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Susceptibility of *Paenibacillus larvae* isolates to a tetracycline hydrochloride and Cinnamon (*Cinnamomum zeylanicum*) essential oil mixture

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Abstract

The antimicrobial activity of tetracycline hydrochloride (OTC) and cinnamon (*Cinnamomum zeylanicum* Nees) essential oil (CEO) was evaluated against six different isolates of *Paenibacillus larvae* (White), the causal agent of American Foulbrood (AFB) disease in honey bee colonies. The bacteria isolates were collected from different localities of Argentine. Minimal inhibitory concentration (MIC) in MYT broth by the tube dilution method was evaluated for each substance and for the combinations of both antimicrobials using Krogstad and Moellering technique in order to establish the possible synergistic effects between these substances. OTC mean MIC values were of 3.67 ± 1.80 μg/ml, while the mean MIC values obtained for CEO were of 41.67±19.17 μg/ml. An inhibitory synergetic effect between these substances was observed with FIC index < 1 on 50% of the on *P. larvae* isolates.

Key words: Tetracycline hydrochloride, cinnamon essential oil, antimicrobial activity, *Paenibacillus larvae*.

Introduction

The bacterial pathogen *Paenibacillus larvae* (White), is the etiological agent of American foulbrood (AFB) disease, an extremely contagious disease of honey bee brood (Genersch *et al.*, 2006). AFB preventive and curative treatments usually consist in the application of antibiotics, such as tetracycline hydrochloride, but their extensive use have led to the accumulation of residues (Bogdanov, 2006) in honey and other beehive products, decreasing their quality and making their marketing more difficult. Besides residue accumulation, antibiotic-resistant isolates of *P. larvae* have been detected in many countries (Alippi, 1996; Miyagi *et al*., 2000; Evans, 2003). The concern for problems arising from microbial resistance is growing and the outlook for the future use of antimicrobial drugs is still uncertain. Therefore, actions must be taken to reduce this problem, for example optimizing the use of antibiotics when legally permitted and/or developing new drugs, either synthetic or natural (Nascimento *et al*., 2000), potentially efficient in the control of this very serious honeybee disease. The use of essential oils, with known antimicrobial properties, can be of great significance in apiary treatments. Since 1988 various studies were carried out to evaluate the effects of diverse essential oils against *P. larvae* (Carta and Floris, 1989; Floris and Carta, 1990; Alippi *et al*., 1996; Floris *et al*., 1996; Bazzoni and Floris, 1999). In the last few years, a number of studies have been conducted to verify the efficiency against *P. larvae* (Fuselli *et al*., 2008, Gende *et al*., 2009a, 2009b) of cinnamon essential oil and its main components (Gende *et al*., 2008a; 2008b; Gende, 2009). The aim of this study was the *in vitro* evaluation of the antimicrobial activity of a tetracycline hydrochloride (OTC) and cinnamon essential oil (CEO) mixture against *P. larvae* isolates, aiming to develop an effective combination of OTC with CEO in apiary, resulting in an overall reduction in the antibiotic employment for apicultural management, consequently reducing the risks of residues accumulation in beehive products and of bacterial resistance appearance, with special regard to the countries where the OTC use is permitted for the control of this bacterial disease.

Materials and methods

Bacterial biomass preparation

The bacterial isolates of *P. larvae* were collected from brood combs of beehives with clinical symptoms of American foulbrood located in Buenos Aires province (Argentina): Mar del Cobo, Sierra de los Padres, La Plata, Vidal, Mar del Plata and Ascasubi (corresponding to strain collection of Arthropods Laboratory, School of Natural and Exact Sciences, National University of Mar del Plata). Isolation was achieved on MYPGP agar (Dingman and Stahly, 1983) and *Paenibacillus alvei* (Cheshire et Cheyne) growth inhibition was ensured by the addition of 9 μg/ml of nalidixic acid. Plates were incubated under microaerobic conditions (5-10 % of CO_2) and strains were identified using biochemical tests (Gordon *et al*., 1973; Alippi, 1992). Pure strains were maintained on MYPGP agar with 15% v/v glycerol until used. Vegetative cells of *P. larvae* previously cultivated on MYPGP agar for 48 h at 35 ± 0.5 ºC were suspended in double distilled sterile water and the suspension was standardized according to FDA method (Merker, 1998). Concentration was adjusted to 0.5 of Mac Farland scale for measuring antimicrobial activity with serial dilution.

