

Review articles

Insight into tick biocontrol with special regard to fungi

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ABSTRACT. The epidemiological and epizootic importance of ticks has been known for a few decades since of the discovery of their role as vectors of many new diseases, and the better detection of those already known. Given the durability of chemical preparations in the environment and the increasing problem of developing tick resistance, natural strategies for biological control are sought. A promising alternative to chemical pesticides is the use of entomopathogenic organisms for effective integrated pest management of low environmental impact. A number of promising microbes have been identified during the search for effective means of controlling the tick population, but the knowledge about the impact of these pathogens on the environment and other non-target organisms is still insufficient. Previous research has still not provided a definite answer about the safety of their use. It is known, however, that the chemicals which are currently used have a negative impact on the environment and/or cause resistance. No efficient biocompound has yet been devised for commercial use. Potential microorganisms for tick biocontrol (mainly bacteria and fungi) are natural tick pathogens, living in the same environment. With their adhesive properties, and their ability to digest the cuticle, they may constitute an appropriate ingredient of bioacaricides. Until now, fungal insecticides have been used only to control crop pests.

Key words: entomopathogenic bacteria and fungi, tick biocontrol, microbial entomopathogens, vector control

Ticks of medical and veterinary importance

The need to control haemotophagous ixodid ticks, which are external parasites of many animals and humans, arises from their medical and veterinary significance. After mosquitoes, ticks are the second most well-known vectors of pathogenic viruses, bacteria and protozoans [1–3]. Ticks – obligate, blood-feeding ectoparasites of vertebrate hosts – are important vectors of livestock and human pathogens. While the tick feeds on blood, many microbial pathogens (viruses, bacteria or parasitic protozoans) enter the tissues and organs of the host. They reproduce, disseminate and often cause tick-borne diseases (TBD), some of which, such as Lyme disease, anaplasmosis, ehrlichiosis, babesiosis, tick-borne encephalitis, Crimean-Congo haemorrhagic fever, Rocky Mountain spotted fever, Colorado tick fever, tick typhus, tularemia, heartwater, East Coast fever as well as Nairobi sheep disease, threaten both global public health and economic development worldwide [4]. For example, cattle fever caused by *Babesia* spp., is widespread in Africa, Australia, South and Central America and the USA [5]. Other epizootics are concerned with *Rhipicephalus sanguineus* and *R.* (*Boophilus*) *microplus*, vectors of TBD of dogs and cattle, ticks with the largest geographical distribution, with a range that covers most of the continents [6].

Another disease which has become the focus of epidemiological research over the past few decades is human borreliosis (Lyme disease, LB), which appears to be one of the most widespread tick-borne bacterial diseases [7]. While, in the north-eastern U.S., the black-legged tick, *Ixodes scapularis*, also known as the deer tick, seems to be the primary vector of LB and other infectious diseases [8], in Poland and the rest of Europe, these are believed to be spread by the common tick, *Ixodes ricinus* [6,9–15].

The wide occurrence of *I. ricinus* presents a challenge for its control. It is the most widespread species in all parts of Poland, and has been

identified, for exumple, in the Lower Silesia [16–19], Mazury [20,21] and Lublin districts [22,23]. It lives in moist woodland where suitable hosts, mainly rodents and game animals, are available [6,9,24], although it is also found during its seasonal activity in anthropogenic habitats such as urban parks or allotments [25–28]. In the Polish climatic zone, it spreads LB (caused by *Borrelia burgdorferi* s.l.) and tick-borne encephalitis (*Flavivirus*) among humans during the period spring to late autumn: mostly April–June and September–October. Cases of Q fewer (*Coxiella burnetii*), anaplasmosis (*Anaplasma phagocytophilum*), tularemia (*Francisella tularensis*) and babesiosis (*Babesia microti*, *B. divergens*) have also been recorded [21,29]. In Poland, and the rest of Europe, *I. ricinus* has been confirmed to play an epizootic role in such animal diseases as Q fever, anaplasmosis, tularemia, as well as listeriosis and Lyme boreliosis [6,24,30].

Tick control nowadays

The current worldwide control of tick populations is mainly based on prevention, followed by chemical acaricides [31,32]. Organochlorines, the first synthetic organic insecticides, as well as organophosphates, carbamate and pyrethroid insecticides are synthetic compounds commonly used as inexpensive acaricides, and can be effective in reducing tick numbers in the environment through treating such livestock as cattle or sheep. Unfortunately, some of the widely used chemical acaricides, such as carbaryl or chloropyrifos, may be toxic to vertebrates, and growing widespread arthropod-vector resistance threatens both public health and the global livestock industries [33]. In addition, the effects of less toxic chemical products such as synthetic pyrethroids, which can be effective in small doses, only become apparent after a short time compared to other insecticidal compounds.

