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REVIEW ARTICLE

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The use of RNA interference for the management of arthropod pests in livestock farms

L'uso di RNA interferente per il controllo degli artropodi infestanti negli allevamenti

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Abstract

Pest management in farm animals is an important action to contain economic damage to livestock production and prevent transmission of severe diseases to the stock. The use of chemical insecticides is still the most common approach followed by farmers; however, avoiding possible toxic effects on animals is a fundamental task for pest control measures compatible with animal well-being. Moreover, legal constraints and insurgence of resistance by target species to the available insecticidal compounds are increasingly complicating farmers' operations. Alternatives to chemical pesticides have been explored with some promising results in the area of biological control or the use of natural products as sprays. The application of RNA interference techniques has enabled the production of new means of pest control in agriculture, and it is opening a promising avenue for controlling arthropod pests of livestock. Transcript depletion of specific target genes of the recipient organisms is based on the action of double-strand RNAs (dsRNA) capable of impairing the production of fundamental proteins. Their mode of action, based on the specific recognition of short genomic sequences, is expected to be highly selective towards non-target organisms potentially exposed; in addition, there are physical and chemical barriers to dsRNA uptake by mammalian cells that render these products practically innocuous for higher animals. Summarising existing literature on gene silencing for main taxa of arthropod pests of livestock (Acarina, Diptera, Blattoidea), this review explores the perspectives of practical applications of dsRNA-based pesticides against the main pests of farm animals. Knowledge gaps are summarised to stimulate additional research in this area.

KEYWORDS
Acari, biopesticides, biosafety, Diptera, honeybees, livestock, pest management, poultry

INTRODUCTION

Arthropods associated with farm animals cause serious threat to farmers' economic activities and animal well-being in direct and indirect manners. Feeding by insects and mites causes bleeding, wounds, tissue consumption and stress to animals; moreover, arthropods may be vectors of pathogens causing serious diseases, especially when they inflict wounds to animals.

Estimates of the economic impact of arthropod pests have been conducted in different countries (Grisi et al., 2014; Kamut & Jezierski, 2014; Rodríquez-Vivas et al., 2017; Taylor et al., 2012). Estimations vary according to farm animal species, production conditions and the end product of the zootechnical activity. Overall, it is quite clear that arthropods are a major source of loss of income for farmers in the order of several hundred million dollars per year.

Establishing economic injury levels for arthropod pests of livestock is quite challenging, due to the complexity of the production system and to the fact that animals can withstand low levels of ectoparasite populations; tolerance to annoyance caused by arthropods may also vary with the age of animals under consideration. Most commonly farmers use insecticides to control pest arthropods based on rough estimates of their populations according to their personal experience or leaving to pest control companies to decide treatment schedules.

Pyrethroids and neonicotinoids are the active ingredients most commonly used in controlling flies or selective acaricides, such as isoxazoline, are used to manage Acarina pests (Sparagano et al., 2022). Most neonicotinoids are currently prohibited in the European Union due to the reported cases of impacts on non-target organisms (Hladik et al., 2018), and a recent assessment of the US Environmental Protection Agency confirming 'likely adverse effects' of three commonly used neonicotinoids on non-target species may trigger further actions in the near future (https://www.epa.gov/pesticides/epa-finalizes-biological-evaluations-assessing-potential-effects-three-neonicotinoid).

Due to their genetic plasticity and the high number of generations, arthropods can rapidly develop insecticide resistance, which is one of the major causes of failure of treatments for insect pests (Mallet, 1989). Cases of resistance to pyrethroids have been reported in different geographic areas (Brito et al., 2019; Freeman et al., 2019; Tainchum et al., 2018), and the increased selection pressure due to their repeated use over time is expected to increase cases of resistant populations. A paradigm case is that of *Musca domestica* L. (Diptera: Muscidae) for which 425 cases of insecticide resistance have been reported so far, involving 65 different active ingredients, including carbamates, organophosphates, organochlorides, neonicotinoids, abamectin and spinosad (https://www.pesticideresistance.org/display.php?page=species&arId=151).

Due to these serious shortcomings of using synthetic pesticides, and considering the expected rise of pest population as a consequence of climate change (Skendžić et al., 2021), the search for more sustainable pest control tactics to protect farm animals is now paramount. Among the additional possibilities offered by insights on insect physiology and genomics, the use of RNA interference (RNAi) seems to be now close to a wide commercial exploitation (Taning et al., 2020).

While several successful cases of application of RNAi in agriculture are now well established (Jiayang et al., 2022; Mezzetti et al., 2020), the possibility of controlling pests of veterinary importance has not yet been pursued with the same emphasis. The aim of this paper is to evaluate the possible applications of RNAi in pest control for farm animals, based on the available information on RNAi mechanisms and their effects on the biology of pests in animal farms.

RNAi: MODE OF ACTION, ADVANTAGES AND LIMITATIONS

RNAi is a natural mechanism that regulates gene expression through the action of double-stranded RNA molecules (dsRNA) targeting specific genome transcripts in the recipient organisms. The process causes the degradation of a messenger RNA with a complementary sequence, inducing a temporary silencing of the gene, resulting in the failure of protein production. The RNAi mechanism was first discovered and characterised in the nematode *Caenorhabditis elegans* by American researchers Craig Cameron Mello and Andrew Fire (Fire et al., 1998) who were awarded the Nobel Prize in Physiology and Medicine in 2006 for these studies. The process occurs naturally in a wide range of plants, mammals, insects and arachnids (Christiaens et al., 2018); it is based on specific mechanisms evolutionarily conserved in eukaryotes that regulate gene expression at the transcriptional or post-transcriptional level, providing a natural defence system targeting invading nucleic acids of hostile organisms. Several species, such as arthropods, are naturally able to exploit this mechanism to their advantage to defend themselves from virus attack; virus nucleic acids are recognised as 'non-self', and consequently a small molecule of dsRNA is synthesised to block protein production. In order for the mechanism to work, it is necessary that this dsRNA molecule recognises specifically the sequence of mRNA produced by the virus, to create a perfectly complementary copy that hampers protein formation.

In brief, the functioning of the RNA machinery in eukaryotic cells is based on the following steps:

- Upon entering the cell, endogenously transcribed (miRNA) or exogenously introduced long dsRNAs are first processed by the RNAse III enzyme Dicer into 21–23 nt small interfering RNA (siRNA).
- The siRNAs are incorporated into the RNA-induced silencing complex (RISC), which consists of an Argonaute (Ago) protein as one of its main components. Ago cleaves and discards the passenger (sense) strand of the siRNA duplex producing an active form of RISC.
- The remaining (antisense) strand of the siRNA guides the RISC to its homologous mRNA, resulting in the cleavage of the target mRNA (for a more detailed description of the mechanism, see Zhu & Palli, 2020).

