1 **Efficacy of double-stranded RNA for the large-scale control and prevention of sacbrood**

2 **virus in *Apis cerana* (Hymenoptera: Apidae) apiaries**

3

4 **Short title: Sacbrood virus (SBV) prevention in *Apis cerana* via SBV RNA interference**

5

6 Mi-Sun Yoo[1,¶], A-Tai Truong[1,2,¶], Hana Jeong[1], Do Hyun Hahn[1], Ju Seong Lee[1], Soon-Seek

7 Yoon[1], Yun Sang Cho[1,*]

8

9 [1] Parasitic and Honeybee Disease Laboratory, Bacterial and Parasitic Disease Division,

10 Department of Animal and Plant Health Research, Animal and Plant Quarantine Agency,

11 Gimcheon, Gyeongsangbuk-do, Republic of Korea.

12 [2] Faculty of Biotechnology, Thai Nguyen University of Sciences, Thai Nguyen, Vietnam

13

14 [*] Corresponding author. E-mail: choys@korea.kr

15 [¶]These authors contributed equally to this work.

16

17

18

19

1

Abstract

Sacbrood virus (SBV) infection in *Apis cerana* has caused tremendous damage in India, Thailand, Vietnam, and China since the 1970s. The disease caused by this virus results in colony collapse disorder in *A. cerana* and is also a devastating disease affecting *A. cerana* in South Korean apiaries. It has almost resulted in the elimination of the species. Therefore, control measures for this emerging threat are urgently needed. SBV RNA interference (RNAi) targeting *VP1* was prepared to test the safety and efficacy of protection and treatment in artificially infected larvae and in infected colonies in South Korean apiaries. The efficacy of *VP1* double-stranded RNA (dsRNA) was confirmed for the protection and treatment of infected larvae by increasing the survival rate in comparison with that in untreated larvae. Furthermore, an optimal application procedure was established for the large-scale RNAi treatment of SBV in apiaries. The protection of healthy colonies from SBV by RNAi was demonstrated in 100% of apiaries, and the treatment results showed that after five administrations, the SBV in infected colonies was mitigated to a safe level at which no symptoms of the disease were observed. Importantly, the low cost of dsRNA production in this study enables its application as a specific drug in large scale in South Korean apiculture.

Keywords: Sacbrood virus, *VP1*, RNA interference, Double-stranded RNA, Honeybee, *Apis cerana*

2

Introduction

Sacbrood virus (SBV) is a positive-sense and single-stranded RNA virus [1] that was first discovered in *Apis mellifera*, in which it causes little damage [2-4]. However, since propagated to *Apis cerana*, the virus has becoming a devastating pathogen and the main cause of the colony collapse of this honeybee species in Vietnam, China, and the Republic of Korea [5-9]. SBV has now become a prevalent pathogen of honeybees and adversely influences the beekeeping industry.

Although various methods for SBV treatment have been tested, an efficient method that is widely application has not been discovered due to the downsides of the methods used thus far. For example, the herbal extract thymol was used to inactivate SBV in *A. cerana*. However, the side effect of this chemical was a decrease in the survival of larvae reared *in vitro* [10]. The use of silver ion has been attempted in South Korea for the treatment and prevention of SBV in *A. cerana* [5]; however, it was not commercialized because of food safety concerns. In addition, lactic acid bacteria have been observed to immunity enhancing effects. However, their application triggers hygienic behavior among nurse bees to eliminate healthy larvae that receive the yogurt [11].

The capacity of insect cells to take up the environmental and artificial double-stranded RNA (dsRNA) via scavenger receptor-mediated endocytosis has been demonstrated [12-14]. The presence of dsRNA in the cytoplasm activates the small interfering RNA (siRNA) pathway

3

58 via which the RNase type III enzyme Dicer-2 cleaves the dsRNA into small fragments of 18–

59 24 nt [15]. The cleavage is then carried out by Argonaute-2, which contains an "RNA induced

60 silencing complex" [13, 16]. The species-specific targeting of RNA interference (RNAi) has

61 become a potential approach for the control of insect pests in crops [17], and the capacity of

62 RNAi to inhibit and control honeybee parasites has been reported, such as the small hive beetle,

63 *Aethina tumida* Murray [18] and mite, *Varroa destructor* [19]. RNAi is also a promising

64 therapy for the prevention of honeybee viral pathogens and could be developed as a drug for

65 field application [7, 20-23].

