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*Original article*

# EVALUATION OF KINETIC BEHAVIOUR OF TWO PREPARATIONS OF TYLOSIN ADMINISTERED IN BEEHIVES FOR AMERICAN FOULBROOD CONTROL

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**Summary**

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We compared the kinetic behaviour of tylosin administered to beehives by dusting or paper-pack placement through three treatment protocols (D, PP, CONTROL). D (dusting): tylosin, divided in four portions, was sprinkled over the ends of the hives' top bars weekly for four weeks (n=3); PP (paper-pack placement): tylosin in paper packs was administered at two-week intervals (n=3); CONTROL (control): the hives were left untreated (n=3). In every inspection, from each of the nine hives, fifty young (2-day-old) larvae were sampled for drug analysis. The concentration of tylosin in the young larvae was determined by a microbiological assay with *Geobacillus stearothermophilus* ATCC 12980 as test organism. The (mean±SD) maximum concentration ($C_{max}$) for D was 136.0±194.0 and for PP – 144.0±187.4 µg/mL; the time to reach $C_{max}$ ($t_{max}$) was 1.5±0.9 h for D and 1.8 ± 1.8 h for PP. The area under the tylosin behaviour kinetics curve between 0–1392 h with D was 308.7±185.2 and with PP: 326.4±141.0 µg/h/mL, indicating no statistical difference between the treatments (P>0.05). The shorter duration of paper-pack-administered tylosin observed in the larvae implied a lower risk of antibiotic residues in the resulting honey.

**Key words**: American foulbrood, beehives, kinetics, tylosin

## INTRODUCTION

American foulbrood disease (AFB), caused by the spore-forming, Gram-positive rod-shaped bacterium *Paenibacillus larvae* (Genersch *et al.*, 2006); is a devastating brood disease that can affect both the larval and pupal stages of honeybees (*Apis*

*mellifera L.*). The unique characteristics of the spores, which can remain viable for long periods of time and survive adverse environmental conditions (Matheson & Reid, 1992; Shimanuki & Knox, 2000), make the AFB a highly contagious and also widespread disease. The control of AFB must be aboard taking into account a global scenario such as infection level, disease incidence, and the use of antibiotics or other natural substances for controlling this pest. In areas where disease incidence in low and infection levels is moderate, a shook swarm methods of treatment is recommended (Von der Ohe, 2003). However in areas where disease incidence is high antibiotic treatment is a possible alternative for the control of the disease. Thus, several honey producers countries (*e.g.* Argentina, Canada, China and USA between others) allow the use of antibiotics to keep the disease under control (Reybroeck *et al.*, 2012). Oxytetracycline (OTC) and tylosin are the only antibiotics approved for the control of AFB in bee hives, nonetheless the high selection pressure because of the intensive use of OTC in professional beekeeping, added to the fact that OTC having been also used against *Melisococcus plutonius* (Oldroy *et al.,* 1989; Thompson *et al.*, 2005) possibly favoured the oxytetracycline-resistant *P. larvae* isolates in Argentina (Alippi, 2000), Canada (Colter, 2000) and the USA (Miyagi *et al*, 2000). In addition, the efficacy of tylosin has already been proven through the use of different application methods (dusting or the placement in paper-packs, syrupy sugar, and patties) by different authors (Hitchcock *et al.*, 1970; Peng *et al.*, 1996; Alippi *et al.*, 2005; Pettis & Feldlaufer, 2005; Reynaldi *et al.*, 2009). This feature makes tylosin an alternative for the control of infected hives with oxytetracycline-resistant isolates.

On the other hand, since honeybees are classified as food producing animals but, as no metabolism exists inside a beehive, maximum residues limit (MRL) of honey cannot be defined (Reybroeck *et al.*, 2012). In the absence of either MRL or reference points for action (RPA), the presence of any detectable (and confirmed) residues in honey prevents this product from being legally placed in a market such as the EU (Anonymous, 2009). For this reason, the study of diverse pharmacokinetics parameters, such as the binding protein (Reynaldi *et al.,* 2010a), minimal bactericidal concentration (MBC), MBC/MIC relationship, killing curves (Reynaldi *et al.,* 2010b) or bioavailability, could help define the best treatment that must be not only efficient, but also safe for honey production.

The aim of the present work was therefore to study the kinetic behaviour of tylosin between the two most common methods of application of tylosin in the field trials, dusting and paper-pack.