All commercial synthetic insecticides are lethal to many invertebrates, including non-target ones including beneficial insects and arthropod predators [4]. As chemical acaricides usually cause drug resistance, new substances are constantly under development. Most *in vitro* studies are carried out away from the host. Despite excellent results, i.e. high tick mortality, the full impact of organochlorine products on non-target organisms remains unknown. Although Oliveira et al. [34] report that fipronil, a component of commercial dog drops/collars, is very effective, the study did not include the impact of the acaricide on the dogs. However, afoxolaner demonstrated high efficiency against ticks (*I. scapularis*) and no effect on beagles [35]. Hunter et al. [36] obtained similar results for fipronil, amitraz and (S)-methoprene against *R. sanguineus*, but their study, like many others, only covered a short time period. Furthermore, as demonstrated by Reck et al. [37] on *R. microplus,* the development of resistance to many new chemical substances made it impossible to determine the effective time of action for each chemical preparation.

Promising ways of tick biocontrol

In view of the many disadvantages of the chemical tick control, new biological, environment-friendly methods are being sought. In tick-abundant areas, attempts are underway to identify natural predators of ticks, including insectivorous birds such as turkey and quail, or rodents. However, the animals consume ticks while grooming, when they are usually engorged after a blood meal. Besides, the effectiveness of these natural predators is often low and poorly predictable [4,32].

The search for new tick control strategies focuses on such biological agents as crystalliferous bacteria, bacilli, and fungi, which have been successfully used to control mosquitoes [38,39]. These entomopathogenic microbes may play a significant role in the environment-safe biocontrol of ticks, as may plant extracts with acaricide properties [40].

Initial attempts, made in the 20th century, relied on the use of parasitic wasps, *Ixodiphagus hookeri*, which laid eggs inside the body of the tick [32]. Subsequent studies in the United States mainly relied on limiting the range of the wild hosts, mainly rodents and game animals, by bio-acaricide treatment: a cost-effective method which reduces the risk of TBD for livestock and humans. In addition, the method can be directed against many species and prevent selection of drug-resistant ticks, when acaricides are repeatedly used [41]. It was used on the white-footed mouse, the main host of *I. scapularis* nymphs and the reservoir of *B. burgdorferi*. The study was based on leaving cotton scraps soaked with bio-acaricide in places accessible to the mice, which used them for nest-building [42]. The adult ticks on the other hand take

deer as their hosts. In the U.S., reducing the populations of white-tailed deer also rapidly reduced the populations of *I. scapularis* [42]. Besides deer hunting, spraying the coat of the deer with acaricides, for example near feeders, can also be used. Acaricides can also be applied directly in tick-abundant places. Soil or lawns can be sprayed with biological acaricides, which is a good solution for protection against ticks in household areas [43].

Entomopathogenic bacteria

Microbial preparations containing entomopathogenic bacteria are very popular and commonly used worldwide for the control of many pest and vector organisms. For a few decades, bacteria such as *Bacillus thuringiensis* (*Bt*) and *B. sphericus* [44–46], with different target spectra, have been used to control mosquitoes and simuliids. The entomopathogenic properties of subspecies and strains of *B. thuringiensis* are highly variable. Most conventional *Bt* products containing specific pathotypes, mainly *B. thuringiensis kurstaki* or *thuringiensis* (pathotype A), *B. thuringiensis israelensis* (pathotype B), as well as *B. thuringiensis tenebrionis* and *B. thuringiensis san diego* (pathotype C) can be toxic or pathogenic to specific groups of insect larvae of the orders Lepidoptera, Diptera and Coleoptera, respectively. Some *Bt* strains are promising bacterial agents against some Protozoa, Trematoda, Nematoda, Hymenoptera and Acari [45].

Bt formulations containing spores and crystal toxic proteins (delta-endotoxin) are typically used in insect control, and need to be consumed by the arthropod to kill it. The formulations are most efficient when applied to larvae or juveniles. In contrast to herbivorous insects such as mosquito larvae, which consume bacterial spores and crystals with water, feeding ticks with *B. thuringiensis* is problematic because they are hematophagous. Attempts to feed hard ticks in the laboratory using artificial membranes (e.g. silicone) to imitate the skin of a host have been successful, and the feeding ticks can be easily infected with the acaricide, but the membrane thickness has to be matched to the hypostome of the tested species, in this case *I. ricinus* [47]. Early studies on the toxicity of *B. thuringiensis* (pathotypes A and C), relying on injecting bacteria into the bloodstream of rats, showed no effect on the health of the rodents [48].

