



In vitro antibacterial activity of plant essential oils against *Staphylococcus hyicus* and *Staphylococcus aureus*, the causative agents of exudative epidermitis in pigs

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Abstract

Greasy pig disease or exudative epidermitis, a generalized or localized skin disease affecting piglets, is mainly caused by *Staphylococcus hyicus*, although other staphylococcal species such as *Staphylococcus aureus* may also induce disease. Piglets with skin lesions can be treated systemically with antibiotics. However, antimicrobial resistance to β -lactam antibiotics are now frequently observed in *S. hyicus* and *S. aureus* isolates. In this study, the antibacterial activity of plant essential oils as well as their ability to potentiate the effect of several antimicrobial compounds against *S. hyicus* and *S. aureus* were investigated with a view to a potential use as skin disinfectants. Among ten essential oils tested, those from cinnamon, thyme, and winter savory were the most active with minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values ranging from 0.078 to 0.313% (v/v). Using a fluorescent probe with DNA affinity, it was found that thyme and winter savory oils act, at least in part, by disturbing the bacterial membrane integrity. At concentrations below the MIC, thyme and winter savory oils reduced biofilm formation by *S. hyicus*. Moreover, a treatment of pre-formed biofilms of *S. hyicus* with cinnamon or thyme oils significantly decreases its viability. Synergistic interactions between essential oils, more particularly from thyme and winter savory, and penicillin G, chlorhexidine or nisin, were observed. This study supports the therapeutic potential of essential oils as topical therapeutic agents against exudative epidermitis.

Keywords Greasy pig · Exudative epidermitis · *Staphylococcus hyicus* · *Staphylococcus aureus* · Biofilm · Essential oil

Introduction

Greasy pig disease or exudative epidermitis is a generalized or localized skin infection affecting mainly neonatal and newly weaned piglets, and characterized by exfoliation, sebaceous exudation and crust formation that may cover the entire body of the animal (Frana 2012). Mortality and

morbidity are high during exudative epidermitis outbreaks. More specifically, death associated with exudative epidermitis mostly results from dehydration. This disease occurs worldwide and the increased incidence may be associated with larger size of units, higher stocking densities, and possibly earlier weaning. Exudative epidermitis is mainly caused by *Staphylococcus hyicus*, although other staphylococcal species such as *Staphylococcus aureus*, may also rarely induce disease (Frana 2012; Foster 2012; van Duijkeren et al. 2007). Pathogenic strains of staphylococci are known to produce exfoliative toxins which can digest desmoglein-1 in the epidermis of porcine skin (Nishifuji et al. 2008). The cleavage of this desmosomal cadherin-type cell–cell adhesion molecule causes the disruption of the cutaneous defense barrier and facilitate bacterial invasion (Nishifuji et al. 2008). Although mature pigs develop resistance to exudative epidermitis with age, they may be carriers of pathogenic *S. hyicus* and serve as a source of contamination for naïve herds. Affected piglets exhibiting skin lesions can be treated with antibiotics used systemically (Davies

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2016). Therapeutic success is increased if the antibiotic is combined with daily applications of antiseptics to the entire body surface. Isolates of *S. hyicus* and *S. aureus* with antibiotic resistance to β -lactams, including penicillin G, ampicillin, and cephalosporins, are now frequently recovered thus making the infection more difficult to treat (van Duijkeren et al. 2007; Park et al. 2013; Wegener and Schwarz 1993). Given the emergence of antibiotic resistance in *S. hyicus* and *S. aureus*, studies aimed to identify novel therapeutic alternatives are highly relevant.

Essential oils are formed by aromatic plants as secondary metabolites, and are extracted from different plant parts (flowers, leaves, roots, seeds, peel, fruits, etc.) by steam distillation, hydrodistillation or solvent extraction (Sharifi-Rad et al. 2017). Essential oils may include up to 100 different compounds; terpenoids and phenylpropanoids being the predominant constituents (Sharifi-Rad et al. 2017). One of the most important properties of essential oils is their ability to exhibit broad spectrum inhibitory activities against pathogenic bacteria and fungi (Sharifi-Rad et al. 2017; Pandey et al. 2016; O'Bryan et al. 2015). The recent emergence of bacteria resistant to multiple antibiotics has spurred research into the use of essential oils as potential alternatives. The aim of this study was to investigate the antibacterial and anti-biofilm properties of plant essential oils against *S. hyicus* and *S. aureus*. The synergistic interactions between essential oils and other antimicrobial compounds (penicillin G, chlorhexidine, nisin) were also examined.

