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THE VIABILITY OF *HAEMONCHUS CONTORTUS* (NEMATODA, STRONGYLIDA) AND *STRONGYLOIDES PAPILLOSUS* (NEMATODA, RHABDITIDA) LARVAE EXPOSED TO VARIOUS FLAVOURINGS AND SOURCE MATERIALS USED IN FOOD PRODUCTION

O. O. Boyko[1], V. V. Brygadyrenko[1, 2]

[1]*Dnipro State Agrarian and Economic University,*
S. Efremova St., 25, Dnipro, 49600 Ukraine
E-mail: boikoalexandra1982@gmail.com
[2]*Oles Honchar Dnipro National University*
Gagarin Ave., 72, Dnipro, 49010 Ukraine
E-mail: brigad@ua.fm

The Viability of *Haemonchus contortus* (Nematoda, Strongylida) and *Strongyloides papillosus* (Nematoda, Rhabditida) Larvae Exposed to Various Flavourings and Source Materials Used in Food Production. Boyko, O. O., Brygadyrenko, V. V. — The objective of this study is to evaluate the viability of larval nematodes of ungulates under the influence of 14 flavourings and source materials approved for use in and on foods. Minimum LD_{50} for L_3 *Strongyloides papillosus* (Wedl, 1856) were observed when using Cinnamaldehyde, α-Terpineol and Benzyl alcohol, for L_{1-2} *S. papillosus* under the influence of Benzyl alcohol, Cinnamaldehyde, L-Linalool and Benzyl acetate, for L_3 *Haemonchus contortus* (Rudolphi, 1803) under the influence γ-Undecalactone and Cinnamaldehyde. Ethyl acetate, β-Ionone и D-Limonene had the lowest effect on all the studied stages of development of the nematodes: larvae did not die during 24 hours even at 1 % concentration of these substances. Further experiments with usage of mixtures of the substances studied in this article will contribute to the development of preparations with a stronger effect on larvae of nematode parasites of the digestive system of vertebrate animals.
Key words: intestinal nematodes, Ruminantia, flavourings, mortality, larvae.

Introduction

Different parasitic diseases have always caused harm to ungulates and brought material loss to farming (Faye et al., 2003; Veneziano et al., 2004; Charlier et al., 2007; Cringoli et al., 2008; Ponomar et al., 2014; Boyko et al., 2016). The condition of animals and their level of infestation are quite difficult to control (Boyko et al., 2016, 2017 b); this is possible to achieve, however, by studying the peculiarities of pathogens, their threshold of sensitivity to the influence of environmental factors.

Today, different antiparasitic preparations are widely used, including extracts of plants (Rahmann et al., 2006; Burke et al., 2009; Cheng et al., 2009; Lu et al., 2010). Food additives are often used against pathogens and fungi (Chiang, 2005; Sato, 2006; Somolinos, 2008; Si, 2009; Belletti et al., 2010). Data exist on the use of these substances against insects and Acari parasitic in animals and plants (Knoblauch et al., 2011; Shen et al., 2012; Belkind et al., 2013).

In medicine, Benzoic acid (E_{210}, Codex Alimentarius) and also its derivatives: E_{211} — Sodium Benzoate, E_{212} — Potassium Benzoate, E_{213} — Calcium dibenzoate and others, is used as an antiseptic and fungicide (Eysker et al., 1994; Beerse et al., 2001; Amborabe et al., 2002; Joshi et al., 2008). One of the additives, methylparabene (E_{218}) is also used as an antiseptic, fungicide and insecticide (Bell, 1990; Shapiro et al., 2002; Posey et al., 2005; Kromidas et al., 2006; Rebbeck et al., 2006; Ishiwatari et al., 2007; Meyer et al., 2007; Gopalakrishnan et al., 2012). Thus, the modern literature includes data on influence of food additives on pathogenic bacteria, fungi, parasitic Acari and insects which are agricultural pests. Katiki et al. (2017) assessed in vitro the ovicidal effect of carvacrol, carvone, eucalyptol, linalool, limonene, thymol, cinnamaldehyde, anethole, vanillin and eugenol using *Haemonchus contortus* (Rudolphi, 1803). However, we found no published data on the impact of food additives on L_3 of the nematodes *H. contortus* and *Strongyloides papillosus* (Wedl, 1856). Therefore, the objective of this study was to evaluate the viability of larval nematodes of ungulates under the influence of flavourings and source materials approved for use in and on foods.