66 The administration of RNAi specific to SBV of *A. cerana* was demonstrated to be

67 effective at blocking viral propagation and increasing the survival rate of larvae *in vitro*;

68 consequently, it could be a promising method for controlling SBV in *A. cerana* [7, 24].

69 However, clinical studies on the capability of RNAi for wide application for SBV treatment

70 and prevention have not been reported. Accordingly, the present study was conducted to

71 analyze the efficacy of RNAi for the prevention and treatment of South Korean SBV in *A.*

72 *cerana*, and a procedure for apiary application was developed. Additionally, this commercially

73 cheap RNAi treatment was produced for wide application in South Korean apiculture.

74

Materials and methods

Isolation of sacbrood virus

77   Larval samples were pulverized using a mortar and pestle, and the presence of SBV

78   and the South Korean genotype of SBV was confirmed by PCR (Table 1). Then, the samples

79   were filtered using 0.45 μm and 0.2 μm syringe filters. After sonicating for 30 s (2 s on and 3

80   s off), the SBV samples were purified via sucrose density gradient centrifugation (10–50%

81   range) at 32,500 rpm for 4 h with an SW 41 Ti Swinging-Bucket Rotor (Beckman Coulter Inc.,

82   Brea, CA). The SBV band that corresponded to the 40% sucrose gradient was harvested and

83   then centrifuged at 25,000 rpm for 12 h; then, the SBV was eluted with ddH$_2$O. Photographs

84   were taken with a transmission electron microscope (H-7100FA, Hitachi Ltd., Tokyo, Japan)

85   to confirm the viral particles of 27.8 ± 0.4 nm in size, which were similar in shape to other

86   picornaviruses (S1 Fig).

87

88   **Table 1. Primers, probes, and PCR conditions for the identification of sacbrood virus**

89   **(SBV)**

90

Target virus	Primer sequences (5′–3′)	Target gene
SBV	F: AGAAGACATTTGATACAGTGGACTC	
	R: GGAATTCCAGATTCTTCGTCCAC	Polyprotein, 131 bp
	FAM-GATTTGTTTAATGGTTGGGTTTCTGGTA-BHQ-1	
Reverse transcription	45℃, 30 min; 95℃, 10 min	
PCR	40 cycles (95℃, 15 s; 62℃, 40 s)	

91

Extraction of viral RNA

5

93   The RNA of SBV was extracted from infected larvae using QIAamp Viral RNA Mini

94   Kit (QIAGEN, Hilden, Germany). Five larvae from each colony were collected in 1 mL

95   phosphate buffered saline (PBS) in a tissue grinding tube with 2.381 mm steel beads (SNC,

96   Hanam, South Korea), and 100 µL of the liquid was used for RNA extraction. For RNA

97   extraction from adult bees, two bees of each colony were collected and put in a tissue grinding

98   tube with 2.381 mm steel beads (SNC). After adding 1 mL of PBS solution the bees were

99   homogenized with a Precellys 24 Tissue Homogeniser (Bertin Instruments, Montigny-le-

100   Bretonneux, France), and 200 µL homogenate was used for RNA extraction. The procedure

101   was carried out according to the manufacturer's instructions. Finally, 50 µL RNA solution was

102   acquired from each sample.

103

Construction of SBV recombinant plasmid

105   The synthesis of SBV VP1 cDNA fragments was carried out using the SuperScript III

106   First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Waltham, MA). A

107   reaction mix (10 µL) consisting of 4 µL of RNA, 5 µL of reverse primer (10 pmol/µL; Table

108   2), and 1 µL of 10 mM dNTP mix was incubated at 65°C for 5 min and then placed on ice for

109   1 min. Then, a solution containing 2 µL of 10× RT buffer, 4 µL of 25 mM MgCl$_2$, 1 µL of

110   RNaseOUT, 1 µL of SuperScript III RT, and 2 µL of ddH$_2$O was added to obtain 20 µL of

111   reaction mixture. Reverse transcription was conducted by incubating the reaction mix at 50°C

6

112   for 50 min, then at 85°C for 5 min, and, finally, after chilling on ice, 1 μL RNase H was added

113   and the mix was incubated at 37 °C for 20 min. The cDNA was stored at −20°C until further

114   use.

