Efficacy and toxicity of VarroMed® used for controlling *Varroa destructor* infestation in different seasons and geographical areas

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Abstract

VarroMed® is a soft acaricide registered for honey bees on the European Union market since 2017 for Varroa control. Researchers involved were partners of different countries of the Varroa control task force of COLOSS Association. Our goal was to evaluate performances (acaricide efficacy and toxic effects on honey bees) of VarroMed® in different climatic conditions. Our results in the tested apiaries showed an efficacy ranging from 71.2 to 89.3 % in summer/autumn, and from 71.8 % to 95.6 % in winter. No toxic effects on bees were observed, except in one apiary, where severe cold climatic conditions played a crucial role. The treatment could be efficiently applied in brood right as well as in broodless colonies. Integrated pest management (IPM) recommendations for beekeepers are provided in order to apply the best Varroa control protocol.

Keywords: VarroMed®; *Varroa destructor*; winter treatment; summer-autumn treatment; queen caging; brood interruption

1. Introduction

The mite *Varroa destructor* [1] is one of the main threats to the European honeybee *Apis mellifera* and thus for the beekeeping sector [2]. The number of suitable veterinary medicine products (VMPs) for the treatment is limited [3] due to the need of low honey bee toxicity [4], the risk of residues in honey bee products [5,6] and the *V. destructor* mite capability to develop resistance to the frequently used active substances [7]. In this context, the availability of new VMPs in the market, as well as upgraded treatment measures or tools (e.i. brood interruption techniques) can be valuable help for beekeepers.

From previous studies it is known that soft acaricides [7] have one or more components as an active ingredient: organic acids like oxalic [8-12], formic [13-15], lactic acid [16], other acids [17], thymol [18-21] and other essential oils [22,23]. Recently, different formulations of natural ingredients were evaluated, like oxalic acid, thymol and oregano oil [24] and some compared or combined with brood interruption technique [25,26]. Since 2017, VarroMed®, an organic acid-based VMP against Varroa, was registered for its use on the honey bees in EU.

In the context of the Varroa control task force of the COLOSS association [27], we decided to test the performances of VarroMed® concerning efficacy against Varroa mite and toxicity for honey bees in different European climatic conditions in agreement with EMA guidelines [27], following the instructions given by the producer.

The aim of our study was to define the best way to apply VarroMed® in order to optimize its use in different conditions, to give detailed and reliable information to European beekeepers in the framework of a Varroa management strategy.

2. Materials and Methods

The active ingredients of VarroMed® (BeeVital GmbH, Handelstrasse 65162 Pbertrum am See, Austria) are formic acid (5 mg/ml) and oxalic acid dihydrate (44 mg/ml). Other ingredients in the product include caramel colour (E150d), sucrose syrup, propolis tincture (20 %), citric acid monohydrate, lemon (*Citrus lemon*) essential oil, star anise (*Illicum verum*) essential oil and pure water. There is no specific range of environmental temperature or humidity for the use of VarroMed® indicated by the producer.

We evaluated the performances of VarroMed® in Italy, Slovenia, Croatia and Belgium in 2018. The protocol was structured in accordance with the European Medicines Agency (EMEA) guidelines on VMPs for controlling *V. destructor* parasitosis in bees [28]. It was designed by the group of participants of the Varroa Control Task Force (COLOSS association). A summary scheme of the activities and their timing is presented in Figures 1-6.

Honey bee colonies were set up in 10-frame Dadant-Blatt (DB), Langstroth Root (LR) or AŽ (Alberti-Žnideršič) hives, and placed in the same apiary each. We established summer/autumn and winter protocols.

The trials were carried out in absence of honey-flow and honey supers. Two experimental groups homogenous in strength and Varroa infestation levels were organized for each trial. In both protocols, the colonies were treated with VarroMed® (treated group), while other group was left untreated (control). The main differences between the two protocols were the absence of brood during the winter protocol versus its presence during the summer/autumn protocol, and the duration of the treatment due to the number of VarroMed® applications. According to label instructions, the treatments in autumn should be performed according to decreasing colony population, 3 to 5 times, 6 days apart. In winter it should be applied once only, at the start of the broodless period and in hives with Varroa infestation.

The summer/autumn protocol was evaluated in Italy, Slovenia, Croatia and Belgium, once per country, and the winter protocol was carried out by three institutions in Italy and one in Croatia. The protocol followed by the participants and the number of honey bee colonies used for each trial are summarized in Table 1.

Table 1. Number of hives used in each trial and location.

Location	Protocol	Apiary code	Number of treated colonies/control colonies	Treatment period

Zutendaal, Belgium	Summer/autumn (x5)[1]	BELGIUM	10/10	23.9.-3.11.2018
Rome, Italy	Summer/autumn (x5)	ITALY_IZSLT	10/10	6.8.-20.9.2018
Brdo pri Kranju, Slovenia	Summer/autumn (x5)	SLOVENIA	8/7[3]	13.9.-15.10.2018
Karlovac, Croatia	Summer/autumn (x3)[2]	CROATIA	10/10	20.7.-2.8.2018
Levico Terme, Italy	Winter	ITALY_FEM	10/10	22.11.-6.12.2018
Rome, Italy	Winter	ITALY_IZSLT	10/10	
Bassano del Grappa, Italy	Winter	ITALY_IZSVE	10/10	17.11.-1.12.2018
Karlovac, Croatia	Winter	CROATIA	10/10	29.1.-20.2.2018

[1] Five applications of VarroMed®.