Material and methods

The faeces of sheep and goats naturally infected with *H. contortus* and *S. papillosus* were collected during summer 2016 on the territory of Dnipropetrovsk Region, Ukraine. They were transported to laboratory in plastic containers at a temperature of 22–24 °C. The experiment used larvae of the third stage (L_3) of *H. contortus* and larvae of first, second and third stages (L_1, L_2, L_3) of *S. papillosus*. The larvae were identified on the basis of morphological parameters (Van Wyk et al., 2004; 2013). Before the experiment, the larvae were cultivated in the faeces during 8 days at 22–24 °C. For this purpose, the faeces containing eggs nematodes were put into sealed Petri dishes, 10 g in each and transferred into thermostat. During 8 days, after each 24 hours, the samples were moistened. L_1, L_2, and L_3 of *S. papillosus* were recorded after 24–72 hours, L_3 of *H. contortus* were identified on the eighth day. To isolate the larvae we used Baermann's method (Baermann test) (Zajac et al., 2011).

The liquid containing the larvae (4 ml) was centrifuged in 10 ml test tubes of (4 minutes at 1500 rpm). The sediment with the larvae was mixed and put in 1.5 ml plastic test tubes of by portions. Then solution of the studied substance was added to the larval culture (0.200 ml, 20–40 individuals) and left for 24 hours at 22–24 °C. After processing by the studied substances, we determined the number of live and dead larvae. For proving death of the larvae, we took into account two factors: immobility and decomposition of intestinal cells.

The larvae were exposed to the flavourings and source materials approved for use in and on foods at three concentrations (1 %, 0.01 %, 0.0001 %) as well as control (distilled water without the addition of flavorings) with eight replications for each variant of the experiment. The experiment used chemically pure p-Anisaldehyde, Benzaldehyde, γ-Undecalactone, Cinnamaldehyde, Ethyl acetate, Benzyl acetate, α-Terpineol, Benzyl alcohol, Citral, L-Linalool, β-Ionone, Citronellol, Acetoin, D-limonene, which are used as flavourings.

The statistical analysis of the results was performed through a set of Statistica 8.0 (StatSoft Inc., USA), the median, 25 % and 75 % quartiles, minimum and maximum values are shown on the plots. LD_{50} (%) was calculated as an average value (x) ± standard deviation (SD).

Results and discussion

The results of the experiment showed that 7 out of 14 studied flavourings and source materials affected the vitality of nematodes at 1 % concentration (figs 1–5, table 1). While studying the effect of the aldehyde flavourings (fig. 1) the registered death rate was 100 % in *S. papillosus* larvae of different developmental stages and L_3 *H. contortus* at 1 % concentration of p-Anisaldehyde, Benzaldehyde, γ-Undecalactone and Cinnamaldehyde.

S. papillosus larvae of different stages of development were affected mostly by Cinnamaldehyde (fig. 1, d). At 0.01 % concentration of this substance 70 % of L_3 and L_{1-2} of *S. papillosus* died.

The influence of 0.01 % and 0.0001 % concentrations of p-Anisaldehyde, Benzaldehyde, γ-Undecalactone did not significantly affect the viability of *S. papillosus* larvae (fig. 1, a, b, c). The *H. contortus* larvae of the third stage appeared to be more resistant to the influence of p-Anisaldehyde, Benzaldehyde, γ-Undecalactone, Cinnamaldehyde than *S. papillosus* larvae. Only 1 % concentration of these aldehyde flavourings significantly affected their viability. At 0.01 % concentration of p-Anisaldehyde and Cinnamaldehyde, 100 % of larvae of this nematode species survived. Under the influence of Benzaldehyde and γ-Undecalactone of the same concentration, 70 % of *H. contortus* larvae of third stage were found to be viable (fig. 1, b, c).

While studying the effect of acetate flavourings on the viability of nematode larvae of Ruminantia, we observed a decrease in the viability of *H. contortus* and *S. papillosus* larvae of the third stage under the influence of Ethyl acetate and Benzyl acetate (fig. 2, a, b). A stronger effect upon the viability of the larvae of the studied nematode species was caused by Benzyl acetate (fig. 2, b). Under its influence at 1 % concentration, 100 % of *S. papillosus* larvae of different stages of development and about 90 % of *H. contortus* larvae of the third stage died. At 0.01 % concentration of Benzyl acetate, 100 % of L_3 *H. contortus* and *S. papillosus* larvae survived. More than 50 % of L_{1-2} *S. papillosus* larvae remained viable at this concentration of Benzyl acetate. 0.0001 % concentration of Benzyl acetate did not affect the viability of larvae of the studied nematode species. 100 % of *H. contortus* and *S. papillosus* larvae of the third stage and about 90 % *S. papillosus* larvae of the first-second stages remained viable under the influence of this concentration of Benzyl acetate. Ethyl acetate did not significantly affect the vitality of *H. contortus* and *S. papillosus* larvae (fig. 2, a). Even at 1 % concentration of Ethyl acetate, 100 % of L_3 *H. contortus* and *S. papillosus* and 60 % of L_{1-2} *S. papillosus* survived.