115

116   **Table 2. Primers with T7 promoter used for manufacturing double-stranded RNA**

117   **(dsRNA)**

Primers	Sequences (5′→3′)	Length (bp)
T7VP1F	taatacgactcactatagggcgaAGATGTGAACGCTTACCCTGAT	596
T7VP1R	taatacgactcactatagggcgaCTCCTCGCATATACACCAAAACTT	

118

119   PCR was performed with the synthesized cDNA, the primer set T7VP1F/R for VP1

120   fragments (Table 2), and TOPsimple DryMIX-HOT PCR premix (Enzynomics, Daejeon,

121   South Korea). The 20 μL PCR mixture was composed of 1 μL of cDNA, 1 μL (10 pmol) of

122   each primer, and 17 μL of ddH$_2$O. PCR conditions were as follows: 94°C for 5 min, followed

123   by 40 cycles at 94°C for 15 s, 62°C for 15 s, 72°C for 15 s, and the final extension at 72°C for

124   10 min. After confirming the presence of the *VP1* band (596 bp; S2 Fig), the DNA fragment

125   was extracted and purified using the QIAquick Gel Extraction Kit (QIAGEN), and a

126   recombinant plasmid carrying the *VP1* fragment was constructed with TOPcloner TA Kit

127   (Enzynomics). The recombinant plasmid was sequenced by GenoTech, Daejeon, South Korea,

128   and compared to the SBV sequences reported in NCBI using the Basic Local Alignment Search

7

129 Tool (BLAST). There was 99% similarity with the South Korean SBV (NCBI accession no.:

130 HQ322114), and the recombinant plasmid was used as a DNA template for the PCR to produce

131 dsRNA.

132

Production of anti-SBV dsRNA

134 The 596 bp dsRNA corresponding to *VP1* of the South Korean SBV (NCBI accession

135 no.: HQ322114; 1596-2191) was synthesized by transcribing the T7 promoter, which was

136 located on the downstream sequence of primers (Table 2), *VP1* dsDNA produced by PCR

137 served as the template, and the *in vitro* Transcription T7 kit (for siRNA synthesis; No. 6140,

138 TaKaRa Bio Inc., Kusatsu, Japan) was used. The dsRNA synthesis was performed according

139 to the manufacturer's instructions. Briefly, the reaction mixture was prepared by mixing 10 µL

140 of NTP, 3 µL of 10× reaction buffer, 1 µg of linear DNA template, and 2 µL of T7 Enzyme

141 Mix (TaKaRa). This was adjusted to 30 µL with nuclease-free ddH$_2$O before incubating the

142 mixture at 42°C for 2 h. Subsequently, 30 µL of LiCl solution was added for 30 min at −20°C

143 to precipitate RNA, the mixture was ultracentrifuged at 16,000 × *g* for 15 min, and the

144 supernatant was discarded. Thereafter, 1 mL of 70% ethanol was added, and the mixture was

145 centrifuged again at 10,000 × *g* for 5 min. After discarding the supernatant, the RNA was

146 dissolved in 30 µL of elution buffer and then stored at −70°C until further use. A large quantity

147 of dsRNA was produced in collaboration with Genolution, Inc. (Seoul, South Korea) to extend

8

148   the clinical application to apiaries in the field.

149

Real-time reverse transcription PCR for evaluation of SBV inhibition by dsRNA

152   Real-time reverse transcription PCR (qRT-PCR) was used to quantitatively evaluate

153   the efficacy of dsRNA for inhibiting SBV in infected honeybees. A specific primer pair,

154   namely, KSBV-PCR-F (5′-GACCAAGAAGGGAATCAG-3′) and KSBV-PCR-123R (5′-

155   CATCTTCTTTAGCACCAGTATCCA-3′), was designed to amplify a 123 bp fragment of

156   South Korean SBV (NCBI accession no.: HQ322114; 2306-2428). The PCR was performed

157   with the One Step TB Green PrimeScript RT-PCR Kit II (TaKaRa), and SYBR Green was used

158   as a fluorescent dye for detection. The corresponding 20 μL reaction mix was composed of 2

159   μL of sample RNA, 1 μL (10 pmol) of each primer, 1 μL of PrimeScript One Step Enzyme

