

1 **Antimicrobial Activity of Natural Agents Coated on Starch-based Films Against**
2 ***Staphylococcus aureus***

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27 **ABSTRACT:**

28 This study investigated the antimicrobial (AM) activity of starch-based films coated
29 with linalool, carvacrol or thymol against *S. aureus* in vitro or inoculated on the
30 surface of Cheddar cheese. In solid media using the agar diffusion method, the
31 inhibitory effect of linalool, carvacrol or thymol coated onto the films increased
32 significantly ($p \leq 0.05$) with the increase in concentration of each AM agent. All the
33 coated films effectively inhibited the growth of *S. aureus* on the surface of Cheddar
34 cheese. The sensitivity of *S. aureus* to the AM agents tested in the concentration
35 range of the study is in the order of thymol > carvacrol > linalool.

36

37 **Keywords:** antimicrobial packaging, linalool, carvacrol, thymol, *Staphylococcus*
38 *aureus*

39

40 **Practical Application:** Biodegradable starch-based films can be used for packaging
41 food products such as Cheddar cheese by adding a coating layer to protect the
42 moisture sensitive starch material. The coating layer can be incorporated with natural
43 antimicrobial agents that are effective against some microorganisms such as
44 *Staphylococcus aureus*. This can potentially extend the shelf life of the food products
45 and offer a more sustainable packaging option for manufacturers and consumers.

46

47

48 INTRODUCTION

49 In recent years, consumers' demand has increased for the provision of fresh, natural
50 foods with minimal addition of preservatives and that are of high quality with an
51 extended shelf life along with advances in the biodegradability of packaging
52 materials (Tharanathan 2003; Cutter 2006; Altskär and others 2008). Therefore, the
53 application of biodegradable polymers coated or incorporated with natural
54 antimicrobial (AM) compounds has the potential of controlling food spoilage as well
55 as enhancing the microbial safety of food products and expanding the functional
56 applications of such polymers in the food industry. Examples of biodegradable
57 materials that have been used in previous studies include starch, alginate, cellulose,
58 chitosan, carageenan, whey protein, corn zein and/or their derivatives (Phan and
59 others 2005; Rodriguez and others 2006). There is also current interest in the use of
60 starch-based materials in the packaging of cheese because these materials are
61 relatively inexpensive and can be incorporated with an AM agent (Pelissari and
62 others 2009; Durango and others 2006). Like many other food products, cheese may
63 be contaminated by undesired microorganisms such as bacteria, yeasts and fungi
64 that may deteriorate the sensory, aesthetic, flavour, odour and/or textual properties
65 (Vermeiren and others 1999; Appendini and Hotchkiss 2002; Gutierrez and others
66 2008; Davidson and Taylor 2007).

67

68 Some of the spoilage or pathogenic microorganisms that may contaminate cheeses,
69 meat, poultry and baked products include *Yarrowia lipolytica*, *Pseudomonas*
70 *aeruginosa*, *L. monocytogenes*, *E. coli* 0157, *Salmonella*, *S. aureus*, *B. cereus*,
71 *Campylobacter*, *C. perfringens*, *A. niger* and *S. cerevisiae* (Singh and others 2003;
72 López-Malo and others 2005; Rydlo and others 2006; Suppakul 2004; Schelz and

73 others 2006). The microorganism *S. aureus* is ubiquitous in nature. It is Gram-
74 positive and has a spherical shape with groups in grape-like clusters. Outside the
75 body it is one of the most resistant non-spore forming human pathogens, being able
76 to survive in a dry state for extended times (Jablonski and Bohach 2001).

77

78 Previous studies have reported that thyme, oregano and basil essential oils (EOs)
79 with their main constituent of thymol, carvacrol and linalool respectively, possess
80 both fungistatic and antibacterial activity against a wide range of microorganisms
81 (Dorman and Deans 2000; Friedman and others 2002; Tepe and others 2004;
82 Olasupo and others 2004; Youdim and Deanes 2000; Lachowicz and others 1998;
83 Suppakul and others 2003a). The AM activity of plant EOs is related to their
84 chemical structure, namely the presence of hydrophilic functional groups such as
85 hydroxyl groups of phenolic components and/or lipophilicity of the components in the
86 EOs and depends on their concentration (Bagamboula and others 2004; Davidson
87 and Naidu 2000; Farag and others 1989; Dorman and Deans 2000). The mode of
88 action for plant EOs such as thyme and oregano with the main constituents thymol
89 and cavarcol respectively is by the alteration of the membrane fatty acid
90 composition for pathogenic or spoilage microorganisms (Di Pasqua and others
91 2006).

