Original Article

IMPACT OF OXYTETRACYCLINE ON *APIS MELLIFERA* COLONIES: PRELIMINARY RESULTS ON RESIDUES AND ANTIBIOTIC RESISTANCE

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Abstract

We tested two summer protocols for oxytetracycline (OTC) administration on beehives to evaluate: toxicity for the bees, residues in honey and presence of genes encoding for OTC antibiotic resistance in bacteria isolated from the gut of adult bees. In July 2020, eighteen healthy colonies were divided into three groups. Two OTC treatment protocols - long and short, were devised, and one group was left untreated. We did not detect any toxicity on adult bees or brood but found OTC residues in the honey of the nest up to seven months after both treatments (210.3±221.2 µg/kg after long protocol; 216.2±238.4 µg/kg after short protocol), even in the untreated group (up to the mean 75.8±173.2 µg/kg). Antibiotic resistance genes were found, even in the untreated group, possibly due to the long exposure to antibiotics. More in-depth studies should be performed to verify how the long-term persistence of antibiotics impacted the beehive during the inactive season.

Keywords: antibiotic resistance, comb honey, oxytetracycline, summer protocol, residues, toxicity

INTRODUCTION

Honey bees (*Apis mellifera*) are of great economic relevance thanks to their colony products and pollination service, improving crop productivity and maintaining plant and animal biodiversity (Klein et al., 2018). Globally, some antimicrobials (oxytetracycline, tylosin tartrate, fumagillin, metronidazole, sulfadimethoxine, trimethoprim) are authorised for the treatment of infectious honey bee diseases (CCRVDF electronic working group on honey, 2010). According to scientific references (Bogdanov

& Fluri, 2000; Reybroeck, 2002; Wallner, 2003; Baggio et al., 2004; European Food Safety Authorithy, 2017, 2018) and the European Food Safety Authority (EFSA reports 2017, 2018), 1-7% of honeys sampled in EU tested positive for antibiotic residues. This data suggest that antibiotics are still in use by European beekeepers (Procedures-Veterinary, 2018).

Oxytetracycline (OTC) is produced by *Streptomyces* spp. and belongs to the tetracyclines group (Buzia et al., 2019). OTC inhibits protein synthesis by preventing the binding of aminoacyl-tRNA to the mRNA-ribosome complex (Önal, 2011). It is a broad-spectrum antibiotic that acts against a wide range of Gram-positive and Gram-negative bacteria. Used since 1967 in Europe (Thompson et al., 2006; Budge et al., 2010) OTC has been adopted for several routes of administration to treat infectious honey bee diseases (Waite et al., 2003; Thompson et al., 2005).

OTC residues in food may be harmful to the consumer due to dysbiosis and trigger allergic reactions or cause liver damage and gastro-intestinal disorders (Thanasarakhan et al., 2011). Antibiotic residues in food can promote the development of antimicrobial resistance (AMR) (Finley et al., 2013). The rise of resistant bacteria and the lack of effective antibiotics against multi-drug resistant bacterial strains, constitute a public health emergency (Gačić et al., 2015) by affecting human, veterinary and environmental sectors and require a One Health Approach. Tetracyclines and Oxytetracycline are the active principles of several commercial beekeeping products for American foulbrood (AFB) and European foulbrood (EFB) control: Terramycin and Pennox 50 in the USA (American Veterinary Medical Association, 2017) Foul Brood Mix, Oxytet-25-S and Oxysol 62.5 in Canada.

