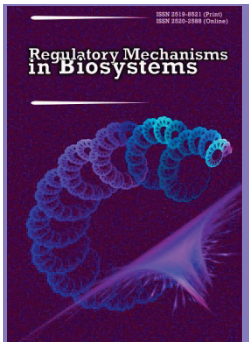

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The impact of certain flavourings and preservatives on the survivability of eggs of *Ascaris suum* and *Trichuris suis*

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The article describes a laboratory study of nematocidal properties of flavourings with antibacterial effect against *Ascaris suum* (Goeze, 1782) and *Trichuris suis* Schrank, 1788. In the experiments, eight concentrations of food additives with antibacterial properties were used: cinnamaldehyde, benzoic acid, formic acid, linalool, citral, β-ionone. Minimum LC_{50} value for eggs of *A. suum* was observed while using cinnamaldehyde and benzoic acid – 1.62 ± 0.37% and 1.69 ± 0.14%, and for eggs of *T. suis* – 0.57 ± 0.03% and 1.80 ± 0.11% respectively. The lowest influence on the development of eggs of nematodes of pigs' *A. suum* and *T. suis* was exerted by formic acid, linalool, citral and β-ionone. In eggs of *A. suum* and *T. suis*, larvae formed in 21 and 50 days even during exposure to 3% emulsions of these substances. The strongest negative impact on the eggs of parasitic nematodes was displayed by cinnamaldehyde flavouring. Further study on nematocidal properties of flavourings, as well as their mixtures, would contribute to the development of preparations which would have a strong effect on eggs and larvae of nematodes of animals and humans.

Keywords: nematodes of pigs; flavourings; preservatives; survivability of eggs.

Introduction

Two of the commonest helminthiases of swine around the globe are ascariasis and trichuriasis. The pathogens of these parasitic diseases cause economic damage to pig farming (Lindgren et al., 2019; Nwafor et al., 2019; Zheng et al., 2020). Today, most farmers use synthetic broad-spectrum anthelmintic preparations against ascariasis and trichuriasis of pigs, though some of them have notable side effects (Jakobsen et al., 2019; Vandekerckhove et al., 2019). At the same time, repeated infection poses a threat of development of nematodes' resistance to antiparasitic preparations (Ceballos et al., 2019; de Oliveira et al., 2019; Palma et al., 2020). Therefore, alternative veterinary and medical preparations with anthelmintic activity are now being searched for all around the world (Boyko & Brygadyrenko, 2019c; Palchykov et al., 2019).

Numerous studies have been published focusing on the ways of combating *Ascaris suum* (Goeze, 1782). Oh et al. (2016) evaluated the effect of several disinfecting preparations on embryogenic development of eggs of *A. suum*. According to the results of their experiment, 3.0% cresol and 0.02% sodium hypochlorite can slow the development, but not inactivate the eggs of *A. suum*. During 6 weeks of the incubation, eggs of these nematodes still completed the embryonic development. Likewise povidone-iodine in 10% solution killed no eggs even during 60 min exposure.

Zhao et al. (2017) determined the influence of medicamental treatment on embryonic development of eggs of *A. suum*. Eggs were taken from the mother of immature ascarids extracted from the intestine of pigs. Zhao et al. (2017) also determined effect of abamectin, doramectin, ivermectin, flubendazole on embryonic development of eggs of *A. suum*. The process of the formation of larvae in eggs of nematodes of pigs which received abamectin, doramectin, and ivermectin reliably did not differ from the control. Flubendazole inhibited the development of embryos of the worms: only in 6.3% of eggs did larvae develop. However, *in vitro* exposure of eggs extracted from the contents of the intestines of pigs which received no anthelmintic preparations had no significant effect on the development of larvae.

Quite often in the literature one can find mentions of the effect of plants or their tinctures on the development of eggs of *A. suum*. Anthelmintic *in vitro* activity of common chicory (*Cichorium intybus* L.) was studied by Williams et al. (2016). They confirmed that methanol extract of plant has significant anthelmintic effect against *A. suum*.

