Evaluating food additives as antifungal agents against *Monilinia fructicola in vitro* and in hydroxypropyl methylcellulose-lipid composite edible coatings for plums

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Abstract

Common food preservative agents were evaluated in *in vitro* tests for their antifungal activity against *Monilinia fructicola*, the most economically important pathogen causing postharvest disease of stone fruits. Radial mycelial growth was measured on PDA petri dishes amended with three different concentrations of the agents (0.01-0.2%, v/v) after 7 days of incubation at 25 °C. Thirteen out of fifteen agents tested completely inhibited the radial growth of the fungus at various concentrations. Among them, ammonium carbonate, ammonium bicarbonate and sodium bicarbonate were the most effective while sodium acetate and sodium formate were the least effective. The effective agents and concentrations were tested as ingredients of hydroxypropyl methylcellulose (HPMC)-lipid edible coatings against brown rot disease on plums previously inoculated with *M. fructicola* (curative activity). 'Friar' and 'Larry Ann' plums were inoculated with the pathogen, coated with stable edible coatings about 24 h later, and incubated at 20 °C and 90% RH. Disease incidence (%) and severity (lesion diameter) were determined after 4, 6, and 8 days of incubation and the 'area under the disease progress stairs' (AUDPS) was calculated. Coatings containing bicarbonates and parabens significantly reduced brown rot incidence in plums, but potassium sorbate, used at 1.0% in the coating formulation, was the most effective agent with a reduction rate of 28.6%. All the tested coatings reduced disease severity to some extent, but coatings containing 0.1% of sodium methylparaben or sodium ethylparaben or 0.2% of ammonium carbonate or ammonium bicarbonate were superior to the rest, with reduction rates of 45-50%. Overall, the results showed that most of the agents tested in this study had significant antimicrobial activity against *M. fructicola* and the application of selected antifungal edible coatings is a promising alternative for the control of postharvest brown rot in plums.

Keywords: Prunus salicina, postharvest disease, brown rot, food additives, antimicrobial agents

1. Introduction

Japanese plums (*Prunus salicina* Lindl.) are stone fruits produced in many geographical regions and consumed willingly worldwide. These fruits are grown commercially in more than 80 countries and total production amount is over 10 million tons (FAO, 2011). Like many other fruits for fresh consumption, plums are quite susceptible to postharvest diseases caused by a number of fungal pathogens (Chen and Zhu, 2011). Brown rot caused by *Monilinia* spp. (syn.: *Monilia* spp.) is one of the most important postharvest diseases that typically affect stone fruits such as plums, peaches, nectarines, apricots or cherries. The casual agents of this disease are mainly three species of the genus *Monilinia*, namely *Monilinia laxa* (Aderh. & Ruhl.) Honey, *Monilinia fructigena* Honey in Whetzel and *Monilinia fructicola* (G. Wint.) Honey (Casals et al., 2010), although other species like *Monilia mumecula, Monilia yunnanensis* and *Monilia polystroma* have recently also been reported as pathogens (Hu et al., 2011; Poniatowska et al., 2013). Few years ago, *M. fructicola* and *M. fructigena* were not distributed over Europe and America, respectively, while *M. laxa* was present in both continents. However, *M. fructicola* has been recently introduced in Europe (e.g. in Spain in 2006) and spread readily to take the place of *M. laxa* as the most frequent cause of brown rot on peaches (Villarino et al., 2013). In Spain, it is first reported in 2012 as the cause of fruit rot in plums (Arroyo et al., 2012). These pathogens are latent parasites that can infect flowers and young fruit in the field, remain latent and show disease symptoms only after harvest. In addition, they are also wound pathogens that require a wound in the skin of mature fruit to enter into contact with susceptible tissue and initiate infection (Spotts et al., 1998). If wounds occur for any reasons (inappropriate harvesting-handling techniques or harsh fruit movements during washing, packaging, or shipping steps), then subsequent infections are greatly favored. Total postharvest losses due to both latent and wound infections may be very high, in

some cases reaching values of 90% if the conditions are favorable for fungal development (Mari et al., 2007).

Brown rot of plums and other stone fruits could be successfully controlled by pre- and/or postharvest applications of some effective fungicides. However, due to the problems regarding fungicide-resistant strains and concerns about the residues on produce and in the environment, the use of many synthetic fungicides has been increasingly restricted or even banned in a number of countries. Therefore, various compounds alternative to synthetic fungicides have been tested to control *Monilinia* spp. in both *in vitro* and *in vivo* studies. Tsao and Zhou (2000) examined the effects of naturally occurring monoterpenoids on spore germination and mycelial growth of *M. fructicola* and *Botrytis cinerea*. Of the 22 compounds tested, carvacrol and thymol were the most potent inhibitors to the pathogens. These compounds completely prevented spore germination and mycelial growth of both pathogens at 100 µg/mL. Yan et al. (2012) tested a berberine-chitosan composite membrane coating for peach fruits against *M. fructicola*. In coated samples, they observed an infection rate of 10%, which was significantly lower than that in the controls after 40 days of storage at 4ºC. Very recently, Feliziani et al. (2013) evaluated many compounds for the control of common pathogens causing postharvest diseases of sweet cherry. They found that the growth of *M. laxa*, *B. cinerea* and *Rhizopus stolonifer* was significantly reduced when potato dextrose agar (PDA) medium was amended with some compounds such as chitosan, benzothiadiazole, oligosaccharides, and an extract from *Urtica dioica*. They also reported that chitosan was the most effective compound in reducing storage decay of sweet cherry with an antimicrobial activity comparable to the fungicide fenhexamid. As an alternative to fungicide treatments, the use of food preservative agents is a relatively new trend for controlling plant pathogens. These agents are natural or synthetic compounds with known and low toxicity and classified as food-grade additives or Generally Regarded as Safe (GRAS) compounds by national/international authorities.

Treating agricultural products with these agents can be achieved generally by dipping (Moscoso-Ramírez et al., 2013; Youssef et al., 2012) or coating (Fagundes et al., 2013; Jin and Niemira, 2011) applications.