92

93 Bagamboula and others (2004) determined the AM effect of thyme and basil EOs
94 with their major constituents thymol, linalool and carvacrol against *Shigella* spp. (*S.*
95 *sonnei* and *S. flexneri*) on lettuce leaves and solid media using the agar diffusion
96 method. They observed a decrease in the *Shigella* spp. after washing the lettuce
97 with 0.5% and 1% (v/v) thymol, linalool and carvacrol. At 1% (v/v) of each agent, the

98 *Shigella* spp population decreased to an undetectable level. They concluded that
99 these EOs showed inhibition of *Shigella* spp. (*S. sonnei* and *S. flexneri*) according to
100 the agar diffusion method. Chiasson and others (2004) evaluated the AM potential of
101 carvacrol, thymol and thyme in minced meat against *E. coli* and *Salmonella Typhi*.
102 Furthermore Seaberg and others (2003) reported the effectiveness of carvacrol as a
103 result of investigating its inhibitory effects against *L. monocytogenes* in ready-to-eat
104 beef slices. The inhibitory effect of carvacrol has also been reported by Ultee and
105 others (2000) who investigated its AM activity against *B. cereus* in the preservation
106 of rice. Ultee and Smid (2001) also investigated the AM activity of carvacrol against
107 toxin production of *B. cereus* in soups. They found that carvacrol reduced the
108 production of *B. cereus* toxin in mushroom soup to a level that could not be detected.

109

110 In view of the current advances in biodegradable materials and the already identified
111 potential of linalool, carvacrol or thymol as effective natural AM agents, the
112 effectiveness of starch-based films coated with linalool, carvacrol or thymol against
113 *S. aureus in vitro* or inoculated on the surfaces of Cheddar cheese samples was
114 investigated in the current study. The AM activity was evaluated for several
115 concentrations of each of the AM agents coated onto a starch-based polymer film.

116

117 **MATERIALS AND METHODS**

118 *POLYMERS*

119 A commercial starch-based film (Biograde-F) supplied by Biograde Ltd., Australia
120 was used in this study. Biograde-F is a biodegradable material based on a blend of
121 thermoplastic starch, aliphatic polyesters and natural plasticisers. Methylcellulose
122 (MC, 18,804-2); hydroxypropyl methylcellulose (HPMC, 42,321-1) and poly(ethylene

123 glycol) (PEG, 20,236-3) were purchased from Sigma Chemical Company Inc.,
124 Milwaukee, WI.

125

126 *ANTIMICROBIAL AGENTS AND CHEDDAR CHEESE*

127 The AM additives were supplied by Sigma-Aldrich Pty. Ltd., Australia and comprised
128 linalool with a purity of 97% (L2602), carvacrol with a purity of 98% (W224502) and
129 thymol with a purity of 99.5% (TO501). Cheddar cheese was purchased from a retail
130 outlet. According to the manufacturer (Woolworths Ltd., Australia), a 100 g sample of
131 the cheese contains as its main components: fat 35.2 g; protein 24.3 g;
132 carbohydrates 0.1 g; calcium 735 mg and sodium 635 mg.

133

134 *MEDIA AND MICROORGANISMS*

135 The media used were nutrient agar (AM 130), 3M Perifilm™ Staph Express Count
136 Plate (6490) purchased from 3M Microbiology Products, USA and plate count agar
137 (AM 144) purchased from Amyl Ltd., Australia. The microorganism *S. aureus* (UNSW
138 056201) was obtained from the culture collection of the University of New South
139 Wales, Australia. Bacteriological peptone (LP0037) was purchased from Oxoid Ltd.,
140 Hampshire, England.

141

142

143 *FILM PREPARATION AND AM COATING*

144 The coating solution was prepared from the MC and HPMC materials.
145 Methylcellulose and HPMC were added slowly to absolute ethanol and heated with
146 magnetic stirring on a hotplate. The heating was discontinued when the temperature
147 reached 65°C. With continuous agitation, a mixture of PEG and distilled water, as a

