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EVALUATION OF MONOTERPENE-CYCLODEXTRIN COMPLEXES AS BACTERIAL GROWTH EFFECTIVE HURDLES

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ABSTRACT

Monoterpenes have antimicrobial properties but are associated with strong smells and flavors that limit their use in foods; therefore, strategies to keep their effectivity using lower concentrations are required. This work tested the antimicrobial capacity of thymol, carvacrol and linalool free or complexed in hydroxypropyl-β-cyclodextrins (HP- β-CDs) using two complexation methods. To this, these monoterpenes were complexed in HP- β-CDs by the solubility method or a microwave-assisted method, spray dried and their antimicrobial capacity was tested on *Escherichia coli* and *Staphylococcus aureus* by determining minimal inhibitory concentration and minimal bactericidal concentration. The results show significant differences ($p < 0.05$) between the complexed and uncomplexed forms. In addition, thanks to the complexation of monoterpenes, the use of lower concentrations of these has been reached to achieve the same level of inhibition than uncomplexed forms. Likewise, it has been found that a lower minimal inhibitory concentration (MIC) is achieved for the solubility method for both microorganisms (3.82 mM for thymol and 2.44 mM for carvacrol in *E. coli;* and 3.91 and 2.61 mM, respectively for *S. aureus*) than for the microwave method. This implies that a lower concentration of these compounds can be used to inhibit microbial growth in foods, which should minimize their effects on their smell and taste.

HIGHLIGHTS

- Monoterpene-cyclodextrin complexes were prepared by two methods.
- Their antimicrobial action was compared with free monoterpenes.
- The solubility method yielded better results than the microwave-assisted method.
- Complexation allows using lower monoterpene concentrations.
- Carvacrol and thymol CD complexes are effective hurdles for microbial grow.

KEYWORDS: thymol, carvacrol, linalool, HP-β-cyclodextrin, antimicrobial.

1. INTRODUCTION

In recent years, the demand for natural compounds in the food industry has grown, as interest has increased in developing, on the one hand, new preservatives with less collateral effects and more biodegradable, that slow down the deterioration of food and avoid proliferation of pathogenic microorganisms, and on the other hand, new "active" packages that incorporate these compounds (El Asbahani et al., 2015; Ribeiro-Santos et al., 2017). This impulse is mainly due to the negative perception that consumers have toward "artificial" preservatives, obtained by chemical synthesis. Usually, the food industry has used essential oils (EOs) as flavoring agents, but numerous works evidence that they contain a big amount of antimicrobial compounds of wide spectrum, supporting their use in food preservation (Hyldgaard, Mygind, and Meyer, 2012).

The EOs are formed by diverse components and at different concentrations. As consequence, their antimicrobial activity cannot be attributed to the action of a single compound, suggesting the employment of their isolated components (Bajpai, Baek, and Kang, 2012). Some of the main components of EOs are monoterpenes, which represent 90% of their total composition. Among them, thymol, carvacrol and linalool, are the main components of the EOs of *Thymus, Ocimum, Origanum, Satureja, Lavandula* and *Monarda* (Hussain et al., 2008, Silva et al., 2012; Licata et al., 2015; Mancini et al., 2015; Sarwar and Latif, 2015).

As it can be found in current literature (Heredia-Guerrero et al., 2018), the antimicrobial action of EOs is due to its ability to penetrate through the bacterial membranes into the cell, causing the inhibition of its living functions (Fisher and Phillips, 2009; Guinoiseau et al., 2010; Bajpai et al., 2012). Recently, it has been postulated that thymol is integrated into the lipid bilayer, causing alterations in the cell membrane (Wang et al., 2017); and at low concentrations, it has been shown to induce adaptive changes in the lipid profile of the membrane, to compensate for the fluidization effect, in order to maintain its structure (Turina et al., 2006; Di Pasqua et al., 2007).

On the other hand, carvacrol affects to a greater or lesser extent the outer membrane of Gram-negative bacteria (La Storia et al., 2011), by promoting the

release of lipopolysaccharides (Helander et al., 1998; Guarda et al., 2011). Regarding linalool, it has been proven that it is capable of destabilizing the membrane, increasing its permeability (Alviano et al., 2005; Silva et al., 2011; Diao et al., 2013).

In summary, the antimicrobial activity described above for some major components of certain essential oils such as thymol, carvacrol and linalool, suggest their application in food preservation. However, their use as preservatives in food technology do not exempt some difficulties. Firstly, they are highly volatile and chemically labile as a result of oxidation processes and other chemical reactions. In addition, due to its poor solubility in water, high concentrations are usually required to achieve the desired effect, which limits their application and effectiveness. It should also be taken into account that the heterogeneous composition of foods where they will exert their preservative effect can reduce their effectiveness, especially the fat and protein content, water activity, pH and enzymes (Burt, 2004; Friedly et al., 2009). And very importantly, their intense aroma and flavor can change the organoleptic properties of the foods (Friedly et al., 2009; Tiwari et al., 2009; Sokovic et al., 2010; Li et al., 2011; Bajpai et al., 2012; Solòrzano-Santos and Miranda-Novales, 2012).

In a previous study, our research group developed a method to incorporate isolated essential oils components (IEOCs) such as thymol, carvacrol and linalool into Hydroxypropyl-β-cyclodextrins (HP-β-CDs) to enhance its water solubility (Rodriguez-López et al., 2019) and in addition, protect the active component from humidity and other adverse environmental conditions (temperature, radiation, oxidation). Also, these complexes were obtained in solid state by spray drying, thus favoring their conservation (Rodriguez-López et al., 2019), as it has recently been described by other authors (Prakash et al., 2018, Al-Nasiri, Cran, Smallridge and Bigger, 2018), improving its stability and viability.

In this work, the effect of the inclusion of IEOCs thymol, carvacrol and linalool in HP-β-CDs by two complexation methods on their antimicrobial activity, as compared with their free form, was evaluated for a further application as natural food preservatives.

2. MATERIALS AND METHODS

2.1 MATERIALS

Thymol (CAS: 89-83-8, 98.8% purity), carvacrol (CAS: 499-75-2, 98% purity) and linalool (CAS: 126-91-0, 97.5% purity), were provided by Sigma (Madrid, Spain). The HP-β-CDs were supplied by AraChem (Eindhoven, The Netherlands). Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA) and buffered peptone water were purchased from Scharlau (Barcelona, Spain). The rest of the chemical products were of analytical grade.

2.2 PREPARATION OF IEOCs-HP-β-CDs COMPLEXES

2.2.1 Complexation by using microwave as energy source (MWI)

The aqueous solutions of HP-β-CDs (0-100 mM) were irradiated in a microwave oven (LG Grill Wavedom, LG Electronics, Madrid, Spain) at 700 W for 30 s, at intervals of 10 s, until solution reached a temperature of 70 ºC, as described by Hernández-Sánchez et al. (2017). Next, the monoterpene under study (thymol, carvacrol or linalool), was added to each one of the samples and, again, irradiated for 30 s at intervals of 10 s, until reaching 70 ºC. Subsequently, the samples were shaken and kept for 12 h in darkness, in sealed vials. Then, following the procedure described above, the samples were irradiated again, until reaching 70 ºC. After that, solutions were filtered through a 0.45 µm nylon syringe filter (Chromafil, Macherey-Nagel, Germany) to remove monoterpene excess (monoterpene not dissolved). Then, the concentration of each monoterpene was determined by GC/MS. In addition, samples with no CD (0 mM) were used as control to test the effect of free (non-encapsulated) monoterpenes.