In recent years, the use of edible films and coatings has emerged as a new, effective, and environmental-friendly alternative mean to extend the shelf life of many products including fresh fruits and vegetables. These coatings or films form a semi-permeable barrier to gases and water vapor that reduce respiration and weight loss. Maintaining the firmness of the fruit and providing gloss to coated products could be other advantages of this treatment (Valencia-Chamorro et al., 2009). In addition; antimicrobial agents, antioxidants, flavors, color pigments, and vitamins can be successfully incorporated into the formulation of these coatings to improve their functional properties. Edible coatings with various antimicrobial agents in their formulations were reported to be effective against some important fungal pathogenic genera such as *Penicillium* (Valencia-Chamorro et al., 2008, 2009), *Aspergillus* (Mehyar et al., 2011; Sayanjali et al., 2011), *Botrytis* (Fagundes et al., 2013; Junqueira-Goncalves et al., 2011; Park et al., 2005), and *Alternaria* (Assis and de Britto, 2011; Fagundes et al., 2013). Different food preservatives or GRAS compounds have been reported as effective to control brown rot disease caused by *Monilinia* spp., generally as dip treatments in aqueous solutions (Casal et al., 2010; Droby et al., 2003, Gregori et al., 2008; Mari et al., 2004). However, no information is available regarding the utilization of these antifungal agents as ingredients of waxes or edible films or coatings for the control of major fungal postharvest diseases of stone fruits.

The objectives of this study were to investigate the *in vitro* activity of various preservative agents, widely used in the food industry, against *M. fructicola* and to evaluate the effects of these agents as ingredients of hydroxypropyl methylcellulose

(HPMC)-lipid composite edible coatings on brown rot disease incidence and severity on plum fruits artificially inoculated with *M. fructicola*.

2. Materials and methods

2.1. Pathogen and fungal inoculum

The strain (MeCV-2) of *M. fructicola* used in this study was obtained from the IVIA CTP culture collection of postharvest pathogens. It was isolated from a decayed peach fruit in a packinghouse in Carlet (Valencia, Spain) and, after isolation and identification, selected among other isolates for its aggressiveness and uniform behavior. The isolate was grown on PDA (Sigma-Aldrich Chemie, Steinheim, Germany) in petri dishes in a growth cabinet at 25 ºC for 7-14 days before each experiment. In *in vitro* studies, mycelial plugs from these cultures produced with a sterilized cork borer (5 mm in diameter) were used. For *in vivo* experiments, high density-conidial suspensions of spores were prepared in Tween 80 (0.05%, w/v; Panreac-Química S.A., Barcelona, Spain) in sterile water. After passing through two layers of cheesecloth, the density of the suspension was measured with a haemacytometer and dilutions with sterile water were done to obtain an exact inoculum density of 1 x 10^3 spores/mL.

2.2. Food preservatives

The names, acronyms, molecular formulas and molecular weights of the antimicrobial agents used in this work are given in Table 1. Most of them are likewise classified as food additives or GRAS compounds by the United States Food and Drug Administration (US FDA). Laboratory reagent grade preservatives (99% minimum purity) were purchased from Sigma-Aldrich Chemie, Fluka Chemie AG (Buchs, Switzerland), Panreac Química S.L.U., or Merck KGaA (Darmstadt, Germany).

Potassium silicate (PSi), as a commercial product Sil-Matrix® (29% PSi) was purchased from PQ Corporation (Valley Forge, PA, USA).

2.3. Fruit

'Friar' and 'Larry Ann' Japanese plums (*Prunus salicina* Lindl.) were purchased from Cooperativa del Camp de Llutxent–Otos S.C.V. (Llutxent, Valencia, Spain). Commercially grown fruits were transported to the laboratory without any postharvest treatments. Before the experiments, fruits were selected, randomized, washed with fruit biodegradable detergent (Essasol V., Didsa, Potries, Valencia, Spain), rinsed with tap water, and allowed for air-dry at room temperature.

2.4. Determination of in vitro antifungal activity of food preservatives

The effect of the agents on mycelial growth of *M. fructicola* was evaluated on 90 mm plastic petri dishes with PDA medium amended at 45-55 ºC with sterile aqueous solutions of the respective antimicrobial agent. Stock solutions of 20% of each preservative were prepared by dissolving the appropriate amount of the agent in sterilized bidistilled water. The concentration of stock solutions was 8% in the case of bicarbonates because of their lower solubility in water. These solutions were used to achieve final concentrations of 0.2, 1.0 and 2.0% (v/v) of the agents in PDA media. In the case of parabens, the final concentrations were selected as 0.01, 0.05 and 0.1% (v/v) because of legal regulations in the European Union (EU) restricting their use in processed fruit and vegetables to a maximum of 0.1% (CR EU, 2011). PDA plates without agents were served as controls. The center of each test plate was inoculated with a 5-mm diameter plug of 7-14 day-old cultures of *M. fructicola* and incubated for up to 14 days at 25 ºC in the dark in a growth cabinet. Radial mycelial growth was determined in each plate by calculating the mean of two perpendicular fungal colony

diameters. These measurements were performed after 3, 5, 7, and in some cases after 14 days of incubation. Results after 7 days are presented. Four replicate plates were used for each agent and agent concentration. The results were expressed as percentage of mycelial growth inhibition according to the formula: (dc-dt)/dc×100, where dc = average diameter of the fungal colony on control plates and dt = average diameter of the fungal colony on agent-amended plates.

2.5. Formulation and preparation of antifungal coatings

HPMC (Methocel E15) was purchased from Dow Chemical Co. (Midland, MI, USA) and beeswax (BW) (grade 1) was supplied by Fomesa Fruitech S.L. (Valencia, Spain). Stearic acid and glycerol were purchased from Panreac Química S.L.U. HPMC-lipid composite edible emulsions were prepared combining the hydrophilic phase (HPMC) with the hydrophobic phase (BW) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. A silicone antifoam agent (FG-1510, Dow Corning Ibérica, Barcelona, Spain) was added into the formulations of the coatings with sodium carbonate (SC) and sodium bicarbonate (SBC). For the coating containing sodium propionate (SP), instead of stearic acid, Tween 80 (Decco, Cerexagri, Cesena, Italy) was used to obtain a stable emulsion. All the emulsions contained 40% BW (w/w, dry basis). HPMC-glycerol (2:1) (dry basis, db) and BW-stearic acid (5:1) (db) ratios and a total solid concentration of 7% were kept constant throughout the study. The concentrations of the agents in the formulations (varied between 0.1-2.0%) were determined according to the effective doses of the agents against the fungus in previous *in vitro* tests. Emulsions were prepared as described by Valencia-Chamorro et al. (2008). Briefly, an aqueous solution of HPMC (5% w/w) was prepared by dispersing the HPMC in hot water at 90 ºC and later hydration at 20 ºC. Water, BW, glycerol, and stearic acid (Tween 80, in case of SP) were added to the HPMC solution and heated at 98°C to melt the lipids. Samples were homogenized with

