Journal of Apicultural Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

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JOURNAL OF APICULTURAL RESEARCH
https://doi.org/10.1080/00218839.2021.1972634

ORIGINAL RESEARCH ARTICLE

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Formic acid combined with oxalic acid to boost the acaricide efficacy against *Varroa destructor* in *Apis mellifera*

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ABSTRACT

Beekeepers need reliable protocols to keep the Varroa infestation to sustainable levels. The goal of our study was to verify if a treatment consisting in the association of two organic acids, commonly used to control Varroa, could be a good strategy to boost their acaricide efficacy without affecting honey bee colonies' survival. We carried out a field trial in Central Italy with 56 colonies divided into seven homogeneous groups of 8 hives each. Honey bee colonies were treated with three different formic acid formulations: liquid formic acid (60%) administered with the Nassenheider Professional® dispenser, MAQS®, and Varterminator®. All of them were combined or not with two oxalic acid treatments (Api-Bioxal®). The mean acaricide efficacy of liquid formic acid (Nassenheider Professional®) alone was 73.2 ± 12.5%, while MAQS® and Varterminator® showed respectively a mean efficacy of 49.3 ± 14.9% and 81.2 ± 16.0%. The combination of the above mentioned treatments with oxalic acid increased the efficacy respectively to 92.7 ± 7.5% (Nassenheider Professional®); 89.5 ± 9.2% (MAQS®) and 89.4 ± 8.0% (Varterminator®). Unfortunately, the higher efficacy reflected an abnormal adult honey bees mortality with respect to the control group in Nassenheider Professional® and MAQS® groups. The combination of organic acids can boost the efficacy of treatments and reduce their variability but colonies strength should be carefully evaluated in order to avoid excessive losses.

ARTICLE HISTORY
Received 19 August 2020
Accepted 17 May 2021

KEYWORDS
Apis mellifera; varroatosis; formic acid; oxalic acid; Integrated Pest Management

Introduction

In recent years, different studies tried to identify efficient *Varroa destructor* (Anderson & Trueman, 2000) management strategies using "soft" acaricides (Gregorc & Sampson, 2019; Rosenkranz et al., 2010) in order to avoid honey residues and to prevent mite resistance (Imdorf et al., 1999; Rosenkranz et al., 2010). In many countries, it is common to use organic acids such as formic, oxalic, lactic, and citric acids to control the infestation level of the mite (Formato & Smulders, 2011; Gregorc & Poklukar, 2003; Gregorc & Sampson, 2019; Melathopoulos & Gates, 2003).

Formic acid is the only active principle that is effective on mites inside the brood cells (Fries, 1990; Rosenkranz et al., 2010). It can be administered to colonies in different concentrations or doses and several dispensers (e.g., NassenheiderProfessional®, Liebig, etc.) have been developed (Eguaras et al., 2003; Fries et al., 1991; Ramos et al., 2010). Mite Away Quick Strips® and Varterminator® are two commercial products that incorporate formic acid into a gel matrix to regulate the evaporation and reduce the handling risks for beekeepers.

Oxalic acid efficacy for varroatosis control has been described for a long time (Büchler et al., 2020; Nanetti et al., 2003; Takeuchi & Harada, 1983). Unfortunately, it has no miticide effect on Varroa during its reproductive phase that takes place inside honey bee brood cells. Trickling sugar solutions of oxalic acid can be administered to the hives in order to distribute the active principle in the colony via trophallaxis and/or direct contact among other bees or in-hive material (Rademacher et al., 2017). In Italy, the registration of a specific veterinary medicine was implemented in 2011 (Api-Bioxal®).

Varroa dynamics into the colonies depend on several factors. Among these, brood rearing and colony growth play a relevant role. Both of them are linked with the queen's egg-laying activity that is often related to foraging sources (Messan et al., 2020). Moreover, the spread of mites can occur by bees swarming, or movements (e.g., migratory beekeeping) operated by beekeepers or during robbing (Peck et al., 2016). In some conditions, population growth rates are so high that it is necessary to perform fast and effective treatments to avoid colony losses.

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/00218839.2021.1972634
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Considering the mode of action of the above mentioned two organic acids, it appears the opportunity to test a combination of these active substances to have, in a short period of time, a boosted acaricide efficacy able to produce a fast decrease of Varroa population. Even though data are available on the efficacy of gel formulations of formic acid (Eguaras et al., 2003; Ramos et al., 2010; Satta et al., 2005) or oxalic acid solutions (Büchler et al., 2020; Nanetti et al., 2003), no data are available on mite fall dynamics and toxicity of combined treatment with the two of them.

