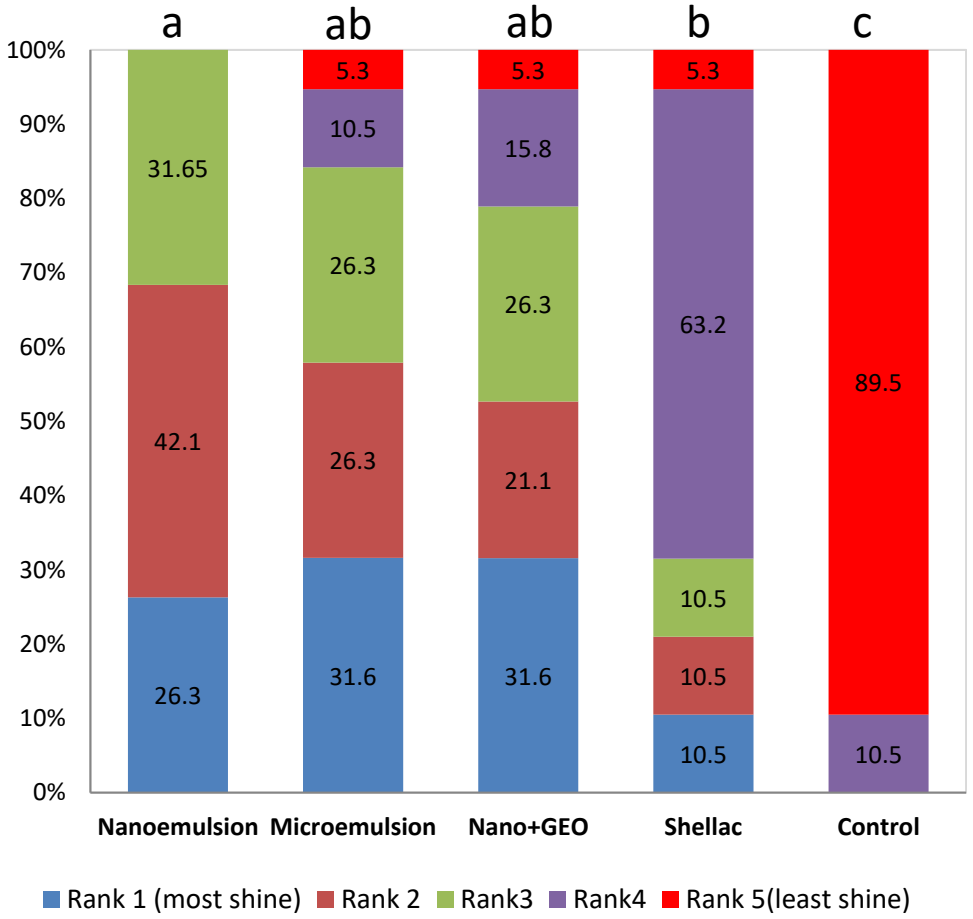
24 h of incubation

stored at 10 °C/14 d; 20 ºC/7 d

# Exp.2: gloss and sensory shine rank test

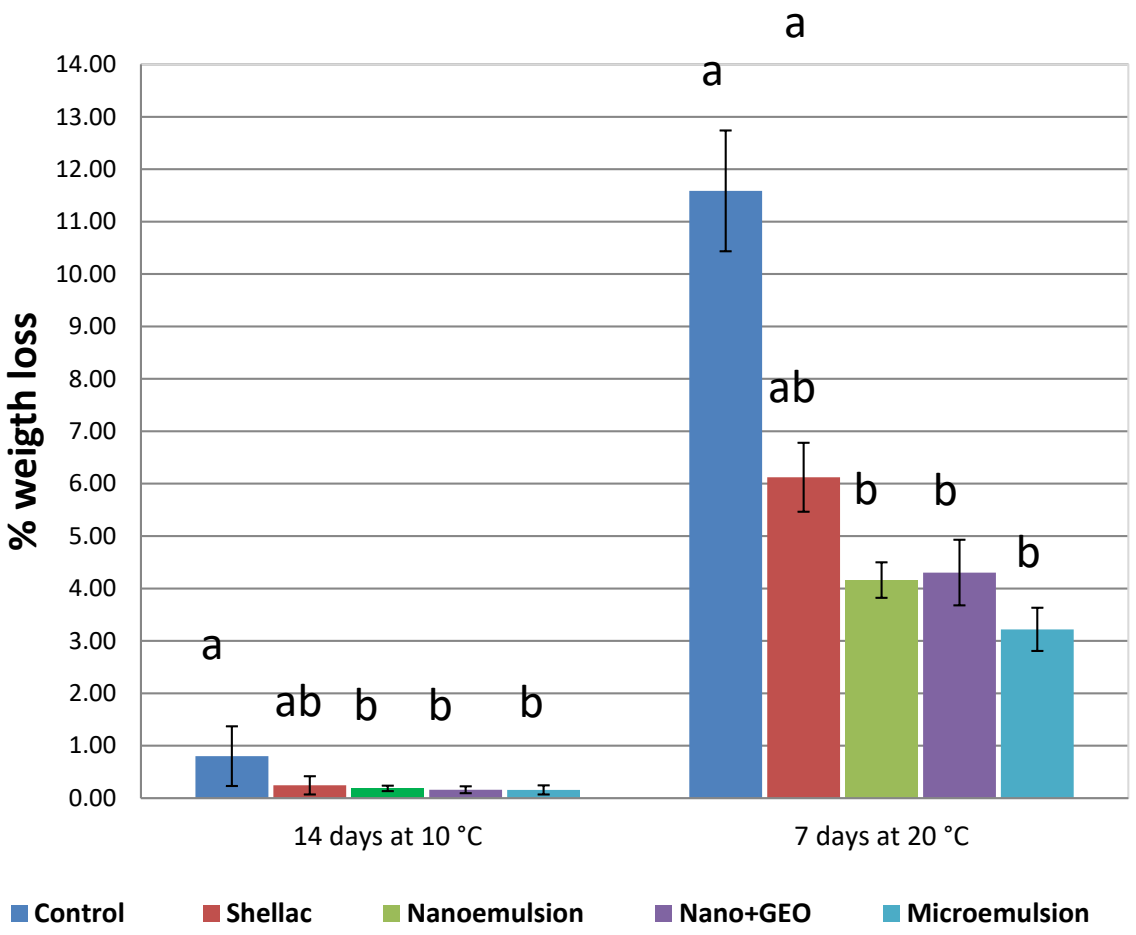



For each storage period, columns with different letters are significantly different by Duncan test (for homocedastic data) or GamesHowell (for heterocedastic data) at p<0.05, applied after Anova.