Materials and methods

Bacteria and growth conditions

Staphylococcus hyicus ATCC 11249 and 84-2978, and *S. aureus* ATCC 25923, 86-184 and 83-4484, kindly provided by M. Gottschalk (University of Montreal, Canada), were used in this study. Bacteria were grown in Trypticase Soy Broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.25% glucose (TSB-G), at 37 °C under aerobic static conditions.

Essential oils

Ten essential oils, including balsam fir (*Abies balsamea*; branch), cinnamon (*Cinnamomum verum*; bark), coriander (*Coriandrum sativum*; seed), Labrador tea (*Ledum groenlandicum*; leaf), myrrh (*Commiphora molmol*; oleogum), peppermint (*Mentha piperita*; flowering herb), sage (*Salvia officinalis*; flowering top), sweet marjoram (*Origanum majorana*; flowering herb), thyme (*Thymus vulgaris*; flowering top), and winter savory (*Satureja montana*; flowering top), were used in this study. Essential oils were purchased from

Hunzaroma (Hunzaroma Inc., Longueuil, QC, Canada). All essential oils were of therapeutic grade and their composition analyzed by gas chromatography.

Determination of minimal inhibitory and minimal bactericidal concentrations

A microplate dilution assay was used to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of essential oils against *S. hyicus* and *S. aureus* strains. To determine MIC values, 24-h bacterial cultures were diluted in fresh culture medium to obtain an optical density of 0.1 at 660 nm (OD_{660}). Equal volumes (100 μ L) of the bacterial suspensions and twofold serial dilutions of essential oils (5–0.0195% v:v) in culture medium were added to the wells of a 96-well microplate. Undiluted essential oils were considered to be at 100%. Wells with no essential oils were used as negative controls (100% growth). The microplate was sealed with an adhesive clear polyester seal film (GE Healthcare, Marlborough, MA, USA) and incubated at 37 °C under aerobic conditions. After a 24-h incubation, bacterial growth was monitored by recording the OD_{660} using a xMark™ microplate reader (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The MIC value was the lowest concentration of essential oils that completely inhibited the bacterial growth, as determined by monitoring the OD_{660} . To determine the MBC values, 5- μ l aliquots from the wells with no visible growth were spread on TSB-G agar plates. After an incubation of 3 days at 37 °C, the MBC value was the lowest concentration at which no colony formation occurred. All assays were performed in triplicates to ensure reproducibility.

Bacterial membrane disruption

The ability of essential oils to disrupt the cytoplasmic membrane of *S. hyicus* 84-2978 and *S. aureus* 25923 was evaluated using the SYTOX Green dye (Life Technologies Inc., Burlington, ON, Canada), which enters the cells and binds to DNA once the cytoplasmic membrane is compromised. Briefly, 1.25 μ M of SYTOX Green dye was added to bacterial cells suspended in 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.0 to an OD_{660} of 0.4. Aliquots of 100 μ L were added to wells of a 96-well black microplate prior to adding 10 μ L of essential oils (cinnamon, thyme, winter savory) at a concentration corresponding to the MIC value. The incubation was carried out in a microplate reader (Synergy 2; BioTek Instruments, Winooski, VT, USA) at 37 °C for 40 min, and the fluorescence resulting from the binding of the dye to bacterial DNA was recorded every 5 min following excitation at 485 nm and emission at 528 nm. A reaction mixture containing HEPES instead of essential oil was used for the negative control.

Biofilm formation, desorption, and killing

The effect of growing *S. hyicus* 84-2978 and *S. aureus* 25923 in the presence of essential oils (cinnamon, thyme, winter savory) on biofilm formation was investigated using the above microplate assay. After incubation for 18 h at 37 °C, spent media and free-floating bacteria were removed by aspiration using a 26-G needle. Biofilms were stained with 0.01% crystal violet (100 µL) for 15 min. The wells were washed with distilled water to remove unbound crystal violet dye and dried for 2 h at 37 °C. After adding 100 µL of 75% (v/v) ethanol to each well, the plate was shaken for 15 min to release the dye from the biofilms and the absorbance at 550 nm (A_{550}) was recorded.