In this paper we report the results of a trial conducted in Central Italy to verify the varroacide efficacy and honey bees toxicity of liquid formic acid (60%) administered with the NassenheiderProfessional® dispenser, MAQS® and Varterminator®, combined or not with two trickled administrations of oxalic acid (Api-Bioxal®).

Materials and methods

The field trial was conducted following the European Medicine AgencyGuidelines (EMA, 2011) from July to October 2015 in one apiary located in central Italy (42°03′41.6″N 12°08′24.0″E − 236 meters above sea level – phytoclimatic area: transition Mediterranean region). Seven experimental groups of 8 hives each, were devised:

1. "Nassenheider Professional®" group: each hive was treated with 290 mL of 60% formic acid administered byNassenheider Professional® for 10 days;
2. "Nassenheider Professional® + Api-Bioxal®" group: each hive was treated with 290 mL of 60% formic acid administered byNassenheider Professional® for 10 days and received two treatments by trickling with Api-Bioxal®: one at the insertion (day 0) and one at the removal of the formic acid dispenser (day 10);
3. "MAQS®" group: each hive was treated with two strips of MAQS® for 7 days;
4. "MAQS®+Api-Bioxal®" group: each hive was treated with two strips of MAQS® for 7 days and received two treatments by trickling with Api-Bioxal®: one at the insertion (day 0) and one at the removal of MAQS® strips (day 7);
5. "Varterminator®" group: each hive was treated for 10 days with two tablets, for a total of two consecutive treatments;
6. "Varterminator®+Api-Bioxal®" group: each hive was treated for 10 days with two tablets, for a total of two consecutive treatments and received two treatments by trickling with Api-Bioxal®: one at the insertion (day 0) and one at the removal of the first pair of tablets (day 10);
7. "Control" group: each hive didn't receive any treatment.

A detailed graph of the protocol is visible in Figure S1 (online supplementary material). All the formic acid administrations started on 4 August 2015.

The liquid formic acid administration consisted of 290 mL formic acid solution at 60% administered byNassenheider Professional® (Joachim Weiland Werkzeugbau GmbH & Co. KG Zimmermannsgasse 215366 Hoppegarten, Germany), a formic acid dispenser to be placed on the top of the frames of the brood chamber, with temperature ranges from +8 °C to +35 °C. We tested this evaporator provided with 290 mL of formic acid 60%, with the larger U-wick size, for a ten-day treatment, according to label indications for Dadant-Blatt beehives.

MAQS® (Mite Away Quick Strips) (NOD Europe Ltd., Europe Branch Office, Grosse Hohl, 67354 Roemerberg, Germany) is a commercial product in strips containing formic acid absorbed in a gel matrix. It is registered in Italy since 2013. Each strip contains 68.2 grams of formic acid. For its administration, the current label indicates to place two strips for 7 days on the top of the frames in the brood chamber or, during the nectar flow, under the queen excluder. As indicated in the leaflet of the product, outside daytime temperature increases in between 10 °C and 29.4 °C on day application, and hot temperatures (33.3 °C) during the first three days may cause excessive brood mortality and queen loss.

Varterminator® (IZO S.r.l., Via San Zeno 99/A, 25124 Brescia, Italy) consists of two gel tablets of 250 grams each containing 90 grams of formic acid to be applied for 10 days on the top of the frames in the brood chamber and replaced with two other tablets to leave in place for 10 days more. The mean daytime temperature ranges to obtain the best performances with Varterminator® go from 15 °C to 35 °C.

Api-Bioxal® (Chemicals Laif, spa, Vigonza, Padova, Italy) is a veterinary medicine for varroatosis control on sale in Italy since 2011 and in the United Kingdom since 2015. It consists of oxalic acid in powder (88.6 g of dehydrate oxalic acid in 100 g), registered both for trickling or sublimating use. The oxalic acid solution adopted consisted of 35grams of Api-Bioxal® melt in 500 mL of syrup (water and sucrose in a 1:1 ratio) and was applied at a rate of 5 mL of syrup for each area between combs occupied by bees.

All treatments were administered according to the indications given by producers.