A synthetic dsRNA can be designed in a complementary way to any gene of interest if the genome of the target organism is known, as the dsRNA is able to identify the sequence of mRNA to be silenced. Information on the genome of other organisms potentially exposed in the receiving environment will facilitate the production of dsRNA molecules highly selective, affecting only the target species, for example, a pest or a disease agent (Christiaens et al., 2021).

The necessity to find insecticidal molecules that have a low impact on farm animals, farm operators and the environment suggests the exploitation of RNAi to counteract the action of insects and arthropods infesting livestock. In addition, their novel mode of action may render them effective candidates to substitute active ingredients towards which pests have developed resistance (Freeman et al., 2019).

The main current limitations to a wider application of RNAi-based pesticides are linked to the variable sensitivity to RNAi among taxonomic groups, the limited availability of genomic information for many economically relevant species, the partial understanding of mechanisms possibly leading to RNAi resistance (Christiaens et al., 2021) and the lack of a specific regulatory framework to approve dsRNA-based products. One important challenge is the development of efficient delivery methods (Whitten, 2019). While injection constitutes a reliable system to confirm as proof of concept the efficiency of gene silencing, dsRNA-based biopesticides need to work efficiently upon ingestion or contact in field conditions. Activity of digestive enzymes however may lead to degradation of dsRNA molecules in some insects, though for other species diets with addition of dsRNA showed a strong silencing effect (e.g., *Ctenocephalides felis* Bouché (Siphonaptera: Pulicidae), Edwards et al., 2018). A fundamental aspect for the activity of RNAi is the ability of the dsRNA molecules to pass cellular membranes in order to allow the uptake of exogenous dsRNA in the cells of the target organism and its subsequent spread. Clathrin-dependent endocytosis is necessary to pass cellular membranes and an additional burden is constituted by the trapping of dsRNA molecules by endosomes inside cells. These cellular mechanisms seem to be the most likely explanation of the lack of effect of RNAi on some insect taxa (e.g., most Lepidoptera) (Christiaens et al., 2020).

DOMAIN OF THE REVIEW

Many recent reviews have addressed important insights concerning the application of RNAi in agriculture or public health domains (Airs & Bartholomay, 2017; Christiaens et al., 2020; Willow et al., 2021; Zotti et al., 2018). Menezes et al. (2022) firstly discussed possible applications of RNAi to livestock production. In particular, the authors discussed in depth the mode of action, possible target genes and delivery methods of dsRNA-based products. Riabinina et al. (2022) describe specifically methodologies for gene silencing in mosquitoes. The focus of our article is on arthropods that are economically relevant pests of farm animals and for which a possible application of RNAi is foreseeable based on their pest status and the available knowledge on RNAi efficacy. The objective is to offer to entomologists, veterinarians and professionals in the animal sector information on how RNAi can provide good support to integrated pest management in zootechnical activities. The selection of taxa to be included in the review was based on three main criteria: threat to livestock, current pest control measures and known sensitivity to RNAi (Table 1). Other arthropod taxa (e.g., Phthiraptera, Phlebotominae, Ceratopogonidae) may cause nuisance to livestock and in some cases economic damage directly, or as vectors of microbial diseases. Though control measures become necessary in certain cases, the effectiveness of gene silencing in these taxa is currently unknown.

Acarina

Due to the lack of antiviral therapies in most tick-borne arbovirus disease, understanding how arboviruses interact with arthropod vectors can provide relevant information for the control of these vectors via gene silencing.

Since the first study demonstrating gene silencing with dsRNA in ticks (Aljamali et al., 2002), treatments of Ixodidae have led to the discovery of several genes that affect host–pathogen interactions. Ticks rely on their immune systems for protection against viral infection; however, tick-borne viruses have evolved to avoid these defences as they establish themselves within the vector (Xu et al., 2021). Ticks can thus harbour pathogens without being affected themselves. RNAi also plays an important role in the efficiency of blood-sucking processes in ticks. Luo et al. (2022) identified a new miRNA (nov-miR-17) in *Hyalomma asiaticum*, which plays an important role in the blood suction process and rate of female's fertility by targeting the *TAB2* gene. The overexpression of nov-miR-17 indicated that this miRNA affected the engorged weight ($p < 0.001$) and spawn rate ($p < 0.001$) of female ticks.

dsRNA in ticks can be administered by injection, immersion, feeding or through viral infection at any stage of development, although adults are the ones most commonly used for reasons of practicality. Soares et al. (2005) firstly demonstrated a successful RNAi via ingestion, obtaining RNAi knockdown in *Ixodes scapularis* Say (Acari: Ixodidae). In this study, a capillary feeding approach was used for nymphs of the species, and a dose of 2 μL of dsRNA (1.2 μg/μL) placed directly in contact with specimens was sufficient to successfully silence the *isac* gene, a salivary anticomplement gene. Phenotypic effects included a 40.6% reduction in weight. Sensitivity to oral RNAi was also demonstrated in another Ixodid tick, namely *Haemaphysalis longicornis* Neumann (Acari: Ixodidae) (Galay et al., 2016). Immersion of *H. longicornis* ticks in a 1 μg/μL dsRNA-containing solution led to efficient knockdown in all stages (Galay et al., 2016; Gong et al., 2009).

F. Wang et al. (2019) injected dsRNA into *Rhipicephalus haemaphysaloides* Supino (Acari: Ixodidae), demonstrating how the silencing of the *RH-Hsc70* gene, expressing an heat shock protein in adult ticks, resulted in a reduction in blood meal fulfilment, and an increase in mortality rates. Lu et al. (2021) silenced the functional ecdysone receptor (*RhECR*/*RhUSP*) of 20-hydroxyecdysone from the salivary gland of the *R. haemaphysaloides* obtaining the inhibition of blood supply and curbed salivary gland degeneration by suppressing caspase-dependent apoptosis.

Unlike ticks, it has not yet been proven whether mites can process dsRNA efficiently. Experimental silencing of essential genes in mites upon feeding has been obtained in several phytophagous or predatory spider mites (Christiaens et al., 2018). Among the species of veterinary interest for which RNAi has been tested, there is the red mite *Dermanysus gallinae* DeGeer (Mesostigmata: Dermanyssidae) that mainly affects poultry and other bird species. Kamau et al. (2013)

TABLE 1 Main groups of economically important arthropod pests of farming animals and possibilities of control using RNA interference.