[2] Three applications of VarroMed®.

[3] Number of colonies and hives was limited due to apiary size.

In order to evaluate the variation of the colony strength due to the VarroMed® treatment, the number of adult bees was estimated [29] immediately before the beginning and after the end of the treatment period.

According to manufacturers' indications, VarroMed® was warmed at 25-35° C, shook before its use and administered by trickling 5 mL per bee space occupied by honey bees. On the day of the VarroMed® treatment (day 0 of the protocol – see Figures 1-6) queen vitality was checked, and colony strength was assessed in both groups. In order to calculate the acaricidal efficacy and plot the mitefall dynamics, mites fallen during the treatment were counted by inserting sticky sheets on the bottom board. The sheets were replaced every 2 to 3 days until 7 days after the beginning of the VarroMed® treatment according to indications given in the label for the summer/autumn and winter treatments (Figures 1-6).
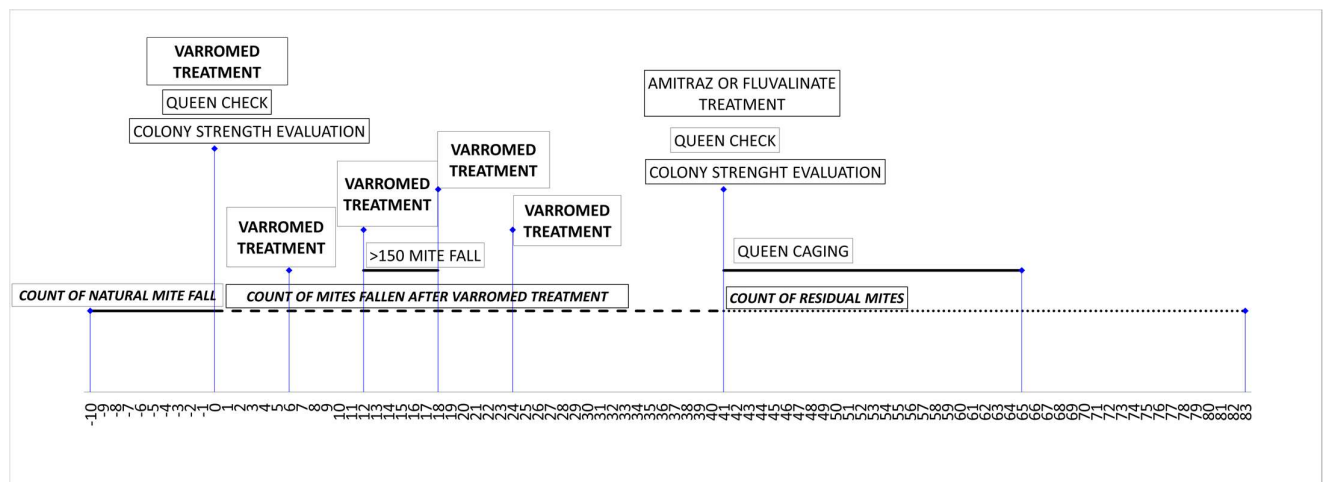
Figure 1. Summer/autumn protocol for the treated group (5 repeated treatments). The following two treatments are additionally applied, if more than 150 mites are detected on the sticky sheets on the bottom boards within 6 days after the third treatment.
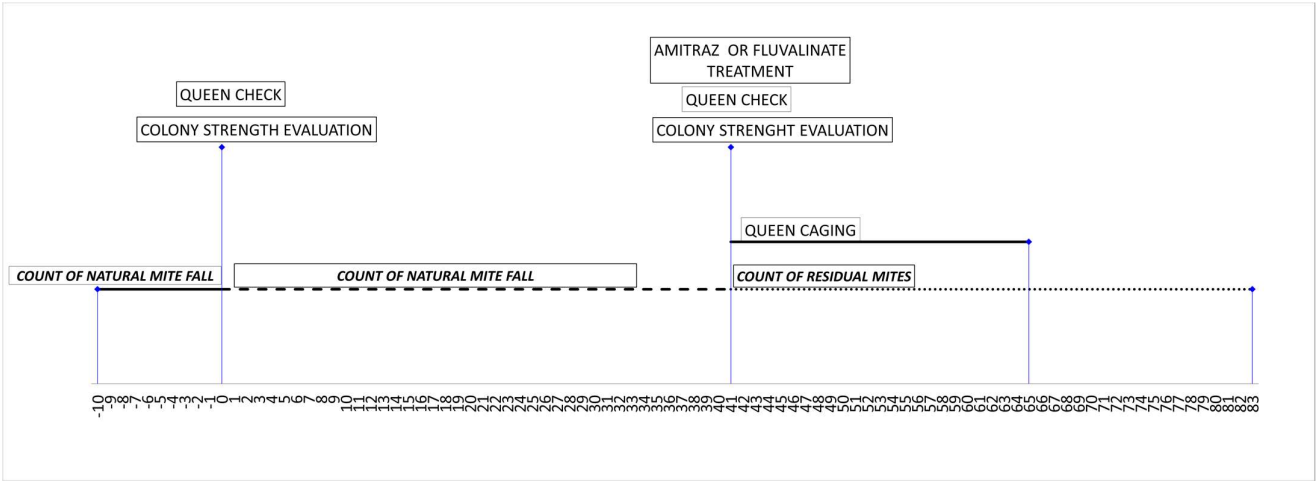
Figure 2. Summer/autumn protocol for the control group (5 repeated treatments).
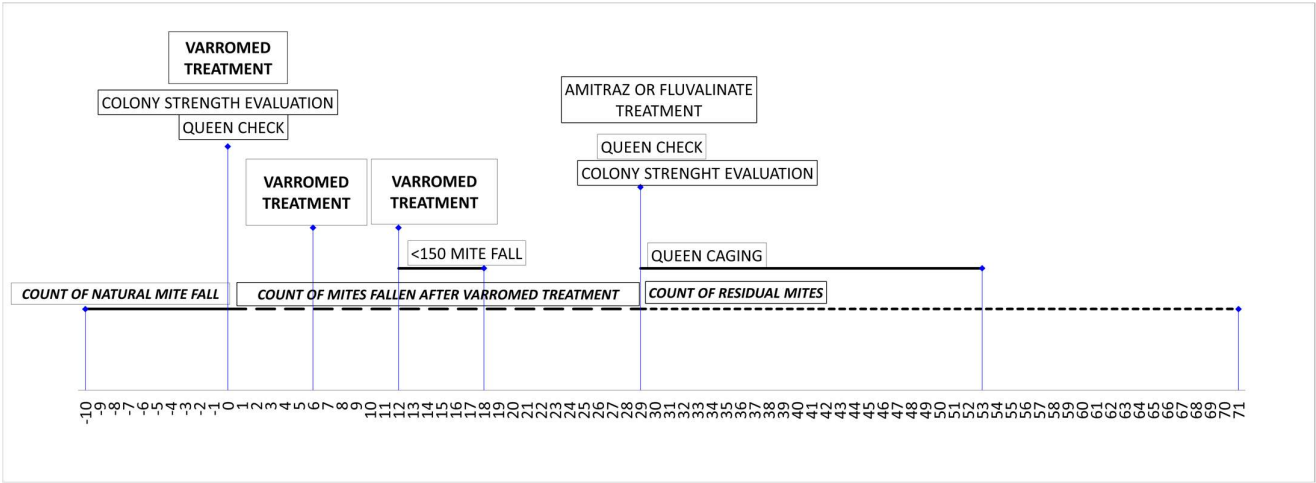
Figure 3. Summer/autumn protocol for the treated group (3 repeated treatments). Only three treatments are needed, if less than 150 mites are detected on the sticky sheets on the bottom boards.
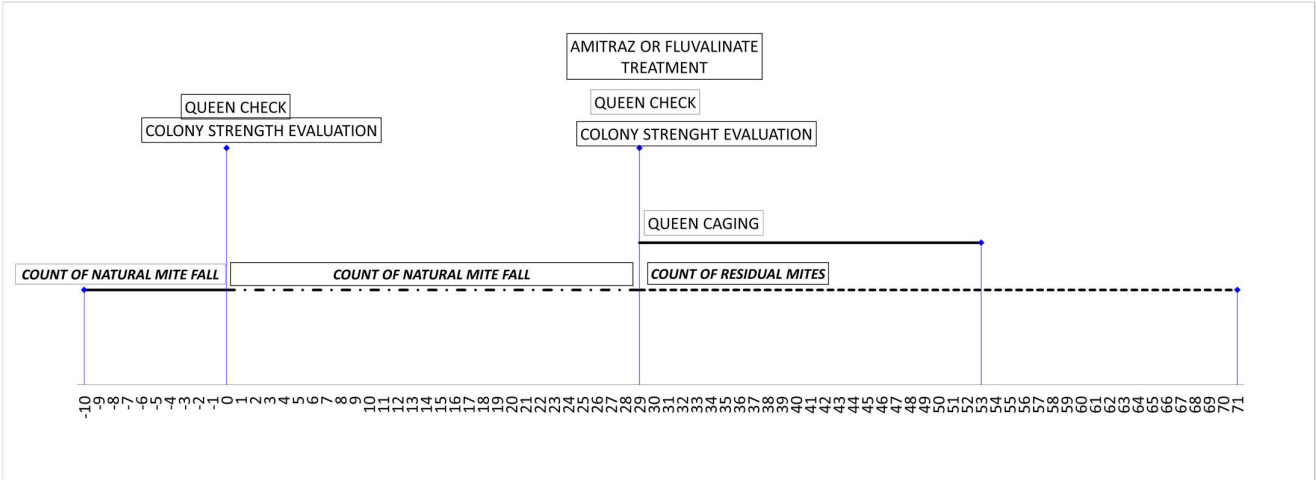
Figure 4. Summer/autumn protocol for the control group (3 repeated treatments).
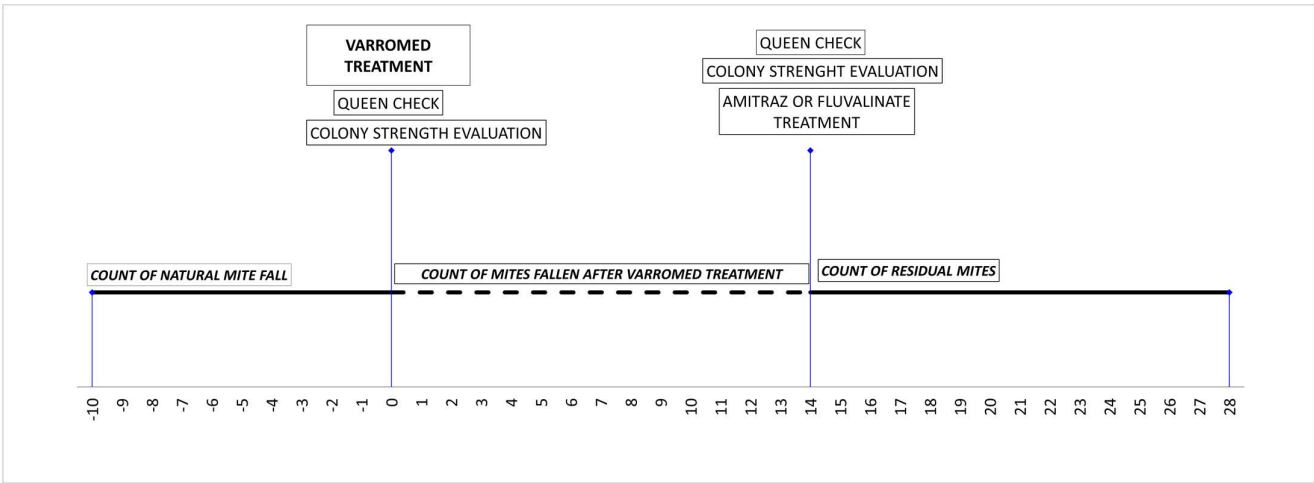
Figure 5. Winter protocol for the treated group in broodless colonies.
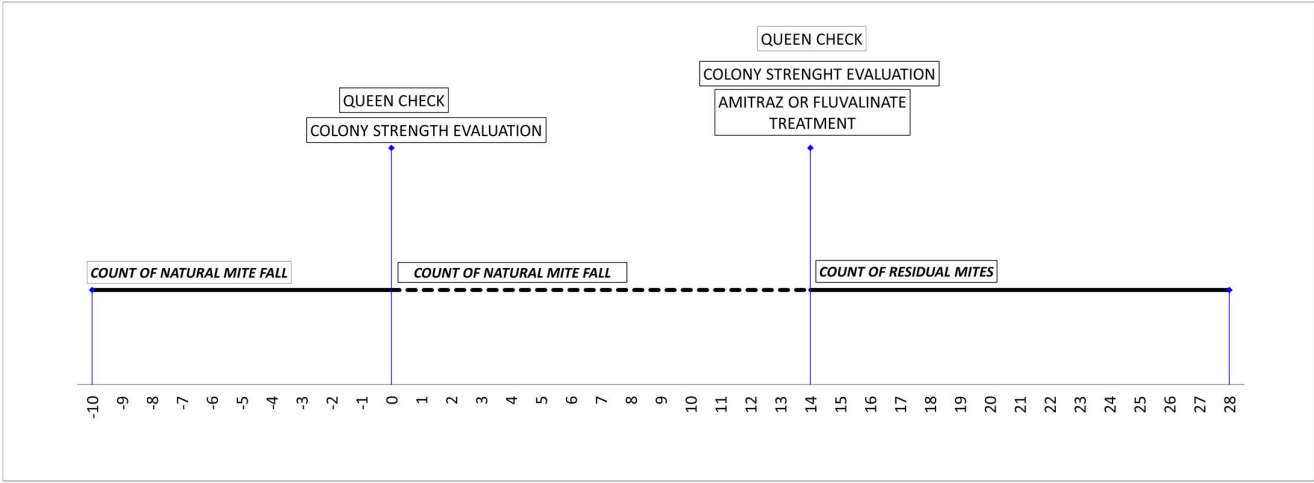
Figure 6. Winter protocol for the control group broodless colonies.