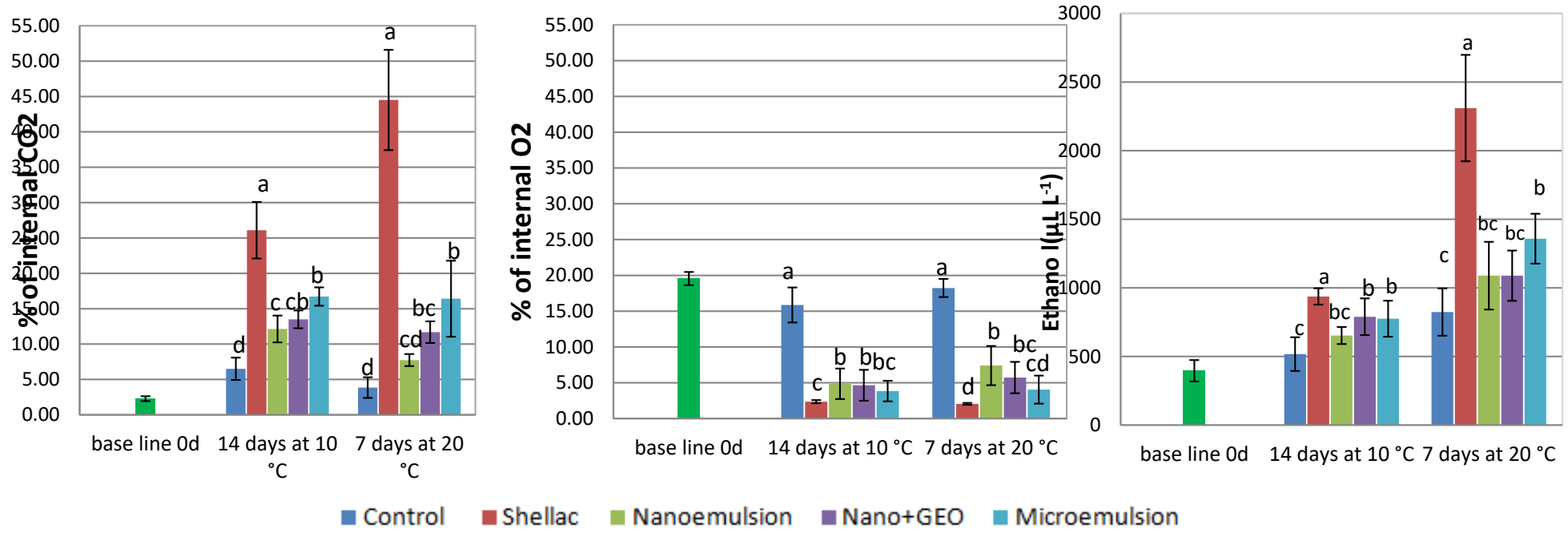

Columns with different letters are significantly different by critical absolute rank sum differences table at p<0.05 (Newell and MacFarlane, 1987),

# Exp.2: Weight loss and Sensory firmness




For each storage period, columns with different letters are significantly diferent by Duncan test ($p<0.05$), applied after Anova.

# Exp.2: Internal $CO_2$ and $O_2$ and ethanol




For each storage period, columns with different letters are significantly diferent by Games Howell test (CO2 and O2 data were hoteroscedastic) or Duncan (homoscedastic data to ethanol) at $p<0.05$, applied after Anova.

- **The lower ethanol level in the nanoemulsion coating +/- GEO, would indicate better flavor compared to shellac or the microemulsion coating, however, uncoated fruit had the lowest ethanol levels and $CO_2$ along with the highest $O_2$ (least anaerobic respiration).**
- **GEO did not significantly change the nanoemulsion barrier properties.**