209a high-shear probe mixer (Ultra-Turrax model T25, IKA-Werke, Steufen, Germany) for
2101 min at 12,000 and 3 min at 22,000 rpm. After adding the corresponding agents at the
211amounts indicated, emulsions were cooled under agitation to a temperature lower than
21225 ºC by placing them in an ice bath and agitation was continued for 25 min to ensure
213complete hydration of the HPMC. Viscosity and pH values of the emulsions were
214determined using a viscosimeter (Visco Star Plus R, Fungilab, S.A., Barcelona, Spain)
215and a pH-meter (Consort C830 multi-parameter analyzer, Turnhout, Belgium),
216respectively. Emulsions were kept overnight at 10 ºC before use. The formulations
217were tested for stability according to the method described by Valencia-Chamorro et al.
218(2008). In brief, the emulsions were placed in volumetric tubes and phase separation
219was assessed after 24 h at 25 ºC.
220
2212*.6. Curative activity of antifungal coatings*
222
223Plums were wounded and inoculated at the same time in the fruit equator surface using
224a stainless steel rod with a probe tip 1 mm wide and 2 mm in length, previously
225immersed once into a spore suspension containing 1 x 10^3 spores/mL of *M. fructicola.*
226After incubation at 20 ºC for 24 h, fruits were individually coated. Two hundred µL of
227coating material was pipetted onto each fruit and rubbed with gloved hands to mimic
228the application of coating machines in the industry (Bai et al., 2002). Coated fruits were
229drained on a mesh screen and allowed for air-dry at room temperature. Inoculated but
230uncoated fruits were used as controls. Coated fruits were placed on plastic trays on
231corrugated cartons and then incubated up to 8 days at 20 ºC and 90% RH. In every
232experiment, each treatment was applied to 3 replicates of 10 fruit each. The
233experiments were repeated once.
234
235The incidence of brown rot was assessed as the number of infected fruit and reported
236as the percentage of incidence reduction with respect to the control treatments.

Disease severity was determined as the diameter of the lesion (mm) and the results were reported as the percentage of severity reduction with respect to the control treatments. Disease development data were used to calculate the area under the disease progress stairs (AUDPS; Simko and Piepho, 2012). Disease incidence and severity were assessed after 4, 6 and 8 days of incubation at 20 ºC.

2.7. Statistical analysis

In vitro data were subjected to a two-way analysis of variance (ANOVA) with agent and concentration as factors. Since significant interactions were found, individual one-way ANOVAs were further performed for the different levels of each factor. *In vivo* data were subjected to one-way ANOVAs. For disease incidence data, the ANOVA was applied to the arcsine of the square root of the percentage of infected fruit in order to assure the homogeneity of variances. Incidence and severity reductions with respect to uncoated controls were calculated as percentages. Non-transformed means are shown. Since the experiment was not a significant factor, means are presented for repeated experiments. Fisher´s protected least significant difference (LSD) test, at the 95% level of confidence (P=0.05), was conducted for means separation. All statistical analyses were performed with the software Statgraphics 5.1 (Manugistics, Inc., Rockville, MD, USA).

3. Results and discussion

Mycelial growth inhibition of *M. fructicola* was determined on PDA petri dishes amended with different concentrations of fifteen antifungal agents that are widely used in the food industry. All the agents tested inhibited the growth of *M. fructicola*, but the effects of the agents varied. In general, the mycelial inhibition increased as the concentrations of the agents increased. Significant interactions were found in the

ANOVA between the factors agent and concentration for the *in vitro* inhibition of fungal radial growth (Table 2). Table 3 shows the inhibition of *M. fructicola* on petri dishes amended with different concentrations of the antimicrobial agents after 7 days of incubation at 25 ºC. According to these results, AC, ABC and SBC were the best agents against *M. fructicola* in these series of *in vitro* experiments. The growth of the fungus was completely inhibited by all concentrations of the agents tested in this study. It is known that the addition of carbonates and bicarbonates have a great effect on the medium pH (Xu and Hang, 1989), and ascending medium pH values might play a crucial role to explain the strong antifungal activity of these compounds. Carbonates and bicarbonates have been shown by other authors to be effective inhibitors of the growth of several plant pathogens. Qin et al. (2006) studied the inhibitory effect of SBC and ammonium molybdate on *M. fructicola*. They found that spore germination and germ tube elongation of the fungus were significantly inhibited by ammonium molybdate at the concentration of 5 mmol/L while SBC was effective at all tested concentrations. Moreover, the inhibitory effect of SBC was observed at relatively low concentrations (0.3-0.6%, w/v) against *B. cinerea* and *P. expansum* (Droby et al., 2003; Palmer et al., 1997). Nigro et al. (2006) reported that a complete inhibition of *B. cinerea* was achieved by ABC at 0.25% after 5 days incubation at 22 ºC. This agent was also reported to inhibit the *in vitro* growth of *Helminthosporium solani* (Olivier et al., 1998), *Uromyces appendiculatus* (Arslan et al., 2006) and *Venturia inaequalis* (Jamar et al., 2007). Our findings with *M. fructicola* are in agreement with the results of these previous studies highlighting the antifungal potential of bicarbonates against several important plant pathogens.

