Novel molecular biopesticides targeting the potassium ion channels of the red flour beetle, *Tribolium castaneum* (Herbst.)

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Abstract

The ion channels of insects are targets for several classes of chemical insecticides and, whilst they are very effective at controlling insect pest populations, they are often accompanied by undesirable effects on non-target organism. RNA interference technology is currently being developed as an alternative means of crop protection. In this study RNAi was used to target two potassium ion channel genes of the beetle *Tribolium castaneum*, namely the *SK* gene, which encodes the small conductance calcium-activated potassium channel and the *SH* gene, which encodes the voltage-gated potassium channel. The results demonstrate that specific double-stranded RNA (dsRNA) targeting these ion channels significantly reduced the levels of gene expression. The oral delivery of dsRNA for both target genes caused a dose-dependent effect, with 100% mortality of third instar larvae at 400 ng dsRNA/mg diet after 10 days. This was accompanied by a decrease in *TcSK* and *TcSH* gene expression of 98% and 83%, respectively, after 3 days continuous feeding. The injection of sixth instar larvae with dsRNAs (62.1 ng/larva) caused 100% and 57.8% mortality for *SK* and *SH*, respectively after 7 days. Moreover, the injection of early pupae, late pupae and adults showed significant ($p < 0.05$) down-regulation in gene expression for both genes, being 93%, 96% and 93% for *SK* and 94%, 92% and 72%, respectively, for *SH*. The toxicity of dsRNA targeting the *T. castaneum SK* and *SH* genes was tested against a beneficial insect, the honeybee *Apis mellifera*. The dsRNA for both targets had no significant effects on bee survival when fed 20 ng/µl of TcdsSK or TcdsSH. Furthermore, there were no effects on expression levels of *SK* or *SH* in the foraging bees, and neither were there any effects on the immunity of the honeybee, as measured by the effects on the titre of the deformed wing virus (DWV). One of the limitations of the applicability of RNAi-based approaches for insect control is that of cost and delivery. We report a technically cost-effective method for the production of dsRNA by the bacterium, *Escherichia coli* HT115. The results show that the toxic effects on larval mortality caused by dsRNA synthesised by *in vitro* transcription compared with that expressed *in vivo* by bacteria were similar. The amount of dsRNA via *in vivo* expression in bacteria was approximately four times that of *in vitro* transcription per reaction. Finally, this study demonstrated that the co-delivery of dsSK or dsSH increased the efficacy of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae.* With both larvae and adults of *T. castaneum*, the effects on mortality with dsSK were synergistic. Overall, this study demonstrates the efficacy of RNAi as a method of insect control and that the ion channels represent good targets. Furthermore, it demonstrates the potentiating effects of the technology with the use of BCAs such as entomopathogenic fungi.

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List of Abbreviations

RNAi	RNA interference
mRNA	Messenger RNA
siRNA	Small interfering RNA
K^+	Potassium ion
Tc	*Tribolium castaneum*
Bt	*Bacillus thuringiensis*
SH	Gene cod voltage-gated potassium channels
SK	Gene cod small conductance calcium-activated potassium channel
cDNA	Complementary DNA
CNS	Central nervous system
dsRNA	double-stranded RNA
dsSH	double-stranded RNA specific for *SH* gene
dsSk	double-stranded RNA specific for *SK* gene
DWV	Deformed wing virus
FAO	Food and Agriculture Organization of the United Nations
h	hour(s)
IPM	Integrated Pest Management
Kana	kanamycin
L: D	light:dark
LC_{50}	Median lethal concentration
mg	milligram(s)
min	minute(s)
ml	millilitre
µl	microliter
mRNA	messenger RNA

µg	microgram
ng	nanogram
SD	standard deviation
RT-qPCR	Reverse transcription-quantitative PCR
v	volume
w	weight
PCR	Polymerase chain reaction
BCAs	biocontrol agents
rh	relative humidity

Table of content

List of Figures

List of Tables

General Introduction

1 General Introduction

1.1 The necessity to protect crops

The rapid growth of the world's population has created a need for sustainable agriculture throughout the world, which can be accomplished by promoting mechanization and new strategies to achieve the maximum production of food. Estimates suggest that food production will have to increase by 70 % globally to feed an additional 2.3 billion people by 2050 (DES, 2013).

Insect pests are the main obstacle to achieving global food needs. Pest infestation causes both direct damage to crops, as well as indirect damage such as the transmission of viral, bacterial or fungal diseases and necrosis of plant tissues or organs. For example, *Helicoverpa zea* larvae feed on plant reproductive tissues, causing severe economic damage (Kitching and Rawlins, 1998), whereas the white fly *Bermisia tabaci* transmits more than 100 plant viruses and thus causes significant damage to crops (Jones, 2003). Overall, pest insects and pathogen infestation cause serious deficits in crop yields.

Worldwide, it is estimated that there are 9000 species of pest insects and mites, 8,000 weed species and 50,000 species of plant pathogens that damage crops; 14% of crop losses are caused by insect pests, 13% by plant pathogens and 13% by weeds (Pimentel, 2009). Another study estimated a 10-16% reduction in agricultural yields globally (Bebber *et al.*, 2013). Bradshaw et al. *(*2016*)* report that invasive insects cost a minimum of $70.0 billion per year globally and $6.9 billion per year due to devastating infectious diseases in humans and livestock. Minimization of these damage levels will ultimately increase the number of crops available for human consumption. However, environmental change as a consequence of global warming leading to increased atmospheric CO2 could promote changes in the distribution of insects and cause additional infestation (Hamilton *et al.*, 2005).

Although insecticides have contributed to increasing food production, they can also have adverse impacts on human health (Guillette Jr. and Iguchi, 2012), wildlife populations, communities and ecosystems (Ramsden *et al.*, 2017). Furthermore, there has been a rise in insect resistance to conventional chemical pesticides. Almost 2000 arthropod species are currently documented to have acquired resistance to one or more pesticides (Arthropod Pesticide Resistance Database, 2017). Combined with more stringent regulations, market

growth has led to an increasing demand for safer, more specific and more efficient insecticides. Hence, there is a need to find plant protection methods that are environmentally friendly and safe to consumers.

Genetic modification (GM) techniques have provided some alternative options; they allow the addition of new traits to crops, which may increase their suitability for environmental conditions including insect interactions. For example, transgenic rice expressing the δ-endotoxin (cry1A) *Bacillus thuringiensis* are more resistant to lepidopteran insects pests than untransformed plants (Fujimoto *et al.*, 1993). Moreover, some insect-resistant transgenic crops express two or more toxic proteins, providing protection against different species of pest insects and reducing the damage to crops (Gatehouse, 2008).

The bacterium *Bacillus thuringiensis* (Bt) is the most successful example of a biological insecticide and produces insecticidal crystal proteins (Habib and Andrade, 1998). These toxins have been applied particularly as biopesticides against Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Isoptera, Orthoptera, Siphonoptera and Thisanoptera (Schünemann *et al.*, 2014). Cry-proteins cause physiological disorders such as changes in nutrient absorption, degenerative transformation, appetite loss and abandonment of food and gut paralysis, leading to insect death (Monnerat and Bravo, 2000). Meanwhile, Bt toxins are safe for non-target organisms (Hammond and Koch, 2012)

Nevertheless, Bt toxins are not effective against some important insect pests, such as aphids, leafhoppers and whitefly (Porcar *et al.*, 2009; Catarino *et al.*, 2015). Furthermore, pests are continuously evolving resistance to Bt crops (Tabashnik *et al.*, 2013). The rise of insect resistance to chemical or biological insecticides, combined with increasing consumer demand, has led to the need to explore different molecular targets and strategies for pest management to secure sustainable crop yields.

1.2 *Tribolium castaneum* as a genetic model insect

The red flour beetle, *Tribolium castaneum* (Figure 1.1), is a global pest of stored agricultural products (Andrić *et al.*, 2010). In a recent post-harvest compendium estimated that *T. castaneum* and *T. confusum* are "the two most common secondary pests of all plant commodities" (Sallam, 2008).

Figure 1.1: The red flour beetle (*Tribolium castaneum) (Meyer, 2003)*

It is distributed worldwide because it can develop at a wide range of environmental temperatures between 22-40˚C. Furthermore, the females live more than 300 days, and a female of *T. castaneum* can lay 360.4±27.9 eggs (Howe, 1962). Development from larvae to pupa to adult takes 2-3 weeks (Arakane et al., 2005).

This species exhibits an ancient short-germ mode of embryogenesis, which is a characteristic of some holometabolous insects such as *T. castaneum* beetle. Another ancestral feature is the development of a larval head and legs, which are not present in *D. melanogaster* (Vilcinskas, 2014). This species provides an ideal model organism for use in study insect development, evolution, and comparative genomics in research, which may be medically as well as economically motivated. Beetle Base (Beetle Base, 2018) is an integrated resource for the *Tribolium* genome. Also, *Tribolium* is amenable to systemic RNAi-mediated gene silencing and other genetic tools for functional gene analyses because it has three *sid-1*-like genes, which encode the SID1 transmembrane protein, the best-characterized protein involved in systemic RNAi in *T. castaneum*. Furthermore, the duplication of Ago-2 (Tc-Ago-2a and Tc-Ago-2b) in *Tribolium* might lead to higher amounts of Tc-Ago2 protein and, thus, an enhanced RNAi response. However, *Drosophila*, which does not show a robust systemic RNAi response, lacks *sid-1*-like genes (Tomoyasu *et al.*, 2008). The robust RNAi effect is systemic in *T. castaneum* and can be found in any tissue and any developmental stage

following the injection of dsRNA into the hemolymph (Tomoyasu *et al.*, 2008; Kalsi and Palli, 2017), or after the oral delivery of dsRNA (El Halim *et al.*, 2016). The induction of RNAi in pupae or adult females causes an RNAi effect in the offspring, a phenomenon known as parental RNAi (Bucher *et al.*, 2002).

1.3 RNA interference

RNA interference (RNAi) is a natural defence mechanism against viruses and other foreign genetic material, which is initiated by the introduction of double-stranded RNA (dsRNA) into a cell leading to the gene silencing process (Hammond *et al.*, 2001; Novina and Sharp, 2004). RNAi involves the cleavage of dsRNA in the cell cytoplasm by the RNase-III enzyme termed Dicer into small interfering RNAs (siRNAs: 20–25 nucleotides) and microRNAs (miRNAs: 21–24 nucleotides) (Meister and Tuschl, 2004). Both can regulate gene expression at the post-transcriptional level. siRNA or miRNA molecules are phosphorylated at their 5′ end and are incorporated into a multiprotein RNA-induced silencing complex (RISC). Short double-stranded RNA is unwound into two single strands, called sense and antisense. The sense strand, known as the "passenger strand" is degraded by the RISC complex, while the antisense strand, which is complementary to the mRNA sequence, guides the RISC complex in recognizing the target mRNA (Hammond, 2005; Moazed, 2009; Kupferschmidt, 2013). When it finds a complementary strand, Argonaute (a protein within RISC) is activated which cleaves the RNA, preventing the mRNA from producing the encoded protein (Scott *et al.*, 2013) (Figure 1.2).

RISC consists of Dicer, RNA binding proteins, protein kinase RNA activator (PACT), transactivation response RNA binding protein (TRBP) and Argonaute proteins (Ago), which form the core component of RISC (Redfern *et al.*, 2013). The functional RNAi machinery has two major components: (1) the core component inside the cells, which includes Dicer and Argonaut; and 2) the systemic component that amplifies the dsRNA via RNA-dependent RNA polymerases (RdRPs) and allows it to diffuse to other tissues within the organism.

Dicer enzymes are endoribonucleases encoded by a changeable number of genes (Firmino *et al.*, 2013). For example, *Drosophila melanogaster* has two paralogues: Dicer-1 (Dcr-1) and Dicer-2 (Dcr-2). Both required for RNAi (Lee *et al.*, 2004). However, in *T. castaneum*, which is a model insect for systemic silencing by RNAi, Dcr-2 plays an important role in the RNAi

pathway, whereas Dcr-1 is involved in wing development, most likely through the miRNA pathway (Tomoyasu *et al.*, 2008). Argonaute proteins contain two domains: a PAZ domain parted in dsRNA binding, which recognizes the 3' end of small dsRNA; and a PIWI-domain which cleaves the single-stranded RNA, mRNA and is guided by siRNA (Meister *et al.*, 2004). The RdRP mediated amplification of siRNA molecules has been reported in plants, nematodes and fungi, but not in humans and insects (Gordon and Waterhouse, 2007). Amazingly, even in the absence of RdRP, insects such as *T. castaneum* exhibit robust systemic RNAi responses (Tomoyasu *et al.*, 2008), thus preventing the mRNA from being translated in proteins, which may be essential for cellular function, causing cellular toxicity and cell death. RNAi is a very specific method because it works only when there is a high level of complementarity between trigger and target.

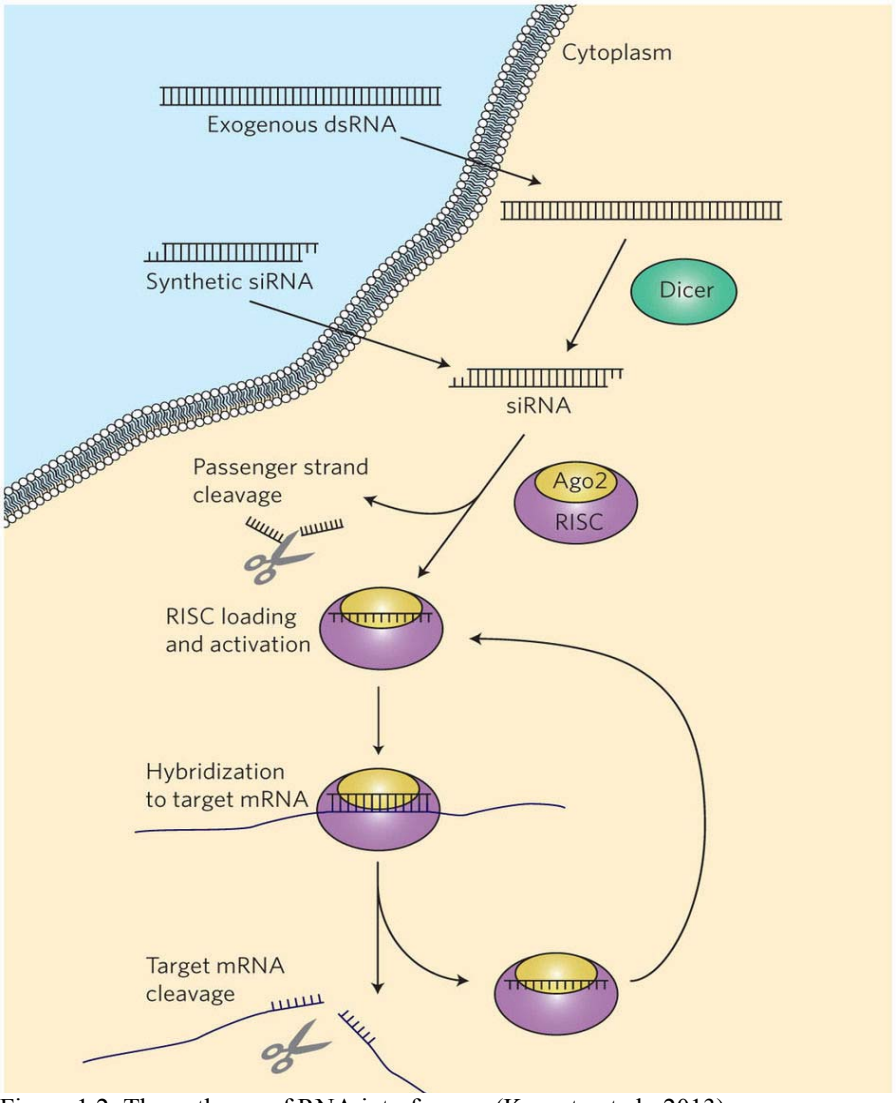

Figure 1.2: The pathway of RNA interference (Kanasty et al., 2013)

1.4 Transport of RNAi information between cells

RNAi is divided into two types: cell-autonomous and non-cell-autonomous (Figure 1.3). As the term suggests, in cell-autonomous RNAi the silencing process is restricted to the cell which is exposed directly to the dsRNA, and therefore, an RNAi effect occurs in the application site of the dsRNA (Grishok, 2005; Whangbo and Hunter, 2008). In the case of non-cell-autonomous RNAi, the silencing signals spread from cell to cell and thus, an RNAi effect occurs in sites different from where of the dsRNA was introduced (Katoch *et al.*, 2013). There are two different categories of non-cell-autonomous RNAi: environmental RNAi and systemic RNAi.

Environmental RNAi refers to the processes by which dsRNA is taken up by a cell from the environment; for example, in the uptake of dsRNA by intestinal lumen cells, followed by the systemic spread of gene silencing to other cells and tissues (Sivakumar *et al.*, 2007).

Systemic RNAi can only take place in multicellular organisms because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another; for example, in the transport of silencing signals from intestinal lumen cells to other tissues like muscle or epidermis (Whangbo and Hunter, 2008).

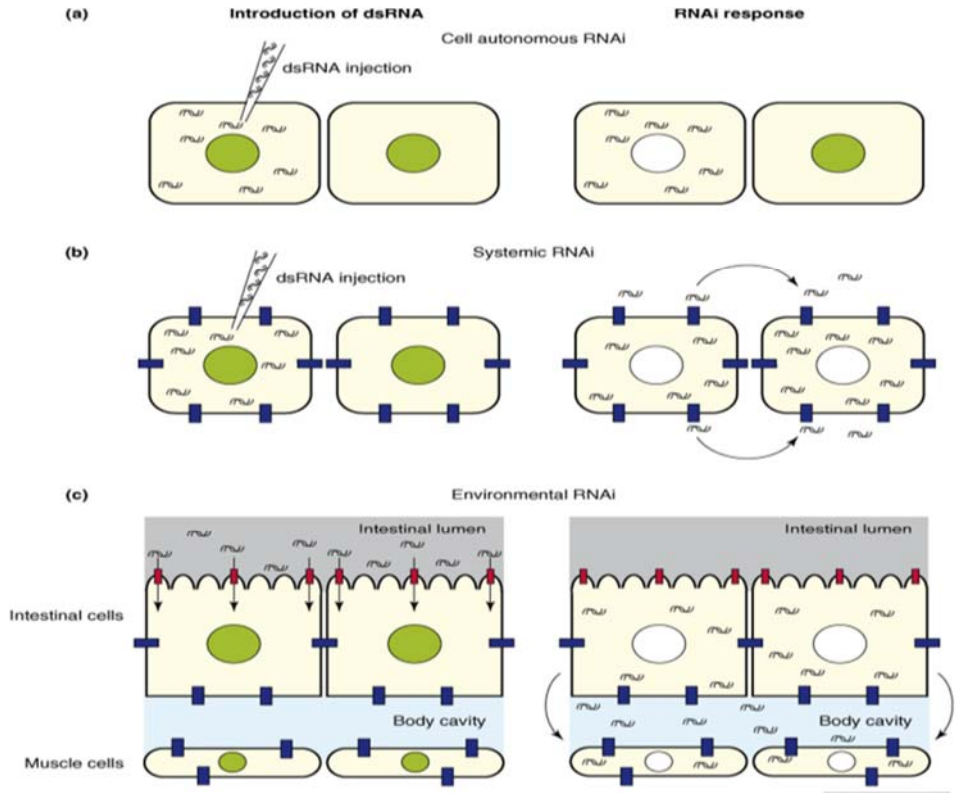

Figure 1.3: Cell-autonomous, systemic and environmental RNA interference (RNAi):| (a) cell autonomous RNAi. (b) systemic RNAi. (c) environmental RNAi (Whangbo and Hunter, 2008)

1.5 RNA interference up take in insects

Two different pathways have been found for systemtic and dsRNA uptake in insect pests. These are the transmembrane Sid-1 channel protein-mediated pathway and the endocytic pathway. Previous studies have reported two proteins involved in systematic RNAi in *Caenorhabditis elegans;* SID-1 and SID-2. SID-1 is essential for the transport of dsRNA into the cells of *C. elegans*; however, it does not export it to the other cells (Winston et al., 2002). Most insect orders have only one *Sid-1*-like gene. However, several coleopteran insects have been identified with 2 or even 3 *Sid-1*-like genes (Tomoyasu et al., 2008). However, lepidopteran *B. more* does not show a robust systemic RNAi response despite containing three *SID,* and systemic RNAi is possible in *Anopheles gambiae* and *D. melanogaster* despite the lack of *SID* genes (Boisson *et al.*, 2006; Saleh *et al.*, 2006). Therefore, an endocytosis-mediated uptake mechanism has been suggested as an alternative dsRNA uptake mechanism existing in insects.

SID-2 is another main protein involved in the uptake of exogenous dsRNA (Winston *et al.*, 2007). One hypothesis concerning the function of SID-2 is that it assists in passing exogenous dsRNA molecules to SID-1, while another suggests that SID-2 activates transport by inducing

a modification to the SID-1 protein, and a third hypothesis suggests that SID-2 participates in an alternative mechanism of the uptake of dsRNA (endocytosis-mediated uptake mechanism) (Whangbo and Hunter, 2008). This suggestion comes from a study of the dsRNA uptake mechanism in *Drosophila* in which includes several proteins involved in endocytosis. Moreover, several scavenger receptors, for example, Eater and Sr-CI, have been discovered to be significant for dsRNA uptake (Saleh *et al.*, 2006; Ulvila *et al.*, 2006).

1.6 Important factors affecting the silencing effect and RNAi efficiency as an insect control technique

Various factors can affect the efficiency of dsRNA uptake and the spread of systemic silencing in different insects. In this section important points are highlighted that must be considered in developing an RNAi approach against insect pests.

1.6.1 Target gene

The selection of the target gene is vital for insect survival or development. It should not exhibit functional redundancy, as in, for example, the loss of function by RNAi in the nicotinic acetylcholine receptor (nAChR) in the *Drosophila melanogaster* nervous system which results in normal insects (Perry *et al.*, 2007). Also, different genes show variation in their susceptibility to dsRNA-induced RNAi. Terenius *et al.* (2011) found that, out of 130 genes, only 50 showed a robust RNAi response.

1.6.2 dsRNA design

The specificity of dsRNA effects is determined by the selection of the target region. SiRNA molecules can inhibit the translation of transcripts with 21–25 bp similarity. Target sites must, therefore, be chosen carefully to avoid the silencing of unintended genes in the target organism (i.e. off-target effects) or adverse effects on non-target insects (i.e. non-target effects) (Araujo *et al.*, 2006).

1.6.3 dsRNA length

In feeding experiments, sequences ranging from 50 to 500 bp have been used to successfully achieve RNAi (de Andrade and Hunter, 2016). The minimum length of dsRNA required to

silence a given gene varies among insect species, but long dsRNA fragments have been shown to be more effective in silencing genes than siRNA (Bolognesi *et al.*, 2012). This may be because the breakdown of dsRNA by Dicer provides a greater diversity of siRNAs available to cause the specific knockdown of the target gene (Scott *et al.*, 2013). In contrast, other studies have shown suppression of genes in different insects via siRNA was more effective than when using dsRNA (Kumar *et al.*, 2009; Upadhyay *et al.*, 2011).

1.6.4 dsRNA concentration

The optimal concentration of dsRNA delivered for every target gene has to be determined to induce efficient silencing. It is not true that exceeding the optimal concentration results in more silencing (Meyering-Vos and Müller, 2007; Shakesby *et al.*, 2009). For example, in the cotton bollworm (*Helicoverpa armigera*), high dsRNA concentrations caused high levels of silencing of chymotrypsin and chitinase, but caused low levels of silencing with the juvenile hormone acid methyl transferase (jhamt) (Asokan *et al.*, 2013). Moreover, when multiple dsRNAs are injected, competition between dsRNAs for the RNAi machinery can occur leading to oversaturation, reducing the RNAi efficiency and further demonstrating that an optimal concentration of dsRNAs depends specifically on the gene in question (Parrish *et al.*, 2000; Miller *et al.*, 2012).

1.6.5 Life stage of insects

RNAi efficiency differs at different insect stages depending on the physiological as well as genetic characteristics of the insects. Most insects show an RNAi response when targeted at younger stages, because of their less developed body and smaller size. For instance, in *Rhodnius prolixus* with the nitropin 2 genes, 42% silencing was observed in second instar larvae whereas no silencing was evident in the fourth instar individuals, (Araujo *et al.*, 2006). Furthermore, in *Spodoptera frugiperda,* which exhibits a more efficient RNAi response, allatoiregulating neuropeptide genes were knockdown in fifth instar larvae but not in adult moths (Griebler *et al.*, 2008). However, Bucher *et al.* (2002) found that parental RNAi is less efficient when younger stages are injected with dsRNA. One of a possible reason for this is that organs in the younger stage are still developing and development is not complete until the pupal stage. However, this will be influenced by the gene in question.

1.6.6 Mode of dsRNA delivery

The efficacy of an RNAi experiment can depend on the mode of delivery of dsRNA. The main dsRNA delivery methods studied in different orders of insects so far include injection, feeding, spraying, soaking and using transgenic plants expressing dsRNA (Figure 1. 4).

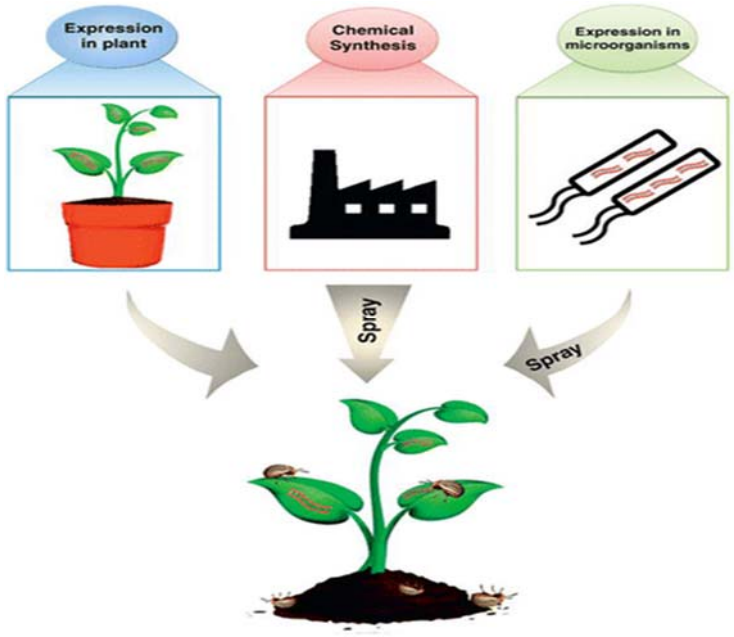

Figure 1.4: Use of RNAi as a future insecticide (Palli, 2014)

The injection of dsRNA directly into the target tissue or hemolymph is a regularly used and effective method (Quan et al., 2002; Xiao et al., 2014). Kennerdell and Carthew (1998) carried out the first successful application of microinjection by downregulating the frizzled and frizzled-two genes in *Drosophila melanogaster*. Subsequently, microinjection has been applied in a wide range of insect species in functional genomics studies, and examples include *T. castaneum* (Brown et al., 1999) and *An. gambiae* (Blandin et al., 2002). Moreover, this technology has been applied to all life stages, including in *Tribolium*, *B. mori, B. germanica* and *A. mellifera* (Guidugli et al., 2005; Martín et al., 2006; Ohnishi et al., 2006; Ulrich et al., 2015). The major advantage of using this method is that an exact amount of dsRNA can be directly introduced into the targeted tissue with a known concentration, in contrast to delivery by other methods. However, this method has disadvantages including cuticular damage caused during injection which stimulates immune functions, which can further complicate the interpretation of the results (Han et al., 1999; Yu et al., 2013). Further, injection is also not

realistically possible in the field (Scott *et al.*, 2013), and so this delivery method has very limited implementation potential in pest control planning.

The delivery of dsRNA by oral feeding is a practical application in insect management and is comparatively simple compared to injection. The feeding of dsRNA has been shown to be an effective gene knockdown method in many insects of the orders Hemiptera, Coleoptera, and Lepidoptera (Nunes and Simões, 2009; Chen *et al.*, 2010; Bolognesi *et al.*, 2012). Also, Whitten *et al.* (2016) reported that ingested recombinant bacteria expressing dsRNA cassettes successfully mediated systemic knockdown phenotypes in *Rhodnius prolixus* and *Frankliniella occidentalis*. However, some studies have demonstrated that the induction of gene silencing by dsRNA feeding is less effective than dsRNA injection because the individuals differ in their consumption and consequently digest variable amounts of dsRNA (Araujo *et al.*, 2006). Recent reports suggest that spraying dsRNA (synthesized in vitro) is an effective delivery method for dsRNA, protecting potato plants from the Colorado potato beetle *Leptinotarsa decemlineata* for 28 days under greenhouse conditions (San Miguel and Scott, 2016).

In plant-mediated or host-induced RNAi approaches (Hi-RNAi), a crop plant is engineered to produce dsRNA against the target gene of the insect pest. Upon feeding on plant tissue parts, dsRNA enters into the insect gut, leading to the induction of RNAi. The advantage of this method is the generation of continuous and stable dsRNA material. Hi-RNAi has also been combined with other transgenic approaches to enhance resistance against pests. For instance, the use of transgenic cotton plants expressing both the P450 gene dsCYP6AE14 and 35GhCP1 (*Gossypium hirsutum* cysteine protease) led to the drastically reduced growth of bollworm larvae *Helicoverpa armigera* (Mao *et al.*, 2013)

1.6.7 Stability of dsRNA in the insect gut

Gut pH is variable among different insect species ranging from very acidic (Coleoptera) to strongly alkaline (Lepidoptera). In addition, pH varies among different gut parts, from acidic in the anterior midgut to basic in the posterior midgut and with distance from the gut epithelium. Both chemical hydrolysis and enzymes play a crucial role in the stability of dsRNA in the insect gut (Hakim *et al.*, 2010). For instance, in feeding assays, the ingested dsRNA must produce siRNA fragments rather than be completely digested into monomers in

order to initiate RNAi (Huvenne and Smagghe, 2010). A recent study of the cotton boll weevil suggests that the chimeric protein PTD-DRBD (peptide transduction domain - dsRNA binding domain) combined with dsRNA forms a ribonucleoprotein particle which protects the dsRNA from gut nucleases and improves the RNAi effect (Garcia *et al.*, 2017). Some insects show a robust RNAi response even in the hostile environment of the insect gut, which may be associated with other factors affecting the stability of dsRNAs in the guts of insects (Xu *et al.*, 2016).

1.7 Improving dsRNA efficiency

RNAi technology has demonstrated the potential to control insect pests; however, the evidence suggests that some modifications may be required to improve RNAi efficiency in certain insects.

1.7.1 Nanoparticles

Nanoparticles can be used to minimize dsRNA degradation and improve the delivery of intact dsRNA as well as for environmental safety (Joga *et al.*, 2016). Polymeric nanoparticles are produced using natural resources such as the animal-derived proteins keratin, collagen and silk protein (Good *et al.*, 2009) or using synthetic resources such as metals or alloys (Kamat, 2007). Zhang *et al.* (2010) reported that two chitin synthase genes, AgCHS1 and AgCHS2 in *Anopheles gambiae* were knocked down by 62.8 and 33.8%, respectively, when their dsRNA mixed with chitosan nanoparticles in the diet fed to larvae. Additionally, a fluorescent cationic core-shell nanoparticle and CHT10-dsRNA incorporated into the diet and fed to the Asian corn borer *Ostrinia furnacalis* larvae led to clear RNAi gene silencing and eventually death. However, non-encapsulated dsRNA treatment had no detectable effect on silencing the gene in the fifth instar larvae (He *et al.*, 2013).

1.7.2 Liposomes

The use of liposomes is another method that can be safely and effectively used to deliver exogenous dsRNA. Liposomes are natural lipids composed of one or more phospholipid bilayers, and they are non-toxic and easily biodegradable (Van Rooijen and van Nieuwmegen, 1980). They are used in various scientific disciplines such as in drug formulation where drugs can be transferred without side effects to an organism (Gregoriadis, 1977). Four different

species of *Drosophila* (*D. melanogaster, D. sechellia, D. yakuba*, and *D. pseudoobscura*) exhibited significant mortality when fed γTub23C-dsRNA encapsulated in cationic liposomes; in contrast, non-encapsulated dsRNA had no RNAi-silencing effects (Whyard *et al.*, 2009). Taning *et al.* (2016) have silenced the V-type proton ATPase subunit E (Vha26) in the spotted wing fruit fly *Drosophila suzukii* by combining dsRNA with liposomes.

1.7.3 Chemical modifications

Chemical modifications to dsRNA by adding methyl-groups to the 2′ position of the ribosyl ring of the 2nd base of the siRNA has been shown to increase specificity (Jackson *et al.*, 2003).

1.8 Potassium ion channels as targets for RNAi

Potassium (K+) channels are present in cell membranes of all species, with the exception for some parasites (Kuo et al., 2005); K+ channels are the largest and most varied of the ion channels. They play crucial roles in controlling the passage of K+ ions the secretion of hormones, resting potential, and the excitability of neurons and muscles. Therefore, these channels are important for the activity of excitable tissues such as the brain, heart and nervous system (Lehmann-Horn and Jurkat-Rott, 1999). There are four major classes of potassium channels based on their structure and functional properties: calcium-activated, inwardly rectifying, voltage-gated and two P domain potassium channels (*www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=133)* (Table 1.1)

Table 1.1: The four main classes of potassium channels

Class	**abbreviation**	**Function**
Calcium-activated	$\mathbf{K_{Ca}}$	Are activated by an increase in intracellular calcium concentrations
Inwardly rectifying	$\mathbf{K_{ir}}$	Secretion of potassium in the malpighian tubules
Voltage-gated	$\mathbf{K_{v}}$	Action potential repolarization
Two P domain	$\mathbf{K_{2p}}$	Contribute to resting potential

Potassium channels are present in humans and other organisms including insects, plants, yeast, and bacteria. All have the same structure, such as the pore-forming α subunits and the β subunits which regulate channel activity, depending on the level of energy of the cell (Jan and Jan, 1997). The sequence similarities of the pore loop provides strong evidence that all potassium channels have the same pore architecture (Miller, 1992). MacKinnon *et al.* (1998) reported that the sequence in the pore region of the K^+ channel is identical between *Drosophila* (*Shaker*) and vertebrate voltage-gated K^+ channels.

1.8.1 Structure of potassium channels

K^+ channels have transmembrane helices (TMs) inserted into the lipid bilayer. The four major classes of channels have different numbers of TMs, ranging from two to six (Buckingham *et al.*, 2005). The inwardly rectifying K^+ channels contain two transmembranes (TM) helices with a P loop between them. The voltage-gated and calcium-activated K+ channels each consist of six TM helices with a P loop between them. The two-pore-domain K+ channels include two repeats of 2TM/P channels, and they are thus composed of four TM and two P loops as shown in Figure 1.5. K^+ channels include two parts: the pore-forming domain and the regulatory domain. The pore-forming domain is responsible for the selective conductance of K^+ ions, and its structure is identical in all types of K^+ channels. The regulatory domain perceives different impulses, and its structure varies among the classes. The active site of K^+ channels consists of a sequence of five amino acid, TVGYG, functioning as a selectivity filter (SF) to efficiently conduct K^+ ions.

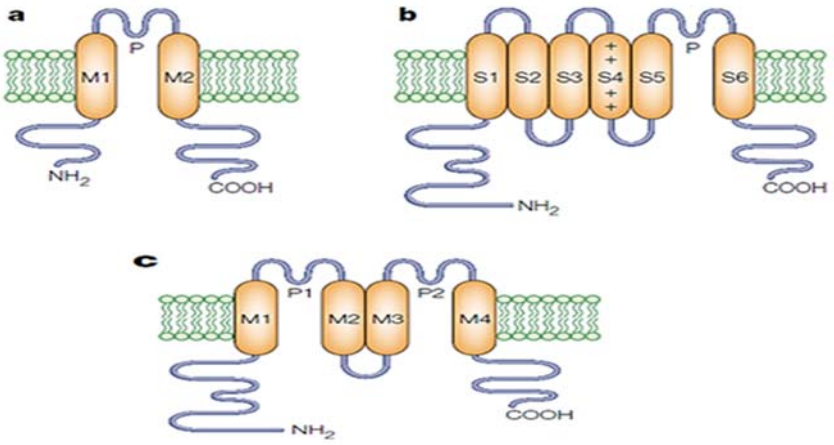

Figure 1.5: The four main classes of potassium channel according their structure:(a) inwardly rectifying K+ channels; (b) voltage-gated and Calcium-activated K+ channels; (c) tandem pore domain K+ channels (Choe, 2002).

1.8.2 Mechanism of selectivity and conductivity of K^+ channels

K^+ ions generally diffuse from the intracellular side (helical bundle), and then enter the central water-filled cavity (Sc), followed by transformation in the SF (S4–S1), and eventually arrive at the extracellular entryway (EC) (Kuang *et al.*, 2015), down the electrochemical gradient (Figure 1.6). Besides this, the feature of conductivity with regard to selectivity by the filter (SF) can join potassium ions at 4 sites (S4–S1), each K^+ ion sits in the middle of two oxygen layers. The K^+ ions are hydrated in the central cavity and extracellular entryway. However, they are dehydrated in the SF.