2.2.2 Complexation by solubility method

For each monoterpene, aqueous solutions of increasing concentrations of HP-β-CDs were prepared (0, 10, 20, 30, 50, 75 and 100 mM), in a total volume of 100 mL. A saturating amount of thymol, or carvacrol, or linalool was independently added to each one of the solutions, and kept in an ultrasound bath (Ultrasons-H with calefactory, 200 W, Selecta, Spain) for 60 minutes in the dark at 25 °C, until reaching equilibrium. Then, the respective solutions were filtered through a nylon filter of 0.45

139 μm to eliminate the excess of monoterpene, and the concentration of each
140 monoterpene was measured by GC/MS. Samples with no CDs (0 mM) were used as
141 control to test the effect of non-encapsulated monoterpenes.

142 From the phase diagrams of thymol, or carvacrol, or linalool (monoterpene),
143 complexed with HP-β-CDs, the efficiency of complexation (CE) and the molar ratio
144 (MR) parameters were determined. CE is the ratio between the dissolved complex
145 and free cyclodextrins (CDs) concentration. It is independent of S_0 (aqueous
146 solubility), and was calculated from the slope of the phase solubility profiles by using
147 the equation (1).

148 $$CE\ (\%) = \frac{[disolved-complex]}{[CD]_f} \qquad (1)$$

149 The MR monoterpene:CD, was calculated using CE values with equation (2).

150 $$MR = \frac{1}{\left(1+\frac{1}{CE}\right)} \qquad (2)$$

151 As shown in Table 1, all IEOCs-HP-β-CDs complexes show the same molar
152 ratio (1:2), indicating that about one of every 2 HP-β-CDs molecules in solution is
153 forming soluble complexes whit thymol or carvacrol (Rodriguez-López et al., 2019).
154 However, the efficiency of complexation obtained for linalool (478.8), is higher than
155 the obtained for carvacrol (272.2) and thymol (139.5).

156 Table 1: Complexation efficiency (CE) and molar ratio (MR) of thymol and carvacrol
157 complexed with HP-β-CDs at different pH ±SD. Standard deviation of triplicate
158 diagrams.

IEOCs-HP-β-CDs complexes	CE (%)	Molar ratio
thymol/HP-β-CDs	139.5 ±12.3	1:2
carvacrol/HP-β-CDs	272.2 ±12.6	1:2
linalool/HP-β-CDs	478.8 ±16.7	1:2

159

160 2.3 SPRAY DRYING

161 The solutions prepared by the solubility method and by MWI method were
162 subjected to an atomization process to obtain complexes in solid state. This process

was carried out using a laboratory-scale atomizer, Mini Spray Dryer Büchi B290 (Flawil, Switzerland). The operating conditions of the drying process were: air inlet temperature 170 ± 2 °C, air outlet temperature 68 ± 2 °C, flow rate 5 mL/min, air pressure 3.2 bar and nozzle diameter 1.5 mm (Lee et al., 1999).

2.4 GAS CHROMATOGRAPHY/MASS SPECTROMETRY ANALYSIS

In order to quantify the amount of monoterpene, the monoterpene-CDs complexes were broken by adding 80% ethanol. Subsequently, each one of the solutions was introduced into a gas chromatograph coupled to a mass spectrometer, (Shimadzu QP 2010), equipped with a Slb-5ms Supelco capillary column (30 m x 0.25 mm x 0.25 mm thickness). The working conditions were: initial temperature 70 °C, increase of 4 °C/min up to 160 °C and 30 °C/min up to 280 °C, which was maintained for 6 min; injector temperature 250 °C, injection type in split mode 1:20 and helium was used as carrier gas.

The analysis and quantification of the component was carried out from the areas obtained after the injection of the samples. The identification of the components was based on the relative elution times and the comparison of the mass spectrum of each compound with the spectrometer database. All measures were carried out by triplicate.

2.5 DETERMINATION OF ANTIMICROBIAL CAPACITY

2.5.1 Bacterial culture

Escherichia coli (CECT 943) and *Staphylococcus aureus* (CECT 239) strains were provided by the Spanish Type Culture Collection (CECT) (Paterna, Valencia, Spain). Strains of *E. coli* and *S. aureus* were activated in TSB medium and were incubated under aerobic conditions at 35 °C for 24 h. The bacteria cultures were preserved in TSA medium at 4 °C for more than 3 months. The working culture was daily prepared, transferring one colony from TSA to 10 mL of TSB, and incubation for 24 h at 35 ºC.

2.5.2 Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) of thymol, carvacrol and linalool in its free and complexed form was determined by the broth dilution method according to Brandt et al. (2010). The MIC analysis was carried out in sterile 96-well flat bottom microtitre plates of 300 µL capacity (MicroWell, Nunc, Thermo-Fisher Scientific, Waltham, MA). First, a suspension of 5.0 $\log_{10}$ colony forming units/mL (CFU/mL) was prepared for each microorganism (*E. coli* and *S. aureus*) in TSB (2X). After that, aliquots of 100 µL of the bacterial suspension were added to each plate well (columns 1-10). Then, 100 µL of antimicrobial solution to be tested (complexed or not with CDs) were added to wells of column 1, and mixed with the same volume of bacterial suspension previously added. Subsequently, serial solutions were carried out transferring 100 µL of each well (starting with column 1), to the next column, and so on until reaching column 10 of the plate well. To test their antimicrobial activity, different aqueous suspensions (from 0.20 mM to 37.75 mM, containing 0.01 g/100 g of Tween 20), of both inclusion complexes and monoterpenes in their free form, were used.

The last plate columns were used as controls for bacterial growth. Thus, the negative control (columns 11) was prepared with antimicrobials, sterile water and TSB (2x) solutions; while the positive control (columns 12) was prepared with inoculum, sterile distilled water, Tween 20 and HP-β-CDs at the test concentrations to rule out any interference of the solvents and/or additives in the optical density measurements or in the antimicrobial activity.

In order to correlate the values obtained by plate count with the optical density values (630 nm), a growth curve of *E. coli* and *S. aureus* was prepared. For that, the bacterial cells were washed for three consecutive times, with peptone water (0.1 g/100 mL), and the assay was carried out as described above.

Once the plates were prepared with the antimicrobial solutions and the bacterial suspensions, the absorbance at 630 nm (OD630) was determined on a SPECTRAmax PLUS plate reader (Molecular Devices LLC, Sunnyvale, CA, USA) at time 0 and each hour for 24 h, remaining the incubation temperature at 35 °C through the process. Those wells where a decrease in absorbance ≤ 0.05 was observed were

222 considered "positive inhibition" and the lowest concentration of the respective
223 antimicrobial agent was considered as their MIC value (Hill et al., 2013).

224 In order to corroborate the results, the bactericidal capacity of all the wells
225 where inhibition was observed was also determined in Petri dishes with TSA. For
226 this, 0.1 mL of each well was added to the plates and incubated for 24 hours at 35
227 °C. The antimicrobial concentrations corresponding to the wells where no growth was
228 observed were labeled as bactericidal, and the lowest of them was taken as the
229 minimum bactericidal concentration (MBC) (Hill et al., 2013).