148 plasticiser, was added slowly to the MC-HPMC dispersion whilst the dispersion
149 cooled (Rardniyom 2008). This resulted in the formation of a uniformly clear coating
150 solution or gel. The AM agent was then added to the coating solution at one of three
151 different concentrations to form the final coating materials with AM agent at target
152 levels of 1, 3 or 5% (w/w). The coating medium was applied to the starch-based
153 material using a roller and the film was then dried at ambient conditions (Cooksey
154 2005). Each of the three natural AM agents: linalool, carvacrol and thymol were
155 coated separately onto the starch-based material. Similarly, starch-based material
156 without any AM agent was prepared as the control. The addition of AM agents and
157 subsequent coating procedures were conducted at a low temperature in order to
158 minimise any significant loss of AM agents due to their volatility. The actual
159 concentration of the AM agents retained in the films after drying was determined on
160 the basis of total dry weight of the coating layer and the total film. The total film
161 thickness of the coating and starch-based film was measured immediately after
162 peeling them off using a hand-held micrometer with a precision of 0.001 mm
163 (Mitutoyo, Japan). The film thickness was measured at 5 different positions and the
164 average thickness was calculated from these readings. After measuring the
165 thickness, the films were wrapped in aluminium foil to prevent further loss of the AM
166 agent before being used.

167

168 *ANTIMICROBIAL ACTIVITY ON SOLID MEDIA*

169 The effectiveness of the AM starch-based films on a solid medium was determined
170 using the agar disc diffusion assay. A bacterial suspension at the level of 10^6 CFU
171 mL^{-1} was prepared in a 0.1% (w/v) sterile peptone solution. The control and AM
172 starch-based films were cut into circular discs (6 mm in diameter) and sterilised

173 using UV light for 2 min (Cooksey 2000). The cut pieces were aseptically placed on
174 nutrient agar plates seeded with 0.1 mL of bacterial solution. The plates were
175 incubated at 37°C for 24 h. After the incubation process, the diameters of the clear
176 zones that formed around the film samples were measured using a Vernier calliper
177 and reported as the zone of inhibition (Suppakul and others 2008). Such a qualitative
178 measurement is sufficient to make meaningful comparisons between the systems
179 studied in this work. **Although not used to analyse the data in the current work,**
180 another approach involves the calculation of an antimicrobial index (*AMI*) defined in
181 equation (1):

182

$$183 \quad AMI = (d_1 - d_2)/d_1 \quad (1)$$

184

185 where d_1 is the diameter of clear zone and d_2 is the diameter of circular film.

186

187 *CHEESE PREPARATION*

188 Cheddar cheese was purchased from a local supermarket and cubes of the cheese
189 were cut weighing *ca.* 20 ± 1 g each (Suppakul 2004; Rupika and others 2005).

190 Samples were divided into four sets, for the control film and the three AM coated
191 films containing linalool, carvacrol or thymol. The cheese samples were then
192 sterilised on all sides for 1 h using UV light. The control and AM films were also
193 sterilised with UV light prior to use.

194

195 *INOCULATION OF S. AUREUS ON CHEDDAR CHEESE*

196 Each of the cheese samples was inoculated on top and bottom surfaces with *S.*
197 *aureus* and then spread using a sterile glass rod to obtain *ca.* 10⁴ CFU g⁻¹ (Suppakul

198 and others 2008) prior to wrapping with the control or an AM test film. The inoculated
199 cheese samples were placed between folded films and then the open sides of the
200 films were sealed. The packaged cheese samples were prepared in duplicate and
201 stored at 15°C for 21 days. The temperature of 15°C was chosen in order to mimic a
202 temperature abuse condition that might arise in the food supply chain (Siragusa and
203 others 1999).

204

205 *BACTERIAL COUNT*

206 The bacteriological analysis was performed periodically on days 0, 1, 3, 5, 7, 10, 15
207 and 21. Two samples from each treatment were aseptically opened on the sampling
208 days. An 11 g sample of cheese was aseptically transferred to a sterile stomacher
209 bag. In accordance with the method described by Rupika and others (2005), 99 mL
210 of 0.1% (w/v) sterile peptone solvent (pH 7.5 ± 0.2 at 25°C) were added to the
211 sample which was then homogenised using a laboratory blender (Seward Stomach®
212 400, Seward Medical, UK) for 3 min. Serial dilutions of the resulting solutions were
213 prepared in a sterile peptone diluent (pH 7.0 ± 0.1 at 25°C) in order to obtain a
214 quantifiable colony count. For the determination of bacteria counts, 1 mL of each
215 serially diluted sample was plated in duplicate on a 3M Perifilm™ Staph Express
216 Count Plate and then incubated aerobically for 24 h at 37°C. The colonies were
217 counted and the results expressed as colony forming units per gram (CFU g⁻¹). Two
218 sets of measurements were taken from each of the bacterium enumeration
219 experiments with the results averaged and the data quoted as a mean.