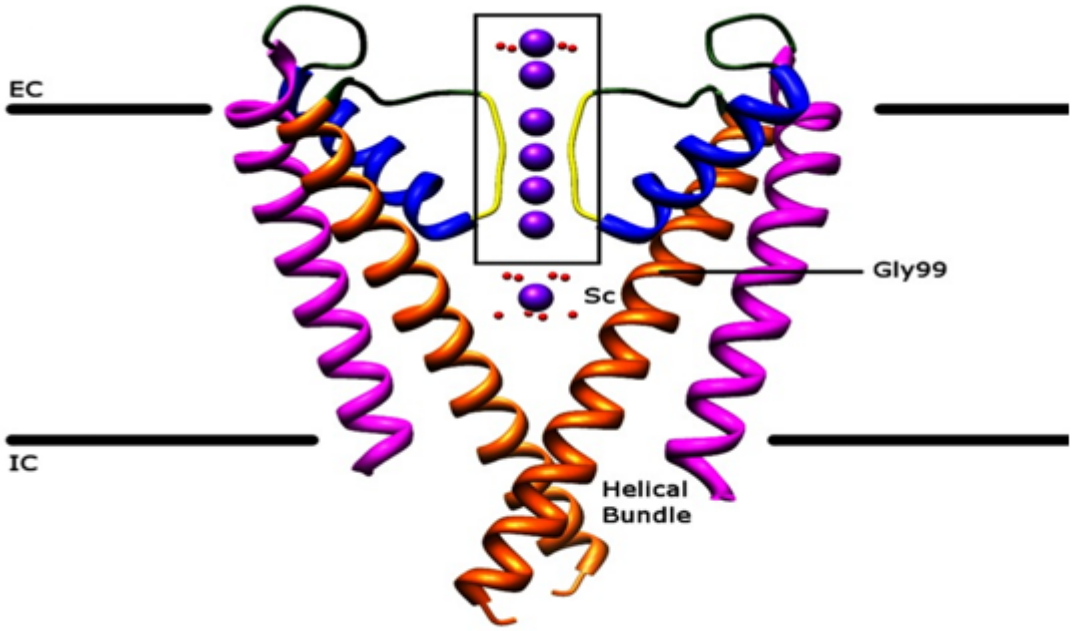

Figure 1.6: The transmembrane part of KcsA, atomic structure of KcsA in the conductive state along the membrane plane. The pore-forming domain consists of the outer helix (magenta), loop regions (dark green), pore helix (blue), SF (yellow), and inner helix (orange). The conducted K+ ions are represented by purple balls with surrounding water molecules in red. EC is extracellular and IC is intracellular (Kuang et al., 2015)

1.9 Ion channels as pesticide targets

The neuronal ion channels of insects have been shown to be the main target site of insecticides (Narahashi, 1996). Five ion channels within the insect nervous system remain the

major targets for the development of small molecule insecticides. These are, firstly, the γ-aminobutyric acid (GABA) receptor, which is the site of action of fipronil, which blocks the inhibitory action of GABA, leading to hyper-excitation which causes paralysis and death. Secondly, the glutamate-gated chloride channel is responsible for the insecticidal action of fipronil, as this compound blocks these channels in addition to its action on the GABA receptors (Narahashi *et al.*, 2010).Thirdly, the insect nicotinic acetylcholine receptor or nAChR is the site of action neonicotinoids. These compounds are efficient agonists of insect neuronal nicotinic acetylcholine receptors (nAChRs) leading to rapid excitatory neurotransmission, causing paralysis which leads to death (Honda *et al.*, 2006). Fourthly, the voltage-gated sodium channel is the site of action of DDT and pyrethroid which cause the prolonged opening of sodium channels preventing them from moving from an activated to an inactivated state. As a result, the cell membrane is depolarised, causing insect paralysis and death (Davies *et al.*, 2007). Finally, the fifth target is the insect ryanodine receptor, which constitutes the Ca^{2+}-activated Ca^{2+} channel and is the site of the binding of ryanodine and the diamides (Qi *et al.*, 2014) (Figure 1.7)

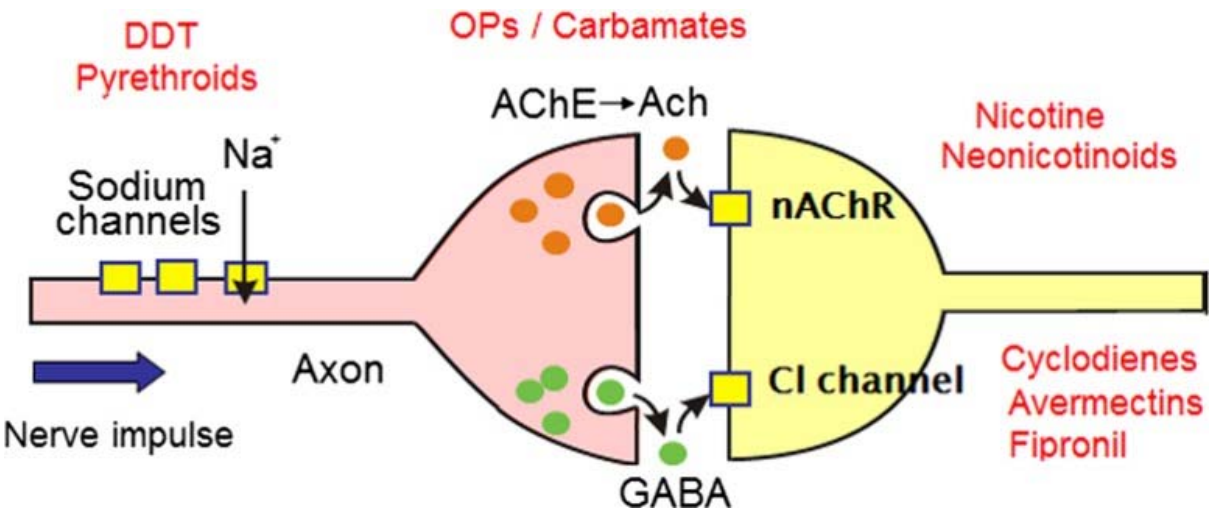

Figure 1.7: Sites of action of the most commonly used classes of insecticide (Field et al., 2017)

However, these ion channel-targeting insecticides have numerous negative effects on human health, including dermatological, neurological, carcinogenic, respiratory and reproductive effects (Nicolopoulou-Stamati *et al.*, 2016) see Figure 1.8.

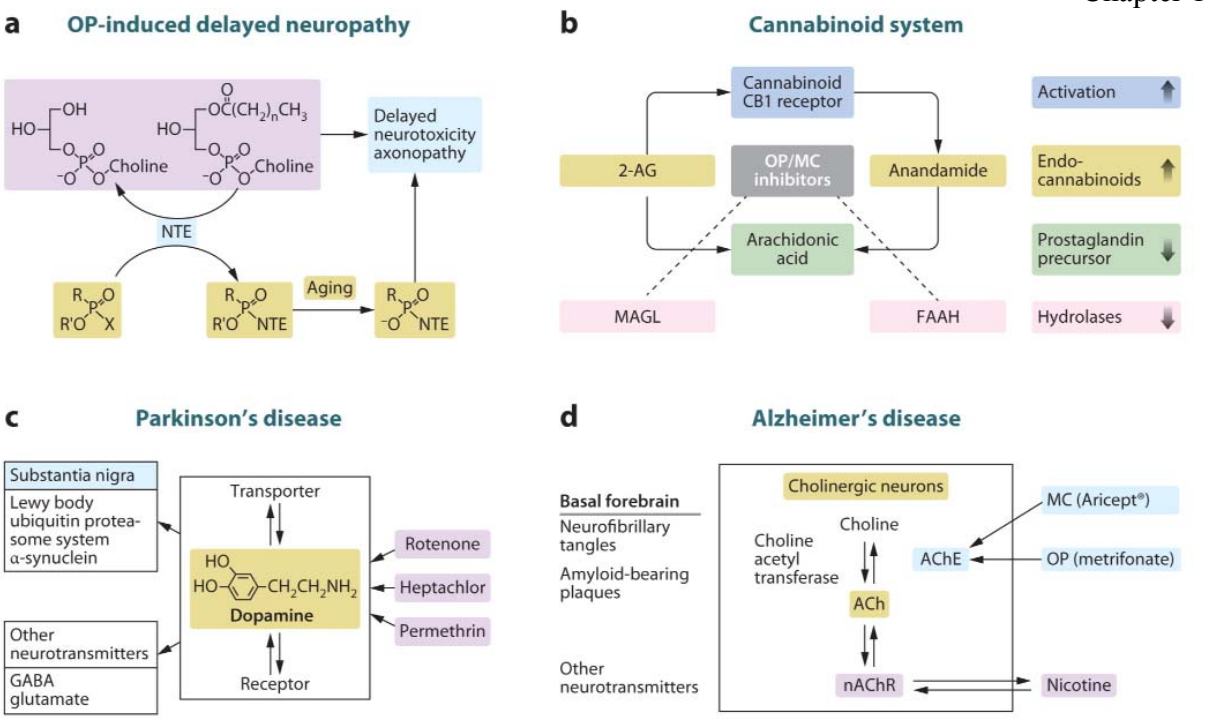

Figure 1.8: Negative health effects of insecticides on humans (Casida and Durkin, 2013)

Additionally, prolonged exposure to insecticides acting on the same insect species can lead to the evolution of resistance in insect populations; currently, there are over 550 different insect species which are resistant to some chemical pesticides. A single amino acid leucine to phenylalanine substitution (L1014F) identified in the domain IIS6 segment of the sodium channel from the grain aphid *Sitobion avenae* has been shown to results in reduced pyrethroid efficacy (Foster *et al.*, 2014). Additionally, four non-synonymous single nucleotide polymorphisms (SNPs) (i.e. V260L, G342A/V, F407Y) in acetylcholinesterase (Ace) and two mutations (W251L/S) in a carboxylesterase (MdaE7) in the housefly *Musca domestica* have been shown to increase resistance to dichlorvos and deltamethrin (Wang *et al.*, 2012).

The important health effects in humans, livestock and other non–target organisms and increasing resistance to synthetic pesticides in insect pest populations, as mentioned above, reveals the urgent need for the investigation of alternative techniques for the control of insect pests. Biological pesticides, or biopesticides, are derived from living organisms like bacteria, fungi and plants and offer alternatives to chemical control. Nakasu *et al.* (2014) demonstrated that the recombinant fusion protein (Hv1a/GNA) isolated from the venom spider *Hadronyche versuta* acts as a calcium channel blocker in the insect central nervous system, leading to the reduced survival of the peach-potato aphid *Myzus persicae* via an artificial diet. There were

also no adverse effects of Hv1a/GNA against non-target species such as *Apis mellifera* which suggests that the calcium channel in aphids is not similar in sequence to *A. mellifera*. Ion channels may also represent an interesting target for RNA interference. It has been shown that dsRNA fragments designed to target the TcNav ion channels in *T. castanuem* shared no homology with those in any other insect species, suggesting that RNAi via dsRNA of this voltage-gated sodium ion channel represents a functional mechanism to target only these pests (El Halim *et al.*, 2016). Consequently, developing insecticides that would target pest ion channels but not those of non-target organisms such as bees would be feasible and provide greater specificity than chemical approaches.

1.10 Regulatory implications of RNAi–based technologies

The latest scientific research has created a high level of anticipation concerning the future role of RNA-mediated traits in GE crops. The Canadian Food Inspection Agency (CFIA) licensed a maize line designated as *MON 87411* for livestock feed on September 26 th, 2016, This line expresses a *D. virgifera* dsSnf7 construct in combination with two Bt constructs, and it has been genetically modified to exhibit insect resistance and herbicide tolerance. Other GE crops in the USA with RNA-mediated traits have already been developed and commercialized, including the silencing of the polygalacturonase gene in tomato for fruit quality and potato resistance to potato leafroll virus (Auer and Frederick, 2009). The regulatory agency the Environmental Protection Agency (EPA) has released a White Paper on human health and an ecological risk assessment of RNAi technology which also considers the fate of dsRNA in the environment and its effect on non–target organisms (EPA, 2014). For example, a transgenic maize line expressing dsRNAs targeted of *Diabrotica virgifera* v-ATPase also targeted v-ATPase in *Diabrotica undecimpunctata* and *Leptinotarsa decemlineata* beetles (Baum *et al.*, 2007). Other studies have reported that rice and corn have siRNA with high similarity to human genomic sequences (Ivashuta *et al.*, 2009). It is worth mentioning; these unwanted effects could indeed represent a real hazard for non-target organisms. European Food Safety Authority (EFSA) is currently in the process of collating findings and information from RNAi –based studies. There are many questions that remain unanswered about the field of RNAi regarding specificity, the fate of dsRNA in the environment, impacts on non-target organisms, the required sequence homology of siRNA to its target region, off-target effects.

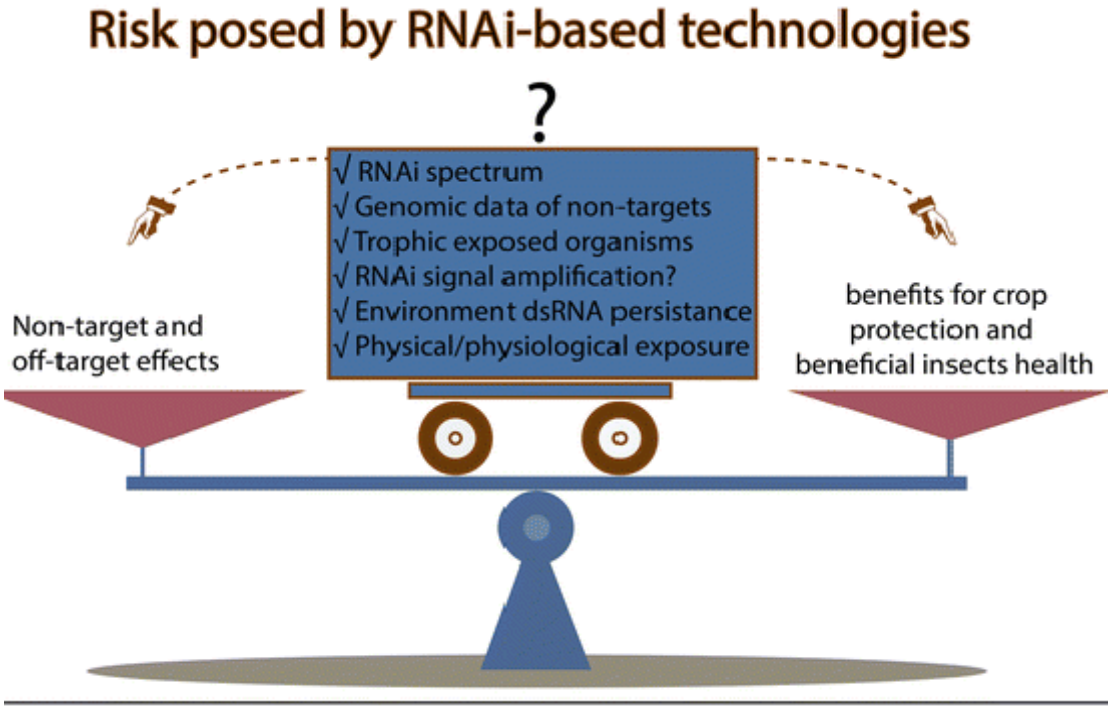

Figure 1.9: Some achievements and concerns about RNAi in insect management (Zotti and Smagghe, 2015)

1.11 Research Rationale

Synthetic pesticides are known to deleteriously affect both target and non-target species, highlighting the need to develop safer and more specific alternatives for crop protection. This study explores the potential of potassium channels as a novel insecticide target using RNAi-based strategies.

1.12 Research Hypothesis

It is hypothesized that

1. The selective knockdown of *SK* encoding small conductance calcium-activated channels and *SH* encoding voltage-gated potassium channels, by RNAi will lead to dose-dependent mortality of *T. castaneum.*
2. dsRNA targeting *T. castaneum SK* and *SH* do not affect the expression of the corresponding genes in the honeybee, nor does it affect bee survival or immunity.

3. The susceptibility of *T. castaneum* to the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* increases following the knockdown of *SK* and *SH* in *T. castaneum*, as a strategy for pest management

1.13 Project Aims

The present project seeks to investigate the suitability of using RNAi technology to disrupt gene expression in *T. castaneum* as a potential control strategy (Figure 1.10). The following specific objectives were as follows:

1. To determine the effects of the knockdown of the *SK* (small conductance calcium-activated channel) and *SH* (voltage gate potassium channel) genes involved in neural transmission by dsRNA produced via in vitro synthesis as a method for the control of *T. castaneum* (Chapter 2).

2. To determine the effects of dsRNA SK and SH of *T. castaneum* against the honeybee (*Apis mellifera*) as a non-target beneficial insect (Chapter 3).

3. To determine the effects of the knockdown of the *SK* and *SH* by dsRNA produced via *Escherichia coli* HT115 lacking in RNase III against *T. castaneum* as a low-cost method (Chapter 4).

4. To evaluate the potential of the targeting of the *SK* and *SH* in *T. castaneum* to increase the susceptibility of this insect pest to pathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Chapter 5).

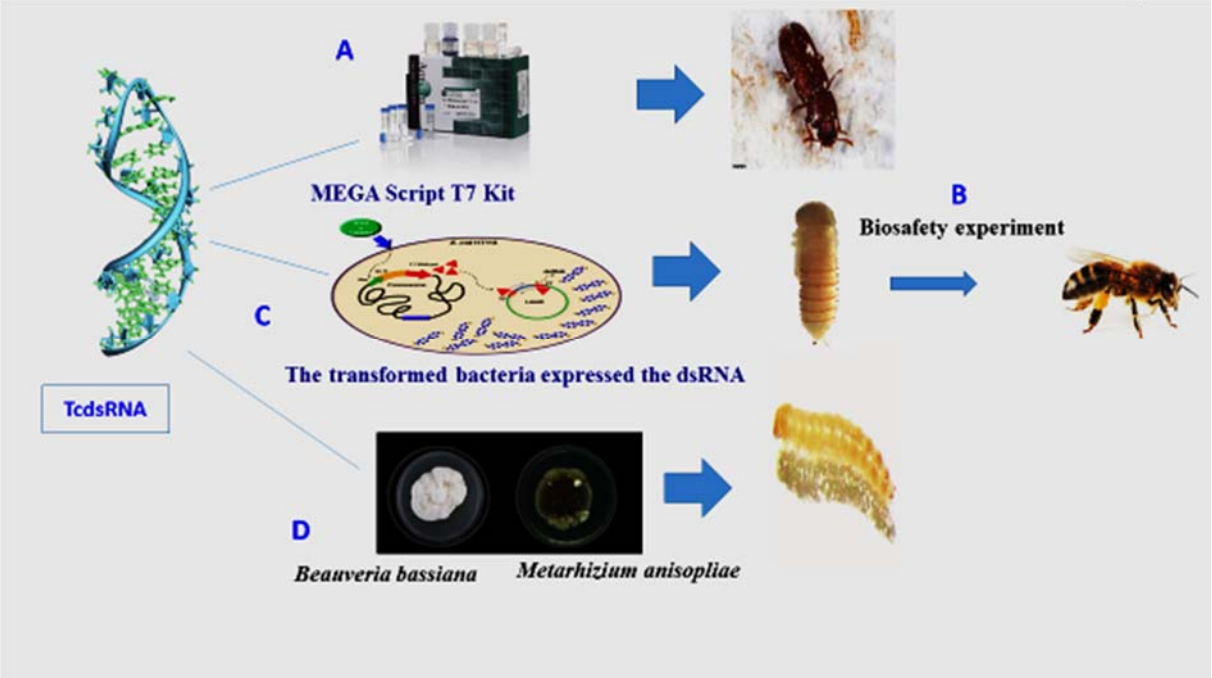

Figure 1.10: Experimental framework of the present study: A) The TcdsRNA produced by a commercial kit was tested for its toxicity against the *Tribolium castaneum*; B) Effects of this dsRNA on honeybees; C) transformed bacteria expressing the dsRNA tested for toxicity against the *T. castaneum*; D) TcdsRNA combined with pathogenic fungi to increase its efficiency.

Chapter two

Targeting the Potassium Ion Channel Genes *SH* and *SK*, as a Novel Approach to Control the Red Flour Beetle *Tribolium castaneum*

2 Targeting the Potassium Ion Channel Genes *SK* and *SH*, as a Novel Approach to Control the Red Flour Beetle *Tribolium castaneum*

2.1 Abstract

Potassium ion channels (K^+) play a critical role in the generation of electrical signals via the selective transport of K^+ ions across neurons. Therefore, the blockage of these channels will create serious effects. Gene silencing techniques such as RNA interference (RNAi) have been suggested as a viable approach to control crop pests and thus increase crop productivity. In this study, genes for the small conductance calcium-activated potassium channel (*SK*) and voltage-gated potassium channels (*SH*), which participate in regulating the action potentials of nerve cell ion channels, were knocked down in *Tribolium castaneum* using the injection and oral delivery of long dsRNA molecules. The delivery orally or by injection delivery of dsRNA for either *SK* and *SH* demonstrated a dose-response effect regarding gene knockdown and mortality. Larvae fed with a diet of 400 ng dsRNA/mg has shown significant knockdown in gene expression what levels of 98% and 83% for *SK* and *SH* respectively. However, the injected delivery of dsSK and dsSH caused 99% and 98% of gene expression knockdown respectively at 248.4 ng/larva. Furthermore, when injected early, late pupae and adults with 248.4ng/insect of dsSK showed knockdown levels in gene expression of 93%, 96% and 93% respectively, compared to 94%, 92% and 72% for dsSH respectively following 48h post dsRNA injection. The expression of both target genes was significantly ($p < 0.05$) reduced in different developmental stages, leading to 100% and 97.78% larval mortality when fed 400.0 ng/mg diet respectively after 7 days. The injected delivery dsRNA of *SK* and *SH* into the larvae caused 100% and 73.4% mortality at 248.4 ng/larvae respectively after 7 days. After injection dsRNA of *SK* at 248.4 ng/insect in early, late pupae and adults of *T. castaneum* with dsRNA of *SK* resulted in 68.9 %, 60.0% and 60.0% mortality respectively, while those treated with the dsRNA of *SH* exhibited 57.80%, 51.20% and 55.55% mortality respectively at the same conditions. LC_{50} values for injection and oral treatment were much lower in *SK* than *SH* with both delivery methods, demonstrating that the knockdown of *SK* had a greater effect on mortality. This study demonstrates the potential power of using RNAi targeted at neural receptors as a technology for the control of *T. castaneum*.

2.2 Introduction

In agricultural systems, insect pests cause severe crop damage resulting in significant economic losses and diminished food resources at a time when the population of the world is increasing rapidly. The United Nations Population Fund (UNFPA) has estimated that, in 2043, the world population will be 9.0 billion (Pimentel *et al.*, 2010). Therefore, it is essential that new, sustainable pest control strategies be developed.

T. castaneum is an insect pest of stored grain which damages the economy by infesting stored agricultural products (Mishra *et al.*, 2012; Knorr *et al.*, 2013). The antioxidant system of *T. castaneum* provides resistance to several insecticides and allows rapid adaptation to extreme temperatures, periods of drought and prolonged periods of fasting (Tabunoki *et al.*, 2016). Chemical pesticides are still the major approach used to control insect pests, but they are associated with significant hazards to the environment, human health and non-target insects (WHO, 2010). In addition, *T. castaneum* has become resistant to the fumigant phosphine which is used extensively worldwide (Perkin *et al.*, 2016). Gene silencing through RNA interference (RNAi), via the specific-post transcriptional down-regulation of gene expression (Hammond *et al.*, 2001), has been proposed as an alternative approach to mitigate the impact of insect pests. RNAi is considered to be one of the breakthroughs of the twenty-first-century molecular biology. Not only it is a powerful tool for understanding basic biological processes, but it can also be exploited for crop protection by targeting specific essential genes. It is considered to represent a specific type of defence mechanism in eukaryotic organisms (Cogoni *et al.*, 1996; Hammond *et al.*, 2001), which can be induced through an *in vivo* application of dsRNA molecules which are homologous to the target gene. This biological process results in the degradation of a target mRNA (Price and Gatehouse, 2008), enabling the analysis of loss-of-function in organisms in which classical genetic analysis is impossible. The ease of RNAi application in the red flour beetle *T. castaneum* has thus made this species a popular and powerful model in evolutionary genetics, and an excellent screening platform for RNAi (Denell, 2008; Dönitz *et al.*, 2014), which can be delivered via sub-cuticular injection and feeding by artificial diet. *Tribolium* has a robust systematic RNAi response through its development, which makes it possible to perform RNAi at the post-embryonic stage by injecting dsRNA into the larval body cavity (Tomoyasu and Denell, 2004). As such, RNAi phenotypes in *Tribolium* are easy to obtain and are highly reproducible. The adult's

insects experience long reproductive lives with high fecundity (Shelby, 1978). In addition, virtually all *Tribolium* tissues can respond to extracellular dsRNA, and all life stages have been induced by RNAi (Knorr *et al.*, 2013; Dönitz *et al.*, 2014). These traits allow researchers to create loss-of-function phenotypes at any desired stage in *Tribolium* by simple larval or pupal injection, making *Tribolium* a good alternative model organism to study gene function outside of classic model organisms.

This technology can be seen as an 'environmentally friendly' approach for the control of insect pests, with a high degree of specificity (Swevers *et al.*, 2013). The knockdown of specific genes has been successful in certain insect species of Diptera (Valzania *et al.*, 2016; Xiong *et al.*, 2016), Coleoptera (Tang *et al.*, 2016; Powell *et al.*, 2017), and Lepidoptera (Quan *et al.*, 2002; Cao *et al.*, 2016; Chikate *et al.*, 2016). Previous studies have revealed that the Coleoptera are more susceptible to dsRNA compared to other arthropod orders (Bellés, 2010), while the Lepidoptera require higher concentrations of dsRNA than Coleoptera according to sensitivity tests (Terenius *et al.*, 2011; Ivashuta *et al.*, 2015). Moreover, Dong *et al.* (2017) showed that the injection of 3 µg/µl of dsASTC or dsASTCC into *Clostera anastomosis* led to suppress ASTC and ASTCC genes, which encode allatostatin C-like peptides playing a role in juvenile hormone (JH) synthesis in the insect. However, in a study of Coleoptera, *T. castaneum* with only injection 500 ng/µl of dsRNA for the *Tc-ff* orphan gene which is importance in epithelial morphogenesis and appendage formation caused the knockdown of this gene (Thümecke *et al.*, 2017)

Potassium ion channels are composed of two parts: The filter allows potassium ions to pass, and the gate opens and closes the channel depending on environmental signals (Castellano *et al.*, 1997; Minor, 2001; Yellen, 2002). These channels are involved in setting and resetting the resting potential in excitable nervous cells (Lim and Dudev, 2016).

The SK gene codes for small conductance calcium activated potassium channels which control the action potential discharge frequency; and are involved in synaptic plasticity, therefore; playing important roles in the learning and memory in insect such as *Drosophila* (Stackman *et al.*, 2002). Meanwhile, the SH gene codes for voltage-gated potassium channels which are integral membrane proteins essential for the correct functioning and repolarization

of the cell and *SH* helps determine an organism's amount of sleep is called mini sleep in insect (Jegla *et al.*, 1995; Cirelli *et al.*, 2005). Both genes are expressed in the CNS in Coloptera.

In this study, RNAi techniques were used to investigate the potential of the targeting of the K^+ channel genes *SK* and *SH* as an effective approach to the control of the coleopteran *T. castaneum*, and that this can provide a novel pesticide target for future control strategies.

2.3 Material and Methods

2.3.1 Insects

Blades Biological Ltd, Kent, TN8 7DX, supplied a culture of *T. castaneum*. The culture was maintained on organic whole flour containing 5% brewer's yeast, at 30 °C, 16:8 h (L:D), and the flour was replaced every 2–4 weeks.

2.3.2 Design of dsRNA

The sequences of the *T. castaneum* potassium ion channel *SK* and *SH* genes were identified using a *Tribolium castaneum* small conductance calcium-activated potassium channel protein in BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?) for the SK gene (gene bank accession number XM-008195295.1) and the *Tribolium castaneum* potassium voltage-gated channel protein shaker for the SH gene (gene bank accession number XM-008192853.1) at NCBI. The E-RNAi web tool (http://www.dkfz.de/signaling/e-rnai3//) selected a region of XM-008195295.1 for the SK gene and XM-008192853.1 for the SH gene. These transcripts had no similarity to other transcript regions in the *T. castaneum* genome. The target sequence for the kanamycin resistance gene (Kana) accession number JN638547 dsRNA, which has previously been described by Tempel Nakasu (2014) was used as a control.

2.3.3 RNA extraction and cDNA synthesis

The total RNA was extracted from 6th larval instar insects using a TRIzol® Plus RNA Purification Kit (Ambion), following the manufacturer's instructions. The integrity and size of total RNA isolates were investigated using 2% agarose gel electrophoresis as described in Sambrook JaR (2001). RNA was quantified on a Nano Drop spectrophotometer (model ND-

1000, Lablech). 1000 ng of RNA was converted to cDNA for each reaction by using SuperScript® II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions to initiate subsequent PCR reactions.

2.3.4 PCR product

The cDNA synthesized in the described above served as a template for PCR. The specific primers were designed using NCBI/ Primer-BLAST software (Table 2.1). All primers were used at a final concentration of 10µM. The primers were designed to amplify the PCR products of 181and 150 bp for *SK* and *SH* respectively (refer to Appendix A for a sequence of *SK* and *SH*). The PCR reaction involves 25µL of PCR Master Mix, 1 µL each of Forward and Reverse Primers, 1 µL of Template DNA, and finally, complete the total volume is made up to 50 µL by adding Ambion® Nuclease-Free Water. After gentle vortexing. The samples were put in a thermal cycler (Applied Biosystems, GeneAmp® PCR system 9700) under the PCR conditions of 95 °C for 3 minutes followed by 35 cycles of 95°C for 30 seconds denaturation, annealing at 57°C for 30 s, then at the extension temperature (72 ˚C for the *SK* for11s and for the *SH* for 9s) and a final extension step of 72 ˚C for 10 min. Following electrophoresis, the bands in the gel were purified using a QIAquick MinElute Gel Extraction kit (Qiagen) following the manufacturer's instructions and were then cloned into StrataClone vector pSC-A-amp/kan (Stratagene) following the manufacturer's instructions. The QIAprep Spin Miniprep Kit (Qiagen) protocol was used to purify the plasmid DNA. These plasmids were sent for sequencing to confirm the cloned insert.

2.3.5 Reverse transcription- quantitative PCR (RT-qPCR)

Gene expression was evaluated via RT-qPCR using SYBR Green (Bioline) following the manufacturer's instructions. The regions to which primer pairs for RT-qPCR (Table 2.1) were designed were distinct from those targeted by dsRNA. RT-qPCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 15 sec. at 95 °C, 30 sec. at 57 °C and 15 sec. at 60 °C. In this studies, three biological replicates of cDNA as described in section 2.3.3 containing 5 pooled insects for each were used, and they were normalised against a reference gene TcRpS6 with gene bank accession number XP_968395.1 (Castellano et al., 1997). The relative transcript quantity was calculated using the ΔΔCq method (Livak and Schmittgen, 2001).

Table 2.1: Primers used for RT-qPCR and PCR analysis in *T. castaneum* for *SK* and *SH*.

Application	Primer ID	Sequence (5' – 3') of primer for *SH*	Primer ID	Sequence of primer for *SK*
RT-qPCR ***T. castaneum***	FA1	CCGGTTCGAGACGCAACTT	FA2	CCTTTGAACTACGAAAGCCC
	RA1	TTGCGAAGCGGGTCGAAGTA	RA2	CAGAGTCCCTGCTCGAGTTC
PCR ***T. castaneum***	FB1	GCTGGACGTCTTCTCCGA	FB2	ACATCCCCATAACAAGAGCG
	RB1	GTACTCGAACAGCAGCCACA	RB2	GTGGGACACGTGGACTACCC
dsRNAsy ***T. castaneum***	FC1	TAATACGACTCACTATAGGG-GCTGGACGTCTTCTCCGA	FC2	TAATACGACTCACTATAGGGG-TGGGACACGTGGACTACCC
	RC1	TAATACGACTCACTATAGGG-GTACTCGAACAGCAGCCACA	RC2	TAATACGACTCACTATAGGGG-ACATCCCCATAACAAGAGCG
dsRNAsy Kanamycin (dsRNA control)	FD1	TAATACGACTCACTATAGGGC-ATTCGCCGCCAAGTTCTTC		
	RD1	TAATACGACTCACTATAGGGTG-CTCGACGTTGTCACTGAA		
RT-qPCR - TcRpS6 (reference gene)	FE1	GAAGCAGGGTGTTCTCACGA		
	RE1	GTTTCCTTTCACCGTCACGC		

2.3.6 Synthesis of dsRNA

dsRNA was synthesized using the MEGA Script T7 Kit (Ambion®) following the manual's instructions. To set up one reaction, 1µg of PCR product was mixed the 10X T7 reaction buffer, T7 enzyme mix and the 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP) and the reaction was incubated at 37°C overnight, and then incubated at 75°C for 5 min. The dsRNA was bound to filter cartridges. Eluted dsRNA was stored at -80 °C before injection and feeding after quantification on a spectrophotometer.

2.3.7 Delivery of dsRNA by injection

Adults, pupae and larvae were injected using a NanojectII^{TM} injector (Drummond Scientific Company) under a dissecting stereomicroscope. An injection needle produced by putting glass capillary tubing in brown micropipette puller (model p-1000) after that the needle was filled with immersion oil and inserted onto the holder of injector, with the rubber completely

inserted into the opening of the needle holder and loading dsRNA into needle. The 6th larval stage insects were injected with dsRNA for each dose (62.1, 124.2, 186.3 and 248.4 ng/larva) into the dorsal side between first and second abdominal segments, while the pupae were injected with dsRNA at 248.4 ng/pupa between the second and third abdominal segments as described by Tomoyasu and Denell (2004). Adults were injected in the dorsal under the elytron at the same concentration of dsRNA as used in the pupae. Injected insects were left for 15 min and then transferred to petri dishes containing white flour supplemented with brewer`s yeast at 30°C. Three biological replicates were used. Each replicate consisted of 15 insects for the survival experiment and 5 insects for the RT-qPCR. The expression of targeted genes was quantified 48 h after exposure to dsRNA. Survival was monitored on a daily basis for 7 days. For all injection assays, 3 controls were used: insects without injection (control 1); insects injected with RNAase free water (control 2); and insects injected with dsRNA Kanamycin at 248.4 ng/insect (dsKana), which is a region of the bacterial resistance gene (control 3).

2.3.8 **Delivery of dsRNA by feeding**

The dsRNA was delivered via flour disks prepared as described by Xie *et al.* (1996). 10µl of flour suspension (dsRNA, 5% brewer`s yeast) was prepared in flat bottom wells of a 96-well microtiter plate and allowed to dry out at room temperature. Next, an individual 3rd instar larva was added to each well. Three biological replicates each consisting of 15 insects for the survival experiments and 5 larvae for each replicate were fed for 72h on flour disks at a range of concentrations (100, 200, 300, 400 ng dsRNA/mg diet) and used to determine gene expression. Three groups of control were used: only flour (control 1), flour with free water (control 2), and flour with free water and a dsRNA fragment of the Kanamycin resistance gene with 200 ng dsKana/mg diet (control 3). The diet was changed every two days to prevent the contamination and degradation of dsRNA (Figure 2.1)

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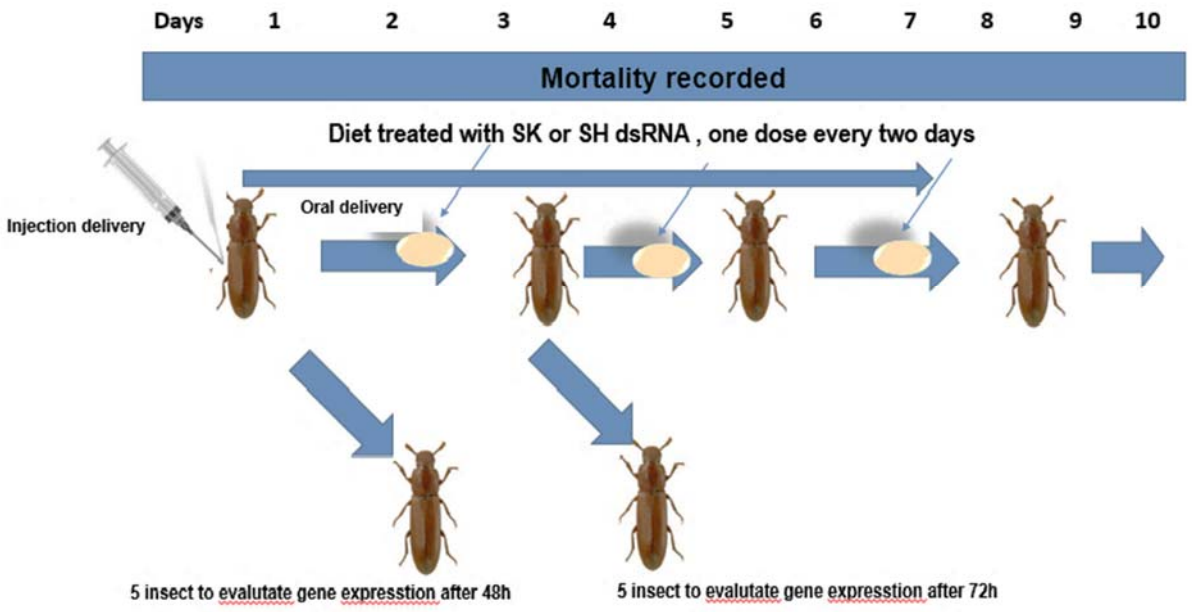

Figure 2.1: Diagrammatic representation of bioassay for dsRNA of *SK* and *SH* via oral and injection delivery with *T. castaneum.*

2.3.9 Statistical analysis

Insect mortality was analyzed using Kaplan-Meier survival analysis and the Sigma Plot program, (version 12.5, Systat. Software Inc., San Jose, USA) and insect mortality was corrected according to Abbott's formula (Abbott, 1987). RT-qPCR results were analyzed treatment with one-way ANOVA followed by the Tukey test to compare differences in the effect of various concentrations of dsRNA (Minitab, State College, PA, USA).