The ability of cinnamon, thyme, and winter savory oils to decrease the viability and promote desorption of *S. hyicus* 84-2978 and *S. aureus* 25923 biofilms was also investigated. Briefly, 18-h biofilms were pre-formed as above, washed once with PBS, and treated for 15 min with essential oils at concentrations corresponding to the MIC value (in PBS). Following these treatments, the biofilms were washed once with PBS. A series of biofilms was stained with crystal violet as above to determine desorption. A second series of biofilms was used to determine bacterial viability using a commercial luminescence assay (BacTiter-Glo™; Madison, WI, USA) that measures adenosine triphosphate (ATP), an indicator of metabolically active viable bacteria. Luminescence was quantified using a Synergy 2 microplate reader. All the above assays were run in triplicates and the means ± standard deviations of two independent experiments were calculated.

Synergistic interactions of essential oils with other antimicrobial compounds

The potential synergistic effects of cinnamon, thyme, and winter savory oils in combination with penicillin G,

chlorhexidine, or nisin were evaluated using the checkerboard method (Eliopoulos and Moellering 1996). Essential oils were serially diluted in TSB-G (100 µL) along the ordinate of a 96-well microplate, while the other antimicrobial compounds were serially diluted in the same medium (100 µL) along the abscissa. Cell suspensions of *S. hyicus* (84-2978) and *S. aureus* (25923 and 86-184), prepared in TSB-G and adjusted to an OD_{660} of 0.1, were used as inoculum. The microplate wells were inoculated with 100 µL of the bacterial suspensions, and the microplate was incubated at 37 °C for 24 h. Wells with no bacteria or compounds were included in the assay. After the incubation period, bacterial growth was assessed by recording the OD_{660} using a Synergy 2 microplate reader. The lowest concentration at which no growth occurred was considered the MIC. The fractional inhibitory concentration index (FICI) was calculated as follows: $FICI = FIC_A + FIC_B = (MIC_{\text{Essential oil in combination}} / MIC_{\text{Essential oil alone}}) + (MIC_{\text{Antimicrobial compound in combination}} / MIC_{\text{Antimicrobial compound alone}})$. An $FICI \leq 0.5$ was considered as indicating a synergistic effect, an $FICI > 0.5$ and ≤ 4.0 as indicating no effect, and an $FICI > 4.0$ as indicating an antagonistic effect. All assays were performed in triplicates in two independent experiments.

Statistical analysis

The mean ± standard deviations analyzed for statistical significance using the Student's *t* test and were considered significant at $p < 0.01$.

Results

MIC and MBC values of ten essential oils against *S. hyicus* (2 strains) and *S. aureus* (3 strains) were first determined (Table 1). Essential oils from cinnamon, thyme and winter

Table 1 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) values of ten essential oils against strains of *S. hyicus* and *S. aureus*

Essential oil	<i>S. hyicus</i> ATCC 11249		<i>S. hyicus</i> 84-2978		<i>S. aureus</i> ATCC 25923		<i>S. aureus</i> 86-184		<i>S. aureus</i> 83-4484	
	MIC (%)	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)
Cinnamon	0.078	0.156	0.078	0.313	0.078	0.078	0.078	0.156	0.078	0.156
Coriander	0.625	0.625	1.25	1.25	0.625	0.625	0.625	2.5	1.25	> 2.5
Balsam fir	2.5	> 2.5	1.25	2.5	2.5	> 2.5	1.25	2.5	> 2.5	> 2.5
Labrador tea	0.625	1.25	0.625	0.63	1.25	2.5	0.625	1.25	1.25	2.5
Myrrh	> 2.5	> 2.5	2.5	> 2.5	2.5	2.5	> 2.5	> 2.5	> 2.5	> 2.5
Peppermint	0.625	0.625	0.625	1.25	0.625	0.625	0.625	> 2.5	1.25	2.5
Sage	2.5	2.5	2.5	2.5	1.25	2.5	1.25	> 2.5	> 2.5	> 2.5
Sweet marjoram	0.625	1.25	1.25	1.250	0.625	0.625	0.625	1.25	1.25	2.5
Thyme	0.078	0.078	0.156	0.156	0.078	0.078	0.156	0.156	0.078	0.156
Winter savory	0.078	0.078	0.156	0.156	0.078	0.156	0.078	0.078	0.078	0.078

savory showed the strongest antibacterial activity with MICs and MBCs ranging from 0.078 to 0.313%. The less effective essential oils were from balsam fir, myrrh, and sage with MIC and MBC values $\geq 1.25\%$. Since cinnamon, thyme and winter savory essential oils were identified as the most efficient against both *S. hyicus* and *S. aureus*, they were selected to further investigate their effects.

