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Review
Pregledni rad

ANTHELMINTIC RESISTANCE IN GASTROINTESTINAL NEMATODES OF SHEEP: CURRENT SITUATION AND NOVEL STRATEGIES

ANTIHELMINTIČKA REZISTENCIJA KOD GASTROINTESTINALNIH NEMATODA OVACA: AKTUELNA SITUACIJA I NOVE STRATEGIJE

Filip Štrbac[a*], Dragica Stojanović[b]

[a]Institute for Multidisciplinary Research, University of Belgrade, Serbia
[b]Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Serbia
*Contact author: filip.strbac@imsi.bg.ac.rs

F.Š. https://orcid.org/0000-0002-2666-7992, D.S. https://orcid.org/0000-0003-4781-2623

ABSTRACT

Gastrointestinal nematodes nowadays represent a major obstacle to sustainable sheep farming due to their negative effect on animal health, welfare and productivity. Commercial drugs such as benzimidazoles, macrocyclic lactones and imidazothiazoles have been used with success in previous decades to control these parasites. However, their irrational application has led to the development of anthelmintic resistance and large economic losses, while the situation is expected to further deteriorate in the future due to the spread of resistance and the emergence of multi-resistant nematode strains. Thus, monitoring is of key importance, which involves the application of various *in vitro* and *in vivo* tests, as well as modern molecular methods in order to early detect the development of resistance and monitor the situation in a certain area. In addition, the problem of the exclusive application of chemical preparations is also reflected in the residues in meat and milk, as well as in the environment. This poses a risk to various organisms, including humans. For these reasons, it is necessary to define new strategies, which are based on the rational application of anthelmintics in terms of targeted treatments, targeted selective treatments, but also combination and rotation of preparations. The introduction of alternative methods into practice, such as phytotherapy, i.e. the use of plant preparations such as extracts and essential oils, direct and indirect biological control, development of vaccines, genetic selection of naturally resistant animals with appropriate management of pastures and nutritional status of animals are also needed, all with the aim of reducing application of commercial drugs. This implies an integrated approach to the control of gastrointestinal nematodes, which is the basis of future treatments.

Keywords: gastrointestinal nematodes, anthelmintic resistance, rational use of anthelmintics, alternative strategies, integrative approach

SAŽETAK

Gastrointestinalne nematode danas predstavljaju glavnu prepreku održivom ovčarstvu zbog njihovog negativnog efekta na zdravlje, dobrobit i produktivnost životinja. Komercijalnu preparati poput benzimidazola, makrocikličnih laktona i imidazotiazola su se sa uspehom koristili prethodnih decenija u kontroli ovih parazita. Međutim, njihova neracionalna primena je dovela do razvoja antihelmintičke rezistencije i velikih ekonomskih gubitaka, pri čemu se u budućnosti očekuje dodatno pogoršanje situacije zbog širenja rezistencije i pojave multirezistentnih sojeva nematoda. Zbog toga je monitoring od ključne važnosti, a koji podrazumeva primenu različitih *in vitro* i *in vivo* testova, kao i savremenih molekularnih metoda u cilju rane detekcije razvoja rezistencije i praćenja situacije na određenom području. Pored toga, problem isključive primene hemijskih preparata se ogleda i u ostacima rezidua u mesu i mleku, kao i životnoj sredini. To predstavlja rizik za različite organizme, uključujući i ljude. Iz ovih razloga je potrebno definisati nove strategije, koje se baziraju na racionalnoj primeni antihelmintika u smislu ciljanih tretmana, ciljanih selektivnih tretmana, ali i kombinacije i rotacije preparata. Od izuzetnog značaja je i uvođennje alternatnvih metoda u praksu poput fitoterapije odnosno upotrebe biljnih preparata poput ekstrakta i etarskih ulja, direktne i indirektne biološke kontrole, razvoja vakcina, genetske selekcije prirodno otpornih životinja uz odgovarajuće upravljanje pašnjacima i nutritivni status životinja, a sve u cilju smanjenja primene komercijalnih preparata. To podrazumeva integrisan pristup kontrole gastrointestinalnih nematoda, koji predstavlja osnovu budućih tretmana.

