Nutritional and Biological Control Synergism against Gastrointestinal Nematodes in Small Ruminants

Sahoo A* and Khan FA
Central Sheep and Wool Research Institute, Avikanagar-304501, Rajasthan, India

*Corresponding author: Sahoo A, Principal Scientist & Head, Division of Animal Nutrition, Central Sheep and Wool Research Institute, Avikanagar, Rajasthan, India 304501, Fax: +91-1437-220163, Tel: +91-1437-220143, E-mail: sahooarta@yahoo.com


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Abstract

Parasitic gastroenteritis caused by gastrointestinal nematodes (GIN) continues to be a major constraint against profitable small ruminant production. The increasing prevalence and severity of anthelmintic-resistant nematodes in many parts of the world has led to a search for non-chemical control options. Approaches alternative or adjunct to chemotherapy for the control of GIN in ruminants are being increasingly important in India because of rapidly developing anthelmintic resistance in nematodes and the increasing concern of chemical residues in animal products and environment. Significant advances have been made in biological control by exploiting nematophagous fungi in different parts of the world including India. Initial studies in India demonstrate that GIN control by larvael trapping fungi looks promising but suitable system for delivering the fungi to ruminants and practical dosing regimes integrated with nutritional and managemental control measures hold promise for sustainable control measure. Synergism of fungal control measures with feeding and nutritional manipulation accentuate correction of nutrient deficiency in grazing animals as well as recuperation of supra-nutritional demand in the event of GIN infection. This papers reviews the work done on both these aspects including delineation of possibilities for a synergistic approach for control of GIN infection in small ruminants. Adoption of this synergistic model for GIN control measures will definitely reduce parasite pathogenesis and the use of chemical anthelmintics, as well.

Keywords: Nutrition; Nematophagous fungi; Gastrointestinal nematodes; Small ruminants

Introduction

Over the last 50 years a number of very effective anthelmintic drugs have been discovered and made available to farmers to successfully control gastrointestinal nematodes (GIN). However, resistance to these anthelmintics is becoming increasingly prevalent in grazing ruminants [1-4] and thus the development of variable degrees of resistance among different species of GIN has been reported for all the major groups of anthelmintic drugs. Frequent usage of the same group of anthelmintic; use of anthelmintics in sub-optimal doses, prophylactic mass treatment of domestic animals and frequent and continuous use of a single drug have contributed to the widespread development of anthelmintic resistance in helminthes [5]. Restrictive legislation regarding the chemical control of nematodes in organic livestock production together with a wish to eliminate drug residues in agricultural products is additional driving force for the interest in alternative parasite control methods [6]. Moreover, anthelmintic resistance has great epidemiological bearing as resistant parasites may be more fecund and pathogenic as compared to susceptible populations of the same. At the same time, they may have an increased establishment rate in the host and the increased survivability of free-living stages. The degree and extent of this problem especially with respect to multidrug resistance in nematode populations is likely to increase. Experience from the development of anthelmintic resistance suggests that modern control schemes should not rely on sole use of anthelmintics, but employ other, more complex and sustainable recipes, including parasite resistant breeds, feeding and nutritional management, pasture management, nematode-trapping fungi, antiparasitic vaccines and botanical dewormers. In this context there is an urgent need to re-evaluate our parasite control measures to ensure the sustainability of small ruminant production necessitating finding possible synergistic alternatives to chemical treatment.

Abbreviations: GIN: Gastrointestinal nematodes; DM: Dry matter; VFI: Voluntary feed intake; FEC: Faecal egg count; EPG: Eggs per gram; CP: Crude protein; P: Phosphorous; Mo: Molybdenum; Cu: Copper; Se: Selenium; CWOP: Copper oxide wire particles; UMMB: Urea-molasses mineral blocks; CT: Condensed tannins; Hb: Haemoglobin; PCV: Packed cell volume; CFB: Complete feed blocks; CSWRI: Central Sheep and Wool Research Institute; NDDB: National Dairy Development Board
Fungal control of gastrointestinal nematodes

The philosophy behind biological control of GIN is that by using one of the natural enemies of nematodes, it will be possible to reduce the infection level on pasture to a level at which the grazing animals avoid both clinical and subclinical effects due to parasitic nematodes. Thus, this method of controlling GIN using nematophagous fungi is fast becoming an area of applied research with the possibility of becoming an important integrated element in developing new sustainable control strategies [7-11]. It is directed against the free-living stages without any effect on the parasitic phase within the host and therefore, the idea of using nematophagous fungi to control parasitic nematodes is based on the reduction of the larval level in the faeces before larvae reach the vegetation, which requires a high density of spores in the faeces. Although no biological control agent will eliminate the number of infective stages to zero, the grazing animals will constantly receive a small amount of parasitic larvae and thereby should be able to develop a natural immune response.