Furthermore, the efficacy of VarroMed® was calculated by counting the fallen mites on the sticky sheets as described above for further 14 days [30,25] after applying a follow-up treatment consisting in the application of synthetic miticides with different mechanism of action based on amitraz and tau-fluvalinate (in combination or in double dose), in order to avoid low efficacy due to possible mite resistance to one of the active substances. We used VPMs registered for honey bees with active substances not present in VarroMed®.

In the summer/autumn protocol, in order to verify the residual number of mites, all queens were caged in VAR-CONTROL cages (Api-Mo.Bru, Campodoro, Padova, Italy – http://www.apimobru.com/en/ppe/ppe.htm) for   21 days and the follow-up treatment was applied at the end of the caging period after queen bee release applying the sticky sheets on the bottom board as previously described [30,25]. The untreated colonies of the control group were checked for the natural mite fall and received the same follow-up treatment.

The percentage of acaricidal efficacy (AE) in each hive was evaluated using the formula: AE=$(V_T/V_{T+follow-up})$*100, where V_T is the total number of mites killed by the VarroMed® treatment, not considering the mites fallen during the queen caging period, and $V_{T+follow-up}$ represents the total number of mites killed by the tested treatment and the follow-up treatments [31].

During the experiments, mean environmental temperatures were recorded by weather stations near the apiaries.

Statistical analysis was performed using XLSTAT™ software [32]. It was verified, if differences in acaricide efficacy were statistically significant using Mann-Whitney Test [33]. The amount of adult honey bees and brood coverage between groups (treated and control) was evaluated using Kruskal-Wallis Test [34]. For the latter, multiple pairwise comparison with Dunn's Test [35] was applied.

3. Results

3.1. Summer/autumn trial

Acaricide efficacy of VarroMed® recorded during the summer/autumn trial ranged from 71.2 %, when applied three times (Croatia), to a maximum of 89.4 % (Belgium) when applied five times (Table 2; Figure 7). All treated groups efficacies differed from control groups (Kruskal-Wallis test: p<0.0001; Dunn's test: p<0.01).