Fitri et al. (2019) performed an in vitro experiment for determining the influence of ground seeds of papaya on the development of embryos in eggs of *A. suum*. The results of their studies suggest decreased ability of the exposed eggs to form vital embryos, in contrast to the control group of eggs. Nyasinge et al. (2018) also studied the influence of the alcohol extracts of plants on *A. suum*. Extracts from the plant *Paullinia pinnata* L. of the Sapindaceae family displayed significant anthelmintic activity towards eggs of *A. suum*. The plant was found to contain saponins, alkaloids, flavonoids, tannins and triterpenoids. The results of these studies on the anthelmintic effect of alcohol extract of the plant on *A. suum* indicate higher mortality of eggs compared with albendazole.

Similar experiments were conducted by Simalango et al. (2018) who studied *in vitro* anthelmintic effect of ethanol extract of seeds of *Nigella sativa* L. (Ranunculaceae) against eggs of *A. suum*: LC_{50} equaled 1.693%.

Williams et al. (2016) studied in vitro anthelmintic properties of traditional medicinal plants of Africa and the Caribbean Basin. The authors performed screening of ethanol extracts from 29 medical plants against *A. suum*. Extracts from *Clausena anisata* (Willd.) Hook. f. ex Benth. (Rutaceae), *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler (Rutaceae) and *Punica granatum* L. (Lythraceae) proved to be the most efficient against *A. suum*. The results of their studies demonstrated the possibility of using these plants as additional variants of the treatment of mammals suffering from nematodes. Sea et al. (2017) report *in vitro* anthelmin-

tic activity of tincture of leaves of holy basil *Ocimum sanctum* L. (Lamiaceae) towards *A. suum*: 15% aqueous tincture exhibited 100% efficiency against eggs of *A. suum* during 36 h exposure. An alternative for treatment against parasitic diseases of agricultural animals may be flavourings and feed additives with antiparasitic activity. Studies in this sphere are becoming popular in modern veterinary medicine (Ullah et al., 2015; Boyko & Brygadyrenko, 2016, 2018). Many additives have already undergone both *in vivo* and *in vitro* tests (Lee et al., 2008; Cheng et al., 2009; Na et al., 2011; Shen et al., 2012; Yi et al., 2016).

Currently, there are no data on the influence of these and other aromatic substances and preservatives on the process of embryonic development of eggs of *A. suum* and *T. suis*. LD_{50} of cinnamaldehyde (C_9H_8O) for rats, mice and guinea pigs orally was 2220, 2225 and 1160 mg/kg respectively (Jenner et al., 1964). LD_{50} of benzoic acid (C_6H_5COOH) for laboratory animals (intravenous administration to rats) equaled 1700 mg/kg, for cats – 300 mg/kg (Bedford & Clarke, 1972; Jakimowska, 1961). LD_{50} of formic acid for laboratory animals equaled 700, 1100 and 4000 mg/kg for mice, rats and dogs respectively (Von Oettingen, 1960, Montgomery, 2000). LD_{50} of linalool ($C_{10}H_{18}O$) for laboratory rats orally was 2790 mg/kg (Jenner et al., 1964). LD_{50} of citral ($C_{10}H_{16}O$) for rats orally was 4960 mg/kg, for β-ionone ($C_{13}H_{20}O$) was 4590 mg/kg (Jenner et al., 1964). These data suggest relatively good tolerance of mammals to these groups of flavourings. The objective of this article was to assess the effect of cinnamaldehyde, benzoic acid, formic acid, linalool, citral, β-ionone on the development of larvae in eggs of nematode pathogens of ascariasis and trichuriasis in pigs.

Materials and methods

In the experiment on ovicidal activity of flavourings, we used non-invasive eggs of two species of pig nematodes: *Ascaris suum* (Goeze, 1782) and *Trichuris suis* Schrank, 1788. Feces of pigs were collected from yards of private households in Dnipropetrovsk Oblast (Ukraine). Manure was surveyed for presence of eggs of nematodes using McMaster method (Zajac et al., 2011). Then, samples of feces were collected in which the quantity of eggs of one species of nematodes in one gram of feces was no less than 500. Samples (separately with eggs of *A. suum* and separately with *T. suis*) were accurately mixed and average samples were extracted.