Ključne reči: gastrointestinalne nematode, antihelmintička rezistencija, racionalna primena antihelmintika, alternativne strategije, integrisan pristup

INTRODUCTION

The small ruminant industry today faces several problems that hinder sustainable production. One of the main obstacles are gastrointestinal nematodes (GINs), which are widespread in the world (1). They parasitize in different parts of the gastrointestinal tract, including the abomasum, small and large intestine, and usually have a direct life cycle. The prevalence and distribution of GIN species depends on geographical and climatic conditions, with *Haemonchus* spp. and *Cooperia* spp. being present in subtropical and tropical regions, *Teladorsagia* spp. and *Nematodirus* spp. in temperate regions, while *Trichostrongylus* spp. are present worldwide (2). In addition to these, *Chabertia* spp., *Oesophagostomum* spp. and *Bunostomum* spp., *Marshallagia* spp., *Strongyloides* spp. and *Trichuris* spp. also occur in Serbia (3,4). Therefore, GINs cause high economic losses in various countries and regions, including the Mediterranean region (5-8).
The extent of GIN infections and their negative effects depend on several factors, such as the age of the animal, the breed, the type of parasite involved and the degree of parasitic infection (8). Although these infections are usually subclinical, they still significantly affect the production of meat, milk and wool (5). With a high worm burden, GINs cause clinical infections with signs of anaemia, diarrhoea, anorexia and edema, which can even lead to animal death (9-11). In addition to the effects on animal health, productivity, fertility and welfare, the costs of anthelmintic treatments also contribute to the negative economic impact (6,12). Due to the various modes of causation, the total economic losses caused by GINs are difficult to quantify precisely, but are estimated to be in the hundreds of millions of EUR (6). In addition, GIN infection is predicted to increase the intensity of greenhouse gas emissions in sheep farms by up to 30 % (13).

THE EXCLUSIVE USE OF COMMERCIAL ANTHELMINTICS AS AN UNSUSTAINABLE APPROACH

Commercial anthelmintics are now the main method of controlling GINs in sheep (14,15). These mainly include benzimidazoles, BZ (e.g. albendazole, fenbendazole), macrocyclic lactones, ML (e.g. ivermectin) and imidazothiazoles, IMZ (e.g. levamisole). These drugs have different modes of action, as benzimidazoles bind to the alpha subunit of the β-tubulin protein, ML bind selectively and irreversibly to the subunits of chlorion channels that are activated by various neurotransmitters, while IMZ act selectively as cholinergic agonists (nicotinic receptors) (15). This list can be fulfilled with monepantel, an amino-acetonitrile derivative (AAD), one of the most recently developed anthelmintics with a novel mode of action based on the nematode-specific ligand-gated ion channel subunit as a potential drug target (16).
Various commercial broad-spectrum drugs have been successfully used in the control of sheep GINs over the last 50 years (17,18). However, their inappropriate use has led to the development of anthelmintic resistance (AR) in the parasites, which now occurs with virtually all commercially available drugs, especially BZ (19). In this context, high treatment frequency, inadequate anthelmintic dosing (especially underdosing) and prolonged use of the same anthelmintic class are considered the main risk factors for the development of AR (20). According to a preliminary report, the annual cost of AR in Europe is around €38 million, with a tendency to increase significantly in the future due to the increasing prevalence of resistant populations and the emergence of helminths resistant to several anthelmintic classes (13). Although monepantel has a different mode of action, resistance has already been reported (21), and the current state of the art

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suggests that AR develops in new drugs as early as 3-9 years after launch (18,22).