Studying the influence of alcohol flavourings showed that the most significant influence upon the viability of larvae of nematodes of Ruminantia was caused by Benzyl alcohol

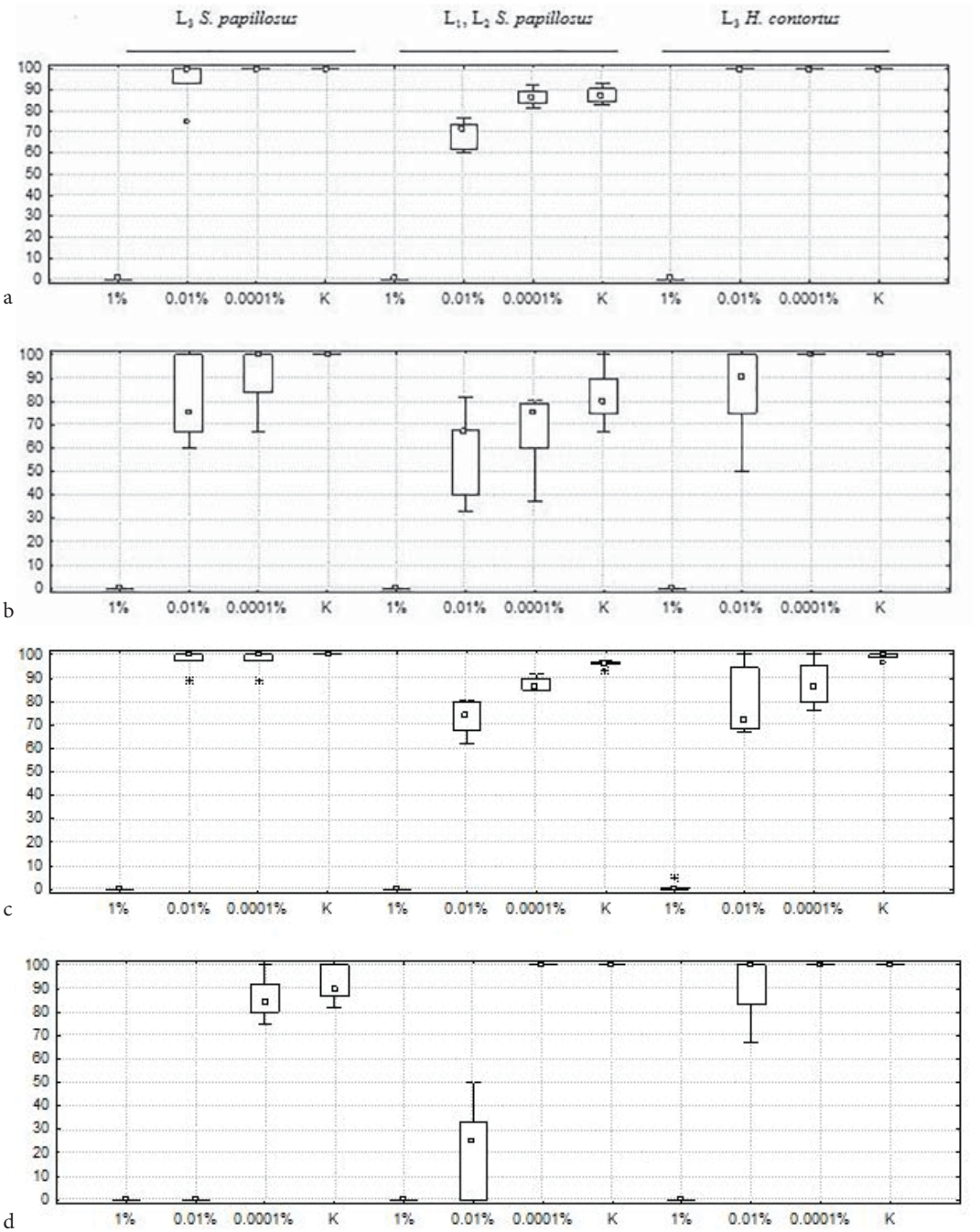

Fig. 1. The effect of aldehyde flavourings on the viability of larvae of nematodes of Ruminantia: a — *p*-Anisaldehyde; b — Benzaldehyde; c — γ-Undecalactone; d — Cinnamaldehyde; the ordinate axis indicates the percentage of living nematode larvae in 24-hour experiment; the abscissa axis indicates the concentration of the active substance (%); (K) control, where the concentration of the active substance is 0 %; (L_3) infective larvae of *S. papillosus*or *H. contortus*; (L_1, L_2) non-infective larvae of *S. papillosus*; small square in the centre corresponds to the median, lower and upper edge of the large rectangle corresponds to first and third quartiles, respectively, the vertical segments, directed upward and downward from the rectangles, correspond to minimum and maximum values (n = 8).