Biological control of parasitism started as early as in the 1940’s but with little success. In the 1960’s evidence was found for predaceous fungi working against parasitic nematodes [12], but further experiments were not taken up. The major breakthrough was achieved at the beginning of the 1990’s when Larsen et al. [13] selected fungi capable of surviving in vitro conditions simulating the passage through the gastrointestinal tract of cattle. Research with nematode-trapping fungi has documented the potential as a biological control agent against the free-living stages under experimental and natural conditions [14]. These fungi occur in the soil throughout the world where they feed on a variety of free-living soil nematodes. These fungi capture nematodes by producing sticky, sophisticated traps on their growing hyphae. The two genera tested, Arthrobotrys and Duddingtonia, displayed the ability of gut survival with the genus Duddingtonia performing significantly better. After passing through the gastrointestinal tract, spores germinate and looped hyphae trap the developing larval stages in the faecal environment. This technology has been applied successfully under field conditions in all livestock species, and is an environmentally safe biological approach for control of worms under sustainable, forage-based feeding systems. D. flagrans displayed excellent gut survival ability and the findings triggered the detailed research in this area and since then many trials have been conducted in several countries in which faecal samples of sheep have been screened for pre-dacious fungi [15-17]. Even after the promising results of the feeding trials with D. flagrans [18-20] researchers con-tinued to work with a wider range of fungi until the end of the 1990’s when trials with fungi other than D. flagrans started to decrease. In the first years, most of the in vivo trials focused on the potential of larval reduction by the fungus without giving any significance to the amount of chlamydospores necessary for a successful reduction.

D. flagrans belonging to the class of Fungi Imperfecti (Deuteromycotina) is a nematode-trapping fungus utilizing nematodes either as the main source of nutrients, or supplementary to a saprophytic existence. The fungus is found world-wide in many different habitats, but is especially frequent in organically rich environments such as compost and faeces. Fungi are naturally picked up by grazing ruminants and subsequently excreted in the voided faeces [15,21-24]. There is necessity of isolating fungi locally as the performance of biological control agent in a particular climatic condition may differ from those prevailing where the fungus originated. Under experimental conditions, the net trapping nematophagous fungus D. flagrans have shown the ability to effectively control the developing GI infective larvae on pasture and subsequent levels of infection in various livestock species. D. flagrans has been used successfully against ruminant GIN and is a promising candidate for biological control of these organisms [25-27]. In India work on biological control of GIN was initiated in 1999 with attempts to isolate local strains of nematophagous fungi, A. oligospora and D. flagrans from fresh faecal samples of grazing small ruminants [28-30]. Both the fungi produce sticky 3-dimensional nets raised above the growth medium (Figure 1). The isolates of D. flagrans had potential to survive passage through the gastrointestinal tract of sheep and subsequently to destroy H. contortus larvae in faecal cultures from fungus fed sheep [30-32]. There is production of large numbers of thick-walled, asexual resting spores that serve as survival propagules, called chlamydospores (Figure 2), which are likely the basis for its survival through the digestive tract of ruminants. Further, Khan et al. [33] delivered 6 million chlamydospores daily and consecutively for six weeks to sheep experimentally infected with Haemonchus contortus and found a very low level of larval count in the pasture compared to control (600 vs 4000 L/kg DM).

Nutritional control measures

Voluntary feed intake (VFI) depression has generally been recognised as a major feature of the pathogenesis of GI infections and reductions of intake between 10 and 30% have been commonly found [34,35]. Dietary modification aimed at increased nutrient availability e.g. nutrient enrichment, can offset the negative impacts of infection even at low levels of feed intake. Similarly, increasing VFI through availability of palatable feed resources/complete feed can make up the deficiency by overcoming anorexia in parasitised animals. It has been demonstrated that extra protein supplementation could overcome some degree of anorexia and pathogenicity in the experiments with H. contortus [36-38]. A reduction in VFI can thus severely influence the protein-energy economy of the host by substantially reducing the total nutrient availability for the anabolic processes [39]. It has been suggested that anorexia may result from pain and discomfort associated with infection or be the result of hormonal feed-back mechanisms from disrupted gastrointestinal function [40]. The degree of anorexia may be affected by the level of infection, species of parasite and its site of infection and by the breed, age and resistance status of the host. Anorexia appears to be greatest during the acquisition phase of immune response [39]. Nutrition of the host could have the potential to affect how rapidly immunity is acquired and expressed in defence of the parasitism. Host protein and energy nutrition can affect greatly, the expression of immunity in sheep a-
nd goats, and in severely undernourished animals that loose protein mass, the acquisition of immunity is severely impaired [41]. Van Houtert et al. [34] observed that supplementation of a hay diet with 150 g protected protein or 100 g fish meal per day affected the rate of worm expulsion after 10 weeks of trickle infection with *Trichostrongylus colubriformis* and the apparent rate of expulsion was related to the level of supplementation. Animals that get challenged by nematode parasites need a surplus of desired nutrients either for an immune response or to maintain performance (e.g. growth, pregnancy, and lactation). Therefore, resistance and performance competes for resources, particularly for protein. Kahn [42] confirmed this phenomenon in a study to state that supplementation of ewes during the peri-parturient period enhanced their ability to resist infection and consequently reduces faecal egg output in animals that are previously selected for nematode resistance. Several ovine studies have shown that improved protein nutrition reduces parasite infestation. This is assumed to be mediated by enhanced host immunity, which may be especially important with selection for immunity to gastrointestinal parasitism [43,44]. The precise regulatory mechanisms through which the improved host nutrition enhances resilience and resistance to parasites are still largely unclear and merits systematic studies. There is need to obtain quantitative information on the nutritional costs of initiating and maintaining the cellular and humoral immune response in parasitized hosts.