2.4 Results

2.4.1 Clone of *SK* and *SH* fragments

The effectiveness of RNAi can be identified using dsRNA. This is a region of the gene of interest designed to induce gene knockdown by RNAi in pest insects, and for this investigation dsRNA molecules of the *SK* and *SH* were synthesized. A 181bp fragment of the *SK* gene and a 150bp fragment of the *SH* gene were cloned in a plasmid vector, and then the

plasmids were sent for sequencing to confirm the identity of the insert. A sequence alignment was applied which confirmed 91% homology between the insert and the *SK*, and 95% homology for the *SH* (Figure 2.2). cDNA was amplified with PCR primers appended with the T7 promoter for both side and this product used for dsRNA synthesis.

Figure 2.2: Output from BLAST alignment tool showing sequences (a) 91% homology between plasmid insert (Query) and *SK* (sbjct); (b) 95% homology between plasmid insert (Query) and *SH* (sbjct). Line between nucleotides point sequence homology, whereas a lack of line points the sequence change at that nucleotide.

2.4.2 Synthesis of the target interference sequence

In order to synthesise a conventional dsRNA that targets the corresponding mRNA of the *SK*, *SH* and *Kana in vitro* transcription was used for both target and the control (kanamycin resistance gene), as described in the section 2.3.6. Fragments of 221bp, 190bp and 508bp for *SK*, *SH* and *Kana* respectively are the result of a 181 bp, 150bp and 468bp amplicons respectively with a 20bp T7 promoter at the 5 end of both the forward and reverse primers

(Figure 2.3). The dsRNA was quantified in order to calculate its concentration so as to prepare the dose that could be delivered and then to examine the effect on gene knockdown and mortality. The large amounts produced were 5000 ng/µl, 3456 ng/µl and 1500 ng/µl in the 100µl volume of dsRNAs corresponding to *SK*, *SH* and the kanamycin resistance genes respectively. It is observed that the migration of dsRNA was slower than the DNA fragment which had the same length because the diameter of dsRNA is 30% larger than the DNA (Van den Hout, 2010).

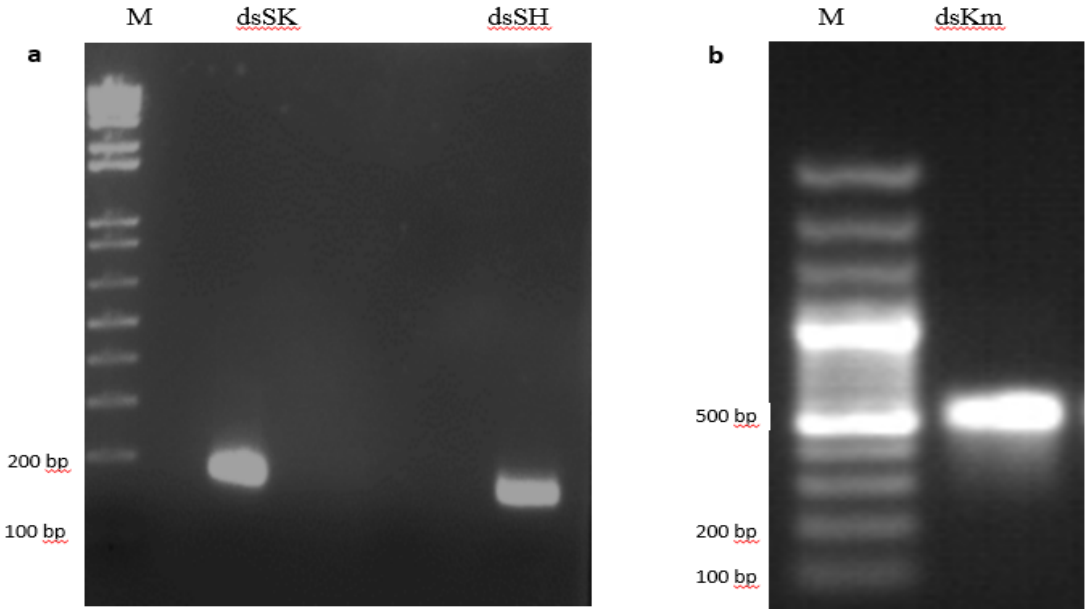

Figure 2.3: dsRNA fragments **a**. *SK* (221 bp) and *SH* (190 bp) genes, **b**. *Kana* (508 bp) tested using 2% Agarose gel electrophoresis, M: 100bp DNA ladder.

2.4.3 **Physiological response of *T. casteneum* to TcK$^+$ dsRNA**

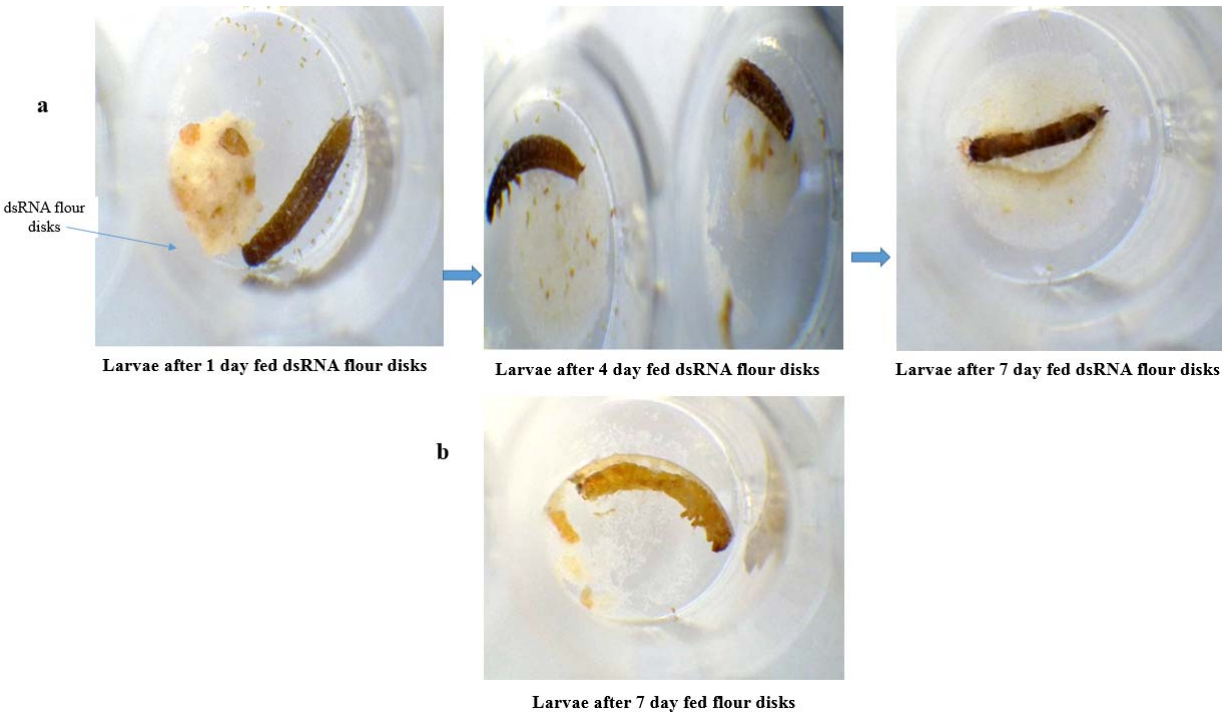

Figure 2.4: The larvae of *T. castaneum* (a) fed on dsRNA flour disks (b) fed on flour disks

It is observed the larvae fed with dsRNA was small size and dark colour comparative with control larvae (Figure 2.4).

2.4.4 Endogenous gene expression

The relative abundance of the *TcSK* and *TcSH* at different developmental stages was examined using RT-qPCR to ensure that subsequent studies were applied on the appropriate stages. RNA was extracted from larvae (n=5) of 1st, 3rd and 6th larvae, early and late pupae and adults. The RNA was converted into cDNA, and the relative abundance of the target genes was investigated using RT-qPCR. TcRpS6 was used as a reference gene for gene expression because it was relevant in this experiment.

Both *SK* and *SH* were shown to be expressed in all developmental stages (1st, 3rd, 6th larvae; early pupae, late pupae and adults). The expression of *SK* and *SH* in the late pupa was 0.91 and 1.1-fold respectively, relative to the adult stage. It is clear the expression of *SH* was slightly higher in the late pupal stage than in the adult. However, the expression was less than the adult in the *SK*. Despite this, there was no significant difference observed in the

expression of these genes between adult and late pupa P>0.05 (Figure 2.5). The expression of *SK* and *SH* in the early pupa was 0.6 and 1 fold respectively, the difference was significant between early pupa and late pupa for *SK*, whereas there was no significant difference between them for *SH*. The expression of both genes was much lower in the larval stage (1^{st}, 3^{rd}, 6^{th}) compared to adult and pupa. A difference that was found to be significant (P< 0.05) showed that the gene expression decreased to 0.07, 0.11 and 0.46-fold in the *SH* and 0.07, 0.30, 0.34 fold change in the *SK* respectively. Thus, the adult, early pupa, late pupa and 6^{th} larval instar would, therefore, suggest to gene knockdown.

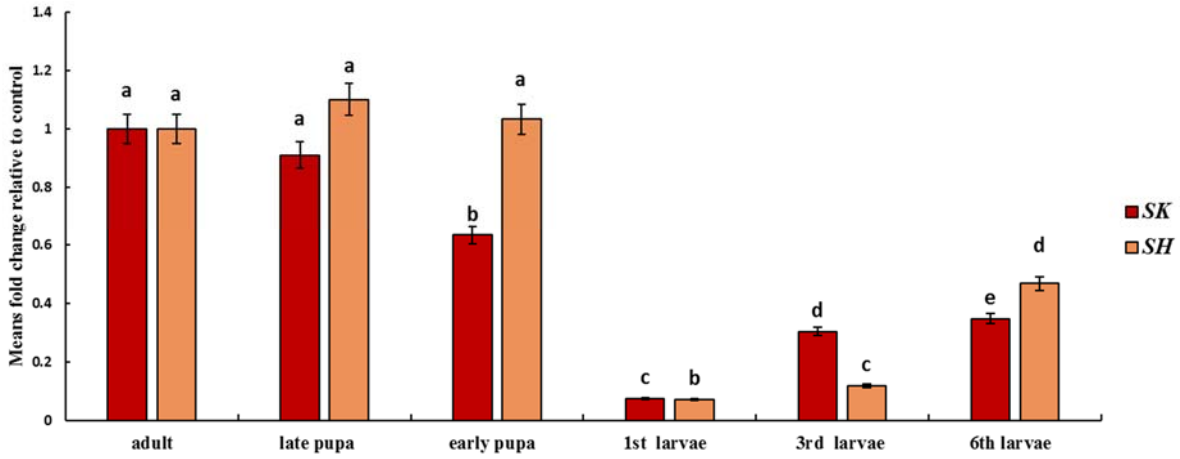

Figure 2.5: Stage-specific *SK* and *SH* expression in the whole body of flour beetles evaluated by using RT-qPCR, *T. castaneum*, n = 5. Mean ± SD of three replications is shown. Means with the different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post-hoc Tukey test) within each gene. Expression levels were normalized against the TcRpS6 gene as an internal standard.

2.4.5 Effects of injection dsRNA on the expression level of target genes

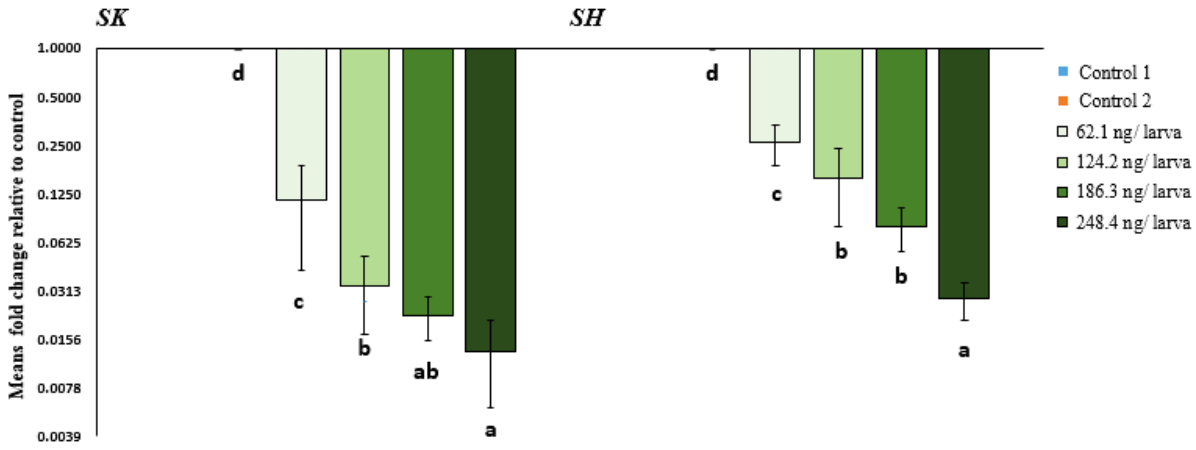

Figure 2.6: Expression of SK and SH mRNA in larvae injected with dsRNA after 48h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= larvae injected with RNAase free water, control 2 = larvae injected with kanamycin resistance dsRNA. Expression levels were normalized against the TcRpS6 gene as an internal standard.

There is a clear trend of decreasing target genes expression levels in the 6th instar larvae at 48h after the corresponding dsRNA injections at 62.1, 124.2, 186.3 and 248.4 ng/larva (Figure 2.6). There were 0.11, 0.03, 0.02 and 0.01-fold respectively for the *SK*, whereas the *SH* was 0.26, 0.15, 0.07 and 0.027-fold at the same concentration respectively. Gene expression in both genes was found to be significantly reduced compared to 1.00 and 0.98 fold in control 1, (injection RANase free water) and control 2 (injection 248.4 ng/larva Kana dsRNA) respectively. However, the reduction in expression of both targets in the two controls was not significant ($p > 0.05$). These results indicate that RNAi can cause knockdown in the expression of the *SK* at levels between 89-99%, while for the *SH* knockdown levels were recorded between74-98%.

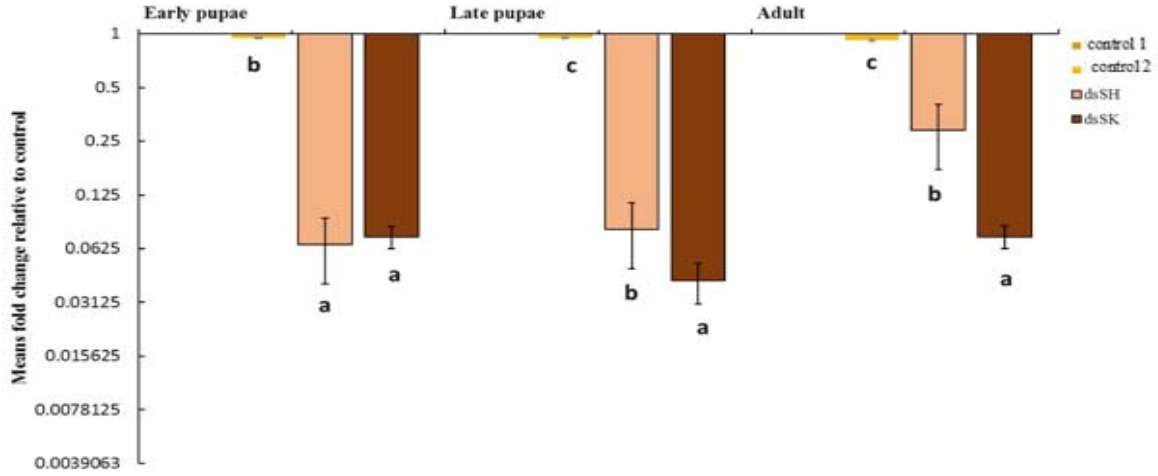

Figure 2.7: Expression of SK and SH mRNA in early, late pupal and adult injected with dsRNA after 48h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) each development stage within each gene. Control 1= insect injected with RNAase free water; control 2 = insect injected with kanamycin resistance dsRNA. Expression levels were normalized against the TcRpS6 gene as an internal standard.

Moreover, RT-qPCR analysis of mRNA transcript levels in early and late pupae injected with 248.4 ng/pupa of dsSK showed that the expression was reduced by 0.07 and 0.04-fold respectively. However, dsSH has recorded down-regulation of 0.066 and 0.080-fold respectively at the same concentration relative to two control groups: control 1 which had 1.00 fold and control 2 had 0.95-fold. Furthermore, the gene expression of adult insect was also down-regulated to 0.07 and 0.28-fold after 48 h that they had been injected with *SK* and *SH* dsRNA respectively at 248.4 ng/adult. These down-regulation results were statistically different from those for dsKana at 248.4ng/insect and in the untreated control, which were 0.92 and 1.00 -fold respectively (Figure 2.7).

These findings show that dsRNA led to a significant reduction in levels of the mRNA of the target genes 48h after injection into larvae, pupae and adults.

2.4.6 Effects of feeding dsRNA on the expression levels of the target genes

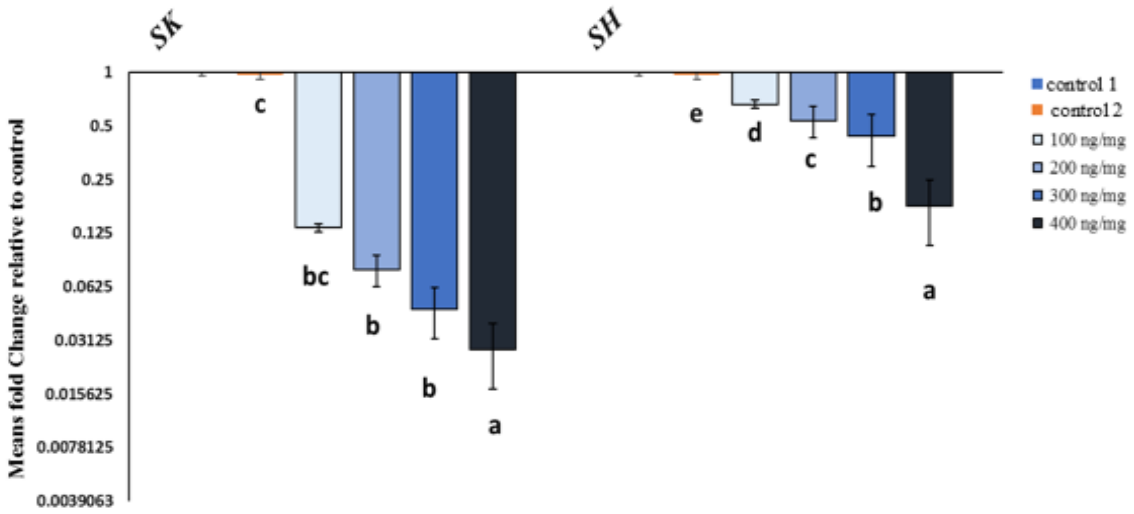

Figure 2.8: Expression of SK and SH mRNA in larvae fed with dsRNA after 72h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= larvae fed on RNAase free water flour disk, control 2 = larvae fed on kanamycin resistance dsRNA flour disk. Expression levels were normalized against the TcRpS6 gene as an internal standard.

Three days of continuous feeding of the 3rd instar larvae on flour disks, containing the dsRNA at a range of different concentrations (100, 200, 300 and 400ng dsRNA/mg diet) caused a decrease in *SK* and *SH* transcript levels by 0.13, 0.07, 0.04 and 0.02-fold respectively for the SK gene, whereas, they were 0.66, 0.53, 0.43 and 0.17 -fold respectively for the SH gene compared to1.00 and 0.96-fold for control 1 and control 2 respectively (Figure 2.8). These results show that the down-regulation of expression of the *SK* reached between 87-98%, while for the SH gene levels between 34-83% were recorded.

From these results, it was determined that the induction of dsRNA for either gene caused the down-regulation in the expression of the gene. However, the induction of non–endogenous dsRNA does not have a significant impact on gene expression.

2.4.7 Injection bioassays targeting potassium channel genes in *T. castaneum*

Experiments were carried out to assess the effects of varying the dose of dsRNA on the survival of different stages of *T. castaneum* by injecting. Larvae were injected with doses of 62.1, 124.2, 186.3 and 248.4 ng/larva dsRNA, whereas early pupa, late pupa and adults were

injected only at the highest concentration. Three biological replicates, 15 insects/ rep, for each dose. Abbott's formula was used to correct survival data relative to control 1 (untreated control).

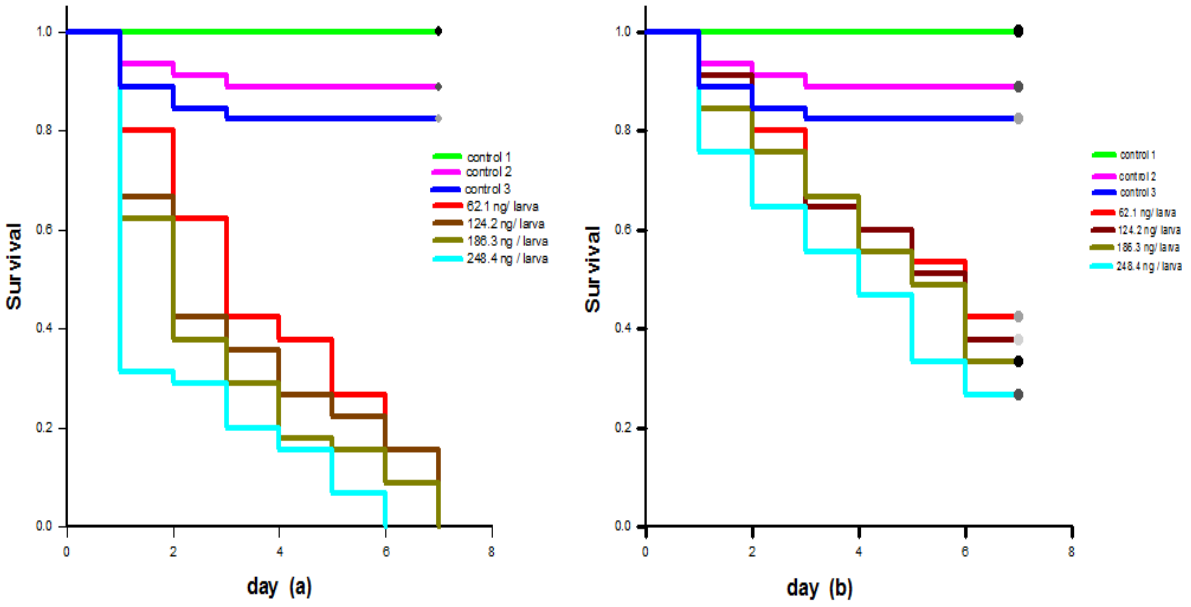

Figure 2.9: Survival of *T. castaneum* larvae injected with dsRNA targeted to *SK* (a) and *SH* (b) ($p<0.001$; n= 45 larva/treatment, as shown by Kaplan-Meier survival analysis). Control 1= untreated larvae, control 2 = larvae injected with RNAase free water, control 3 = larvae injected with kanamycin resistance dsRNA.

It was found that the dose-response effect of the injection of dsRNA targeted of both the *SH* and *SK* at concentrations 62.1, 124.2, 186.3 and 248.4 ng/larva caused significantly higher mortalities ($p<0.001$) for 6th instar larvae compared with the 3 control groups. No mortality occurred in control 1. Moreover, 11.2% and 17.8% mortality were found in control groups 2 and 3 respectively; statistical analysis revealed no significant difference among these values ($p >0.001$). Final mortality was recorded as 100% after 7 days with the *SK* dsRNA at all concentrations (Figure 2.9a). While there was 57.8%, 62.3%, 66.7% and 73.4%, after 7 days for SH dsRNA at the respective doses (Figure 2.9b). The lowest dose of dsRNA, 62.1ng/larva caused a significant reduction in survival of larvae compared to the controls ($p<0.001$). The LC_{50} of the larvae injected with *SK* and *SH* dsRNA were 2.38 and 34.93 ng/larva respectively on day 6.

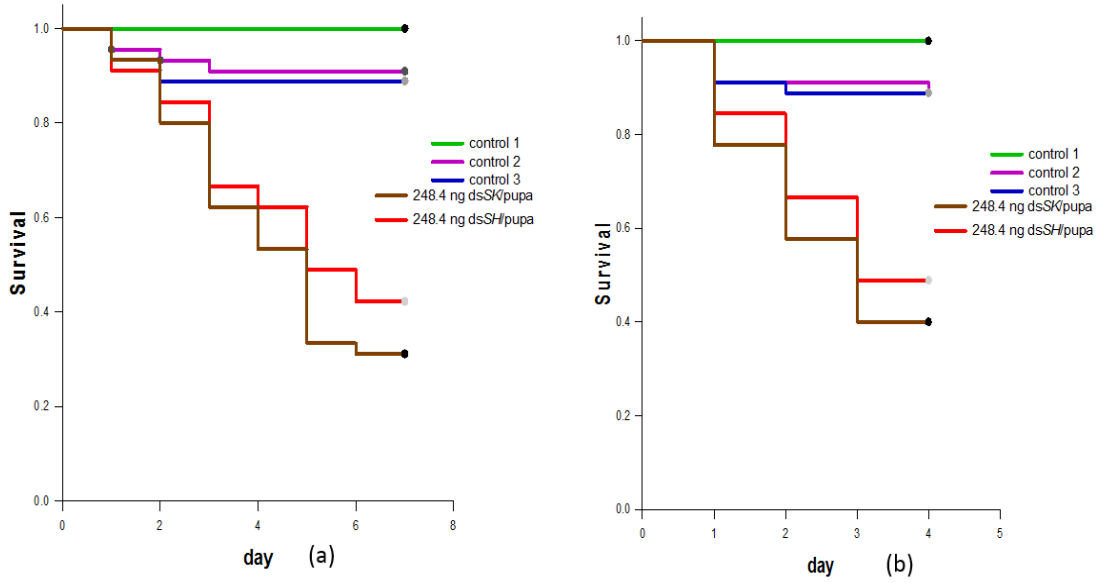

Figure 2.10: Survival of *T. castaneum* early pupae (a) and late pupae (b) injected with dsRNA targeted of the *SK* and *SH* ($p<0.001$; n= 45 pupa/treatment, as shown by Kaplan-Meier survival analysis). Control 1= untreated pupae, control 2 = pupae injected with RNAase, control 3 = pupae injected with kanamycin resistance dsRNA.

The dose-response effects of dsRNA for both target genes for the survival of the pupae were investigated. The mortalities have been measured 7 days after injection early pupal (zero day-old) and 4 days injected late pupal stages (three day-olds) at 248.4 ng/pupa dsRNA. These mortalities were significantly different relative to three control groups ($p<0.001$). The control results indicated that no mortality occurred in control 1 for early and late pupae. However, control 2 and control 3 had recorded 9.1% and 11.5% respectively for early pupa, while there was 11.11% mortality for both controls for late pupa. The differences among the three control groups were not significant ($p>0.001$). When injected with dsRNA, early pupal mortalities were 68.9% and 57.8% for *SK* and *SH* dsRNA respectively (Figure 2.10a), while late pupal mortality was 60% and 51.2% respectively (Figure 2.10b).

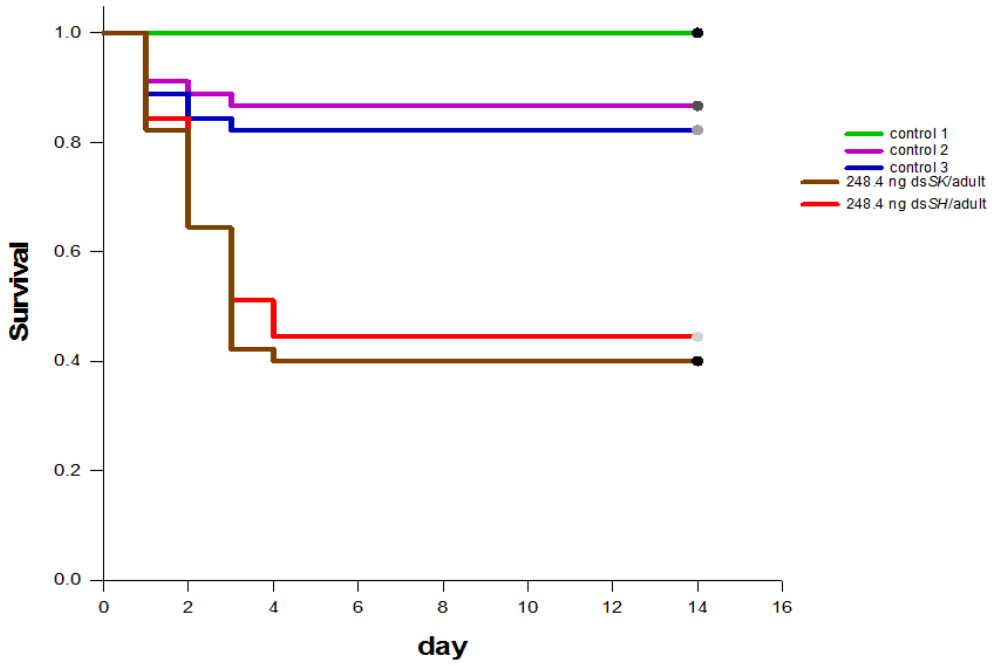

Figure 2.11: Survival curves of *T. castaneum* adults injected with dsRNA targeted to *SK* and *SH* ($p<0.001$; n= 45 adult/treatment, as shown by Kaplan-Meier survival analysis). Control 1= untreated adults, control 2 = adults injected with RNAase free water, control 3 = adults injected with kanamycin resistance dsRNA.

The mortality of adults beetles injected with 248.4 ng/adult *SK* and *SH* dsRNA after 2 weeks was 60.0% and 55.55% respectively compared to 13.33 % and 17.3% mortalities for control groups 2 and 3 respectively and 0.0 % for control 1. The differences were found to be significant ($p<0.001$) between dsRNA treatment and control groups. However, there were no significant differences among control groups ($p>0.001$), as indicated in Figure 2.11.

2.4.8 **Oral bioassays targeting a potassium channel gene in *T. castaneum***

The induction of RNAi via oral delivery was applied by feeding 3rd instar larvae on flour disks containing dsRNA (Figure 2.11) at different concentrations (100, 200, 300 and 400 ng/mg diet). Experiments were carried out to assess the effects of varying dose of dsRNA on survival. Three biological replicates, 15 insects/ rep, for each dose were used, Abbott's formula was used to correct survival data relative to control 1 (untreated control).

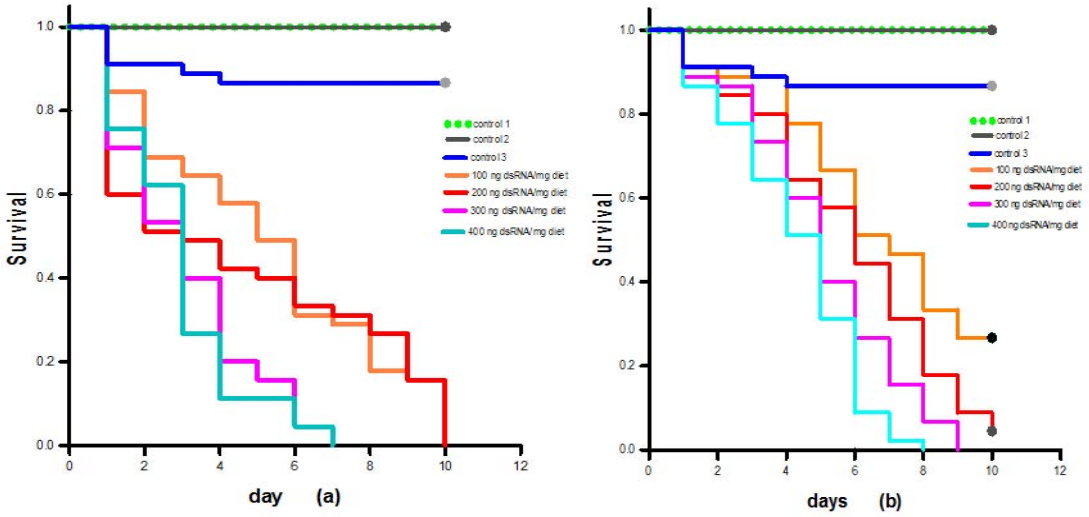

Figure 2.12: Survival of *T. castaneum* larvae fed with dsRNA targeted to *SK* (a) and *SH* (b) ($p<0.001$; n= 45 larva/treatment, as shown by Kaplan-Meier survival analysis), Control 1= larvae fed on flour, control 2 = larvae fed on RNAase flour disks, control 3 = larvae fed on kanamycin resistance dsRNA flour disks.

There is a clear trend of decreasing survival of 3rd instar larvae with increasing of the concentrations when delivering both *SK* and *SH* dsRNA as shown in Figure 2.12. Meanwhile, no mortality occurred in control 1 and 2, and there was only 13.33% mortality for control 3, but significant mortality ($p<0.001$) were recorded in dsRNA treated insects. Ten days after exposure to the dsSK, its mortality reached 100 %, for all the 4 doses of 100, 200, 300 and 400 ng/mg (Figure 2.12a). In the dsSH treatments, mortality were recorded at 73 % and 91% at 100 and 200 ng/mg dose respectively and 100% at 300 and 400 ng/mg after 10 days (Figure 2.12b). The comparative effectiveness of each dsRNA dose in killing *T. castaneum* populations was estimated in a probit analysis which showed that the LC_{50} of *SK* and *SH* were 65 and 117.01 ng/mg respectively at day 6. From these results, it is clear that the increased mortality in all experiments was caused specifically by the dsRNA of *SH* and *SK* instead of the off-target dsRNA such as dsRNA of kanamycin or due to the injection process itself.

2.4.9 Emerge of adults from injected pupae

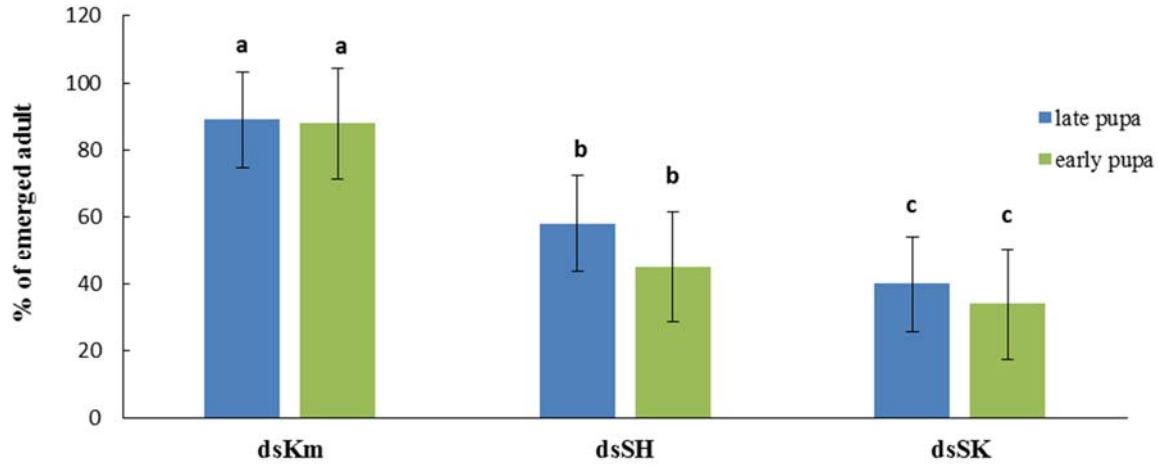

Figure 2.13: Percentage of emerged adults of *T. castaneum* from the injected early and late pupae. Mean ± SD of three replications is shown. Means with different letters are significantly different ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene.