Then, the eggs were rinsed. For this purpose, a fecal mass of 10 g was put into a glass cup, the sample was accurately stirred to uniform mass and 100 mL of water was added. The eggs were rinsed several times until pure sediment was obtained. Then, the formed sediment was centrifuged (1,500 rpm) for 2 min. Supernatant fluid was removed. Sediment with eggs from centrifuged test tubes was put by 0.1 mL (on average by 10–20 eggs) into plastic tubes (1.5 mL). Then, 1 mL of solutions (or emulsions) of cinnamaldehyde, formic acid, linalool, citral, β-ionone, suspension of benzoic acid, and also distilled water (control) were added in eight-fold replication. All the reagents were manufactured by Hebei Qige Biotechnology Co., Ltd. (China), Pro Analysi (>99% purity). Immature eggs were exposed to the tested emulsions and solutions in various concentrations (0.1, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 3.0%) for 24 h. Then, eggs of nematodes were rinsed and covered with distilled water. Test tubes were put into a thermostat. The eggs were cultivated at a temperature of 28 °C: *A. suum* – 21 days, *T. suis* – 50 days. Ovicidal activity of the substances was expressed as a percentage of the amount of formed eggs (eggs with larvae inside). The statistical analysis of the results was performed through the set of Statistica 8.0 (StatSoft Inc., USA). The figures show the median, 25% and 75% quartiles, minimum and maximum values. LC50 is expressed as a %: average (x) ± standard deviation (SD).

Results

The experiment revealed that the eggs of *A. suum* were the most resistant to the studied substances. Formation of larvae was observed in more than 90% of eggs exposed to formic acid, linalool, citral and β-ionone in different concentrations (up to 3%) with the exposure of 24 h (Fig. 1).