# Volatiles for Unique Tangors

| Class | Compound | Base line | Control | Shellac | Nano carnauba | Nano carnauba + GEO | Micro carnauba | Control | Shellac | Nano carnauba | Nano carnauba + GEO | Micro carnauba |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 day | 14 days at 10 °C | | | | | 14 days at 10 °C + 7 days at 20 °C | | | | |
| Aldehydes | Acetaldehyde | 0.708 | 0.850* a | 0.514 c | 0.723 b | 0.694 b | 0.680 b | 0.895 a | 0.634 b | 0.850 a | 0.823 a | 0.872 a |
| | Hexanal | 0.177 | 0.293 c | 0.541 a | 0.366 bc | 0.435 b | 0.405 b | 0.237 d | 1.112 a | 0.765 b | 0.451 cd | 0.698 bc |
| | Octanal | 0.028 | 0.026 b | 0.031 a | 0.026 b | 0.027 b | 0.026 b | 0.019 d | 0.048 a | 0.028 c | 0.021 cd | 0.037 b |
| | Decanal | 0.475 | 0.436 b | 0.586 a | 0.598 a | 0.503 ab | 0.563 a | 0.808 b | 1.921 a | 0.676 b | 0.612 b | 0.757 b |
| Alcohols | Methanol | 4.948 | 2.617 a | 1.971 b | 2.273 ab | 2.349 ab | 2.253 ab | 2.646 a | 1.666 c | 2.362 b | 2.571 ab | 2.401 b |
| | Ethanol | 431.321 | 602.892 c | 937.194 a | 652.624 bc | 740.511 b | 682.339 bc | 872.189 d | 2293.789 a | 1190.604 c | 1011.583 cd | 1407.279 b |
| | 2-MethyPropanol | 0.035 | 0.015 bc | 0.025 b | 0.020 bc | 0.059 a | 0.012 c | 0.011 c | 0.165 a | 0.079 b | 0.014 c | 0.029 c |
| | Hexanol | 0.226 | 0.192 b | 0.278 a | 0.261 a | 0.266 a | 0.233 ab | 0.212 b | 0.327 a | 0.225 b | 0.208 b | 0.349 a |
| | cis-3-Hexenol | 0.573 | 0.207 c | 0.372 a | 0.283 b | 0.267 bc | 0.277 bc | 0.297 bc | 0.984 a | 0.446 b | 0.246 c | 0.462 b |
| | trans-2-Hexenol | 0.003 | 0.005 d | 0.012 ab | 0.008 c | 0.009 bc | 0.012 a | 0.021 a | 0.044 b | 0.026 a | 0.022 a | 0.026 a |
| | Linalool | 0.562 | 0.626 a | 0.907 a | 0.763 a | 1.052 a | 0.772 a | 0.782 b | 1.126 ab | 1.427 a | 0.745 b | 1.445 a |
| | Octanol | 0.419 | 0.218c | 0.425 ab | 0.333 bc | 0.536 a | 0.383 b | 0.482 cd | 1.211 a | 0.674 bc | 0.423 d | 0.821 b |
| | Terpinen-4-ol | 0.281 | 0.240 a | 0.265 a | 0.288 a | 0.270 a | 0.254 a | 0.225 c | 0.346 a | 0.259 bc | 0.241 bc | 0.311 ab |
| | α-Terpineol | 0.154 | 0.307 a | 0.330 a | 0.342 a | 0.307 a | 0.209 b | 0.193 a | 0.243 a | 0.252 a | 0.174 a | 0.201 a |
| Terpenes | α-Pinene | 0.107 | 0.062 c | 0.105 ab | 0.090 b | 0.114 a | 0.119 a | 0.120 cd | 0.245 a | 0.150 c | 0.108 d | 0.205 b |
| | Sabinene | 0.015 | 0.009 c | 0.016 b | 0.013 b | 0.021 a | 0.014 b | 0.017 d | 0.048 a | 0.030 bc | 0.024 cd | 0.036 b |
| | Myrcene | 0.548 | 0.313 c | 0.544 ab | 0.475 b | 0.601 a | 0.626 a | 0.592 c | 1.223 a | 0.764 bc | 0.627 c | 0.901 b |
| | Limonene | 11.333 | 5.695 c | 10.924 ab | 9.217 b | 11.803 ab | 13.097 a | 12.082 c | 32.170 a | 16.750 c | 11.882 c | 22.600 b |
| | γ-Terpinene | 0.003 | 0.002 ab | 0.002 ab | 0.002 b | 0.003 a | 0.002 ab | 0.003 c | 0.006 a | 0.004 bc | 0.002 c | 0.005 b |
| | Valencene | 10.162 | 7.130 b | 8.930 ab | 9.141 a | 9.226 a | 8.922 ab | 7.038 b | 9.058 ab | 9.267 a | 8.711 ab | 8.136 ab |
| Esters | Methyl Butanoate | 0.038 | 0.053 a | 0.054 a | 0.015 b | 0.049 a | 0.046 a | 0.052 a | 0.067 a | 0.052 a | 0.055 a | 0.060 a |
| | Ethyl Butanoate | 0.047 | 0.034 b | 0.071 a | 0.029 a | 0.084 b | 0.029 b | 0.155 b | 0.520 b | 0.224 b | 0.127 b | 2.419 a |
| | Ethyl Hexanoate | 0.032 | 0.025 b | 0.030 ab | 0.023 b | 0.034 a | 0.034 a | 0.044 c | 0.085 a | 0.050 bc | 0.036 c | 0.061 b |
| | Ethyl 3-hydroxyhexanoate | 23.731 | 24.494 b | 32.742 a | 29.672 ab | 36.645 a | 32.600 a | 24.292 c | 37.992 a | 32.693 b | 37.763 a | 40.781 a |
| | Ethyl Acetate | 0.683 | 1.250 a | 1.155 a | 1.086 a | 1.096 a | 1.275a | 1.294 b | 2.248 a | 2.232 a | 1.498 b | 1.553 b |
| Ketones | Acetone | 149.691 | 216.931 c | 327.512 a | 236.466 bc | 256.123 b | 237.134 bc | 303.940 d | 721.868 a | 414.981 bc | 351.979 cd | 485.116 b |