Table 2. Acaricide efficacy recorded during the summer/autumn trial in the apiaries and relative mite fall during the same period in control group (%).

Statistics	VARROMED X5 (BELGIUM)	VARROMED X5 (ITALY_IZSLT)	VARROMED X5 (SLOVENIA)	VARROMED X3 (CROATIA)	CONTROL (BELGIUM)	CONTROL (ITALY_IZSLT)	CONTROL (SLOVENIA)	CONTROL (CROATIA)
Minimum	81.5	73.9	83.4	13.3	6.8	28.2	21.4	0.4
Maximum	100	99.2	93.8	80.7	32.8	75.3	69.2	1.5
1st Quartile	83.6	82.9	84.9	76.1	9.8	32.1	28.8	0.5
Median	89.2	89.7	88.6	77.3	12.9	39.8	46.7	0.6
3rd Quartile	94.1	98.2	91.3	78.9	13.5	53.7	48.7	0.7
Mean	89.4	88.2	88.3	71.2	15.3	44.6	42.7	0.7
Variance (n-1)	0.5	0.9	0.2	4.2	0.9	2.7	3.2	0
Standard deviation (n-1)	7.2	9.3	4.0	20.4	9.7	16.3	17.9	0.4
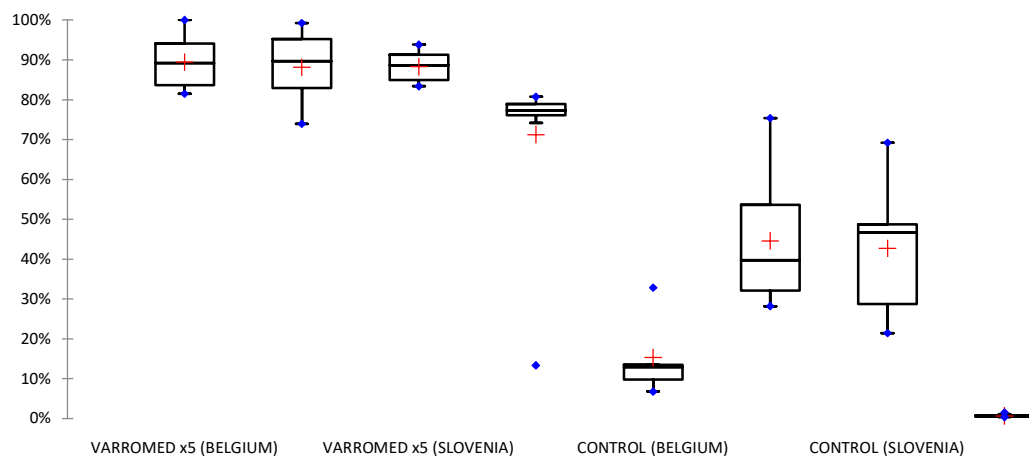
Figure 7. Box plot of acaricide efficacies recorded during the summer/autumn trial and relative mite fall during the same period in control group (%). The horizontal line in boxes shows median. The box shows 1st and 3rd quartile. Whiskers extend to 1.5 x interquartile range (IQR). Diamonds mark the outliers outside the 1.5 x IQR. Additionally, the pluses show mean values.

The amount of adult bees was reduced in treated groups, compared to the control ones, in Belgium, Italy and Slovenia, ranging from -130.1 % to -12.1 %, and increased in Croatia +8.8 % (Table 3 and Figure 8). These differences were statistically significant only

in one apiary (Belgium) (Dunn's test p value: 0.0001) where the colonies were weak and three of them died after the treatment.

Table 3. Variation of adult honey bee coverage in all groups recorded during the summer/autumn trial compared to the beginning of the treatment (%).

Statistics	VARROMED X5 (BELGIUM)	VARROMED X5 (ITALY_IZSLT)	VARROMED X5 (SLOVENIA)	VARROMED X3 (CROATIA)	CONTROL (BELGIUM)	CONTROL (ITALY_IZSLT)	CONTROL (SLOVENIA)	CONTROL (CROATIA)
Minimum	7.5	11.5	15.3	69.7	92.4	56.4	10.9	53.3
Maximum	122.2	219.4	63.2	94.6	242.7	448.1	109.2	111.7
1st Quartile	23.1	55.2	18.5	76.5	152.3	61.9	23.7	61.2
Median	44.0	90.1	20.4	80.2	180.0	117.5	28.8	70.8
3rd Quartile	63.7	121.2	38.4	85.2	208.2	126.4	.48.5	78.1
Mean	49.6	94.2	30.4	80.9	179.7	137.8	42.5	72.1
Variance (n-1)	15.3	37.0	3.5	0.5	25.1	147.1	12.7	2.9
Standard deviation (n-1)	39.1	60.8	18.8	7.3	50.1	121.3	35.6	16.9
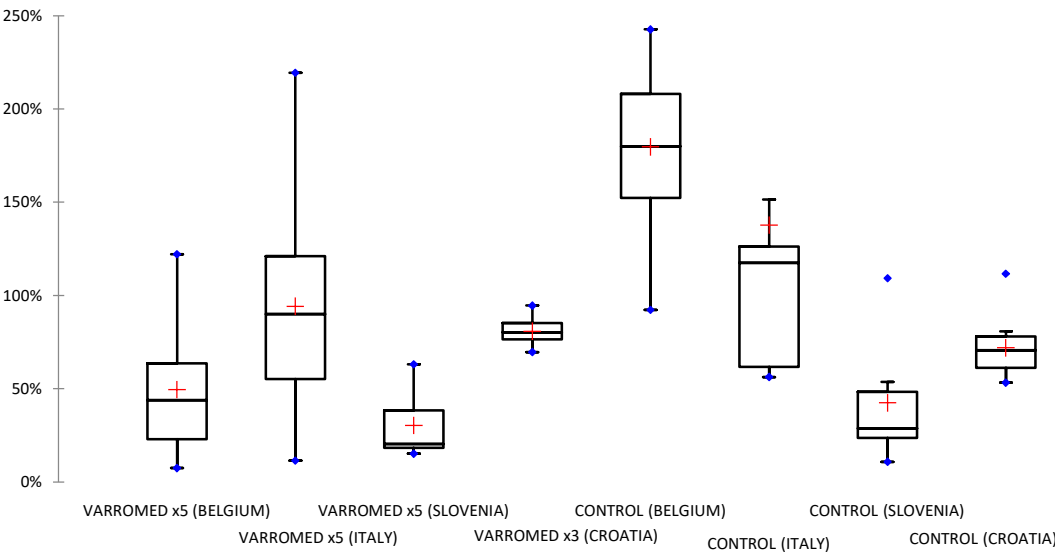
Figure 8. Box plot of adult bee coverage in all groups recorded during the summer/autumn trial compared to the beginning of the treatment (%). The horizontal line in boxes shows median. The box shows 1st and 3rd quartile. Whiskers extend to 1.5 x interquartile range (IQR). Diamonds mark the outliers outside the 1.5 x IQR. Additionally, the pluses show mean values.