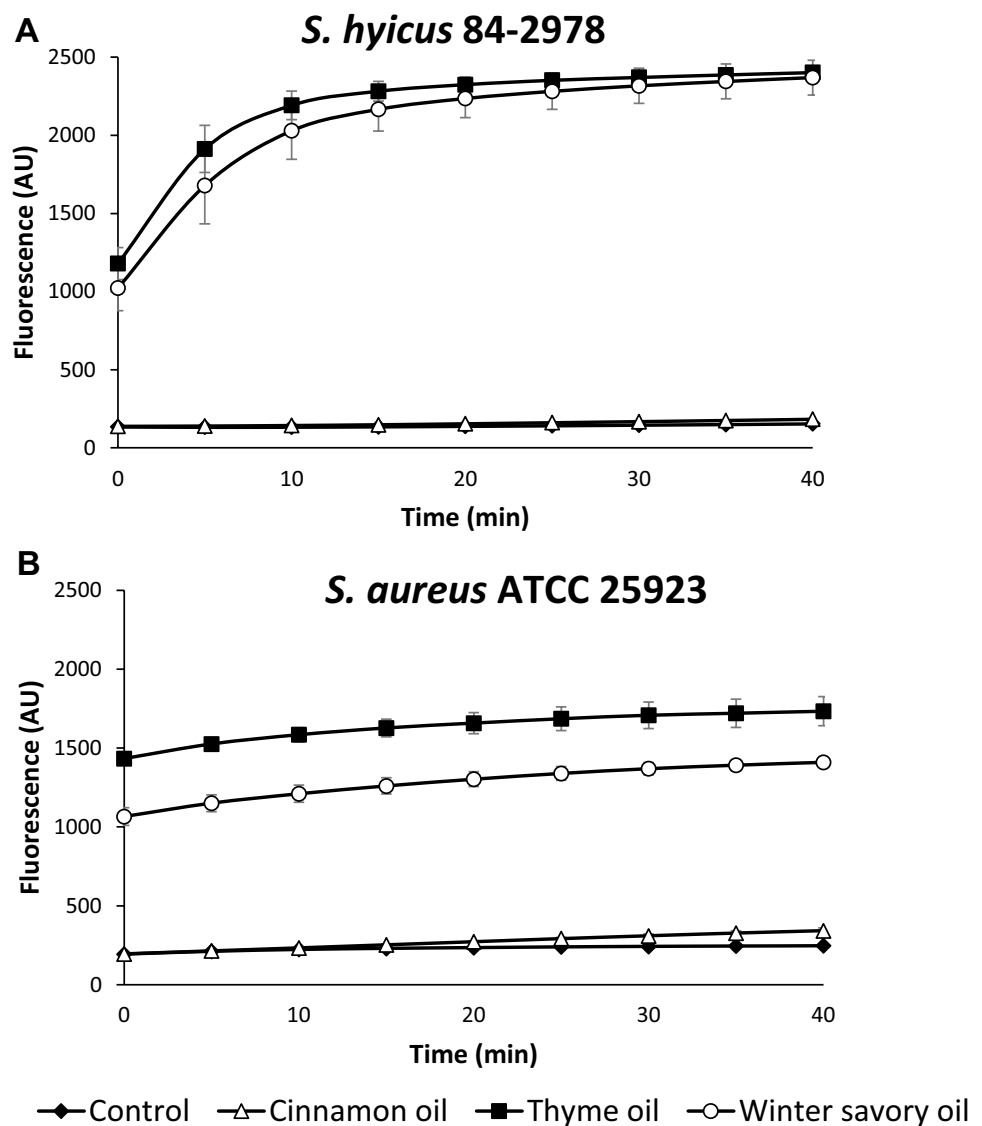
The effect of cinnamon, thyme and winter savory essential oils on membrane integrity of *S. hyicus* 84-2978 and *S. aureus* 25923 was investigated using the SYTOX[®] Green dye. As shown in Fig. 1, following the addition of thyme oil and winter savory oil, an increase in fluorescence was observed time-dependently. This increase in fluorescence occurred immediately after adding essential oils suggesting that permeabilization of the membrane was rapid. No such effect was observed when cells of *S. hyicus* and *S.*

aureus were incubated with cinnamon oil. Moreover, no increase in fluorescence occurred in the negative control.