Further attempts have been made at the external treatment of ticks with a suspension of *B. thuringiensis kurstaki*. Immersing *R. microplus* or *I. scapularis* in a solution of bacilli spores and crystals. or spraying their eggs with it, resulted in a high mortality rate of up to 79% after 20 days, and a decreased number of hatched eggs [46,49]. It is suggested that the mechanism of action of *Bt* delta-endotoxins on ticks may be different from what it was understood to be for a number of decades.

The pathogenicity to different arthropod taxa is determined by two main classes of the crystalline delta-endotoxins: the Cry (crystal) and Cyt (cytolic) proteins. These toxins are produced and stored inside the *Bt* cell as parasporal inclusions during the stationary phase. In addition, some strains of *B. thuringiensis* can also produce other useful virulent factors, often with a narrow activity spectrum, such as Vip proteins, P20 protein, Sip proteins and chitinase. These toxins or enzymes produced during the *Bt* vegetative growth stage can enhance the invasiveness and penetration of bacteria into the body of the pest or vector and increase the toxicity of delta-endotoxins [45,50]. Many *Bt* strains, including those used in commercial *Bt* products (*Bt* subsp. *kurstaki* HD-1 or *Bt* subsp. *israelensis* HD-567), have functional genes coding for other virulence factors, known from *B. cereus*, such as degrading enzymes (phospholipases C, shingomyelinase, proteinases, collagenase, nucleases), cytotoxic proteins (enterotoxins, except the emeric toxin, haemolysins), as well as cell surface proteins (flagellin, S-layer proteins) and secondary metabolites. Several strains of *Bt* can produce a broad spectrum of vegetative virulence factors, i.e. beta-exotoxins which effectively control fly larvae, bacteriocins, antibiotics (zwittermicin A) and hydrolytic enzymes [45]. Despite the genetic and phenotypic similarity of *B. thuringiensis* and *B. cereus*, the *Bt* products seem to be safe for humans, animals and the environment [51,52].

The mode of exposing the targets, including ticks to the delta-endotoxins or a combination of spores and delta-endotoxins, for example, oral administration or injection, or direct target sites, can have a significant effect on the pathogenic action of these toxins. Habeeb and Abou El-Hag [53] demonstrated that some of *B. thuringiensis thuringensis* toxins could be lethal to the haemoplast cells of a hard tick, *Hyalomma dromedarii*, and propose an alternative method of tick control using *Bt* products. They suggest that it is possible to transfer bacillus spores from the environment into the tick haemocoel,

where the bacteria germinates and extensively reproduces, causing septicaemia and the death of the host organism. Hassanain et al. [54] showed three commercial *Bt* products containing spore/crystal mixtures of *B. thuringiensis kurstaki*, *B. thuringiensis israelensis* or *B. thuringiensis thuringiensis* to have a potential toxic effect against both soft and hard ticks. Several environmental *Bt* strains (*B. thuringiensis kurstaki*) were shown to be highly toxic to an ixodicid resistant tick: *R. microplus* [46]. Similar results indicating high mortality of adult *R. microplus* females in the presence of *B. thuringiensis kurstaki* strains were obtained by Martinez et al. [40].

Other candidates for tick biocontrol are the symbiotic gram-negative gamma probacteria *Xenorhabdus* spp. and *Photorhabdus* spp. transmitted by two important nematode groups: Steinernematidae and Heterorhabditidae, isolated from the soil [55]. Their life cycle includes a free-living stage in the soil (invasive larva), which is capable of active search for a host, e.g. arthropod pest or vector. After entering the host body through natural integument orifices, the nematode releases its symbiotic entomopathogenic bacteria. These multiply rapidly, overcome the defensive system of the host and produce various virulent substances, including intracellular protein crystals and antibiotics [56], causing the death of susceptible hosts such as weevils – *Hylobius excavatus*, *Liparus glabrirostris* or beetle larvae – *Cetonia aurata* after 24–48 h [57]. These bacteria are heat-sensitive. Increasing the temperature to 80°C and maintaining it for 15 minutes inhibits their activity [58]. The virulence of individual nematodes and their bacteria varies, depends on the tick species and development stage [59–61]. Experiments with *Ixodes ricinus* [60] showed a low mortality of up to 40%, to be associated with three species: *Steinernema carpocapse*, *S. feltiae* and *Heterorhabditis bacteriophora*; most of the mortality was observed for the first species, with unengorged females. The efficiency of nematodes in the biological control of ticks is highest under optimum conditions: high humidity, 20–30°C and little exposure to UV radiation. The nematodes may lose their ability to infect ticks when stored at high temperature or in liquid-containing tanks for a long time [62].