(fig. 3, b). 100 % of *S. papillosus* larvae of different stages died after being affected by 1 % concentration of Benzyl alcohol. About 20 % of L_3 of *H. contortus* survived at this concentration. 0.01 % concentration of this substance caused death of 50 % of *S. papillosus* L_3 and about 92 % of L_{1-2}. 0.0001 % concentration of Benzyl alcohol was able to affect only the L_{1-2} of *S. papillosus* (fig. 3, b). The number of larvae that survived at this concentration was only 35 %. The third-stage nematode larvae of this species remained 100 % viable at this concentration. L_3 of *H. contortus* were more resistant to the effect of Benzyl alcohol than *S. papillosus* larvae. At 0.01 % concentration, 100 % of larvae of this species survived.

The effect of α-Terpineol at 1 % concentration (fig. 3, a) caused death of 90 % of L_{1-2} and L_3 of *S. papillosus*. Only 50 % of *H. contortus* larvae died under the influence of this substance.

Citral was able to affect only the L_{1-2} of *S. papillosus* (fig. 3, c). Its solution at 1 % concentration caused death of 100 % of these nematodes. Only about 50 % of L_3 of this species died at 1 % concentration of Citral. 0.01 % concentration did not greatly affect the viability of *S. papillosus* larvae of different stages and *H. contortus*. About 60 % of L_{1-2} and more than 80 % of L_3 *S. papillosus* survived in such cases (fig. 3, c). L_3 of *H. contortus* were the most resistant to this substance. In 100 % of cases, they survived at different concentrations of Citral.

About 75 % of L_{1-2} and 90 % of L_3 of *S. papillosus* died after the influence of 1 % concentration of L-Linalool (fig. 3, d). 0.01 % concentration did not cause significant effect on *S. papillosus* L_3. Only about 40 % of them died at such conditions. Almost 100 % of *H. contortus* L_3 survived at all concentrations of L-Linalool that we studied (fig. 3, d).

β-Ionone (fig. 4, a) had no significant influence on larvae of all studied stages of development of *S. papillosus* and *H. contortus*. More than 60 % of L_{1-2} and 90 % of L_3 o *S. papillosus* and also 100 % of L_3 of *H. contortus* survived under the influence of 0.01 % concentration of β-Ionone.