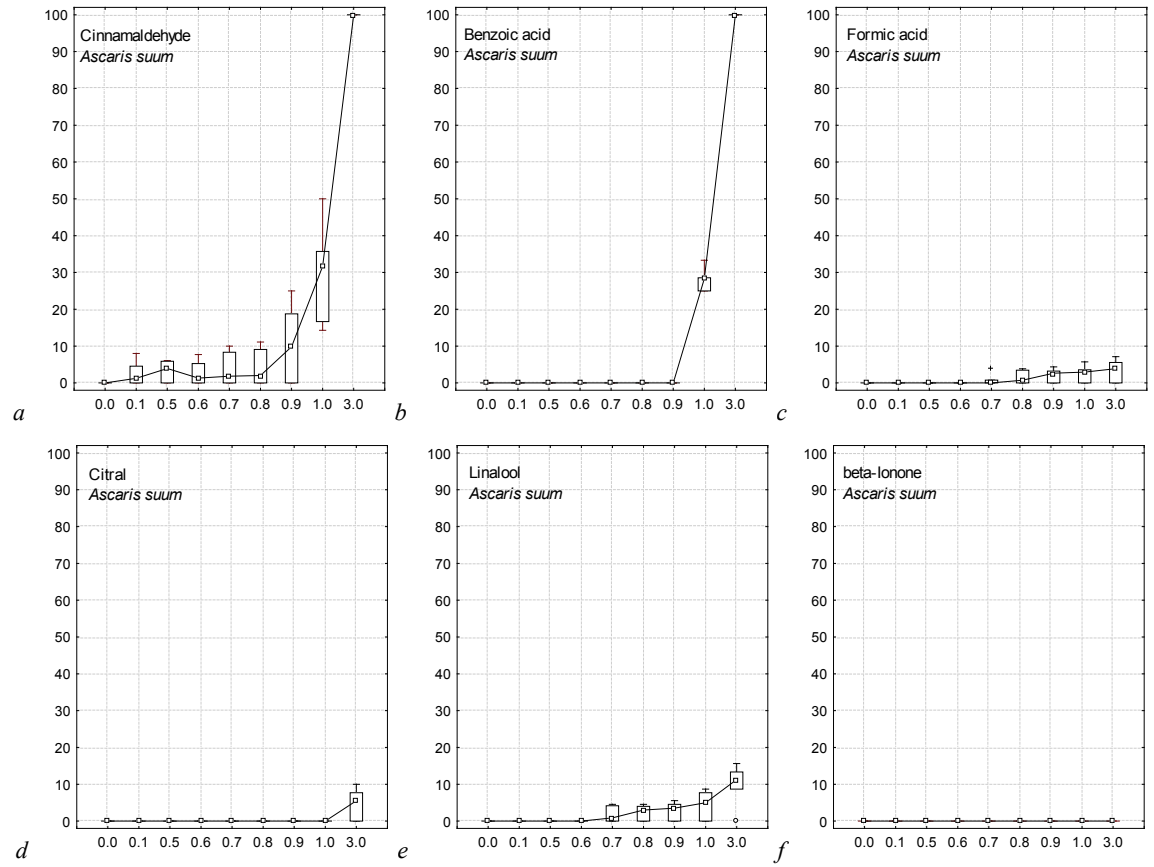

Fig. 1. The impact of certain flavourings and preservatives on vitality of eggs of *Ascaris suum* (Goeze, 1782): the ordinate axis indicates the percentage of living nematodes eggs in the course of the 24-hour experiment; the abscissa axis indicates the concentration of the solution's or emulsion's of active substance (%); the small square in the centre corresponds to the median, the 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)

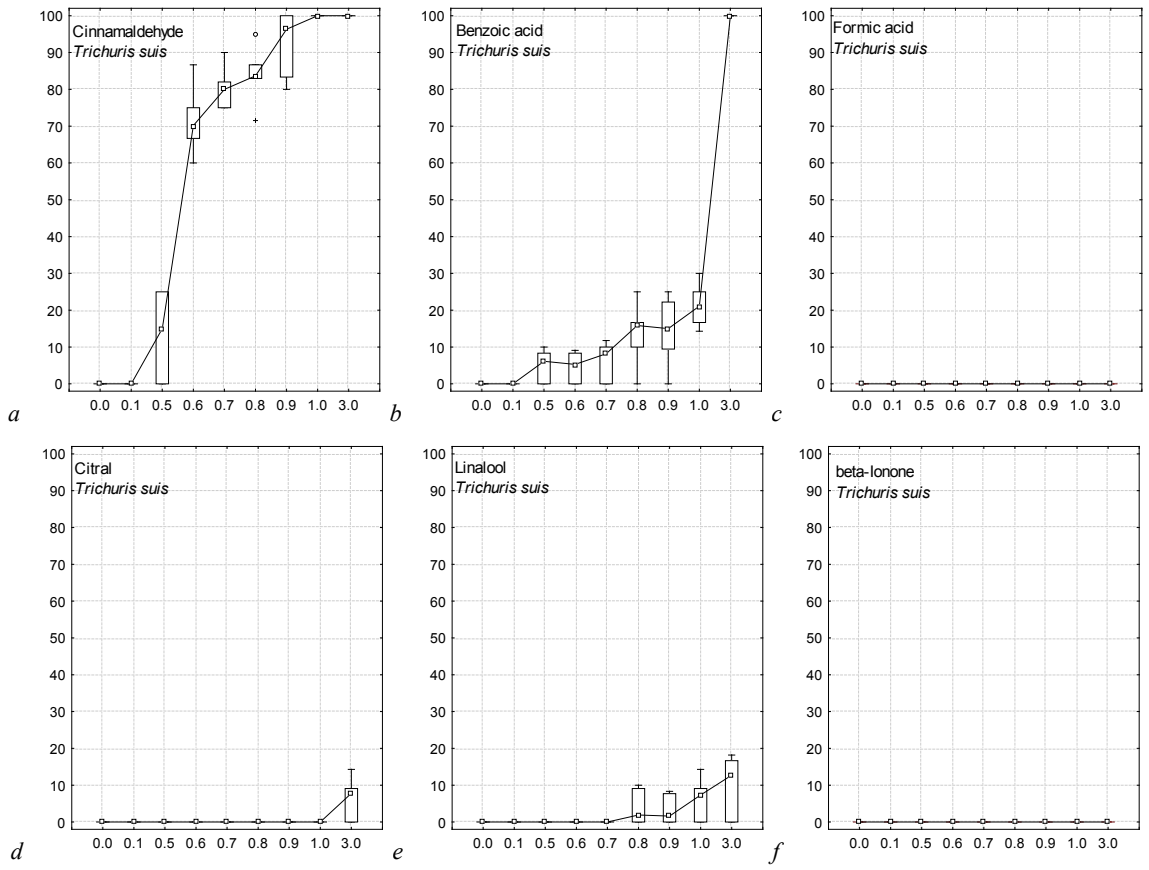

Fig. 2. The impact of certain flavourings and preservatives on vitality of eggs of *Trichuris suis* Schrank, 1788: the ordinate axis indicates the percentage of living nematodes eggs in the course of the 24-hour experiment; the abscissa axis indicates the concentration of the solution's or emulsion's of active substance (%); the small square in the centre corresponds to the median, the 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)