220

221 *DETERMINATION OF MICROBIAL DEATH RATE BY THE AM FILMS*

222 The death rate of *S. aureus* bacterium inoculated onto the cheese samples during
223 storage was determined in accordance with the calculation procedures described by
224 Bachrouri and others (2002). Accordingly, a specific death rate, μ , can be
225 determined from equation (2):

226

$$227 \quad N = N_0 e^{-\mu t} \quad (2)$$

228

229 where, N is the population surviving at any time t , N_0 is the initial population. The
230 specific death rate is obtained from the gradient of a plot of the natural logarithm of N
231 (expressed either in units of CFU mL⁻¹ or CFU g⁻¹) versus time. Whence, taking
232 natural logarithms of both sides of equation (2) results in equation (3):

233

$$234 \quad \ln(N) = \ln(N_0) - \mu t \quad (3)$$

235

236 In the present study, the decadic logarithm of the surviving population of *S. aureus* in
237 the presence of the three different AM agents was plotted as a function of the time of
238 storage. A linear regression analysis was performed on each curve to obtain the
239 specific death rate, μ' , where $\mu' = \mu/\ln(10)$. The values of μ' were subsequently used
240 to compare the AM activity of the films.

241

242 *DATA ANALYSIS*

243 Experiments on solid media and Cheddar cheese were performed in triplicate and
244 duplicate respectively. Individual experiments for each of the AM agents (linalool,
245 carvacrol and thymol) coated onto starch-based films were performed separately and

246 by comparing the three levels of AM agent added to the starch-based material. Data
247 points are represented by the mean of the results obtained for each AM agent
248 coated onto the substrate. Bacterial colony counts were converted into decadic
249 logarithm values. The latter were subjected to analysis of variance (ANOVA) at the
250 0.05 confidence level. Differences amongst the treatments were examined by least
251 significant differences tests using SAS (Version 9.5, SAS Institute, Cary, NC).

252

253 **RESULTS AND DISCUSSION**

254 *RETENTION OF AM AGENT IN THE COATING LAYER*

255 The incorporation of linalool, carvacrol or thymol into the MC-HMPC coatings at
256 different concentrations did not significantly change the thickness of the AM films.
257 The average thickness of the coated starch-based films is ca. 138 μm . **The residual**
258 **concentrations of the AM agents in the starch-based films containing 1, 3 and 5%**
259 **(w/w) linalool, carvacrol or thymol in their coating retained 0.48%, 1.43% and 2.38%**
260 **(w/w) respectively after drying. The retention of the AM agent in the coating layer**
261 **suggests that the coating procedure resulted in only a minimal and acceptable loss**
262 **of AM agent.**

263

264 *ANTIMICROBIAL ACTIVITY OF AM STARCH-BASED FILMS ON SOLID MEDIA*

265 The AM starch-based films were initially tested on solid media using an agar disc
266 diffusion assay in order to provide preliminary information about the potential AM
267 activity of the active agents against *S. aureus*. The presence of a clear zone of
268 inhibition around the test films was taken as an indication of AM activity for the film
269 formulation. Figure 1 shows the AM activity of starch-based film coated with linalool,
270 carvacrol or thymol against *S. aureus* at 37°C on the solid media in terms of the clear

271 inhibition zones. These zones are visible in the systems containing the AM agent.
272 The film containing no AM agent (control) did not inhibit the growth of *S. aureus* on
273 the solid medium, as expected.

274

275 The average values of the zones of inhibition for each of the AM films are presented
276 in Table 1. These data confirm the visual observations made in Figure 1 in that the
277 starch-based films coated with linalool, carvacrol or thymol are effective in inhibiting
278 the growth of *S. aureus* as revealed by the agar disc diffusion method. It can also be
279 observed from these results that the inhibitory effect of these agents when coated
280 onto the films increased significantly ($p \leq 0.05$) with the increase in concentration of
281 the agent.

282

283 All the films coated with carvacrol demonstrated a positive AM activity against *S.*
284 *aureus* in this study. A detailed statistical analysis of the results suggests that the
285 inhibitory effect of the film containing 0.48% (w/w) carvacrol was significantly ($p \leq$
286 0.05) lower than the inhibitory effect of the films containing 1.43% (w/w) and even
287 more so than the film containing 2.38% (w/w) carvacrol. A similar concentration
288 dependence of carvacrol activity against *S. aureus* on solid media was observed by
289 Rupika and others (2005) who evaluated the AM activity of polyethylene films
290 containing carvacrol within the bulk of the film against *S. aureus*, using the agar disc
291 diffusion assay. In the present study, the inhibitory activity of linalool-coated starch-
292 based film against *S. aureus* increased notably with increasing concentration. The
293 zone of inhibition data for each of the concentrations showed that the films
294 containing 2.38% (w/w) linalool, carvacrol or thymol had a higher inhibitory activity
295 than those containing any of the lower concentrations of these agents in their

296 coatings and were all effective against *S. aureus* on solid media. The greatest
297 inhibition for the starch-based coated films occurred with 2.38% (w/w) thymol. This
298 observation is consistent with the work of Sivropoulou and others (1996) who studied
299 the AM activity of thymol and reported its significant activity against *S. aureus*. Tepe
300 and others (2004) have also reported a significant AM activity of thymol against *S.*
301 *aureus* also *in vitro*.