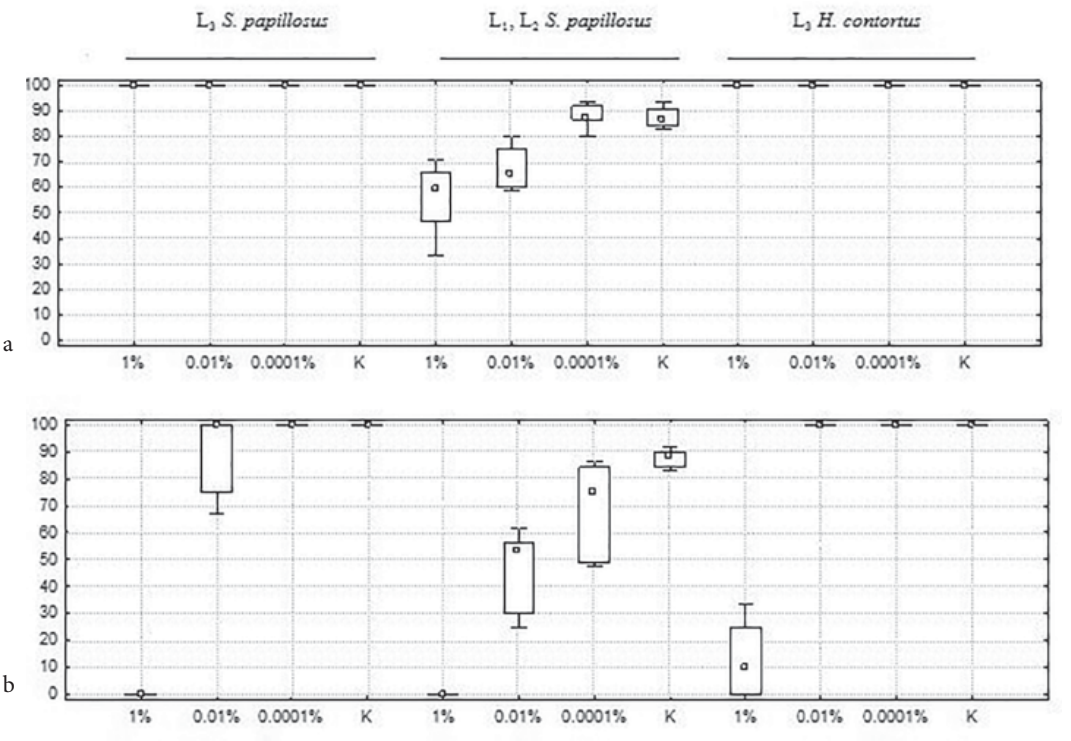

Fig. 2. The effect of acetate flavourings upon the viability of larvae of nematodes of Ruminantia: a — Ethyl acetate, b — Benzyl acetate; for explanations see fig. 1.

Citronellol affected only L_{1-2} and L_3 of *S. papillosus* (fig. 4, b). Almost all larvae of this species died at 1 % concentration of Citronellol. 80 % and 100 % of *S. papillosus* larvae survived at 0.01 % concentration of this substance. More than 50 % of *H. contortus* L_3 remained viable after 24 hours of exposure, even at 1 % concentration of Citronellol solution (fig. 4, b).

Acetoin did not cause significant mortality of third-stage larvae of the studied species of nematodes; at 1 % concentration, on average 80 % of L_{1-2} and L_3 of *S. papillosus* larvae died (fig. 4, c).