Like fungi, invasive forms of nematodes can be obtained from the soil, their natural habitat, using insect bait methods. The only difference is that the soil moisture should be higher than in the fungi isolation [63].

Entomopathogenic fungi

Fungi are the most commonly used and effective agents in invertebrate pathology, mainly in the control of mites and insects as forestry pests [64]. Many different species of pathogenic fungi are used in pest and vector biocontrol: *Beauveria bassiana*, *B. microplus*, *Metarhizium anisopliae*, *M. flavoviride*, *Isaria fumosorosea* (first described as *Paecilomyces fumoroseus*), *I. farinose* (first described as *Paecilomyces farinosus*), *Lecanicillium* sp., *Simplicillium lamellicola*, *Verticillium* sp., *Aspergillus parasiticus* and *A. flavus*. The most important candidate species for tick control are *B. bassiana* and *M. anisopliae* [4,40]. They are commonly found in the environment, staying in saprogenesis for a very long time [65]. Under natural conditions, spores which have come into contact with the tick cuticle sprout and insert their invasive threads into the body of the host. The fungus is capable of producing cuticle-degrading enzymes such as proteases and esterases, produced in the first 24 hours, as well as chitinases and lipases 4–5 days after infection [66]. The hyphae then start to multiply, resulting in the death of the host. The death of the host usually takes place after the exhaustion of nutrient reserves, but some fungi are able to produce toxic metabolites which accelerate death.

Entomopathogenic fungi can be isolated in a few ways: directly from ticks [60], from soil, mulch or plants using selective media [67] or using insect bait method [68].

Acaropathogenic fungi infect ticks naturally, however, live ticks are their hosts for a short period. As the fungi are usually found in dead ticks [60,63], it is recommended to collect dead arachnids from the area. Typically, moisture and temperature conditions do not allow the fungus to overgrow the host cuticle and produce spores. The surface of the collected host should be disinfected [69] with sodium hypochlorite (NaClO) and 70% ethanol. Ticks prepared in this way are placed in Petri dishes at high humidity and room temperature to allow fungal growth in the cuticle. Spores can be collected in a few days.

Attempts at isolating microorganisms from the soil, mulch or plants may yield a wide variety of fungi, bacteria and actinomycetes. To avoid contamination by non-entomopathogenic microorganisms, selective media should be used [70]. Antibiotics such as tetracycline, chloramphenicol or

streptomycin can be applied for bacterial inhibition [63]. Gram-positive bacteria can be inhibited by applying crystal violet, while non-pathogenic fungi can be eliminated by adding dodine, or cyclohexamide to the media [63,67,71]. Adding benomyl can inhibit growth of *B. bassiana* and *M. anisopliae* [67]. Collected samples (e.g. soil) or a diluted (soil) solution should be put directly on the media. Incubation should be carried out in the dark, at room temperature, for 5–7 days [71]. Each Petri dish should contain 5–15 g of soil: using portions which are too small (1 g) may result in no fungi being present in the sample, as the distribution of the fungi in their natural habitat is usually clustered [63].

Meyling [63] describe a very sensitive detection method using 5–10 live larvae of *Galleria mellonella*, *Acanthocinus aedeilis*, *Tribolium destructor* or *Tenebrio molitor*. They are placed in soil-filled containers at room temperature [69]. The first larvae usually die after a few days [71]. Studies of this kind should be closely monitored, because the mycelium growth on the dead larvae produces spores which will re-infect the soil. Moreover, high levels of soil moisture cause infection by nematodes, not fungi [63]. Dead larvae should be collected and treated as described by the first of the described methods of isolating fungi. When the mycelium overgrows the cadaver, the larva should be only washed in distilled water (not disinfected).