In the US, the Food and Drug Administration (FDA) has recognised the use of 0.2 g/colony of OTC, applied every four to five days for three treatments for AFB and EFB control in bees. The honey super withdrawal period is fixed between 54 and 57 days from the first day of treatment, and maximum residue limits (MRLs) are established at 750 µg/kg (Richards et al., 2021). Different MRLs are used in Australia and India (300 µg/kg) or Brazil (20 µg/kg) (Buzia et al., 2019), and in the EU there are no registered antibiotic products for honey bees. However, OTC can be used through the "cascade system", as an off-label manner, (European Parliament, 2001; European Parliament and the Council of the European Union, 2019), with an MRL of 100 µg/kg (European Commission, 2018), which should include the sum of parent drugs and its four epimers (European Commission, 2010). Off-label use consists of the prescription and use of a drug without strictly respecting the conditions of destination species and pathology indicated in the package insert. Where there are no authorized veterinary medicinal products, the veterinarian in charge may exceptionally and under their own responsibility prescribe a medicinal product by derogation in order to avoid obvious states of suffe OTC administration to the bees ring to the animal, (European Parliament and the Council of the European Union, 2019). According to the label indication (Tasmanian Government, 2016; Lafrenière et al., 2018; Richards et al., 2021), OTC treatments can be applied to beehives when beekeepers see symptoms of AFB and/or EFB. This usually occurs in spring and, with lower frequency, in summer, due to poor weather conditions (Rowland et al., 2021).

Antibiotics can impact composition and abundance of the honey bee microbial gut community (Raymann et al., 2017). Honey bee gut microbiota has many functions, including nutrients biosynthesis, digestion and detoxification of secondary plant compounds and other xenobiotics. It promotes detoxification enzyme expression in the midgut. Moreover, intestinal bacteria impact the neurophysiology and regulation of honey bees' social behaviour (Liberti & Engel, 2020). Gut microbiota is also involved in the mucosal immunity by controlling intestinal homeostasis and activating intracellular pathways. The host releasing defensins, lectins, reactive oxygen, bacteriocins controls the expansion of pathogens. In addition, gut microbiota induces immune responses by

upregulating the expression of antimicrobial peptides, such as apidaecin (Nowak et al., 2021). Dysbiosis can decrease the honey bee life span and promote such pathogen invasions as *Lotmaria passim*, *Nosema* spp., *Ascosphaera apis*, and *Serratia marcescens* (Raymann et al., 2017).

The aim of this study was to evaluate the impact of two different OTC summer protocols on honey bee colonies through the assessment of 1) toxicity on adult bees and brood, 2) residues in honey taken from the nest, 3) honey bee gut microbiota and 4) OTC-related antibiotic resistance genes.

MATERIAL AND METHODS

Trial setup

Antibiotic treatments were performed in July 2020 in an experimental apiary in Rome (Central Italy). Research was carried out on eighteen healthy colonies divided into three similarly sized groups, based on the number of frames covered by bees and brood. 180 ml of sucrose solution (1:1) and 0.576 grams of OTC active ingredient (Unione Commerciale Lombarda S.p.A, Brescia, Italy – A.I.C. 102781010) were poured in six Petri dishes (Steris, diameter 90 mm and height 16.2 mm). Each plate containing 30 ml of OTC sucrose solution was placed on top of the frames of each hive and fitted with a mesh to reduce contact with bees and prevent them from drowning (Fig. 1). In OTC administration to the bees group 1, OTC was applied once a week for five weeks in a row ("*long protocol*"), and in group 2, OTC was applied *semel in die* (*SID*) for five days in a row ("*short protocol*"). Each colony was treated with the same amount of OTC, corresponding to 2.88 grams. (Tasmanian Government, 2016). Group 3 ("*control*") received only the sucrose solution. The remaining amount of sucrose solution was measured at each administration and all Petri dishes were replaced (Fig. 1). OTC toxicity in colonies was evaluated before and after the treatment application through the counting of frames occupied by adult honey bees and brood (Chabert et al., 2021). Moreover, the queen's presence was checked each time the colonies were visited.