Mean brood area compared to the control group increased after VarroMed treatment in Italy and Slovenia (+71.1 % and +3.0 % respectively) and reduced in Croatia (-24.7 %) (Table 4; Figure 9). Brood was not estimated and control groups in each country, variations were not statistically significant.

Table 4. Variation of brood area in all groups recorded during the summer/autumn trial compared to the beginning of the treatment (%).

Statistics	VARROMED X5 (ITALY_IZSLT)	VARROMED X5 (SLOVENIA)	VARROMED X3 (CROATIA)	CONTROL (ITALY_IZSLT)	CONTROL (SLOVENIA)	CONTROL (CROATIA)
Minimum	1.5	1.3	34.0	42.6	0	19.0
Maximum	170.5	23.8	71.4	450.0	29.1	36.7
1st Quartile	40.1	4.5	39.5	76.8	3.0	23.3
Median	75.9	8.6	50.4	115.6	12.1	26.5
3rd Quartile	117.6	16.2	62.8	173.3	24.7	28.8

Mean	81.1	10.7	51.1	152.3	13.7	26.4
Variance (n-1)	33.7	0.8	1.9	154.5	1.7	0.3
Standard deviation (n-1)	58.0	8.9	13.8	124.3	13.0	5.5
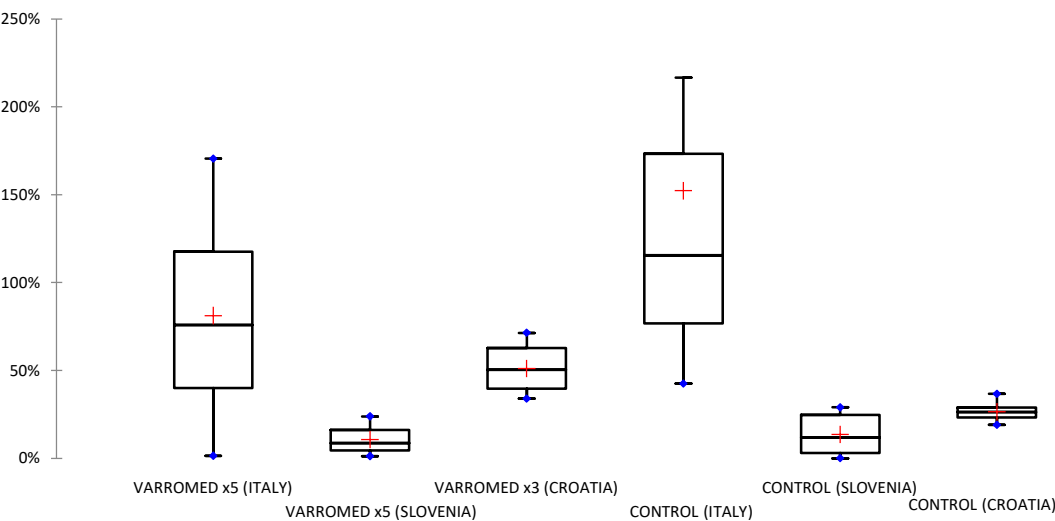
Figure 9. Box plot of brood area in all groups recorded during the summer/autumn trial compared to the beginning of the treatment (%). The horizontal line in boxes shows median. The box shows 1st and 3rd quartile. Whiskers extend to 1.5 x interquartile range (IQR). Diamonds mark the outliers outside the 1.5 x IQR. Additionally, the pluses show mean values.

3.2. Winter trial

In colonies where VarroMed was applied during wintertime, in the absence of brood, the recorded acaracide efficacy ranges from 71.8 % to 95.6 % (Table 5; Figure 10). Efficacies in all treated groups differed when compared to control groups (Kruskal-Wallis test: p<0.0001; Dunn's test_ p<0.01).

Table 5. Acaricide efficacy recorded during the summer/autumn trial in the apiaries and relative mite fall during the same period in control group (%).