Other problems associated with the exclusive use of chemical drugs include the environmental consequences of anthelmintic use and public health risks. Most of these drugs are excreted in the faeces and urine, and some of them, such as ML, are not fully biotransformed in the animal organism. They also tend to accumulate in soil and groundwater and thus pose a high risk to many non-target organisms such as beneficial arthopods, including dung beetles, various flies, but also fish and other aquatic organisms (23). Residues of such pharmaceuticals can contaminate meat, milk and by-products and thus pose a serious risk to human health (23,24). For this reason, the European Community has enacted a law that requires systematic and uniform control of veterinary medicines, including anthelmintics, in food in all member countries (25). In addition, the prices of these drugs, such as albendazole and mebendazole, continue to rise despite all these negative effects (26)

MONITORING OF ANTHELMINTIC RESISTANCE

The use of various *in vitro* and *in vivo* methods for the early detection of resistance development is crucial for the definition of appropriate strategies. However, monitoring the presence of resistance in a given area and worldwide is not an easy task, considering the complex mechanisms of resistance development and certain limitations of existing methods. Currently, the most appropriate and widely used method is the faecal egg count reduction test (FECRT), an *in vivo* method based on the collection of faecal samples before and at different time points after treatment and the measurement of the reduction in the number of parasite eggs, usually expressed as eggs per gram (EPG) (27). Several techniques can be used for FECs, including McMaster ili Cornel-Wisconsin or some novel ones such as mini-FLOTAC (28) or FECPAK (29), highly sensitive and practical techniques. In contrast to the other tests, the FECRT allows the evaluation of drug efficacy for all anthelmintic classes in all animal species and for multiple parasite species without the need to sacrifice the animals. In addition, it can be performed on-site without the need for a diagnostic reference laboratory or specialised equipment and/or expertise (27). According to the instructions of the World Association for the Advancement of Veterinary Medicine (WAAVP), a reduction of GIN eggs below 95% indicates the presence of drug resistance. The results of the FECRT should be interpreted carefully when the FEC is low and the results do not always correlate with the worm burden in adult animals, although they are very reliable in young animals (27,30,31). The other *in vivo* method is the controlled efficacy test (CET), which is even more precise than the FECRT, in which the animals are artificially infected, treated and then slaughtered and the worm burden is counted. However, it is very intensive and requires the sacrifice of animals, which makes it less practical (32).

Among the *in vitro* methods, the egg hatch test (EHT) and the larval development test (LDT) are the most commonly used. The EHT is used to measure the ovicidal effect of decreasing concentrations of anthelmintics. After a 48-hour incubation of the eggs with anthelmintics at 27 °C, the remaining eggs and the hatched larvae (L_1) are counted and the inhibition of hatchability is calculated. It is one of the standardized tests (33) recommended by the WAAVP and it was developed only for the detection of AR with benzimidazoles, as they have ovicidal effects (18). However, it is the most commonly used laboratory test in practice. The only *in vitro* test that allows the detection of resistance to all groups of anthelmintics regardless of their mechanism of action is the LDT (30), which is based on the ability of larvae to survive and develop in different concentrations of anthelmintics (18). This test is more intensive that EHT and there are several ways to perform it, including the preparation of suspensions and incubation under conditions of increased humidity for 48 hours, the addition of increasing concentrations of the drug and a re-incubation of 5 days, after which the L_3 are counted to determine the survival of the larvae. The other method is a micro-agar LDT based on the preparation of drug concentrations, their dissolution in dimethyl sulfoxide and dilution in distilled water. After 7 days of incubation of the eggs, drug concentration and culture medium containing yeast extracts and salt solutions, the procedure is the same (30). Both EHT and LDT include the calculation of the IC_{50} parameter, which is used to interpret the results and for comparison.

The other *in vitro* tests used to detect AR are the larval motility test (LMT), the larval paralysis test and the adult worm motility test (AWMT), which are used less frequently than EDT and LDT but can confirm their results. Recently, various molecular techniques based on the detection and/or analysis of nucleic acid molecules (DNA or RNA) have also been used. These tools are able to directly measure the genetic differences between susceptible and resistant populations and can therefore also be used as a means to quantify the composition of the parasite community (34). Listed below are the most common advanced methods that are efficiently used to detect AR: Restriction fragment length polymorphisms (RFLPs), allele-specific PCR (A-SPCR), diagnostic PCR, real-time PCR, direct sequencing, pyrosequencing, deep amplicon sequencing, microsatellite markers, loop-mediated isothermal amplification (LAMP), P-glycoprotein probe, and transmembrane functional analysis (35).