*Figure 1:* Three dimensional hyphal loop of *Duddingtonia flagrans* after inoculation of *Haemonchus contortus* infective larvae on agar medium

*Figure 2:* Chlamydospores of *Duddingtonia flagrans* obtained from barley culture (×400)
**Role of macronutrients**

The nutritional status of the host has long been considered as an important factor in influencing the host-parasite relationship and the pathogenesis of parasite infections. While the mechanisms underlying this fact are not clear, energy balance and supply is increasingly considered to be an important factor in the immune response [45]. An improvement in energy supply was reported to improve resistance to *Teladorsagia circumcincta* infection of growing lambs [46] and, a similar response to supplementary feeding was found to natural GI infections in Criollo kids [47]. The effect of *D. flagrans* as a biological control agent appeared clearly enhanced both in magnitude and duration by energy supplementation [48]. Studies on the role of fat reserves on the immune response to GI infection in sheep [49] have shown that fat mass stored by ewes was related to the response of immunity to GI parasites and this commenced at an early stage of infection affecting the establishment and development of incoming larvae, thereby reducing faecal egg excretion and affecting pasture infectivity. Furthermore, the differential response on FEC and worm counts between fungitreated groups that showed a similar level of fat reserves at slaughter, appears to be in line with the fact that the nutritional effects of controlling the number of worms established was found to be more effective at lower than at higher levels of infection. In the same line, Gomez-Rincon *et al.* [48] observed the interaction between fungus and barley supplement on EPG and total worm burden to establish that the combination had a greater effect as a method of parasite control than when applied separately.

A variable degree of protein deficiency does occur in grazing ruminants depending on seasonal availability of pasture and other forages. Van Houtert *et al.* [50] showed that protein supplement reduced production losses, enhanced expulsion, reduced the need for drenching significantly, increased wool production and fibre diameter and reduced FEC. In field studies Knox and Steel [51] observed increased live-weights of lambs at weaning, increased reproduction rates in maiden ewes and reduction in faecal egg output in grazing sheep supplemented with urea-molasses blocks. Coop and Holmes [43] reviewed several studies and was of the opinion that protein supplementation appears to have no influence on the initial parasite establishment but on established infection and the immune response, which is indicated by a reduced FEC and worm burdens and by enhanced resistance to re-infection. Numerous studies have examined the interactions between protein nutrition and the response to nematode parasitism in small ruminants [52-58]. Houdijk *et al.* [59] opined that optimized host protein nutrition could be able to reduce the establishment of parasites around the periparturient loss of immunity. Protein intake does not influence initial infections in naïve sheep, but with trickle infections (serial doses of infective larvae more comparable with natural transmission), experimental evidence for sheep showed that higher protein intake (>120 g CP/kg DM) increased immune responses as measured by reduced egg counts and worm burdens [43]. Several studies thus suggest that under drought conditions, species unable to maintain adequate nutrition, mainly low-quality feeders, are less able to cope with gastrointestinal parasite infections. In particular, during dry periods, reduced protein intake seems to be associated with declining resilience and resistance to infection. Louvardini *et al.* [55] reported that diet supplementation with high protein improved resilience and resistance to natural infection by endoparasites during the rainy season and in the dry season there was a decrease in both of these traits, which were intimately linked to the quality of available forage under tropical conditions. Inadequate uptake of CP is considered to be the principal determinant in grazing animals with sub-clinical GIN infection [35].