Chemical Analysis

OTC residues were determined through analysis of a piece of honeycomb (5 cm x 4 cm) cut out of the nest from the upper part of the frames, right under the Petri dishes ("worst-case hypothesis"), before the first OTC application, after one and three weeks, as well as after one, two, three, five and seven months from the last OTC administration. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) was used to investigate the presence of OTC residues. All reagents used for HPLC analysis were of analytical grade. The tetracyclines standards with a 99% degree of purity were obtained from the Sigma-Aldrich

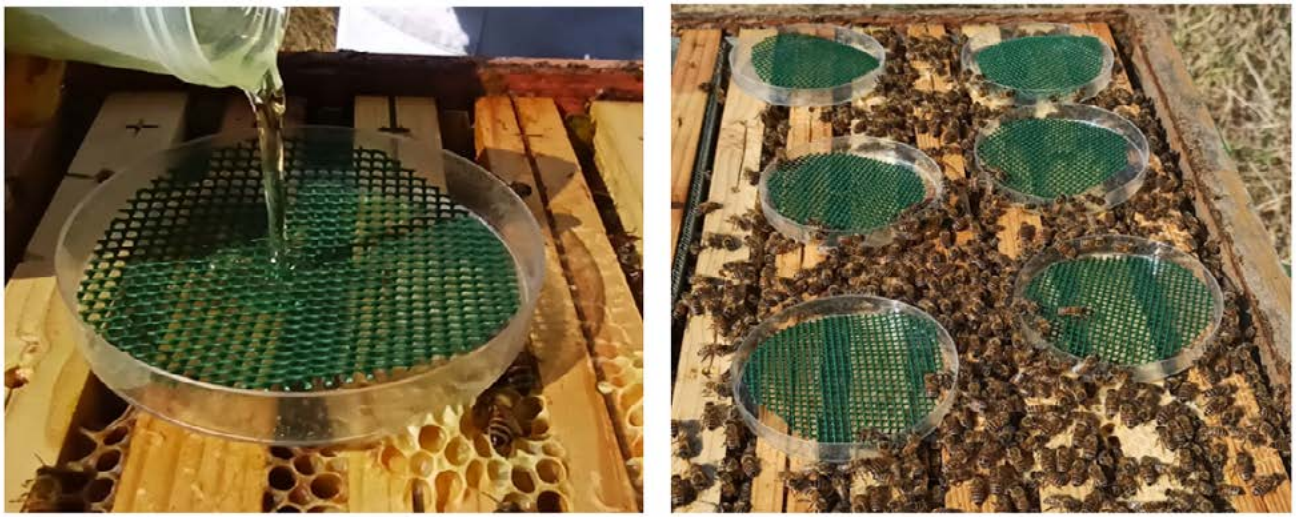


Fig. 1. OTC administration to the bees.

Chemical Company (USA). Acetonitrile, methanol and formic acid were provided by Carlo Erba Reagents (Milan, Italy). Oasis HLB (60 mg, 3 ml) polymeric cartridges were from Waters (Waters, Milan Italy). Water was purified with the use of the Milli-Q system. Stock standard solution (1 mg/ml) was prepared through the weighing of 10.0±0.1 mg of standard substances and dissolving them in 10 ml of methanol in an amber glass. Working standard solutions (100 µg/ml, 10 µg/ml) were prepared in acetonitrile through the diluting of a suitable aliquot of stock standard. Working standard solutions in the mobile phase were prepared on the day of analysis. An aliquot of 2 grams of honey was collected from the sampled honeycomb and mixed with 10 ml McIlvaine-EDTA for 15 min in an ultrasonic bath. The extract was charged on Oasis HLB (60 mg) SPE cartridge previously activated with 3.0 ml of methanol and 3.0 ml of deionised water, while the SPE cartridge was washed with 3.0 ml of deionised water, 2.0 ml of methanol-water 5:95 v/v. Tetracyclines were eluted with 3 ml of methanol containing 0.1% of formic acid, and the eluted liquid was evaporated at 40°C under a nitrogen stream. The residue was finally dissolved in 0.5 ml of methanol-water 50:50 v/v and injected into the LC-MS/MS system. Analyses were carried out with an QTRAP 5500 tandem mass spectrometer detector (AB Sciex, MA, USA) equipped with a 1260 Infinity high performance liquid chromatography (Agilent Technologies, CA, USA). The instrument was set in positive electrospray ionisation mode with a capillary voltage of 5.5 kV and a source temperature of 500°C. Ultra-pure air was used as a nebulizer gas, and ultra-pure nitrogen was both a curtain and collision gas. Positive ions were acquired in multiple reaction monitoring (MRM) mode, acquiring two or more diagnostic product ions from the chosen precursors to obtain high specificity and sensitivity. The chromatographic separation of the analytes was achieved on a Kinetex Biphenyl column (50 mm x 3.0 mm, 2.7 µm, Pnenomenex) with a mobile phase consisted of 0.1% formic acid in water (mobile phase A) and acetonitril (mobile phase B) at a flow rate of 0.3 ml min^{-1}, in gradient mode. The presented quantitative method is very sensitive and specific for the analysis of tetracyclines residues and their epimers in honey (limit of detection, Limit Of Detection - LOD - 1 µg/kg).