In the present study, SB and PBC provided 100% inhibition at the highest concentration tested (2.0%). Complete inhibitions of *M. fructicola* were observed at concentrations of SEP and SMP of 0.05% or higher, and at concentrations of SC, SP, PC, PS, SDA, and PSi of 1.0% or higher. In the literature, strong inhibitions of conidial

germination of *B. cinerea* (Yildirim and Yapici, 2007) and some toxigenic *Fusarium* spp. and *Penicillium* spp. (Thompson et al., 1993) in *in vitro* assays with parabens were reported. Droby et al. (2003) tested calcium propionate and observed a distinctive inhibitory effect at 2.5% on the radial growth of *B. cinerea* and *P. expansum*. Gregori et al. (2008) determined the Minimum Inhibitory Concentration (MIC) of PS for conidial germination and mycelial growth of *M. laxa* as 260 and 1250 mg/L, respectively. We observed complete inhibition of *M. fructicola* with PSi at concentrations of 1.0 and 2.0%. Biggs et al. (1997) evaluated another silicate (calcium silicate) and determined that it reduced the growth of *M. fructicola* on amended PDA (600 mg Ca/L) by approximately 65% compared with the control. In contrast, Adaskaveg et al. (1992) showed no *in vitro* toxicity of calcium silicate to *M. fructicola*. Fagundes et al. (2013) found complete *in vitro* growth inhibitions of *B. cinerea* and *A. alternata* on PSi-amended PDA medium. Differences among these results could be presumably due to the different silicate doses used for media amendment. The dose-dependent efficacy of PSi was also observed in the present study (Table 3). Bekker et al. (2009) claimed that the inhibitory effect of PSi against plant pathogens was due to its direct fungitoxic activity. Li et al. (2009) observed some morphological changes (mycelium sparsity and asymmetry, hyphal swelling, curling, and cupped shape) and ultrastructural alterations (thickening of the hyphal cell walls, cell distortion, and cavity) in silicate-treated hyphae of *Fusarium sulphureum*.

The complete inhibition of *M. fructicola* obtained with some agents was maintained throughout the incubation period and even lasted on the fourteenth day of incubation (data not shown). These were the cases for AC, ABC, and SBC at all concentrations; for SC, SP, PC, PS, and SDA at the concentrations of 1.0 and 2.0%, and for SEP and SMP even at the lowest concentration of 0.05%. Our results showed that SA and SF only inhibited the growth of *M. fructicola* at rates lower than 95% even with the highest concentration tested (2.0%). Furthermore, at concentrations of 1.0 and 2.0%, the

inhibition obtained with these agents was significantly lower (P<0.05) than that observed with the rest of evaluated agents. Similarly to our results, Nigro et al. (2006) observed relatively limited efficacy of SA and SF against *B. cinerea* and found that MIC values for these agents were higher than 2% in a colony growth assay. Fagundes et al. (2013) reported that SA and SF not only did not reduce, but even increased the growth of *A. alternata* at concentrations below 2%. Biggs et al. (1997) tested calcium salts of formic and acetic acids and reported that calcium formate could not significantly inhibit the growth of *M. fructicola* on amended PDA and that calcium acetate had a moderate effect on the fungus (35% growth inhibition). Therefore, it can be concluded from the results reported here and those from other studies that the salts of acetic and formic acids are not good candidates to be used as antifungal agents for the control of various phytopathogenic fungi, including *Monilinia fructicola*.

Since the *in vitro* control of fungal growth obtained with SA and SF was relatively lower compared to that observed with the other salts, these agents were discarded and not evaluated in subsequent *in vivo* tests. All the other agents were used at their minimum effective concentrations for the formulation of emulsions and these emulsions were applied to fresh fruit as edible coatings. The emulsion containing 1% SC had a very high viscosity (over 350 cp) and it was impossible to apply as a coating material. The emulsions with 1% PC and 2% PBC were unstable and phase separation occurred soon after preparation. Moreover, when applied to plums, these emulsions with PC and PBC left apparent whitish residues on the fruit surface. For these reasons, SC, PC and PBC were also discarded and only ten agents, namely SBC (2%), SDA (2%), PS (1%), SB (1%), SP (1%), PSi (1%), AC (0.2%), ABC (0.2%), SEP (0.1%), and SMP (0.1%), were selected for use in further *in vivo* tests.

Incidence and severity reductions of brown rot disease in plums coated with HPMC-lipid edible coatings can be seen in Fig. 1. Complete reduction of disease incidence

was not achieved with any treatments, showing that none of the antifungal coatings tested was able to prevent the onset of the disease on plums inoculated with *M. fructicola* about 24 h before coating. Further, incidence reduction percentages were generally low. Coatings containing AC, PSi, SDA, SB, and SP did not reduce brown rot incidence at all compared to uncoated controls. On the other hand, coatings containing bicarbonates (ABC and SBC) and paraben salts (SMP and SEP) significantly reduced brown rot incidence within a range of 10-18% (P<0.05). In agreement with our findings, bicarbonates have been reported in the past to reduce brown rot caused by *Monilinia* spp. in sweet cherries or peaches (Droby et al., 2003; Feliziani et al., 2013; Kitteman et al., 2008; Qin et al., 2006). According to the results of the present study, PS was the most effective agent in reducing brown rot incidence in plums among the agents used in coating formulations. Incidence reduction was 28.6±7.1% in plums coated with HPMC-BW formulations containing 1% PS (Fig. 1). In this respect, our results agree with those by Gregori et al. (2008) who observed high efficacy of PS against *M. laxa* in peaches and nectarines. They reported that immersion of naturally infected fruit into a PS solution (15 g/L for 120 s) reduced brown rot disease over 80% in 4 of 5 trials. In addition, Mari et al. (2004) reported that PS at 1.5% was able to significantly reduce *M. laxa* infections in sweet cherries, apricots and nectarines, with reduction values with respect to controls of 61.6, 78.5, and 31.8%, respectively. Furthermore, Palou et al. (2009) found that among a variety of food preservatives tested as aqueous solutions, PS at 200 mM was the most effective dip treatment in reducing brown rot in peaches wound inoculated with *M. fructicola* 24 h earlier. The effectiveness of this treatment significantly increased when the solution was heated to 55 or 60 ºC. Thus, our results confirmed the findings from previous research in which the strong antifungal activity of PS against *Monilinia* spp. had been reported. PS is well known for its potent antifungal function and has been used in many food systems for controlling the growth of molds thus extending product shelf-life (Park et al., 2005). Biggs et al. (1997) reported that brown rot incidence and severity in peaches were significantly correlated with

polygalacturonase activity of *M. fructicola*. According to Gregori et al. (2008) the mode of action of PS against *M. laxa* depends on the inhibition of polygalacturonase activity. Indeed, many authors reported secretion of polygalacturonase (a cell wall-degrading enzyme) by some species of *Monilinia in vitro* (Willet et al., 1977) and *in vivo* in fruits such as apple (Snape et al., 1997) or peach (Lee and Bostock, 2007). Fielding (1981) suggested that some natural inhibitors could prevent the secretion of pectolytic enzymes by a number of plant pathogenic fungi as a result of a natural wound defence mechanism. It is possible that some of the agents tested in this study could inhibit the infections of *M. fructicola* through a similar mode of action.