**Role of micronutrients**

Types of diet and availability of vitamins, minerals and other nutrients are directly related with susceptibility of animal to the parasites. Supplementation of minerals and vitamins can play a key role in affecting ruminant susceptibility. Minerals like zinc, iron, cobalt, sodium, potassium, phosphorus, etc. are very much essential for proper functioning of cellular and immunological response to develop functional immunity against the parasites [60]. Many nutritional deficiencies, specifically, zinc deficiency causes atrophy of lymphoid tissues leading to lower numbers of immune cells and functional defects during antigen-specific responses [61,62]. Given the paramount role of the gastrointestinal-associated lymphoid tissues in inducing and regulating immune responses to intestinal parasites and in orchestrating responses in the spleen and peripheral circulation, it may be concluded that zinc deficiency (in association with energy restriction) exerts profound effects on the gut mucosal immune system, leading to changes in systemically disseminated immune responses and, importantly, to prolonged parasite survival. Studies on the relationship of phosphorous (P) with *T. vitrinus* worm burden in sheep suggest that increased P supply decreases mean worm burden [63]. It was observed that the weight gain of lambs infected with parasites increased by 40% when the P level of the diet was 0.28% compared to those fed a low P (0.18%) diet [64]. Addition of molybdenum (Mo; 0.05 mmol/kg DM) to the diets of lambs reduced the number and length of *T. vitrinus* worms established and the FEC and number of *H. contortus* worms recovered from the abomasum [65] after trickle infections with the two parasites. McLure *et al.* [66] suggested a critical dietary Mo concentration of 6-10 mg Mo/kg DM for maximum host resistance to *T. colubriformis*. Sheep on this Mo intake showed a 90% reduction in FEC and worm burdens compared to lambs offered diets with Mo concentrations below or above this range. It may be suggested that Mo could enhance the inflammatory response by increasing superoxide radical concentration in the mucosa either directly or by reducing the effectiveness of local trace element dependent anti-inflammatory enzymes. Trace elements like Cu, Mo, and Se are known to influence the host resistance to nematode infection with a probable direct anthelmintic effect and by influencing host metabolism [52]. They found reduced worm burden (*H. contortus, O. circumcincta*) in lambs by administering copper oxide wire particles (CWOP) in the diet. Iron supplements are also recommended where animals are affected by blood sucking worms, like *Haemonchus* spp. Burke *et al.* [67] evaluated the efficacy of combined treatment of CWOP and *D. flagrans*.
against GI nematodes in lambs in USA. There was no adverse effect of COWP on the ability of fungus to inhibit residual larval development after COWP treatment. Cobalt deficiency may also promote parasitism as it is essential for vitamin B12 synthesis.

Vitamins A, D, and B complex are integral in developing immunity to parasites. Vitamin A is essential to improve the intestinal epithelial integrity [68] and its deficiency most likely disrupts the intestinal immune system and weakens the host defense against intestinal parasites. Vitamin B12/Co deficient animals may become predisposed to high FEC in pregnant sheep [69]. The co-enzyme forms of the vitamin—adenosyl cobalamin and methyl cobalamin – are important in the conversion of propionate to glucose and the methylation of homocysteine to form methionine, respectively. Failure of either pathway could decrease critical amino supply to the immune system and the homocysteine–cysteine pathway may be particularly important.

Looking at the essentiality of minerals and vitamins, the grazing animals should always have access to mineral block and vitamin supplements to compensate for the deficiencies in pastures. These licks can form a vehicle of oral inoculation of fungal zoospores for lowering the parasitic larvae in the grazing pasture. We need to evaluate survival of fungal zoospores and accordingly, the dosage schedule for administering through salt licks, urea-molasses mineral blocks (UMMB), urea molasses liquid supplement, urea-molasses semi-solid cake, etc.