160   Mix (TaKaRa), 10 μL of 2× One Step RT-PCR buffer (TaKaRa), and 5 μL ddH$_2$O. The CFX96

161   Touch Real-time PCR Detection System (Bio-Rad, Hercules, CA) was used for qRT-PCR.

162   Reverse transcription was performed at 42°C for 30 min, followed by PCR at 95°C for 5 min,

163   then 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. To identify positive results,

164   the peak melting curves were analyzed. To estimate the number of DNA copies, standard

165   curves representing the relationship between the cycle threshold of amplification and the initial

166   number of DNA copies, namely, from 10^{10} to 10^1 (10-fold dilution), were established in

9

167   triplicated PCRs (S3 Fig).

168

Efficacy of dsRNA in vitro

170   The safety and efficacy of dsRNA were evaluated *in vitro* with artificially reared

171   larvae. Honeybee larvae were transferred into the wells of 24-well plates for cell culture, and

172   then incubated at 35°C and 80% humidity. An artificial nutrient solution composed of 6% D-

173   glucose, 6% D-fructose, 1% yeast extract, 33% Gibco Grace's insect medium (Thermo Fisher

174   Scientific), and 50% royal jelly was provided to each larva at a dose of 100 µL/day.

175   To assess the efficacy of dsRNA in the inhibition of SBV *in vitro*, we examined the

176   effects of treatment with dsRNA on SBV infection. Three-day-old larvae were artificially

177   infected with SBV. Field larvae that were confirmed to have been infected with SBV, based on

178   clinical signs and via RT-PCR (Table 1), were ground until they were liquefied, and then, they

179   were used to prepare feeding solution by adding other nutrients with ratios similar to those

180   described above. The feeding solution with 10^8 copies of SBV was supplied to each of the larva

181   being reared in one dose. The experiment was designed with four groups of larvae. Group 1

182   contained larvae in four 24-well plates ($n = 96$) that were orally administered with dsRNA 1

183   day after SBV infection. The dsRNA solution was prepared by mixing 1 µg of dsRNA with the

184   feeding solution, and it was supplied to each larva in one dose. The SBV-infected larvae in the

185   other four plates of Group 2 were not fed dsRNA and served as the control. In Group 3, the

10

186   larvae were fed only dsRNA in feeding solution (no SBV infection) to evaluate the safety of

187   administering dsRNA to larvae *in vitro*. In Group 4, only feeding solution (no dsRNA and no

188   SBV) was supplied. The number of living larvae was counted daily until the 8th day of the

189   inoculation period to calculate the survival rate of larvae.

190

Safety and efficacy under controlled conditions

192   Two hives of *A. cerana* were tested in different isolated rooms. The first hive was

193   sprayed with a feeding solution that was contaminated with SBV. The feeding solution was

194   prepared by mixing 990 mL of ddH$_2$O, 600 g of sucrose, 170 g of honey, and 6 mL of apple

195   vinegar. The SBV-contaminated solution was prepared by grinding 25 SBV-infected larvae in

196   5 mL of feeding solution. The number of RNA copies of SBV in the 5 mL spraying solution

197   was estimated by qRT-PCR to be approximately 3.58×10^{12} equivalent copies. The other hive

198   served as a control and was treated with the SBV-free feeding solution. Feeding solution was

199   sprayed to the bees every day, and room temperature was retained at 25℃ during the

200   experimental period. Larvae and adults were collected at 1, 5, 8, and 12 days after infection

201   (i.e. day 0), and the level of SBV infection was confirmed by Korean SBV (kSBV) real-time

202   PCR. Five larvae or five adult honeybees were randomly collected at each time for the

203   quantitative detection of SBV, and SBV DNA copy/bee was finally calculated. Adults in the

204   control group were collected at the same time, and their level of SBV infection was compared

11

205   with that of the honeybees in the SBV infection group.

206   The other hive of *A. cerana* was placed in another isolated room and was sprayed once

207   with 5 mL of a feeding solution that contained 10 mg *VP1* dsRNA. Three days after the

208   administration of dsRNA, larvae were artificially infected with SBV by spraying them with 5

209   mL of a feeding solution that contained the same quantity of SBV as described above. The

210   changes in the SBV infection level were measured over time (before infection, on the infection

211   date, and 2, 4, 7, and 10 days after infection) by SBV qRT-PCR.