In order to divide the experimental groups evenly according to the strengths of the hives and the

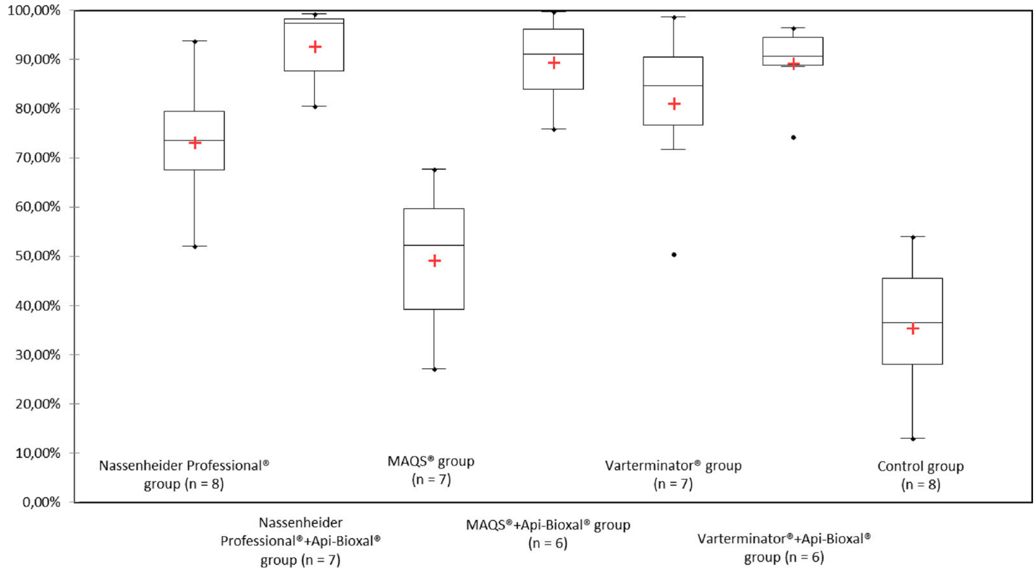

Figure 1. Box-plot of acaricide efficacy registered in the tested groups.

Varroa infestation level, before starting the trial, honey bee populations were estimated according to Delaplane et al. (2013) noting the number of bees and the amount of brood cells (open and closed brood).

The Varroa infestation level was evaluated by counting the natural mite fall replacing sticky sheets every 2–3 days in the bottom boards for 15 days before the application of the treatment. During the treatments, the mites' death was counted every 2–3 days in order to build up mite fall curves. The mites mortality by the treatments inside the brood cells was calculated by counting the mite fall for 12 days after the end of each formic acid treatment.

The residual number of mites was evaluated with a follow-up treatment carried out with:

- two trickled oxalic acid solutions (Apibioxal®, Chemicals Laif, spa, Vigonza, Padova, Italy) administrations, one at the beginning and one at the end of a queen caging period of 21days;
- a double dose (4 strips/hive) of Apistan® (tau-fluvalinate; Vita Europe Ltd, Basingstoke, Hants, United Kingdom), administered for all the queen caging period, until 7 days after the queen release.

To calculate the acaricide efficacy (AE) obtained in each hive, the following formula was used:

$$AE = \left(V_F/V_{(F+FOLLOWUP)}\right) * 100 \text{ (Dietemann et al., 2013)}$$

where V_F is the total number of mites died by each formic acid treatment, considering also the combination of oxalic acid in the groups where applied, and $V_{(F+FOLLOWUP)}$ represents the sum of the mites in total died in the hive (including the follow-up treatments).

To evaluate the effect of treatments on colonies, the number of adult bees and brood were estimated accordingly to Delaplane et al. (2013) before and at the end of treatments application.

Temperature and humidity have been monitored during the formic acid treatment period placing a data logger (iButtonHygrochron Temperature/ Humidity Logger DS1923 – Maxim Integrated, San Jose, CA 95134 USA) into an empty hive near the experimental groups.

Statistical analysis was performed to verify differences in acaricide efficacy and reduction of bees and brood coverage. Mann-Whitney Test (Mann & Whitney, 1947) was used to carry out single comparisons between acaricide efficacies obtained by all groups. Kruskal-Wallis Test (Kruskal & Wallis, 1952) was applied to verify the statistical differences between groups in the amount of Varroa mite fall before treatment and in reduction of adult honey bees and brood coverage after treatments. For the last case, multiple pairwise comparison with Dunn's Test (Dunn, 1961) was applied.