Evaluation of the antimicrobial activity

The classification of susceptible was made based on the methodology described by Alippi *et al.* (2007). The CEO was mixed in water and emulsified with 8% (v/v) propylene glycol (1,2-propanediol, Budavari *et al*., 1996), and the OTC antibiotic was mixed in water, separately. To evaluate the combination of these two antimicrobials, we used Krogstad and Moellering technique (Koneman *et al*., 1999), known as checkerboard test. Dilutions of each antimicrobial were chosen in the middle of the concentration of each drug, 9 each, in relation to previous studies (Gende *et al*. 2008b, Gende 2009). The concentrations evaluated were in the range of 5 dilutions below the MIC, the MIC and twice over the MIC (Eliopoulos and Moellering, 1996). For broth microdilution, 100 µl of MYT broth (Gende *et al*., 2008c) were placed in each of the microtitre plates. Drug A (OTC) was diluted in series in the direction of the ordinates, while the B (CEO) was diluted along the abscissas axis. The resulting grid provided all possible combinations of two antibiotics, from a well that contained the highest concentration of each to the lowest concentration in the opposite corner (figure 1). Then, microbial biomass suspension was added to each well. Inhibitory concentration was directly evaluated by turbidity observation. Positive and negative controls (with microorganisms and water, respectively) were used. Microtiter plates were incubated at 35 ± 0.5 ºC for 48 h. Antimicrobial activity was tested by triplicate analyses. When two antibiotics are given together, their effects can be: a) additive, when corresponding to the sum of the effects that each of them produces separately; b) antagonistic, when resulting in a lower effect than the sum of the single effects produced by each agent separately; c) synergistic, when the resulting effect is greater than the sum of the separate effects of each drug. The interpretation of results in the evaluation of these two antimicrobial products was based on the calculation of fractional inhibitory concentrations (FIC) and FIC index (Davidson and Parrish, 1989): FIC A = (MIC of A in the presence of B) / (MIC of A); FIC B = (MIC of B in the presence of A) / (MIC of B); FIC index = FIC A + FIC B; FIC index value allow to determine whether the mixture is additive, antagonistic or synergistic. A mixture is additive if the FIC index value is equal or approximately equal to 1; antagonistic if FIC index is > 1; synergistic if FIC index is < 1.

Results and discussion

Results for antimicrobial activity of OTC and CEO, alone or in combination, against *P. larvae* strains isolated from different Argentinean geographic areas are shown in table 1. The majority of the analyzed isolates (66.7%) can be considered susceptible to the action of OTC MIC values < 4 µg/ml. While, the remaining percentage (33.3%) showed MIC values of 6 µg/ml, being considered as intermediate Alippi *et al.* (2007). On the bases of the calculated FIC index values for each isolates, it was determined that for 33.3% of the strains the antimicrobial effect against *P. larvae* was antagonistic, with FIC index values > 1; for the blend, the effect was additive, with an FIC index value ≈ 1 in 16.7% of cases; finally, synergism was observed, with FIC < 1, for 50% of the isolates. This variability in results could be mainly due to a difference in the OTC antibiotic suscep-