230 2.6 DATA PROCESSING

231 Growth data was fitted to the Baranyi and Roberts (1994) model using the
232 DMFit shareware package for Excel as follows:

233 $$\ln N = \ln N_{max} + \ln\left(\frac{-1+e^{\mu max\,\lambda}+e^{\mu max t}}{-1+e^{\mu max t}+e^{\mu max\lambda+\ln N_{max}-\ln N_0}}\right) \quad (3)$$

234 where N_{max} (absorbance) is the upper asymptotic value and approximately equal to
235 the maximal population density; t (h) is time; μ_{max} (absorbance h^{-1}) is the maximum
236 growth rate; λ (h) is the latency time, and N_0 (absorbance) is the lower asymptotic
237 value and approximately equal to the initial population density.

238 All determinations were run by triplicate and analyzed by t-student test by
239 means of SPSS Statistics 24 (IBM, USA).

240 **3. RESULTS AND DISCUSSION**

241 3.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration
242 (MBC) of free monoterpenes

243 First, the antimicrobial activity of thymol, carvacrol and linalool was measured
244 by the broth dilution method, in the absence of CDs. The study was carried out in
245 TSB with an initial inoculum of approximately 5.0 $\log_{10}$ CFU/mL per well in a final
246 volume of 300 mL, with monoterpene solutions prepared by the MWI and solubility
247 methods (Table 1).

248 As shown in Table 2, thymol in free state only exhibited antimicrobial activity
249 against *S. aureus*, being 1.4 times more effective ($p < 0.05$) when the monoterpene

was prepared by the solubility method in comparison with the MWI method. Concerning carvacrol in the free state, it showed antimicrobial activity against both pathogens, showing significant differences ($p < 0.05$) between the complexes prepared by the solubility method and MWI, needing a lower concentration of free monoterpene (3.4 times for coli and 2.2 times for S. aureus) by the method of solubility, as was evidenced by thymol. However, linalool did not show antimicrobial activity in the free state. This fact could be justified by the low concentration of linalool present in the medium and its chemical instability. To corroborate the results obtained, the MBC was determined in all the wells where inhibition was observed, using Petri dishes with TSA. The MBC test was applied at free monoterpenes (in absence of CDs), obtained by both solubility and MWI methods, not reaching in any case, the MBC that justifies its bactericidal action.

Since monoterpene solutions were prepared at concentrations up to the solubility limit, which were in some cases not enough to exert antimicrobial activity, these results evidence the necessity of searching for ways to increase their aqueous solubility. In this sense, a complexation with CDs could be an alternative to this aim. In fact, this approach has been previously described by Tao et al., 2014, arguing that the employ of the β-CDs not only increase the solubility of thymol, but it is also possible to improve the mechanism of antimicrobial action.

3.2. *Effect of encapsulated monoterpenes on growth curves*

Once established the antimicrobial activity of each monoterpene in absence of CDs, a study on the effect of the presence of HP-β-CDs on antimicrobial activity was conducted. In order to carry out the study with *E. coli* and *S. aureus*, the atomized complexes in solid state, prepared by both methods were dissolved in sterile distilled water (1:1, w/v). Figures 1-4 show the growth curves of *E. coli* and *S. aureus*, noting that the maximal growth occurred in control curves (without IEOCs), while increasing concentrations of HP-β-CDs lead to growth inhibition. This behavior suggests that the presence of solid complexes in the culture medium assure a higher concentration of thymol, carvacrol and linalool in the reaction medium, with respect to the assay in the absence of CDs. This is the pursued effect and it is a consequence of the increase in solubility caused by HP-β-CDs.

281 3.2.1 *Effect of encapsulated monoterpenes on growth curves of E. coli*

282 As can be seen in Figures 1-2, both complexed thymol and carvacrol had the
283 same behavior; lower concentrations were required to inhibit the growth of *E. coli*
284 when the complexes were obtained by the solubility method, than by the MWI
285 method. However, when the inhibitory capacity of each compound (obtained by MWI
286 method), against *E. coli* was compared, complexed carvacrol required (14.60 mM) to
287 achieve total growth inhibition, while for complexed thymol more than double is
288 required (37.75 mM).

289 As expected for linalool due to its structure and reactivity, the inhibitory activity
290 for the complexes obtained by both methods was less marked than in the case of
291 thymol and carvacrol. The results (Figures 1-2) show that the complexation favors the
292 antimicrobial activity of this monoterpene, since the MIC was not reached when it
293 was tested in its free state. In fact, the linalool complexes obtained by the MWI
294 method involved 69% growth inhibition for *E. coli* at a concentration of 3.53 mM after
295 24 h of incubation. With respect to the complexes obtained by the solubility method,
296 78% inhibition was obtained, at a concentration of 12.92 mM.

297 Therefore, it is demonstrated that the linalool complexes behave differently from
298 the thymol and carvacrol complexes, where the inhibitory effect was more
299 pronounced for the complexes obtained by MWI. Since it required lower
300 concentrations (approximately 3 times less) to reach a similar inhibitory rate.

301 From the respective growth curves (see Figures 1-2), the growth rate (μ), the
302 lag phase (ʎ) and Adjusted R-square (R^2) for *E. coli* (Table 2), were determined using
303 the Baranyi and Roberts (1994) model. The values of μ obtained from the Baranyi
304 model were used to determine the effect of the complexation of thymol, carvacrol and
305 linalool by both methods on the growth kinetics of *E. coli* and (equation 3). The
306 values of R^2 (Table 3), as well as the graphical evaluation of the fitting curve (Figure
307 1-2), indicated a good adjustment of the model to the effect of complexation on the
308 growth of microorganisms was described.

309 The results obtained showed that microbial growth rate decreased as the
310 concentration of complexed monoterpene increased, until reaching a complete

311 inhibition (Figures 1-2). Thus, at a fixed concentration of thymol (3.84 mM), the μ
312 decreased 4.2, for the complexes obtained by the MWI method, being more
313 significant ($p < 0.05$) the reduction of μ (20.6 fold) when the complexes obtained by
314 the solubility method were evaluated, requiring up to 50% less active principle in the
315 medium (1.99 mM).

316 In the case of carvacrol complexes obtained by the MWI method, a lower
317 concentration (2.49 mM) than for thymol was required to achieve a similar decrease
318 in μ (4.3 times). In the test performed with carvacrol complexes obtained by the
319 solubility method, the reduction of μ (4.6 times) was less marked than for thymol
320 (Table 3).

321 For linalool (see Table 3), μ decreases 3.97 fold for *E. coli*, for complexes
322 obtained by MWI, whereas in the case of complexes obtained by solubility method,
323 the results were significantly improved, obtaining decreases in μ values of 6.18 fold,
324 at linalool concentrations of 0.40 mM and 1.67 mM, respectively. However, it should
325 be noted that the effect on the growth rate seems to be antagonistic (favoring the
326 growth rate), at concentrations higher than 3.6 mM for *E. coli*. This fact was probably
327 due to the increase of the linalool molecules in the medium favors the interaction
328 between them, being able to give rise to intramolecular transpositions (through
329 carbocation), rearrangement of olefinic double bonds, even forming cyclic derivatives,
330 changing completely the initial compound activity and, therefore, the actual
331 concentration of linalool in the reaction medium (Sell, 2003).