The coatings containing antifungal agents tested in this study were generally more efficient in severity reduction than in incidence reduction. A similar trend was observed in previous research work by Fagundes et al. (2013). Under our experimental procedure, the variable disease incidence measures the amount of infections that take place from free conidia deposited in the infection courts (fruit wounds) during artificial inoculation. In contrast, the variable disease severity quantifies the growth rate of the pathogen once the infection has been initiated. Therefore, in general, the curative effect of the antifungal coatings was higher against the ability of fungal hyphae to grow and multiply in the infection wounds than against the capacity of free spores to germinate or recently-germinated spores to initiate infections in these wounds. Obviously, the period of time between inoculation and coating application may influence disease control ability. In our case, a period of 24 h was selected to simulate the time between the production of common field infections by *M. fructicola* that can take place in superficial fruit wounds inflicted by pickers during harvesting and the application of postharvest antifungal treatments in the packinghouse (Palou et al., 2009). Disease severity was best reduced by parabens (SMP and SEP) at rates of about 50%. These compounds are alkyl esters of *p*-hydroxybenzoic acid and were reported to have a strong antimicrobial activity over a wide pH range of 4-8 (Thompson,

1994). Their mode of action is attributed to an uncoupling of oxidative phosphorylation, inhibition of NAD+ and FAD-linked mitochondrial respiration, or the reduction of mitochondrial membrane potential (Soni et al., 2002). Propyl paraben, the most widely investigated member of this group, has been shown to inhibit many fungi from important genera such as *Penicillium* (Thompson et al., 1993), *Fusarium* (Torres et al., 2003), *Alternaria* (Mills et al., 2004), and *Aspergillus* (Barberis et al., 2010). When incorporated into edible films, this agent is also effective in controlling green and blue molds in citrus fruits (Valencia-Chamorro et al., 2009), and gray mold and black rot in tomatoes (Fagundes et al., 2013). However, propyl paraben (E-216) and its sodium salt (E-217) have been recently excluded from The List of Permitted Food Additives in the EU because of potential health hazard issues (CR EU 2011). According to this current legislation, SMP and SEP are allowed for uses in processed fruits and vegetables at a maximum level of 0.1%. After comparing the individual MICs of four parabens (butyl, ethyl, methyl, and propyl parabens) against toxigenic species of *Penicillium*, *Fusarium*, and *Aspergillus*, Thompson (1994) found SMP as the least effective one. In the present study, severity reduction obtained with SMP was slightly higher than that obtained with SEP, but the difference between these values was not statistically significant ($P>0.05$). Our study showed that PS, the most effective compound in incidence reduction, had a mild effect in severity control with a reduction rate of 35% (Fig. 1). Close results were reported by Palou et al. (2009). They observed that brown rot incidence and severity were reduced by 35 and 25%, respectively, on PS-treated peaches after 7 days of incubation at 20 ºC. Application of this agent has been previously shown to markedly reduce silver scurf (a disease caused by *Helminthosporium solani*) severity on potato tubers (Olivier et al., 1998, 1999). When applied 2 and 4 days after inoculation, PS reduced silver scurf severity by 83 and 60%, respectively (Hervieux et al., 2002). It was also showed in this work that that ammonium carbonates (AC and ABC) and sodium salts of benzoic and propionic acids (SB and SP) significantly reduced disease severity, with a reduction range of 37-46%. It was suggested that the antifungal action

of benzoate is caused by an accumulation of active ingredient at low external pH, which lowers the intracellular pH (Krebs et al., 1983). This inhibits the glycolysis and causes a depletion of ATP and a consequent limitation in microbial growth. Brock and Buckel (2004) studied on the mode of action of sodium propionate using *Aspergillus nidulans* as a model organism. They claimed that pyruvate dehydrogenase inhibition is the most important means for fungal inhibition as this enzyme directly affects glucose and propionate metabolism. SDA and SBC were the least effective agents in reducing disease severity. Severity reduction values obtained with these agents (15.6 and 19.3%, respectively) were significantly lower than those obtained with the other agents. To the best of our knowledge, this is the first report evaluating the effect of SDA on the growth of *Monilinia* spp. SDA, the sodium acid salt of acetic acid, can be effective in preventing the growth of several mold strains, thus prolonging the shelf life of many foods (EPA, 1991). Sagedhi Mahounack and Shahidi (2001) evaluated the antifungal effect of different concentrations of SDA against some species of *Aspergillus*, *Rhizopus*, and *Penicillium*. They found that this agent at 5000 ppm inhibited mold growth up to the last day (5^{th} day) of the experiment. Stiles et al. (2002) reported that the growth of 33 out of 42 mold strains tested was affected by the presence of SA in deMan Rogosa Sharpe medium, with *Fusarium* strains as the most sensitive. In an earlier study, SA was also reported to inhibit the *in vitro* growth and aflatoxin production of *Aspergillus parasiticus* (Buchanan and Ayres, 1976). Therefore, it can be concluded that the antifungal activity of acetic acid salts considerably varies when they are applied against different pathosystems.

Measuring disease progress is important for understanding the interactions between the host and the pathogen and the temporal effects of an antifungal treatment. Traditionally, the area under the disease progress curve (AUDPC) has been frequently used to combine values from multiple observations of disease severity into a single value (Shaner and Finney, 1977). Even though AUDPC is a widely used means to

measure disease progress, in recent years this approach has been claimed to severely undervalue the effects of the first and last observations. Therefore, the area under the disease progress stairs (AUDPS) was reported as??? a new method to improve the estimation of disease progress by giving a weight closer to optimal to the first and last observations (Simko and Piepho, 2012). Average AUDPS values from plums artificially inoculated with *M. fructicola* and incubated at 20 °C and 90% RH for 8 days are shown in Fig. 2. All AUDPS values obtained from coated fruit were significantly lower than that obtained from the uncoated control. Coatings containing the agents AC, ABC, SEP, and SMP induced the lowest AUDPS values. Treatments with these agents resulted in 38-44% reductions in AUDPS values compared with the non-treated control. Reductions in AUDPS values obtained with SDA, PSi, SP, PS, and SB ranged from 16 to 36%. SBC-coated plums showed the highest AUDPS value (statistically not different from SDA), indicating that this coating was one of the least effective in reducing disease throughout the entire incubation period. Likewise, Casals et al. (2010) reported that SBC at any concentrations tested (1-4%) failed to control brown rot caused by *M. laxa* in either nectarines or peaches.