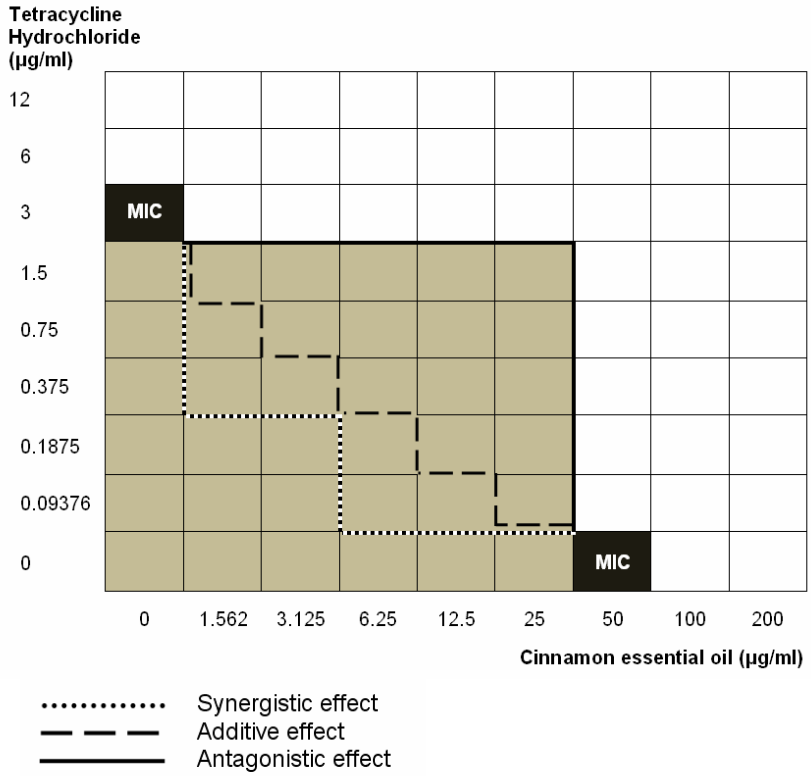

Figure 1. Checkerboard titration to evaluate antimicrobial synergism. Each box represents a tube or microtiter tray.

Table1. Antimicrobial activity[1] of oxitetracycline, *C. zeylanicum* essential oil and of both substances together against *P. larvae* strains isolated from different Argentinean geographic areas.
MIC values were in µg/ml expressed as mean and standard deviation values; FIC A = (MIC of A in the presence of B) / (MIC of A); FIC B = (MIC of B in the presence of A) / (MIC of B); FIC index = FIC A+ FIC B.
P. l Cb: *P. larvae* Cobo; *P. l* SdP: *P. larvae* Sierra de los Padres; *P. l* LP: *P. larvae* La Plata; *P. l* V: *P. larvae* Vidal; *P. l* Tp: *P. larvae* Mar del Plata; *P. l* As: *P. larvae* Ascasubi.

Antimicrobial agent		*Paenibacillus larvae* isolates					
		P. l Cb	*P. l* SdP	*P. l* LP	*P. l* V	*P. l* Tp	*P. l* As
OTC	MIC	3 ± 0 S	3 ± 0 S	2 ± 0.87 S	2 ± 0.87 S	6 ± 0 I	6 ± 0 I
	FIC A	0.13	0.06	0.38	0.09	1	1
Cinnamon	MIC	66.67 ± 28.87	33.33 ± 14.43	41.67 ± 14.43	25 ± 0	50 ± 0	33.33 ± 14.43
	FIC B	0.02	0.05	0.60	0.13	0.03	0.05
FIC index		< 1 Sy	< 1 Sy	≈ 1 Ad	< 1 Sy	> 1 An	> 1 An

[1] Antimicrobial activities were determined by triplicate analyses.
S: OTC sensible; I: OTC intermediate; Sy: synergistic effect; Ad: additive effect; An: antagonistic effect.