332 In general, and taking into account the results obtained for the three compounds
333 under study, the differences observed between both complexation methods may be
334 due to the speed in which the monoterpene is released from the HP-β-CDs cavity
335 into the reaction medium (Hedges et al., 1995), showing greater antimicrobial
336 capacity the solid complexes of thymol and carvacrol prepared by the solubility
337 method. This effect has been previously described by Tao et al. (2014), in which they
338 evaluated the antimicrobial activity of thymol/β-CD complexes, as well as those
339 obtained with different EOs by several methods, evidencing that the MIC not only
340 depends on the preparation method of the inclusion complexes, but also on the
341 speed of release of the compound under study.

In addition, it was observed that for thymol and carvacrol, ʎ of *E. coli* (3.58 h) increased, according as μ decreased. This behavior could be justified by the increase of its concentration in the inclusion complexes, with respect to the control, changing for the complexes obtained by MWI method to 8.53 h (thymol) and 7.34 h (carvacrol) for *E. coli*. Similar trend was observed for complexes obtained by the solubility method, shifting to 6.59 h (thymol) and 8.08 h (carvacrol) for *E. coli*.

Regarding the effect of linalool complexes in the growth of both pathogens, this was less pronounced (6.38 h for *E. coli* for complexes obtained by MWI method), not observing growth retardation with respect to the control of *E. coli*, with the complexes obtained by the solubility method. In spite of that as commented previously, it has a favorable influence (retardation) on the microbial growth rate until a certain linalool concentration, passing to exert an antagonistic effect when overcoming this cut-off concentration.

As can be seen in Table 3, from a concentration of 3.97 mM of thymol and 3.78 of carvacrol for the complexes obtained by the solubility method, the growth of both *E. coli* was completely inhibited over the 24 hours of the study, while for linalool, this effect was not observed.

3.2.2 *Effect of encapsulated monoterpenes on growth curves of S. aureus*

To carry out the study with *S. aureus*, the same procedure that for *E. coli* (see section 2.5.2) was applied, obtaining similar results to the previous ones, since the presence of solid complexes in the culture medium supposed a higher concentration of thymol, carvacrol and linalool in the reaction medium, with respect to the assay in the absence of CDs, which had a favorable effect on the MIC in all cases.

In addition, the same behavior for complexes of thymol and carvacrol (Figures 3-4) was observed, that is, a lot of lower concentration to inhibit the growth of *S. aureus* is required, when complexes were obtained by the solubility method (3.97 mM). In the case of linalool, a similar effect is observed in *E. coli*, since the high reactivity of this compound determines its ability to be included in the hydrophobic cavity of the cyclodextrin and, consequently, justify its lower capacity to inhibit the growth of *S. aureus*. Even so, the complexation makes it possible for linalool to exert

antimicrobial activity, that was not observed in the absence of HP-β-CDs. Thus, for the complexes of linalool it is possible to reduce the growth of *S. aureus* by 75% and 85% at concentrations 0.78 mM (MWI method) and 5.95 (solubility method), respectively, reducing its inhibition capacity at concentrations higher than described for the complexes obtained by both methods.

In Table 4, it is observed that as the thymol concentration in the medium decreases the growth rate of *S. aureus*, increasing its inhibitory capacity, until it reaches complete inhibition. Thus, at a concentration of 3.84 mM thymol, the μ decreases 2.85 times for the complexes obtained by the MWI method, while for the complexes obtained by the solubility method, the μ decreases 3.24 times, requiring 50% less active matter in the medium (1.99 mM). With respect to carvacrol, it is required at a lower concentration (2.49 mM) than with thymol, so that the μ will experience a similar delay (2.8 times) for the complexes obtained by the MWI method. In the test carried out with the complexes acquired by the solubility method, a reduction of μ (3.9 times) is observed with respect to that of thymol, requiring a concentration 1.89 mM of carvacrol, lower by 0.10 mM to thymol.

In the case of linalool assay (Table 4), we observed that μ for *S. aureus* decreases 2.17 times (MWI) and 3.78 times (solubility method) at 0.40 mM and 1.67 mM, respectively, although it should be noted that the effect on speed it seems to be antagonistic (favoring the growth rate), at complex concentrations higher than 5.95 mM (solubility method) and 1.15 mM (MWI), probably because an increase in the mean of the linalool molecules, favors the interaction among them, as described in the case of *E. coli*; completely changing the activity of the initial compound and, therefore, the concentration of linalool in the reaction medium.

As it happens in *E. coli*, ʎ of *S. aureus* (6.86 h) for thymol and carvacrol increases as μ decreases, coinciding with the increase in the medium of complexes, with respect to the control, moving to 8.06 h for the MWI method and to 9.02 and 8.58 h for the method of solubility, for thymol and carvacrol respectively; being less accused for linalool (7.46 h by the MWI method and 8.21 h by the solubility method).

As seen in Table 4, from a concentration of 3.97 mM thymol and 3.78 mM carvacrol for the complexes obtained by the solubility method, we managed to completely inhibit the growth of *S. aureus*.

A comparison of results related to the antimicrobial activity against *E. coli* and *S. aureus* for the complexes obtained by both methods yielded statistically significant differences ($p < 0.05$). Thus, it was evidenced that both thymol and carvacrol complexes obtained by the solubility method exerted greater antimicrobial activity than those prepared by MWI method. This statement could be set since a $\mu = 0$ value was obtained for 10 mM of HP-β-CDs, for the solid complexes of thymol (3.97 mM) and carvacrol (3.78 mM) for both bacteria. In addition, lower concentrations against *E. coli* of thymol (9.5 fold) and carvacrol (3.8 fold) complexes, or *versus S. aureus*, requiring 9.5 times less of thymol and 6.6 times less of carvacrol, to those required for the complexes obtained by MWI. However, in the case of linalool, although the growth rate slows, a $\mu = 0$ value was neither reached.

In the case of thymol, although the MIC is not achieved in the absence of CDs; this was successful reached for HP-β-CDs-thymol complexes, obtaining a MIC for *E. coli* at 6.68 mM for MWI complexes and 3.82 mM for complexes obtained by the solubility method. However, for *S. aureus*, the MIC of thymol was 4.83 mM for the MWI method and 3.91 mM for the solubility method, a value similar to that obtained for the free monoterpene (5.59 mM).

In contrast, the MIC values of carvacrol obtained for the MWI method are three units higher than those described for thymol, both in its free and complexed form; nevertheless, by the solubility method, that of carvacrol is approximately two tenths lower than that of thymol. These results are in agreement with those obtained for Helander et al. (1998), which demonstrated that carvacrol and thymol showed inhibitory effects against the growth of *E. coli* at a similar concentration.

Since thymol and carvacrol are isomers obtained by hydroxylation of their natural precursor *p*-cymene, it could be assumed that their antimicrobial action should be similar. However, evaluating the results obtained in this study, it is verified that the thymol MIC is greater (it needs a higher concentration to exert the

antimicrobial activity) for MWI complexes, than the one required for carvacrol. These results justify that the complexation method could exert a marked influence in its antimicrobial action.

3.3 *Minimum bactericidal concentration of free and complexed monoterpenes*

Once the MIC for each compound was determined, the MBC was evaluated in Petri dishes on TSA. Thus, to those wells where inhibition was observed in the MIC assay, the bactericidal capacity was evaluated by diffusing 0.1 mL solution of each well, containing the corresponding concentration of complexed carvacrol or thymol, in Petri dishes with TSA.