Statistics	VARROMED (ITALY_FEM)	VARROMED (ITALY_IZSLT)	VARROMED (ITALY_IZSVE)	VARROMED (CROATIA)	CONTROL (ITALY_FEM)	CONTROL (ITALY_IZSLT)	CONTROL (ITALY_IZSVE)	CONTROL (CROATIA)
Minimum	73.1	87.8	64.8	42.9	1.1	0.6	1.7	0
Maximum	100	98.9	98.7	87.3	19.6	23.8	33.3	35.7
1st Quartile	79.3	93.8	84.6	64.6	5.4	2.8	2.4	3.4
Median	94.6	97.0	93.7	74.1	6.6	6.5	14.4	16.0
3rd Quartile	99.8	97.6	96.0	82.3	10.2	11.8	23.3	20.3
Mean	89.9	95.6	89.7	71.8	8.3	8.6	15.0	14.8
Variance (n-1)	1.2	0.1	1.1	1.9	0.3	0.5	1.6	1.5
Standard deviation (n-1)	11.1	3.5	10.5	13.7	5.3	7.3	12.7	12.0
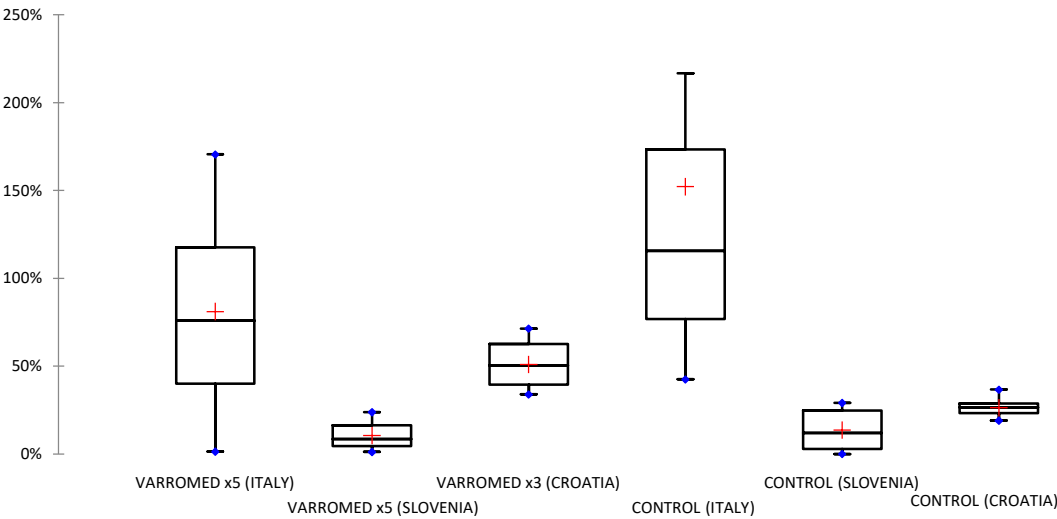
Figure 10. Box plot of acaricide efficacies recorded during the winter trial and relative mitefall during the same period in control group (%). The horizontal line in boxes shows median. The box shows 1st and 3rd quartile. Whiskers extend to 1.5 x interquartile range (IQR). Additionally, the pluses show mean values.

Small variations of conerage of adult honey bees were recorded in the treatment groups and compared to the control ones (from -13.6 % in Italy_FEM to +7.0 % in Croatia). There was no statistical significant difference (Table 6; Figure 11). No queen mortality and no clinical signs of other economically important diseases were observed.

Table 6. Variation of adult honey bee coverage in all groups recorded during the winter trial compared to the beginning of the treatment (%).

Statistics	VARROMED (ITALY_FEM)	VARROMED (ITALY_IZSLT)	VARROMED (ITALY_IZSVE)	VARROMED (CROATIA)	CONTROL (ITALY_FEM)	CONTROL (ITALY_IZSLT)	CONTROL (ITALY_IZSVE)	CONTROL (CROATIA)
Minimum	70.6	62.4	84.6	81.1	71.6	80.6	91.3	85.7
Maximum	199.6	95.4	120.7	100	187.6	93.8	112.5	100
1st Quartile	98.7	88.9	97.3	85.7	81.9	83.3	95.7	99.0
Median	122.9	92.2	101.3	88.3	93.4	90.3	98.2	100
3rd Quartile	135.6	94.2	104.2	99.5	124.1	90.9	105.9	100
Mean	120.2	88.1	101.9	91.1	106.6	88.3	100.3	98.0
Variance (n-1)	13.1	1.1	1.0	0.6	13.4	0.2	0.5	0.2
Standard deviation (n-1)	36.2	10.4	10.2	7.6	36.6	4.8	7.0	4.5
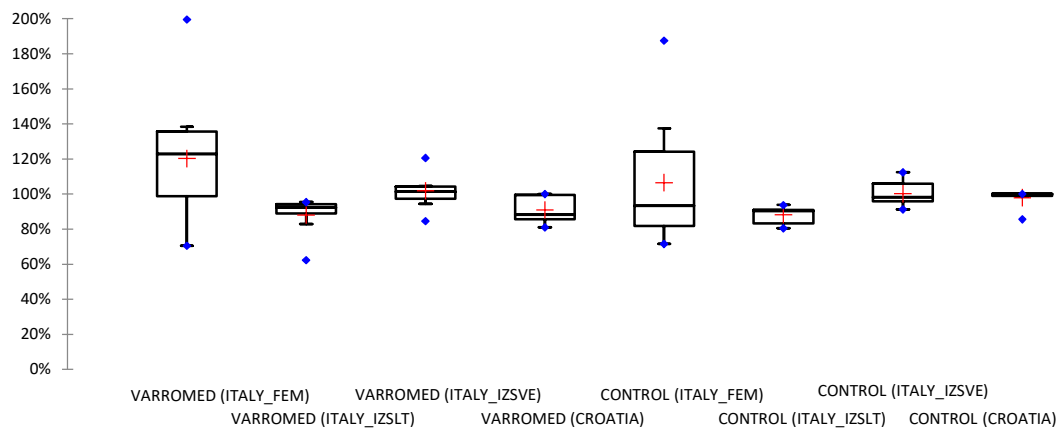
Figure 10. Boxplot of adult honey bee coverage in all groups recorded during the winter trial and compared to the beginning of the treatment (%). The horizontal line in boxes shows median. The box shows 1st and 3rd quartile. Whiskers extend to 1.5 x interquartile range (IQR). Additionally, the pluses show mean values.