During 24 h exposure to cinnamaldehyde, mortality of 100% of non-invasive eggs (eggs with undeveloped larvae) was seen in the conditions of exposure to 3% emulsion. None of the remaining concentrations of cinnamaldehyde caused death to even 5% of eggs of *A. suum* over 24 h.

Also, a significant influence on the development of eggs of *A. suum* was exerted by benzoic acid. Twenty-four hour exposure to 3% suspension of this acid caused 100% death to eggs of ascarids. During exposure of non-invasive eggs of *A. suum* to benzoic acid in less saturated concentrations, nematode larvae formed in over 70% of cases.

Eggs of *A. suum* were quite resistant to formic acid. Formation of larvae was recorded at 24 h exposure to different concentrations in more than 95% of eggs. Similar results were observed during the influence of citral and β-ionone.

Less resistant to the impact of the studied substances were the eggs of *T. suis*. A significant impact on the development of larvae in eggs was recorded during the exposure to cinnamaldehyde and benzoic acid (Fig. 2). During 24 h exposure to 3% and 1% emulsions of cinnamaldehyde, 100% death of eggs of *T. suis* was observed. The number of invasive eggs gradually increased with gradual decrease of concentration of this substance. A total of 85% of eggs remained vital during exposure to 0.5% cinnamaldehyde. This substance in 0.1% solution took no effect on the development of larvae in 100% of cases.

Benzoic acid showed a lower effect: during exposure to 3% suspension no larvae were seen to form in 100% of eggs. However, the effect of 1% benzoic acid differed from the effect of the previous substance in this concentration: in 80% of cases in eggs of *T. suis* we observed the formation of larvae. Less than 10% of embryos in eggs exposed to lower concentrations of this substance (0.7, 0.6, 0.5 and 0.1%) were observed to be dead. At the exposure of eggs of *T. suis* to formic acid, no effect was observed. Almost all the eggs formed larvae inside regardless of the concentration of this acid. Similar results were obtained also during the influence of β-ionone. Exposure of eggs of *T. suis* to 3% citral and linalool was accompanied by death of about 10% of eggs. The rest of the studied concentrations of these substances took no effect on the development of eggs in 100% of cases.

Discussion

The antimicrobial properties of cinnamaldehyde, benzoic acid, formic acid, linalool, citral, β-ionone are actively studied (Saddiq & Khayyat, 2010; Manu, 2016; Ding, 2017; Brütsch et al., 2017; Alborzi et al., 2018; Durgadevi et al., 2019; Ren et al., 2020). Manu (2016) assessed the antimicrobial efficiency of cinnamaldehyde and geraniol against *Escherichia coli* O157:H7 and *Salmonella enterica*: cinnamaldehyde (2.0 μL/mL) exerted the highest bactericidal effect against both pathogenic bacteria. This author has also determined that cinnamaldehyde has a great potential for the control of the growth of pathogenic bacteria of food origin in cooled fruits and vegetable juices. Brütsch et al. (2017) studied the antimicrobial properties of formic acid produced by wood ants and determined that this acid can also increase antimicrobial properties of tree-collected resin used by ants. Ren et al. (2020) determined the potential of formic acid and monolaurin as an alternative to antibiotics in the diet of piglets infected by ETEC (enterotoxigenic *Escherichia coli*): combination of formic acid and monolaurin in the diet of piglets infected by ETEC can become an alternative to antibiotics for reduction of the inflammatory process.

As an antimicrobial preparation, various authors also use benzoic acid. Ding (2017) assessed antibacterial properties of benzoic acid against *Escherichia coli* O157:H7 and determined that benzoic acid in combination with other substances can increase the safety and quality of fresh products.

Alborzi et al. (2018) also studied antimicrobial properties of different substances and determined the antimicrobial effect of benzoic acid and ethylenediaminetetraacetic acid. They assessed these substances as poten-

tial antimicrobial preparations against bacteria *Escherichia coli* O157:H7 and *Listeria innocua*.