Results

Natural mite fall during 15 days before the application of the treatments showed no statistical differences between groups ($K = 6.293$; $p = 0.391$).

Acaricide efficacy of the Nassenheider Professional® treatment was 73.2 ± 12.5%, while MAQS® and Varterminator® showed respectively a mean efficacy of 49.3 ± 14.9% and 81.2 ± 16.0%. The combination with two oxalic acid treatments increased the efficacy of Nassenheider Professional®, MAQS® and Varterminator®, respectively to 92.7 ± 7.5% (Nassenheider Professional®); 89.5 ± 9.2% (MAQS®) and 89.4 ± 8.0% (Varterminator®) (Table 1; Figure 1). Efficacies obtained in the Nassenheider Professional®and Varterminator®groups were

Table 1. Acaricide efficacy. Descriptive statistics.

Variable	N	Minimum	Maximum	Mean	Std. deviation
Nassenheider Professional® group (n = 8)	8	52,0%	93,8%	73,2%	12,5%
Nassenheider Professional®+Api-Bioxal® group (n = 7)	7	80,5%	99,3%	92,7%	7,5%
MAQS® group (n = 7)	7	27,1%	67,7%	49,3%	14,9%
MAQS®+Api-Bioxal® group (n = 6)	6	75,9%	99,7%	89,5%	9,2%
Varterminator® group (n = 7)	7	50,5%	98,7%	81,2%	16,0%
Varterminator®+Api-Bioxal® group (n = 6)	6	74,3%	96,4%	89,4%	8,0%
Control group (n = 8)	8	12,9%	54,0%	35,5%	14,3%

Table 2. Acaricide efficacy. P-values of Mann-Whitney comparisons.

	Nassenheider Professional® group (n = 8)	Nassenheider Professional®+ Api-Bioxal® group (n = 7)	MAQS® group (n = 7)	MAQS®+ Api-Bioxal® group (n = 6)	Varterminator® group (n = 7)	Varterminator®+ Api-Bioxal® group (n = 6)	Control group (n = 8)
Nassenheider Professional® group (n = 8)	–	**0,005**	**0,013**	**0,024**	0,183	**0,033**	**0,001**
Nassenheider Professional®+Api-Bioxal® group (n = 7)	**0,005**	–	**0,001**	0,520	0,125	0,284	**0,000**
MAQS® group (n = 7)	**0,013**	**0,001**	–	**0,001**	**0,011**	**0,001**	0,118
MAQS®+Api-Bioxal® group (n = 6)	**0,024**	0,520	**0,001**	–	0,353	0,936	**0,001**
Varterminator® group (n = 7)	0,183	0,125	**0,011**	0,353	–	0,353	**0,002**
Varterminator®+Api-Bioxal® group (n = 6)	**0,033**	0,284	**0,001**	0,936	0,353	–	**0,001**
Control group (n = 8)	**0,001**	**0,000**	0,118	**0,001**	**0,002**	**0,001**	–

statistically similar ($p = 0.183$). MAQS®efficacy was the lower of treated groups and not so different from the control group ($p = 0.118$). The application of two additional treatments with Apibioxal® permitted to obtain similar efficacies in all boosted groups (Table 2). Mitefall dynamics were plotted (Figure 2) to understand the different behaviour of treatments.

The strengths of the colonies and the Varroa infestation level before starting the trial were similar: Varroa pre-treatment ($K = 6.293$; $p = 0.391$); adult bees population ($K = 5.208$; $p = 0.517$); brood coverage ($K = 3.007$; $p = 0.808$).The number of adult honey bees after the treatments resulted in statistically significant lower respect for the control group in the "Nassenheider Professional® + Api-Bioxal®" (−47.5%) and "MAQS®+Api-Bioxal®" (−55.6%) groups (Tables 3, 4). The brood coverage received a significant reduction in"Varterminator®+Api-Bioxal®" group (−83.9%) respect the Nassenheider Professional® group (Tables 5, 6).