As can be seen in Table 5, thymol and carvacrol complexes not only have a bacteriostatic effect against *E. coli* and *S. aureus*, but also exert a bactericidal action on both pathogens, since no growth was observed on the plates. The obtained results agree with those described by Kamimura et al. (2014) for carvacrol microencapsulated in HP-β-CDs prepared by kneading (KN) and freeze-drying (FD) methods, evidencing that encapsulation process improved the antimicrobial activity of carvacrol against *E. coli* and *Salmonella spp*. Similar effects were observed for Tao et al. (2014) for *E. coli* with thymol and thyme essential oil complexed in β-CDs, and other authors (de Oliveira et al., 2010; Ait-Ouazzou et al., 2011; Pesavento et al., 2015; Sakkas and Papadopoulou, 2017), which demonstrate the antimicrobial action of thyme, oregano and rosemary EOs, as well as of its main components thymol and carvacrol, against *S. aureus* and *Listeria monocytogenes*. Therefore, the encapsulation of thymol and carvacrol with CDs not only does not affect their antimicrobial activity, moreover acts as an activity enhancer, since both complexes exert their action against both *E. coli* and *S. aureus* at much lower concentrations than those corresponding to free monoterpenes, or their essential oils (Tao et al., 2014; Marchese et al., 2016).

The increased in antimicrobial efficacy of the complexed forms of monoterpenes could be related to the slow release of thymol and carvacrol from the CDs complex, acting HP-β-CDs as a dosing pump that allows a prolonged liberation in time to the reaction medium. In contrast, free monoterpenes are very volatile and their

461 concentration drops fast; consequently, the volatilized amounts of the compounds
462 would not be available to exert their antimicrobial action (Marques, 2010).

463 Despite the behavior described by some authors for certain EOs such as
464 coriander (Silva et al., 2011), justifying a greater antimicrobial effect against Gram
465 negative bacteria than for Gram positive bacteria due to differences in bacterial
466 cover; the antimicrobial activity results obtained for thymol/HP-β-CDs, carvacrol/HP-
467 β-CDs and linalool/HP-β-CDs complexes on *E. coli* (Gram -) and *S. aureus* (Gram +)
468 were similar, showing no differences in the MIC values against both bacteria for the
469 complexes obtained by solubility method, being less effective in the case of linalool
470 complexes, probably due to its structural differences and stability.

471 In fact, as described by Ultee et al. 2002, the presence of the hydroxyl group on
472 both carvacrol and thymol isomers play an important role, acting as an electron
473 delocalization system able to disrupt the cell membrane potential, the proton motive
474 force system, and the electron transport chain; hence it decreased the production of
475 intracellular ATP. Indeed, when they access in cytoplasmic membrane changes their
476 physical and chemical properties and disrupts both lipid ordering as well as bilayer
477 stability, leading in an increase of proton passive flux across the membrane. Although
478 both monoterpenes are biosynthesized from *p*-cymene, this precursor lacked of
479 hydroxyl groups, needing higher concentrations of *p*-cymene to obtain the same
480 microbial grow reduction as that obtained with carvacrol and thymol (Ultee et al.
481 2002). In addition, the benzene ring structure of carvacrol and thymol, not present in
482 linalool, enhance its antimicrobial activities, as has been previously described
483 (Veldhuizen et al., 2006).

484 **4. CONCLUSIONS**

485 The complexes of thymol, carvacrol and linalool with HP-β-CDs obtained by the
486 solubility and microwave irradiation methods, decreased the MIC and MBC,
487 increased the lag phase and decreased the growth rate of *E. coli* and *S. aureus* in
488 comparison to the effects of these compounds in their free state. Therefore, the
489 complexation with HP-β-CDs favors the antimicrobial capacity of monoterpenes. In
490 practice, this implies that a lower concentration of these compounds is required to

inhibit microbial growth in foods, while minimizing their potential adverse effects on certain organoleptic parameters such as smell and flavor. From the two complexation methods evaluated, the solid complexes of thymol and carvacrol obtained by the solubility method showed higher antimicrobial activity for both *E. coli and S. aureus*. However, although a decrease in the growth rate for both microorganisms with linalool complexes was observed, in any case the minimum inhibitory concentration was reached. These results advise the use of thymol/HP-β-CDs and carvacrol/HP-β-CDs complexes in nutritional or therapeutic applications. For industrial food formulations, its dosage as additive in the form of solid complexes is recommended, forecast widespread applications not only as food flavoring agents, but as preservatives to prevent bacterial growth.

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689

690 **FIGURE CAPTIONS**

691 **Figure 1**. Effect of the concentration of IEOCs complexed with HP-β-CDs by
692 microwave-assisted method on their antimicrobial capacity over *E. coli*: A) Thymol
693 MWI, B) Carvacrol MWI, C) Linalool MWI.

694 **Figure 2**. Effect of the concentration of IEOCs complexed with HP-β-CDs by
695 solubility method on their antimicrobial capacity over *E. coli*: D) Thymol, E) Carvacrol,
696 F) Linalool.

697 **Figure 3**. Effect of the concentration of IEOCs complexed with HP-β-CDs by
698 microwave-assisted method on their antimicrobial capacity over *S. aureus*: A) Thymol
699 MWI, B) Carvacrol MWI, C) Linalool MWI.

700 **Figure 4**. Effect of the concentration of IEOCs complexed with HP-β-CDs by
701 solubility method on their antimicrobial capacity over *S. aureus*: D) Thymol, E)
702 Carvacrol, F) Linalool.

703

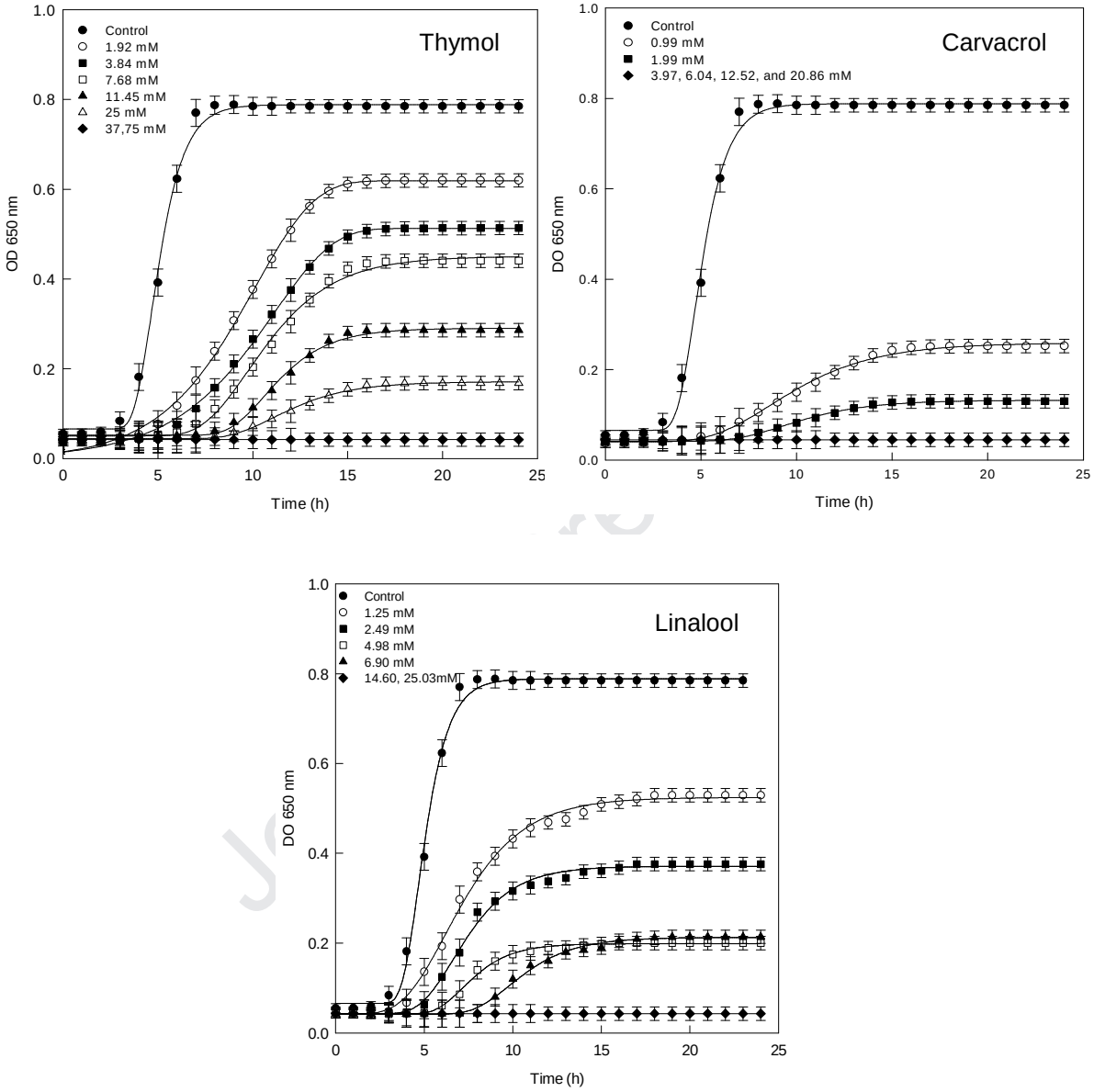
Thymol
Control
1.92 mM
3.84 mM
7.68 mM
11.45 mM
25 mM
37,75 mM
OD 650 nm
Time (h)
Carvacrol
Control
0.99 mM
1.99 mM
3.97, 6.04, 12.52, and 20.86 mM
DO 650 nm
Time (h)
Linalool
Control
1.25 mM
2.49 mM
4.98 mM
6.90 mM
14.60, 25.03mM
DO 650 nm
Time (h)