METHODS FOR DELAYING ANTHELMINTIC RESISTANCE AND SUSTAINABLE CONTROL OF NEMATODES – RATIONAL USE OF ANTHELMINTICS

The methods for delaying AR refers to the strategies for sustainable control of GINs in sheep, which are described in our previous review paper (36). Here, an attempt will be made to explore these strategies further with new facts and from a slightly different aspect, especially regarding on how to reduce an application of commercial anthelmintics. As mentioned above, AR is considered an inevitable phenomenon caused by rare mutations, and since it is impossible to stop this process completely. However, there are certain strategies that can be used to delay the development of resistance and slow its spread so that the efficacy of drugs can be maintained for as long as possible. These include strategies for the rational application of commercial anthelmintics based on refugia, that refers untreated hosts or environments that allow drug-susceptible parasites to survive despite exposure to drugs (37). The best known of these strategies are targeted treatments (TT) and targeted selective treatments (TST). TT refers to treating all animals in a given herd at the most appropriate times to reduce the number of treatments in a herd and thus minimize contamination of the pasture with resistant genotypes. This can result in longer intervals between treatments while achieving the same effects (38). The TST is even more specific as it refers to the individual selection of animals within the herd for treatment and uses logical, specific criteria by which this selection is made (39). In this way, only the animals that are susceptible to GIN infection or contaminate the pasture the most are treated, which is a continuous source of worms in refugia (38). The selection criteria are not easy to determine as many different markers can be used as indicators of TST:

1) Parasitological indicators (FEC and determination of EPG)
2) Pathophysiological indicators (anaemia caused by hematophagous parasites – FAMACHA score, DAG score, DISCO score, body condition score)
3) Performance-related indicators (milk production, live weight gain) (39,40)

Each of these markers has advantages and disadvantages, and therefore their combination is most appropriate. For example, in a study by Soto-Barrientos et al. (41), the combination of FEC, FAMACHA and body condition score in sheep and goats in Mexico led to the avoidance of 70% of unnecessary treatments.

Combinations of anthelmintics with similar spectrum of activity and different mechanisms of action and resistance are widely used in several regions of the world for the control of sheep nematodes. This strategy is proposed to enable effective control of nematodes in the presence of single or multiple resistance and to slow down the development of resistance to the individual anthelmintic classes. Three main outcomes are possible when combining drugs: indifference, antagonism, additive or synergistic effects. The aim is therefore to achieve the third option, where the additive effect is given as $(A + B) = 1 - ((1 - a) \times (1 - b))$, (the efficacy of one drug is a and that of the other is b). In contrast, a synergistic effect exists when the response obtained after their combined administration is greater than additive (42). In any case, a fixed-dose combination of anthelmintics could slow down the development of AR by allowing the highest possible elimination of nematodes, with parasites surviving one agent being eliminated by the other. However, the use of anthelmintic combination products does not eliminate the significant resistance risk posed by dosing strategies that allow cattle to graze on clean (low contamination) pastures after treatment (43).

Rotation of different classes of anthelmintics approximately annually has also been widely promoted and adopted as a strategy to delay AR development in nematodes. This strategy is based on the expectation that resistant worms have lower ecological fitness than susceptible worms (at least in the early stages of selection), so that a return to susceptibility can be expected in years when a different class of anthelmintic is used (44). The rotation of drugs also aims to prolong the efficacy of each of them and allow susceptibility to return when they are not used. However, this only appears to be possible when AR is at an early stage of development, even before it can be detected, when natural selection can reduce the number of resistant parasites. Therefore, this strategy can be implemented in novel or future drugs, considering the level of AR development in currently used anthelmintics (45).