Taxon	Threat to farm animals	Cases of insecticide resistance	Available pest control methods	Sensitivity to RNAi
Acarina Ixodidae (ticks)	Direct, due to feeding activity, and indirect due to the transmission of pathogens (protozoan, viruses, bacteria [including rickettsiae] and fungi).	Numerous cases of resistance to pesticides have been reported (e.g., BHC/cyclodienes, organochlorines, carbamates, pyrethroids, and so forth).	Integration of hygienic measures and acaricide use. Treatment with acaricides still provides the most widely used means to control or prevent tick attacks.	Known for several species, for example, *Ixodes scapularis* particularly important as vector of microbial agents to humans, rodents and cattle; *Haemaphysalis longicornis* important pest and vector of animal (severe fever with thrombocytopenia syndrome virus) and human diseases.
Acarina Varroidae	Due to the feeding activity of *Varroa destructor*, individual bees are damaged in many ways (body weight reduction, decreased flight performance, reduced longevity and reduced ability to navigate). The activity of this ectoparasite is a contributing cause to colony losses. *Varroa* mites are also vectors of numerous bee viruses.	*V. destructor* was reported as resistant to pyrethroids, amitraz and coumaphos.	Organophosphates, pyrethroids, formamidine, cymiazole can be applied via fumigation, trickling or with impregnated plastic strips. Alternatively organic acids or essential oils can be used.	*V. destructor* can be successfully controlled feeding bees with dsRNA-based sucrose syrup. Active dsRNA persists in bee haemolymph and is ingested by mites feeding on adult bees.
Acarina Dermanyssidae	*Dermanyssus gallinae*, the red poultry mite, is a cosmopolitan species and is especially a problem in the Palearctic region and in the United States where it commonly occurs in poultry houses or around buildings where pigeons are nesting. Bites can induce skin dermatitis and allergic reactions. Chicken can experience reduced egg production and develop sleep disturbance, inactivity and unusual behaviours. Newly hatched chicks are particularly vulnerable.	The species has shown resistance to amitraz, pyrethroids, DDT and trichlorfon.	Integration of preventive measures (e.g., isolation of attacked individuals) and acaricide use.	Treatments of mites with dsRNA against *V-ATPase* via ingestion obtained significant silencing of the targeted gene.
Diptera: Muscidae	Due to feeding activity, flies cause discomfort, injure skin, affect growth and well-being of animals, and transmit pathogenic diseases (viruses, bacteria, and so forth)	The house fly *Musca domestica* has a tremendous record of resistance to insecticides involving 65 different active ingredients, including carbamates, organophosphates, organochlorides, neonicotinoids, abamectin and spinosad.	IPM plans are available aimed at the reduction of infestation levels, usually combining preventive measures, use of traps and a regular use of insecticides. Biocontrol agents (i.e., parasitoid wasps) are available, though their use is currently limited.	Sensitivity was proven for larvae of several species, including *M. domestica*. Adults have been tested only by injection.

TABLE 1 (Continued)

Taxon	Threat to farm animals	Cases of insecticide resistance	Available pest control methods	Sensitivity to RNAi
		Several strains of other species of veterinary importance like *Haematobia irritans*, *Musca autumnalis* and *Stomoxys calcitrans* were reported as resistant to DDT, pyrethroids, carbamates, organophosphates, ivermectin and BHC/cyclodienes.		
Diptera: Culicidae	Mosquitoes are a cause of irritation, blood loss and allergic reactions in animals. Their presence disrupts normal behaviour of livestock. Indirect damage is caused by their role as vectors of several microbial diseases, particularly viruses. The genus *Culicoides* is a vector of blue tongue disease. Species of *Aedes*, *Anopheles* and *Culex* are involved in the transmission of equine encephalomyelitis virus.	Proven records of resistance to insecticides are mainly available for species vectors of human diseases. *Aedes aegypti* has become resistant to 42 active compounds. *Anopheles messeae*, one of the vectors of malaria has shown resistance to BHC/cyclodienes, DDT, fenthion and malathion.	An effective mosquito control is aimed at targeting larvae in water bodies (e.g., with *Bacillus thuringiensis*) or adults (either via sprays or insecticidal nets). Sterile insect technique, including novel biotechnological approaches (e.g., modelling the *Wolbachia* incompatible insect technique), is available for some vector mosquito species.	Known for several species including *Ae. aegypti*, *Anopheles gambiae* and *Culex pipiens pallens*.
Blattodea	Cockroaches serve as intermediate hosts for a number of parasitic worms of animals. Sometimes these relationships are of economic importance.	*Blattella germanica* has shown resistance to 45 active Ingredients. There are 115 reported cases from all continents. Resistance to organochlorines is known for *Blatta orientalis* (Europe) and *Periplaneta brunnea* (United States).	Reliance on side effects of insecticide treatments used for controlling flies. Use of baited traps.	Known for several species (e.g., *B. germanica*, *Periplaneta americana*).

Abbreviations: BHC, Benzene exachloride; DDT, para-dichlorodypheniltrichloroethane; dsRNA, double-strand RNAs; IPM, integrated pest management; RNAi, RNA interference.
Source: Michigan State University, Arthropod pesticide resistance database (www.pesticideresistance.org).

obtained successful knockdown of histamine releasing factor (*HRF*) gene and *cathepsin D* after 16 h of immersion of mites in dsRNA; however, no phenotypic observations were conducted in the study. W. Chen et al. (2018) treated *D. gallinae* with dsRNA against *V-ATPase* via ingestion, resulting in significant silencing of the targeted gene. Effects were visible 24 h after administration, showing a high sensitivity of the mite to dsRNA treatment.

Marr et al. (2018) targeted the *Psoroptes ovis* Hering (Astigmata: Psoroptidae) mite, responsible for psoroptic mange, in which the dsRNA was administered by immersion, resulting in significant reduction in gene transcription. Encoding group 2 allergen (*PSO* or 2), mu-class glutathione S-transferase (*PoGSTmu-1*) and beta-tubulin (Poβtubulina), a fundamental component of cytoplasm, were selected as target genes. The study led to a first demonstration in *P. ovis* of mining genomic and transcriptomic data for selecting a target gene for the possible control of this ectoparasite. Fernando et al. (2022) treated *Sarcoptes scabiei* L. (Astigmata: Sarcoptidae) eggs with dsRNA pre-treated with NaOCl to guarantee a greater absorption and successfully silenced three single copy genes of *S. scabiei*, designated *Ss-Cof*, *Ss-Ddp* and *Ss-Nan*, whose homologues in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) had been shown to be essential for normal development.

A significant reduction in the expression of glutathione-S-transferase mu-class 1 enzyme (*DpGST-mu1*) in *Dermatophagoides pteronyssinus* Trouessart (Astigmata: Pyroglyphidae) (dust spider mite) was achieved by Marr et al. (2015) following overnight immersion in dsRNA, although no detrimental phenotypic changes were observed

following silencing. The selection of *DpGST-mu1* gene as a target is an interesting alternative approach to pest control based on dsRNA. Indeed, glutathione-S-transferase enzymes are active in the degradation of glutathione for the purpose of detoxification. Ingestion of dsRNA targeting the involved effector genes weakens the defensive ability of a pest to withstand exposure to noxious molecules, making pests more vulnerable to several possible stressors, such as suboptimal diets or acaricides, and may reduce the risk of insurgence of resistance.