Figure 1

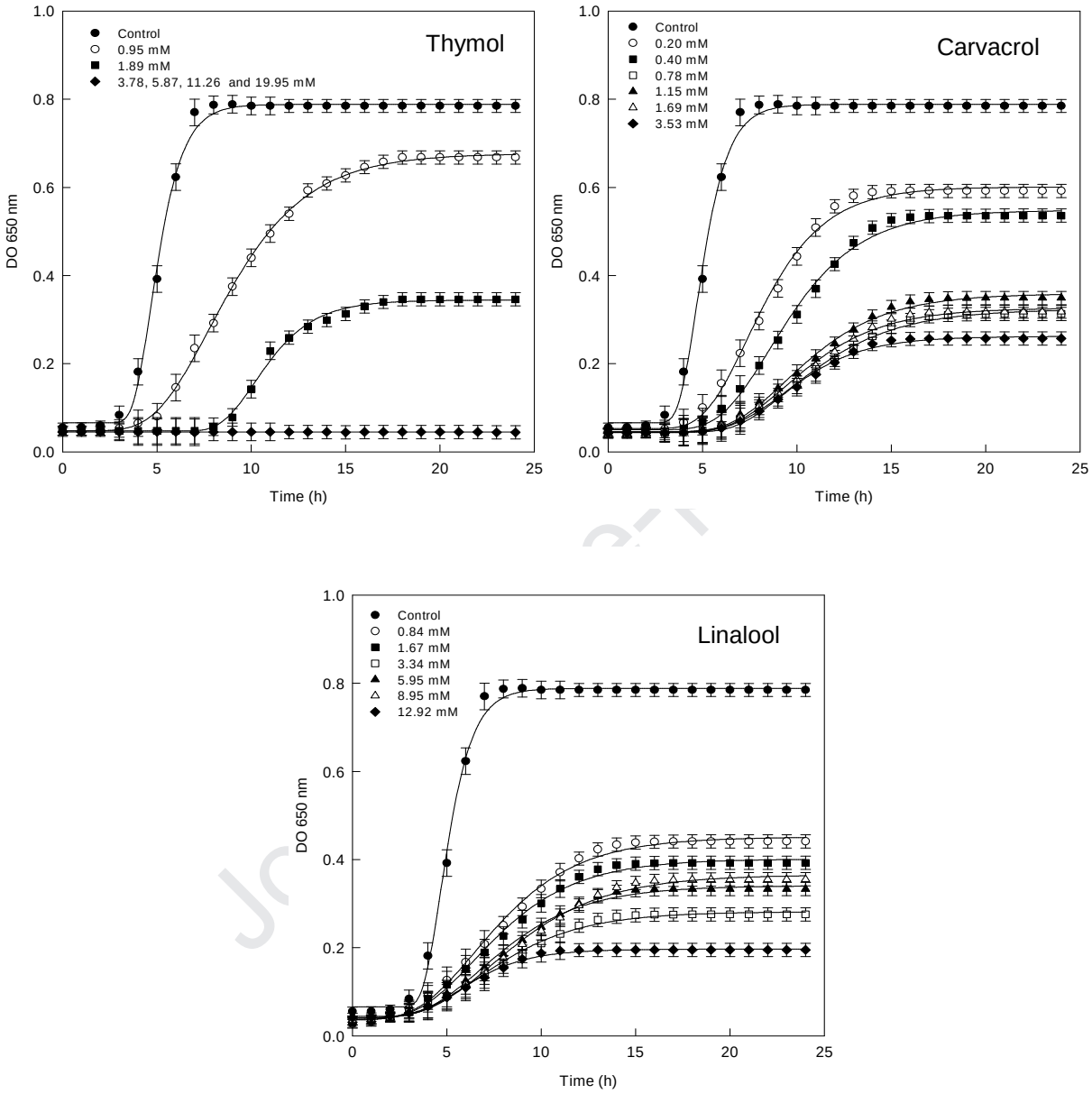
Thymol
Control
0.95 mM
1.89 mM
3.78, 5.87, 11.26 and 19.95 mM
Carvacrol
Control
0.20 mM
0.40 mM
0.78 mM
1.15 mM
1.69 mM
3.53 mM
Linalool
Control
0.84 mM
1.67 mM
3.34 mM
5.95 mM
8.95 mM
12.92 mM
DO 650 nm
Time (h)