Environmental temperatures ranged from a minimum of 16 °C to a maximum of 44 °C during the whole trial (Figure 3). Considering the days of administration of formic acid products tested, it was possible to observe that the minimum daily temperature recorded in the apiary never exceeded the minimum temperature ranges indicated by the producers. The mean daytime temperatures indicated for the treatments with Nassenheider Professional® and Varterminator® were respected too. A part (5 of 10 days) of Nassenheider Professional® and all (7 days) MAQS® treatment period was carried out when environmental temperatures were higher than those suggested by the manufacturers for the proper application of the products. Relative humidity increased after 6 days from the beginning of the treatments due to weather conditions.

Discussion

Varroa control using synthetic acaricides like fluvalinate, coumaphos, flumethrinor amitraz should be considered for sporadic treatments due to the increase of resistance phenomena (Eizen et al., 2001, Floris et al., 2001; Elzen et al., 2000; Elzen & Westervelt, 2002; Milani, 1999).

Most promising alternatives seem to be represented by organic acids like oxalic acid and formic acid, still used by beekeepers for decades that so far did not evidence resistance in Varroa. Those mentioned acids have intrinsic usage limitations like ineffectiveness of oxalic acid on reproductive mites (Charriere & Imdorf, 2002; Gregorc & Planinc, 2001, 2002) and side-effects like excessive toxicity or queens' mortality due to quick increase in evaporation rates of formic acid (Bolli et al., 1993; Elzen et al., 2004; Pietropaoli & Formato, 2019).

Acaricide efficacy of the tested formic acid commercial products/dispensers (Nassenheider Professional®, MAQS® and Varterminator®) varied between treatments (from 49.3% to 81.2%) and within each group (standard deviations from a minimum of 12,5% to a maximum of 16,0%). Those values could be not always able to guarantee the over-wintering survival of the colonies due to an excessive Varroa (and virus) load. Formic acid at the concentration of 60% reported a lack of efficacy on colony overwintering survival according to Beyer et al. (2018).

The combination with two oxalic acid treatments increased the final efficacy of all formic acid