Among the strains tested in this study, *S. hyicus* 84-2978 and *S. aureus* 25923 were found to form a dense biofilm as determined by crystal violet staining following growth in 96-well microplate. We thus investigated the ability of cinnamon, thyme and winter savory essential oils at sub-MIC values to prevent biofilm formation. While cinnamon oil had no significant effect on biofilm formation by both bacterial species (data not shown), thyme and winter savory oils reduced biofilm formation by *S. hyicus* 84-2978 at concentrations that did not reduce bacterial growth (Fig. 2). No such effect was observed for *S. aureus* 25923.

Thereafter, we evaluated the effect of a 15-min treatment with cinnamon, thyme and winter savory essential oils at a concentration corresponding to the MIC value on desorption and viability of pre-formed biofilms (18 h) of *S. hyicus*

Fig. 1 Effect of cinnamon, thyme and winter savory oils on membrane permeabilization of *S. hyicus* 84-2978 (panel A) and *S. aureus* 25923 (panel B). Bacterial cells were incubated with essential oils and SYTOX[®] Green dye. The fluorescent dye enters the cells and binds to DNA once the bacterial membrane is compromised. Arbitrary units of fluorescence were monitored following excitation at 485 nm and emission at 528 nm



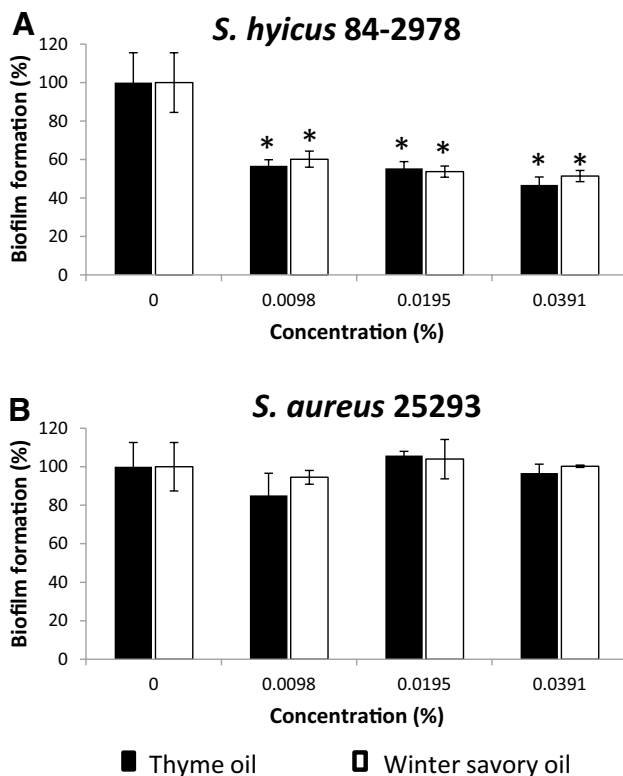


Fig. 2 Effect of thyme and winter savory oils on biofilm formation by *S. hyicus* 84-2978 (a) and *S. aureus* 25923 (b) as determined in a microplate assay and crystal violet staining. *Significantly different from control at $p < 0.01$

84-2978 and *S. aureus* 25923. As reported in Fig. 3, none of the essential oils induced a significant desorption of the pre-formed *S. aureus* biofilm, while thyme oil caused a slight but significant desorption of the pre-formed *S. hyicus* biofilm. The essential oils significantly reduced the biofilm viability to various extents. Cinnamon oil decreased biofilm viability of *S. hyicus* by 91% while having no effect on the viability of the *S. aureus* biofilm. The essential oils from thyme reduced the biofilm viability of *S. hyicus* 84-2978 and *S. aureus* 25923 by 95 and 71%, respectively.

Last, we investigated the synergistic interactions between essential oils (cinnamon, thyme and winter savory) and other antibacterial compounds. Table 2 reports the MIC and MBC values of penicillin G, chlorhexidine, and nisin against *S. hyicus* (84-2978) and *S. aureus* (25923 and 86-184). *S. hyicus* 84-2978 and *S. aureus* 86-184 were found to be resistant to penicillin G (MIC > 250 $\mu\text{g}/\text{mL}$). For the three strains tested, MIC values for chlorhexidine were in the range of 0.98–1.95 $\mu\text{g}/\text{mL}$, while MIC values for nisin ranged between 3.91 and 31.3 $\mu\text{g}/\text{mL}$. Using the checkerboard method, the effect on potency of the combination of compounds in comparison with their individual activities, represented as the FICI value, was evaluated. As shown