Role of phytoadditives

The therapeutic use of medicinal plants as a substitute to synthetic drugs for controlling GIN in small ruminants can be envisaged through strategic feeding regimen at times of higher seasonal incidence or as part of daily feed supplements/additives. The herbal supplements also possess some unique active constituents that preclude worms to develop resistance against them. Different medicinal plants have been investigated by researchers for their adulticidal, larvicidal and ovicidal activity to GIN [70-73]. Sujon et al. [74] screened ten indigenous medicinal plants having anthelmintic action and found significant and potent antinematodal effect in ethanol extracts of Labanga, Neem, Karolla and Pineapple at dose of 100mg/kg. Balamurugan and Selvarajan [75] evaluated methanolic extract of Indigofera tinctoria and observed a significant anthelmintic property in a dose dependent manner giving shortest time of paralysis and death with 100 mg/ml concentrations, comparable with the standard drugs. Phytochemical analysis of the crude extract revealed presence of polyphenolic compounds, tannins, as one of the phytoconstituent and they correlated this activity to some synthetic phenolic anthelmintics e.g. niclosamide, oxyclozanide and bithionol that are shown to interfere with the energy generation in helminth parasites by uncoupling oxidative phosphorylation [76]. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal [77] or glycoprotein on the cuticle of the parasite and cause death [78]. Condensed tannins (CT) have shown biological effects that may aid in the control of dewormer-resistant internal parasites and thus forages rich in CT have potential to be a component of GIN control programs [79-82]. Zaman et al. [83] evaluated the anthelmintic activity of a herbal formulation based on aqueous extracts of leaves of Azadirachta indica and Nicotiana tabacum, flowers of Calotropis procera and seeds of Trachyspermum ammi and found a maximum of 96.2% reduction in FEC at a dose rate of 4mg/kg body weight to sheep naturally parasitized with mixed species of GIN. Salifou et al. [84] studied the efficacy of an aqueous extract of leaves of Chenopodium ambrosioides against nematode parasites in goats and observed significant decrease of faecal strongyle eggs, over 70% in 3 days and almost 100% in 5 to 6 days. They observed a parasite clearing effect in the abomasum and small intestine and recommended a dose as low as 1 ml/kg body weight for three days to be appropriate for prescriptive control of GIN. Pathak et al. [58] incorporated leaf meal mixture of Ficus insetorcaria and Psidium guajava in the ratio of 70:30 to provide CT (1.5% of DM) in H. contortus infected sheep and observed a discernible positive impact on N-retention and haematological parameters (Hb and PCV) and an inhibitory effect on intensity of H. contortus infection. Similarly, Kimani et al. [85] found significant reduction in FEC (84%) when sheep fed herbal leaf mixture containing Entada leptostachya and Prosopis juliflora (500 mg/kg body weight).

A number of several other plants that have been used sporadically for controlling internal parasites in ruminants but most of this information is anecdotal and needs further verification if these plants/herbs are to be used effectively. Many botanical dewormers that could be added in animal feed includes garlic, pumpkin seed, worm wood (Artemisia spp.), neem seed, tansy (Tanacetum vulgare), wild gingers (Asarum canadense), mustard, common juniper (Juniperus communis), male fern radishes, raw grated turnips or horseradish, pyrethrum (Chrysanthemum cinerariifolium) [86,87]. Thus, use of herbs/medicinal plants in routine feeding practice as a preventative/treatment measure without allowing the GIN to establish resistance may be considered a feasible approach. However, more extensive studies are needed to be directed towards experimental validation of plants, isolation and characterization of active constituents, clinical evaluation and ascertaining of their specific mode of action against GIN so as to establish as an effective alternative to chemical anthelmintics. Further, incorporation of these phytochemical rich plant biomass or their active principles in the dietary of animals would probably ensure ‘organic consumerism’ of the livestock products from small ruminants not exposed to chemical drugs. The feed supplements/additives can be included in the ration as complete feed (block/pellet), through concentrate mixture, nutrient enriched pellets or blocks (CFB, UMMB etc.) to provide both nutrition as well as therapeutic/preventive control of GI parasitism. Amalgamation of biological control through nematophagous fungi in this delivery system may further widen the scope of sustainable parasite control programme in grazing ruminants.
Combining biological and nutritional control strategy

Delivering fungal zoospores

There are two possible ways to reach high spore density in faeces, the first is to artificially inoculate the faeces and the second way is to administer the spores orally. Since the first way seems not viable the only possibility adheres to discovering fungal zoospores that are able to survive the gastrointestinal tract of ruminants and then defecated onto the pasture. This complex problem may have been one reason for the rather slow progress of science concerning this topic. The only delivery system is incorporating the fungal spores into supplemental feedstuffs that must be fed daily to ensure that all animals consume an equivalent amount of feed for a standard period no fewer than 60 days. This eventually involves feeding and nutritional management, which can be directed at GIN control strategy. Thus, both biological and nutritional control measures would act synergistically against parasite pathogenesis and sustain small ruminant productivity.