Diptera

D. melanogaster Meigen has been widely used for testing the effects of dsRNAs and silencing genes is considered a basic tool for studying gene expression for many different metabolic pathways. The systematic review by Christiaens et al. (2018) reported that 1243 studies of RNAi on this species had been published by then.

The oral route can be effective in triggering RNAi in *D. melanogaster*, as demonstrated by targeting the gamma-tubulin gene (Whyard et al., 2009). Furthermore, inducing gene silencing in *D. melanogaster* has allowed the discovery of resistance factors to insecticides. The use of transgenic lines of *D. melanogaster* allowed for the targeted knockdown of genes putatively involved in DDT resistance and has validated the role of several cuticular proteins (*Cyp4g1* and *Lcp1*), cytochrome P450 monooxygenases (*Cyp6g1* and *Cyp12d1*) and ATP binding cassette transporters (*Mdr50*, *Mdr65* and *Mrp1*) in DDT resistance (Gellatly et al., 2015).

Among species of veterinary importance, the house fly *M. domestica* has been the subject of several studies where RNAi was used as a tool to discover functions of important genes (Q. Wang et al., 2020; W. Wang et al., 2022; Zhuang et al., 2021).

Genes involved in the immune system of *M. domestica* have been extensively studied, leading to the discovery of potential new targets for insecticides. Y. Zhang et al. (2019) found that trehalose-6-phosphate synthase (*TPS*) transcription was up-regulated following bacterial challenge by *Escherichia coli* or *Staphylococcus aureus*. Silencing *mdPAP1* gene on *M. domestica* larvae led to a significant reduction in the survival rate of the bacterially infected housefly, suggesting that *mdPAP1* may have a role in antimicrobial immune response (Zhuang et al., 2021).

In a perspective of possible applications of RNAi for pest control, Sanscrainte et al. (2018) treated groups of 50–65 females of *M. domestica* with injections of 5 μg of dsRNA targeting *actin-5c* or ribosomal protein transcripts *RPL26* and *RPS6*. Screening the possible effects on oviposition and ovarian morphology, a significant reduction of fertility per female was observed in house flies injected with dsRPL26 (8.4 ± 6.3, mean ± SE, $p = 0.002$) and dsRPS6 (1.4 ± 1.4, $p = 0.002$) when compared with flies injected with the control dsGFP (green fluorescent protein 135.7 ± 15.7). The dsActin-5C treated flies did not show a significant reduction in clutch size (98.2 ± 26.1, $p = 0.237$). Mortality rates were low for all treatments. Gene expression levels were significantly and specifically reduced in the dsRNA-injected groups but remained unchanged in the control group, demonstrating the targeted effect. Injections of flies with an *Aedes aegypti* L. (Diptera: Culicidae) conspecific dsRNA designed against *RPS6* did not impact fecundity, demonstrating species specificity of the RNAi response.

A recent study by Jiao et al. (2022) showed that knockdown of the *MdCht2* gene, belonging to group VII chitinases, resulted in high rates of mortality and abnormal eclosion in adults *M. domestica* after dsRNA injection. Compared with the control used (dsGFP), the expression of *MdCht2* was reduced by 82%, 93% and 74% in the integument, trachea and whole body, respectively. Conversely, administration of dsMdCht2 did not affect the expression of non-target genes *MdCht4*, *MdCht5*, *MdCht7*, *MdCht9* and *MdCht10*. Knockdown of *MdCht2* resulted in increase of the chitin content, leading to abnormal eclosion and high mortality rates indicating this gene as a potential target for the control of *M. domestica*.

Investigations in *Ae. aegypti* mosquitoes provided the first demonstration that RNAi could be induced in insects by topical application of dsRNA (Pridgeon et al., 2014). The possible application of RNAi in public health entomology was reviewed by Airs and Bartholomay (2017) who summarised studies in a number of vector mosquito species. Among the physiological aspects clarified using gene silencing, there are olfaction and sensation, blood feeding, morphogenesis and development.

Identification of possible target genes successfully silenced via RNAi included *IAP1*, a cellular inhibitor of apoptosis protein, resulting in activation of apoptosis and rapid mortality in *Ae. aegypti* (Puglise et al., 2016), chitin synthase genes *AgCHS1* and *AgCHS2* in *Anopheles gambiae* Giles (Diptera: Culicidae) larvae, which induced a reduced chitin content. In addition, larvae feeding nanoparticles assembled from *AgCHS1* and *AgCHS2* dsRNA showed increased susceptibility to diflubenzuron and white calcofluor or dithiothreitol, suggesting that dsRNA could be used as an adjuvant of certain insecticides (X. Zhang et al., 2010). Reducing the number of female mosquitoes in a population constitutes an important target with the aim of reducing disease transmission. Taracena et al. (2019) tested RNAi expression pattern in *Ae. aegypti* larvae of the *dsx* (doublesex) gene involved in sex determination in insects. Following oral administration of dsRNA, a reduction in *AgdsxF* expression, a significant reduction (>66%) in female *dsx* transcript mRNA and a reduction in the number of female larvae reaching adulthood occurred. The control groups produced a balanced 52:48 males:females ratio, while the *AgdsxF* dsRNA-treated groups had 72.1% males versus 27.8% females. Adult females showed reduced fertility (37.1%) compared with controls. The treatment had no impact on the number of viable adult males, in *dsx* male transcripts or in male fitness parameters (i.e., longevity, body size), proving to be highly selective towards females.

Occurrences of mosquito-borne diseases are increasing, a consequence of the reduced sensitivity of mosquito strains to pyrethroids, and many studies focused on the search for target genes that could hinder insecticide resistance. Three mosquito strains of *Culex quinquefasciatus* Say (Diptera: Culicidae) (S-Lab, HAmCqG0 and MAmCqG0) were treated with dsRNA to identify the role of the GPCR (G-protein-coupled receptors) regulatory pathway in mosquitoes by characterising the precise function of FPCR, G Protein, AC and PKA. G-protein

receptors are involved in a number of fundamental physiological functions in insects, such as development, locomotion, reproduction and immune reactions. Silencing of the *GPCR020021* gene in S-Lab caused a significant increase in susceptibility of mosquitoes to permethrin and reduced expression of downstream genes (*AC007240*, *PKA-018257* and *PKA00789*) and *P450* genes involved in resistance to permethrin (Liu, 2015).

Khalil et al. (2021) examined the effect of RNAi on 10 target genes selected from the genome of *C. quinquefasciatus*. dsRNA was administered to both larvae and adults via soaking and nanoparticles. Silencing of *chitin synthase-1*, *apoptosis inhibitor 1* and *vacuolar adenosine triphosphatase* (*V-ATPase*), which are produced by highly conserved genes in eukaryotes overseeing cell functioning, resulted in high mortality in both instars, while medium to low mortality was obtained with the others.