Genetic analysis

After five and a half and eight months from the last OTC administration, we sampled ten live adult honey bees from the central frame of the brood chamber of each hive to isolate gut bacterial strains. Adult workers have a relatively stable set of bacterial species in their gut (Kwong & Moran, 2016). We divided the bees into three groups according to each administration method. Due to COVID-19 and restrictions imposed by the SARS-CoV-2 pandemic at the national level, live honey bees could not be sampled before and immediately after OTC administration.

The guts of ten live bees from each pool were extracted in sterile conditions and placed in a tube with 1 ml of a 0.90% (w/v) sodium chloride solution. Subsequently, each sample was sown with a 10µl single-use loopful, with the use of the triple smear technique, on two Sabouraud plates (incubated for 24-48 hours at 37°C +/- 2°C and at 25°C for 5 days) and on three Blood Agar plates (incubated at 37°C +/- 2°C) in aerobic, anaerobic and microaerophilic conditions for 48/72 hours, respectively). The growing bacterial colonies on each plate were identified according to their morphology, oxidase test (with oxidase strips relevant to the cytochrome oxidase enzyme), catalase test (by hydrogen peroxide at 3%), Gram stain and biochemical identification tests (API Biomeriéux for *Enterobacteria,* Non-Enterobacteria, *Bacillus*, Corynebacteria, Anaerobes and Mycetes). Dubious biochemical identification of the Pasteurellaceae family was identified by the Gram stain with the use of the Standard Procedures ("England, P.H. UK Standards for Microbiology Investigations (UK SMI): General Information," 2014). All detected and identified microorganisms were placed in microbanks and stored a -80°C freezer for subsequent Antibiotic resistance genes (ARGs) molecular analyses.

Isolates were sub cultured at a selective pressure with the use of tetracycline. Bacterial DNA was extracted with an automated system (QIAsymphony SP Qiagen) and the DSP Virus/Pathogen Mini Kit, according to the manufacturer's instructions. The extracted DNA was subjected to nine simplex PCR assays for the detection of tetracycline resistance genes tet(A), tet(B), tet(C), tet(D), tet(G), tet(K), tet(L), tet(M) and tet(O) (Trzcinski et al., 2000; Aarestrup et al., 2003). Products of amplifications were detected with the use of QIAxcel capillary electrophoresis (Qiagen). PCR were considered positive with amplification size reported in the references.

Statistical analysis

Statistics were analysed with XLSTAT™ software (Addinsoft, 2010). Kruskal-Wallis test was computed to verify differences in colony strength or OTC residues between groups. Dunn's test compared significant values (p-value<0.05) multiple times applying Bonferroni correction. Fisher's exact test was used to evaluate differences in the frequency of antibiotic resistant genes induced by both protocols.