It was demonstrated that the fungus was effective in reducing larval numbers in faeces in sheep that were offered blocks containing D. flagrans [87]. They suggested that supplementary feeding or block administration might provide options for product delivery. In another study, Waller et al. [14] demonstrated that D. flagrans that was pressed into an intraruminal controlled-release device retained viability for up to 9 months of storage at 4°C. In addition, the device was effective at substantially reducing the number of infective larvae that developed in cultures of faeces collected from sheep infected with H. contortus for up to 3 weeks after treatment. Such a device would provide an option for animals that are either not supplemented or are housed in remote locations (i.e., range conditions). In Malaysia pen feeding trials were conducted using naturally and artificially infected H. contortus and local isolate of fungus either in a grain supplement or incorporated into feed blocks [24]. Fungus survived the gut passage in sheep and at about 1x 10⁶ spores/animal/day, reduced the number of developing larvae in faecal cultures by more than 90%. Chandrawathani et al. [88] found satisfactory results while assessing the efficacy of fungus feeding daily as a feed supplement or incorporated into feed blocks in a small scale field trial. Young sheep receiving the fungus through both the methods did not suffer clinical haemorrhage and control animals required the salvage anthelmintic treatment. Long term field studies were conducted on two government managed sheep farms in different agro climatic regions in Malaysia [89]. The results have shown a benefit of using fungus as a daily feed supplement with rapid rotational grazing. In Brazil, Rocha et al. [90] assessed the efficacy of local isolate of D. flagrans against trichostrongyle infections in sheep at pasture throughout 6 months. Pellets prepared from fungus mycelia in liquid culture medium were mixed with the diet at a concentration of 1g pellet per 10 kg live weight twice a week. Such feeding of animals with pellets containing fungus had no benefit to the prophylaxis of nematode infections. Aguillar et al. [91] evaluated the use of multinutritional pellets (MP) containing urea, wheat coat, soy meal, molasses, and chlamydospores as vehicles for oral administration in sheep against H. contortus. The workers concluded that the use of MP containing D. flagrans chlamydospores could be considered as a viable alternative control tool against sheep haemorrhage. Santurio et al. [92] observed a significant reduction in the number of nematode eggs per gram of feces and in the larval availability on herbage (difference of 37.6%) in comparison to the control group after the oral administration of lyophilized chlamydospores of D. flagrans. Control animals needed to be dewormed three times during the experiment, whereas the fungus-treated animals maintained a low parasite load, independent of seasonal variation.

Like other countries Indian researchers too made efforts to find out a suitable and cheap method for delivering fungus, which could be integrated with existing animal husbandry practices without causing problems to the farmers (Table 1). The occurrence of night housing of animals in India provides the practical option of deploying fungus spores in a feed supplement. The incorporation of chlamydospores of D. flagrans in concentrate feed pellets has been trialed at National Dairy Development Board (NDDB), Anand, however, with a very poor shelf life of spores [93]. The fairly long shelf life of desiccated chlamydospores led to being used for top dressing of concentrate feeds. A device was developed and evaluated at Central Sheep and Wool Research Institute (CSWRI), Avikanagar (Rajasthan) to deliver nematophagous fungus D. flagrans to sheep by using local fungal isolate. Two isolates of nematophagous fungi, Arthrobotrys oligospora [94] and one isolate of D. flagrans have been obtained from the fresh faeces of sheep [33]. Bulk production of D. flagrans was tried on soaked and sterilized wheat, maize and barley grains. After 9 weeks of growth, estimation of chlamydospores from cultures was done and chlamydospore concentration was found to be 2, 10000, 560000 and 800000 per gram of grains of wheat, maize and barley, respectively confirming barley as the suitable substrate for harvesting fungal zoospores [95]. Chlamydospores were obtained from the grains in powder form by lyophilization. The powder was filled in capsules in order to develop a delivery device (Figure 3). Capsules were fed to H. contortus infected sheep and effect of fungus was evaluated on the development of infective larvae in faeces. In another approach, sterilized barley grains supporting the fungal growth were incorporated into straw based complete feed blocks (CFB, Figure 4). The viability of fungal spores was assessed for the biological control of ovine H. contortus. Two groups (fungus-treated and control without fungus), each consisting of five which were artificially infected with H. contortus. The treated group received fungus incorporated CFB (400 g block per lamb delivering 6 million chlamydospores) daily during 6 weeks. Fungal chlamydospores survived well in CFB with very little moisture (11.8%) when stored at room temperature. Mean larval development was significantly (P<0.001) lower compared to control from day 1 to the end of the feeding. Larval recovery from the grass samples started from 2nd week of the experiment and it increased markedly on the plot grazed by the control group reaching as high as 4000 L/Kg DM compared to less than 600 L/Kg DM in the plot grazed by experimental group fed on CFB with fungal spores. No significant difference was observed in the number of eggs per gram of
faeces between both the groups. However, significant (P<0.001) reduction in developed larvae on coprocultures was seen for fungal spore incorporated group. Low levels of larval counts were maintained in the plot grazed by fungus treated group throughout the experiment. The study demonstrated that CFB feeding might prove a potential means of delivery of *D. flagrans* to sheep for successful biological control of *H. contortus*.

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**Table 1**: Biological and nutritional control measures against gastrointestinal parasitism in India

**Figure 3**: Capsules containing chlamydospores of *Duddingtonia flagrans*

**Figure 4**: Fungus incorporated complete feed blocks (CFB)
Activity of D. flagrans

The challenge remains to increase the effectiveness of D. flagrans and its optimal delivery for effective parasite control strategy. Oral administration of the fungal spores through feeding of CFB allowed simultaneous deposition of fungal spores and parasite eggs and since, D. flagrans is only active within the faecal deposit it prevented transmission of third-stage larvae from the faecal deposit onto the pasture [20,96] thereby reducing subsequent FEC in the treated animals. In the process, it might take an extended period of time to gain the cumulative effect of this strategy depending on the initial infection level of the pasture in question and continuance of this biological control measures.