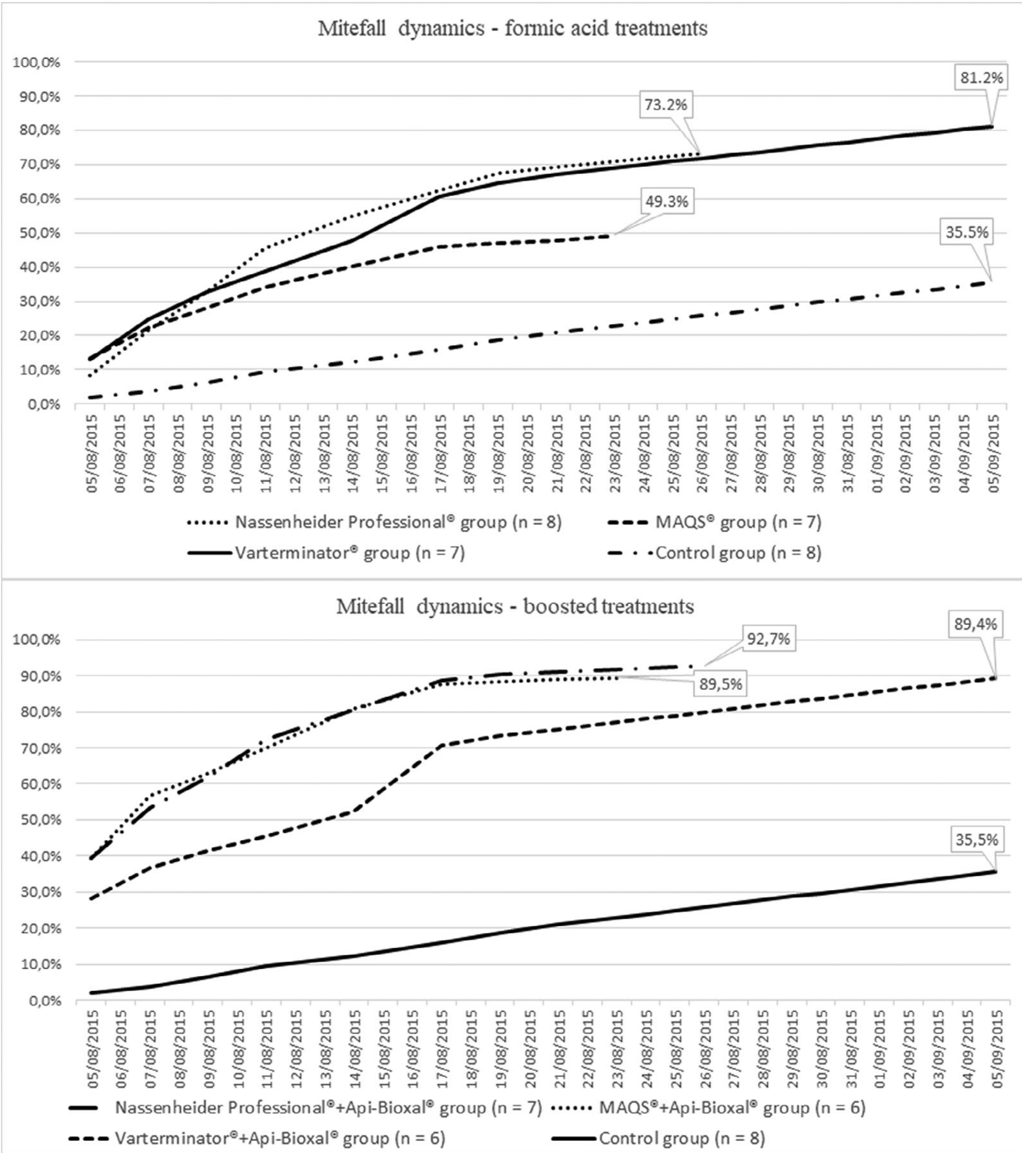

Figure 2. Mitefall dynamics.

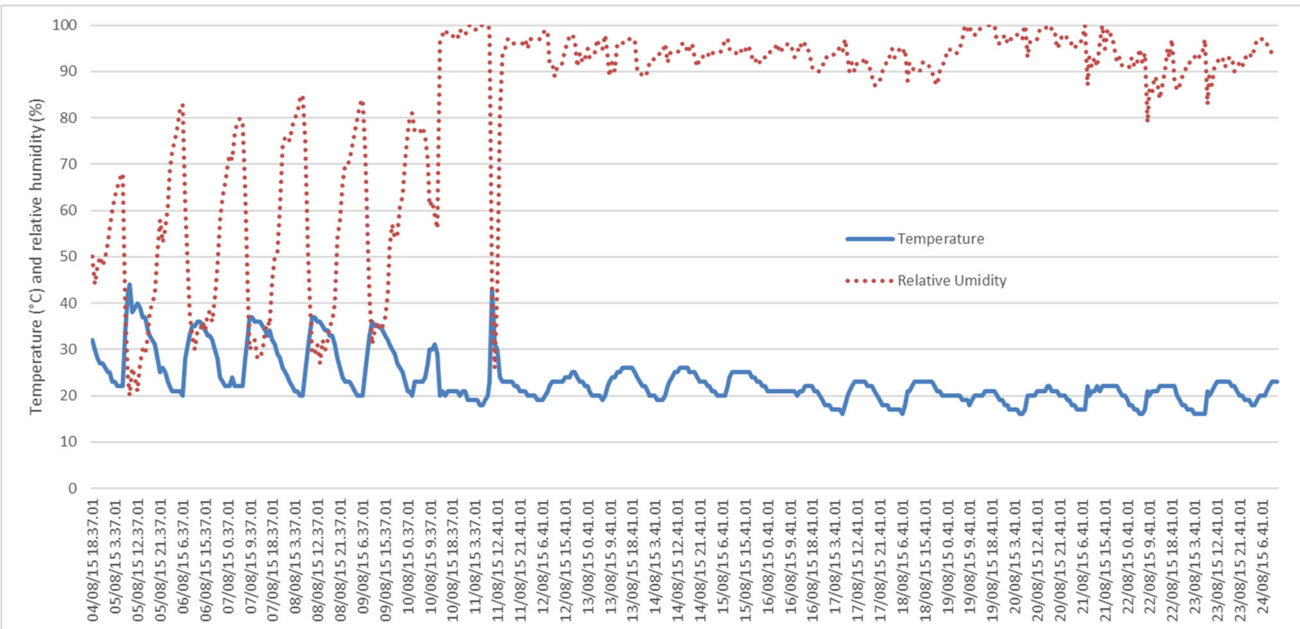

Figure 3. Environmental temperature and humidity recorded during the trial.

Table 3. Adult honey bee variations after treatment. Descriptive statistics.