At the termination of the bioassay, the reductions rates in adult emergence from injected early and late pupae at higher concentration of dsSK was 34% and 40% respectively, whereas, the corresponding rates were 45% and 58% respectively from dsSH injected into early and late pupae, respectively. These effects were significantly different relative to the control groups (Figure 2.13).

2.5 Discussion

Targeting potassium ion channels using RNA interference is a novel and potentially highly exploitable way to control *T. castaneum* and other coleopteran insects. This study has demonstrated for the first time that the expression of the two genes *SK* and *SH* which encode K^+ channels in the nervous system of *T. castaneum* can be knocked down by the injection and feeding of dsRNA. The RNAi effect was seen in different life stages. Knocking down these genes resulted in the ordinary interaction between the insects and their environment being obstructed, ultimately causing their death.

The resistance of insects to insecticides poses a serious threat to the ability to control the spread of insects. RNAi is a novel approach to control insects where resistance likely develops much slower. Applying artificial double-stranded RNA molecules for all or part of a specific gene transcript leads to down-regulation of the expression of that gene (Price and Gatehouse, 2008). K^+ channels are present in most cells of eukaryotic and prokaryotic organisms, and they control a variety of cellular functions (Jessell *et al.*, 2000; Hille, 2001). These ion channels are targeted by pyrethroid insecticides such as permethrin, cypermethrin, fenvalerate and cyfluthrin, which disrupt the normal transmission of nerve impulses (Coats, 2012). Inward-rectifying potassium channels (K_{ir}) of *Aedes aegypti* have been blocked by the injection into the hemolymph of adult females of VU573 which is a synthetic organic molecule inhibiting of mosquito K_{ir} channels, resulting in disruption to the production and excretion of urine, so that the mosquito is eventually incapacitated being (flightless or dead) within 24 hours (Beyenbach *et al.*, 2015). Thus, it has been suggested here that targeting these channels is a novel approach to control the development of *T. castaneum* by knocking down the *SK* and *SH* which are responsible for these channels. Previous studies have shown robust RNA_i responses and knockdown effects for multiple genes in *T. castaneum* (Tomoyasu and Denell, 2004).

2.5.1 Gene expression

This study first determined the transcript abundance of *SK* and *SH* in *T. castaneum* at different developmental stages (1st, 3rd and 6th larval instars, early and late pupae and adults). The data reported here clearly show that *SK* and *SH* are highly expressed in late pupal and adult stages. This might be expected given the extraordinary levels of phenotypic differentiation in this beetles and the neuronal remodelling processes which occur during metamorphosis that are necessary for some functions, such as the mushroom body neuron remodelling which is necessary for adult memory (Boulanger and Dura, 2015). Therefore, the gene expression of potassium ion channels was highest in insects at their most developed life stage, but the differences in expression between adults and pupae were not significant ($p > 0.05$). On the other hand, gene expression was low in the larval stages. Furthermore, transcript levels of the two genes were present in all developmental stages of this insect, but relative expression levels were different based upon the stage. The findings for the voltage-gated potassium channel reported here are similar to the expression of the *T. castaneum*

voltage-gated sodium ion channel (El Halim *et al.*, 2016), where the two genes which are responsible for coding these channels was highest expression in late pupae, followed by adults and larvae. However, it was highest in the adult, followed by pupa and larvae for the conductance calcium-activated potassium channel.

The present study shows RNAi results in the down-regulation of the target genes. The transcription of both genes was down-regulated in the 3rd instar larvae, where the *SK* was recorded at 87%-98%, while the *SH* was down-regulated to 34%-83% via the oral delivery of dsRNA after 72h feeding the 3rd larvae of *T. castoreum.* Our results showed that oral delivery of dsRNA could induce sufficient RNAi to knock down of target genes similar effect to injection delivery of dsRNA. Reductions for the *SK* were also between 89%-99%, while the *SH* was down-regulated to 74%-98% after 48h post injection of 6th instar larvae. Both delivery systems being effective and similar observations for our lab strain of *T. castaneum* have been reported by Laudani *et al.* (2017), where transcript levels for the ecdysone receptor gene were reduced to 93 % and 34% by both injections and feeding respectively using a single dsRNA dose at 150 ng. Also, El Halim *et al.* (2016) mentioned that injection and the feeding of larvae showed 60% and 43% knockdown respectively in sodium ion channel paralytic A gene expression at doses of 160 ng/larva and 150 ng/mg dsRNA, respectively, both these results compared to the control after 48h post-injection and 72h feeding corresponding dsRNA. These results are in agreement with those of a previous investigation by Whyard *et al.* (2009) who observed that orally delivered dsRNA (*vATPase*) at 2.5 µg/g diet caused gene knockdown in *T. castaneum*. However, another research group failed to knock-down the gene by delivering dsRNA through the diet of *T. castaneum* (Spit et al., 2017). A possible explanation for these results may be the use of different strains of *T. castaneum,* causing differences in the efficiency of RNAi (Swevers *et al.*, 2013).

The success of RNAi in *T. castaneum* is most likely due to several major factors. Firstly, the genome of several coleopteran insects includes 2 or even 3 of the *Sid-1*-like genes which are necessary for RNAi pathways (Miyata *et al.*, 2014). Secondly, Cappelle *et al.* (2016) indicated that coleopterans have another pathway called receptor-mediated endocytosis which is involved in dsRNA uptake. The findings of this study provide evidence that potent knockdown occurred using RNAi at different developmental stages of *T. castaneum.* the present data consistent with findings reported by Tempel Nakasu (2014), where larvae of *T.*

castaneum exhibited 6-fold downregulation of the V-ATPase when orally exposed to dsRNAs, whereas the injection of dsRNA produced 2 and 3 fold downregulation of V-ATPase and calcium channel genes(*Ca1*) respectively. Moreover, similar results were obtained in *T. castaneum* for the RNAi mediated knockdown of a chymotrypsin-like peptidases (TcCTLP), by injection of *TcCTLP*-dsRNA into penultimate instar larvae caused a significant reduction in transcript levels after 72 h possessed compared to the negative control *Ver* dsRNA (Broehan *et al.*, 2010). Data presented by Noh *et al.* (2014) demonstrated that dsRNA for the 2 cuticular proteins *TcCPR27* and *TcCPR18* injected into the larvae of *T. castaneum* caused significant down-regulation to their corresponding mRNA. As with larvae, suppression of the transcript levels of *SK* and *SH* were also observed in early and late pupae and adults of *T. castaneum* via injection. These results match those of Broehan *et al.* (2013), who found that transcript levels of the ATP-binding cassette (ABC) were significantly silenced after injection with *TcABC* dsRNA in penultimate larvae, pre-pupae and adults of *T. castaneum*. Li *et al.* (2016) revealed that the injection of dsRNAs for the blimp-*1* required for metamorphosis into larvae or pupae depleted about 90% of the mRNA of itself in *T. castaneum.*

2.5.2 **Survival post-treatment**

Another important finding of this study is that 34-99% down-regulation of both genes resulted in significant larval mortality between 52-100% by using the two methods of delivery of both genes. No significant correlation between mortality and treatment used was found [r (358) = 0.075, p=0.155] from the injection of 6th larval instar with dsRNA of *SK* and *SH*, while a significant positive correlation was found in the feeding test of the 3rd larval instars on flour containing dsRNA of *SK* and *SH* [r (358) = 0.241, p=0.01]. The LC_{50} of the larvae injected with *SK* and *SH* dsRNA were 2.385 and 34.93 ng/larva respectively, whereas they were 65 and 117.01 ng/mg diet, respectively on day 6 by feeding treatment. This compares with LC_{50} of dsRNA targeting the sodium ion channel paralytic A (TcNav) gene in *T. castaneum* of 79.89 ng/larva by injection and 150.23 ng/mg by feeding at day 6 (El Halim *et al.*, 2016). Another study has shown that the oral delivery of dsRNA targeted against calcium channel genes is not viable in inducing mortality in *T. castaneum* via RNAi (Tempel Nakasu, 2014). Therefore, it can be concluded that the impact of dsRNA targeting the potassium channel *SK* and *SH* is greater and more effective than targeting sodium or calcium ion channel genes in *T.*

castaneum. In addition, the results indicate that the targeting small conductance calcium-activated potassium channel is more efficient than voltage-gated potassium channels. These findings further support the idea that calcium-activated potassium channels could be significant target sites for the control insects. A study with cockroaches, *Periplaneta americana*, found that the neurotoxic effect of dimethyl disulfide (DMDS) on calcium-activated potassium channels occurred through complex regulatory pathways increasing the intracellular calcium concentration responsible for the abrogation of this channel, leading to higher toxicity (Gautier *et al.*, 2008).

The down-regulation in *SK* and *SH* caused significant decreases in insect survival. One possible reason for this is that lose function of *SK* and *SH* gene causing the delay in repolarization of neurons, leading to constant firing and, eventually, neurodegeneration. Another likely reason for mortality is that the expression of K^+ channels was knocked down in the muscle. In *Drosophila*, Ca^{2+}-activated K^+ channels, have been identified in the adult and larval muscles and also in the larval neurons (Elkins *et al.*, 1986), and interfering with both of these would result in death by physiological disruption inside the body.

The present findings show that larvae injected with 248.4 ng/ larva dsRNA of *SK* and *SH* caused 100% and 73% mortality respectively within 4 days after eclosion. In comparison, Lu *et al.* (2012) revealed that the injection of late larvae *T. castaneum* with acetylcholinesterase (*TcAcel)* dsRNA at 400 ng led to 100% mortality within 2 weeks after eclosion. This is also comparable with the findings of Sang *et al.* (2016), who demonstrated that the injection of 200 ng of the dsRNA of insulin receptor genes *T.cas-ir1* and *T.cas-ir2* in the late larvae of *T. castaneum* caused 100% and 42.0% insect death respectively within 4 weeks after eclosion. These findings indicate that potassium ion channels may represent an effective target for pesticide action. The reason for this is that the time to 100% and 73% mortality achieved with the dsRNA of *SK* and *SH* is less than that for the other genes mentioned. As with the larvae, high mortality was also detected by injection in the early and late pupal stages, the effect extended to the prevention of a majority of injected pupa emerging adults.

The present study has demonstrated a systemic dose-dependent RNAi response in various developmental stages. Most studies have based their criteria for selecting the dose of dsRNA on the use of a low dose to examining the injection-based RNAi, and using a high dose in

feeding to induce insect mortality (Lu *et al.*, 2012; Broehan *et al.*, 2013; Xiao *et al.*, 2014; Li *et al.*, 2016).

In conclusion, this study has demonstrated the selective knockdowne of two types of potassium ion channels in *T. castaneum*, which caused significant mortality in different stages of the insect. It has been shown for the first time that targeting potassium ion channels by RNAi may provide a novel insecticide to control this insect.

Chapter three

Biosafety of dsSK and dsSH targeting the red flour beetle (*Tribolium castaneum*) towards honeybee (*Apis mellifera*)

3 Biosafety of dsSK and dsSH targeting the red flour beetle (*Tribolium castaneum*) towards honeybee (*Apis mellifera*)

3.1 Abstract

Pesticides commonly used in agriculture are known to be toxic to living organisms, especially bees, resulting in either killing the bees or contaminating their products. Moreover, the sublethal effects of some pesticides such as neonicotinoids can negatively affect some parameters of bee performance such as reproduction and olfactory learning, eventually leading to a reduction in the viability of the hive. Because of these non-target effects, many alternative solutions to control insect pests safely have been proposed. One of the solutions is to use RNA interference (RNAi) in a novel approach to protect crops from pests by using double-stranded RNA (dsRNA). This technique results in the specific knockdown of target genes within the pest. The current study evaluates the biosafety of dsRNA targeted to the potassium ion channels, *SK* and *SH* in *T. castaneum* as an effective method for controlling this insect pest. The results demonstrate that there is no significant difference between the mortality of honeybees fed on sucrose solution containing the dsSK and dsSH of *T. castaneum* with those of control-fed adult bees. Furthermore, there was also no significant difference in the levels of gene expressions of *SK* and *SH* in the adult bee. Similarly, there was no significant difference in the titre of deformed wing virus (DWV), used as a measure of immune suppression between these treatments and controls. This work provides evidence of the biosafety of RNAi technology to control *T. castaneum*.

3.2 Introduction

The honey bee *Apis mellifera* is an essential pollinator of approximately 30% of all vegetables and fruits (Klatt et al., 2014). Some studies have estimated that bee pollination could be worth £92.1 million across the UK (Garratt et al., 2016) and it represents a global economic worth about in the region of $215 billion in food production (Gallai et al., 2009). Unfortunately, the abundance of insect pollinator populations has declined in recent decades, particularly in *A. mellifera* (Goulson et al., 2008); and one of the main causes for colony losses is attributed to exposure to pesticides. Compared to many other insects, honey bees are highly sensitive to pesticides and this is thought to be due to the lack genes encoding

detoxification enzymes, including cytochrome P450 monooxygenases (P450s), glutathione-S-transferases (Elbashir et al., 2001) and carboxylesterases (Claudianos et al., 2006).

Systemic insecticides are of particular concern concerning bees because they can be translocated to pollen and nectar (Byrne et al., 2014; Kasiotis et al., 2014). The forager bees are particularly vulnerable to exposure to pesticide residues in pollen and nectar. Furthermore, they can transport the contaminated food source back to the colony, which is then fed to other castes such as larvae and the queen (Orantes-Bermejo et al., 2010). Sublethal doses of pesticides can have various other effects on the honeybee's life cycle. For example, feeding honeybee larvae on pollen contaminated with chlorpyrifos reduce the emergence of queen bees (DeGrandi-Hoffman et al., 2013). Another study found that wild bees (*Osmia bicornis*) exposed to sublethal levels of the neonicotinoids, thiamethoxam and clothianidin resulted in a 50% reduction in offspring production as well as a predominance of males in the generation. Previous studies have reported that newly emerged adults of *A. mellifera* and *A. cerana* exposed to diflubenzuron showed reduced weight gain and suppressed the development of hypopharyngeal glands influencing the development of queen bees and larvae (Gupta and Chandel, 1995). All of these effects result in reducing the fitness of individual bees and that of the colony. To protect insect pollinators from insecticides, the European Union has banned the use of three neonicotinoid compounds, clothianidin, imidacloprid and thiamethoxam, all of which are thought to affect bee behaviour and survival (Carrington, 2013). Because of their importance both to agriculture and the natural environment, the non-target effects of new insecticidal molecules have to be tested with honeybees as part of the registration process.

Recent developments in the pest control area include the search for new tools which are environmentally friendly. RNAi is a novel potential tool for the control of insect pests in agriculture. This strategy assumes that inducing dsRNA in the insect can disrupt the expression of essential genes by blocking the expression of its homologous gene (Schwinghammer et al., 2011). Currently, the genome of 5000 insect species is available at http://arthropodgenomes.org/wiki/i5K (Scott *et al.*, 2013). This information provides the ability to synthesise dsRNA that is similar to specific genes in target insect pests. Many insect species from different orders, including Coleoptera (Prentice *et al.*, 2017), Hemiptera (Luo et al., 2017), Orthoptera (Yu et al., 2017), and Lepidoptera (Meng et al., 2017), have been shown to be sensitive to dsRNA. Several studies indicate that the RNAi machinery is present

in the different honeybee developmental stages (Nunes and Simões, 2009; Nilsen et al., 2011), since the honeybee genome includes the necessary components for RNA interference machinery such as the two Dicer enzymes and the RNA-induced silencing complex proteins (Weinstock et al., 2006). RNAi has been applied successfully in both adult bees and larval bees in gene function analyses (Amdam et al., 2003; Schlüns and Crozier, 2007). Viruses can attack at different developmental stages and castes of honeybees, including the eggs, larvae, pupae, and adult worker bees, adult drones, and queens. Recently, the Israeli acute paralysis virus (IAPV) and the Chinese sacbrood virus (CSBV) was silenced by dsRNA ingestion (Maori et al., 2009; Liu et al., 2010). Furthermore, field studies have indicated that feeding honey bees on IAPV-specific dsRNA resulted in improving bee health (Hunter et al., 2010).

It is, therefore, reasonable to assume that more rigorous testing for dsRNA toxicity to pollinating insects should be implemented to identify risks before its application in the field to reduce potential environmental impacts. In this work, the targeting of the K^+ ion channels in *T. castaneum* using RNAi represents a novel mode of action for biopesticides. However, the question is, are there any effects on non-target organisms, and in particular these that are considered to be beneficial?

Here we assess the potential risks associated with the use of dsSK and dsSH targeting *T. castaneum* against honeybees, *A. mellifera,* as a non-target model organism.This is primarily to follow the Organisation for Economic Co-operation and Development (OECD) regulations on pesticide registration.

3.3 Material and Methods

3.3.1 Honeybees

Honeybees, *A. mellifera* used in this study were obtained from Tyneside Beekeepers Association, Newcastle upon Tyne. For the survival test, four treatments were used, each with five biological replicates. Each of replicate contained ten foragers. The bees were fed daily with 2 ml of diet as follows: treatment 1, 50% (w/v) sucrose solution containing 20 ng/ul *T. castaneum* dsSK; treatment 2, 50% (w/v) sucrose solution containing 20 ng/ul *T. castaneum* dsSH; control 1, 50% (w/v) sucrose solution; and control 2, 50% (w/v) sucrose solution

containing bacterial dsKana (Figure 3.1). Bees were reared in an incubator at 34ºC and 75-80% rh in the 24h dark. Survival was monitored daily for 10 days. Bees clearly attracted and consumed dsRNA solution as it was mixed with the sucrose solution compared to water consumption (Figure 3.1). For survival studies, n = 50 per treatment; for gene expression studies by RT-qPCR, n = 25 per treatment.

Figure 3.1: Cages used for carrying out *A. mellifera* bioassays.

3.3.2 **Design of primers**

Sequences for the *A. mellifera* potassium ion channel genes *SK* and *SH* were identified using a *Apis mellifera* small conductance calcium-activated potassium channel protein in BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?) for the SK gene (gene bank accession number XM-016914844) and the *Apis mellifera* potassium voltage-gated channel protein shaker for the SH gene (gene bank accession number XM-016914894) at NCBI. The primer sequence for β-actin and DWV has previously been described elsewhere (Di Prisco *et al.*, 2016). The specific primers were designed using NCBI/ Primer-BLAST software (Table 3.1). β-actin was used as the house keeping gene.

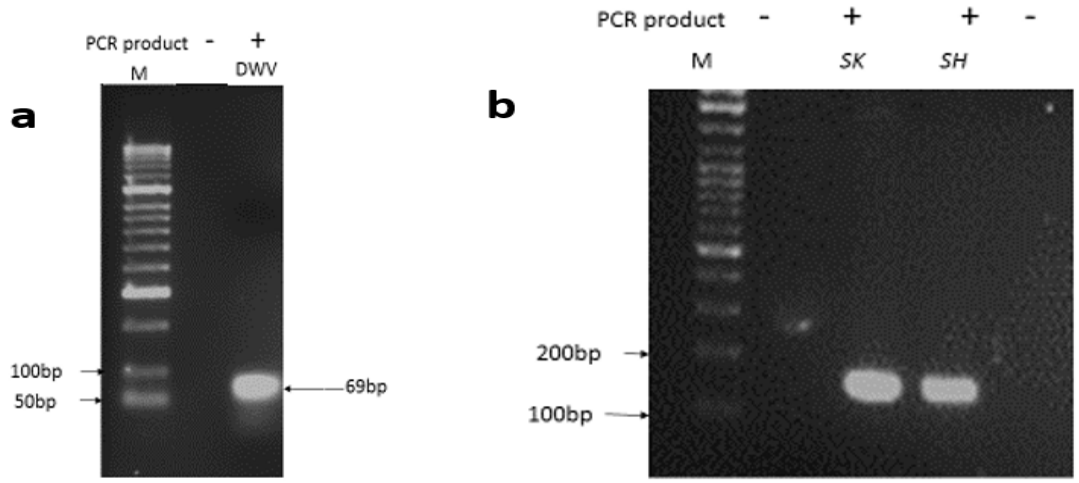

Figure 3.2: PCR products of (**a)** deformed wing virus (69bp), (**b)** *SK* (178bp), *SH* genes (170bp) of *A. mellifera* visualized on 2% agarose.

Table 3.1 Primers used for RT-qPCR and PCR analysis in *A. mellifera* for *SK* and *SH*.

Application	Primer ID	Sequence of primer for *SH* gene	Primer ID	Sequence of primer for *SK* gene
qPCR *Apis mellifera*	FE1	GCCGAACCCACATACCCAAT	FE2	CGAGGAGTTGACCAGGTGTC
	RE1	CCCGCTCACGTTAATGACGA	RE2	GTGTCGGGGTGAAGGAAGTT
qPCR Deformed wing virus	FF1	GCGCTTAGTGGAGGAAATGAA		
	RF1	GCACCTACGCGATGTAAATCTG		
qPCR β-actin (reference gene)	FG2	GATTTGTATGCCAACACTGTCTT		
	RG2	TTGCATTCTATCTGCGATTCCA		

3.3.3 Reverse transcription- quantitative PCR (RT-qPCR)

The transcription levels of *SK* and *SH* (Figure 3.2) and deformed wing virus genome copies in adult honeybee were determined by SYBR Green RT-qPCR using conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 15 sec. at 95 °C, 30 sec.at 60 °C and 0.07 sec. at 60 °C. β-actin was used as a reference gene. The total RNA for RT-qPCR was isolated from honeybees study from (Laboratory, 2016) showed that more than 95% of honeybee colonies were infected with DWV. The standard curve was established by plotting the logarithm of eight 10-fold dilutions of a starting solution containing 21.9 ng plasmid DNA using a Strata Clone PCR cloning kit (Agilent Technologies) with a DWV insert (from 21.9 ng to 2.19 fg),

against the corresponding Cq values as the average of three repetitions (Di Prisco *et al.*, 2016). The relative transcript quantity of the DWV gene from honeybees fed on dsRNA targeting *T. castaneum SK* and *SH* was calculated by plotting Cq values on the standard curve (refer to Appendix B for the number of virus) to obtain the amount, according to the following equation (Staroscik, 2004):

Number of copies = (amount * 6.022x10^{23}) / (length * 1x10^{9} * 650)

3.3.4 Stability of dsRNA in sucrose solutions containing *T. castaneum* dsRNA targeting *SH* and *SK.*

The stability of dsRNA in sucrose solution was evaluated by incubating 1 µg of dsRNA for *SH* and *SK* in 10 µl of 50% (w/v) sucrose solution at 34°C (Powell *et al.*, 2017) at the following time points: 0, 6, 12, 18, 24, and 48 h. The integrity of the dsRNA was analysed by separation on 2% (w/v) agarose gels, and bands were visualized by ethidium bromide staining under UV.

3.3.5 Statistical analysis

The data were analysed as described in section 2.3.9.

3.4 Results

3.4.1 Bioinformatics analysis of targeted gene sequences

The results of the MegaBLAST homology searches conducted with both different insect species and human sequences confirmed that there was no significant similarity to any other insect species or human sequence for the dsRNA of *T. castaneum* for *SK* used in the present study. Moreover, there was no significant similarity with *A. mellifera*. However, there was a

15.2% nucleotide sequence similarity between the dsRNA of *T. castaneum* for *SH* used in the present study and the *SH* gene of *A. mellifera (*Figure 3.3)

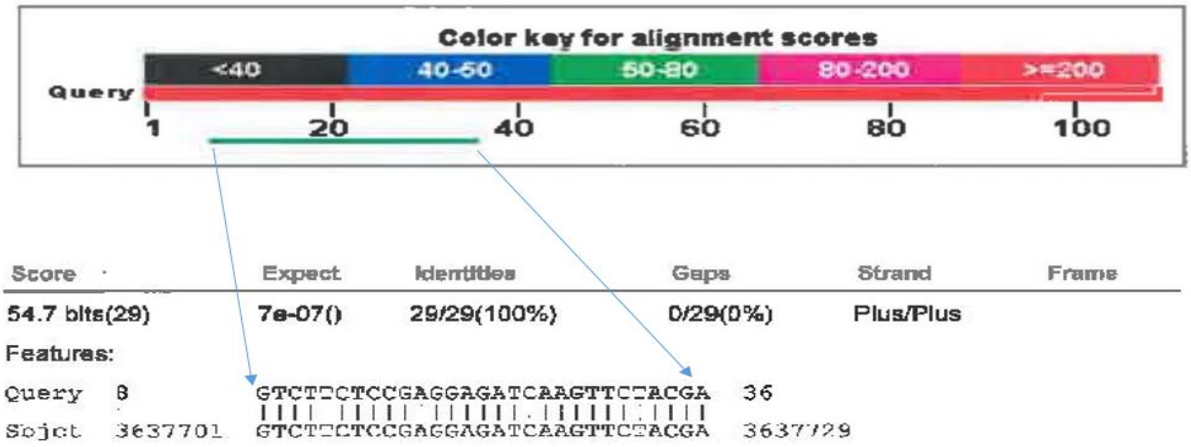

Figure 3.3: The MegaBLAST homology between dsRNA of *T. castaneum* for *SH* gene (Query) and its corresponding gene in *A. mellifera* (sbjct).

3.4.2 Effects of dsSK and dsSH of *T. castaneum* on the survival of *Apis mellifera*

Experiments were carried out in order to test whether or not dsRNA of *T. castaneum* for *SK* and *SH* would affect *Apis mellifera*, and a simple bioassay method was set up. The honeybee foragers were fed with 2 ml 50% sucrose solution containing dsRNA of *T. castaneum* at 20 ng/µl, the amount 40µg dsRNA, previous study indicated 20 ng/µl of dsRNA induce RNAi in bee by oral feeding (Maori et al., 2009; Liu et al., 2010). Five technical replicates of 10 bees, for each treatment were used. The survival of the honeybees was recorded every day for 10 days.

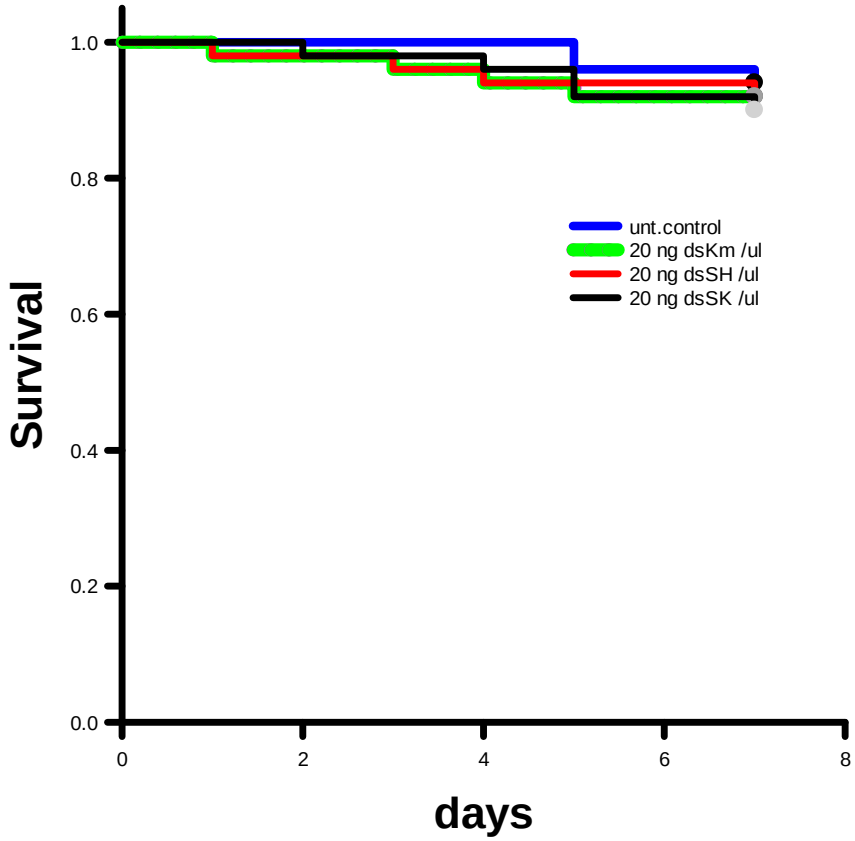

Figure 3.4: Survival curves of *A. mellifera* foragers fed on sucrose 50%. Containing TcdsRNA targeting *SK* and *SH* ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). unt.control = foragers fed only sucrose 50%. dsKana control = foragers fed sucrose 50% containing 20ng/µl bacterial dsKana.

The results of this study (Figure 3.4) show that honeybee foragers fed on 50% sucrose solution containing *T. castaneum* dsSK and dsSH at 20 ng/µl caused 10% and 8% mortality respectively after 10 days. Interestingly, they were no significant differences ($p>0.001$) between the mortality of honeybees fed on dsSK or dsSH with either of the control fed bees, where the untreated control (50% sucrose) and the dsKana control (50% sucrose containing 20 ng/µl dsKana) caused 6% and 8% mortality, respectively. Honeybee survival was unaffected by the treatment.

3.4.3 Gene expression levels of *SK* and *SH* in *A. mellifera*

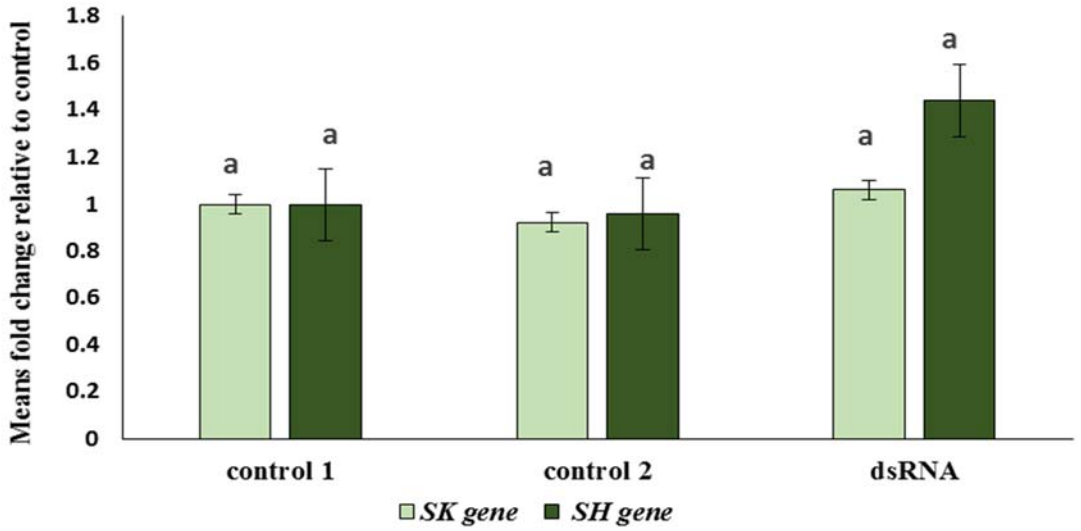

Figure 3.5: Expression of SK and SH mRNA in foragers fed on TcdsRNA after 72h. Mean ± SD of three replications are shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test). Control 1 = foragers fed only sucrose 50%, control 2 = foragers fed sucrose 50% containing 20ng/µl bacterial dsKana, dsRNA = foragers fed sucrose 50% containing 20 ng/µl TcdsSK or dsSH. Expression levels were normalised against β-actin gene as an internal standard.

The knockdown of *SK* and *SH* expression in honeybee foragers was investigated after continuous feeding for a period of 72h. The oral uptake of off-target dsRNA (control 2) did not affect the expression of either *SK* or *SH*, relative to those fed on the basic diet (control 1). This dsRNA molecule was designed towards a 468bp fragment of the microbial kanamycin resistance gene (*Kana*). RT-qPCR revealed that the expression of *TcSK* and *TcSH* in control 2 fed honeybees was 0.92-fold and 0.96-fold respectively, relative to that of the control 1 group.

When honeybee foragers were fed either dsRNA targeting *T. castaneum SK* or *SH* (20 ng/µl), again there were no significant differences in gene expression ($p > 0.05$) compared to either control group over this same time period, with an increase in expression of 1.06 fold and 1.4 fold for *SK* and *SH* respectively, relative to control 1 (Figure 3.5).

3.4.4 **Expression of the deformed wing virus (DWV) in honey bee**

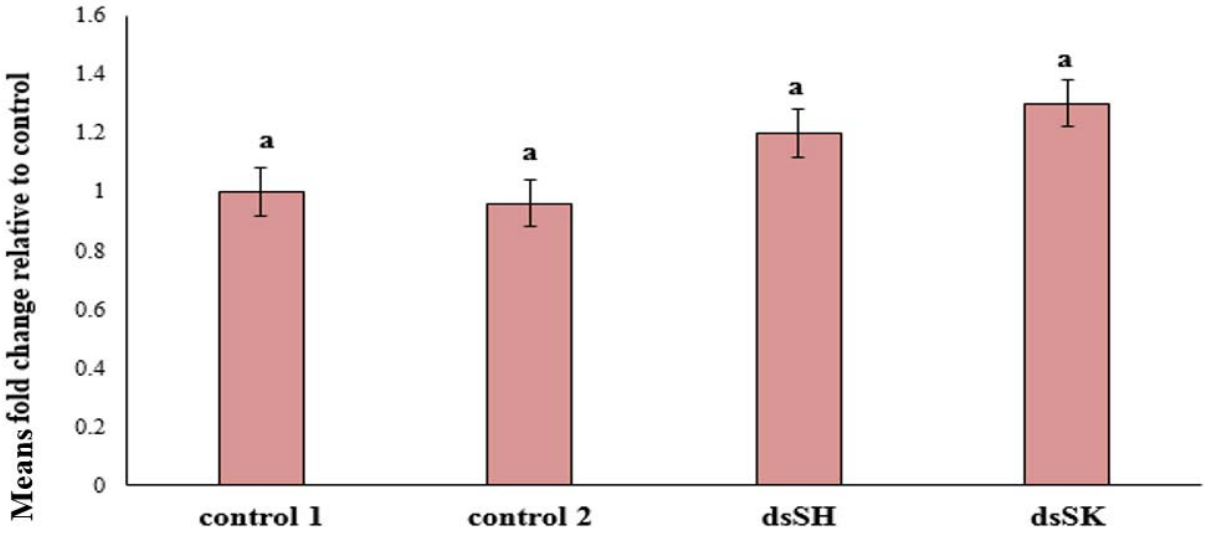

Figure 3.6: Expression of deformed wing virus in foragers fed on TcdsRNA for 72h. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test). Control 1= foragers fed only sucrose 50%, control 2= adults fed sucrose 50% containing 20 ng/µl bacterial dsKana, dsSH = foragers fed sucrose 50% containing 20ng/µl TcdsSH, dsSK = foragers fed sucrose 50% containing 20ng/µl TcdsSK. Expression levels were normalised against β-actin gene as an internal standard.

Studies were carried out to investigate the effects of TcdsRNA targeted to *SK* and *SH* on the immunocompetence of forager honeybees by evaluating expression levels of DWV in bees fed for 72 h an either TcdsSK or TcdsSH. RT-qPCR analysis revealed that DWV transcript levels in foragers fed on 50% sucrose containing 20 ng/µl TcdsRNA were not significant ($p > 0.05$), with mRNA levels for DWV being 1.3 and 1.2-fold for dsSK treatment and dsSH treatment, respectively (Figure 3.6), relative to the control groups. Again there was no difference between the two control groups, with expression in the control 2 group being 0.97-fold relative to control 1.

Table 3.2: Deformed wing virus titre in honeybee foragers fed on the dsSK and dsSH of *T. castaneum* and bacterial dsKana as a control.

Treatment	**Viral titre (copies/bee)**
Control 1	$2.80 \pm 0.005 \times 10^4$
Control 2	$2.79 \pm 0.003 \times 10^4$

Honey bee fed dsSK	2.82±0.003 x10^4
Honey bee fed dsSH	2.81±0.004 x10^4

Control 1 = foragers fed on only sucrose 50%.Control 2 = foragers fed on sucrose 50% containing 20 ng/µl dsKana

The level of DWV in bees fed on sucrose solution containing 20 ng/µl of TcdsRNA of *SK* and *SH* was quantified and normalised to actin, with titres for DWV being 2.82±0.003 x10^4 and 2.81±0.004 x10^4copies/bee respectively. Titers for control groups were 2.80±0.005 x10^4 and 2.79±0.003x10^4copies/bee for control 1 and 2, respectively (Table 3.1). There was no significant difference ($p > 0.05$) between the two treatments or the group controls.

3.4.5 Stability of dsRNA in sucrose solution

The stability of the dsRNA in the diet was investigated over a period of 48 h. The results indicate that the incubation of 1 µg of dsSK and dsSH of *T. castaneum* with 10 µL of 50% (w/v) sucrose solution at 34°C had a little adverse effect on the stability of the dsRNA after 0, 6, 12, 18, 24, 48h incubation. The results of agarose gel band intensities of dsRNA (Figure 3.7) estimated by Image Lab software show a slight degradation of dsRNA over time (Figure 3.8). There was 13% degradation for the dsSK. However, only 2% degradation for the dsSH.