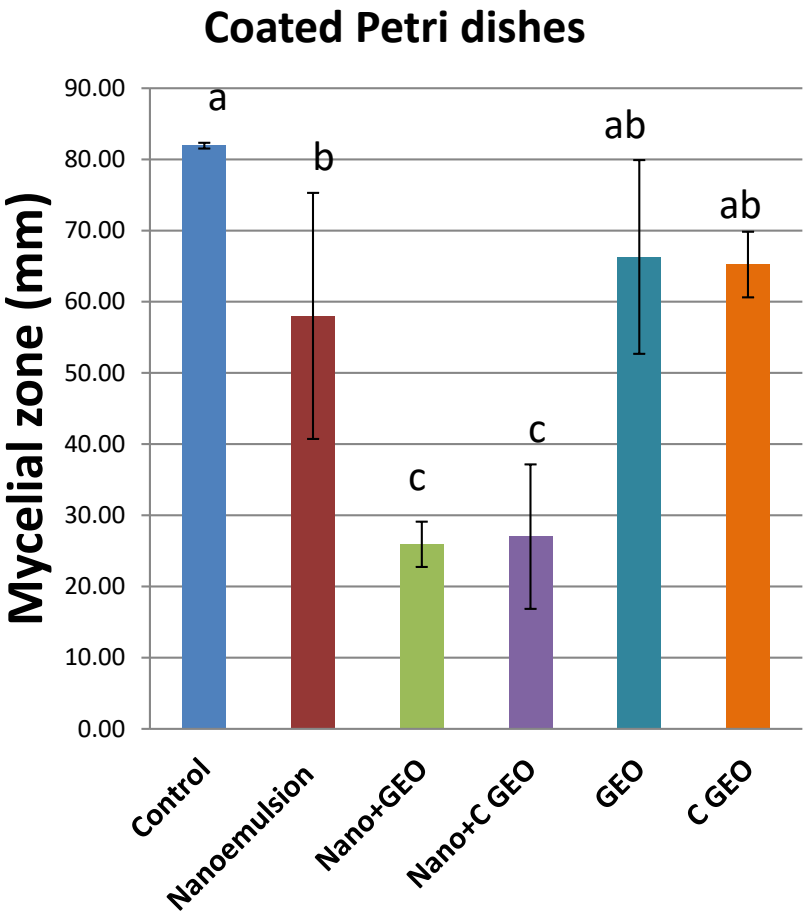
*For each period of storage, values followed by the same letter within rows are not significantly different by Duncan´s test at the 0.05 level.

# Exp.3: Coated Petri-dishes

- 10 uL spore suspension $10^6$ was placed on PDA in Petri dishes
- After 24h incubation, 1 mL of each coating from the 3rd expt. was placed and spread on Petri dishes, then incubated for 7 days
- Sterile water were control treatment

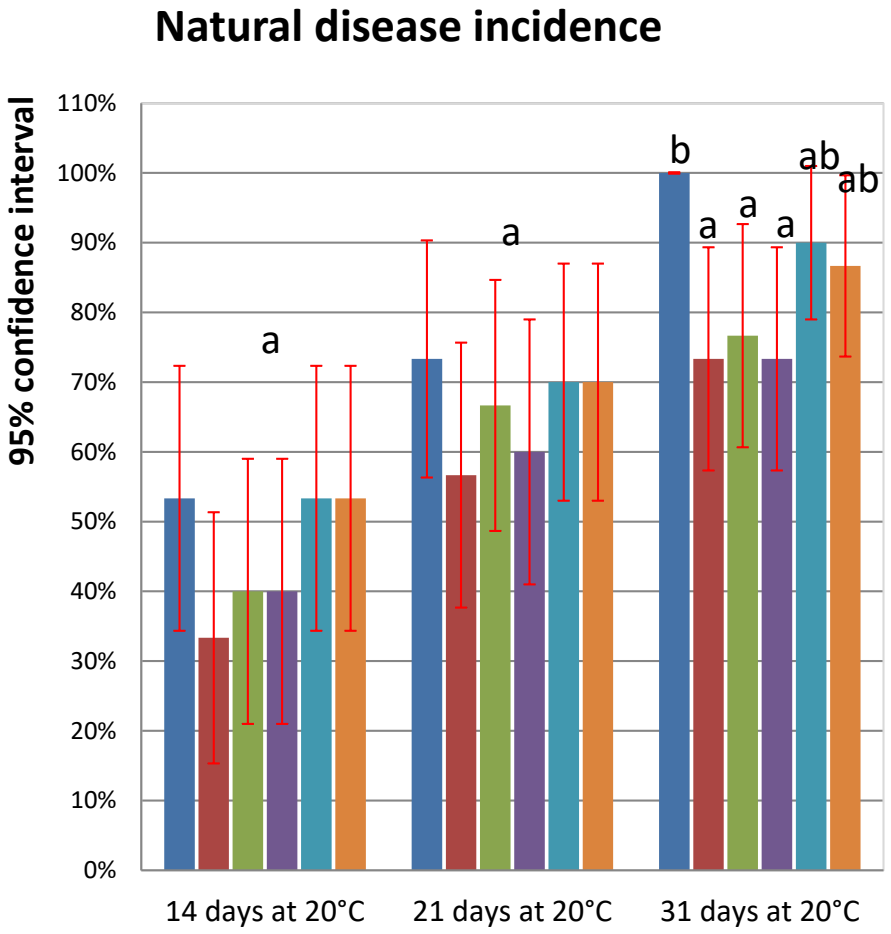
➢**For the *in vitro* assay, the nanoemulsion coating improved the antimicrobial activity of the GEO**