Statistic	Nassenheider Professional® group (n = 8)	Nassenheider Professional®+ Api-Bioxal® group (n = 7)	MAQS® group (n = 7)	MAQS®+ Api-Bioxal® group (n = 6)	Varterminator® group (n = 7)	Varterminator®+ Api-Bioxal® group (n = 6)	Control group (n = 8)
Minimum	31,5%	21,0%	51,5%	20,8%	45,4%	58,0%	61,0%
Maximum	100,6%	86,7%	89,8%	62,6%	116,7%	108,9%	116,0%
1st Quartile	58,5%	32,7%	60,4%	27,9%	67,9%	67,6%	82,2%
Median	77,2%	43,4%	68,4%	32,8%	74,1%	84,3%	93,2%
3rd Quartile	92,9%	46,2%	73,8%	38,8%	81,0%	89,4%	102,4%
Mean	73,3%	44,1%	68,3%	36,0%	76,3%	81,5%	91,6%
Variance (n-1)	6,0%	4,6%	1,8%	2,2%	4,7%	3,5%	3,4%
Standard deviation (n-1)	24,5%	21,4%	13,3%	14,7%	21,6%	18,7%	18,4%

Table 4. Dunn's multiple comparisons on adult honey bees amounts. P-values.

	Nassenheider Professional® group (n = 8)	Nassenheider Professional®+ Api-Bioxal® group (n = 7)	MAQS® group (n = 7)	MAQS®+ Api-Bioxal® group (n = 6)	Varterminator® group (n = 7)	Varterminator®+ Api-Bioxal® group (n = 6)	Control group (n = 8)
Nassenheider Professional® group (n = 8)	1	0,027	0,621	0,007	0,948	0,608	0,202
Nassenheider Professional®+Api-Bioxal® group (n = 7)	0,027	1	0,096	0,571	0,027	0,011	**0,001**
MAQS® group (n = 7)	0,621	0,096	1	0,030	0,588	0,338	0,084
MAQS®+Api-Bioxal® group (n = 6)	0,007	0,571	0,030	1	0,007	0,003	**0,000**
Varterminator® group (n = 7)	0,948	0,027	0,588	0,007	1	0,662	0,243
Varterminator®+Api-Bioxal® group (n = 6)	0,608	0,011	0,338	0,003	0,662	1	0,503
Control group (n = 8)	0,202	**0,001**	0,084	**0,000**	0,243	0,503	1

Bonferroni corrected significance level: 0,0024.

Table 5. Descriptive statistics of brood coverage variations after treatment.

Statistic	Nassenheider Professional® group (n = 8)	Nassenheider Professional®+ Api-Bioxal® group (n = 7)	MAQS® group (n = 7)	MAQS®+Api-Bioxal® group (n = 6)	Varterminator® group (n = 7)	Varterminator®+ Api-Bioxal® group (n = 6)	Control group (n = 8)
Minimum	35,0%	8,2%	70,7%	67,0%	28,9%	51,6%	39,0%
Maximum	287,2%	121,7%	202,9%	446,7%	112,9%	73,6%	374,4%
1st Quartile	101,6%	70,1%	90,4%	72,0%	63,9%	56,7%	79,2%
Median	130,7%	84,7%	100,0%	82,2%	93,5%	67,9%	99,4%
3rd Quartile	177,0%	92,2%	159,1%	162,8%	108,6%	72,8%	153,5%
Mean	148,6%	77,0%	124,6%	156,1%	82,9%	64,7%	135,2%
Variance (n-1)	69,2%	12,6%	27,8%	223,4%	10,6%	1,0%	109,2%
Standard deviation (n-1)	83,2%	35,5%	52,7%	149,5%	32,5%	9,8%	104,5%

treatment up to 89.4% of Varterminator®, 89.5% of MAQS® and 92.7% of Nassenheider Professional®, reducing always the variability within all groups (standard deviation from a minimum of 7,5% to a maximum of 9,2%). Adding two oxalic acid treatments also permitted to strongly increase the efficacy of MAQS®, making the final acaricide efficacy similar to the other boosted groups.

Mitefall curves showed that Varterminator® final efficacy is due to the longer administration period and the replacement of the tablets. In the case of combination with oxalic acid, the slope of the mite fall curve increases even more. On the contrary, the curves of Nassenheider Professional® and MAQS® both combined with Apibioxal® are very similar and they reach a higher efficacy in fewer days respect the Varterminator® group.