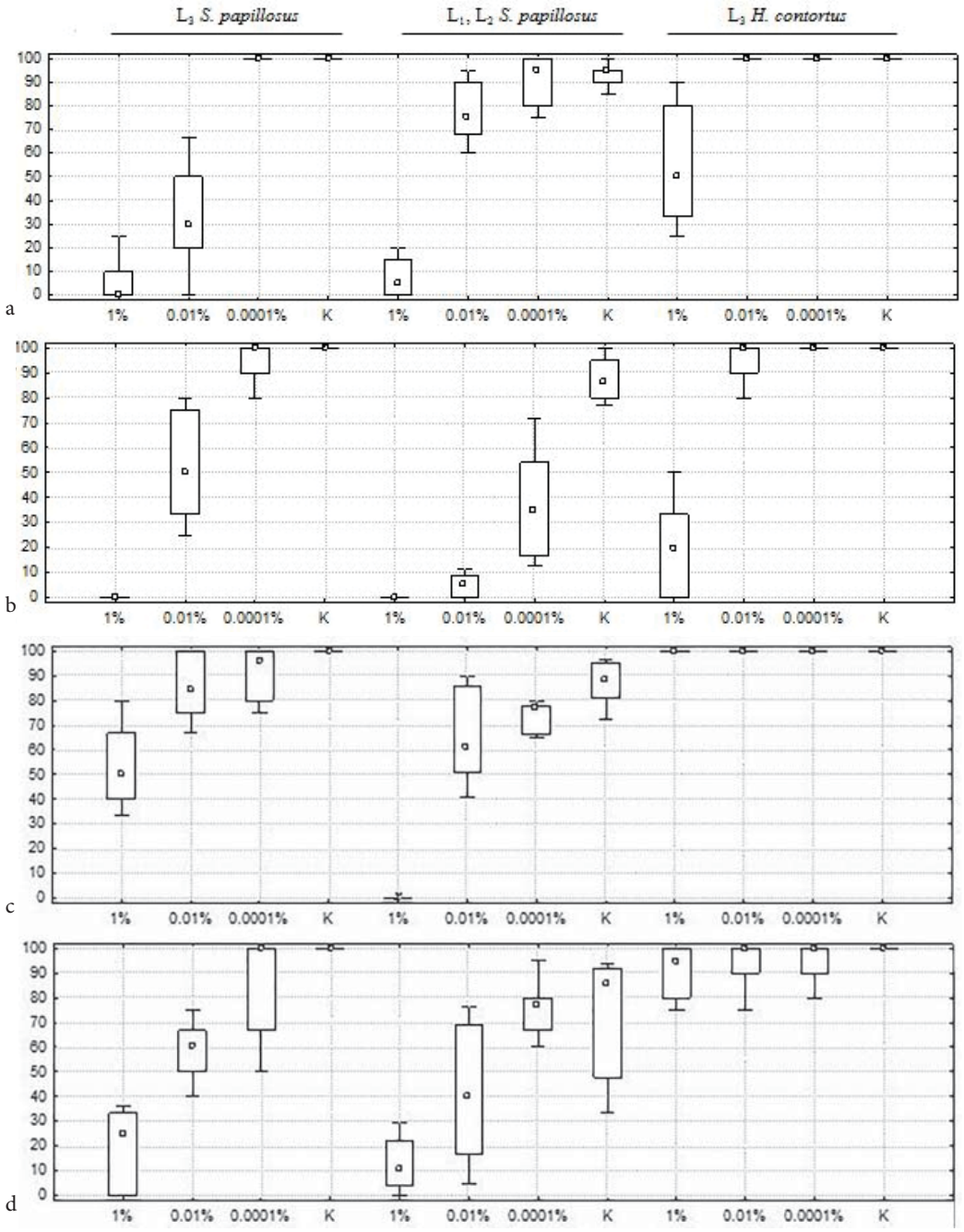

Fig. 3. The effect of alcohol flavourings and their compounds on the viability of larvae of nematodes of Ruminantia: a — α-Terpineol, b — Benzyl alcohol, c — Citral, d — L-Linalool; for explanations see fig. 1.

D-limonene (fig. 5) had no significant effect on all studied stages of *S. papillosus* and *H. contortus*. More than 80 % of L_{1-2} and L_3 of *S. papillosus* and 100 % of *H. contortus* larvae survived at 1 % concentration of D-limonene.

Minimum indicators of LD_{50} (%) were registered for L_3 *S. papillosus* exposed to Cinnamaldehyde, α-Terpineol and Benzyl alcohol, for L_{1-2} *S. papillosus* with Benzyl alcohol, Cinnamaldehyde, L-Linalool, Benzyl acetate, Acetoin and Citral, for L_3 *H. contortus* in solution of γ-Undecalactone and Cinnamaldehyde (table 1). Minimum effect on all studied developed stages of nematodes was achieved with Ethyl acetate, β-Ionone, D-limonene and Acetoin (table 1).

Thus, the viability of L_3 nematodes from the alimentary tract of Ruminantia is significantly affected by the flavourings and source materials: p-Anisaldehyde, Benzaldehyde, γ-Undecalactone, Cinnamaldehyde, Benzyl acetate, Benzyl alcohol and α-Terpineol. 1 % concentrations of these substances significantly influenced the viability of *S. papillosus* and *H. contortus* larvae of different stages. Katiki et al. (2017) determined LD_{50} for eggs of *H. contortus* under the impact of cinnamaldehyde, linalool and limonene. For cinnamaldehyde, this indicator was the lowest, equaling 0.018 g/l. For linalool it was almost 10 times higher — 0.29 g/l. The highest LD_{50} for the eggs of *H. contortus* was recorded after using limonene — 207.5 g/l. According to the results of our studies, the best results against larvae