302

303 Figure 2 shows the variation in the zone of inhibition as a function of concentration of
304 linalool, carvacrol or thymol AM agents. The AM activity of the starch-based films is
305 highly linear between ca. 0.48% (w/w) and 1.43% (w/w) as revealed by the linear
306 regression analysis data listed in Table 1. These regression data, namely the
307 gradient and the vertical axis intercept, pertain to the zone of inhibition data obtained
308 in the latter concentration range. The respective gradients of the regression lines
309 are indicative of the sensitivity of the test microorganism to changes in the
310 concentration of the three AM agents in the film coating. The order of concentration
311 sensitivity is thus thymol > carvacrol > linalool. Such linearity in the response of *S.*
312 *aureus* to changes in AM concentration does not, however, seem to be maintained in
313 the region between the control sample and ca. 0.48% (w/w) of AM agent as shown
314 by the dashed line. The latter is consistent with the observations made by
315 Bagamboula and others (2004) who reported a non-linear relationship between the
316 zone of inhibition of *Shigella sp.* with the concentration of thyme and basil EOs with
317 their main constituents: carvacrol, thymol and linalool. However, it is important to
318 note that the non-linearity reported by Bagamboula and others (2004) was observed
319 over the concentration range spanning several orders of magnitude (0.01 - 10% w/w)
320 and that the data seem reasonably linear in the AM concentration range used in the

321 present work. Possible causes of this phenomenon include the role of diffusion
322 kinetics in the observed non-linear relation between the zone of inhibition and the
323 concentration of AM agents in the polymer coating. Furthermore, the non-linear
324 relationship may be due to factors that limit the applicability of the method at low
325 concentrations of AM agents (Suppakul and others 2003a).

326

327 *ANTIMICROBIAL ACTIVITY ON CHEDDAR CHEESE - A CHALLENGE TEST*

328 Further to the above *in vitro* study using the agar diffusion technique, the effect of the
329 AM starch-based films was explored when placed in contact with a particular
330 foodstuff. In particular, the films were used to package samples of Cheddar cheese
331 in order to assess their effectiveness against *S. aureus* that was inoculated on the
332 surface of the samples and in order to attempt to identify low concentrations where
333 effective growth control of the microorganisms occurs. Clearly, a low concentration of
334 additive is preferable since the higher the concentration of the natural plant extracts
335 the greater is the concern about off-flavour issues for food packaging applications
336 (Suppakul and others 2003b). Figure 3 is a plot of the decadic logarithm of the
337 population counts of *S. aureus* that were determined on the surface of the cheese as
338 a function of storage time (i.e. the “death” curves) at 15°C for starch-based AM films
339 containing carvacrol. Data for the control film are also plotted for comparison.
340 Similar trends were observed for the death curves obtained using thymol and
341 linalool-coated starch-based films (not shown). The specific death rates were
342 determined from the gradients of the plots for all the systems. These data are listed
343 in Table 2 along with the other linear regression analysis data.

344

345 The data shown in Table 2 confirm the consistency in the initial inoculation
346 procedure as reflected by the consistency in the vertical axis intercepts of the plots.
347 A more detailed analysis of the specific death rate data can be obtained by plotting
348 these values as a function of the concentration of the AM agent for each of the
349 experimental systems. Such a plot is shown in Figure 4 where a linear relationship
350 exists between the specific death rate and the concentration of each of the AM
351 agents across the entire range of studied concentrations. This, contrasts slightly to
352 the behaviour exhibited in the solid media where a non-linear relationship was
353 observed between the zone of inhibition and the AM concentration at low
354 concentrations. These observations suggest that the specific death rate μ' may in
355 fact be a pseudo-first order rate constant where $\mu' = \alpha[\text{AM}]$, where $[\text{AM}]$ is the
356 concentration of AM agent and α is a second-order rate constant. The second order
357 rate constants can be obtained from the gradient of the plots in Figure 4.