tibility of the different tested isolates. MIC values obtained for OTC against *P. larvae* isolates ranged between 2 to 6 µg/ml. The MIC values of susceptible strains obtained were comparable to those reported by Piccini and Zunino (2001). On the other hand, Alippi *et al.* (2007) found that the OTC-susceptible strains showed MIC values lower than 4 µg/ml, considering that some of the isolates analyzed were intermediate to the antibiotic. Results of CEO antimicrobial activity were similar to those found by Gende *et al.* (2008b). In the attempt to enhance the antimicrobial activity and reduce the development of resistance, the *in vitro* administration of antibiotic in combination with another active ingredient was conceived. In this particular case, trials aimed to determine whether it is possible to maintain efficacy decreasing the dose of OTC when administered in a mixture with CEO. The results indicate that it is not possible to generalize the combined use of these antimicrobials in apiary, since synergism was observed only in 50% of the cases, while effects were antagonistic for 33% of the isolates studied. In practice, this variability suggests to conduct laboratory tests before adopting antibiotic cinnamon essential oil mixture application against *P. larvae* as an effective alternative to control the AFB in apiary.

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References

ALIPPI A. M., 1992.- Deteccion de *Bacillus larvae* en poblaciones mixtas de esporas bacterianas a partir de restos larvales.- *Microbiologia SEM*, 8: 115-118.

ALIPPI A. M., 1996.- Caracterización de aislamientos de *Paenibacillus larvae* mediante tipo bioquímico y resistencia a oxitetraciclina.- *Revista Argentina Microbiologia*, 28: 197-203.

ALIPPI A. M., RINGUELET J. A., CERIMELE E. L., RE M. S., HENNING C. P., 1996.- Antimicrobial activity of some essential oils against *Paenibacillus larvae*, the causal agent of American foulbrood disease.- *Journal Herbs Spices Medicinal Plants*, 4 (2): 9-16.

ALIPPI A. M., LÓPEZ A. C., REYNALDI F. J., GRASSO D. H., AGUILAR O. M., 2007.- Evidence for plasmid-mediated tetracycline resistance in *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) disease in honeybees.- *Veterinary Microbiology*, 125: 290-303.

BAZZONI E., FLORIS I., 1999.- Azione in vitro di diversi oli essenziali contro *Paenibacillus larvae* e *Ascosphaera apis*, pp. 191-197. In: *Atti del Convegno Apilombardia 98, Giornate di studio sull'apicoltura* (SABATINI A. G., COLOMBO M., SPREAFICO M., Eds), 25-27 September 1998, Minoprio, Italy.

BOGDANOV S., 2006.- Contaminants of bee products.- *Apidologie*, 37: 1-18

BUDAVARI S., O'NEIL M. J., SMITH A., HECKELMAN P. E., KINNEARY J. F., 1996.- *The Merck index: an encyclopedia of chemicals, drugs, and biologicals, 12th ed.*- Merck & Co., Whitehouse Station, NJ, USA.

CARTA C., FLORIS I., 1989.- Prospettive di controllo della peste americana delle api con oli essenziali. Prove preliminari.- *Atti Società Italiana di Ecologia*, 8: 183-187.

DAVIDSON P. M., PARRISH M.E., 1989.- Methods for testing the efficacy of food antimicrobials.- *Food Technology*, 43: 148-155.

DINGMAN D. W., STAHLY D. P., 1983.- Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components.- *Applied Environmental Microbiology*, 46: 860-869.

ELIOPOULOS G. M., MOELLERING R. C., 1996.- Antimicrobial combinations, pp. 330-383. In: *Antibiotics in laboratory medicine, 4th edidion* (LORIAN V., Ed.).- Lippincott Williams & Wilkins, Baltimore, USA.

EVANS E., 2003.- Diverse origins of tetracycline resistance in the honey bee bacterial pathogen Paenibacillus larvae.- *Journal of Invertebrate Pathology*, 83: 56-50.

FLORIS I., CARTA C., 1990.- In vivo activity of *Cinnamomum zeylanicum* Nees essential oil against *Bacillus larvae* White.- *Apicoltura*, 6: 57-61.

FLORIS I., CARTA C., MORETTI M. D. L., 1996.- Activité in vitro sur *Bacillus larvae* White de plusieurs huiles essentielles et essai d'utilisation au le rucher.- *Apidologie*, 27: 111-119.