RESULTS

Honey bees consumed all the medicated syrup administered in each colony. Concerning the strength of the colonies, no statistically significant differences were found in the number of frames covered by bees and brood after treatment in the three groups (Fig. 2A, 2B). No queen mortality was observed in the three groups. Before treatment, OTC residues in honey taken from the nest were below the LOD of 1 µg/kg in all groups. The average amount of OTC in honey post treatment per each group is reported in Fig. 3A, 3B, 3C.

Mean OTC residues at the last sampling date were in the "*long protocol*" group 210.3±221.2 (n=6), in the "*short protocol*" group 216.2±238.3 (n=6) and below the LOD in the "*control*" group (n=6).

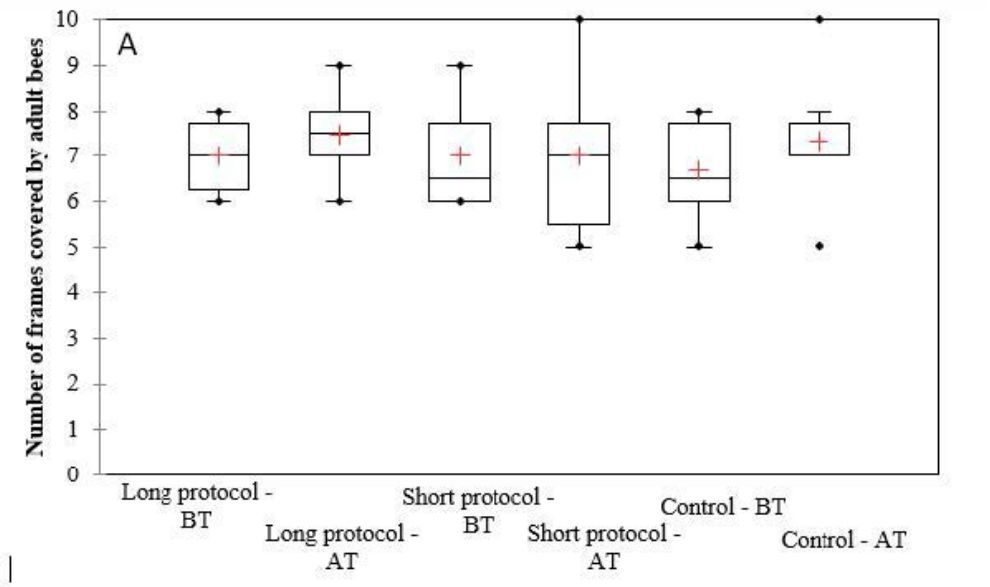
OTC residues significantly differed during trials in the long protocol group (K Obs=14.406; K Critical=12.592, DF=6; p-value=0.025) and in the control group (K Obs=24.651; K Critical=12.592, DF=6; p-value<0.001). Conversely, no significant differences in OTC residues were found in the short protocol group (K Obs=11.929; K Critical=12.592, DF=6; p-value=0.064). Post hoc test showed that in the long protocol group from day 03/09/2020 to 16/03/2021 (after 194 days) OTC residues significantly decreased within each group. In the "control", group OTC residues increased at 03/09/2020 and 18/09/2020 and significantly decreased only after 138 and 194 days (at day 19/01/2021 and 16/03/2021).

The Italian National Reference Laboratory for Antibiotic Resistance (IT-NRL-AR) investigated the presence of OTC-related resistance genes in the bacterial strains isolated from the gut of the adult bees after 5,5 months and 8 months from the end of the OTC treatments. Three ARGs were found in the first sample and fourteen in the second one (Tab. 1). Specifically, one ARG was found in the first sample and nine in the second sample of the "*long protocol*" group. Two ARGs were found in the first sample and four in the second sample of the "*short protocol*" group. A single ARG was found in the "*untreated*" group, and only in the second sampling (Tab. 1). The overall one-tailed probability (including the probability of extreme values) was identical in both sampling frames (Fisher's exact test p=0.24) of the three groups.