Figure 2

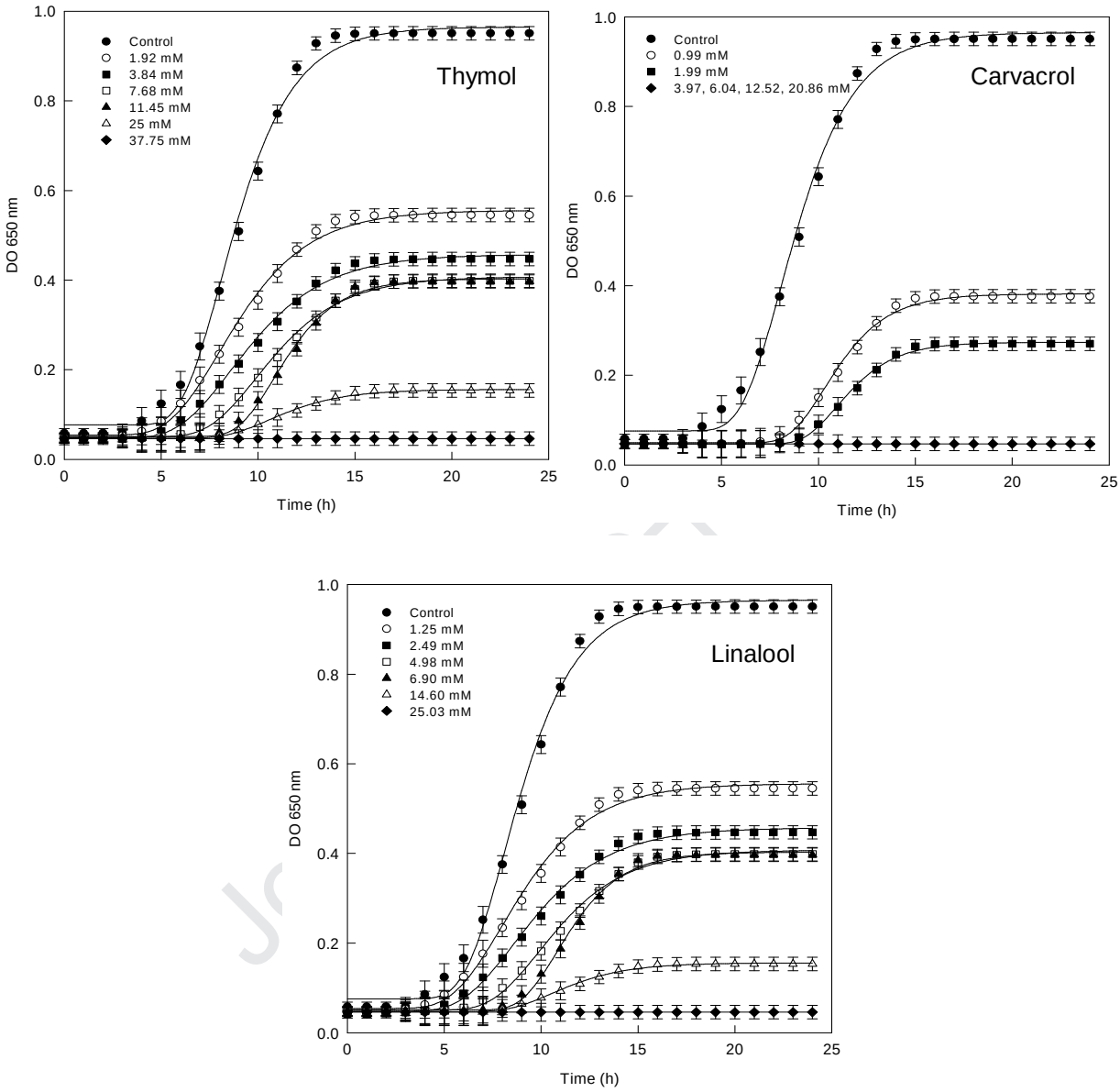
Thymol
Control
1.92 mM
3.84 mM
7.68 mM
11.45 mM
25 mM
37.75 mM
DO 650 nm
Time (h)
Carvacrol
Control
0.99 mM
1.99 mM
3.97, 6.04, 12.52, 20.86 mM
DO 650 nm
Time (h)
Linalool
Control
1.25 mM
2.49 mM
4.98 mM
6.90 mM
14.60 mM
25.03 mM
DO 650 nm
Time (h)

Figure 3.

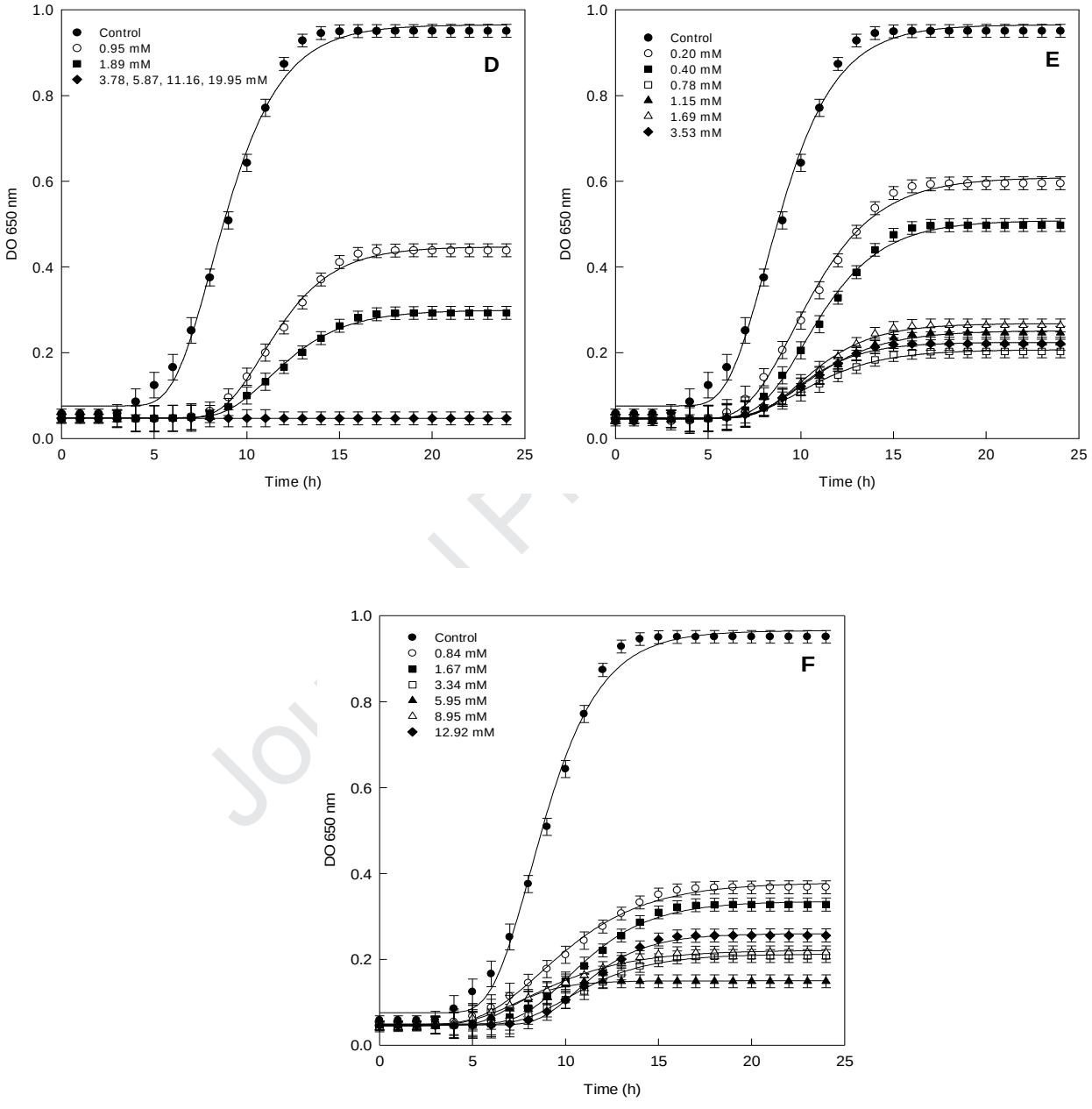
Control
0.95 mM
1.89 mM
3.78, 5.87, 11.16, 19.95 mM
D
DO 650 nm
Time (h)
Control
0.20 mM
0.40 mM
0.78 mM
1.15 mM
1.69 mM
3.53 mM
E
DO 650 nm
Time (h)
Control
0.84 mM
1.67 mM
3.34 mM
5.95 mM
8.95 mM
12.92 mM
F
DO 650 nm
Time (h)

Figure 4

Table 2. Minimum inhibitory concentration (MIC, mM) for *E. coli* and *S. aureus* in absence of CDs with monoterpene solutions prepared by a microwave-assisted method (MWI) and the solubility method.