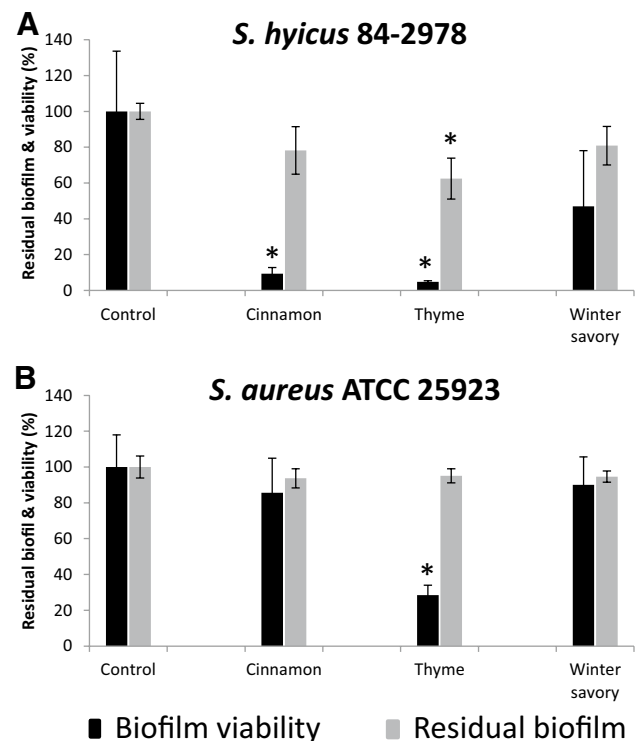


Fig. 3 Effect of cinnamon, thyme and winter savory oils on biofilm viability and desorption by *S. hyicus* 84-2978 (a) and *S. aureus* 25923 (b). Biofilm desorption was evaluated by crystal violet staining. Biofilm viability was assessed using a commercial luminescence assay (BacTiter-Glo™) that quantifies ATP, an indicator of metabolically active viable bacteria. *Significantly different from control at $p < 0.01$

in Table 3, thyme and winter savory oils acted in synergy with penicillin G, chlorhexidine, and nisin against penicillin-resistant *S. hyicus* 84-2978. For the penicillin-resistant *S. aureus* 86-184, synergy was only observed with winter savory oil and chlorhexidine. In the case of *S. aureus* 25923, several combinations of essential oils with either penicillin G, chlorhexidine or nisin showed synergy.

Discussion

Staphylococcus hyicus and *S. aureus* producing exfoliative toxins are implicated in the development of exudative epidermitis characterized by lesions ranging from localized lesions to a generalized condition covering the entire body of piglets (Frana 2012; Foster 2012). Infections often follow abrasions produced by scratches, bites or rough bedding that allow bacteria to invade the epidermis. This disease is frequently treated with antibiotics given systemically and consequently this may favor the occurrence of antibiotic resistance among isolates. Given the risk for transfer of antibiotic resistances from animal bacteria to human pathogens, researchers have been looking for alternatives to antibiotics

Table 2 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) values of penicillin G, chlorhexidine, and nisin against *S. hyicus* and *S. aureus*

Antimicrobial compound	<i>S. hyicus</i> 84-2978		<i>S. aureus</i> 86-184		<i>S. aureus</i> ATCC 25923	
	MIC	MBC	MIC	MBC	MIC	MBC
Penicillin G (µg/mL)	> 250	> 250	> 250	> 250	0.03	3.91
Chlorhexidine (µg/mL)	0.98	1.95	1.95	7.81	1.95	3.91
Nisin (µg/mL)	31.3	31.3	3.91	3.91	15.6	15.6

Table 3 Interactions between cinnamon, thyme and winter savory oils in combination with either penicillin G, chlorhexidine, or nisin against *S. hyicus* 84-2978, *S. aureus* 86-184, and *S. aureus* ATCC 25923, as determined by the checkerboard method

Antimicrobial compound	<i>S. hyicus</i> 84-2978 (Pen ^R)			<i>S. aureus</i> 86-184 (Pen ^R)			<i>S. aureus</i> ATCC 25923		
	Cinnamon	Thyme	Winter savory	Cinnamon	Thyme	Winter savory	Cinnamon	Thyme	Winter savory
Penicillin G	S	S	S	NE	NE	NE	NE	S	S
Chlorhexidine	NE	S	S	NE	NE	S	S	S	NE
Nisin	NE	S	S	NE	NE	NE	S	S	S