METHODS FOR DELAYING ANTHELMINTIC RESISTANCE AND SUSTAINABLE CONTROL OF NEMATODES – ALTERNATIVE STRATEGIES

Phytotherapy

Phytotherapy has been defined by the World Health Organization (WHO) as the medical discipline that allows the correct use, for preventive or curative purposes, of medicinal plants and their derivatives (phytotherapics or phytomedicaments), in relation to the pharmacological properties of their chemical constituents (46). In the context of the treatment of gastrointestinal nematodes in sheep, this practice is a relatively new approach on which studies have been conducted in the last 20-25 years, especially in recent years (47). Herbal anthelmintics include different types of preparations that can be used in animals in different ways. First, some reports claim that sheep infected with gastrointestinal nematodes consume more plants containing bioactive anthelmintic compounds than uninfected animals, which is known as zoopharmacognosy or self-medication (48). In these cases, animals on pasture focus on, for example, tannin-rich legumes to restore or maintain their health. It has been shown that sheep infected with *H. contortus* eat more tannin-rich *Lysiloma latisiliquum* (Tzalam) than uninfected animals (49). Villalba et al. (50) demonstrated that parasitized lambs showed a greater preference for alfalfa (*Medicago sativa*) and a mixture of 90 % alfalfa and 10 % quebracho tannin than non-parasitized animals, while these differences disappeared when the parasite load was removed by regular therapy. A better understanding of this bihevioral mechanism can help researchers develop innovative and more sustainable management strategies to improve ruminant health and welfare (48).

These findings can also be applied in the context of supplementary feeding of animals to treat or prevent diseases caused by GINs (51). Although bioactive metabolites can reduce the digestibility of food, this approach has been shown to reduce the number of nematode eggs in feces, but also the number of parasites present in general, and also negatively affect the development of some species, including *H. contortus* and *T. colubriformis* (52). This includes a large number of plant species, such as hornworts and greater trefoil (*Lotus corniculatus*, *L. pedunculatus*), sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychis viciifolia*) (51). In some cases, bioactive molecules can enter the feces and interfere with the larval hatching process or even the mobility of infective larvae, which means a reduction in the potential infectivity of eggs in the feces and less contamination of the pasture by larvae (52).

Herbal products such as extracts or essential oils can also be used to treat GIN infections. Plant extracts are defined as concentrated preparations of liquid, solid or viscous consistency containing a variety of secondary metabolites in different concentration ranges (53). These include various alkaloids, glycosides, phenols, terpenoids and flavonoids. Extracts can be categorized as aqueous or alcoholic, and various methods can be used to obtain plant extracts, such as maceration, infusion, percolation and decoction, but also Soxhlet extraction, microwave or ultrasound-assisted extraction, etc. (54). On the other hand, essential oils (EOs) are highly concentrated substances extracted from different parts of plants (leaves, stems, flowers, seeds, roots, etc.) and contain a mixture of volatile compounds produced by the secondary metabolism of aromatic plants (55). Therefore, EOs are pure and highly concentrated, while extracts are diluted but still highly potent, and this is the main difference between these two plant products (56). EOs also contain a large number of bioactive components from different chemical groups, such as hydrocarbon terpenes, terpenoids (which can be various phenols, alcohols, aldehydes, ketones, oxides, esters), phenylpropanoids, etc. (57). Like extracts, EO can also be obtained by various extraction methods, including distillation (hydrodistillation and the so-called steam distillation), solvent extraction, supercritical fluid extraction, etc. However, both plant extracts and EOs have the similar problems with the variability of their chemical composition depending on the many factors such as genetic characteristics of plants, climatic and microclimatic

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factors, soil properties, the presence of some organisms and microorganisms in plant environment, the time of harvest and extraction methods, which can hinder the standardization of these products (58). Also, their active ingredients are degradable and can be inactivated to some extent in animal organism, which can affect the efficacy *in vivo*. However, there are different ways on how to overcome this problem listed in our previous review manuscripts (36,47), which includes besides all the use of encapsulation techniques to protect the active ingredints or the application of extracts or EOs with other methods in integrated approach.