Monoterpene	*E. coli*		*S. aureus*	
	MWI	Solubility	MWI	Solubility
Thymol	--*	--	5.59	3.92
Carvacrol	8.20	3.73	8.20	2.39
Linalool	--	--	--	--

* MIC was not reached.

794 **Table 3**. Effect of the concentration of monoterpenes complexed in HP-β-CDs by microwave-assisted method (MWI) and solubility method on the
795 growth rate and lag phase of *E. coli*.

Monoterpene	MWI			Monoterpene	Solubility		
(mM)	$\mu_{máx}$ (abs/h)	λ (h)	R^2	(mM)	$\mu_{máx}$ (abs/h)	λ (h)	R^2
Thymol				Thymol			
Control	0.2367 ± 0.001	3.59 ± 0.18	0.999	Control	0.2367 ± 0.001	3.59 ± 0.18	0.999
1.92	0.0705 ± 0.003	5.19 ± 0.45	0.999	0.99	0.0203 ± 0.002	5.09 ± 0.21	0.999
3.84	0.0564 ± 0.008	5.97 ± 0.63	0.999	1.99	0.0115 ± 0.003	6.39 ± 0.15	0.999
7.68	0.0519 ± 0.010	6.99 ± 0.39	0.999	3.97	0	24	--
11.45	0.0404 ± 0.006	8.51 ± 0.28	0.999	6.04	0	24	--
25.00	0.0179 ± 0.004	8.54 ± 0.31	0.999	12.52	0	24	--
37.75	0	24	0	20.86	0	24	--
Carvacrol				Carvacrol			
1.25	0.0608 ± 0.002	3.17 ± 0.19	0.988	0.95	0.0679 ± 0.014	4.31 ± 0.17	0.997
2.49	0.0547 ± 0.005	4.43 ± 0.21	0.988	1.89	0.0508 ± 0.013	8.04 ± 0.26	0.991
4.98	0.0326 ± 0.006	5.42 ± 0.17	0.993	3.78	0	24	--
6.90	0.0253 ± 0.007	7.34 ± 0.13	0.988	5.87	0	24	--
14.60	0	24	0	11.26	0	24	--
25.03	0	24	0	19.95	0	24	--
Linalool				Linalool			
0.20	0.0754 ± 0.001	4.67 ± 0.20	0.999	0.84	0.0429 ± 0.003	2.79 ± 0.16	0.999
0.40	0.0596 ± 0.003	5.50 ± 0.26	0.999	1.67	0.0383 ± 0.007	2.83 ± 0.19	0.997
0.78	0.0289 ± 0.008	6.17 ± 0.14	0.999	3.34	0.0246 ± 0.001	2.86 ± 0.22	0.999
1.15	0.0347 ± 0.005	6.11 ± 0.16	0.999	5.96	0.0315 ± 0.008	3.19 ± 0.29	0.999
1.69	0.0318 ± 0.007	6.17 ± 0.25	0.999	8.95	0.0311 ± 0.004	3.43 ± 0.25	0.999
3.53	0.0288 ± 0.006	6.38 ± 0.19	0.999	12.92	0.0233 ± 0.006	3.09 ± 0.18	0.999

796 μ: potential maximum rate, λ: lag phase, R^2: Adjusted R-square statistics.
797

798 **Table 4**. Effect of the concentration of monoterpenes complexed in HP-β-CDs by microwave-assisted method (MWI) and solubility method on the
799 growth rate and lag phase of *S. aureus*.
800

Monoterpene (mM)	MWI			Monoterpene (mM)	Solubility		
	$\mu_{máx}$ (abs/h)	λ (h)	R^2		$\mu_{máx}$ (abs/h)	λ (h)	R^2
Thymol				Thymol			
Control	0.1368 ± 0.004	6.86± 0.21	0.999	Control	0.1368 ± 0.004	6.86± 0.21	0.999
1.92	0.0618 ± 0.006	5.00 ± 0.32	0.999	0.99	0.0580 ± 0.008	8.26± 0.52	0.999
3.84	0.0481 ± 0.004	5.46 ± 0.29	0.999	1.99	0.0422 ± 0.006	9.02± 0.32	0.999
7.68	0.0462 ± 0.007	7.09 ± 0.22	0.999	3.97	0	24	0
11.45	0.0599 ± 0.009	8.71 ± 0.43	0.999	6.04	0	24	0
25.00	0.0159 ± 0.005	8.06 ± 0.37	0.999	12.52	0	24	0
37.75	0	24	0	20.86	0	24	0
Carvacrol				Carvacrol			
1.25	0.0618± 0.003	5.00 ± 0.27	0.999	0.95	0.0598 ± 0.007	8.42± 0.33	0.999
2.49	0.0481± 0.008	5.46 ± 0.35	0.999	1.89	0.0350 ± 0.003	8.58± 0.41	0.999
4.98	0.0461± 0.006	7.09 ± 0.26	0.999	3.78	0	24	0
6.90	0.0599± 0.008	8.71 ± 0.30	0.999	5.87	0	24	0
14.60	0.0159 ± 0.007	8.06 ± 0.40	0.999	11.26	0	24	0
25.03	0	24	0	19.95	0	24	0
Linalool				Linalool			
0.20	0.0718± 0.005	6.73 ± 0.37	0.999	0.84	0.0338 ± 0.004	5.06± 0.28	0.999
0.40	0.0628± 0.004	7.46 ± 0.30	0.999	1.67	0.0362 ± 0.002	7.17 ± 0.27	0.999
0.78	0.0204± 0.009	7.00 ± 0.43	0.999	3.34	0.0210 ± 0.008	7.17 ± 0.34	0.999
1.15	0.0264± 0.011	7.21 ± 0.47	0.999	5.96	0.0206 ± 0.003	5.04 ± 0.31	0.999
1.69	0.0319± 0.008	7.41 ± 0.35	0.999	8.95	0.0218 ± 0.006	4.20 ± 0.40	0.999
3.53	0.0288 ± 0.006	6.37 ± 0.19	0.999	12.92	0.0326 ± 0.009	8.21 ± 0.29	0.999

801 μ: potential maximum rate, λ: lag phase, R^2: Adjusted R-square statistics.

802 **Table 5**. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *E. coli* and *S. aureus* in
803 presence of monoterpene/HP-β-CDs complexes prepared by microwave-assisted method (MWI) and solubility method.

804

Monoterpene	*E. coli*				*S. aureus*			
	MIC (mM)		MBC (mM)		MIC (mM)		MBC (mM)	
	MWI	Solubility	MWI	Solubility	MWI	Solubility	MWI	Solubility
Thymol	6.68	3.82	13.37	3.87	4.83	3.91	4.83	6.12
Carvacrol	4.63	2.44	9.26	2.51	7.04	2.61	7.04	3.14
Linalool	--*	--	--	--	--	--	--	--

805 * MIC and MBC were not reached.

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