Integrated Pest Management (IPM), as defined by Food and Agriculture Organization (Vetek et al., 2017), means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. Referred to our context, the IPM strategy considers the contemporary treatment of two different active substances to enhance the final miticide efficacy, guaranteeing, at the same time, quality of hive products by using low environmental impact medicines. This innovative method could be effectively considered by beekeepers, among the other possible protocols for Varroa control. In conclusion, beekeepers should adopt the best treatment considering: the beehive infestation level, the time of the year, and the strength of the colony.

Concerning toxicity treatments, it should be noticed that all formic acid products tested reported a reduction of the number of adult honey bees from

Table 6. Dunn's multiple comparisons on brood coverage. P-values.

	Nassenheider Professional® group (n = 8)	Nassenheider Professional®+ Api-Bioxal® group (n = 7)	MAQS® group (n = 7)	MAQS®+ Api-Bioxal® group (n = 6)	Varterminator® group (n = 7)	Varterminator®+ Api-Bioxal® group (n = 6)	Control group (n = 8)
Nassenheider Professional® group (n = 8)	1	0,047	0,639	0,257	0,094	**0,002**	0,426
Nassenheider Professional®+Api-Bioxal® group (n = 7)	0,047	1	0,142	0,456	0,765	0,263	0,224
MAQS® group (n = 7)	0,639	0,142	1	0,506	0,242	0,011	0,764
MAQS®+Api-Bioxal® group (n = 6)	0,257	0,456	0,506	1	0,647	0,072	0,691
Varterminator® group (n = 7)	0,094	0,765	0,242	0,647	1	0,159	0,365
Varterminator®+Api-Bioxal® group (n = 6)	**0,002**	0,263	0,011	0,072	0,159	1	0,020
Control group (n = 8)	0,426	0,224	0,764	0,691	0,365	0,020	1

Bonferroni corrected significance level: 0,0024.

−26.7% of Nassenheider Professional® to −31.7% of MAQS®, always higher respect the natural reduction of honey bees of the control group (−8.4%) related to the seasonal climatic conditions. Even though such reductions were not statistically significant due to the high standard deviations reported in all treated groups, they agree with previous studies (Giusti et al., 2017; Moro & Mutinelli, 2019; Pietropaoli & Formato 2018, 2019). Oxalic acid is usually relatively safe for *A. mellifera* at the colony level (Rademacher et al., 2017) even though mortality of a small number of adult bees is always present due to the attractiveness of sucrose solutions (Rademacher et al., 2017). In our field trial, the number of adult honey bees was significantly reduced after the combination of two Apibioxal® treatments with both the NassenheiderProfessional® and "MAQS® while the brood coverage was reduced in the "Varterminator®+Api-Bioxal®" group. Possible alternatives to reduce the toxic effects of a combined treatment could be the substitution of sugar with glycerol in the oxalic acid solution (Rademacher et al., 2017; Rademacher & Harz, 2009). Moreover, further studies could evaluate the positive effects on bees survival, reducing the number of trickling administrations (one instead of two).

From the practical point of view, it should be underlined that the combination of formic and oxalic acid requires a small amount of time (especially for the disposable products like MAQS® and Varterminator®) and permits to avoid cage the queen, that is an alternative way to boost the efficacy of oxalic acid and other compounds (Cengiz, 2018; Giacomelli et al., 2016; Wagnitz, 2009; Wagnitz & Ellis, 2010).

The reduction of the number of treatments for Varroa control during the year is a milestone of an Integrated Pest Management strategy that focuses also on rotating all active principles to avoid resistance phenomena. In order to achieve this goal, in conjunction with a selection of most resistant/adapted colonies, it is fundamental to provide beekeepers well-timed effective summer and winter treatments to manage Varroa population (Beyer et al., 2018).

Further studies are necessary to verify the overwintering of colonies, in different climatic conditions, after the above mentioned or similar boosted treatments.

Acknowledgements

The authors would like to thank the Latium Region for funding this study, Apimondia, in the person of its Secretary-General Riccardo Jannoni-Sebastianini, for providing precious comments and suggestions, the technician Alessandra Giacomelli and the beekeeper Stefano Grazioli for their zeal and willingness in participating to the field trials.

Disclosure statement

The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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