Because the solubility of benzoic acid in water equals 0.29 g/100g of water, the acid perhaps penetrates through the biological membranes of the eggs of nematodes in undissociated form, It is interesting that in our experiment, 0.1% benzoic acid caused no death to eggs of *A. suum* and *T. suis*.

Benzoic acid in the concentration of 0.5% (0.29% in it were in dissociated form in the solution, and 0.21 g/100 mL of water were the weighed amount at the bottom of the test tube) killed around 5% of eggs of *T. suis* and no eggs of *A. suum*. However, in 1% benzoic acid which contained 0.29 as solution and the rest as suspension at the bottom, death of the embryos occurred much more intensively: 20% eggs of *T. suis* and around 28% – *A. suum*. This allows us to state that eggs are affected by both dissociated and undissociated acids (suspension of small undissolved particles of this substance)

According to the results of our previous studies on the influence of cinnamaldehyde and benzoic acid (E_{210}, Codex Alimentarius) on the vitality of invasive eggs of *A. suum*, the lowest LC_{50} parameters were observed for benzoic acid (Boyko & Brygadyrenko, 2017). However, in a previous experiment we observed differences in the LC_{50} readings for cinnamaldehyde and benzoic acid: 0.2437% and 0.1240%, respectively. Perhaps such results are associated with the use of eggs in the previous experiment at the development stage of 21–23 days, and in this experiment – 1–2 days.

In our previous experiments, we studied also antiparasitic properties of these substances against nematode larvae L_{1-3} *Strongyloides papillosus* (Wedl, 1856) and L_3 *Haemonchus contortus* (Rudolphi, 1803) (Boyko & Brygadyrenko, 2019a, 2019b): notable nematocidal activity was displayed by cinnamaldehyde, benzoic acid and formic acid towards larvae of *S. papillosus* and *H. contortus*.

Similar experiments were conducted on eggs of *H. contortus*. Katiki et al. (2017) reported nematocidal properties of cinnamaldehyde. At the same time, the authors indicated antiparasitic effect of linalool against eggs of *H. contortus*. However, this substance had less notable anthelmintic effect. The results of our studies also showed notable anthelmintic effect of cinnamaldehyde against eggs of *A. suum* and *T. suis*. Our experiment with linalool did not show such impact on eggs of *A. suum* and *T. suis*, as on eggs of *H. contortus* in the study by Katiki et al. (2017). Around 90% of eggs of *A. suum* and *T. suis* had formed larvae in the end of the experiment after exposure to 3% emulsion of linalool.

Allowable daily consumption of benzoic acid for humans is 0.005 g/kg (Zu et al., 2017), whereas LD_{50} of this substance for rats equals 1.7 g/kg (Bedford & Clarke, 1972; Jakimowska, 1961). According to the results of our experiments, to kill eggs of *A. suum* and *T. suis* during disinfection of the environmental objects in the housings of agricultural animals, LD_{50} equals 16.9 and 18.0 g/kg respectively. LD_{50} of cinnamaldehyde for agricultural animals, particularly swine, was 1.16 g/kg. This parameter for rats was significantly higher accounting for 2.22 g/kg (Jenner et al., 1964). In our experiment, the value for *A. suum* and *T. suis* was 16.2 and 5.7 g/kg respectively. Thus, eggs of *A. suum* and *T. suis* were more resistant to cinnamaldehyde and benzoic acid than other organisms. Their LD_{50} values several times exceeded this parameter for other organisms.

Conclusion

One of the new spheres in agriculture is the study of anthelmintic properties of flavourings against nematodes of animals and humans. In practice, the food additives with antibacterial effect (cinnamaldehyde and benzoic acid) in intense pig farming could be used against eggs with nematodes of *A. suum* and *T. suis*, and also complexly take effect on pathogenic microorganisms in the housings of agricultural animals (after application to the litter, walls, places of watering, etc). These substances can stop the development of larvae in eggs of helminths and can be used in the future for obtaining preparations for disinfecting objects of the environment. At the same time, in pig farming complexes, use of cinnamaldehyde and benzoic acid would allow reduction in the use of synthetic substances against ascariasis and trichuriasis.

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