In *Culex pipiens pallens* L. (Diptera: Culicidae), the primary vector of lymphatic filariasis and epidemic encephalitis, two *AK* (Arginine kinase) genes essential for energy metabolism were identified as targets; their silencing led to high mortality (74%) and reduced fertility in adult females together with a reduction in blood meal completion (Qian et al., 2022).

Blattodea

The studies performed with RNAi show how insects belonging to the Order Blattodea are sensitive to dsRNA upon injection: the use of 1 μg/μL of RNA injected into the abdomen of late-stage female nymphs of *Blattella germanica* L. (Blattodea: Ectobiidae) reduced the nymphal development of *BgEcR-A* expression and did not allow moulting. All nymphs treated with *dsBgEcR-A* that transformed into adults showed different deformities in the fore and hind wings. In addition, a small number of specimens treated with *dsBgEcR-A* showed correctly modelled hind tarsi, but shorter than the controls (Cruz et al., 2006). Li et al. (2021) used *Periplaneta americana* L. (Blattodea: Blattidae) and developed protocols based on the different developmental stages and their dietary characteristics to induce dysfunctional phenotypes. The oral administration of specific dsRNA on *B. germanica* indicates that a better stability of dsRNAs can be obtained through the use of lipoplexes. The encapsulation of dsRNA in liposomes allowed to counteract the action of nucleases at the level of the midgut improving gene silencing. Continuous administration of liposome-encapsulated dsRNA reduced the expression of a *α-tubulin* in the midgut, resulting in significant mortality (Huang et al., 2018). Lin et al. (2017) used 1 μL of dsRNA solution (2 or 4 μg/μL) for injection into the abdomen of male *B. germanica*, which proved to be an effective method to block the expression of α-tubulin. dsRNA administered by ingestion twice a day with 4 μL of lipoplexes dsRNA solution (0.0625 μg/μL dsRNA with liposome) or bare dsRNA solution (0.0625 μg/μL without liposome) had milder effects, if any, apparently because ingested dsRNA is rapidly degraded by the RNA core. The study, however, showed that liposomes can be a protective vehicle for dsRNA against the degradation that occurs in intestinal juice when it is administered orally.

Experimental details relative to successful experiments with dsRNA are given in Table 2.

Outside of these main taxa, studies of RNAi involving insects of veterinary importance were conducted on *C. felis* Bouché, known for causing discomfort mostly in pets (dog and cats), but this host-specific group of arthropods usually do not cause parasitic problems in farm animals. Though dogs might represent a possible vehicle of infections to livestock, they are not commonly treated for ectoparasites in farms. On the contrary, in the order Phthiraptera there are several species that can cause problems to livestock, but no specific studies with RNAi have been published so far.

RNA MACHINERY AND PHYSIOLOGY OF ARTHROPODS PESTS

There are several physiological mechanisms acting against the completion of the RNAi process before dsRNA enters cells (Cooper et al., 2019). In arthropods, the main mechanism leading to failure of the RNAi process is linked to the digestive system of the target organisms. Extracellular stability of dsRNA in the insect body can be hindered by nuclease activity in the saliva, midgut and haemolymph of the recipient organism. From the very beginning of the digestive process, salivary enzymes may digest double-stranded ribonucleic acids as demonstrated in *Lygus lineolaris* Palisot de Beauvois (Hemiptera: Miridae) (Allen & Walker III, 2012), several aphid species (Christiaens et al., 2014), and *Nezara viridula* (Hemiptera: Pentatomidae) (Sharma et al., 2021). Insect midgut is the main organ where digestion processes take place, and among many enzymes active during this process, nucleases are abundant. Fan et al. (2021) demonstrated high expression of a Lepidoptera-specific nuclease in the gut and capable of degrading dsRNA in the moth *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae). Degradation of dsRNA in midgut of *N. viridula* L. was also observed by Sharma et al. (2021), though the process is less efficient than in saliva.

Additional mechanisms leading to dsRNA stability may be the cause of lack of effect of RNAi in other insect species. Spit et al. (2017) described the identification and characterisation of two nuclease genes expressed in the gut of the beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) that limited the efficiency of RNA silencing; however, removal of nuclease activity of a gene with similar function in the locust *Schistocerca gregaria* did not lead to an improvement of the RNAi response.

Even though not all possible mechanisms have been elucidated, it is clear that sensitivity to RNAi is very variable among arthropod species (Willow & Veromann, 2021) and even within the same species (Sugahara et al., 2017). Consequently, predicting possible effects based on taxonomic similarity is not a guarantee of safety for non-target organisms. Conversely, a genetic similarity between species may trigger specific biosafety studies, because in laboratory tests highly RNAi-sensitive predatory lady beetle species (belonging to the same order of Coleoptera) were found when fed with *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) specific V-ATPase dsRNA (Pan et al., 2020).

TABLE 2 Experimental details of successful gene silencing experiments via RNA interference in arthropod pests of veterinary importance.

Target species	Instar	Target gene(s)	Delivery method	dsRNA concentration	Measured endpoints	References
Ixodes scapularis (*Acarina*)	Nymphs	*isac*	Ingestion	1.2 μg/μL	Weight	Soares et al. (2005)
Haemaphysalis longicornis (*Acarina*)	Adults	*HlYkt6*	Injection	2 μg/μL	Engorgement rate, engorged body weight, mortality and oviposition rate	Gong et al. (2009)
Varroa destructor (*Acarina*)	Adult females	*α-tubulin*, *RNA polymerases*, *V-ATPase*, *Na+/K+ atpase* and *Apoptose inhibitors*	Ingestion	40 μg/μL	Number of mites	Garbian et al. (2012)
Dermanysus gallinae (*Acarina*)	Unspecified	*HRF* *Cathepsin D*	Immersion	Not specified	No phenotypic observations other than gene silencing	Kamau et al. (2013)
Dermatophagoides pteronyssinus (*Acarina*)	Adult males	*DpGST-mu1*	Immersion	2.5 μg/μL	No phenotypic observations other than gene silencing	Marr et al. (2015)
H. longicornis (*Acarina*)	All stages	ds*Hlfer1*	Immersion	1 μg/μL	Blood feeding period, engorged body weight, pre-oviposition period, weight of egg mass and egg conversion ratio	Galay et al. (2016)
D. gallinae (*Acarina*)	Mixed stages and sexes	*V-ATPase*	Ingestion	100 ng/μL	No phenotypic observations other than gene silencing	W. Chen et al. (2018)
Psoroptes ovis (*Acarina*)	Adults	*PSO* or 2 *PoGSTmu-1* Poβtubulina	Immersion	0.05 μg/μL	No phenotypic observations other than gene silencing	Marr et al. (2018)
Rhipicephalus haemaphysaloides (*Acarina*)	Adult females	*RH-Hsc70*	Injection	2 μg/μL	Mortality, engorgement rate and engorgement weight	F. Wang et al. (2019)
R. haemaphysaloides (*Acarina*)	Adult females	*RhECR*/*RhUSP*	Injection	2 μg/μL	Engorgement rate, engorgement weight, oviposition rate and egg hatching rate	Lu et al. (2021)
Sarcoptes scabiei (*Acarina*)	Eggs	*Ss-Cof*, *Ss-Ddp* and *Ss-Nan*	Immersion	2.5 μg/μL	Hatchability	Fernando et al. (2022)
Hyalomma asiaticum (*Acarina*)	Adult females	*TAB2*	Injection	2 μg/μL	Engorged weight and spawn rate	Luo et al. (2022)
Anopheles gambiae (*Diptera*)	Larvae	*AgCHS1 e AgCHS2*	Ingestion	32 μg/μL	No phenotypic observations other than gene silencing	X. Zhang et al. (2010)
Aedes aegypti (*Diptera*)	Adult females	*IAP1*	Injection	188 ng/μL	Apoptosis and mortality	Puglise et al. (2016)