MERKER R. L., 1998.- Media and reagents, Appendix 3. In: *Food and Drug Administration bacteriological analytical manual*, 8th ed. (revision A) (CD-ROM version).- AOAC International, Gaithersburg, MD, USA.

FUSELLI S. R., GARCIA DE LA ROSA S. B., EGUARAS M. J., FRITZ R., 2008.- Chemical composition and antimicrobial activity of Citrus essences on honeybee bacterial pathogen *Paenibacillus larvae*, the causal agent of American foulbrood.- *World Journal of Microbiology & Biotechnology*, 24: 2067-2072.

GENDE L. B., 2009.- Principales componentes de aceites esenciales con actividad antimicrobiana frente a *Paenibacillus larvae*. 233 pp. *Doctoral thesis*, Buenos Aires University, Argentina. (In Spanish).

GENDE L. B., PRINCIPAL J, MAGGI M. D., PALACIOS S. M., FRITZ R., EGUARAS M. J., 2008a.- Extracto de paraíso (*M. azedarach*) y aceites esenciales de *C. zeylanicum*, *M. piperita*, *L. officinalis,* como control de *Paenibacillus larvae*.- *Zootecnia Tropical*, 26 (2): 151-156.

GENDE L. B., FLORIS I., FRITZ R., EGUARAS M. J., 2008b.- Antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) essential oil and its main components against *Paenibacillus larvae* from Argentine.- *Bulletin of insectology*, 61 (1): 1-4.

GENDE L. B., EGUARAS M. J., FRITZ R., 2008c.- Evaluation of culture media to support growth of *Paenibacillus larvae* subsp. *larvae* applied to studies of antimicrobial activity.- *Revista Argentina de Microbiología*, 40: 147-150.

GENDE L. B., BAILAC P., MAGGI M. D., PONZI M., FRITZ R., EGUARAS M. J., 2009a.- Antimicrobial activity of *Pimpinella anisum* and *Foeniculum vulgare* essential oils against *Paenibacillus larvae*.- *Journal of Essential Oil Research*, 21: 91-93.

GENDE L. B., MAGGI M. D., DAMIANI N., FRITZ R., EGUARAS M., FLORIS I., 2009b.- Advances in the apiary control of the honeybee American Foulbrood with Cinnamon (*Cinnamomum zeylanicum*) essential oil.- *Bulletin of Insectology*, 62 (1): 93-97.

GENERSCH E., FORSGREN E., PENTIKAINEN J., ASHIRALIEVA A., RAUCH S., KILWINSKI J., FRIES I., 2006.- Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation.- *International Journal of Systematic and Evolutionary Microbiology*, 56: 501-511.

GORDON R. E., HAYNES W. C., PANG H. N., 1973.- *The genus Bacillus*.- United States Department of Agriculture, Agricultural Handbook No.427, Washington, DC, USA.

KONEMAN E. W., ALLEN S. D., JANDA W. M., SCHRECKENBERGER P. C., WINN W. C., 1999.- Pruebas de sensibilidad a agentes antimicrobianos, pp. 763-832. In: *Diagnóstico Microbiológico, 5ª ed*.- Ed. Médica Panamericana SA., Buenos Aires, Argentina.

MIYAGI T., PENG C. Y. S., CHUANG R. Y., MUSSEN E. C., SPIVAK M. S., DOI R. H., 2000.- Verification of oxytetracycline-resistant American Foulbrood pathogen *Paenibacillus larvae* in the United States.- *Journal of Invertebrate Pathology*, 75: 95-96.

NASCIMENTO G. G. F., LOCATELLI J., FREITAS P. C., SILVA G. L., 2000.- Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria.- *Brazilian Journal of Microbiology*, 31: 247-256.

PICCINI C., ZUNINO P., 2001.- American Foulbrood in Uruguay: Isolation of *Paenibacillus larvae larvae* from larvae with clinical symptons and adult honeybees and susceptibility to oxytetracycline.- *Journal of Invertebrate Pathology*, 78: 176-177.

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