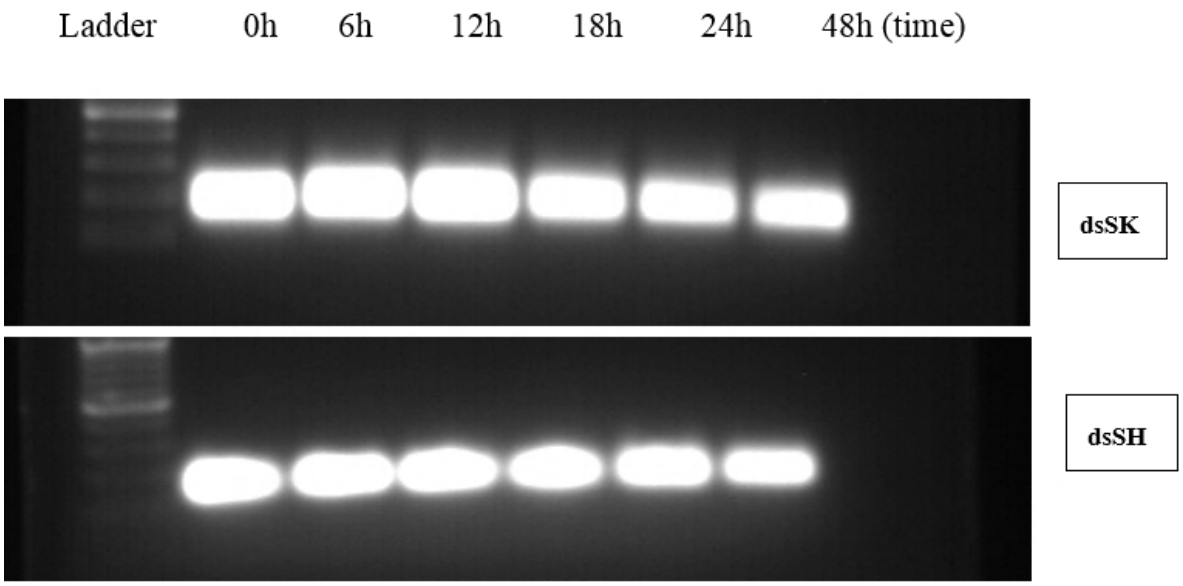

Figure 3.7: Stability of dsRNA in sterile sucrose solution containing dsRNA. Samples taken at specified time points were analyzed for integrity by 2% agarose gel electrophoresis.

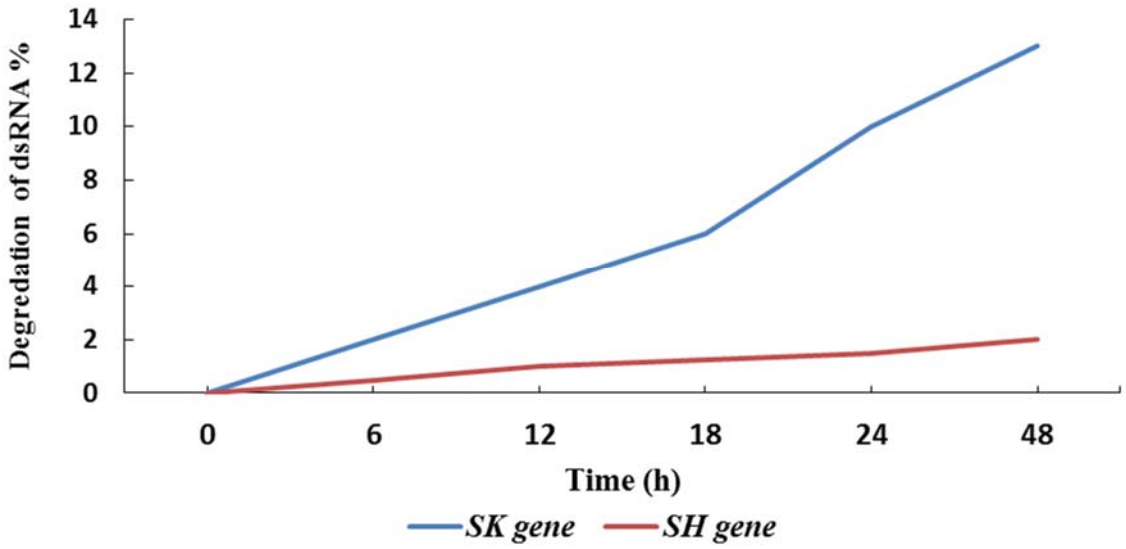

Figure 3.8: Percentage of degradation of dsSK and dsSH of *T. castaneum* in sucrose solution relative to time 0h, based on the intensity of dsRNA band on gel estimated by Image Lab software (Bio-Rad)

3.5 Discussion

The results of this study show that dsRNA targeting the potassium ion channels of *T. castaneum* is safe for non-target organisms (NTOs) such as the honeybee. There were no significant effects on survival, immunity or gene expression in honeybees fed with *T. castaneum* dsRNA targeting either *SK,* which codes for the small conductance calcium-activated potassium channel and *SH* which codes for the voltage-gated potassium channel.

The use of RNAi technology could pose a risk to NTOs. For example, the consumption of sufficient quantities of dsRNA may lead to the induction of the RNAi machinery of NTOs and the suppression of a corresponding mRNA transcript homologous to the sequence (Roberts *et al.*, 2015), which may cause a loss of gene function, and adversely impact on the NTO (Bachman *et al.*, 2013). The safety of RNAi technology can be predicted only if bioinformatics data demonstrate that the dsRNA used does not show sufficient sequence similarity with non-target species. Both Elbashir *et al.* (2001) and Tijsterman and Plasterk (2004) indicated that activation of the RNAi machinery in the organism's cells requires the introduction of dsRNA matching around 21-25 bp of mRNA. Honeybees have been shown to exhibit a high response to RNAi (Costa *et al.*, 2016).

In this study, the delivery of dsRNA designed to target two different potassium ion channels in *T. castaneum* was toxic to that insect and was effective at inducing an RNAi response. Our data are consistent with other findings (Zhu *et al.*, 2011; Bolognesi *et al.*, 2012; Linz *et al.*, 2014). Small doses of dsRNA can cause 90% gene knockdown in coleopterans, and the effect may stay for the long-term and be passed on to subsequent generations(Joga *et al.*, 2016).

The present study provides some important insights to support the biosafety of this technology as an alternative strategy to conventional pesticides, as illustrated below**.**

3.5.1 **Survival and gene expression**

We checked the biosafety of the use of dsRNA designed to target *T. castaneum* against the honeybee. In this study, the alignment of *A. mellifera* and dsRNA of *T. castaneum* for *SH* and *SK* indicated the presence of more than 29 nucleotides of identical regions between the dsRNA of *T. castaneum* for the *SH* and *SH* of *A. mellifera* but no homology between the *SK* genes. Honey bees are known to be highly sensitive to RNAi (Amdam *et al.*, 2003), which could theoretically imply that there may be an effect on the honeybee. However, we found that the survival of honeybees fed on sucrose solution containing 20 ng/µl of dsRNA for *SK* or *SH* was not affected their survival and was not knockdown either of the target genes in *A. mellifera*. A similar observation in *A. mellifera* has been reported by Powell *et al.* (2017), who demonstrated that injections into honeybees of 50 ng of dsRNAs *Laccase 2* and vacuolar-*ATPase V-type subunit A,* designed to target *Aethina humidity* had no effect on their survival and did not induce the suppression of either of the target genes. An alignment of *A. mellifera* and *A. tumida Laccase 2* and *V-ATPase subunit A* mRNAs indicated sequence identities of 74 % and 68 %, respectively. These present findings appear to be consistent with those of other studies which have found that, although the nucleotide sequence identities between *Diabrotica virgifera* and *Leptinotarsa decemlineata* were 83 % for *V-ATPase subunit A*, there were no effects of *D. virgifera V-ATPase subunit A* dsRNAs on the survival of *L. decemlineata* (Baum *et al.*, 2007). It is difficult to explain this result, but is likely to be related to the specific segment of mRNA not shared between the target and non-target insects, thus preventing disrupting the expression of the non-target insect.

3.5.2 Effect on honey bee immunity

In the invertebrate body, there is a close association between the nervous system and the immune system (Pavlov and Tracey, 2015). The target genes *SK* and *SH* have a role in the nervous system. Therefore, we checked the effect of dsRNA on the immune system of the honeybee, and especially on levels of the deformed wing virus (DWV) that they carry. DWV causes atrophied wings or paralysis of the legs and wings of adult honeybees in colonies infested with Varroa mites (Bowen-Walker et al., 1999). The varroa mite has been shown to cause amplified levels of deformed wing virus from 10 % to 100 % (Martin et al., 2012). In the absence of mites, the virus is thought to persist in bee populations as a covert infection transmitted orally between adults (nurse bees), since the virus can be detected in hypopharyngeal secretions (royal jelly) and brood food and is transmitted vertically through the queen's ovaries and drone's sperm. The results provided in this study demonstrated that there are no significant differences in viral titre between honeybees fed on the dsRNA targeting the genes and the controls. The treated and control honeybees contained approximately 2.8×10^4 copies of virus per bee, whereas that of symptomatic honeybee was recorded at 3.3×10^{10} copies of virus per honeybee. A study by Highfield et al. (2009) estimated the level of DWV to fluctuate between $<10^2$ and 4.2×10^9 copies per asymptomatic worker. However, for symptomatic honeybees, recorded values range from 1.8×10^{10} to 6.9×10^{11} DWV per worker. Therefore, these results provide further support for the use of dsRNA targeting these genes for controlling *T. castaneum* populations in the field.

3.5.3 DsRNA stability assays

One of the limitations identified of this technology concerns stability of dsRNA. Stability studies of dsRNA provided proof that the dsRNA of the target genes was not degraded significantly during 48 h. Our result agrees with those of a study by Li *et al.* (2011a), which indicated that three dsRNAs targeting different sites within a gene encoding vacuolar ATP synthase subunit E in *Nilaparvata lugens* were found to be stable in 0.1 g mL−1 sucrose solution at 22h. The *Ap_ST1* dsRNAs (sugar transporter gene) from an aphid kept for a week at room temperature was not degraded, thus demonstrating its stability (Alotaibi and Abdullah, 2011). However, 1 µg of *V-ATPase subunit* dsRNA targeting *A. tumida* in 10 µl of 50% after 8 h is completely degraded (Powell *et al.*, 2017). These contrasting results

demonstrate the importance of evaluating the stability of specific dsRNAs under the appropriate conditions.

In conclusion, this study has successfully demonstrated an environmentally safe use of dsRNA specifically targeting potassium ion channels to control *T. castaneum*, without adverse effects on the survival of NTOs such as *A. mellifera*. Furthermore, RT-qPCR analysis provided evidence that there are no adverse effects on the suppression of *SK* and *SH* in the honeybee. Additional, safety tests and more studies on other NTOs such as bumble bees and parasitoid wasps would help to establish more comprehensive knowledge of the degree of biosafety of the use of this technique in the field.

Chapter four

A Transformed Bacterium Expressing Double-Stranded RNA Specifically to Target Potassium Ion Channels Genes (*SH* and *SK*) in the Red Flour Beetle, *Tribolium castaneum*

4 A Transformed Bacterium Expressing Double-Stranded RNA used Specifically to Target Potassium Ion Channels Genes (*SH* and *SK*) in the Red Flour Beetle, *Tribolium castaneum*

4.1 Abstract

The transformed bacterium *Escherichia coli* HT115 was used to express dsRNA to control *T. castaneum*. An *in vivo* system was developed to produce dsRNA for either *SK* and *SH* fragments, and this bacterial expression system allows the generation of large amounts of dsRNA less expensively than with in vitro synthesis techniques. The delivery of oral and injection of dsRNA for either *SK* and *SH* was found to cause the suppression of gene expression in the larvae and adults of *T. castaneum*. The results showed that the LC_{50} values of the larvae injected with *SK* and *SH* TcdsRNA extracted from bacteria were 2.98 and 36.03 ng/larva respectively; as opposed to 66.04 and 119.21 ng/mg diet for larvae fed with *SK* and *SH* dsRNA respectively at day 6. In addition, the analysis of data demonstrates that the feeding of bacterially expressed dsRNA for both target genes to larvae and adults had toxic effects. The results show that the amounts of dsRNA developed from the *in vivo* system were greater than those from the equivalent *in vitro* system per reaction. The use of bacteria to produce dsRNA could mitigate the high costs that may prohibit this technology from being used as a novel topically applied biopesticide.

4.2 Introduction

The RNAi pathway is triggered by specific gene dsRNA, leading to a reduction in the expression of target genes. This technique has the potential to be used for the control of insect pests in agriculture (Castellano *et al.*, 1997). There are two approaches currently available to synthesize dsRNA. The prototype method is in vitro transcription using commercially available kits. In this technique, the gene sequence of interest should be cloned into a plasmid vector flanked by two T7 RNA polymerase promoters or a fragment of the target sequence can be produced by PCR using the incorporation primers in a 5' terminal T7 RNA polymerase. However, it is expensive when large amounts of dsRNA for RNAi experiments need to be produced. The second approach: utilises an endogenous *in vivo* system to produce the dsRNA. The *E. coli* strain HT115 used for synthesis contains a recombinant plasmid, which is a

transcription plasmid which has two convergent T7 promoters flanking the multicloning site (Timmons and Fire, 1998). To increase the effectiveness of the bacterial expression dsRNA, modifications were made to dsRNA-specific endonuclease RNaseIII (lacking in RNase III), which cleaves dsRNA in the bacteria. Moreover, T7 RNA polymerase was designed to be induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (Timmons *et al.*, 2001).Thus, the formulation of dsRNA in bacteria may give benefits to allowing many challenges to be overcome, including it is a cheaper method (Timmons *et al.*, 2001).

The study by Timmons and Fire (1998) was the first to try to induce RNAi in bacteria, using the nematode *Caenorhabditis elegans* as a model fed bacteria expressing a dsRNA segment from *unc-22* gene which encodes an abundant muscle filament protein leading to the partial loss of function in the *unc-22* gene. Tian *et al.* (2009) demonstrated that *Spodoptera exigua* fed with *E. coli* expressing dsRNA specific to the chitin synthase gene A (*CHSA)* gene resulted in significant mortality rates between 62–88%. In a recent study using bacterially expressed dsRNA the knockdown of gene expression of chitinase (*MseChi1* and *MseChi2)* caused high mortality and reduced body weight in *Mythimna separate* larvae via feeding studies(Ganbaatar et al., 2017).

T. castaneum is an excellent model for RNAi studies, and there is a growing body of literature which demonstrates the sensitivity of this insect to dsRNA molecules (Tempel Nakasu, 2014; Li *et al.*, 2016). The use of dsRNA targeting potassium ion channels could provide the specific control of *T. castaneum* without any impact on non-target species encouraged us to look for a method of producing dsRNA on a large scale and at a cost that is more reasonable. Recombinant bacteria can provide significant RNAi efficacy with the large-scale production required for commercial application (Thammasorn *et al.*, 2015). Tenllado *et al.* (2003) found that an RNase III-deficient strain of *E. coli* produced massive amounts of virus-derived the dsRNA of two viruses, the Pepper mild mottle virus (PMMoV) and the Plum pox virus (PPV), which are common plant viruses in nature. Ongvarrasopone *et al.* (2007) found that large amounts of dsRNA (40–50 µg) could be produced from a 1 OD_{600}/ml *E. coli* culture and the cost of production of 30 mg dsRNA by the transformed bacteria was approximately one-third of that of in vitro synthesized dsRNA. Aalto *et al.* (2007) estimated that it might be possible to produce quantities of dsRNA of the order of kilogram using this method in conjunction with industrial-scale bioreactors.

This study aims to demonstrate a practical approach to the production of considerable quantities of the *SK* and *SH* dsRNA that could be used as an insecticide against *T. castaneum.*

4.3 Materials and Methods

Insect rearing, RNA extraction, cDNA Synthesis, PCR products, RT-qPCR and statistical analysis were described in chapter two.

4.3.1 Cloning of dsRNA specific to *SK* and *SH*

Fragments of *SK* and *SH* generated by end PCR were cloned into the TOPO® vector (pENTR) following the instructions of manufacturer pENTR/D-TOPO cloning Kit. The QIAprep Spin Miniprep Kit Protocol was used to purify the plasmid DNA. To produce the expression clone, the entry clones had to be sub-cloned into a destination vector, PET161-DEST that contains all the sequence information necessary for expression. LR Clonase was used for the sub-cloning between the entry clones and the destination vector in order to generate the final expression clone, all without the need for restriction enzymes and ligase. These plasmids were transformed into *E. coli* HT115-competent cells that express dsRNA in the presence of IPTG (Figure 4.1).

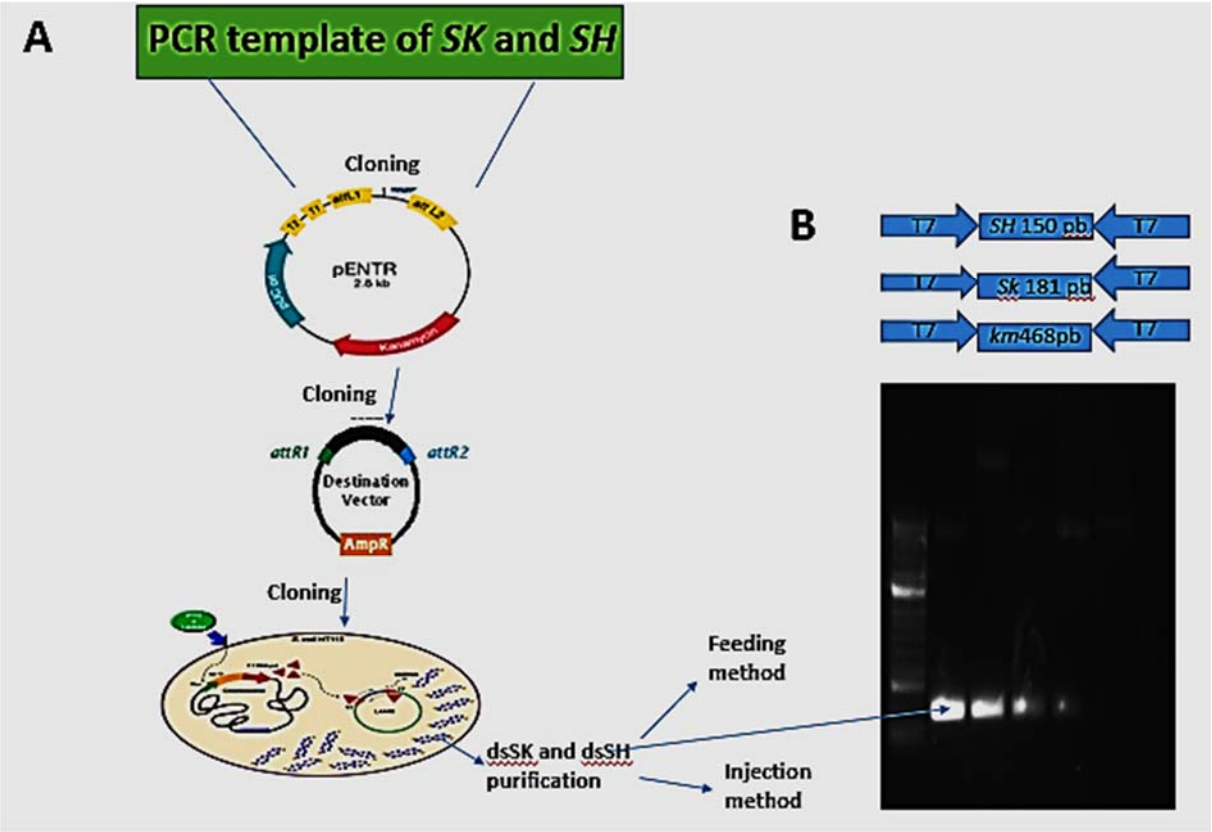

Figure 4.1: Genetic interference following injection and feeding *T. castaneum* with dsRNA-expressing bacteria. **A**. general scheme for dsRNA production. The 5′-terminal portions of the genes were amplified by PCR and cloned into pENTR and then cloned into the destination vector PET161-DEST for dsRNA transcription. The multicloning site of the vector is bidirectionally flanked by T7 promoters driving the synthesis of dsRNA. The recombinant plasmid was transfected into the bacterium HT115, allowing dsRNA purification in order to conduct bioassay experiments with the expressing bacteria, **B**. constructs performed in this work. Three independent DNA fragments were cloned and used for RNAi experiments, including *SK*, *SH* and *Kana*.

4.3.2 **Bacterial expression of dsRNA**

To produce the dsRNA, single colonies of the bacteria were transformed with PET16-DES-*SK* and PET16-DES-*SH* plasmids which were grown overnight at 37 ºC in 5 ml LB media containing 100 µg/ml ampicillin and 12.5µg/ml tetracycline. 25 ml LB were inoculated with 250 µl in an overnight culture using the same antibiotic at 37 ºC until the OD_{600} reached 0.4. T7 RNA polymerase and hence dsRNA synthesis was induced by the addition of 0.5 mM IPTG. The culture was incubated by shaking at 37 ºC until the value of OD_{600} reached 1, after that the bacterial cells were harvested by centrifugation at 2,720 xg for 10 min at 4°C (Posiri *et al.*, 2013). To prepare crude recombinant bacteria expressing dsRNA, the pellet was resuspended in fresh culture medium.

4.3.3 Pretreatments of the transformed bacteria

Two different pretreatments were applied to the bacterial cells. A heat treatment used 65°C for 20 min, and sonication was applied using an ultrasonicator, Soniprep 150 at 10 cycles of 10 min bursts separated by 2 min gaps. Bacterial viability was tested by plating 50 µL of the treated bacterial sample on LB media containing ampicillin and left to grow overnight at 37˚C.

4.3.4 Extraction of dsRNA

The dsRNA was extracted from bacterial pellets using TRIzol® Reagent. Cells were lysed by boiling for 2 min in 5 ml 0.1% SDS in 1x PBS and immediately placed on ice. To eliminate other bacterial RNA, 1.5 ml 300 mM sodium acetate, 10 Mm Tris-HCL, and pH 8.0 Mm EDTA buffer containing 100 µg RNase A was added to the 5 ml lysate (6.5 ml final volume). Lysates were incubated at 37°C for 30 min. dsRNA for *SK* and *SH* was extracted with 500 µl TRIzol following the manufacturer's instructions (Posiri *et al.*, 2013) and stored at-80°C until use.

4.3.5 Quantification of dsRNA produced by the transformed *E. coli*

To quantify the dsRNA extracted from bacteria or expressed in the bacteria, the dsRNA sample was separated on 2% agarose gel and visualized with ethidium bromide. A standard curve was established according to the known amounts of dsRNA was synthesized by the in vitro transcription method at serial dilution. Values of intensity are used in the standard curve. The gel band intensities were estimated using Image Lab software (Bio-Rad). The intensity value of the sample on the y-axis corresponds to a concentration value on the x-axis and concentration of the dsRNA extracted from bacteria or expressed in bacteria can be determined from the intensity values using a standard curve.

4.3.6 Bioassay of the transformed bacteria expressing dsRNA

To assess the insecticidal efficacy of bacteria expressing dsRNA or dsRNA produced by bacteria specific to *SK* and *SH*, three bioassay experiments were applied to adults and larval

stages of *T. castaneum*. In the first bioassay experiment: dsRNA extracted from bacteria was delivered via flour disks prepared as described by Xie *et al.* (1996). 10µl of suspension flour (dsRNA, 5% brewer`s yeast) were prepared into each flat bottom well of a 96-well microtitre plate and dried out at room temperature. Individual 3rd instar larvae or adult were added to each well. Three biological replicates each consisted of 15 insects for the survival experiments and 5 insects each were used to determine gene expression at a range of concentrations (100, 200, 300, 400 ng dsRNA/mg diet). In the oral delivery experiment, three groups of controls were used: flour only (control 1), flour with free water (control 2), and flour with free water and dsRNA Kanamycin in 200 ng dsKana/mg diet (control 3). The diet was changed every two days to prevent the contamination and degradation of dsRNA.

The second bioassay experiment used crude bacteria. The transformed bacteria were fed to adults and larvae of *T. castaneum* at the same concentrations of dsRNA as used with the dsRNA extracted from bacteria. The pellet was heated at 65°C for 20 min and resuspended in fresh culture medium. Bacterial viability was tested by plating 50 µl of the treated bacterial sample on LB media containing ampicillin and left to grow overnight at 37˚C to confirm all bacteria killed. Quantify the dsRNA in resuspended fresh culture medium after that prepared a range of concentrations (100, 200, 300, 400 ng dsRNA/mg diet) and vortex with 100 mg flour and amount of nuclease free water depend on dsRNA stock and add 10 µl of suspension flour were prepared into each flat bottom well of a 96-well microtitre plate and dried out at room temperature.

In the third bioassay experiment, adult and 6th larval stage were injected using a NanojectII™ injector (Drummond Scientific Company) under a dissecting stereomicroscope. Insects were injected with dsRNA for doses of 62.1, 124.2, 186.3 and 248.4 ng/larva. For the injection experiment, three controls were used: insects without injection (control 1), insects injected with RNAase-free water (control 2), and insects injected with dsRNA Kanamycin at 248.4 ng/insect (dsKana) which is a region of bacterial resistance gene (control 3).

4.4 Results

4.4.1 Transformed bacteria expressing dsRNA specific to *SK*, *SH* and *Kana*

A PCR of target genes was inserted in pENTR vector, and then the plasmids were sent for sequencing to confirm the identity of the insert. A sequence alignment was applied which confirmed 91% homology between the insert and the *SK* (Figure 4.2a), and 95% homology between the insert and the *SH* (Figure 4.2b). We have obtained an entry clone containing the gene of interest, then perform an LR recombination reaction between the entry clone and a destination vector the *SK*, *SH* and *Kana* fragment was inserted between two T7 polymerase of destination vector and then the plasmids were sent for sequencing to confirm the identity of the insert. A sequence alignment was applied which confirmed 91% homology between the insert and the *SK* (Figure 4.2c), and 95% homology between the insert and the *SH* (Figure 4.2d). The recombinant vector was used to transform *E. coli* HT115 competent cells that are expressing dsRNA in the presence of β-D-thiogalactoside (IPTG). The amount of produced dsRNA was quantified according to known amounts of dsRNA was synthesized by the in vitro transcription.

a

Download GenBank Graphics

PREDICTED: Tribolium castaneum small conductance calcium-activated potassium channel protein (LOC657962), transcript variant X6, mRNA

Sequence ID: XM_015980284.1 Length: 3337 Number of Matches: 1

Range 1: 293 to 475 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
244 bits(132)	2e-63	166/183(91%)	0/183(0%)	Plus/Minus

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Query  87   aaaCATCCCCATAACAAGAGCGTAATCACTAATTCttttttcttttttCAAAAAGCGCTTT  146
            ||||||||||||||||||||||||||||||||||| ||||| || ||||||||||||| ||
Sbjct  475  AAACATCCCCATAACAAGAGCGTAATCACTAATCCTTTTCCTCTTTTCAAAAAGCGCCTT  416

Query  147  ACGCCTGCCTAACCGATAGCCTACATTGGGTTTGTGCTTGAGACTTCCAGACGAAGGCCC  206
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Sbjct  415  GCGCCGGCCTAACCGATAGCCCACGTTGGGTTTGTGCTTGAGACTTCCAGACGAAGGCCC  356

Query  207  CTTGTACACACCACTCCCTATAGTCCTTTCTTCCATAAACCGGGGGTAGTCCACGTGTCC  266
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Sbjct  355  TTTGTAGACGCCGCTTCCTATAGTCCTGTCCTCCATGTACCGGGGGTAGTCCACGTGTCC  296

Query  267  CAC  269
             |||
Sbjct  295  CAC  293
```

b

Download GenBank Graphics

PREDICTED: Tribolium castaneum potassium voltage-gated channel protein Shaker (LOC100142279), transcript variant X11, mRNA

Sequence ID: XM_015978048.1 Length: 1614 Number of Matches: 1

Range 1: 558 to 708 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
235 bits(127)	1e-60	143/151(95%)	0/151(0%)	Plus/Plus

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Query  104  GCTGGACGTCTTCTCCGAGGAGATCAAGTTCTACGAATTGGGCGAGCTGGCGATCAACAA  163
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Sbjct  558  GCTGGACGTCTTCTCCGAGGAGATCAAGTTCTACGAGCTGGGGGAGCTGGCGATCAACAA  617

Query  164  ATTCCGCGAGGACGAGGGCTTCATCAAGGAGGAGGAGAAACCTCTGCCGTCGCACGAGTT  223
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Sbjct  618  GTTCCGCGAGGACGAGGGCTTCATCAAGGAGGAGGAGAAGCCGCTACCCTCGCACGAGTT  677

Query  224  CCAGCGCAACGTGTGGCTGCTGTTCGAGTAC  254
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Sbjct  678  CCAGCGCAACGTGTGGCTGCTGTTCGAGTAC  708
```

Figure 4.2: Output from BLAST alignment tool showing sequences. Cloned in pENTR vector (**a**) 91% homology between plasmid insert (Query) and *SK* (sbjct); (**b**) 95% homology between plasmid insert (Query) and *SH* (sbjct). Cloned into destination vector PET161-DEST (**c**) 91% homology between plasmid insert (Query) and *SK* (sbjct); (**d**) 95% homology between plasmid insert (Query) and *SH* (sbjct)Line between nucleotides point sequence homology, whereas a lack of line points the sequence change at that nucleotide.

4.4.2 Synthesis of the target interference sequence

dsRNA for *SK*, *SH* and *Kana* was prepared using specific dsRNA expressed in *Escherichia coli* HT115 lacking in RNase III, as described above. The bacteria used in this study were prepared with a recombinant vector containing a fragment of *T. castaneum*. The transformed *E. coli* was induced to express dsRNA under LacZ promoter by adding an isopropyl β-D-thiogalactoside (IPTG) inducer. The dsRNA was extracted and quantified depending on the known amounts of dsRNA synthesized by the *in vitro* transcription method. The large amounts produced were 20 µg/µl, 19 µg/µl and 13 µg/µl in 100µl of dsRNAs corresponding to the *SK*, *SH* and kanamycin resistance genes respectively (Figure 4.3). However, the respective amounts were 21 µg/µl, 9µg/µl and 8 µg/µl in the 100µl volume of dsRNAs expressed in *E. coli* HT115 for the *SK*, *SH* and kanamycin resistance genes respectively.

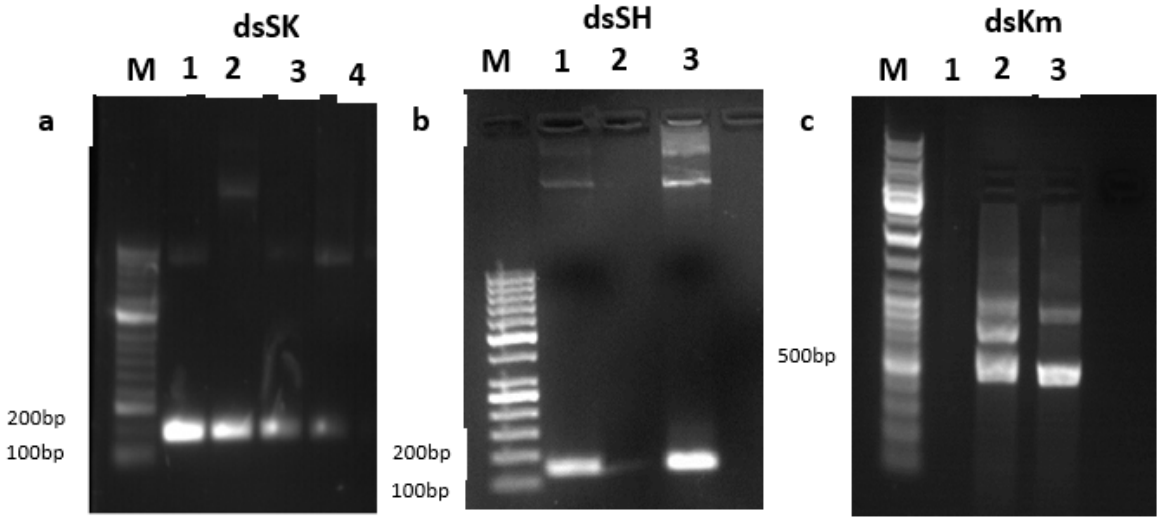

Figure 4.3: Different concentrations of dsRNA of *T. castaneum* produced by *E. coli* HT115 visualized on a 2% agarose gel; marker (100bp): **a**.dsSK (221bp) lanes 1(20 µg/µl), lanes 2 (10 µg/µl), lanes 3(5µg/µl), lanes 1(3µg/µl); **b**. dsSH (190bp), lanes 1(13 µg/µl), lanes 3(16 µg/µl); **c**. dsKana (508bp), lanes 2 (10 µg/µl), lanes 3 (13 µg/µl).

4.4.3 Effect of pretreatment on dsRNA-expressing bacteria

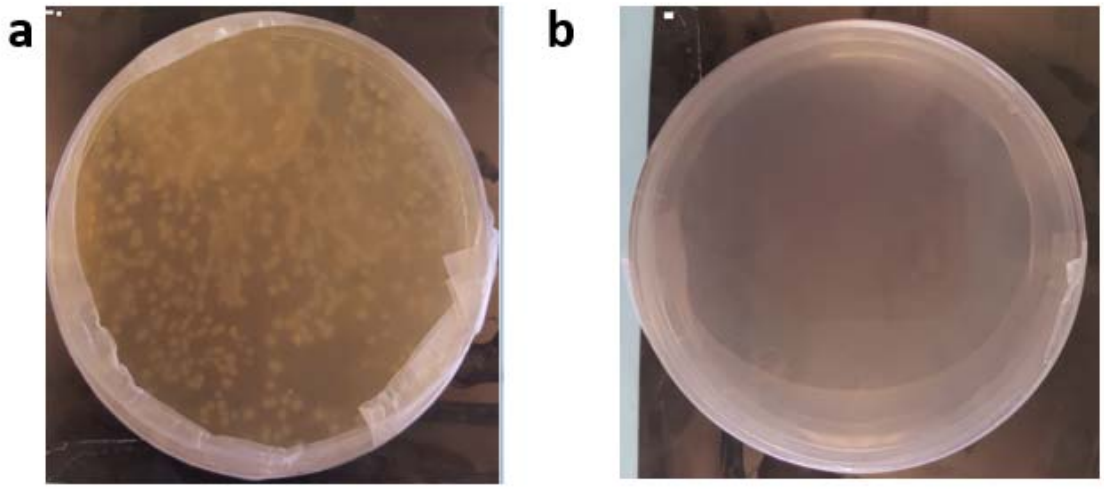

Figure 4.4: Pretreatment effect of dsRNA –expressing *E. coli* HT115 **a.** live bacteria **b.** killed bacteria after heat and sonication.

To release dsRNA from the bacteria, they were treated with heat (65 °C for 20 min) and sonication. After pretreatment, the bacterial suspension was plated, and cultured overnight at 37°C. Heat treatment killed all the bacteria (Figure 4.4).

4.4.4 Effects of the injection dsRNA extracted from *E. coli* on the expression levels of the target genes

Analyses of potassium channel gene expression were carried out with larvae and adults of *T. castaneum* collected 48h and 72h after injection and feeding respectively, with dsRNA produced by *E. coli* HT115 against *SK* and *SH*. Three biological replicates were carried out, and the knockdown of the targeted genes was demonstrated using RT-qPCR.

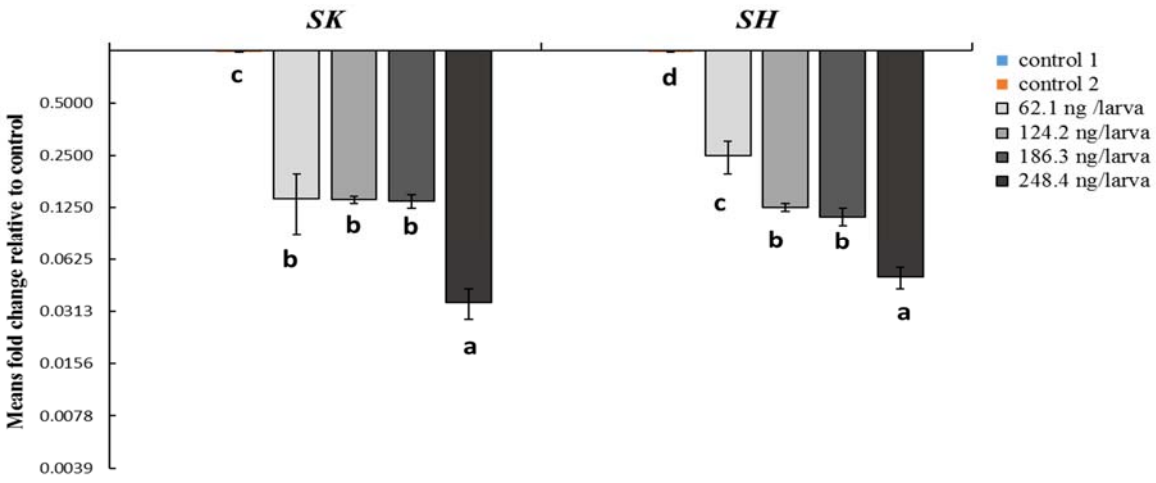

Figure 4.5: Expression of SK and SH mRNA in larvae injected with dsRNA (bacteria) after 48h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= larvae injected with RNAase free water, control 2 = larvae injected with kanamycin resistance dsRNA. Expression levels were normalized against the TcRpS6 gene as an internal standard.