The evaluation of the anthelmintic efficacy of essential oils against GINs was also the topic of our research group. In two separate studies, more than twenty EOs were tested against GINs in sheep. To evaluate their anthelmintic potential *in vitro*, an egg hatch test was carried out in which oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), winter savory (*Satureja montana*), summer savory (*Satureja hortensis*), fennel (*Foeniculum vulgare*), basil (*Ocimum basilicum*), peppermint (*Mentha x piperita*), wild mint (*Mentha spicata*) and hyssop oil showed the highest efficacy. The effect varied depending on the oil used, the chemotype of the plant and the concentrations used. At some concentrations, the effect of some of these EOs was higher than that of thiabendazole, the positive control applied at standard concentration. Five EOs were selected also for an *in vivo* study performed by using faecal egg count reduction test. Oregano and peppermint showed the strongest effects when applied intraruminally, which meant the highest field efficacy for some of the plant products at the tested dose (150 mg/kg). The chemical composition was determined for all EOs tested, and carvacrol, thymol, anethole, p-cymene, γ-terpinene, carvone, pinocamphone and linalool were identified as the bioactive compounds most responsible for the anthelmintic effect. Both laboratory and experimental tests were performed on animals with natural mixed infections, identifying the genera *Haemonchus*, *Trichostrongylus*, *Teladorsagia* and *Chabertia* in the coproculture study. Importantly, physical observation and analysis of hematological and biochemical blood parameters in the animals during the field trials did not reveal any toxic effects or side effects at the doses tested, suggesting the safety of these natural products (59-63).

Despite some limitations, the use of EO and plant extracts in the control of sheep GIN has many advantages. Firstly, their rich chemical composition can contribute to high efficacy against different parasite stages (eggs, larvae, adults), as shown in various studies so far (47,59-63). So far, they are known to have signicant negative effects on the survival, reproduction, development, behavior and metabolic pathways of nematodes (64). Since their bioactive compounds belong to different chemical groups with different mechanisms of action, the likelihood of developing AR is probably lower than with commercial drugs. Environmental and public health aspects are also in favor of herbal anthelmintics, as they are natural and biodegradable. They are also often considered safer for hosts than synthetic chemical compounds. Finally, the large number of plant species offers the possibility to find the most suitable ones, also from a financial point of view, especially in countries with a developed biodiversity such as Serbia (47,59-63).

Biological methods

Biological control (or biocontrol) is defined as any activity of one species that reduces the adverse effect of another (64). In the context of sheep GINs, this refers to the use of their natural enemies to reduce the number of free-living stages on pasture. Biocontrol can be direct, when the other species have a direct effect on the GINs, or indirect, when they destroy the habitat of parasites (12). Several organisms can be used for direct biocontrol of GINs. The most studied are nematophagous fungi, carnivorous fungal species found worldwide. Their mechanisms of interaction with nematodes can be divided into four groups:

1) nematode-feeding fungi that use sticky traps (nets),
2) endoparasitic fungi,
3) opportunistic fungi that colonise nematode eggs, cysts and females (this applies above all to plant-parasitic nematodes), and
4) toxin-producing fungi (65)

The first two groups are considered to have the greatest potential, especially the nematode-feeding fungi (genera *Duddingtonia*, *Arthrobotrys* and *Monacrosporium*). They can be used to reduce the number of infective GINs larvae on pasture (12). In most studies, these fungi are administered via the feed in the form of sodium alginate pellets. Since the spores only activate and attack the GIN larvae after they have passed through the gastrointestinal tract and are excreted in the faeces, they have no side effects for the hosts (66). Among these fungi, *D.*

flagrans shows the highest potential, achieving an effect of 37.6–91.5 % on sheep GINs (12).
Other organisms that can be used for direct control are *Bacillus thuringiensis*, which produces δ-endotoxin (crystal protein) that shows high efficacy *in vitro* against adult *H. contortus*, *T. colubriformis* and *O. circumcinta*. Various viruses, the soil amoeba *Theratromyxa weberi* and protozoa are also mentioned (67). Free-living nematodes such as *Butlerius butleri* and the mite *Lasioseius penicilliger* have also shown potential for biocontrol of GINs, but may pose a risk to non-target organisms as they are not selective (12). Dung beetles and earthworms are most often mentioned in the context of indirect control, as they feed on and decompose faeces that contain GIN larvae. During feeding, both can consume nematodes in the soil or faeces or actively participate in the destruction of eggs and larvae. They can also transport them to deeper soil layers, where it is practically impossible for the larvae to reach the surface as infective larvae (67).