## MATERIALS AND METHODS

### *Field assay*

The assay was conducted at the experimental field of the School of Agricultural Science, UNLP, La Plata (35.8º S, 5.8º W). All honeybee colonies, derived from *Apis mellifera ligustica* L., were standardised to contain 25,000 adult bees, 6 combs of brood (2 open and 4 sealed broods), 2 combs containing honey and pollen, and 2 combs of wax. The queens were marked and their wings clipped to avoid swarming. The colonies were distributed in a completely randomised design and none had been treated with antibiotics for the last ten years. Three treatments were then randomly assigned to the hives. (1) Treatment PP (paper-pack pla-

cement) involved a combination of 114 g of confectioner's sugar, 5 g of cherry, and 1 g of tylosin tartrate (Vetifarma®). The entire admixture (121 g) was divided between two paper-packs of about 60 g each, those being applied over the ends of the top bars of the hives for a total of four weeks at two-week intervals. (2) Treatment D (dusting) consisted of combining 114 g of confectioner's sugar, 5 g of cherry jelly (used as an attractant to promote consumption), and 1 g of tylosin tartrate (Vetifarma®). The admixture was divided into four 30 g portions. Each application consisted in a sprinkling one portion over the ends of the top bars for a total of four weeks at one-week intervals. The control group consisted of a set of untreated hives. Three hives were randomly assigned to each experimental group. The present work was approved by the Ethic Committee of the Faculty of Agricultural Sciences and Forestry, National University of La Plata, Argentina

*Sampling and preservation of samples*

In every inspection, fifty young (2-day-old) larvae (YLs) from each of the nine hives were sampled for drug analysis. For the interval of delivery of the antibiotic during the first four weeks, with both forms of administration (*e. g.*, dusting and paper-pack), samples were taken on days 2, 4, and 6 of each week; and from the fifth week onwards, the collection of larvae was performed weekly until the end of the three months of the experiment. Samples were stored at –20 °C until further analysis.

*Quantification of the antibiotic*

The concentrations of tylosin tartrate in YL were determined by microbiological assay using *Geobacillus stearothermophilus* ATCC 12980 as test organism (Herbst, 1982; Rule *et al.,* 2014). Each sample was assayed for tylosin by an agar diffusion bioassay with Mueller Hinton Agar (Lab. Britania, Argentina) seeded with the *Geobacillus stearothermophilus* ($1\times10^6$) and allowed to solidify on 23×28 cm glass plates. Duplicate 25-µL portions of each sample and of the standards were then placed in 6 mm wells cut into the seeded agar. The tylosin standards (20.0, 10.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.125, 0.06, 0.03, 0.01, 0.006, 0.003, 0.001 µg/g) were prepared in YL taken from an untreated hive. After incubation of the assay plates for 6 to 8 hours at 64 °C, the zone of inhibition around each well was measured and standard curves were prepared. Sampling before administration of the antibiotic showed no bacterial inhibition. The correlation coefficient for the standard curves, the intra- and interassay coefficients of variation, the sensitivity and quantification limits of the assay were calculated according to Reynaldi *et al.* (2010a). Briefly, the correlation coefficient for the standard curves prepared for all the experiments was >0.98. Intra- and interassay coefficients of variation were lower than 8%. Sensitivity and quantification limits of the assay for YL was 0.001 µg/mL. No inhibition zones were observed in samples from control beehives.

*Pharmacokinetic analysis and statistics*

The pharmacokinetic analysis of the data was performed by means of a non-compartmental model, according to the methods described by Gibaldi & Perrier (1982). The maximum concentration ($C_{max}$) of tylosin in YLs and the time to reach $C_{max}$ ($t_{max}$) are empirical values taken directly from the analytical data. The area under the curve (AUC) of concentration with respect to time was calculated by trapezoidal methods. The statisti-

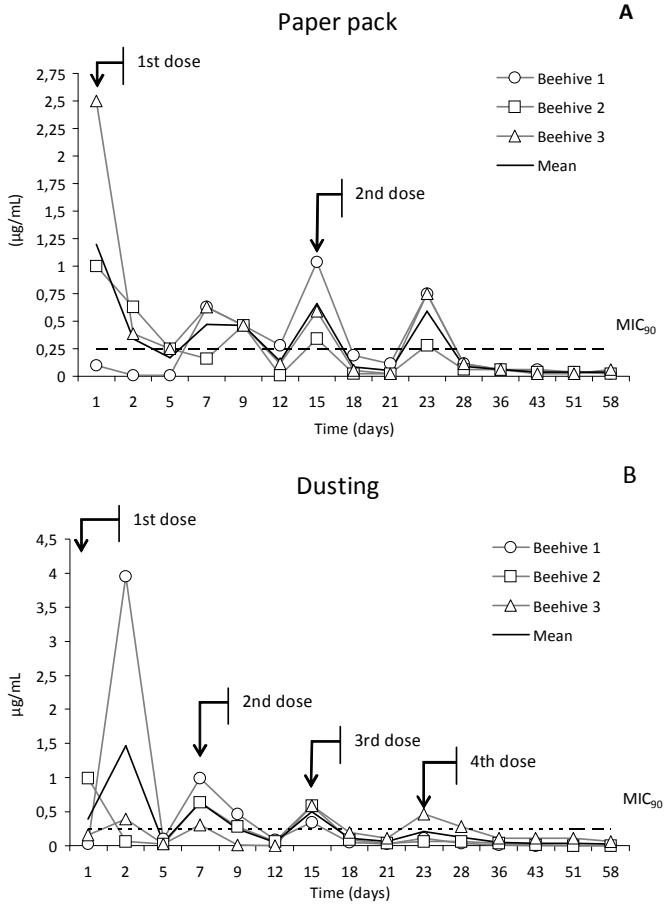


**Fig. 1.** Concentration-time curves of tylosin in young *Apis mellifera* larvae administered either in a paper pack (A) or by dusting (B). The mean larval tylosin concentration (µg/mL) is plotted on the ordinate as a function of time in days on the abscissa. The minimum inhibitory concentration ($MIC_{90}$) for the growth of *Paenibacillus larvae* (Alippi *et al.*, 2005) is indicated by a horizontal line. The arrows above the curves, point the days on which tylosin doses were added to the hives.

cal comparisons of the pharmacokinetic parameters were made by means of a two-way analysis of variance (Statgraphic Plus 7.0). A probability level of less than 0.05 was considered statistically significant.