S synergy, *NE* no effect

in the agriculture field. Through their antimicrobial properties, essential oils may represent compounds of high interest. Interestingly, since essential oils contain a large variety of chemical compounds that may possess different modes of action and act in synergy (Burt 2004), the appearance of antimicrobial resistance in bacteria is unlikely. Indeed, evidence has been brought suggesting that prolonged exposure to essential oils does not induce resistance in *Helicobacter pylori* (Ohno et al. 2003; Ali et al. 2005). To the best of our knowledge, the effects of essential oils on the etiologic agents of exudative epidermitis have not been investigated. In this study, we investigated the antibacterial and antibiofilm properties of plant essential oils against *S. hyicus* and *S. aureus* as well as their ability to act in synergy with other antimicrobial compounds (penicillin G, chlorhexidine, nisin).

Among the ten essential oils tested, those from cinnamon, thyme and winter savory showed the strongest antibacterial activity against *S. hyicus* and *S. aureus*. On the one hand, thyme and winter savory oils appear to exert their antibacterial activity by disturbing the bacterial membrane. It has been previously reported that the main actions of essential oils is to affect the permeability of the bacterial cell membrane (O'Bryan et al. 2015). On the other hand, cinnamon oil involves an additional mechanism. Bouhdid et al. (2010) brought evidence that the antibacterial activity of cinnamon oil may rely on its ability to alter the aerobic metabolism, in addition to alteration in membrane permeability.

Our results indicating that cinnamon oil was highly active against staphylococci are in agreement with the study of Zhu et al. (2016) who reported that cinnamon oil was an effective antimicrobial agent against pathogens

causing bovine mastitis, including *S. aureus*. Cinnamaldehyde is the main constituent of cinnamon oil and is well known for its antimicrobial activity. Interestingly, cinnamaldehyde has been reported to upregulate the expression of tight junction proteins in intestinal porcine epithelial cells thus increasing the epithelial barrier function (Sun et al. 2017). Since tight junction proteins, which seal the intercellular spaces between cells, are involved in skin barrier, studies aimed to investigate whether cinnamaldehyde or cinnamon oil may increase tight junction protein expression in dermal epithelial cells are of high interest. Such an effect may prevent dermal tissue invasion by *S. hyicus* and *S. aureus*, in addition to cause bacterial killing.

Thyme and winter savory essential oils were found to attenuate biofilm formation by *S. hyicus* at concentrations that did not reduce bacterial growth. This may be associated with the ability of certain essential oils to inhibit quorum sensing-mediated signaling in bacteria (O'Bryan et al. 2015). Interestingly, Singh et al. (2017) recently reported that thyme oil can reduce biofilm formation and impair virulence of *Xanthomonas oryzae* through a decreased production of quorum sensing factors.

Among the potential strategies proposed to fight against the emergence of *S. hyicus* and *S. aureus* with antibiotic resistance, combining antimicrobial agents with essential oils may be a valuable approach. We showed synergistic interactions against penicillin-resistant *S. hyicus* 84-2978 and *S. aureus* 25923 by combining penicillin G with essential oils. This is in agreement with Palaniappan and Holley (2010) who reported that natural antimicrobials, including active ingredients of essential oils, can potentiate the

effects of conventional antibiotics to which bacteria were normally resistant.

Nisin is a bacteriocin (lantibiotic class) naturally produced by *Lactococcus lactis* that has received GRAS (generally recognized as safe) status (Hansen 1994). It is mainly active against Gram-positive bacteria and is currently used as a food preservative specially in dairy products in more than 50 countries, including the United States of America and several countries within the European Union (Hansen 1994). In our study, synergistic interactions between essential oils and nisin were observed against penicillin-resistant *S. hyicus* 84-2978 (thyme and winter savory) and *S. aureus* 25923 (cinnamon, thyme and winter savory). Synergism between essential oils from thyme and cinnamon, and bacteriocins including nisin has been previously reported against foodborne pathogens (Turgis et al. 2012).

In summary, this study brought evidence that essential oils, more specifically those from cinnamon, thyme, and winter savory, may contribute to develop an herbal treatment against exudative epidermitis in piglets. This may reduce the incidence of the disease and decrease the use of antibiotics to treat this infection.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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