RT-qPCR analysis showed a positive relationship between the amounts of dsRNA injected and decreases in the abundance of SK and SH mRNA transcript. Gene knockdown at 48 h post injection of 6th instar larvae was shown to be significantly different ($p < 0.05$) between the treatments and control, with the expression of *SK* reduced by 0.14, 0.13, 0.13 and 0.03-fold at 62.1, 124.2, 186.3 and 248.4 ng/larva, respectively. However, the reduction in expression of the *SH* were 0.24, 0.12, 0.11 and 0.04-fold relative to the control groups; there was no difference ($p < 0.05$) between the two control groups. Control 2 (larvae injected with

dsKana) which was 0.98-fold relative to control 1 (Figure 4.5). These findings show that RNAi can effectively down-regulate the expression of the *SK* by between 86-97%, while for the *SH* down-regulate reached 76-96% in dose-dependent manner.

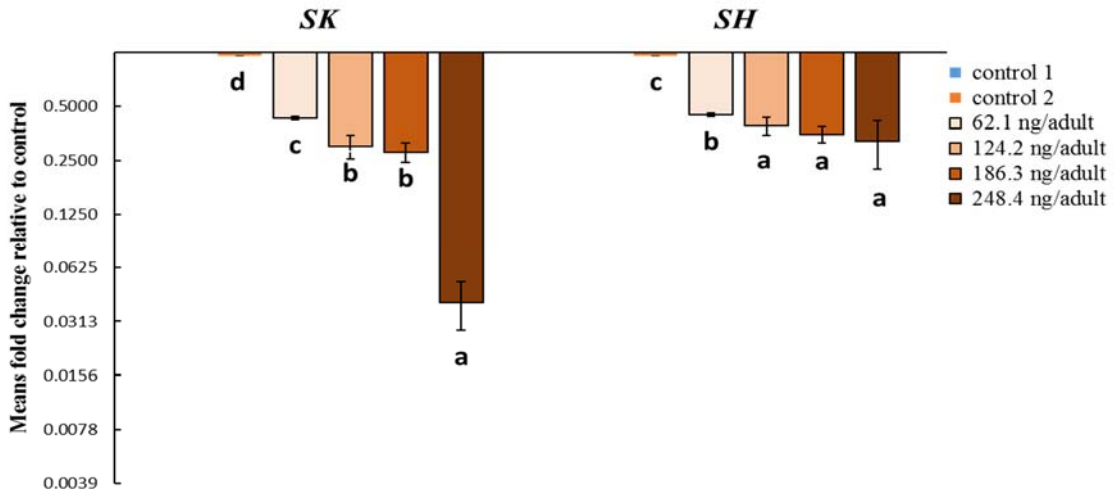

Figure 4.6: Expression of SK and SH mRNA in adults injected with dsRNA (bacteria) after 48h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= adults injected with RNAase free water, control 2 = adults injected with kanamycin resistance dsRNA. Expression levels were normalized against the TcRpS6 gene as an internal standard.

The expressions of both *SK* and *SH* were significantly down-regulated at 48 h post injection. The reduction in expression was 0.43, 0.30, 0.28 and 0.04-fold for the *SK* and 0.45, 0.39, 0.35 and 0.32-fold for the *SH* relative to the control groups, in response to injection concentrations of dsRNA at 62.1, 124.2, 186.3 and 248.4 ng/adult, respectively. While it was 0.96 fold in control 2 relatives to control 1, there was no difference ($p < 0.05$) between the two control groups (Figure 4.6). These results indicate that RNAi can knockdown the expression of the *SK* between 57-96%, while the down-regulation of the *SH* was recorded 55-68%.

4.4.5 Effects of feeding dsRNA extracted from *E. coli* on the expression level of the target genes

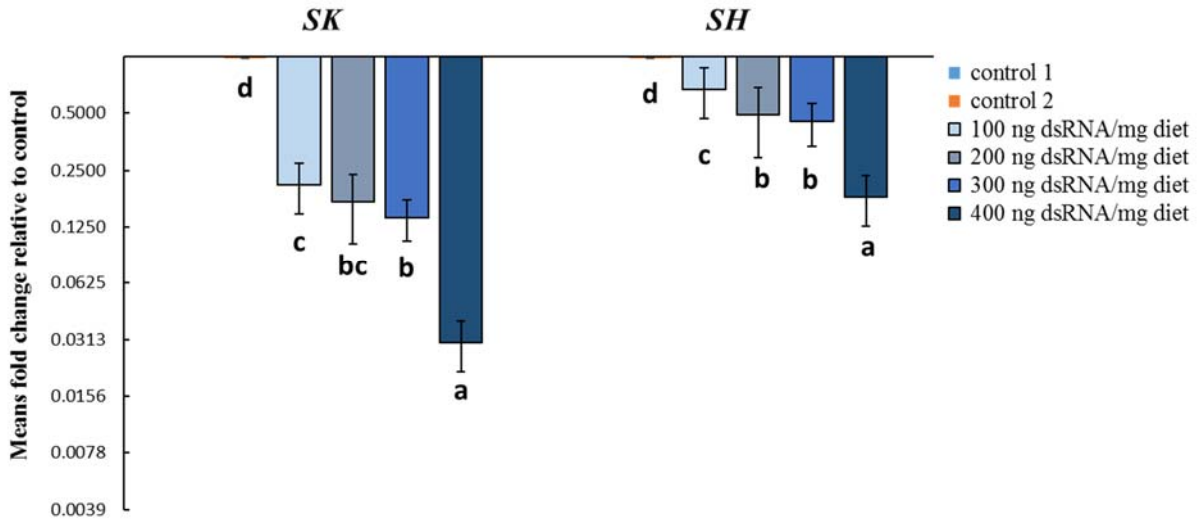

Figure 4.7: Expression of SK and SH mRNA in larvae fed with dsRNA (bacteria) after 72h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= larvae fed on RNAase free water flour disk, control 2 = larvae fed on kanamycin resistance dsRNA flour disk. Expression levels were normalized against the TcRpS6 gene as an internal standard.

After three days of continuous feeding of the 3rd instar larvae with flour containing 100, 200, 300 and 400 ng/mg dsRNA-producing bacteria, the transcript levels of SK and SH mRNA were significantly ($p < 0.05$) down-regulated to 0.21, 0.17, 0.14 and 0.03-fold respectively for the *SK*. While the reduction in expression of the *SH* were 0.67, 0.49, 0.45 and 0.18-fold respectively at the same concentration, relative to 0.98-fold for larvae fed on the kanamycin dsRNA flour disks (control 2) and 1-fold for control 1 again there was no difference ($p < 0.05$) between the two control groups (Figure 4.7). These results indicate that RNAi can knock down the expression of *SK* between 79-97%, while the down-regulation of the *SH* was recorded 33-82%.

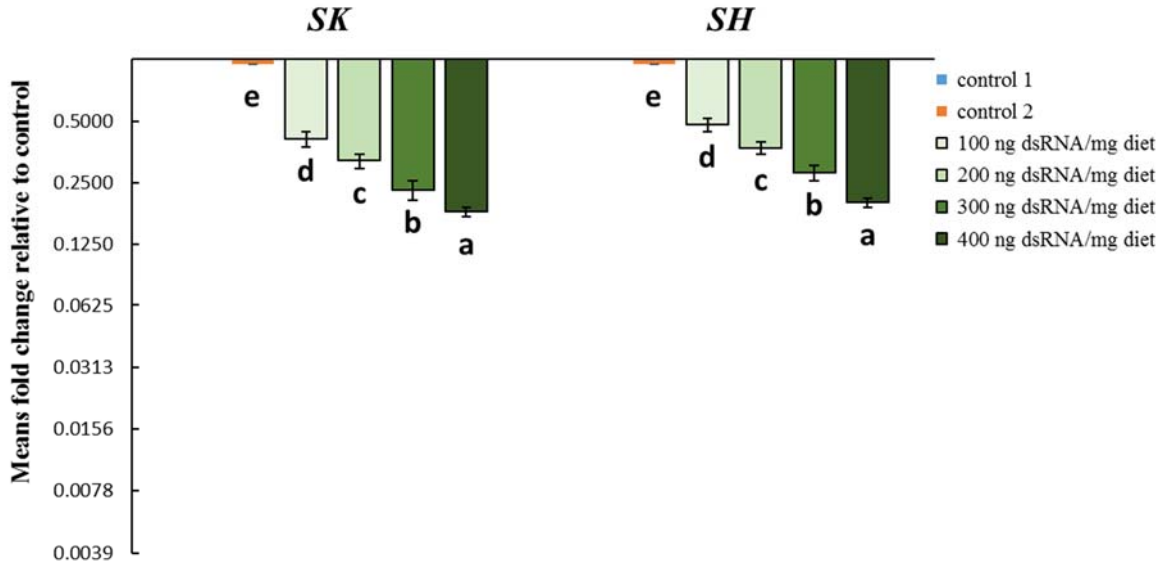

Figure 4.8: Expression of SK and SH mRNA in adults fed with dsRNA (bacteria) after 72h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= adults fed on RNAase free water flour disk, control 2 = adults fed on kanamycin resistance dsRNA flour disk. Expression levels were normalized against the TcRpS6 gene as an internal standard.

RT-qPCR analysis revealed that mRNA transcript levels in adult fed on flour containing 100, 200, 300 and 400 ng/mg dsRNA produced from recombinant *E. coli* HT115, were also significantly ($p < 0.05$) down-regulated at 72h post-feeding. The measured mRNA levels for the *SK* were 0.41, 0.32, 0.23 and 0.18-fold respectively, whereas the mRNA levels were 0.48, 0.37, 0.28 and 0.20-fold respectively at the same concentration for *SH* relative to control groups. Again there was no significant difference in gene expression ($p > 0.05$) compared to either control group over this same period. The expression of target genes was 0.95-fold in control 2 relative to that of the control 1 group (Figure 4.8). This dsRNA molecule was designed towards a 468 bp fragment of the microbial kanamycin resistance gene, which was synthesized and fed at 200 ng/ insect. Insects feeding on flour disks containing dsKana did not show any significant reduction in expression of either target gene ($p > 0.05$) compared to the group consuming the control diet. These results indicate that RNAi can knock down the expression of the *SK* between 59-82%, while for the *SH* knockdown values were recorded 52-80%.

4.4.6 **Effects of feeding bacteria expressing dsRNA on the expression levels of the target**

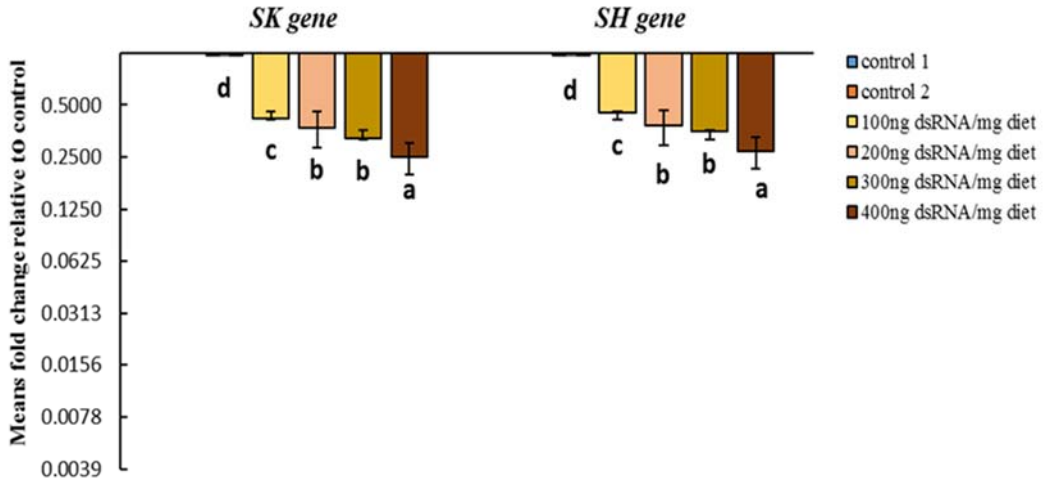

Figure 4.9: Expression of SK and SH mRNA in larvae fed with dsRNA expressed in bacteria after 72h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= larvae fed on RNAase free water flour disk, control 2 = larvae fed on kanamycin resistance dsRNA flour disk expressed in bacteria. Expression levels were normalized against the TcRpS6 gene as an internal standard.

The relative expression levels of the targeted transcripts were then examined in larvae fed on a diet with bacteria expressing dsRNA for the *SK* and *SH* (Figure 4.9). The transcript expression was significantly reduced ($p < 0.05$) in the dsRNA treatments after 72h. However, the expression levels of genes not targeted by a specific dsRNA treatment did not show reduced expression (control 2), indicating that the specific action of the dsRNA is triggered on the targeted transcripts. The transcript level of *SK* was 0.42, 0.37, 0.32 and 0.25-fold at 100, 200, 300 and 400 ng/mg respectively, whereas the transcript level of *SH* was o.45, 0.38, 0.35, and 0.27-fold at the same concentration respectively. Larvae fed on dsRNA expressed in bacteria showed significant ($p<0.05$) knockdown in gene expression between 58-75% for *SK* and 55-73% for *SH*.

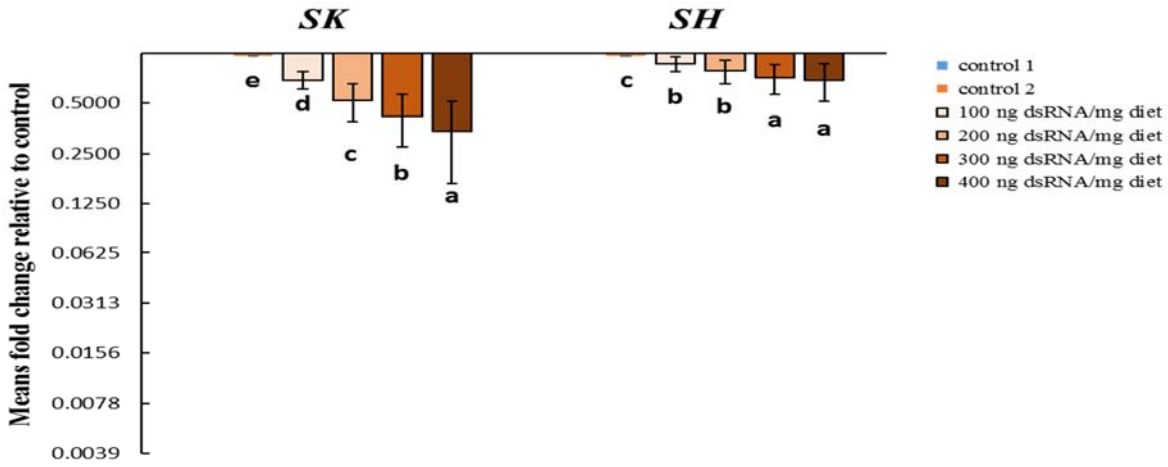

Figure 4.10: Expression of SK and SH mRNA in adults fed with dsRNA expressed in bacteria after 72h, n=5. Mean ± SD of three replications is shown. Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene. Control 1= adults fed on RNAase free water flour disk, control 2 = larvae fed on kanamycin resistance dsRNA flour disk expressed in bacteria. Expression levels were normalized against the TcRpS6 gene as an internal standard.

Figure 4.10 showed that there was a significant difference ($p < 0.05$) in gene expression for both targets genes between the controls and the treatments where adults fed dsRNA expressed in bacteria. However, there was no significant difference ($p > 0.05$) between two the controls, indicating that Kana dsRNA did not have the effect of silencing *SK* and *SH*. At 100, 200, 300 and 400 ng/mg the mRNA levels for the *SK* were 0.69, 0.52, 0.42 and 0.34-fold respectively, whereas the levels was 0.80, 0.78, 0.71 and 0.69-fold respectively at the same concentration for the *SH* compared to 0.95- in control 2. Expression was also significantly reduced in adults following feeding with bacteria expressing dsRNA, causing the knockdown of the gene between 31-66% for *SK* and 20-31% for *SH.*

4.4.7 Injection bioassays of dsRNA extracted from *E. coli* targeting potassium channel genes in *T. castaneum*

In order to test the efficacy of the dsRNA produced from *E. coli* HT115 against *T. castaneum*, bioassay experiments were carried out by the injection or feeding larvae and adults of T. *castaneum* at the same concentrations used in the *in vitro* dsRNA synthesis of dsRNA. Three biological replicates were used at each dose and the mean survival calculated for 15 insects/rep, for each dose.

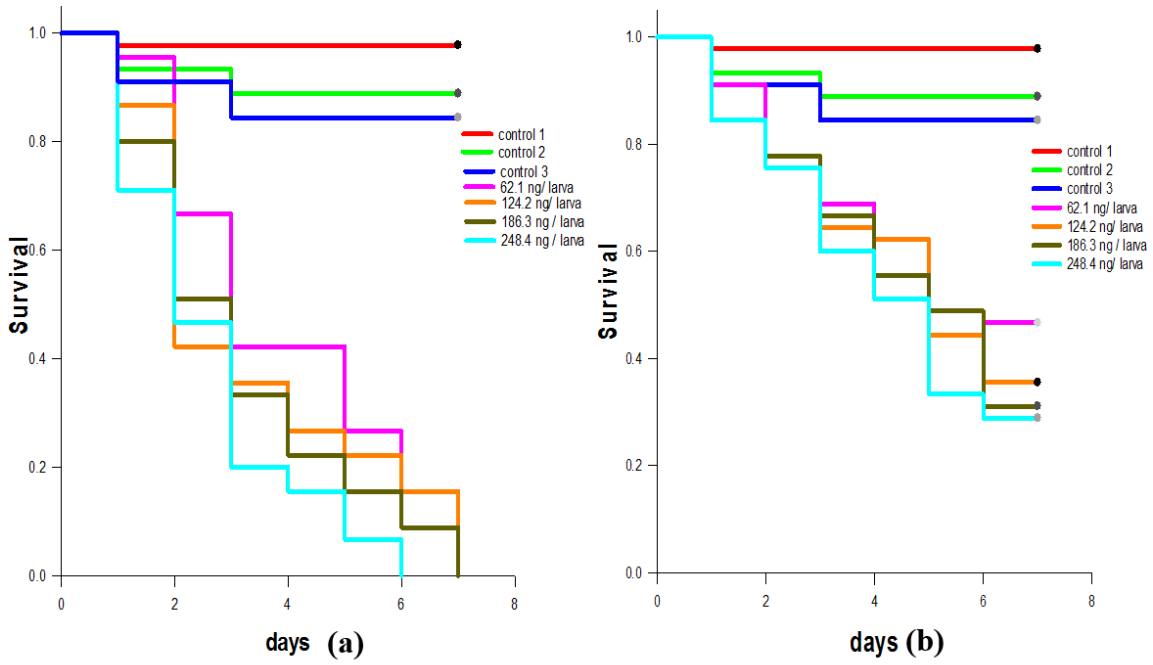

Figure 4.11: Survival curves of *T. castaneum* larvae injected with dsRNA produced by the bacteria targeted at the *SK* (a) and *SH* (b) ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). Control 1= untreated larvae, control 2 = larvae injected RNAase free water, control 3 = larvae injected with kanamycin resistance dsRNA.

Injection of dsRNA from the bacteria in the 6th instar larvae of *T. castaneum* caused significant dose-dependent decreases in survival ($p<0.001$) compared with the 3 control groups: the mortalities were 4.4%, 11.11% and 15.55% for controls 1, 2 and 3 respectively, but these differences among controls were not significant ($p > 0.001$). dsSK injected in larvae has caused a mortality 100% at concentrations 62.1, 124.2, 186.3 and 248.4 ng/larva (Figure 4.11a), while it was 51.18%, 60.48%, 67.45% and 69.78% at 7 days post-injection with dsSH respectively (Figure 4.11b). There was a significant difference observed in survival in experimental treatments compared to controls. Despite this, statistical analysis revealed no significant differences among treatments ($p > 0.001$) for the *SK*. The values of LC_{50} of the larvae injected with dsRNA for the *SK* and *SH* were 2.989 and 36.03 ng/larva respectively on day 6.

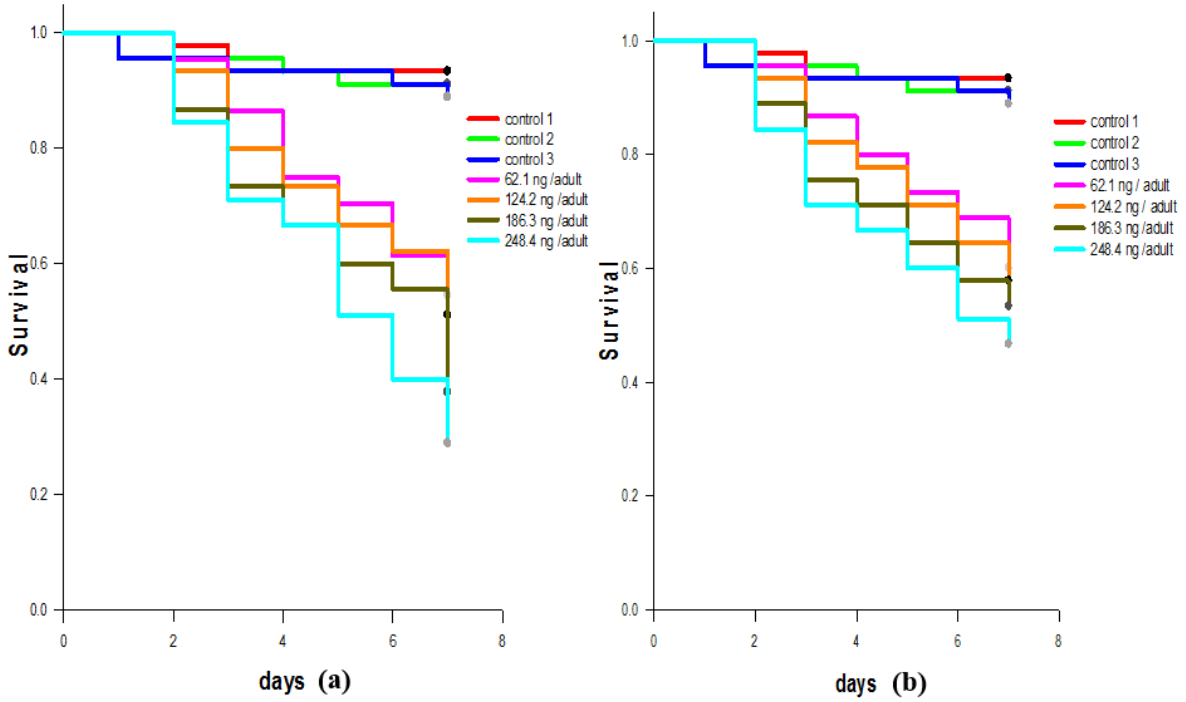

Figure 4.12: Survival curves of *T. castaneum* adult injected with dsRNA produced by the bacteria targeted at the *SK* (a) and *SH* (b) ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). Control 1= untreated adult, control 2 = adult injected with RNAase free water, control 3 = adult injected with kanamycin resistance dsRNA.

The injection of dsRNA extracted form the recombinant bacteria in adults of *T. castaneum* resulted in a significant ($p<0.001$) impact on adult survival relative to mortality rates of 6.6%, 8.8% and 11.11% for control 1, 2 and 3 respectively, with no significant difference among the three controls at day 7. The mortality was found in the groups that received dsRNA was 42.89%, 45.27%, 59.55% and 66.68% at 7 days of exposure to dsSK at 62.1, 124.2, 186.3 and 248.4 ng/adult respectively (Figure 4.12a). The corresponding rates were 35.76%, 38.14%, 42.89% and 50.03% respectively for dsSH at the same concentration (Figure 4.12b). The values LC_{50} of the adults injected with dsRNA of the *SK* and *SH* were 220.23 and 235.76 ng/adult respectively on day 6.

4.4.8 Oral bioassays of dsRNA extracted from *E.coli* targeting potassium channel genes in *T. castaneum*

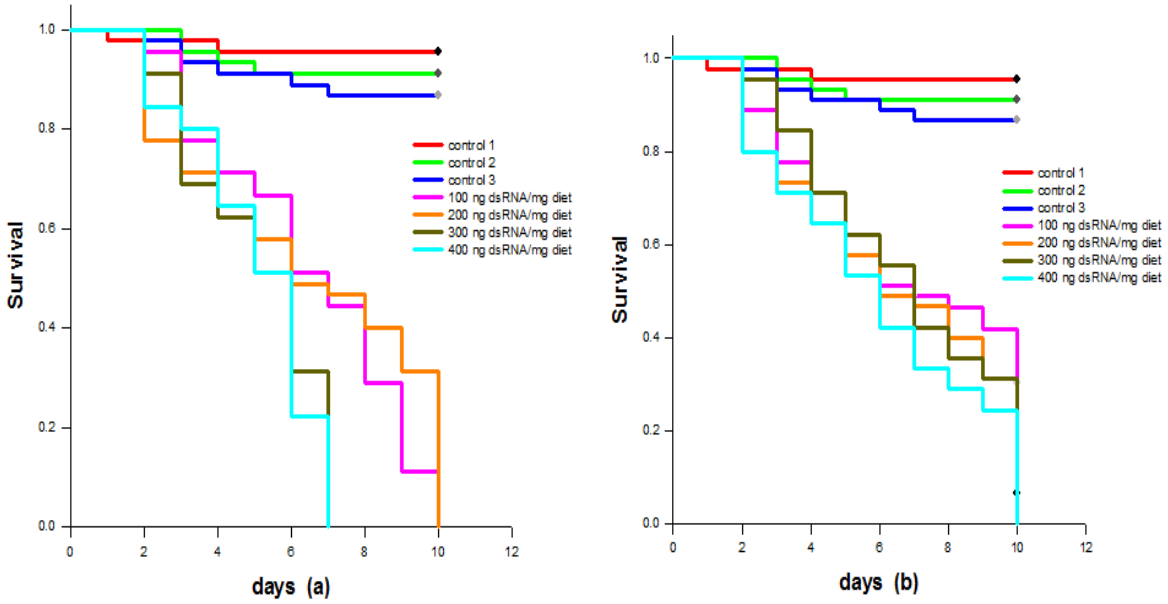

Figure 4.13: Survival curves of *T. castaneum* larvae fed with dsRNA produced by the bacteria targeted at the *SK* (a) and *SH* (b) ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). Control 1= larvae fed on flour, control 2 = larvae fed with RNAase flour disks, control 3 = larvae fed with on kanamycin resistance dsRNA flour disks.

There is a clear decrease in survival when using dsRNA for both genes in third instar larvae of *T. castaneum*. The mortality of control groups were 8.8%, 11.11% and 13.3% for controls 1, 2 and 3 respectively were observed over the assay period. With no significant differences among the control groups. In contrast, the mortality with dsSK was 100% at both 100 and 200ng/mg after 10 days as well as at 300 and 400ng/mg after 7 days (Figure 4.13a). However, dsSH mortality was only 72.50%, 92.50 % at 100, 200 ng/mg respectively, 100% at 300, and 400 ng/mg (Figure 4.13b). There were be significant differences in survival between larvae receiving dsRNA and the control treatments. Further analysis showed that the values of LC_{50} for the *SK* and *SH* were 66.04 and 119.21 ng/mg respectively on day 6.

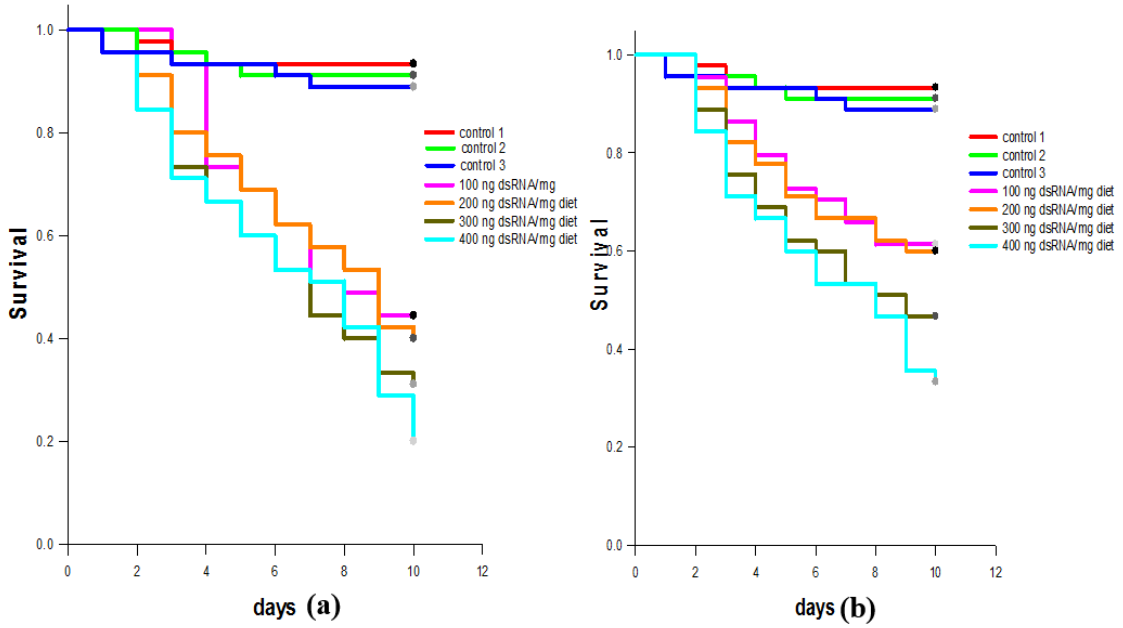

Figure 4.14: Survival curves of *T. castaneum* adult fed with dsRNA produced by the bacteria targeted at the *SK* (a) and *SH* (b) ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). Control 1= adults fed on flour, control 2 = adults fed on RNAase free water flour disks, control 3 =adults fed on kanamycin resistance dsRNA flour disks.

The oral delivery of dsRNA caused higher adult mortality which was significantly different relative to the 3 control groups (($p<0.001$). Controls 1, 2 and 3 insects recorded 2.2%, 13.33% and 15.5% adult mortality, with statistical analysis indicating no significant difference among the control groups ($p > 0.001$). However, there were significant differences between the effects of dsRNA treatments and the controls. The mortality of adults fed dsSK extracted from the recombinant bacteria at 100, 200, 300 and 400ng/mg reached 53.99 %, 59.10 %, 67.28% and 79.55% respectively after 10 days (Figure 4.14a), while for dsSH the mortalities were 36.37%, 38.65%, 52.28 % and 65.91% respectively at the same concentrations and time of assay (Figure 4.14b). All mortality results indicate that larval instars are more susceptible to the RNAi than adults. The values of LC_{50} of the adults fed with *SK* and *SH* dsRNA were 290.29 and 300.76 ng/mg respectively on day 6. All mortality were corrected using Abbott's formula relative to control 1 (untreated control).

4.4.9 Oral bioassays of bacteria expressing dsRNA targeting potassium channel genes in *T. castaneum*

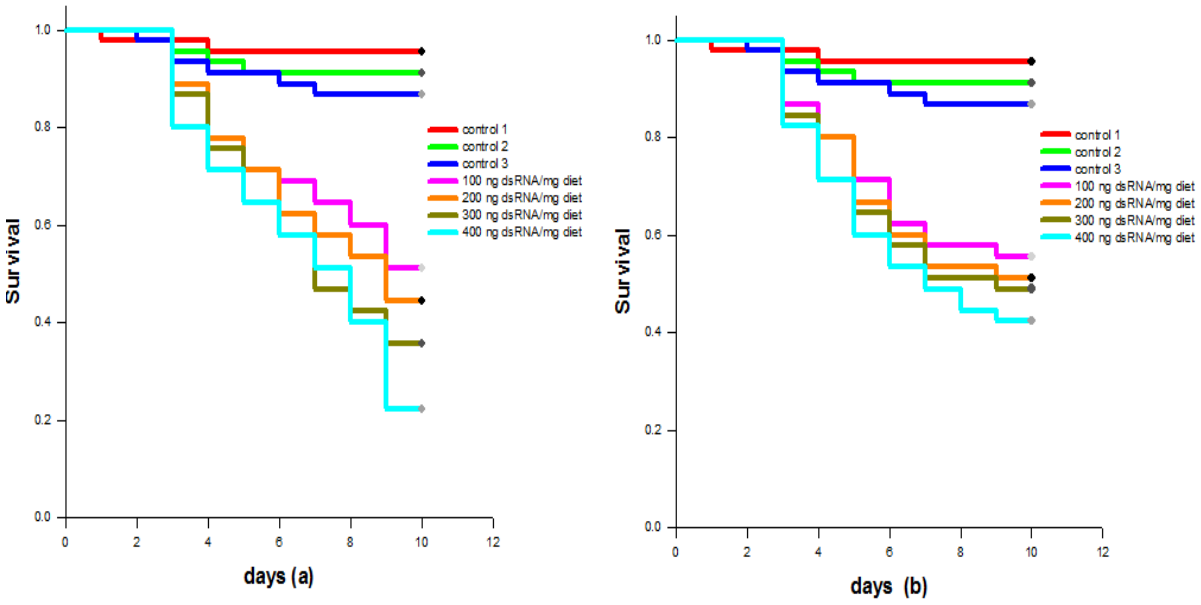

Figure 4.15: Survival curves of *T. castaneum* larvae fed with bacteria expressing dsRNA targeted at the *SK* (a) and *SH* (b) ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). Control 1= adults fed on flour, control 2 = adults fed on RNAase free water flour disks, control 3 =adults fed on kanamycin resistance dsRNA flour disks.

The induction of RNA interference by the oral delivery of the bacteria expressing dsRNA for both target genes was applied by feeding larvae on flour disks containing the dsRNA at a range of concentrations (100, 200, 300 and 400 ng/mg of diet). Insect in the control groups yielded mortality of 1, 2 and 3 resulted in 6%, 9% and 11% mortality respectively, and the differences were significant ($p > 0.001$). The mortality of larvae that had received bacteria expressed dsRNA of the *SK* were 49%, 56%, 55 % and 77% respectively (Figure 4.15a), while rates were 45%, 49%, 51 % and 58 % respectively of larval mortality for dsSH treatment at the same concentration (Figure 4.15b) for the 5 doses at 10 days. The lowest dose of dsRNA of 100 ng/mg diet led to significant reductions in survival compared to the control groups ($p<0.001$). However, there was no significant differences in mortality among dsRNA treatments for the *SH*.

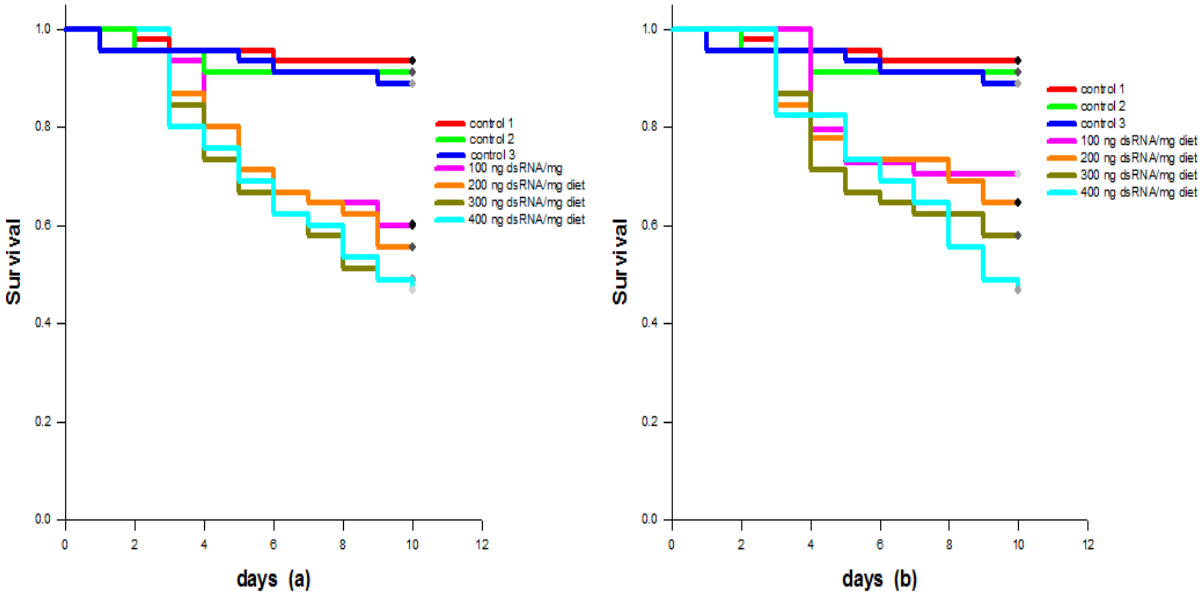

Figure 4.16: Survival curves of *T. castaneum* adults fed with bacteria expressing dsRNA targeted at the *SK* (a) and *SH* (b) ($p<0.001$; SigmaPlot v.12.5, as shown by Kaplan-Meier survival analysis). Control 1= adults fed on flour, control 2 = adults fed on RNAase free water flour disks, control 3 =adults fed on kanamycin resistance dsRNA flour disks.

dsRNA treatment resulted in a significant ($p<0.001$) impact on adult survival, and there were significant differences ($p<0.001$) between the survival curve of the control groups and those curves of dsRNA treatments. However, the differences among three control groups were found not to be significant. Which recorded 5%, 7% and 9% adult mortality. The mortality of adults that had received a bacteria expressed dsRNA for the *SK* of doses of 100, 200, 300 and 400 ng/mg of diet were 40%, 45%, 52% and 53% respectively (Figure 4.16a). Meanwhile the rates were 30%, 36%, 42% and 53% for the *SH* gene (Figure 4.16b).