358

359 The plots shown in Figure 4 indicate that the inhibition of *S. aureus* on the surface of
360 Cheddar cheese when packed in starch-based films coated with these AM agents is
361 ca. 1.6 times more sensitive to changes in the concentration of thymol compared to
362 that of linalool. The sensitivity of this microorganism to changes in carvacrol
363 concentration is between those of the other two AM agents. The relative order of the
364 sensitivity determined in these storage experiments at 15°C was similar to that found
365 in the solid media experiments conducted at 37°C suggesting that the relative order
366 remains unchanged across this range of temperatures.

367

368 In the present study the starch-based films containing thymol demonstrated the
369 strongest inhibitory effect on the growth of *S. aureus* on the cheese when compared

370 to the control film (see Table 2). The seemingly natural rate of decrease of the *S.*
371 *aureus* count observed in the control film might be due to the depletion of oxygen
372 during the test and/or other factors such as the preservatives originally present in the
373 Cheddar cheese. During the first 5 days, the AM films containing 0.48% (w/w)
374 thymol in their coatings extended the lag phase of *S. aureus* growth and reduced by
375 24% the *S. aureus* population on the cheese after 21 days of storage. The AM films
376 containing thymol at 1.43% (w/w) and 2.38% (w/w) in their coatings further extended
377 the lag phase and reduced the *S. aureus* count on the surface of the Cheddar
378 cheese by 33% and 43% respectively. A high AM activity of thymol has also been
379 reported by Olasupo and others (2003) and is consistent with the present findings.

380

381 The population count of *S. aureus* on the cheese packaged in the starch-based film
382 containing 0.48% (w/w) linalool decreased by 22% after 21 days of storage at 15°C
383 (see Table 2). It can also be seen from the results in Table 2 that increasing the
384 concentration of linalool contained in the film to 1.43% or 2.38% (w/w) had a
385 significant effect; the population of *S. aureus* on the cheese was reduced by 26%
386 and 33% respectively after 21 days of storage. Kim and others (1995) observed a
387 similar dose-related activity of linalool against *S. aureus*. Mazzanti and others (1998)
388 as well as by Dorman and Deans (2000) have also reported the overall effectiveness
389 of this agent against *S. aureus*. The observations in the present study are also
390 consistent with the results of Rupika and others (2006) who found that linalool
391 exhibited an inhibitory effect against *S. aureus* on Cheddar cheese packaged in
392 polyethylene-based AM films. Moreover, the observed inhibition of *S. aureus* on the
393 surface of Cheddar cheese by carvacrol is consistent with the work of Rardniyom
394 (2008) who reported the AM activity of carvacrol against the growth of *E. coli* on

395 Cheddar cheese. The linear response in the inhibition of *S. aureus* with the
396 concentration of carvacrol (see Figure 4) is in accordance with the observations
397 made by Ultee and others (1998) who found a concentration dependence of
398 carvacrol against *B. cereus* as well as those of Periago and others (2004) who
399 observed a dose-dependence of carvacrol activity also against *L. monocytogenes* in
400 carrot juice.

401

402 **CONCLUSIONS**

403 The results of the present study suggest that the AM agents linalool, carvacrol or
404 thymol, can be successfully coated onto starch-based films to produce packaging
405 materials that exhibit activity against *S. aureus*. In solid media using the agar
406 diffusion method, all of the AM films formed clear zones of inhibition against *S.*
407 *aureus*. All AM films containing linalool, carvacrol or thymol effectively inhibited the
408 growth of *S. aureus* on the surface of Cheddar cheese. The AM activity of these
409 agents against *S. aureus* was found to be dependent on the concentration of AM
410 agent coated onto the film samples. The order of effectiveness found was thymol >
411 carvacrol > linalool. The concentration dependence of the effectiveness was found
412 to be linear at the higher concentrations of the AM agent (above ca. 0.48% (w/w))
413 and can be used as a measure of the sensitivity of the test microorganism to the AM
414 agent. Starch-based films containing linalool, carvacrol or thymol have a potential for
415 applications in AM packaging systems and may reduce the risk of food-borne illness
416 associated with microbial contamination in hard cheeses. The results also infer that
417 starch-based AM films containing these agents may, in the future, be used to extend
418 the shelf life of packaged hard cheeses. Further research is underway to study the
419 AM effect of linalool, carvacrol or thymol coated on starch-based films at low

420 concentration in order to attempt to identify systems that could be commercially

421 attractive.

422

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424

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560 **Table 1:** Analysis of the zone of inhibition data in solid media for *S. aureus* at 37°C in
 561 the presence of starch-based film coated with the AM agents: carvacrol, linalool or
 562 thymol.