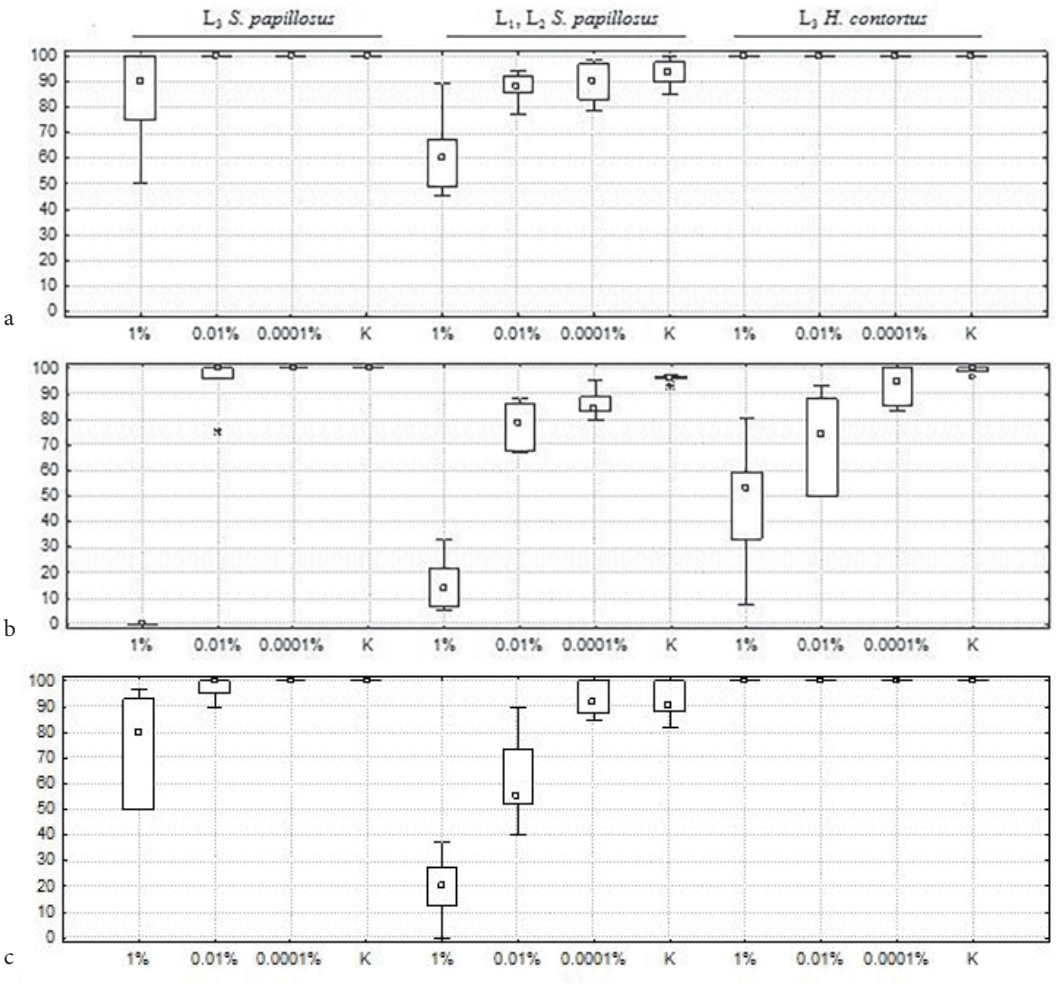

Fig. 4. The effect of alcohol flavourings and their compounds on the viability of larvae of nematodes of Ruminantia: a — β-Ionone, b — Citronellol, c — Acetoin; for explanations see fig. 1.

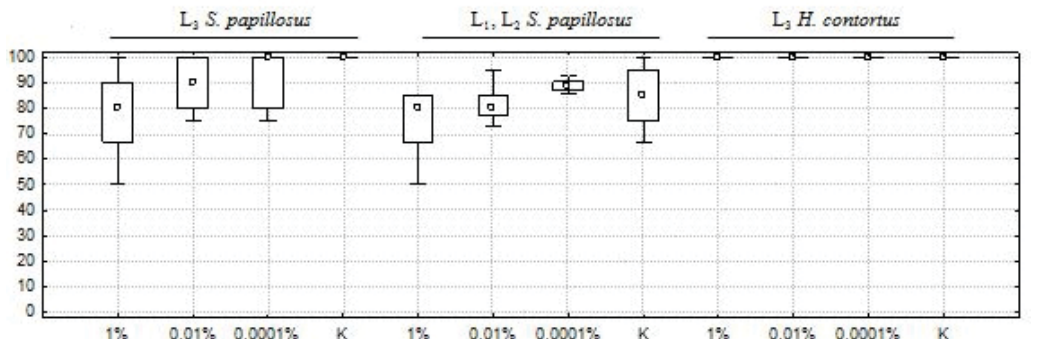

Fig. 5. The effect of D-limonene on the viability of larvae of nematodes of Ruminantia: for explanations see fig. 1.

of *H. contortus* were demonstrated by cinnamaldehyde. We obtained similar results earlier while studying the effect of this substance on the larvae of *Strongyloides ransomi* Schwartz & Alicata, 1930 parasitic in alimentary tract of pigs (Boyko, Brygadyrenko, 2017 a). The larvae of this species also could not survive at 1 % concentration of Benzaldehyde. Our research coincides with the works of a number of authors concerning the effect of this flavoring on other agricultural pests. The experiments of Ullah et al. (2015) also showed insecticidal activity of Benzaldehyde against *Galleria mellonella* (Linnaeus, 1758), they recommend using this substance in developing of insecticidal preparations against agricultural pest insects. Lee et al. (2008) also proved that this flavoring had an insecticidal effect on *Sitophilus oryzae* (Linnaeus, 1763) (Coleoptera, Curculionidae): LD_{50} at 48 hours exposure was 0.004–0.200 mg/cm^2.

There are numerous data in the literature on the use of Cinnamaldehyde against parasitic organisms, and also against pest insects. Shen et al. (2012) used Cinnamaldehyde experimentally against parasitic Acari of the genus *Psoroptes*. Its LD_{50} after 24 hours was 107 mg/ml. Also Na et al. (2011) studied the influence of Cinnamaldehyde on *Dermanyssus gallinae* (De Geer, 1778), poultry mites. After one day, LD_{50} was 0.54 mg/ml. Cinnamaldehyde also significantly affects insects. Cheng et al. (2009) proved the influence of Cinnamaldehyde on mosquito larvae. LD_{50} of this substance was 40.8 mg/ml. Lee et al. (2008) also proved the insecticidal properties of Cinnamaldehyde.