Most of the studies were performed on adult ticks. Questing ticks were collected with flagging method, while engorged ones were collected from cattle pastures. Collecting engorged females made it possible to test the effect of fungal strains on eggs, measuring both the rate of egg production, or the number of hatched larvae, and larvae [72,73]. Testing the fungal preparations on the larvae requires an excellent knowledge of the life cycle of the tick species. It is important to capture the moment when the female begins to lay eggs, as well as the exact time of hatching of the larvae, at which point, it is important to maintain adequate relative humidity (>80%) to avoid the eggs drying. Besides, larvae derived from the same female constitute good material for this type of research, due to their homogeneity and the large number of individuals, which can be as many as a few thousand, depending on the species. However, their small size and fast movement may cause difficulty during the tests. Biocontrol of ticks feeding on the host was also attempted [74]. However, the efficiency of the fungus species used (*Metarhizium brunneum*) was low; it was most effective against the larvae which failed to transform into nymphs (30.1%) despite the presence of the host. Spraying with the substance had a significant effect on the quantity of consumed blood, resulting in a shorter feeding time, at each developmental stage of the tick. No death of feeding females was observed, however, compared to the control groups, the number of harvested eggs was slightly lower.

Overall, the greatest efficiency is observed when fungi were applied to the engorged females, with the egg production rate being much lower than in the control groups. Also the number of unhatched eggs was high [75]. The differences in the mortality between unengorged and engorged ticks in every developmental stage was found to depend on the species of pathogen and tick [60,76–78]. Furthermore, individual isolates of the same species can show different virulence. Fernandes [65] notes that the most virulent strain, the only isolate with synnemata growth, was the one isolated from a human infection.

Most bioassays used *I. scapularis,* a vector tick of LB from the Northern Hemisphere. Both *in vitro* and *in vivo* studies involve spraying the land with solutions containing fungal conidia [43]. Arachnids were collected by the flagging method from an area where the collection was made the previous year without spraying. A significant reduction in the population of *I. scapularis* was noted in the studied areas (74.5–90%).

Only few studies deal with biological control of the common tick, *I. ricinus*, which is the main TBD vector in Poland and Central Europe [60,79]. The studies included all the life stages of the tick, and the following species of fungi: *B. bassiana*, *M. anisopliae* and *Paecilomyces fumosoroseus. M. anisopliae*, isolated from lepidopteran species, was found to be the most effective strain. The greatest mortality (80%) was observed among the unengorged nymphs. The absence of effects observed in the case of engorged nymphs and larvae is accounted for by the changes in the epidermis which impede penetration of substances through the cuticle. *B. bassiana* and *P. fumosoroseus* were slightly less effective. However, different strains of the same species acted with varying degrees of effectiveness.

It is important in *in vitro* experiments that the ticks should be decontaminated in 1% sodium hypochlorite solution [73], physiological saline or distilled water with 0.1% Tween 80 [72] before

using the spores/conidial suspension. The most common way to infect ticks with fungal pathogens is to immerse them in a spore/conidial suspension for 1–3 minutes, dry them on sterile gauze and place them on Petri dishes at room temperature and relative humidity >80%. It should be noted that excessive temperature and insufficient relative humidity have an adverse impact on the survival of hungry ticks [24]. It is therefore necessary to determine the optimal conditions for both ticks and fungi.

To infect ticks with fungi, specimens are placed on a leaf or gauze soaked with conidial or blastospore suspension [60,78], or immersed in the solution for a few minutes, then placed on Petri dishes [75], and incubated at room temperature and 80–100% relative humidity. A conidial suspension based on oil is much more effective than that based on distilled water, because oil has better affinity to the lipophilic and hydrophobic tick cuticle [78].

Preliminary identification of fungi is based on macroscopic observations, noting the colour, shape, size and texture of the colony. Moreover, in microscopic observations, the shape of the conidia is important. However, because polymorphism is common even within populations, the ultimate identification of the fungal species can be obtained through polymerase chain reaction (PCR) [65].

Tick biocontrol in practice

Research continues in the United States, which first initiated biocontrol of tick populations [43,69,80], and other countries, such as Mexico, Brazil or Australia, follow this trend [65,72,81]. In Europe, only a few experiments have been performed on biological acaricides, tested *in vitro* on *I. ricinus* [60,79].

Until now, in Poland, tick control is mainly based on prevention and the use of chemical acaricides for personal or pet protection (e.g. Fiprex, Autan plus). None of the acaricides currently in use is specific to ticks. No fungal or bacterial strains have been isolated directly from *I. ricinus* which could be candidates for its effective biocontrol. Moreover, commercially available preparations such as Boverin, Botani Gard, Es Naturalis O, Metabeave Beauveria, Met 52 Bio-insecticide or Biosar Bio Insecticides used for control of agricultural pests, could also be used in this case. However, although the ones which have already been tested are characterised by high efficiency, as demonstrated in previous studies, many other aspects remain to be researched.

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