During the summer/autumn trials, the mean environmental temperatures recorded during VarroMed treatments were as follows: 30.2±2.5° C in Croatia; 23.6±1.3° C in Italy; 15.6±4.2° C in Slovenia; 12.4±2.7° C in Belgium. During winter trials we recorded the following mean environmental temperatures: 7.4±3.4° C in Italy_IZSVE; 6.4±3.2° C in Italy_IZSLT; 4.2±4.6° C in Croatia, and 3.3±2.6° C in Italy_FEM.

4. Discussion

Varroa mite is a major cause of overwintering honey bee (*A. mellifera*) colony losses across the globe [36]. In recent years, several surveys were conducted to analyze beekeepers' treatment practices and overwintering [36-39]. One of the findings of the above-mentioned studies was that the worst scenario concerning overwintering ability was a combination of weakness of honey bee populations, low food reserves and high Varroa infestation levels [37].

In our study, we administered VarroMed® in several apiaries with different climatic conditions. The average temperatures near the experimental apiaries in Croatia, Italy, Slovenia and Belgium show a versatile range (30.2 – 12.4 ° C) only in summer/autumn treatment period in 2018. Our results show that the efficacy of VarroMed® treatment was satisfactory, regardless of the presence of honey bee brood and environmental temperature, despite some relevant differences between minimum and maximum efficacy values in some sites as shown in Tables 2 and 5.

The protocols of the treatments against *V. destructor* may require quite a long time (over 40 days), to reach a high acaricide efficacy, especially in brood right colonies with high levels of infestation [31]. The field trial was carried out in medium infested colonies and the treatment was therefore appropriate. In experimental apiaries, the efficacy ranged from 71.2 to 89.3 % in summer/autumn treatment. Tlak Gajger & Sušec [40] reported even higher acaricide efficacy (91.5 %) in summer, after three consecutive applications of the oxalic acid based complementary feed HiveClean® (former product, like VarroMed®). In winter treatments reported in this paper, the overall acaricide efficacies ranged from 71.8 to 95.6 %. According to the report of the European Medicines Agency [41], the efficacy of winter treatment with VarroMed® was 88 %. The level of efficacy of VarroMed® treatment in summer/autumn and winter was found sufficient, which can be supported by the average number of mite fall after queen caging and follow-up treatment.

Another critical aspect to the use of mixture of organic acids (oxalic and formic acid) and additional substances against Varroa mites that should be considered, is a possible toxicity to honey bees and colonies. The use of oxalic acid by trickling, evaporation and

spraying, honey bee tolerability and efficacy has been reviewed by Rademacher & Hartz [12]. In general, the application of oxalic acid in a formulation with sugar syrup increases the efficacy against Varroa mites. However, there are some negative effects of oxalic acid on queen health [43,44], and on worker bees' digestive system [44].

Formic acid operates through inhibition of mitochondrial energetic metabolism of Varroa mites binding the cytochrome C oxidase enzyme [45], as well as through a significant neuroexcitation process [46]. It also affects honey bee colony as it reduces longevity of worker bees [47] and affects brood survival [48]. There is a report on increased number of dead bees in front of the hive, queen rejection and decrease of honey yield during treatment [49].

Considering the above-mentioned toxicity aspects, we evaluated the effects on the strength of the colonies after VarroMed® treatments. We found that the reduction in number of honey bees and brood in treated hives was very low or insignificant in all countries and in all application's seasons except in Belgium, where less honey bees were observed in treated colonies and some of them even died. This finding could be explained by the lack of an ideal combination of temperature, treatment and colony condition at the time of field trials.

It is important to highlight that our data showed that there is no need to treat in absence of brood (e.g. applying the queen caging) as a high acaricide efficacy was observed in both cases (with or without brood). The treatments were repeated several times and the duration of the treatment covered the period longer than worker developmental stage from egg to emerging. However, we want to underline the repeated applications of the product, that demand extended efforts of the beekeeper in terms of time and costs and result in higher toxicity to adult honey bees, especially in case of environmental temperatures lower than 15°C.

Finally, it is noteworthy that the producer of VarroMed® leaves the decision to apply an extra-treatment or not to the beekeepers, based on the Varroa infestation levels obtained after the previous treatment. This approach to the treatment against Varroa mites based on an integrated pest management (IPM) includes the implementation of good beekeeping practices and beekeepers' education to a sustainable and successful beekeeping.

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