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Treatment	Zone of inhibition/mm <i>S. aureus</i>			Gradient dz/dc	Intercept	Correlation coefficient (R ²)
	0.48% (w/w)	1.43% (w/w)	2.38% (w/w)			
Linalool	9.2 ± 0.3	13.3 ± 0.6	18.6 ± 1.0	5.98	-4.69	0.969
Carvacrol	10.3 ± 1.1	15.3 ± 1.7	21.9 ± 1.7	7.06	-5.78	0.977
Thymol	11.3 ± 1.8	16.7 ± 0.9	23.8 ± 1.8	7.69	-6.29	0.977

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577 **Table 2.** Analysis of the “death” curve data for *S. aureus* on the surface of Cheddar
 578 cheese packaged and stored at 15°C in starch-based films coated with AM agents:
 579 carvacrol, linalool or thymol.

580

Treatment	AM agent concentration in coated film % (w/w)	Specific death rate μ' /day ⁻¹	Intercept	Correlation coefficient R ²	Population of <i>S. aureus</i> on cheese log CFU g ⁻¹	
					Day 0	Day 21
Control	0	0.041	4.82	0.951	4.76 ± 0.08	4.06 ± 0.48
	0.48	0.047	4.74	0.976	4.66 ± 0.10	3.66 ± 0.26
Linalool	1.43	0.056	4.70	0.989	4.66 ± 0.10	3.47 ± 0.25
	2.38	0.072	4.64	0.979	4.74 ± 0.07	3.17 ± 0.13
Carvacrol	0.48	0.048	4.75	0.977	4.73 ± 0.09	3.7 ± 0.15
	1.43	0.062	4.70	0.980	4.71 ± 0.02	3.36 ± 0.23
	2.38	0.079	4.60	0.952	4.71 ± 0.08	2.9 ± 0.44
	0.48	0.054	4.76	0.984	4.73 ± 0.05	3.6 ± 0.11
Thymol	1.43	0.071	4.70	0.994	4.67 ± 0.05	3.12 ± 0.40
	2.38	0.089	4.64	0.984	4.65 ± 0.04	2.65 ± 0.50

581

582 **Figure Captions**

583

584 **Figure 1.** Inhibition of *S. aureus* on solid media at 37°C after 24 h on starch-based
585 coated films containing: (a) no AM agent, (b) 2.38% (w/w) linalool, (c)
586 2.38% (w/w) carvacrol, and (d) 2.38% (w/w) thymol.

587

588 **Figure 2.** Zone of inhibition of *S. aureus* at 37°C versus AM agent concentration for
589 starch-based films containing in their coating: no AM agent (□), linalool
590 (●), carvacrol (△) and thymol (○).

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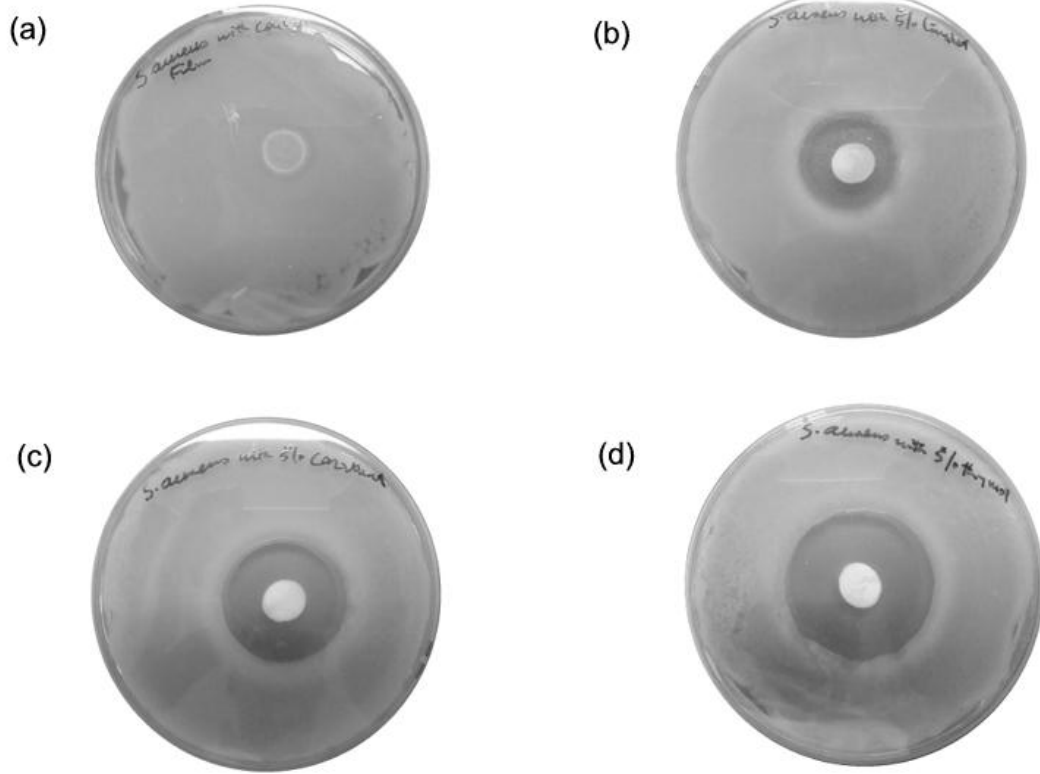
592 **Figure 3.** Inhibition of *S. aureus* on Cheddar cheese packaged and stored at 15°C
593 in starch-based coated films containing: no AM agent (□), 0.48% (w/w)
594 carvacrol (△), 1.43% (w/w) carvacrol (○), and 2.38% (w/w) carvacrol (■).