DISCUSSION

Our study aimed at verifying how antibiotic treatments for late infections of foulbroods impacted colony strength, OTC residues and presence of antibiotic resistance genes (Milbrath, 2021; Rowland et al., 2021). Even though during our trial honey bee colonies were able to consume all the medicated syrup quickly, OTC administration during summer was risky due to the possibility of robbing and the drifting behaviour that could increase cross-contamination among hives.

Although Peng et al. (1992) reported that an



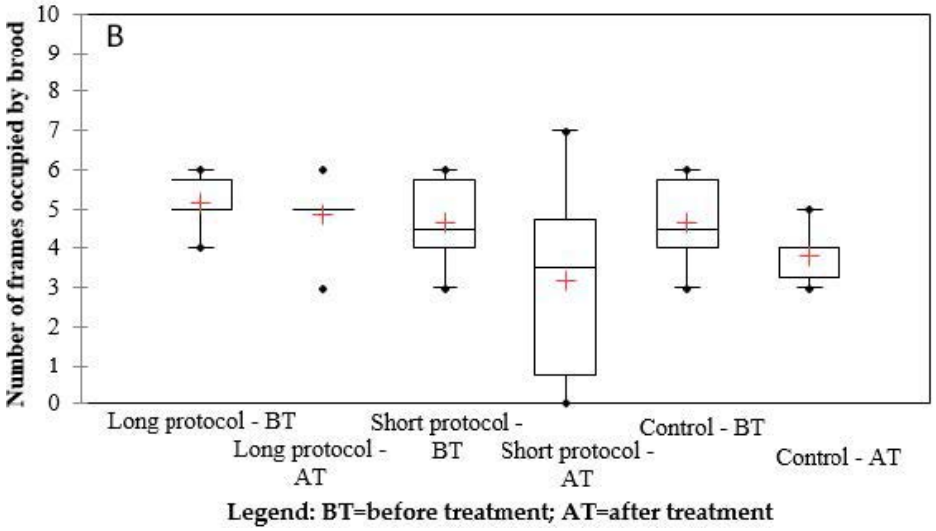


Fig. 2. Box plots of number of frames covered by adult bees (A) or occupied by brood (B) before and after treatment.

in-vitro toxicity of chlortetracycline on bee larvae caused retarded larval growth and development, during our study no toxic effects of oxytetracycline were observed on adult honey bees nor in brood coverage. Alippi et al. (1999) used 1.20 grams of oxytetracycline hydrochloride and observed a similar result. Moreover, no mortality of queens was recorded in the three groups.

OTC MRL for honey is 100 µg/kg (European Commission, 2010). Our study showed that OTC residues in honey collected from the brood-box persisted at concentrations greater than 100 µg/kg for more than seven months after the end of the treatments. *P*-values of multiple comparisons showed that OTC residues in the "long protocol" significantly decreased only after 6.5 months from the end of the treatment, conversely to what had been observed in the "short protocol". Unfortunately, residue levels were always above the accepted MRLs. The "short protocol" OTC administration period was thirty days shorter than the long protocol one. During this time span, "short protocol" colonies were not treated with antibiotics, which reduced the risk of antibiotic resistance