Presumably, the inhibition of *M. fructicola* by antimicrobial agents both *in vitro* or as ingredients of coatings might be related to pH variations. The pH values of PDA medium amended with various concentrations of antimicrobial agents and HPMC-lipid composite emulsions containing the agents are given in Table 4. Only the pH values of the coatings containing PS, SDA, SP, and SMP were within the range of pH of amended-PDA. Therefore, it can be assumed that not only the agents determined the pH of the coatings, but other components (HPMC, stearic acid, glycerol, etc.) had also an influence. It is also known that the effectiveness of most of the agents used in this study is pH-dependent. For instance, the fungistatic effect of PS is greater at low pH (Kitagawa and Kawada, 1984). The antimicrobial activity of SBC was attributed to increased pH and the presence of HCO_3^- in the dissociation of $NaHCO_3$ at high pH (Xu

and Hang, 1989). Contrarily, Biggs et al. (1997) reported that the activity of various calcium salts against *M. fructicola* was not affected by the pH of the medium. Bekker et al. (2009) investigated the effect of pH on mycelial growth of 11 pathogenic fungi on PSi-amended PDA and concluded that the direct inhibitory effect of the agent clearly overrode the effect of pH. In our opinion, many internal and external factors (other than pH) could also play an important role on this antimicrobial action. According to Talibi et al. (2011), inhibition of microorganisms by organic/inorganic salts might be caused by a reduction of cell turgor pressure with collapse and shrinkage of spores and hyphae or by alteration of cell-transport function and inhibition of enzymes involved in the glycolytic pathway.

In the current study, the inhibitory effects of the agents tested *in vitro* or *in vivo* as ingredients of edible coatings considerably differed. For instance, SBC, one of the best agents against *M. fructicola* in the *in vitro* experiments, failed in controlling incidence and severity of brown rot disease in plums. Notable differences in the effectiveness of antimicrobial agents between *in vitro* and *in vivo* experiments have been reported many times in the past (Fagundes et al., 2013; Nigro et al., 2006; Park et al., 2005). According to Park et al. (2005), such differences might be explained by actual exposure of fungal structures to different amounts of the agents. While in radial growth tests spore suspensions in petri dishes were fully exposed to the agents, in coating applications the agents might have gradually diffused into the surface of the fruit to interact with spores, thus limiting the antifungal action. After conducting simulation experiments with parabens, Chung et al. (2001) reported that the release of the chemical from a polymer coating into food-simulating solvents depended on the complicated interactions among the agent, the coating, and the solvents. Also, it is likely that the diffusion of the agent is affected by some other factors such as solubility and partition coefficient of the agent or the structure of the fruit skin. For these reasons, appropriate and cost-effective coatings should be specifically developed for particular

517fruit species or even cultivars (Valencia-Chamorro et al., 2009). According to Vargas et
518al. (2008), antimicrobial coatings have advantages over the application of
519antimicrobials by dipping, dusting or spraying because they could be designed to slow
520down the diffusion of the active ingredient from the coating to the commodity. By
521slowing its diffusion from the coating, the preservative activity on the food surface is
522maintained. In this sense, recent research is focusing on the development of coatings
523with micro or nano-encapsulation of the active ingredients to effectively control their
524release (Lucera et al., 2012).
525
526In conclusion, we have demonstrated in this study the potential of several food
527additives as antimicrobial agents against *M. fructicola*. AC, ABC and SBC were found
528to be the best agents in *in vitro* tests, as they completely inhibited the mycelial growth
529of *M. fructicola* on PDA at all concentrations tested. However, any of the agents could
530not prevent the onset of brown rot in plums, although the coating containing 1% of PS
531was able to reduce the disease incidence by 28.6%. All the coatings tested could
532significantly reduce the disease severity in plums while the best results were obtained
533with the coatings containing AC, ABC, SEP and SMP. Further research is needed to
534determine the effect of the application of HPMC-lipid composite edible coatings
535containing these antifungal agents on quality parameters and storability of plum fruit.
536The mechanisms for antimicrobial action of the effective coatings are also needed to be
537clarified by further investigations.
538
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540
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752**Table 1**

753Characteristics of antimicrobial food agents tested *in vitro* or *in vivo* as coating

754ingredients for inhibition of *Monilinia fructicola*

Antimicrobial agent	Acronym	Molecular formula	E-code[a]	MW[b]
Ammonium carbonate	AC	$(NH_4)_2CO_3$	E-503(i)	114.1
Ammonium bicarbonate	ABC	NH_4HCO_3	E-503(ii)	79.06
Potassium carbonate	PC	K_2CO_3	E-501(i)	138.21
Potassium bicarbonate	PBC	$KHCO_3$	E-501(ii)	100.12
Potassium silicate	Psi	K_2SiO_3	E-560	154.26
Potassium sorbate	PS	$C_6H_7O_2K$	E-202	150.22
Sodium carbonate	SC	Na_2CO_3	E-500(i)	105.99
Sodium bicarbonate	SBC	$NaHCO_3$	E-500(ii)	84.01
Sodium acetate	SA	CH_3COONa	E-262(i)	82.03
Sodium diacetate	SDA	$C_4H_7O_4Na$	E-262(ii)	142.09
Sodium benzoate	SB	$C_7H_5O_2Na$	E-211	144.11
Sodium formate	SF	$HCOONa$	E-237	68.01
Sodium propionate	SP	CH_3CH_2COONa	E-281	96.06
Sodium methylparaben	SMP	$C_8H_7O_3Na$	E-219	174.13
Sodium ethylparaben	SEP	$C_9H_9O_3Na$	E-215	188.16

755[a] E-code = code number for food additives approved by the European Union.

756[b] Molecular weight.