595

596 **Figure 4.** Specific death rate of *S. aureus* on the surface of Cheddar cheese versus
597 AM agent concentration for cheese packaged and stored at 15°C in
598 starch-based films containing in their coating: linalool (●), carvacrol (△)
599 and thymol (○).

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601 **Figure 1.**

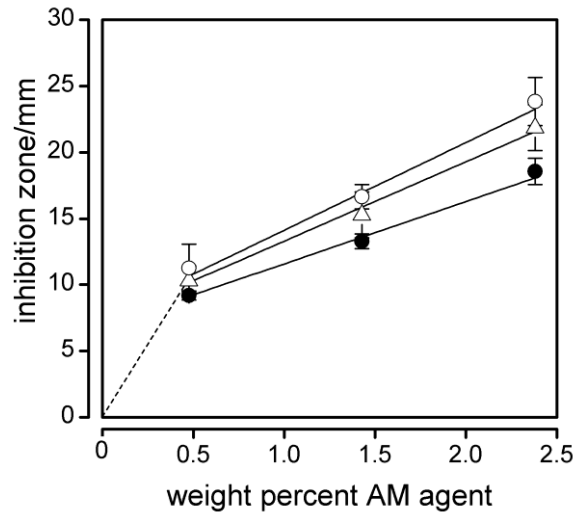


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604 **Figure 2.**

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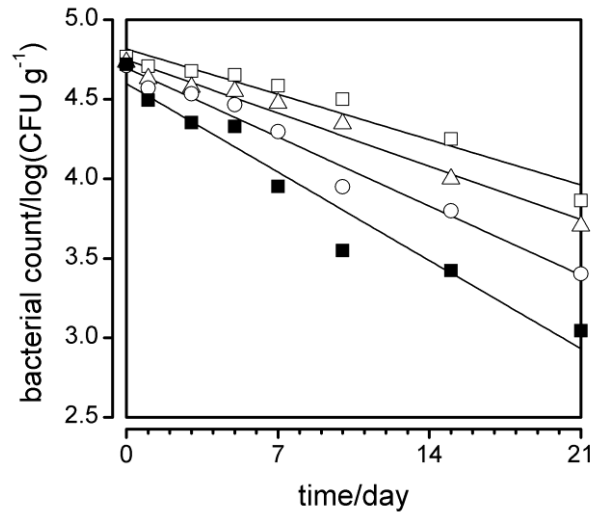
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609 **Figure 3.**

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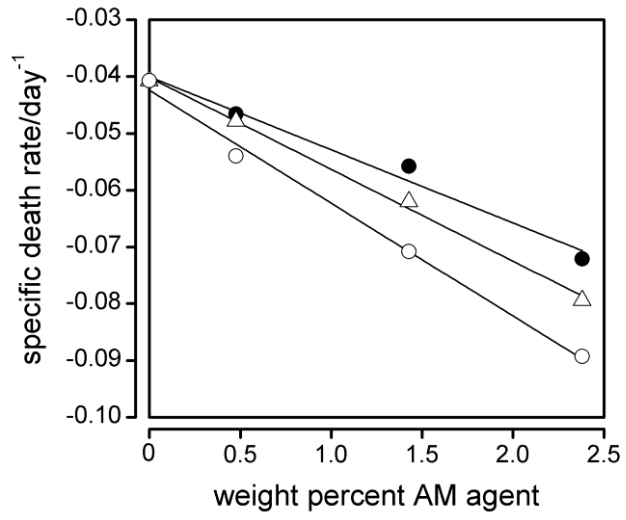


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613 **Figure 4.**

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