Dosage: A continuous uptake of fungal zoospores by the host animal that can be released onto the pasture through faeces to exert its nematophagous activity is considered important while assessing the dose and its scheduling. Single and sustained dosing experiments with locally isolated D. flagrans at the dose rate between 5×10^6 and ×10^8 chlamydospores per day resulted in more than 80% reduction in larval number in faecal cultures [97]. During a pasture plot study, where faeces collected from sheep given a daily dose of chlamydospores at a rate of 5×10^6 was deposited on pasture plots, Faedo et al. [20] found significantly lower numbers of larvae recovered from pasture contaminated with faeces from fungus fed sheep than from plots contaminated by control group of sheep. Knox and Faedo [8] conducted a field study to determine the effect of feeding 2×10^6 daily for 6 months to lambs on pasture and found reduced FEC as well as improvement in live weight gain. Feeding trials were conducted by Sanjal and Mukhopadhyaya [98] with stall-fed sheep parasitised with H. contortus. Animals were offered concentrate feed which had been top-dressed with desiccated chlamydospores for 10 days. It was observed that sporé level of ×10^6/kg body weight virtually eliminated larvae from both the pasture and the faecal cultures. In another study in New Zealand, the fungus has been found effective in reducing larval numbers on pasture grazed by goats fed a daily dose of chlamydospores at the rate of 5×10^6 per animal per day [11]. Terrill et al. [99] tested various levels of daily dosing (ranging from 5×10^4 to 5×10^7 spores/kg body weight mixed in a complete diet) to goats in the Southeastern U.S. and found a significant dose response for controlling parasitic larval development in goat faeces. In a second experiment, the researchers fed 2.5×10^6 spores to does every day, every other day, or every third day and found that daily fungal spore feeding provided more consistent larval reduction than intermittent feeding. In France, the ability of the fungus to reduce the number of infective nematode larvae in coproculture was investigated in goats using different doses (0, 1.25 × 10^7, 2.5 × 10^7, 5 × 10^7 chlamydospores per kg body weight daily) by oral administration or by voluntary consumption in the feed during natural or experimental infections with nematodes [100]. The results indicated that a minimum daily dose of 5×10^7 chlamydospores per kg body weight must be used to ensure a high treatment efficacy and that daily administration is preferable for maintenance of efficacy over time in farm conditions. The effectiveness of combination of selective anthelmintic treatments and fungus feeding (at the dose rate of 0.5×10^8 spores per kg body weight daily) was evaluated in goats in South Africa [101]. Although no improvement in parasite control was evident by the deployment of fungus, only very few animals required the anthelmintic treatment in fungus fed group. Gomez-Rincon et al. [48] observed excellent reduction in trichostrongyle infection in lambs given the fungus at the dose rate of 0.5×10^7 chlamydospores per kg body weight daily mixed in 100 g of barley. We [95] at CSWRI also evaluated the dosage scheduling in sheep and found effective delivery of 6×10^6 chlamydospores daily for 6 weeks for controlling H. contortus larvae in pasture.

Biological control and animal performance

Research results are available from all over the world showing parasitological as well as animal performance benefits of the strategic use of D. flagrans against GI nematodes of small ruminants. We can assess the relevance of fungus in controlling GI nematodes by looking into the research results from temperate/ sub temperate and tropical/ subtropical regions of the world. Among temperate/ sub temperate regions, Denmark ranks first where Githigia et al. [19] assessed the preventa–tive effects of D. flagrans under field conditions in lambs. With regards to the tropical/ subtropical regions of the world, a survey conducted yielded 16 isolates of D. flagrans from fresh faecal samples collected from grazing livestock in Australia [15]. In USA, Pena et al. [102] found the fungus highly effective in reducing H. contortus larvae in faeces of sheep. In Louisiana (USA), daily feeding of fungus to grazing ewes reduced larval population in faeces which reduced pasture infectivity and subsequently nematode burden in tracer lambs [103]. The daily feeding of spores was found effective in reducing a predominantly H. contortus population on pasture. From 2002 to 2004, three field trials carried out by Larsen [104] demonstrated a positive effect on production with increased weight gains in lambs after feeding chlamydospores daily to ewes and lambs for two months in the first half of the grazing season. Fungus could pass well through both sheep and goat and was equally effective against GI nematodes in laboratory faecal cultures [105]. The evaluation of D. flagrans on the two farms in Sweden from 2001 to 2003 was the only set of trials that had overall positive results. FEC and worm burdens were comparatively low throughout the evaluated time, indicating that the farms had reasonably good helminths control strategies [106]. In the mountain regions of Spain, D. flagrans spores fed to sheep at critical times of GIN epidemiology resulted into significant reduction in larval numbers on pasture, which could further improve lamb performance [48].