Table 2

Two-way analysis of variance of the in vitro inhibition of Monilinia fructicola (percentage of colony diameter reduction) on PDA plates amended with different concentrations of food preservative agents after 7 days of incubation at 25 ºC

	SS	df	MS	*F*-ratio	*P*-value
A: Agent	39473.9	14	2819.56	312.62	0.0000
C: Concentration	67765.4	2	33882.7	3756.82	0.0000
A x C	42546.1	28	1519.5	168.48	0.0000
Error	1217.56	135	9.01899		
Total	151003.0	179			

761**Table 3**

762Percentage inhibition of radial growth of *Monilinia fructicola* on PDA petri dishes

763amended with different concentrations of food preservative agents after 7 days of

764incubation at 25 ºC [a]

Antimicrobial agent	Inhibition of *Monilinia fructicola* (%) [b]		
	Agent concentration		
	0.2%	1.0%	2.0%
Ammonium carbonate	100.00 iA	100.00 eA	100.00 cA
Ammonium bicarbonate	100.00 iA	100.00 eA	100.00 cA
Potassium carbonate	81.76 gA	100.00 eB	100.00 cB
Potassium bicarbonate	89.22 hA	98.01 dB	100.00 cC
Potassium silicate	11.08 bA	100.00 eB	100.00 cB
Potassium sorbate	49.42 efA	100.00 eB	100.00 cB
Sodium carbonate	96.37 hiA	100.00 eB	100.00 cB
Sodium bicarbonate	100.00 iA	100.00 eA	100.00 cA
Sodium acetate	22.64 cA	62.21 bB	93.98 bC
Sodium diacetate	35.83 dA	100.00 eB	100.00 cB
Sodium benzoate	42.34 deA	91.92 cB	99.67 cC
Sodium formate	0.00 aA	60.44 aB	92.50 aC
Sodium propionate	54.37 fA	100.00 eB	100.00 cB
Sodium methylparaben[c]	24.85 cA	100.00 eB	100.00 cB
Sodium ethylparaben[c]	27.80 cA	100.00 eB	100.00 cB

765[a] Means in lines with different capital letters and means in columns with different 766lowercase letters are significantly different by Fisher´s protected LSD test ($P<0.05$) 767applied after an ANOVA.

768[b] Colony diameter reduction with respect to control treatments (non-amended PDA 769plates).

770[c] The doses of the agents tested were 0.01, 0.05 and 0.1% (CR EU, 2011).

771**Table 4** Some characteristics of PDA medium amended with agents and HPMC-lipid

772composite edible emulsions containing agents

Antimicrobial agent	PDA medium amended with agents		HPMC-lipid composite edible emulsion containing agents		
	Concentration (%)	pH	Concentration (%)	pH	Viscosity (cp)
Ammonium carbonate	0.2	8.12	0.2	7.67	64.8
	1.0	8.61			
	2.0	8.75			
Ammonium bicarbonate	0.2	7.84	0.2	7.53	68.9
	1.0	8.39			
	2.0	8.52			
Potassium carbonate	0.2	9.69	1.0	10.79	43.7
	1.0	10.85			
	2.0	11.13			
Potassium bicarbonate	0.2	7.59	2.0	8.16	40.5
	1.0	8.45			
	2.0	8.70			
Potassium silicate	0.2	8.28	1.0	11.35	58.9
	1.0	10.06			
	2.0	10.82			
Potassium sorbate	0.2	6.35	1.0	6.78	70.5
	1.0	6.53			
	2.0	6.84			
Sodium carbonate	0.2	9.74	1.0	10.50	377.2
	1.0	10.52			
	2.0	10.63			
Sodium bicarbonate	0.2	7.47	2.0	8.83	54.4
	1.0	8.14			
	2.0	8.35			
Sodium acetate	0.2	6.10		- [a]	
	1.0	6.52			
	2.0	6.83			
Sodium diacetate	0.2	4.64	2.0	4.63	37.8
	1.0	4.56			
	2.0	4.58			
Sodium benzoate	0.2	5.82	1.0	6.50	65.8
	1.0	6.12			
	2.0	6.29			
Sodium formate	0.2	5.83		- [a]	
	1.0	6.02			
	2.0	6.25			
Sodium propionate	0.2	6.30	1.0	6.88	46.2
	1.0	6.71			
	2.0	7.01			
Sodium methylparaben	0.01	5.99	0.1	7.69	92.9
	0.05	7.13			
	0.1	7.84			
Sodium ethylparaben	0.01	5.89	0.1	7.85	90.2
	0.05	6.89			
	0.1	7.52			

773[a] Coating was not prepared.

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775**Fig. 1**. Reductions of the incidence and severity of brown rot on plums artificially 776inoculated with *Monilinia fructicola*, coated 24 h later with HPMC-lipid composite edible 777coatings containing the following agents with the concentrations indicated in the 778parenthesis, and incubated for 8 days at 20 ºC and 90% RH: sodium bicarbonate 779(SBC, 2%), sodium diacetate (SDA, 2%), potassium sorbate (PS, 1%), sodium 780benzoate (SB, 1%), sodium propionate (SP, 1%), potassium silicate (PSi, 1%), 781ammonium carbonate (AC, 0.2%), ammonium bicarbonate (ABC, 0.2%), sodium 782methylparaben (SMP, 0.1%), and sodium ethylparaben (SEP, 0.1%). Incidence and 783severity reductions were determined with respect to control fruit (inoculated but 784uncoated). Disease incidence and severity in control treatments were 90% and 90-110 785mm, respectively. Means are from two experiments. For disease incidence reduction, 786the ANOVA was applied to arcsine-transformed values. Non-transformed means are 787shown. Columns with different letters are significantly different according to Fisher´s 788protected LSD test (P<0.05) applied after the ANOVA.

789

790**Fig. 2**. Area under the disease progress stairs (AUDPS) for brown rot on plums 791artificially inoculated with *Monilinia fructicola*, coated 24 h later with HPMC-lipid edible 792composite coatings containing the following agents with the concentrations indicated in 793the parenthesis, and incubated for 8 days at 20 ºC and 90% RH: sodium bicarbonate 794(SBC, 2%), sodium diacetate (SDA, 2%), potassium sorbate (PS, 1%), sodium 795benzoate (SB, 1%), sodium propionate (SP, 1%), potassium silicate (PSi, 1%), 796ammonium carbonate (AC, 0.2%), ammonium bicarbonate (ABC, 0.2%), sodium 797methylparaben (SMP, 0.1%), and sodium ethylparaben (SEP, 0.1%). Control fruit 798(CON) were inoculated but uncoated. Columns with different letters are significantly 799different according to Fisher´s protected LSD test (P<0.05) applied after an ANOVA. 800Means are from two experiments.