TABLE 2 (Continued)

Target species	Instar	Target gene(s)	Delivery method	dsRNA concentration	Measured endpoints	References
Musca domestica (*Diptera*)	Adult females	*Actin-5c*, *RPL26* and *RPS6*	Injection	5 μg/μL	Fertility	Sanscrainte et al. (2018)
Ae. aegypti (*Diptera*)	Larvae	*dsx*	Ingestion	2.3 μg/μL	Fertility	Taracena et al. (2019)
Culex quinquefasciatus (*Diptera*)	Larvae and adults	*Chitin synthase-1*, *apoptosis inhibitor 1*, *V-ATPase*	Immersion	5 μg/μL	Mortality	Khalil et al. (2021)
M. domestica (*Diptera*)	Larvae	*mdPAP1*	Injection	370 ng/μL	Mortality	Zhuang et al. (2021)
M. domestica (*Diptera*)	Adults	*MdCht2*	Injection	2 μg	Chitin content, eclosion and mortality	Jiao et al. (2022)
Culex pipiens pallens (*Diptera*)	Adult females	*CpAK1*, *CpAK2*	Injection	200 ng	Mortality and fertility	Qian et al. (2022)
Blattella germanica (*Blattodea*)	Nymphs, adults	*BgEcR-A*, *α-tubulin*	Injection	1 μg/μL	Development, morphological observations	Cruz et al. (2006), Lin et al. (2017)
B. germanica (*Blattodea*)	Adult males	*α-tubulin*	Ingestion	0.0625 μg/μL	No phenotypic observations	Lin et al. (2017)

Abbreviation: dsRNA, double-strand RNAs.

DEVELOPING DELIVERY SYSTEMS FOR dsRNA IN ANIMAL FARMS

In order to foresee a possible practical application of dsRNA-based pesticides in commercial farms, specific delivery strategies need to be developed.

Acarina

The mites or Acari are a subclass of Arachnida, within the orders Ixodida, Mesostigmata, Thrombidiformes and Sarcoptiformes: these are species that cause health problems to farm animals (Mullen & O'Connor, 2019). Due to the wide atherogenicity of this group, their biological cycle is different between orders and it is adapted to the host. A common feature however is their ectoparasitic habits and a feeding regime based on the assumption of blood or haemolymph from the host as food source; in addition, post-embryonic development mostly takes place after egg hatching, from which hexapod larvae are born. A few hundred species of mites are recognised as the cause of health-related problems for humans and domestic animals (Mullen & O'Connor, 2019). Only a few species commonly cause economic damage to livestock, thereby requiring control measures. For example, *D. gallinae* causes direct negative effects due to its feeding habits and indirect effects as a vector of diseases (e.g., fowl pox; Sparagano & Giangaspero, 2011). When preventive measures are not sufficient to avoid symptoms on farm animals, treatments of individuals with acaricide sprays are necessary and they still provide the most widely used means to control or prevent tick infestations (Yawa et al., 2020). Intensive use of acaricides has resulted in insurgence of resistance in many populations of ticks (Rodriquez-Vivas et al., 2018). Due to their ability to conceal within animal hair close to the skin, treatments need to be directly targeted to infested animals and the whole herd they live in.

The availability of molecules that do not cause non-target effects, as the case of dsRNA-based products, may offer an important support to controlling these pests in animal farms. Current knowledge indicates that several economically important tick species have shown sensitivity to treatments with dsRNA (see above); however, a possible delivery strategy to mimic currently used pesticides needs to better define sensitivity of target species to topic application of the molecule, with ingestion being a secondary possible exposure route.

Diptera

Diptera brachycera belonging to the families Tabanidae, Hyppoboscidae and Muscidae are pests capable of relevant economic losses to livestock (Baldacchino et al., 2018). While the first two families are biting flies, the females of Tabanidae and both sexes in the Hyppoboscidae family are blood feeders, and Muscidae adults rely on sugars from off-host sources or occasionally from secretions from host animals (Mullen & O'Connor, 2019).

In addition to their role as vectors of pathogens, all these species cause discomfort and induce host-defensive reactions in cattle with effects on animal growth rates (Maciel et al., 2015) and milk production (Taylor et al., 2012). Damage provoked by biting flies is obviously more pronounced; an accurate estimate of economic thresholds is

therefore required (Baldacchino et al., 2018). While using biological control of flies with inundative release of Hymenoptera parasitoids represents an important sustainable alternative (Rutz & Patterson, 2021), the combination of chemical insecticides with the application of traps remains the most common means of controlling flies in animal farms (Cook, 2020; Malik et al., 2007; Trout Fryxell et al., 2021).

The immature development of Tabanidae and Muscidae larval instars is linked to the presence of abundant organic matter as an optimal biotope. While larvae of Tabanidae have generally a carnivorous diet and do not necessarily live in the vicinity of farms, larvae of the family Muscidae are saprofages and commonly develop in the farm preferring manure and straw bedding as pabulum (Baldacchino et al., 2018). A different life cycle is characteristic of Hyppoboscidae, whose females are larvivorous; larvae develop in the maternal uterus and are laid only when close to pupation (Mullen & O'Connor, 2019). Larvicides can be applied to animal manure to control fly immature stages, while adulticides can be applied directly on the animals or in selected areas of the farm, toxic baits can be used successfully, to limit exposure to pesticides for farm animals and workers. The attract-and-kill strategy is successfully applied to control Dipteran pests including mosquitoes and house fly (Airs & Bartholomay, 2017; Amin et al., 2017; Klick et al., 2019; Vaníčková et al., 2017).