Contrary to this, another field trial undertaken in Northern Germany investigated the influence of D. flagrans on the infection risk for sheep and goats under set-stocked conditions and found no significant differences in FEC or pasture infectivity between control and fungus receiving sheep [107]. Similarly, Eysker et al. [108] evaluated the efficacy of feeding fungal spores on parasitic gastroenteritis in lambs kept under an evasive grazing system in the Netherlands and observed a high reduction in yield as a result of fungal application in the faecal cultures but no differences between groups for FEC, pasture larval counts, worm counts and weight gain. Faessler et al. [109] also found significant suppression of larval development in faecal cultures during the fungus feeding period but no significant effect on the parasitological parameters when adult sheep were given fungus chlamydospores on three farms in Switzerland.
Combinatorial approach

In most of parasitism, the economic losses are actually not attributable to mortality but due to impaired productivity of animals. In view of increasing incidence of anti-parasitic drug resistance in animals, there is an urgent need to design sustainable parasite control strategy which must include on the host as well as off the host control measures to harvest the maximum productivity from the animal [110]. Combination of more than one strategy always plays a significant role to control GIN infection in grazing ruminants, but a very few systematic studies have been conducted so far. In India, CSWRI has undertaken some studies to assess the effect of biological (nematophagous fungi, D. flagrans) control in association with dietary supplementation strategy by delivering the fungus in complete feed block (CFB) post-grazing [95]. Since night housing of sheep and goat is a common practice in our production system, administration of fungus through CFB seems to be a practical way of employing biological control agent. It may thus be recommended that in times of deficit feed intake from grazing, the delivery of nematophagous fungi through CFB would be of added advantage of meeting the minimum feed and nutrient intake, specifically the CP requirement in grazing ruminants, thereby integrating nutritional and biological control. Further, drenching animals with an effective antinematicidal medicinal plant before releasing them for grazing is a main factor to control nematodiasis as the pasture is less likely to be contaminated with nematode larvae for subsequent infection. Further, combining nutritional supplementation and phytotherapy with biological control seems more practical, feasible and suitable to combat GI parasitism in grazing ruminants. The likely responses are i) presence of indirect synergism between both substitutes (supplementary diets improve immune system of infected animals, whilst ethnobotanicals kill worms); ii) nematophagous fungi lessens pasture worm burden; iii) better nutritional status provides increased resilience; iv) supplemental feeding post-grazing ameliorates periodic and hidden nutritional deficiency. Besides, the alternatives are easily available, economically reasonable, culturally acceptable by the farmers, environmentally friendly, relatively safe and has no residual effects on food chain. We believe strongly that this combined approach will solve the essence of the problem including anthelmintic resistance if accompanied with pasture rotation.

Conclusion

Increasing incidence of anthelmintic resistance, increasing awareness of issues involving environmental contamination with chemicals and consumer pressure to reduce drug residues in meat and meat products demand an alternate non-chemical parasitic control strategies. D. flagrans is considered the most promising species for biological control against GIN infestation of livestock. The success of this strategy depends on survival of fungus in the gut and pass out onto the pasture through faeces prevent infective larval development for subsequent control in grazing animals. The nutrition-parasite interaction and the fact that long-term effect of D. flagrans on animals’ levels of infection could be affected by a differential immune response, emphasizes to explore to what extent an enhancement in the animals immune response through nutritional intervention could improve the fungus efficacy as a practical biological control agent of parasitic nematodes in pastured ruminants. In this endeavour, combining nutritional input and ease in delivery of fungal zoospore through supplementation of feed block has promise to provide both protection against parasitism and resilience in sheep flock that are subjected to seasonal feed deprivation and thereby sustaining production.

Reduction in pasture infectivity will be an important advancement in small ruminant production and D. flagrans has the potential to be used for the purpose. However, it should not be considered as an agent to completely control GIN in livestock. It must be a part of an integrated control system utilizing minimum chemotherapy, grazing management, vaccine and other novel methods such as condensed tannins and utilizing genetically resistant breeds of sheep. The advantage that the fungus will have is that it has the potential to reduce the dependence on anthelmintics thereby addressing the concerns of drug resistance development, residues in meat and increased environmental toxicity.

Authors’ Contributions

First author contributed to nutritional role and combinatorial approach while the second author to biological control of GIN. Both the authors read and approved the final manuscript.

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