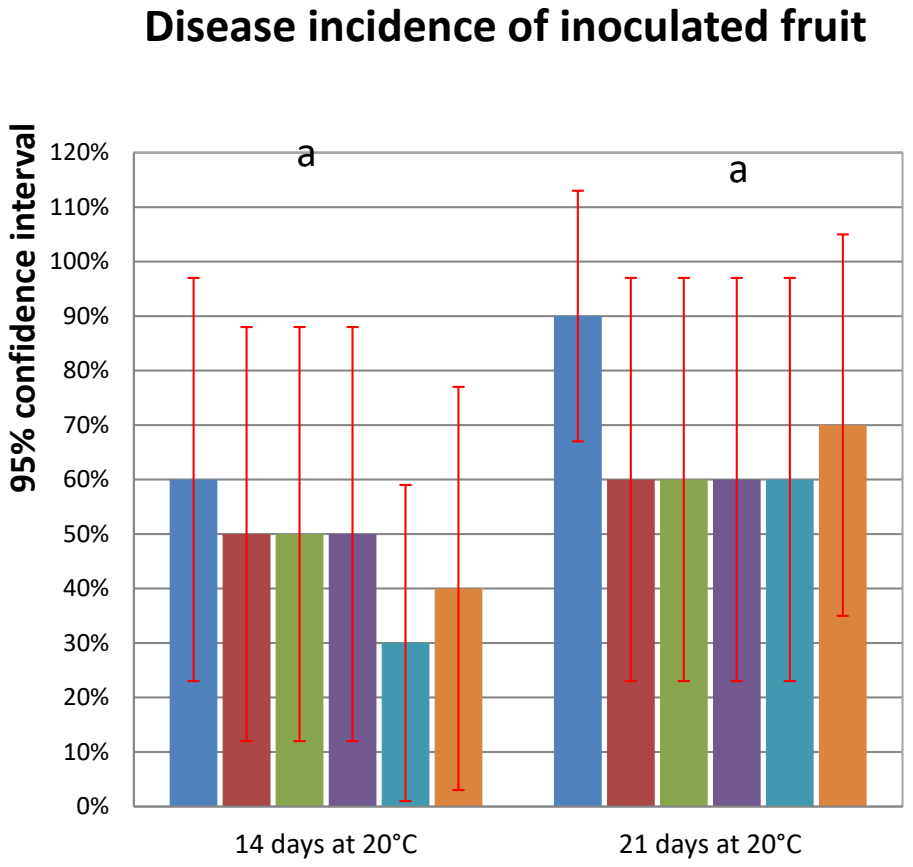
➢**The Commercial GEO (C GEO) had similar activity to extracted GEO in this assay**




Columns with different letters are significantly diferent by Games Howell ($p<0.05$) applied after Anova.

# Exp.3: Natural disease and *P. digitatium* inoculated disease incidence




Control Nanoemulsion Nano+GEO Nano+C GEO GEO C GEO

Columns with different letters are significantly different by 95% confidence interval, $p<0.05$.

# Conclusions

- The GEO generally exhibited the most antimicrobial activity among the ginger extracts

- Two times *Peniciulium digitatium* MIC (0.8%) added to nanoemulsion carnauba wax coating was not enough to significantly reduce decay on tangor fruit after 31 d of storage

- The nanoemulsion carnauba coating performed as well as or better than the microemulsion and better than shellac for water loss and formation of off-flavor ethanol indicator

- The combination of nanoemulsion + GEO was more effective in coated petri dishes than the nanoemulsion or the GEO alone, but not on fruit, where the nanoemulsion showed antimicrobial ability on its own w/ or w/o GEO and better than GEO alone

- Higher concentrations of GEO in nanoemulsion coatings will be tested as GEO shows promising antimicrobial ability for application in edible coatings for fruits

- The coatings did not affect fruit sugar and acid levels, however aroma volatiles from the fruit were analyzed, and showed changes in the aroma profile in addition to ethanol.

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