The intensive use of insecticides for fly control has induced two main issues, the rapid development of insecticide resistance in fly populations and the negative impact on non-target insects such as dung beetles or pollinators (Baldacchino et al., 2018). Biopesticides based on dsRNA molecules are a promising alternative, though cases of practical applications in field conditions are still lacking. The possible use of dsRNA-based pesticides against larvae may be complicated due to the biotopes immature flies live in (e.g., manure, straw bedding) and could be difficult to reach. Alternatively, a possible approach to the control of their populations in livestock could consider the adult stage as a target. As naked RNAs have a very short half life in the environment (Arpaia et al., 2021), the use of carriers in developing RNA-based pesticides may allow them to maintain their biological activity on sprayed surfaces for 20–30 days (Mitter et al., 2017). This may allow their use on, for example, perimeter walls or sphere traps. Commercial formulations of dsRNA-based insecticides should also enable the preparation of toxic baits to be used in different types of traps. However the effects on the adult flies upon ingestion need to be confirmed in further studies. Knowledge gaps need to be filled in order to assess the effective dosage to trigger response in adults, and find appropriate formulations that guarantee persistence of the product in farming conditions in a bacteria-rich environment, either as sprays or as toxic compounds for baits.

Current approaches to control vector mosquitoes include an integration of methods to reduce larval sources in body waters (e.g., using *Bacillus thuringiensis*, Brühl et al., 2020) and topical and contact applications for adults (e.g., residual spraying and long lasting insecticidal nets, World health Organization, 2009). Insecticide resistance among mosquito species vectoring human and animal diseases is seriously threatening the control of such vectors (Airs & Bartholomay, 2017). The possible role of sterile insect technique and incompatible insect technique is being currently analysed in different environments with promising results (Bouyer et al., 2020; Foley et al., 2021); however, its regulatory status still needs to be defined. The most practical way to apply dsRNA-based bioinsecticides could be directed to those areas where water stagnation can occur, for example, drinking troughs, washing tanks, irrigation pumps and wells, slurry collection tanks, and so forth. Based on the wealth of knowledge being accumulated with the use of dsRNA against mosquito vectors of human diseases, Lopez et al. (2019) developed a dsRNA-based bioinsecticide for the containment of *An. gambiae* by targeting chitin synthase A and B. dsRNA expressed in *E. coli* provided to larvae achieved specific knockdown of target proteins. Treatment resulted in significantly reduced levels of larval survival associated with reduced *CHs* transcript levels. The bioinsecticide was directly supplied without a carrier to tap water containing first instars, indicating that a delivery method in farming systems in body waters where immature mosquitoes thrive would be feasible. The bioinsecticide also showed an insecticidal adjuvant effect, probably due to its weakening of the cuticle and intestine of the mosquito.

Blattodea

Cockroaches represent the intermediate hosts of numerous parasites, in particular, belonging to the class of Nematodes of the Order Spirudida. Infestations of Blattodea commonly do not cause significant economic damage, but constitute an important problem mainly linked to the indirect damage of parasite transmission for farm animals. In North America and Europe, *B. germanica* and *P. americana* can spread nematode infections in chicken and turkey farms, causing damage of various degrees, for example, to the digestive or the ocular systems. *B. germanica* is also the responsible intermediate host of *Gongylonema pulchrum*, a nematode of the digestive system of livestock (Brenner & Kramer, 2019). The discomfort caused by Blattodea in livestock farms does not cause serious economic losses, despite their control may be still necessary to maintain a clean and healthy environment.

When necessary, pesticides, such as pyrethroids and organophosphates, are applied to cockroach harborage sites and areas frequented by foraging individuals (Rust et al., 1995). Juvenile hormones may hamper exposed individuals to reach sexual maturity. The use of baits containing several of the active ingredients can be profitable to control cockroaches. Huang et al. (2018) in feeding experiments with *B. germanica* used liposomes as protective vehicles in oral delivery of dsRNA against degradation in the gut. This technology allowed to induce mortality in cockroaches with administrations of low doses (0.5 μg per day) of dsRNA. This opens the possibility of using dsRNA as a poisonous agent in baited traps for controlling Blattodea in both indoor and outdoor farm environments.

THE CASE OF HONEYBEES

Several pests and diseases are threatening apiaries worldwide, and the Federation of Veterinarians of Europe warns that not enough veterinary medicines are available for bees (https://fve.org/publications/honey-bees-health-vets-are-vital). Here we briefly discuss why RNAi

constitutes a valid alternative to control bees' pests and diseases and contribute to maintaining bees' economic and ecological services.

The first field application of this technology aimed to heal *Apis mellifera* L. (Hymenoptera: Apidae) infected with the Israeli acute paralysis virus by synthesising specific homologous dsRNA (Hunter et al., 2010). Garbian et al. (2012), investigated the bidirectional transfer of dsRNA between honey bees and the parasitic mite *Varroa destructor* Anderson & Trueman (Mesostigmata: Varroidae) to assess the effects of dsRNA against this ravaging pest of apiaries. After treatment of the bee colony with dsRNA designed to target *Varroa* genes, the parasite population was reduced by 60% while bees did not suffer any adverse effects. Collected data demonstrated that movement of dsRNA along the food chain did not alter its biological activity and that bees were quite insensitive to interference. Additional studies confirmed a generalised non-sensitivity of honey bees to environmental dsRNA (A. Chen et al., 2015; Hollowell & Rieske, 2022; Tan et al., 2016).

GreenLight Biosciences Inc. has recently added to its portfolio a patent for a dsRNA-based product to control *V. destructor* infestations (Inberg & Mahak, 2016). Their dsRNA is a 372 base pair homologous to a sequence region within the mite *CaM* gene that encodes calmodulin, an essential calcium-binding protein that regulates multiple protein products. The product is formulated as an 80% sucrose solution to be placed in the hive; mites could thus be exposed to dsRNA through contact with the solution and/or through ingestion of the haemolymph of larvae or adult bees. The Varroa-targeting dsRNA has a 99% nucleotide match with the calmodulin mRNA and a 74% nucleotide match with *A. mellifera* calmodulin mRNA. However, there are no contiguous overlaps of 21 or more nucleotides (considered to be the optimal match length for efficient silencing) between the Varroa-active dsRNA and the bee genome (Krishnan et al., 2021). Muntaabski et al. (2022) produced high levels of dsRNA in vivo through the use of bacteria. dsRNA expressed in bacteria solution administered to honeybees induced silencing of several genes involved in inhibition of apoptotic process in *V. destructor*, inhibiting their expression levels by 50%. Worker bees that were fed *Varroa*-targeted dsRNA showed no survival differences compared with control bees.