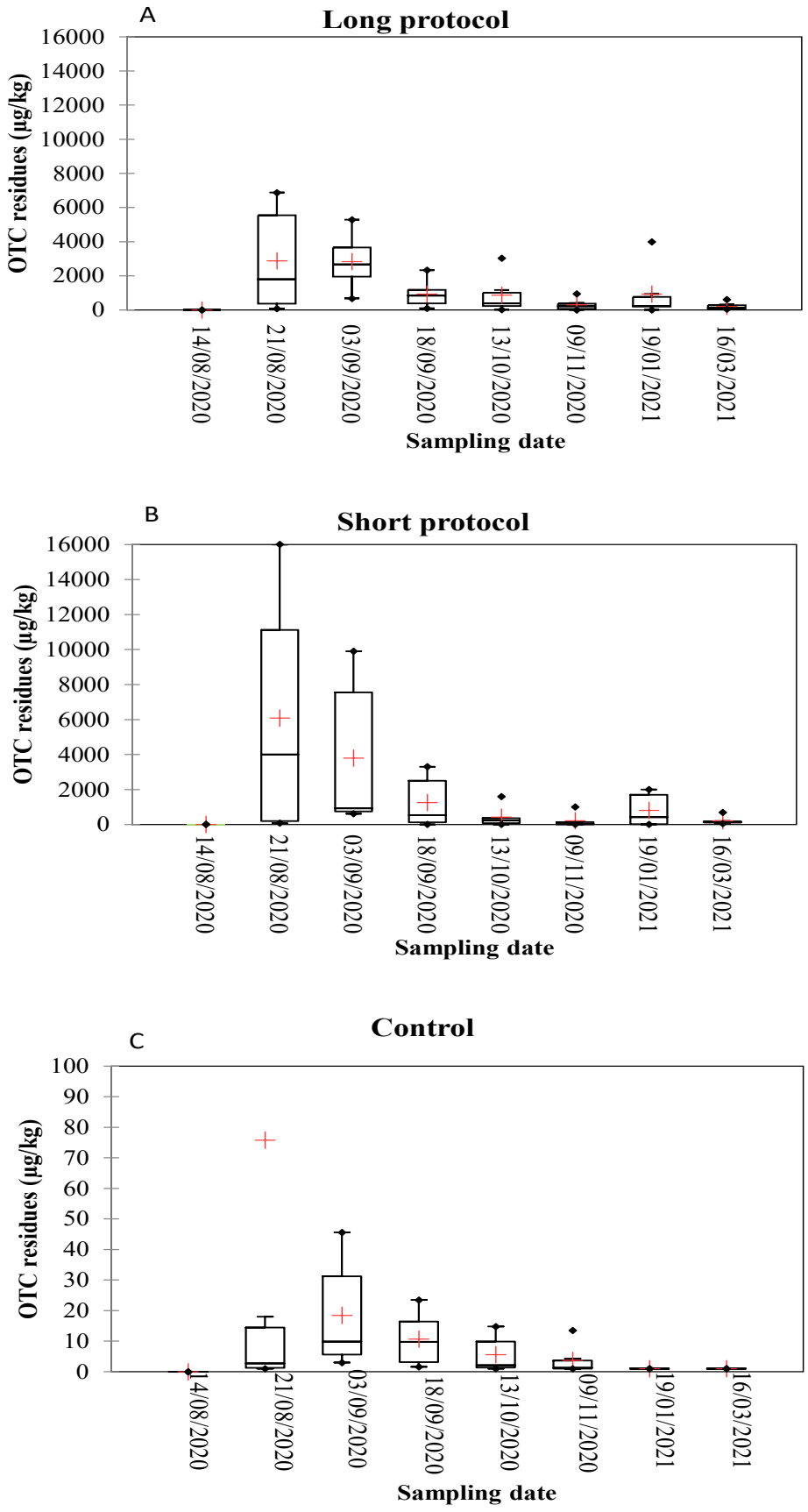


Fig. 3. Box plots of OTC residues (µg/kg) in honey taken from the nest of the group 1 (A), group 2 (B) and group 3 (C).

Table 1.

Bacterial strains and ARGs found in the three groups 5.5 and 8 months after treatments