4.4.10 Emerged of adult from larva fed bacteria expressing dsRNA

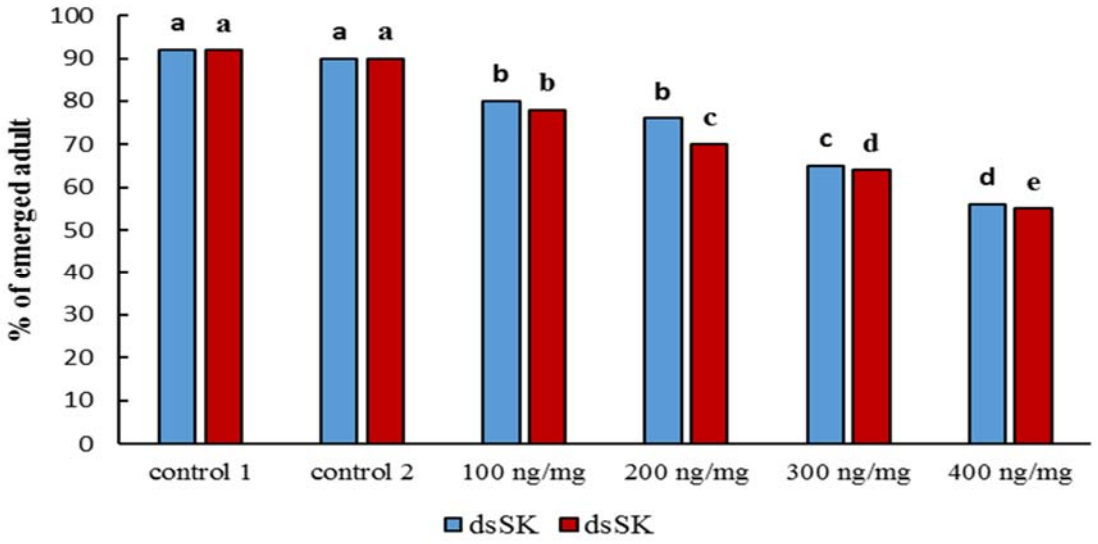

Figure 4.17: % of emerged adults of *T. castaneum* from larva fed bacteria expressing dsRNA Means with different letters are significantly different in the expression level of the target gene ($p < 0.05$, one way ANOVA, post- hoc Tukey test) within each gene.

The reduction in adult emergence from the larva stage fed with a range of concentrations bacteria expressing dsRNA (100, 200, 300 and 400 ng/mg of diet) were 80%, 76%, 65% and 56%, respectively, corresponding rates for the *SH* gene were 78%, 70%, 64% and 58% . The dsRNA caused significant reductions in survival compared with the control groups (Figure 4.17).

4.4.11 Comparison of the amount and purified dsRNA produced via the *in vitro* dsRNA produce and the *in vitro* dsRNA produce

dsRNA can be prepared using both *in vitro* transcription and *in vivo* expression in bacteria. The large amounts of dsRNA produced via *in vivo* expression in bacteria are larger than in the *in vitro* transcription. The large amount obtained from dsRNA processing *in vivo* were 20 µg/µl, 19 µg/µl and 13 µg/µl in the 100µl volume of dsRNAs corresponding to the *SK*, *SH* and kanamycin resistance genes respectively. However, 5000 ng/µl, 3456 ng/µl and 1500 ng/µl in the 100µl volume of dsRNAs respectively obtained via dsRNA processing *in vitro*. The faint

smeared background of the dsRNA produced by *in vivo* expression is shown in Figure 2.3. However, no smearing was observed with *in vitro* transcription (Figure 4.2).

4.4.12 Comparison insecticidal activity of *in vitro* prepared dsRNA with dsRNA prepared from bacteria delivered by oral feeding of larvae

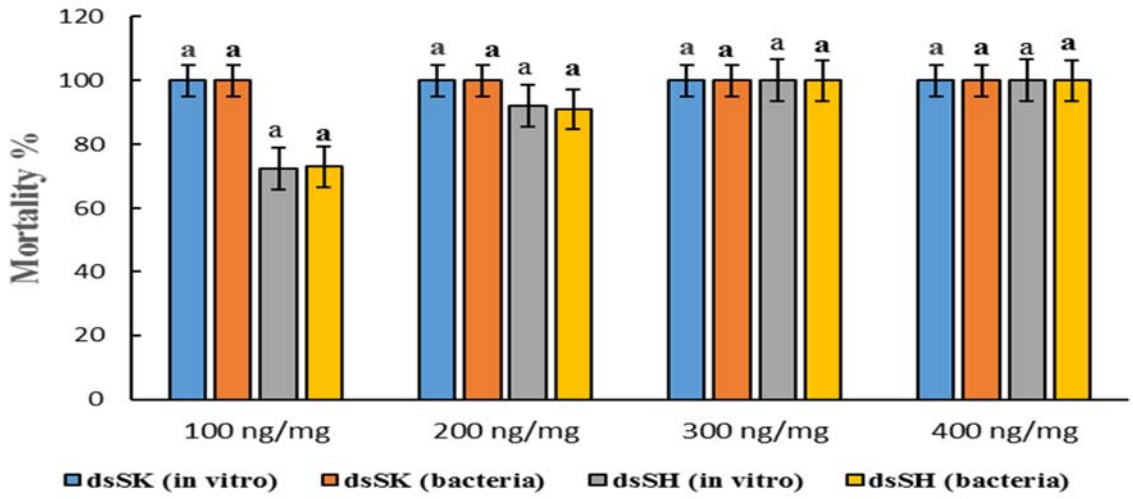

Figure 4.18: Comparison larval mortality in *T. castaneum* by oral delivery of two types of dsRNAs prepared by *in vitro* transcription or by the recombinant bacteria at 10 days. Means with different letters are significantly different ($p < 0.05$, one-way ANOVA, post- hoc Tukey test) within each concentration of two methods.

The efficacy of dsRNA produced by the recombinant bacteria was compared with that of dsRNA prepared *in vitro* (Figure 4.18). Both treatments caused high mortality in third instar larva by oral delivery at 10 days. In addition, RNAi efficiency appeared to be similar in both treatments in the range of concentrations.

4.4.13 Comparison of in vitro prepared dsRNA with dsRNA prepared from bacteria in the suppression of the target gene with delivery larval feeding

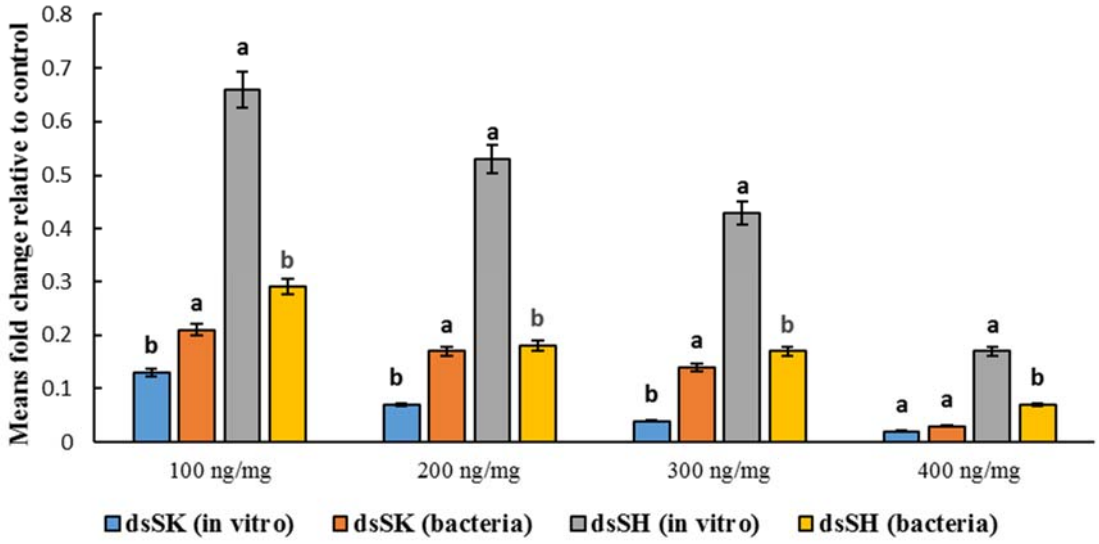

Figure 4.19: Comparison of suppression of target gene (*SK* and *SH*) expression levels in larvae of *T. castaneum* by two types of dsRNAs prepared by in vitro transcription or by the recombinant bacteria via oral delivery. Means with different letters are significantly different ($p < 0.05$, one-way ANOVA, post- hoc Tukey test) within each concentration of two methods.

Figure 4.19 shows a significant decrease in *SK* and *SH* mRNA levels in third instar larvae of *T. castaneum* fed with dsRNAs prepared by *in vitro* transcription or by the recombinant bacteria. More importantly, it was observed that the reduction in expression levels of both target genes was different between the two methods at the same concentrations.

4.4.14 Comparison of insecticidal activity of in vitro prepared dsRNA with dsRNA prepared from bacteria delivered by injection of larvae

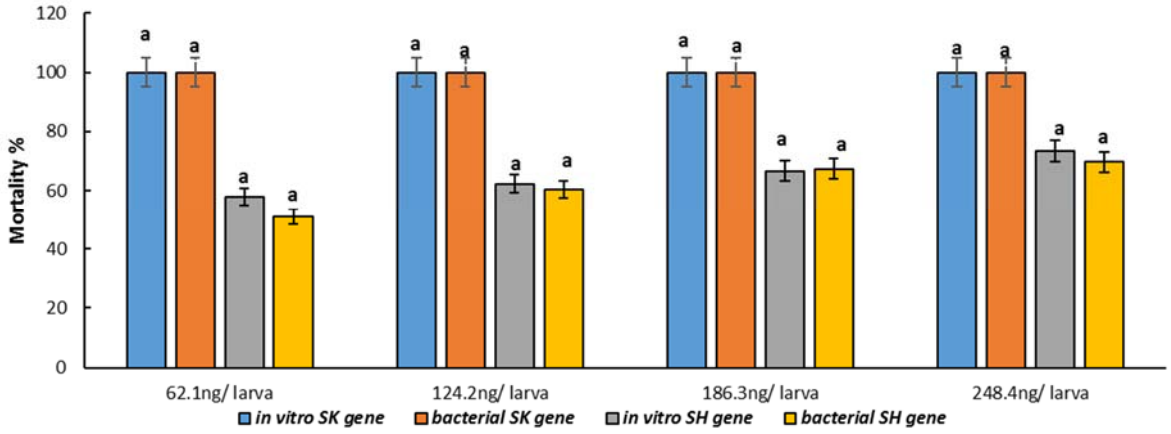

Figure 4.20: Comparison of larval mortality in *T. castaneum* with delivery by injection of two types of dsRNAs prepared by *in vitro* transcription or by the recombinant bacteria at 7 days. Means with different letters are significantly different ($p < 0.05$, one-way ANOVA, post- hoc Tukey test) within each concentration of two methods.

Figure 4.20 shows that the dsRNA produced by *in vivo* expression in bacteria was as effective as the *in vitro* transcription. For the *SK* recorded the same mortality via both methods. However, for the *SH* a slight difference in survival was found between methods.

4.4.15 Comparison of in vitro prepared dsRNA with dsRNA prepared from bacteria in the suppression of target gene by larval injection delivery

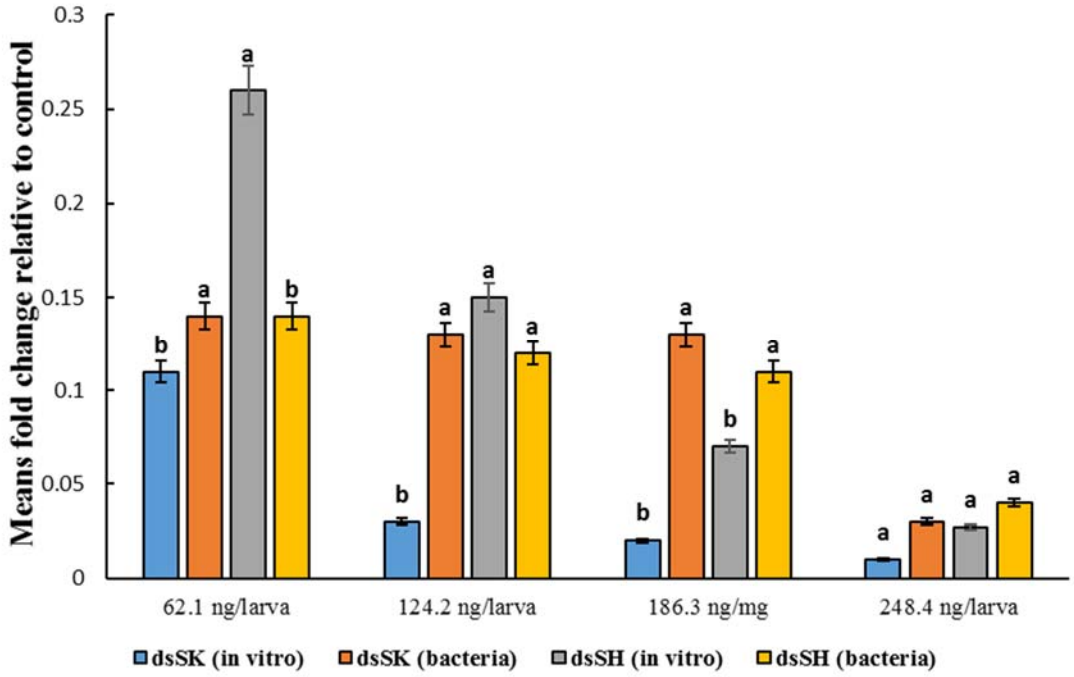

Figure 4.21: Comparison of suppression of target gene (*SK* and *SH*) expression levels in larvae of *T. castaneum* by two types of dsRNAs prepared by in vitro transcription or by the recombinant bacteria, with delivery by injection. Means with different letters are significantly different ($p < 0.05$, one-way ANOVA, post- hoc Tukey test) within each concentration of two methods.

RNAi mediated the effects on the expression levels of both target genes by two methods produce dsRNA. There is a decrease in the expression level of the target encoding two types' potassium ion channels. However, there was the difference between two methods at the same concentration in 6th instar larva via injection delivery (Figure 4.21).

4.1 Discussion

This is the first study to report an advantage in using a recombinant bacterium to express dsRNA specific to the potassium ion channels of *T. castaneum*, giving significant insecticidal activity against larval and adult instars. The amount of dsRNA produced via *in vivo* expression in bacteria was approximately four times that of the *in vitro* transcription. The *in vivo* method to generate dsRNA is comparatively easy, including *E. coli* culturing, the RNase A treatment of the lysed cells and dsRNA extraction.

RNAi is a powerful technique for functional genomics in insects, showing a potential ability for pest control (Huvenne and Smagghe, 2010). To improve insecticidal activity, it is important to select target genes that are vital for the survival of the insects. In this study, *SK* and *SH* were selected as targets via *in vitro* synthesis dsRNA and disrupted both genes expression and induced significant mortalities in adults and larvae. Although the *in vitro* system of dsRNA production is highly effective and require less molecular cloning work than the *in vivo* alternative (Rouhana *et al.*, 2013). However, the costs remain high (Zhang *et al.*, 2013).

Therefore, this study includes an alternative more cost-effective method to knock down both genes in *T. castaneum,* which involves the expression of dsRNA in *E. coli* HT115 with plasmids containing RNA promoters. This is shown to be a more cost-effective method. To facilitate the release of dsSK and dsSH from transformed *E. coli* cells, they were treated with heat and sonication before the extraction of dsRNA. Heat treatment kills all bacteria for environmental safety purposes, and the sonication treatment disrupts the bacterial membrane (Kim *et al.*, 2015), and thus the damage to the membrane may ease the release of dsRNA from the bacteria. In this study, a large quantity of dsRNA was obtained from the *in vivo* system, which was four times greater than from the equivalent the *in vitro* system per reaction. In this regard, the bacterial method to express dsRNA might be a better alternative. The spraying of bacteria expressing dsRNA in the field could be used against the Colorado potato beetle, and preliminary work has recently been underway (Palli, 2014). Most recently, symbiont bacteria *Rhodococcus rhodnii* was engineered to produce dsRNA as a biocontrol against agricultural pest species (Whitten *et al.*, 2016). The dsRNA produced *in vivo* could be used as an alternative method to produce the large amounts of dsRNA which are necessary for practical spraying method.

The present study provides more evidence to support this method, as illustrated below**.**

4.1.1 Gene Expression

The gene expression results confirm that there is a significant drop in the transcript levels of target genes in *T. castaneum* larvae after that the injection or feeding of the dsRNA of *SK* and *SH* extracted from the recombinant bacteria. The larval feeding results showed a 79-97%

knockdown for the *SK*, while for the *SH* was 33-82% knockdown in gene expression recorded. Expression was also reduced in adults, causing 59-82% down-regulation for the *SK*, and 52-80% for the *SH* at 72h post feeding. However, feeding *T. castaneum* with bacteria transformed with a plasmid vector expressing dsRNA-targeting *SK* and *SH* caused less knockdown in the gene expression of both of the target genes. Our results are exciting because they showed that the oral delivery of dsRNA expressed in bacteria could induce sufficient RNAi knockdown of target genes to reduce the survival of insect pests.

The results of RT-qPCR indicate that both *in vivo* and *in vitro* treatments suppressed *SK* and *SH* transcript levels in *T. castaneum*. However, the results of these two experiments slightly different, even though the *in vitro* dsRNA experiment was conducted under the same conditions for that with the dsRNA extracted from bacteria. In the *in vitro* treatments, the oral delivery of dsRNA caused significant reductions in transcript levels of the *SK* and *SH* at 72 h post-feeding of 3rd instar larvae, where the levels were down-regulated by 98% and 83% respectively at 400 ng dsRNA/mg diet, whereas they were down-regulated by 97% and 82% respectively at the same concentration during *in vivo* treatments. In addition, in the *in vitro* treatment, the injection delivery also caused significant knockdown in transcript levels of *SK* and *SH* at 48 h post-injection of 6th instar larvae, where the levels were down-regulated by 99% and 98% respectively at 248.4 ng/larva. Meanwhile, they were down-regulated by 97% and 96% respectively at the same concentration during *in vivo* treatments. A slight difference in a knockdown in transcript levels of *SK* and *SH* was found between methods and this due to the differences in the quantification of dsRNA produced by two methods.

These findings indicate that RNAi could effectively knock down the expression of target genes in *T. castaneum* larvae and adults, resulting in a loss of gene function. That is because the insects lose the interferon-regulated immunity pathway which is responsible for the protection of the organism from dsRNA molecules, as invertebrates (Geiss *et al.*, 2001).

Moreover, dsRNA extracted from the recombinant bacterial treatment was more effective in killing the target insects and knocking down gene expression compared to treatment with the recombinant bacteria expressing dsRNA caused 75% and 73% knockdown for *SK* and *SH*, respectively, whereas the dsRNA extracted from bacteria caused 97 % and 82 % knockdown for *SK* and *SH* respectively at 400 ng/mg diet after 72 h post-feeding of 3rd instar larvae.

The application of this technology has been tried in the control of the fruit fly *Bactrocera dorsalis* and showed effective suppression of four different target genes, Rpl19, V type ATPase D subunit, Noa, and a small GTPase Rab11, after feeding with dsRNA-expressing bacteria (Li *et al.*, 2011b). The present results are consistent with those reported for the beet armyworm *Spodoptera exigua* by Kim et al. (2015), which indicated that bacterially expressed dsRNA specific to the β-subunit of integrin (*SeINT*) gene which is associated with cellular immune responses and larval development gave a significant reduction in *SeINT* expression and caused significant mortality in the insect larvae.

In this study, the *in vivo* production of dsRNA using an *E.coli* bacterial system can be effectively employed with RNAi for insect pest control.

4.1.2 **Survival**

The present data indicate that the use of dsRNA expressed in *E. coli* HT115 lacking in the RNase III of either gene efficiently reduced the gene expression when the feeding delivery method was used, resulting in a significant impact on larval and adults survival. In an effort to increase the toxicity of the bacteria expressing the dsRNA of the *SK* and *SH* in the current study, an attempt was made to improve the efficiency of the dsRNA released from the live *E. coli* by pretreatment of the bacteria with heat and ultra-sonication to disrupt the bacterial cell wall then extraction of dsRNA. There was a difference in insecticidal activity between the dsRNA expressed in *E. coli* and the dsRNA extracted from *E. coli*. dsRNA extracted from the bacteria gave significantly enhanced efficacy in killing insects than the dsRNA-expressing bacteria themselves. The larvae fed with the recombinant bacteria resulted in larval mortality rates 77% and 58% for the *SK* and *SH* respectively fed at 400 ng/mg. Meanwhile, mortality was 100% at the same concentration for either gene after the larvae were fed with dsSK or dsSH extracted from the bacteria. These results suggest that the disruption by sonication pretreatment helps the release of dsRNA from the bacteria. Moreover, the free dsRNAs could easily enter the midgut epithelial cells of *T. castaneum*.

The results also indicated that the RNAi technique is more effective against larvae than adults. Morever, dsSK had a greater effect than dsSH. Additionally, the *in vitro* and *in vivo* dsRNA for both targets were effective in killing the target insects. The values of LC_{50} of the larvae

injected with the *in vitro* dsRNA of *SK* and *SH* were 2.385 and 34.93 ng/larva, respectively, whereas the corresponding values were 65 and 117.01 ng/mg respectively by larval feeding at day 6. However, The LC_{50} values of TcdsRNA of *SK* and *SH* prepared from bacteria were 2.989 and 36.03 ng/larva respectively by larval injection and 66.04 and 119.21 ng/mg by feeding on the same day. No significant differences were found between the LC_{50} of TcdsRNA by the two production methods. In addition, the control efficacy in killing *T. castaneum* was 100% larval mortality at 400 ng dsRNA/mg diet via oral delivery for both targets for both methods. Again, there was no difference in larval mortality rates compared two methods for the *SK* via injection delivery. However, the mortalities were 73.4% and 69.7 % at 248.4 ng /larva via the *in vitro* and *in vivo* methods respectively for the *SH*. The differences in mortality between the two methods may have contributed to differences results of the survival of control 1 between the two-bioassay studies.

There was a positive relationship between time after delivery and mortality, suggesting an accumulation of dsRNA molecules over the time. Loss of the functions relevant could cause death in *T. castaneum*. The results indicate that bacterially produced dsRNA might be viable for the application of RNAi in the field for pest control. Any possible resistance that might develop by mutation of the target sequence in the insect could be overcome by designing dsRNAs for a different sequence of the same gene or targeting different genes. It is unlikely that resistance will develop from mutations in the core of RNAi machinery because this system is a central component in eukaryotic gene regulation and helps in protecting against viruses.

In a similar study of bacterial treatment in the Colorado potato beetle, *Leptinotarsa decemlineatam,* the RNAi mediated gene was silenced by the feeding of bacterially expressed dsRNA targeted at the Vacuolar ATP synthase subunit E gene, causing significant mortality and reduced body weight in the insect (Zhu *et al.*, 2011). Another successful study of the use of RNAi from bacterially expressed dsRNA was shown in *Chilo suppressalis* larvae, the expression of four genes involved in ecdysteroidogenesis was significantly affected resulting in high larval mortality at 72h after oral delivery (Zhu *et al.*, 2016).

Our data have demonstrated the level of insect control through mortality or gene knockdown is not affected by the method used to synthesize the dsRNA. The simple molecular biology

used to construct the dsRNA vectors and purification of the dsRNA molecules in the bacterially expressed system favours it as the method of choice over the traditional *in vivo* bacterial expression system regarding both the low cost of reagents and the amounts involved. If dsRNA were to use as a topically applied a biopesticide, the low cost of the bacterially expressed molecules would make it the system of choice. Therefore, our result represents an opportunity to potential use of bacteria, as an active producer of the RNAi response against *T. castaneum*, and may be able to compete with current insecticides regarding efficacy and cost.

Chapter 5

The potential of RNAi to enhance the efficacy of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* against the Red Flour Beetle, *Tribolium castaneum*

5 The potential of RNAi to enhance the efficacy of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae,* to control the Red Flour Beetle, *Tribolium castaneum:* A case study

5.1 Abstract

The potential of RNAi to enhance the use of biocontrol agents (BCAs) such as the entomopathogenic fungus *Beauveria bassiana* and *Metarhizium anisopliae* for the control of the storage beetle *T. castaneum* was investigated. If successful using this dual approach could reduce the levels of dsRNA required. RNAi was demonstrated to be effective in controlling *T. castaneum* through the silencing of the small conductance calcium-activated potassium channel (*SK*) and voltage gate potassium channel (*SH*) (see chapter 2). The dual treatment of dsRNA in combination with entomopathogenic fungi was more effective than either the fungi alone or the administration of dsRNA alone. Knockdown of the *SK* together with a range of fungal dosages (3×10^2, 3×10^3 and 3×10^4 spores/ml) of *B. bassiana* resulted in 100% mortality of 3rd instar larvae after 10 days compared to knockdown of the *SH* in the presence of the fungus at the highest concentration (3×10^4 spores/ml), where the mortality decreased to 86%. The result also demonstrated that a combination of dsRNA (at the LC_{50} value) was more effective with *B. bassiana* compared with *M. anisopliae* for both larvae and adults, with larval mortality being 88% and 64% for dsSK and dsSH, respectively, with *M. anisopliae*. Adult mortality for the combination of dsSK and dsSH with *B. bassiana* was 86 %, and 72%, respectively, while dsSK and dsSH combined with *M. anisopliae* was 76% and 50%, respectively. In all cases, the effects of combining RNAi technology with the use of entomopathogenic fungi significantly, enhanced the mortality of *T. castaneum*. For dsSK, the effects with either fungus and irrespective of the developmental stage of the insect were synergistic.

5.2 Introduction

RNA interference (RNAi) is seen as a new strategy for the control of crop pests which is environmentally-friendly. Genome sequencing programmes for different pest species have created a wealth of information useful for the design of dsRNA to target specific genes within pest species. Moreover, it has been noted that *T. castaneum* is very susceptible to this technique. Several studies have revealed that RNAi in *T. castaneum* is efficient in preserving the silencing impact for a long period, and even in inducing gene silencing in the postembryonic stages (Gao *et al.*, 2017; Xie *et al.*, 2017). Despite advances, the technology has not been commercialised yet as a biopesticide due to the production costs of the synthesis of dsRNA, although alternative forms of delivery via bacteria are being investigated. The Monsanto Company have licenced the technology versus transgenic crops; for example, SmartStax PRO (Shukia *et al*., 2016; San and Scott. 2016). To date, RNAi technology has been used predominantly to understand the function of genes in insects (Gotoh *et al*., 2016; Noh *et al*., 2016; Liu *et al*., 2016). Despite problems encountered with the technology regarding its method of deployment, for example in transgenic crop expression of dsRNA and the associated costs, the technology has the potential to be highly species-specific, thus reducing the non-target effects seen with many other crop protection strategies. RNAi targeting *SK* and *SH* by dsRNA was shown to reduce transcription levels of mRNA for *SK* and *SH* and induce high levels of mortality in *T. castaneum* (see chapter 2). We selected the two entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* as a second control strategy to combine them with dsRNA targeting *SK* and *SH* as part of an of integrated pest management approach (IPM).

The use of entomopathogenic fungi such as *Beauveria bassiana* (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) to control stored product insects has been applied extensively in laboratory studies and the field (Khan and Selman, 1988). The fungi used are very specific to insects, many to particular kinds, and do not infect plants or other animals. Moreover, fungi are very safe for the environment and therefore are considered by many to be promising alternatives for insect control (Sahayaraj and Tomson, 2010). Both *B. bassiana* and *M. anisopliae* are fungi that develop naturally in soils throughout the world, and they work as a parasite on various insects species, causing white muscardine disease by *B. bassiana* and green muscardine disease by *M. anisopliae*

(Erler and Ates, 2015; Sree and Varma, 2015). They produce germ tubes that germinate over the surface of the insect cuticle and penetrate in the intersegmental region of the cuticle, which is a relative weakness, thus infection easily be achieved (Deacon, 2013). The setae or bristles present on larvae and adults of *T. castaneum,* facilitate the adherence of the spores (Wakefield, 2006; Dönitz *et al.*, 2013). However, entomopathogenic fungi are still less effective than conventional chemical control methods. We hypothesise that the use of RNAi technology in combination with the use of entomopathogenic fungi will have synergistic effects and therefore improve the efficacy of these BCAs.

The present study aims to evaluate the compatibility of the knockdown of *SK* and *SH,* by RNAi with the use of *Beauveria bassiana* and *Metarhizium anisopliae* against *T. castaneum*, as a strategy of pest management that may be more affordable in the field than the application of synthesised dsRNA.

5.3 Materials and methods

5.3.1 Treatments and formulations of fungal suspensions used for bioassays

Met52 granules containing *M. anisopliae* and liquid of *B. bassiana* were obtained from Fargro Ltd, Littlehampton, West Sussex and Belchim Crop Protection Limited, Eaton Soco, respectively. The granular product contained 2% w/w *M. anisopliae* var.anisopliae strain F52, and the oil dispersion formulation contained 7.16% w/w *B. bassiana* ATCC 74040, the oil dispersion formulation is vegetable oil or methylated seed oils, ideally the active ingredient is uniformly suspended in the oil phase. Each formulation was applied separately by spraying the insects with a conidial suspension. Two doses of dsRNA targeting *SK* and *SH* at their LC_{50} (see chapter 2 and 4) for larvae and adults were used in these studies. In two separate experiments, spore suspensions of *M. anisopliae* and *B. bassiana* were sprayed on third instar larvae and adults of *T. castaneum*. The first bioassay involved treatments with three different spore concentrations made from the primary suspension and a control. The concentration used were 3×10^2, 3×10^3, and 3×10^4 spores/ml using a hand-held sprayer. Each replicate comprised 10 insects in one small Petri dish. Thus, each group of 50 insects was treated by direct spraying with about 3ml of solution while they stood on the large glass Petri dish (200-mm). After 10 minutes to allow the insect dry to prevent contamination to dsRNA then the

insects were transferred to a 96-well microtiter plate. For the second bioassay, dsRNA was delivered via flour disks prepared as described by Xie *et al.* (1996). 10ul of suspension flour (dsRNA, 5% brewer`s yeast, free nuclease water) were prepared into flat bottom wells of a 96-well microtiter plate and dried at room temperature. Insects were transferred to these plates (one insect per well) and allowed to feed on the diet of flour treated with of dsRNA (65, 117 ng dsRNA/mg diet) of targeting either *SK* or *SH* respectively for larvae, whereas (290, 300 ng dsRNA/mg diet) of either *SK* and *SH* respectively, for adults. Feeding was assessed by observing food consumption every 2 days. Appropriate controls were carried out for both larvae and adults for both genes. Three groups of controls were used: (control 1) only flour; (control 2) RNAase free water with flour; (control 3) dsRNA Kanamycin with flour. The same experiment was repeated in the absence of fungi and used only dsRNA compare with controls 1, 2 and 3 and the same experiment carried out without dsRNA only fungi relative to control 4 (insect was sprayed with distilled water). Records of insect mortality started 24 hours after the treatments were applied and continued for 10 days. Every day the number of insects that had died in the previous 24 hours were noted and removed. The number of insects surviving was also recorded daily. All treatments for the three experiments were done at 25 °C at the same time.

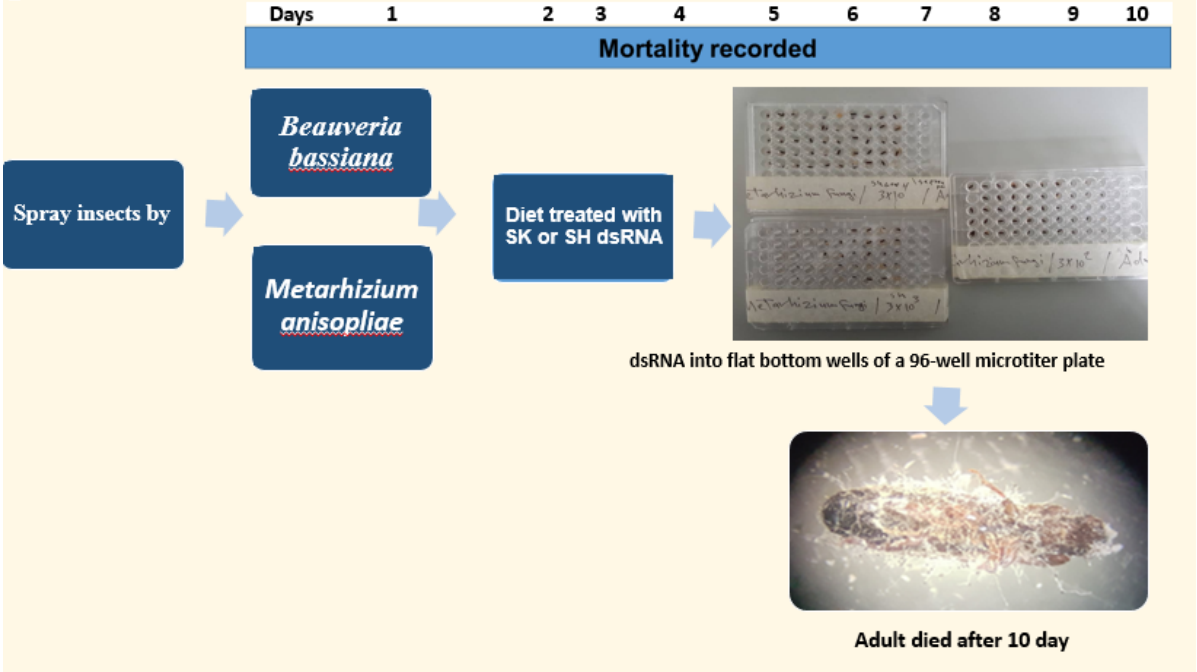

Figure 5.1: Diagrammatic representation of bioassays for dsRNA with fungi.

5.3.2 Statistical analysis

Insect mortality was analyzed using the SIGMAPLOT program, version 12.5 (Kaplan-Meyer survival analysis) and corrected according to Abbott's formula. Moreover, Stata (version 13) was used to determine any potential synergistic effects.

5.4 Results

The present study investigated the survival of 3rd instar larvae and adult stages of *T. castaneum* treated with dsSK, dsSH either alone or in combination with *B. bassiana* and *M. anisopliae* fungi for all treatments with dsRNA. The levels used were those that had previously been shown to cause 50% mortality (see chapters 2 and 4).

5.4.1 Survivorship of 3rd instar larvae treated with dsRNA targeting *SK* or *SH* and *Beauveria bassiana*

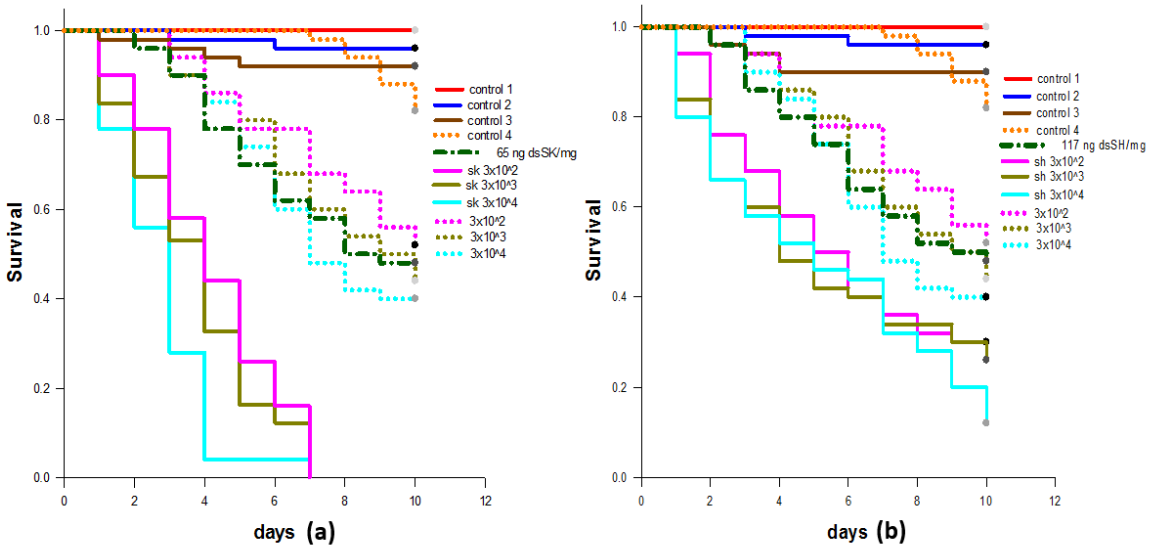

Figure 5.2: Survival curves of *T. castaneum* larvae fed on dsRNA targeted to *SK* (a) and *SH* (b) with *B. bassiana* ($p<0.001$; Sigma Plot v.13, as shown by Kaplan-Meier survival analysis). Control 1 = larvae fed on flour, control 2 = larvae fed on RNAase free water flour disks, control 3 = larvae fed on kanamycin resistance dsRNA flour disks, control 4 = larvae were sprayed with distilled water.