Vaccine development
Vaccination is considered a sustainable intervention strategy to control GIN infections in livestock, including sheep (68). They are considered environmentally friendly and, similar to phytotherapy, are likely to be less susceptible to the development of AR (69). Currently, progress has been made in researching vaccines against GINs in many economically important species such as *H. contortus*, *O. ostertagi*, *T. circumcincta*, *C. oncophora* and *T. colubriformis* (68). In general, these vaccines can be classified as follows:
1) Hidden antigens, which are generally found in the intestines of the parasites and are not recognized by the sheep's immune system
2) Natural antigens that are expressed during infection and recognized by the sheep (70)
The list of potential antigens is long and includes H11, H-gal-GP, ES15/24, thiol-binding fraction (ES), AC-5 (ES), rPEP1, rMEP1, rMEP3, rMEP4, rHc23, rES15/24, TciAPY-1 + TciMEP-1, mTciAPY-1 + TciMEP-1, somatic antigen, etc. These antigens were isolated from adult GINs and different larval stages ($L_{3\text{-}5}$), but also from other nematodes (*Caenorhabditis elegans*), bacteria (*E. coli*, *P. pastoris*) or insect cells. The vaccines also contain various adjuvants and are administered i.m., s.c. or intrarectally at different intervals depending on the vaccination schedule. However, only one vaccine called "Barbervax" (https://barbervax.com/) against one of the GIN species (*H. contortus*) in sheep has been commercialized so far, which is based on natural antigens derived from the glycoproteins of the intestine of adult worms (68). This vaccine is available in Australia, the UK and South Africa and showed an effect on FECRT of more than 80 % with a high antibody titer in two different feeding regimens (71). Vaccines investigated so far were only directed against one GIN species, which is a problem as mixed infections occur in most practical cases. The main problem is the genetic variations and immunoregulatory properties of gastrointestinal nematodes, which complicate the development of vaccines (72). However, with the development of bioinformatics and methods such as reverse vaccinology, the immunoprophylactic and immunotherapeutic spectrum to combat these parasitic pathogens is expected to expand (70).

Genetic selection
One of the methods to mitigate the effects of GINs in sheep is also to select animals that are naturally resistant to these infections in order to increase host resistance. This is only possible if the desired trait must have non-zero heritability (a trait with $h^2 = 0$ has no additive genetic variance and a trait with $h^2 = 1$ is completely determined by additive genetic effects). In this context, breeding selection is based on two traits: resistance, which refers to the ability of an animal to reduce parasite load, and resilience, which represents the ability of an animal to maintain its performance in the face of infection. Similar to the TST, various markers can be used to measure resistance (73). For the FEC, various qualitative reviews show that the heritability of resistance in animals ranges from 0 to 0.65 (74), with typical values of 0.2 to 0.4 (75). For FAMACHA scores and nematode-specific antibody responses, the heritability is 0-0.4 (76,77). Since higher resistance is associated with higher animal production, breeding for higher resistance should also lead to better performance. For example, some studies have already shown that genetic selection has reduced FEC by almost 70 % (78). However, these measures are difficult to implement on small production farms, and technical and infrastructural issues should also be considered in genetic selection programs, which are a particular problem in less developed countries (Zvinorova et al., 2016).

Other methods

Pasture provides the environment for parasite eggs and larvae. Therefore, certain pasture management practices that attempt to keep the pasture clean result in lower worm infestations (79). The aim of these strategies is to provide low-risk pastures (with low larvae numbers) for the most susceptible animals (weaning and lactating ewes, young animals). The aim is also to avoid exposure that would lead to clinical disease and loss of production, while allowing sheep to build up immunity to parasites (45,79). Three main strategies are proposed to reduce pasture contamination: a preventive strategy, in which animals are prevented from contaminating pasture by moving clean animals to clean pastures; an avoidance strategy, in which moderate existing infection is eliminated by anthelmintic treatment and movement of treated animals to clean pastures; and a dilution strategy, in which resistant adult animals are grazed together with susceptible animals to dilute their parasite egg-enriched feces. Based on these strategies, the following grazing management practices are proposed (79):