## RESULTS

Fig. 1 shows the concentration-time curves of tylosin in YLs after either treatment PP (A) or treatment D (B). The mean±SD values for $C_{max}$ for treatments PP and D were 136.0±194.0 µg/mL and 144.0± 187.4 µg/mL respectively. The $t_{max}$ of tylosin was 1.5±0.9 h (treatment PP) and 1.8±1.8 h (treatment D). No statistical differences in either the $C_{max}$ or the $t_{max}$ between treatments were found. The AUC obtained from time 0 to 1392 h for treatment PP was 308.7±185.2 µg/h/mL and

for treatment D: 326.4±141.0 µg/h/mL, with likewise no statistical difference occurring between the two modes of tylosin administration ($P \geq 0.05$). With treatment PP the antibiotic became undetectable on day 62, while with treatment D the corresponding time was day 76.

## DISCUSSION

The efficacy of several antibiotics such as tylosin, lincomycin (Okayama *et al.*, 1996; Kochanski *et al.*, 2001) or even more recently tilmicosin (Reynaldi *et al.*, 2008) have been tested against OTC-resistant strains (Alippi, 2000; Colter, 2000; Miyagi *et al.*, 2000). Among them, tylosin appeared as an attractive alternative exhibiting both a bactericidal effect and the best *in vitro* inhibition of *P. larvae* in terms of the MIC, MBC, and MBC/MIC values (Peng *et al.*, 1996; Alippi *et al.*, 2005; Reynaldi *et al.*, 2010b). Furthermore, the field efficacy of tylosin had already been demonstrated by several authors (Elzen *et al.*, 2002; Alippi *et al.*, 2005; Pettis & Feldlaufer, 2005). However, the consumption of tylosin was a trouble, for this reason, Peng *et al.* (1996) recommended the use of cherry jelly as an attractant to improve the palatability of tylosin treatment. More recently, Reynaldi *et al.* (2009) have demonstrated the effectiveness of 1 g of tylosin tartrate as the lowest dose for eliminating disease symptoms in beehives, with no recurrence being observed for up to a year.

In general, an antibiotic would need to remain for a certain time within the hive, after which period the agent should be removed from the hive so as to reduce the chance of leaving residues in the honey. A limited period of exposure to the antibiotic would also avoid the chance of selecting resistant mutant bacteria because, leaving antimicrobial concentrations inside the mutant window selection described by Drilca (2003), is expected to enrich resistant subpopulations. Even though the kinetics parameters showed no statistical differences between treatments, treatment PP remained less time below the MIC, thus minimising the chance of enabling mutant selection. Antibiotics *per se* do not induce a resistance, but simply provide the selective force to promote the Darwinian process of natural selection. The mutation potential, the genetic interchange between bacteria, added to the limited time of bacteria generating together with a wrong therapeutic application can rapidly produce resistant populations (Drilca, 2003). For this reason, between the two field treatments (paper-pack and dusting) with the same resulting efficiencies of action, the treatment of choice would be the use of paper packs because the concentrations of the antibiotic remained in the hive for 14 days less than with the application by dusting.

## CONCLUSION

The intention of the present study was to determine the kinetic behaviour of tylosin in beehives in order to generate a useful strategy for the employment of antibiotics as bactericidal agents for the control of American Foulbrood in beehives. Accordingly, the concentrations of tylosin in the YLs indicated that although both forms of delivery – via paper pack or by dusting – had proven effective in controlling AFB in previous field trails (Hitchcock *et al.*, 1970; Peng *et al.*, 1996; Alippi *et al.*, 2005; Reynaldi *et al.*, 2010b), the more rapid elimination of tylosin applied by paper pack made this treatment the better one. The more time a microorganism spends at sub-inhibitory concentrations,

the greater the chances of selection for a resistant microorganism. Treatment PP proved to be easy to use by the beekeepers; moreover the treatment required only two visits to the apiary, as opposed to the four needed with dusting.

To date, the use of antimicrobial agents in apiculture has been indiscriminate and the pharmacological and chemotherapeutic data available in bees are limited. Consequently, all the information related to this topic such as relative bioavailability studies, percentages of binding protein for antibiotics, MIC, MBC, the MBC/MIC ratio, killing curves and the kinetic behaviour presented here would be useful in developing treatments that should be both efficient and safe for honey production.

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