Figure 5.2 shows the survival of three bioassy experiments with 3rd instar larvae of *T. castaneum*. Firstly, using dsSK, dsSH alone. Secondly, using entomopathogenic fungi, *B.*

bassiana and *M. anisopliae* alone.Thirdly, using dsSK, dsSH combination with *B. bassiana* and *M. anisopliae* fungi . There is a clear decrease in larval survival when dsRNA for either *SK* or *SH* is used with *B. bassiana* rather than using just dsRNA or fungi; larval mortalities reached 100% at 3×10^2, 3×10^3 and 3×10^4 spores/ml for *B. bassiana* with 65 ng/mg for *SK* dsRNA by day 7 (Figure 5.2a). In contrast, *SH* dsRNA at 117ng/mg with the same fungal concentration gave larval mortalities of 70%, 74%, 86%, respectively after 10 days (Figure 5.2b). Meanwhile, *SK* and *SH* dsRNA only treatments recorded 52 % and 50 % mortality, respectively. Larval mortality of both dual treatments (fungus and *SK* dsRNA) and dsRNA treatment was significantly different relative to control 1 (larvae fed on flour disk), control 2 (larvae fed on RNAse free water flour disk) and control 3 (larvae fed on Kana dsRNA 100 ng/mg flour disk). No larval mortality occurred in control 1, with only 4 % for control 2 and 8 % for control 3 but there was no significant difference ($p<0.001$) in mortality among the three controls. The mortality of larvae treated with *B. bassiana* only after 10 days was 48%, 56% and 60% at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively relative to control 4 which recorded 17% mortality. The statistical analysis revealed that there were significant differences between combined treatments (fungus and *SK* dsRNA) and treatment only with fungus or dsRNA ($p<0.001$). However, in respect of the *SH* there was not all combined treatments were significantly different from the single treatments. Regression analysis was carried out on the mortality data for all the treatment groups, and the results indicated that the effect on larval mortality was synergistic in the combined treatment for both *SK* and *SH* ($p=0.0012$ and $p=0.000$, respectively).

5.4.2 Survivorship of 3rd instar larvae treated with dsRNA targeting *SK* or *SH* and *Metarhizium anisopliae*

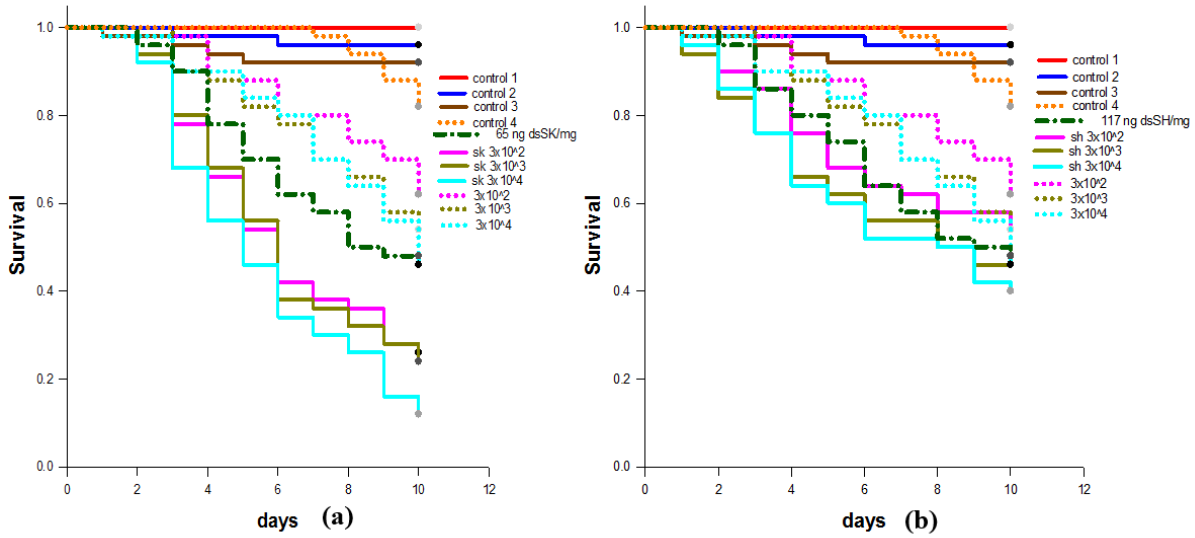

Figure 5.3: Survival curves of *T. castaneum* larvae fed on dsRNA targeted to *SK* (a) and *SH* (b) with *M. anisopliae* ($p<0.001$; Sigma Plot v.13, as shown by Kaplan-Meier survival analysis). Control 1= larvae fed on flour, control 2 = larvae fed on RNAase free water flour disks, control 3 = larvae fed on kanamycin resistance dsRNA flour disks, control 4=larvae were sprayed with distilled water.

The data clearly show that, at each time point, the level of mortality increased with increasing concentration of the entomopathogenic fungus *M. anisopliae*. The mortality of larvae treated with *SK* dsRNA at 65 ng/mg and *M. anisopliae* at 3×10^2, 3×10^3 and 3×10^4 spores/ml after 10 days reached 74%, 76% and 88%, respectively (Figure 5.3a); these values were 46%, 54% and 64% mortality respectively for SH dsRNA at 117 ng/mg with the fungus in the same concentration range (Figure 5.3b). Whereas larval mortality was 52% and 50% when fed with SK and SH dsRNA at concentrations of 65 ng/mg and 117 ng/mg, respectively. The combined treatment and dsRNA treatment relative to controls 1, 2 and 3. No mortality occurred in control 1, with only, 4 % for control 2 and 8 % for control 3, and the differences among control groups were found not to be significant ($p > 0.001$). The mortality of larvae treated with only *M. anisopliae* after 10 days was 38%, 46% and 52% at 3×10^2, 3×10^3 and 3×10^4 spores/ml respectively compared with control 4 had 17% larval mortality , whereas larval mortality was 52% and 50% when fed with SK and SH dsRNA at concentrations of 65 ng/mg and 117ng/mg, respectively. The statistical analysis revealed that there were significant

differences between the combined treatments and single treatments of fungi or dsRNA. However, with *SH*, there were no significant differences between all dual treatments and single treatments of either fungus or dsRNA. In addition, all treatments caused significant mortality ($p<0.001$) for the insect larvae compared with four control groups. Significant interactions were found between dsSK, and fungus treatment ($p=0.000$) and regression analysis showed that the effects were synergistic, whereas the effect of dsSH and fungus was additive ($P=0.42$).

5.4.3 Survivorship of adults treated with dsRNA targeting *SK* or *SH* and *Beauveria bassiana*

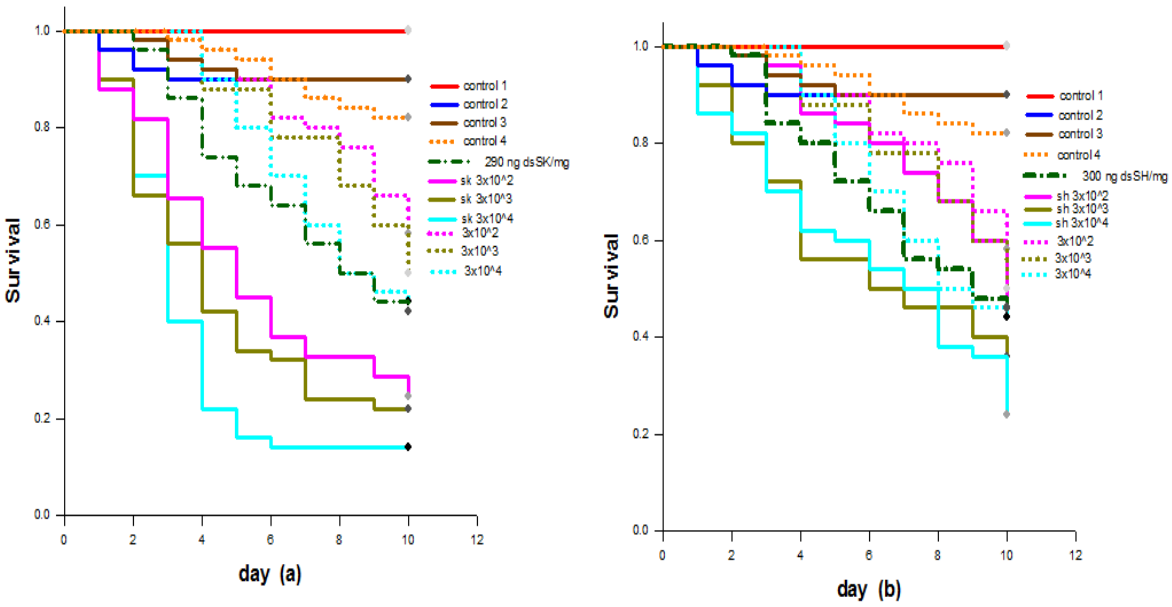

Figure 5.4: Survival curves of *T. castaneum* adult fed on dsRNA targeted to *SK* (a) and *SH* (b) with *B. bassiana* ($p<0.001$; Sigma Plot v.13, as shown by Kaplan-Meier survival analysis). Control 1= adults fed on flour, control 2 = adults fed on RNAase free water flour disks, control 3 =adults fed on kanamycin resistance dsRNA flour disks, control 4=adults were sprayed with distilled water.

The mortality of adults treated with SK and SH dsRNA with *B. bassiana* after 10 days was 76%, 78% and 86% at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively for the *SK* (Figure 5.4a). However, the mortality values 56%, 64% and 72% at the same concentrations, respectively for the *SH* (Figure 5.4b). Whereas SK and SH dsRNA treatments at concentrations of 290 ng/mg and 300 ng/mg, respectively showed 58 % and 54% mortality, respectively. Both treatments compared to 10 % for control 2 and control 3 and 0.0 % for

control 1. The fungal treatments mortality were 44%, 50% and 56% at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively relative to the control 4, which had 15% adult mortality, whereas for SK and SH dsRNA treatments at concentrations of 290 ng/mg and 300 ng/mg, respectively (these represent LC_{50} value, chapter 4) led to 58 % and 54% mortality, respectively. Statistical analysis revealed that there were significant differences between the dual treatment of SK dsRNA with fungus, and fungal treatments alone or dsRNA treatments. In contrast, there were no significant differences between all the dual treatments of SH dsRNA with fungus, and single treatments in the survival of adult. In addition, all treatment caused significant mortality ($p<0.001$) for adults compared the control groups. Results of the regression analysis of the *T. castaneum* adult mortality data indicated that the effect was synergistic for dsSK and fungus ($p=0.00003$). For dsSH and fungus, the effects were additive ($p=0.25$).

5.4.4 Survivorship of adults treated with dsRNA targeting *SK* or *SH* and *Metarhizium anisopliae*

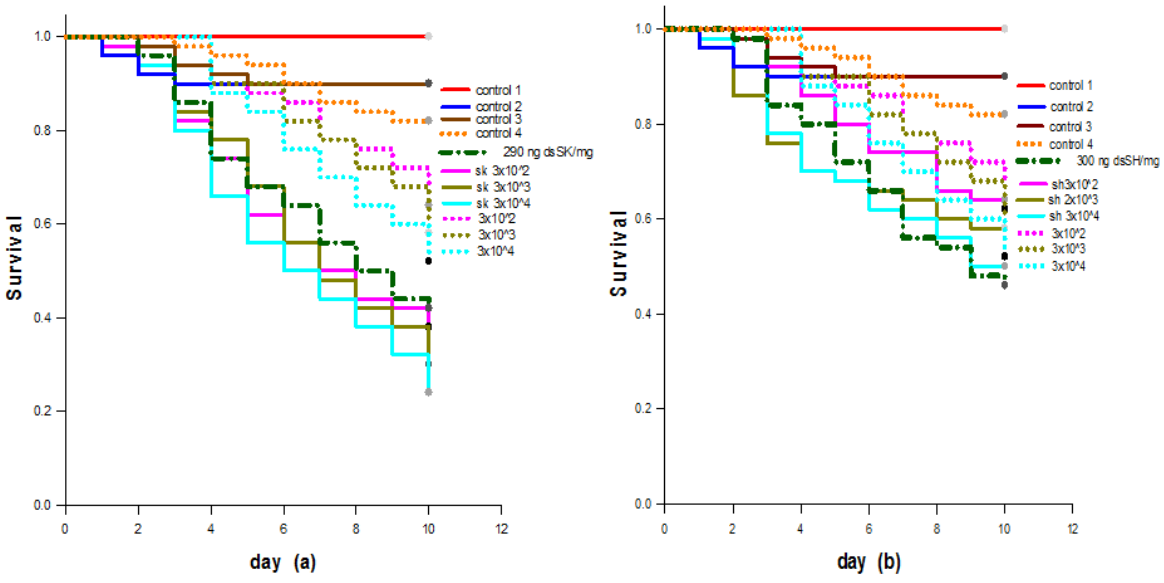

Figure 5.5: Survival curves of *T. castaneum* adult fed on dsRNA targeted to *SK* (a) and *SH* (b) with *M. anisopliae* ($p<0.001$; Sigma Plot v.13, as shown by Kaplan-Meier survival analysis). Control 1= adults fed on flour, control 2 = adults fed on RNAase free water flour disks, control 3 =adults fed on kanamycin resistance dsRNA flour disks, control 4=adults were sprayed with distilled water.

Mortality was measured after 10 days for both genes. Adult mortality was 62%, 70% and 76% for SK dsRNA with *M. anisopliae* at 3×10^2, 3×10^3 and 3×10^4 spores/ml respectively (Figure 5.5a), whereas it was 38%, 46% and 50% for SH dsRNA and the fungus at the same concentrations respectively (Figure 5.5b). These mortalities were significantly different relative to the control groups ($p<0.001$), where controls 2, 3 recorded 4% and 6% adult mortality, and no mortality occurred in control 1. The fungal treatments recorded 36%, 42% and 48% mortality at 3×10^2, 3×10^3, and 3×10^4 spores/ml respectively compared to control 4 which had 16% mortality. Statistical analysis shows that there were significant differences between the dual treatment of fungus and *SK* dsRNA compared to fungus alone ($p<0.001$). However, no significant differences were found between the dual treatment of fungus and *SH* dsRNA or single treatment. A synergistic interaction was found between dsSK and *M. anisopliae* ($p=0.0006$) whereas there was only an additive effect from the combined dsSH and *M. anisopliae* treatment ($p=0.6$).

5.5 Discussion

The results of this study indicate that the effectiveness of dsRNA targeting the potassium channel *SK* and *SH* in combination with *B. bassiana* and *M. anisopliae* isolates was more toxic towards *T. castaneum* third instar larva and adults than when either technology was used alone. Suppression up to 50% of *SK* or *SH* by RNAi led to the hypothesis that the down-regulation of these genes will increase the susceptibility of *T. castaneum* to *B. bassiana* and *M. anisopliae*. The dsSK and dsSH enhanced efficiency of the entomopathogenic fungi, *B. bassiana* and *M. anisopliae* led to up to 38% and 23% third instar larval mortality respectively, compared with fungal treatment at 10 days. The effects of most of the combinations were synergistic effects, indicating that combinational treatments caused greater mortality than a single treatment, dsRNA had either a synergistic or additive effect when combined with the fungal strains compared to when the dsRNA was applied alone, leading to improving efficacy against the storage pest *T. castaneum.* As far as we are aware, the current study is the first demonstration that such synergistic effects can occur when dsRNA is combined with entomopathogenic fungi.

Another finding of this study is that, while a combination of *SH* dsRNA with *B. bassiana* was effective for both the larva and adult stages, it was not as effective as *SK* dsRNA combined

with fungus. However, *M. anisopliae* with *SH* dsRNA did not have a significant effect on mortality compared with either single treatment on the adults. The results generally showed that dsRNA combined with *B. bassiana* isolates was more toxic to insects than *M. anisopliae* isolates with dsRNA. In addition, treatment with *B. bassiana* isolates alone caused higher mortality than treatment with *M. anisopliae* isolates, which is consistent with the findings of Mohammadbeigi and Port (2013) who demonstrated that *B. bassiana* and *M. anisopliae* fungal isolates are capable of causing the infection and mortality of *Uvarovistia zebra* either via contact or ingestion, with *B. bassiana* being more effective. Interestingly, no comparative toxicity studies with these two entomopathogenic fungi appear to have been carried out on *Tribolium* species. The data shown here indicate that third instar larvae were more susceptible than an adult to infection by both *B. bassiana* and *M. anisopliae*. It has previously been observed that the larvae of *Tribolium* species are more susceptible to entomopathogenic fungi than the adult life stage (Michalaki *et al.*, 2006).

In invertebrates, the nervous system regulates innate immune responses through the release of neurotransmitters, neuropeptides and neurohormones (Kawli *et al.*, 2010). Since *SK* and *SH* are expressed in the nervous system of *T. castaneum,* as mentioned before in chapter 2. It is anticipated that the knockdown of these genes will make this insect more susceptible to pathogens. Work presented in this chapter shows that combinations of RNAi and fungal treatment are synergistic or additive, depending on different concentrations. Previous studies have shown that the knockdown of ion channels in insects leads to mortality (Tempel Nakasu, 2014; El Halim *et al.*, 2016). Other studies have also demonstrated that the knockdown of specific genes in *T. castaneum* caused mortality, and the silencing of TcTre1-4 or TcTre2 (trehalase inhibitor gene), which plays a role in chitin synthase in the insect cuticle and midgut causing 40% larval mortality (Tang *et al.*, 2016). The silencing of TcDad1, which encodes an essential subunit of the OST complex catalyzing the attachment of the N-glycan precursor to a polypeptide, caused 96.4% larval mortality and inhibited larval growth (Walski *et al.*, 2016).

The second mode of insect mortality that has been demonstrated is via the use of pathogenic fungi (Maniania et al., 2008; Vega et al., 2008; Roberts and St. Leger, 2004). In the present study, *B. bassiana* and *M. anisopliae* were applied. When the spores of these fungi come into contact with the body of an insect host, the hyphae penetrate the cuticle by the production of

an extracellular chitinase, lipases and proteases that are thought to participate in the degradation of the insect cuticle (Joshi *et al.*, 1995; Kang *et al.*, 1999). The hyphal bodies grow in the haemocoel, leading to insect death within 3-4 days, and a possible explanation for this might be that of the depletion of nutrient resources. Another explanation is that the hyphae secrete toxic-like bassiacridin (Quesada-Moraga and Alain, 2004) and secondary metabolites are produced by this fungus, such as beauvericin, bassianolide, and beauverolides, and polyketide-derived pigments (Halo *et al.*, 2008; Vega *et al.*, 2008; Feng *et al.*, 2015). Subsequently, the fungus emerges from the insect and produces new spores. In the present study, both fungal isolates showed potential as control agents for this insect, with *B. bassiana* being more effective than *M. anisopliae*. This finding corroborates those of studies by Erler and Ates (2015), who reported that *B. bassiana* was slightly more effective than the *M. anisopliae* and caused 79.8% and 71.6% mortality in young and older larvae of *Polyphylla full* respectively. *M. anisopliae,* on the other hand, caused 74.1% and 67.6% for young and old larvae respectively.

The use of biologcal control agents such as entomopathogenic fungi, Pseudomonas fluorescens has been well documented (Inglis *et al.*, 2001). While the use of such fungi have been shown to be successful in niche environments such as glasshouses, their efficacy in the field is much less reliable and certainly not as effective as synthetic pesticides for the control of pests (Lovett and St. Leger, 2018). To date, numerous studies have been carried out to genetically engineer these insect pathogens to significantly improve their virulence and hence efficacy as insect pest control agents. For example, recent studies by Zhao *et al.* (2016) demonstrated that engineering fungi could not only increased the virulence and efficacy of these fungi but also fungal tolerance to biotic stresses. As an alternative to genetically engineering entomopathogenic fungi, we hypothesized that using these BCAs in conjunction with RNAi-based technology would enhance their efficacy.

Based on the findings of this study, we can conclude that using dsSK together with *B. bassiana* or *M. anisopliae* was more efficient than ds*SH* with either fungal strain to control *T. castaneum.* All effects were either additive or synergistic, and these effects were observed for both genes with both fungi. Thus, these results suggest that utilizing dsRNA in combination with entomopathogenic fungi has the potential to be used as a powerful tool, in the management of *T. castaneum* and could provide alternatives to chemical insecticides.

General discussion

6 General discussion

This study provides the first evidence to support the potential use of RNA interference (RNAi) to target potassium ion channels as an approach to control members of the Coleoptera, many species of which are major agricultural pests including *Tribolium castaneum (*Herbst.). To test the hypothesis that these potassium ion channels represent viable targets, the *TcSK* and *TcSH* genes were silenced using RNAi. These particular genes were selected because they participate in generating action potentials in nerve cells. This study demonstrated that the knockdown of these genes caused a significant reduction in the survival of the target insects.

Insect pests of stored grain damage the economy by infesting stored agricultural products. According to a recent estimate, the rice weevil *Sitophilus oryzae* L. and red flour beetle *T. castaneum* are responsible for a worldwide loss of 10-40% of stored grain annually. Chemical pesticides are still the major approach used to control insect pests. However, they are associated with significant hazards to the environment, human health and non-target insects (WHO, 2010). Gene silencing methods such as RNA interference, which involves the specific down-regulation of gene expression by double-stranded RNA (dsRNA), has been suggested as an alternative approach to decrease crop damage by insect pests. It is a powerful technique for carrying out functional genomic in insects, in addition to its potential ability for the targeted control of insect pests. As such it could offer an environmentally friendly bioinsecticides for the control of pest insects and the protection of beneficial insects, especially the honey bee (Xue *et al.*, 2012).

In Chapter 2, RNAi was induced following the administration of a range of concentrations of the dsRNA, delivered via injection or feeding to different developmental stages of *T. castaneum*. In all the bioassays performed, the percentage mortality, produced after 10 days feeding on dsRNA in the diet or 7 days post-injection with dsRNA, was significantly higher compared to control treatments. The analysis of mortality in the injected nuclease-free water control group confirmed that the injection technique was successfully performed, and that mortality was not due to injection *per se*. In addition, comparison against other controls, for example, those fed on the normal beetle feed, showed that environmental factors did not affect the treatments. A third control group was included whereby the larvae were treated with

the dsRNA targeted to the kanamycin gene, which is not present in *T. castaneum*, in order to assess whether the presence of any dsRNA affected mortality, rather than the dsRNA specifically targeting *SK* and *SH*.

The findings from RT-qPCR with cDNA from the insects treated with dsRNA targeting the *SK* and *SH* genes gave strong evidence that the knockdown of *SK* and *SH* was the cause of increased mortality compared with the control groups. Furthermore, a comparatively low concentration of dsSK, delivered via injection or feeding, caused 100% larval mortality, suggesting the potential of RNAi to control this pest species before it reaches the reproductive stage. A similar observation by Knorr *et al.* (2018) found that 28 of 50 dsRNA targets caused 100% mortality in *T. castaneum*. There are several possible explanations for the observed sensitivity of Coleoptera to RNAi, including the fact that the expression of genes coding for DNase is lower in this insect order when compared to that with other orders where the dsRNA is less stable within the insect gut (Baum *et al.*, 2007; Baum and Roberts, 2014). Another explanation for the sensitivity of *T. castaneum* to RNAi may be due to duplication of Argonaute2 in this insect species (Wang et al., 2013; Xiao et al., 2015). All these factors are likely to contribute to the successfull oral delivery of dsRNA in *T. castaneum*, as seen in the present study.

In this study, larval mortality rates were greater than pupae and adults. However, pupal and adult treatments could still be viable, as RNAi can still induce mortality in the first generation offspring from treated pupa and adults (Bucher *et al.*, 2002). In a further investigation in the present study (Chapter 2), levels of *SK* and *SH* expression level were significantly reduced in *T. castaneum* at different stages using both delivery methods. These results confirmed the potential of using RNAi as an alternative control method for *T. castaneum*.

The present study included the oral delivery of dsRNA to mediate gene silencing because it is thought that the successful applications of RNAi for crop protection will require a robust oral RNAi response to induce mortality in the target insect (Knorr *et al.*, 2018). Two potential issues relating to the oral delivery of dsRNAs to insects are the ability of the organism's cells to uptake dsRNA and whether or not the effects are continuous. However, the high efficacy of oral delivery found in this study supports the results of previous research targeting both vATPase and $TcNa_v$ genes in *T. castaneum* (Whyard *et al.*, 2009; El Halim *et al.*, 2016),

possibly because the knockdown of genes via RNAi occurs within 24 hours after feeding with dsRNA (Li *et al.*, 2018) .

In the light of the findings of this study, the use of potassium channels as targets for insecticides has the potential to selectively control insect pests. A few products for the control of coleopteran pests by RNAi are expected to be commercially available in the near future. However, several aspects regarding the practical application of dsRNA still have to be investigated, including its stability after delivery and the costs of large-scale production. Studies on dsRNA degradation by dsRNases have found that the rate of degradation of dsRNA is variable among insects in different orders as well as among different insect species within the same order (Singh *et al.*, 2017). Regular molecular biology kits are not affordable for the production of large amounts of dsRNA for field application. Although the costs of producing dsRNA are diminishing, for example from $12500 USD in 2008 to $60 USD today for 1 g of dsRNA, the amounts required per hectare are estimated to be 2–10 g. However, this amount may vary depending on the sensitivity of each species to RNAi, systemic RNAi and the efficiency of dsRNA delivery (Moises Zotti and Olivier Christiaens, 2018). The delivery method is also very important in bringing RNAi technology nearer to field application. For example, transgenic delivery has proved to be successful in the protection of crops from pest insects. The United States Environmental Protection Agency recently registered four products containing an RNAi-based Plant-Incorporated Protectant (PIP) called 'SmartStax PRO', a genetically modified seed of corn, that will help US farmers to control western corn rootworms (Head *et al.*, 2017).

These results suggest that the dsRNA of the *SK* and *SH* genes of *T. castaneum* can be an effective oral biopesticide for *T. castaneum*. Although this technique has been successfully used in *T. castaneum*, it is not known whether the application of this dsRNA would lead to unintended effects in non-target insects, and in particular beneficial species. Therefore, as discussed in Chapter 3, the effects of the dsRNA targeting *T. castaneum* on the non-target species, *A. mellifera* was studied. However, after ten days of continuous feeding on Tc*SK* and Tc*SH* dsRNA, bee survival rates were the same as compared to the control groups. Moreover, the results did not show significantly reduced transcript levels of the *SK* and *SH* genes in honey bees compared with control treatments. Further experiments confirmed that TcdsRNA did not influence the immune system of honeybees using DWV level as an indicator for a

comparison between treated and untreated bees. Potential impacts on the immune system are highly pertinent since effects on the nervous system may affect immunity.

The current study found that dsSK and dsSH of *T. castaneum* are stable molecules. The stability of TcdsSK and TcdsSH was examined by running 1 µg of the dsRNA on an agarose gel for 0-48h, and only slight degradation was found. Moreover, dsSH was degraded less than dsSK. This could be because the GC content is higher in dsSH compared to dsSK meaning that it contains more hydrogen bonds, being 57% and 53% respectively. It can thus be suggested that the nucleotide sequence plays a role in the stability of dsRNA.

What is surprising is that despite there being a 29 nucleotide sequence identity between dsRNA of *T. castaneum* (190bp) of *SH* gene used in the present study with the *SH* gene of *A. mellifera*, that there was was no impact on honeybee survival; furthermore, dsSH of *T. castaneum* did not induce the knockdown of the corresponding target gene in *A. mellifera*. Less surprising was the fact that dsSK of *T. castaneum* had no effect either on gene expression of the corresponding honeybee gene nor mortality since there was no sequence identity between the two insects regarding this particular gene. Thus, further work is required to determine the relative amounts of mismatch between the target and non-target organisms that cause a loss of effectiveness. However, with the lack of genomic data for most exposed non-target organisms, it becomes difficult to minimize effects on non-target organisms. As such, it is currently difficult to predict possible dangers of RNAi-based insecticides compared to the advantages of this technology for the protection of crops.

dsRNA can be synthesised *in vitro* by a reaction with RNA polymerases and DNA templates via the chemical polymerisation of nucleotides. However, these methods are expensive and, from a practical application perspective, the limiting factor is reasonable cost and the production of large quantities of dsRNA (Palli, 2014). This is particularly important as dsRNA is not amplified in the insect body and so a continuous supply of dsRNA is necessary. Therefore, as presented in Chapter 4, an alternative method for producing dsRNA, using transformed *E. coli* HT115 was investigated. This study showed that the transformed *E. coli* expressing dsSK and dsSH exhibited significant insecticidal activity against larval and adult instars. The treated larvae and adults had significantly reduced levels of *SK* and *SH* expression due to the specific RNAi. A comparison of the results between dsRNA prepared *in*

vitro compared to that produced *in vivo* shows that there was a difference in the reduction of gene expression caused, with the dsRNA produced via *in vitro* being more effective in the most of treatments. Although the study was conducted under the same conditions for each group, this might be due to differences in the purity of the dsRNA. The agarose gel image shows that there is a smear in the background of the dsRNA produced by the bacteria, while no smearing was noticed on *in vitro* dsRNA. However, despite these differences, both methods of dsRNA synthesis resulted in significant reductions in target gene expression, and interestingly, both methods caused high mortality in both injection and feeding bioassays. These results support those of other studies which suggest that both bacteria-expressed and *in vitro* synthesized dsRNA delivered by feeding successfully knocked down the expression of five target genes and caused significant mortality in *Leptinotarsa decemlineata* (Zhu *et al.*, 2011). The use of RNAi technology has been applied to protect *A. mellifera* from Israeli Acute Paralysis Virus, by the specifically targeting the virus (Hunter *et al.*, 2010). Zhu *et al.* (2011) suggested that the implementation of bacterially expressed dsRNA for control of agricultural insect pests may be accepted by the public because the dsRNA could be purified from the bacteria as a safe method of application of the technology.

The results presented in Chapters 2 and 4 offer clear evidence for the potential of using RNAi for controlling *T. castaneum*. However, RNAi has not yet been applied commercially in the field for pest control. Therefore, in order to reduce costs for practical purposes, and to increase the efficacy of BCAs, dsRNA could be combined with entomopathogenic fungi such as *B. bassiana* and *M. anisopliae*. Entomopathogenic fungi generally invade the host insects through the cuticle and penetrate the haemocoel. In the present study, oral application of dsSK or dsSH of *T. castaneum*at their LC_{50} values significantly increased the efficacy of these two entomopathogenic fungi. In the case of TcdsSK, the effects with both fungal species were synergistic. It can thus be suggested that the increased efficiency of dsRNA for both target genes resulted from the combination with *B. bassiana* and *M. anisopliae*, which means that reduced amounts of dsRNA will be necessary to control of *T. castaneum*, thereby reducing production cost.

6.1 Conclusions

The work carried out in this thesis has shown that selectively down-regulating the *SK* and *SH* genes of the potassium ion channels in *T. castaneum* causes significant insect mortality. This may reduce the number of potassium ion channels involved in excitable cells. Additionally, both *in vivo* and *in vitro* synthesized dsRNA were shown to be functionally active in various developmental stages of *T. castaneum.* Interestingly, the effect of the lowest concentration of dsRNA used in the bioassays conducted proved to be the most effective. The analysis of the results obtained in this research shows that TcdsSK and TcdsSH are relatively stable molecules. Importantly, TcdsRNA exhibited no detectable effects on the honeybee, *A. mellifera* survival. Similarly, it did not have any effects on the silencing of the *SH* and *SK* genes in the honeybee. Application of RNAi technology targeting the potassium ion channels in *T. castaneum* significantly increased the efficacy of the fungal biopesticides *B. bassiana* and *M. anisopliae.* Using a combined approach, i.e. RNAi together with the use of BCAs may provide a cheaper option that using RNAi alone for control of. *T. castaneum,* or indeed other insect pests

6.2 Future work

- Further testing using transgenic plants, which express the *T. castaneum* specific dsRNA, will be necessary to demonstrate applicability in the field. Some important aspects to take into consideration in the future will be the biosafety of this technology.

- It is not thought that this TcdsRNA will cause major hazards to honeybees. It would be desirable to test dsSK and dsSH for possibility effects against beneficial species from other insect orders, such as ladybirds from Coleoptera and predatory Heteroptera from Hemiptera.

- Using transgenic fungal entomopathogens has the potential to improve the effectiveness of applications of RNAi technology for pest control in the field (Hu and Wu, 2016).

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Appendix

Appendix A

Table 1. The sequence of *SK* and *SH* fragments

Application	Length of fragments	Sequence
SK	**181bp**	GTGGGACACGTGGACTACCCCCGGTACATGG AGGACAGGACTATAGGAAGCGGCGTCTACA AAGGGCCTTCGTCTGGAAGTCTCAAGCACAA ACCCAACGTGGGCTATCGGTTAGGCCGGCGC AAGGCGCTTTTTGAAAAGAGGAAAAGGATTA GTGATTACGTCTTGTTATGGGGATGT
SH	**150pb**	GCTGGACGTCTTCTCCGAGGAGATCAAGTTC TACGAGCTGGGGGAGCTGGCGATCAACAAGT TCCGCGAGGACGAGGGCTTCATCAAGGAGG AGGAGAAGCCGCTACCCTCGCACGAGTTCCA GCGCAACGTGTGGCTGCTGTTCGAGTC

Appendix B

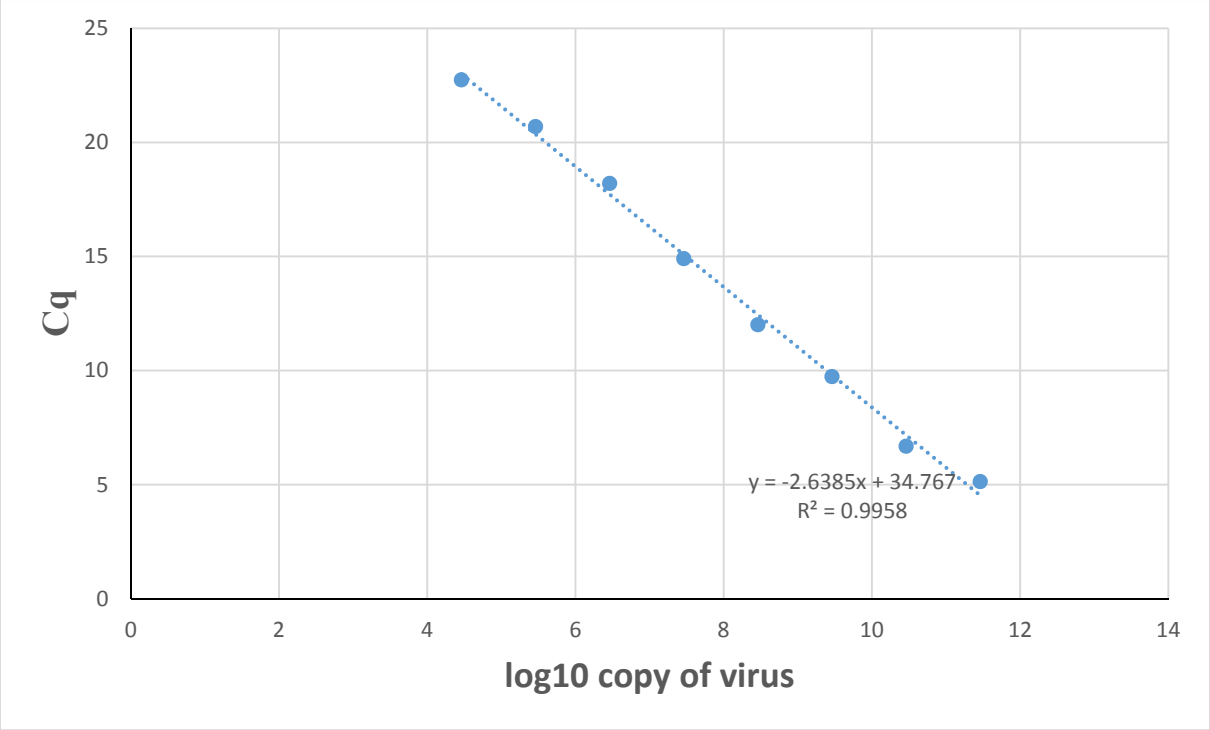

Figure 8.1: Stander curve for calculation number of deformed wing virus.