Europe has recently been invaded by an alien coleopteran species, *Aethina tumida* Murray (Coleoptera: Nitidulidae) the small hive beetle, whose damages in the newly invaded area is causing serious problems to apiculture (Granato et al., 2017). A study by Powell et al. (2016) reported a significant dose-dependent mortality of *A. tumida* following injections of 2–12.5 ng of dsRNA targeted to *Laccase 2*, an essential gene for the sclerotization and pigmentation of the cuticle, and to the subunit A of the *V-ATPase* (vacuolar) into 7-days old larvae. Oral administration of the V-ATPase A subunit resulted in 50% larval mortality, but a poor reduction in gene transcription levels. We have recently developed a testing protocol to evaluate possible effects upon ingestion of artificial diet by young larvae using two dsRNAs specifically aimed at silencing essential genes in *A. tumida* (*V-ATPase* and *RPS13*). Treated larvae suffered from a decrease in the rate of development, a slowdown in the biological cycle and surviving adults experienced a significant reduction in fertility (Arpaia et al., 2022).

Benefitting from the availability of the full genome of *A. mellifera*, the species has been used as a non-target organism during the environmental risk assessment of dsRNA-based products. For instance, honeybees were tested during the safety assessment of the genetically modified (GM) maize event MON 87411, which is now commercially available; both larvae and adults proved to be insensitive to diet containing high dose of dsRNA targeting the *DvSnf7* gene of *D. virgifera virgifera* (Bachman et al., 2016). Similarly, honeybees were among the non-target organisms tested for safety during the development of the new dsRNA-based bioinsecticide Ledprona®, currently being evaluated by the US Environmental Protection Agency for use in the field as a sprayable pesticide. Overall, existing literature studies indicate the exploitation of RNAi technology as an appealing alternative for beekeepers to control pests and diseases in apiaries.

SAFETY OF dsRNA FOR FARM ANIMALS

Before any commercial application of RNAi is authorised, regulators consider possible human and environmental health and safety issues, to rule out possible effects due to silencing of target or off-target genes of exposed organisms (Christiaens et al., 2021). The main risk scenario of dsRNA-based pesticides use in livestock concerns possible adverse effects due to ingestion or topical exposure of the herd and of workers performing farming operations.

RNA molecules, such as siRNAs and miRNAs, are present in commonly consumed plant and animal-derived foods (Dávalos et al., 2019); therefore, there is an established history of their safe consumption. A number of small RNAs with perfect complementarity to human and animal genomes have been identified in crops largely used as food or feed ingredients, such as soybean, corn and rice (Ivashuta et al., 2009). There is ample evidence that biological barriers in mammals are able to prevent negative effects due to ingested RNAs (O'Neill et al., 2011; Petrick et al., 2013). During the digestion process along the gastro-intestinal tract, dietary RNA molecules are broken down by a series of enzymes starting from the nucleases present in the saliva (Park et al., 2006). The stomach of mammals represents a most hostile environment to nucleic acids, whose digestion occurs under a low pH, which favours their denaturation. Also, in different parts of the intestine, the few remaining molecules of RNA are further degraded by digestive enzymes produced by the pancreas (O'Neill et al., 2011). When naked RNA or DNA were fed to ruminating lambs, little or none survived to the abomasum, and when they were provided via diet to steers, RNA and DNA disappeared to extents of 89% and 80%, respectively, between the abomasum and the terminal ileum, irrespective of the diet (Razzaque & Topps, 1972).

Additionally, rare intact molecules of nucleic acids present along the gastro-intestinal tract of mammals can hardly be absorbed from the lumen of the intestine, due to a series of cellular membrane barriers of gastro-intestinal epithelial cells and the vascular epithelium. Even in case of a successful cellular uptake of RNA, molecules have to escape endosomes, known to sequester most RNA molecules entering a cell. Likewise, physical barriers (e.g., skin, fur) limit dermal absorption if exposure occurs (Ibaraki et al., 2019; Kasiewicz & Whitehead, 2019).

These defensive mechanisms ubiquitous in mammals are also considered to be the main obstacle to the development of efficient RNA therapeutics for humans (Gilleron et al., 2013; Rodrigues & Petrick, 2020).

In summary, available studies indicate that the amount of dietary silencing RNAs absorbed after ingestion can be considered negligible in humans and animals, unless chemical modifications of the dsRNA molecule or the use of co-formulants aimed at facilitating their delivery are introduced in the final product. Therefore, the safety of these products for mammals needs to be confirmed using the commercial products when testing on non-target organisms are performed.

CONCLUDING REMARKS

Applications of RNAi in support to agriculture are steadily increasing, demonstrating a high potential for applications in food/feed production and in pest management (Taning et al., 2020). GM plants based on the RNAi technology have already appeared on the market in some countries. Non-GM-based approaches (e.g., the production of dsRNA-containing biopesticides) seem generally more appealing in terms of public perception, and requests for approval of such products are currently under evaluation at the US Environmental Protection Agency.

Degradation of dsRNA by nucleases in the environment and within the insect's body is a major barrier to successfully induce RNAi in target insects (Spit et al., 2017). Therefore, there are several approaches taken by both academic and industry researchers to find formulation technologies compatible with the biological active ingredient in order to improve uptake of dsRNA by recipient organisms, ensure the integrity of dsRNA molecules in target insects and product stability in the field, for example, to extreme heat, UV light or soil nucleases (Christiaens et al., 2020).

For experimental use, in vitro transcription of dsRNA using purified RNA polymerases and nucleotides has been obtained routinely. However, their high cost (ca. 500 Euros per mg dsRNA) is an important limit for large-scale application in the field. Fermentations of transformed *E. coli* strains producing dsRNA have allowed to strongly reduce production costs (ca. 1 Euro/gram). More recently, a cell-free production platform has been set up by GreenLight Biosciences, based on the use of endogenous cellular RNA. Using this method, the production cost is estimated to be $0.50 per gram, making it more appealing for the market (He et al., 2022).

The livestock sector seems to lag a little behind in pursuing RNAi technology; however, problems of pest control in livestock are worsening due to the reduced availability of low-risk pesticides, increasing cases of insecticide resistance and to a generalised increase of pest populations along with climate change (Howard et al., 2022).

Several features of dsRNA molecules indicate their profitable use in animal farms, since they are quite specific against target organisms and practically innocuous for mammals. Lack of knowledge exists in better assessing their effects on specific arthropod pests and especially in optimising a delivery strategy that could ensure the efficacy of dsRNA-based products in farming conditions. Research should try to fill these gaps and propose valuable alternatives conforming to the goals of a more sustainable farming.

AUTHOR CONTRIBUTIONS

Salvatore Arpaia: Conceptualization; writing – original draft; writing – review and editing; methodology; validation; formal analysis; supervision; data curation. **Valeria Bonina:** Investigation; validation; writing – review and editing; writing – original draft; data curation.

CONFLICT OF INTEREST STATEMENT

The authors declare there are no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available by reasonable request to the authors https://www.ncbi.nlm.nih.gov/pmc/.

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