The influence of β-Ionone upon living organisms was studied by Boussaada et al. (2008). They proved the influence of this substance on microorganisms. Nevertheless, β-Ionone did not work as a fungicide. Our research also did not find a relationship between the viability of nematode larvae of the alimentary tract of Ruminantia and β-Ionone. Compounds of this

Table 1. LD_{50} **(%, x ± SD) for *S. papillosus* and *H. contortus* larvae in laboratory experiment during 24 hours; (–) the experiment did not achieve death of 50 % of the larvae (needed concentration of over 1 %)**

Substance	*S. papillosus*, L_3	*S. papillosus*, $L_1 + L_2$	*H. contortus*, L_3
p-Anisaldehyde	0.59 ± 0.21	0.05 ± 0.03	0.50 ± 0.25
Benzaldehyde	0.31 ± 0.17	0.12 ± 0.08	0.56 ± 0.19
γ-Undecalactone	0.53 ± 0.19	0.08 ± 0.04	0.04 ± 0.03
Cinnamaldehyde	0.0064 ± 0.0023	0.0008 ± 0.0002	0.0638 ± 0.0341
Ethyl acetate	–	–	–
Benzyl acetate	0.52 ± 0.24	0.02 ± 0.02	0.62 ± 0.29
α-Terpineol	0.007 ± 0.004	0.06 ± 0.05	1.00 ± 0.65
Benzylalcohol	0.010 ± 0.005	0.00008 ± 0.00005	0.68 ± 0.41
Citral	1.00 ± 0.41	0.04 ± 0.03	–
L-Linalool	0.19 ± 0.15	0.008 ± 0.006	–
β-Ionone	–	–	–
Citronellol	0.42 ± 0.23	0.06 ± 0.05	–
Acetoin	–	0.038 ± 0.014	–
D-limonene	–	–	–

substance and aldehydes have a significant effect on microorganisms — infectious agents (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) (Sharma et al. 2012).

Despite the fact that L-Linalool had no significant effect on $L_3$of *H. contortus*, *S. papillosus* larvae of different stages died in our experiment at 1 % concentration of this substance. The toxicity of L-Linalool was proved also by Yi et al. (2016) against the larvae of *Cotesiaglomerata* (Linnaeus, 1758). Lee et al. (2008) and also determined the level of insects' survivability under the influence of not only Cinnamaldehyde, Benzaldehyde, but also under the influence of Linalool, Limonene, and α-Terpineol. These results partly coincide with our research on the effect of α-Terpineol on invertebrates. At 1 % solution of α-Terpineol, over 50 % of larvae of the studied species of nematodes died.

Research by Chalquest (2002) supports our results concerning the anthelminthic effect of Benzyl alcohol. They indicated the significant influence of compounds which included Benzyl alcohol upon the nematodes and their eggs. It should be noted that it is non-phytotoxic. It is recommended to be used as a solvent for antiparasitic preparations by Pedersen and Woldum (2011). This substance can be used in compounds which have insecticidal properties. According to data by Taylor (2009), such compounds are active against fleas, common parasites of mammals. The effect of Benzyl acetate upon pathogens has been studied by a number of authors. Kurkal-Siebert et al. (2015) created an antibacterial preparation which includes Benzyl acetate.

Many researchers have proven the antibacterial effect of Citral, Linalool, γ-Undecalactone, and also p-Anisaldehyde (Demirci, 2008; Saddiq, 2010; Abi-Zaid, 2015; Carrasco, 2016). According to Lee (2004), p-Anisaldehyde also has acaricidal properties. The results of these studies are partly supported by our experiments on the effect of these substances on living organisms, including *H. contortus* and *S. papillosus* larvae.

Using flavourings which are used in human food as antiparasitic preparations for animals is a promising area for further research, for this can reveal new mechanisms never studied before, which regulate the influence of familiar organic compounds on living organisms. On the one hand, this may possibly stimulate the easier introduction of preparations based on these flavourings into veterinary practice, and on the other hand it may cause restrictions of using the most active of them in the food industry.

Further experiments with using the compounds studied in this article (which include β-Ionone, aldehydes, Benzyl alcohol and others) will lead to development of their compounds which will have a stronger effect on nematodes parasitic in the digestive system of vertebrates.

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