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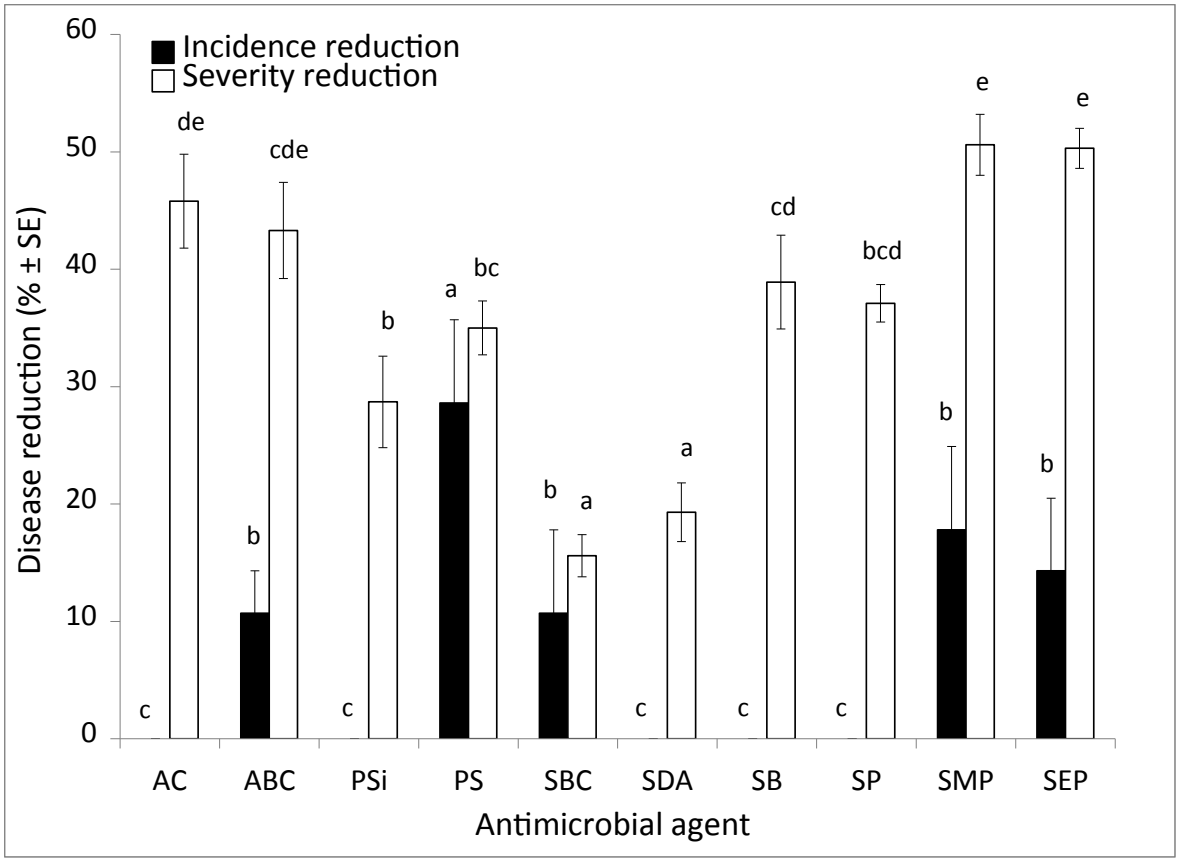

805 Fig. 1

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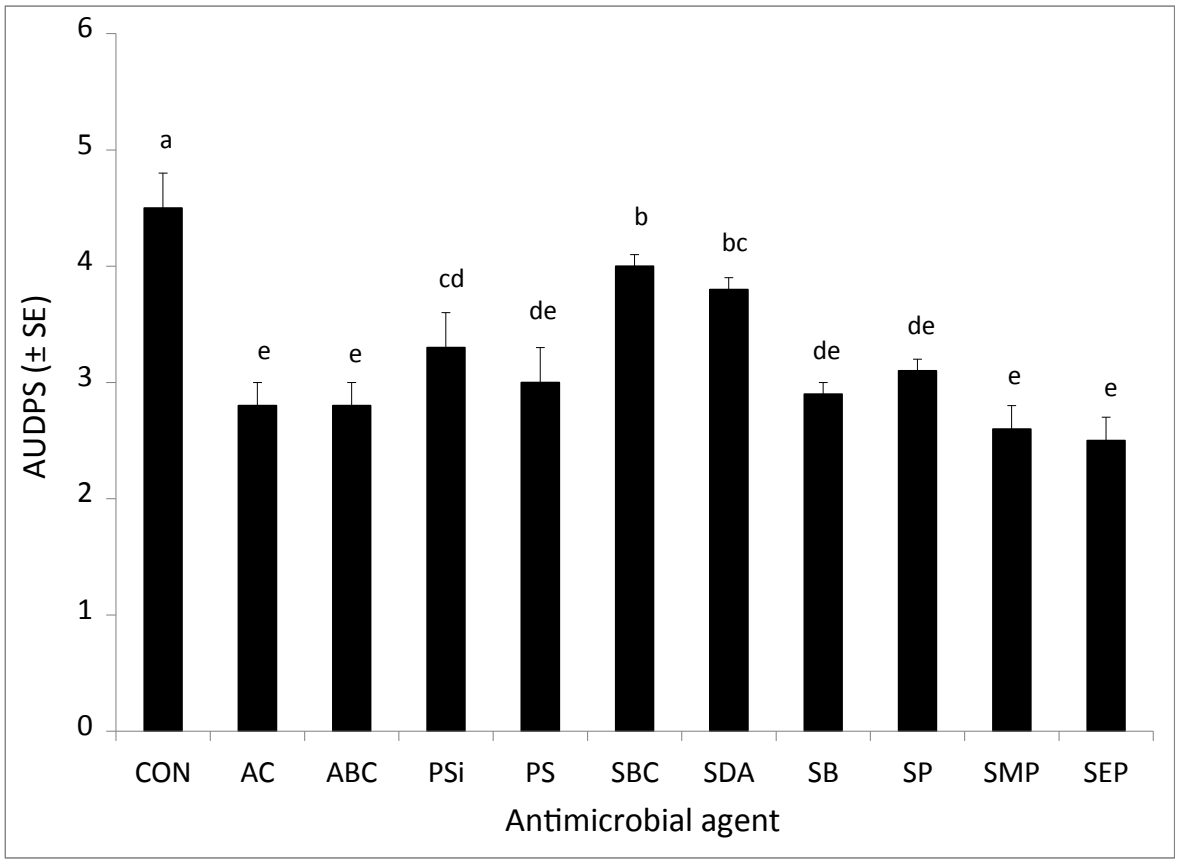

812 Fig. 2

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