	1st Sampling 5.5 months after treatment		2nd Sampling 8 months after treatment	
GROUPS	**Strains**	**ARGs**	**Strains**	**ARGs**
"Long protocol"	*Mannheimia haemolytica*		*Bacillus licheniformis*	
	Pantoea spp.		*Serratia plymuthica*	
	Pasteurella pneumotropica		*Klebsiella oxytoca*	
				TET-A
	Pasteurella pneumotropica/ Brucella/Psychrobacter		*Psychrobacter phenylpyruvicus*	TET-M
				TET-O
	Pasteurella pneumotropica/ Mannheimia/Bibersteinia	TET-M	*Pasteurella* spp.	TET-B
	Aeromonas-Vibrio-Pseudomonas luteola		*Moraxella* spp.	TET-M
	Bacillus spp. (*B. thuringiensis*)		*Aeromonas hydrophila*	TET-A
	Acinetobacter		*Mannheimia haemolytica/ Bibersteinia trehalosi*	TET-M
				TET-O
			Citrobacter koseri	TET-A
"Short protocol"	*Proteus* spp.		*Fusobacterium* spp.	
	Bacillus lentus		*Proteus vulgaris*	TET-D
	Bacillus firmus	TET-O	*Aeromonas hydrophila*	TET-A
	Bacillus megaterium	TET-O	*Mannheimia haemolytica/ Bibersteinia trehalosi*	TET-M
				TET-O
Control	*Bordetella* spp.		*Kluyvera* spp.	
	E. coli		*Vibrio vulnificus*	
	Brevibacillus		Aeromonas hydrophila/ *caviae*	TET-A
	Kodamaea ohmeri		*Brevundimonas vesicularis/ Mannheimia haemolytica/ Bibersteinia trehalosi*	
	Paenibacillus spp.		*Brevundimonas vesicularis/ Mannheimia haemolytica/ Bibersteinia trehalosi*	
	Pasteurella spp.			
	Vibrio perahemolyticus			

development. Thompson et al. (2005 and 2006) reported amounts of OTC in comb honey after the administration of 1 gram OTC in 200-250 mL of sucrose liquid equal to 3700 µg/kg.

Similarly to what Ricchiuti et al. (2019) observed, we noted that OTC residues were also found in honey sampled from the brood-box of the untreated group. These residues significantly increased in September, probably due to the robbing of treated hives caused by the lack of food sources in the environment. Moreover, residues significantly decreased at the end of winter/beginning of spring, probably

due to increased internal consumption of stored contaminated honey for feeding the brood and the dilution effect of new honey flow. Sick colonies showed suppressed feeding behaviour, which could negatively interfere with the antibiotic intake, but at the same time could be more subject to robbing, increasing the possibility of transmitting antibiotic residues to stronger colonies of the same or neighbouring apiaries. The replacement of combs with the shook swarm method would possibly reduce the risk of residues in honey.

Several factors may interfere with the honey bee intestinal flora: pathogens, pesticides, air pollution, microplastics, apiary site, seasonal variations, diet, phytochemicals, aging, etc. (Nowak et al., 2021). Contrarily to a report by Manfredini et al. (2019), we observed in the three groups the abundant presence of the Pasteurellaceae family in both samples, after the end of treatments. A dysbiosis related to antibiotic use could be the driver of this microbiological pattern but further studies, including honey bee sampling before treatment, should be performed. Promising opportunities are arising from the use of probiotics for the prevention and control of honey-bee foulbroods (Kuzyšinová et al., 2016; Iorizzo et al., 2022; Pietropaoli et al., 2022) and in vitro trials could be conducted to verify the positive effects of probiotics after an antibiotic treatment of honey bees. Regarding antibiotic resistance, statistical tests did not show any difference in the expression frequency of ARGs in the isolated strains from both sampling frames of the three groups. Although the second sampling detected one ARG in the untreated group, the same ARG was present in the two treated groups.

Further field tests on larger populations are needed to deepen these preliminary findings and evaluate antibiotic resistance by phenotypic antibiogram and targeted molecular investigations. In the use of antibiotics in beekeeping, especially in summer, the possible risks of cross-contamination between treated and untreated hives should be carefully considered. Late summer treatments, more than spring treatments, could risk robbing in autumn and a longer persistence of antibiotic residues inside the treated hives during the inactive season leading to the transmission of ARGs and residues among colonies. Further studies are needed to confirm our preliminary results.

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