1) Pasture rotation system – this involves subdividing the pasture where each part is grazed for a short period and then rested for a longer period to induce death of larvas;
2) Combined cropping/livestock system – larvae numbers are reduced over time by resting the pasture for a period of time while hay/silage is grown and harvested;
3) Alternate grazing system - grazing different species, e.g. sheep with cows, as they do not share common parasite species;
4) Dose & Move – moving animals from pasture to pasture after deworming;
5) Stocking density – lowering the stocking density, as a high number of animals increases the larval load
6) Grass height – as the majority of larvae live in the first 5 cm of vegetation, the animals should be grazed on longer (10 cm) vegetation;
7) Grazing time – limit the grazing time in strong sunlight, as the larvae move to the top of the grass when light intensity is low;
8) Zero grazing – practiced for fat lamb production, where animals are kept in a dry barn without grass.

In addition to these strategies, supplementary feeding could be used to increase the resistance of sheep to GINs. Indeed, a balanced diet provides an adequate source of nutrients and an acceptable parasite load that allows for optimal productivity levels. This refers to various vitamins (especially A, D and B-complex), minerals (zinc, iron, cobalt, sodium, potassium and phosphorus) and proteins, which are crucial for the development of functional immunity against parasites. In this way, a reduction in the pathophysiological effects of parasite infestation and improved productivity could be achieved while reducing the use of anthelmintics (52). Copper oxide wire particles (COWP) have also shown promising anthelmintic activity against GINs in small ruminants when administered as an oral bolus. Although the exact mechanism of action is unknown, it is known that COWP reduces both fertility and the number of *H. contortus* worms in the abomasum. Importantly, oral administration of COWP has also been shown to be a safe and effective method (80).

Combination of different strategies – *integrated approach*

Since all of the above strategies have their advantages, but also limitations and the exclusive use of commercial drugs is no longer sustainable, many researchers agree that a combination of different methods should be performed in GINs management programs. Firstly, the alternatives mentioned can enhance the effect of the regular drugs. For example, it has been shown that the combination of carvacrol in conjunction with nicotinic acetylcholine receptor agonists such as imidazothiazole or GABA receptor agonists such as avermectins and piperazine could have a synergistic effect against various nematodes (81-82). Specifically, *Nigella* sativa oil has been shown to enhance the effect of ivermectin against *H. contortus*, but also against some other parasites such as *Moniezia expansa* and *Fasciola gigantica*, compared to independent use (83). In a study by Vilela et al. (84), the combination of *D. flagrans*-based pellets and levamisole hydrochloride (5%) was shown to have a synergistic effect, as the group of animals receiving both treatments had lower EPG levels compared to the isolated application of the drug. Copper chloride and copper sulphate in combination with nitroxynil showed up to 52% increase in efficacy beyond the expected additive results, indicating a synergistic/drug enhancing interaction (85).

In some studies, different alternatives in combination showed high anthelmintic activity. For example, Bombou et al. (86) investigated the combination of mixed grazing and dietary supplementation to improve the protective response of the host and the production performance of goats.

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Both strategies had a positive effect - FEC was significantly lower in the mixed grazing groups, while PCV, body condition score and live weight were significantly higher in the supplemented animals, suggesting that this combination can improve animal productivity. In a study by Werne et al. (87), the synergistic effect of combining a relatively resistant breed and a diet rich in condensed tannins against sheep GINs could not be clearly demonstrated, but a proportion of only 55% sainfoin in the diet resulted in a lower FEC compared to the control group. The combination of the ethanol extract of *Ananas comosus*, drenched weekly for 6 weeks using a stomach tube (100 mg/kg), with 1 g of the fungal product *Clonostachys rosea* given daily, effectively reduced faecal egg and larva counts, larval development and the number of infectious larvae (L_3) in the pasture (88). It can be concluded that an integrated approach, based on the rational use of anthelmintics and the use of alternative strategies, should form the basis for the future treatment of GIN infections in order to reduce the use of commercial drugs, combat anthelmintic resistance and achieve sustainable control.

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