212

Efficacy in the field

214   The prevention efficacy of dsRNA against SBV was first evaluated in 78 colonies

215   across 17 apiaries, of which 18 colonies were first treated with 10 mg of dsRNA/hive by

216   spraying or oral administration. Afterward, dsRNA application was extended to 60 other

217   colonies at 20 mg/hive via oral administration. Presence of SBV in the colonies was confirmed

218   by qRT-PCR, with 7.77×10^5–2.11×10^8 copies of SBV DNA per larva. However, no clinical

219   sign of SBV disease was observed in the colonies. The hives were administered with dsRNA

220   five times, with a 1-week interval. In addition, seven colonies from four apiaries were not

221   supplied with dsRNA, and these were used as the controls. The apiaries were located near the

222   disease-outbreak regions (approximately 6 km).

223   Finally, dsRNA was applied for the prevention and treatment of SBV in large-scale in

12

224   269 colonies belonging to 33 different apiaries in South Korea, with oral administration of 20

225   mg dsRNA/hive. In the colonies, the queens were not confined during the period of dsRNA

226   application.

227

228   **Data analysis**

229   Survival rates in the dsRNA treatment group and the control groups *in vitro* test were

230   analyzed using the Kaplan–Meier method [25, 26]. The comparison was also performed to

231   verify the efficacy of dsRNA in inhibiting SBV in infected colonies in the field by comparing

232   the SBV DNA copies before and after treatment. The Student's *t*-test was used to test for

233   significant difference in Microsoft Excel 2019 (Microsoft Corp., Redmond, WA). Probabilities

234   below 5% were considered to indicate significance.

235

236   **Results**

237   **Efficacy of dsRNA in vitro**

238   The efficacy of dsRNA for inhibiting SBV was demonstrated in the survival rate of

239   larvae reared *in vitro*. The survival of SBV infected larvae without dsRNA treatment rapidly

240   decreased from 97.5% at the 3rd day of inoculation to 24.0% at the 8th day of inoculation.

241   Meanwhile, the infected larvae that received the dsRNA treatment showed a higher rate of

242   survival ($p = 0.02$), namely, 97.5% 3 and 4 days after inoculation, before decreasing to 87.8%

13

243   on the 5th day and to 60.5% at the end of the inoculation period (Fig 1). In addition, oral

244   administration of dsRNA was safe for larvae. This was demonstrated by the survival rate of the

245   group that was fed only dsRNA (without SBV) not significantly differing from that of the

246   larvae that only received the feeding solution without SBV ($p = 0.13$; Fig 1).

247

248   **Fig 1. Treatment of sacbrood virus (SBV) using *VP1* double-stranded RNA (dsRNA) in**

249   **honeybee larvae reared *in vitro*.** Survival probability of larvae was calculated according to

250   the Kaplan-Meier method for groups that were artificially infected with SBV and treated with

251   dsRNA (SBV+dsRNA) and not treated with dsRNA (SBV). Two other groups of larvae not

252   infected with SBV were fed dsRNA mixed with feeding solution (dsRNA) and feeding solution

253   only (feeding solution).

254

255   ## Prevention of SBV disease in controlled conditions

256   After the artificial infection of *A. cerana* colonies with SBV, the number of SBV DNA

257   copies peaked (approximately 10^{10} copies/bee) 5 days after inoculation and remained at that

258   level until the 12th day after inoculation (Fig 2A). In the colonies that were treated with dsRNA

259   3 days prior to SBV infection, the development of SBV occurred and remained high

260   (approximately 10^{10} DNA copies/bee) from the 2nd to the 7th day after SBV infection. The

261   capability of dsRNA to inhibit SBV was demonstrated after 7 days of infection by the

14

262   considerable decrease in SBV to around 10^4 copies/adult bee and 10^2 DNA copies/larva by the

263   10th day following infection (Fig 2B). Afterward, the colony was placed in natural conditions

264   and observed for 6 months, with no SBV disease symptoms observed. In addition, the infected

265   colony with no dsRNA administration showed colony collapse after 2 months.

266

267   **Fig 2. Prevention of sacbrood virus (SBV) using *VP1* double-stranded RNA (dsRNA).** The

268   experiment was conducted in two different colonies; qRT-PCR was used to evaluate the

269   efficiency of dsRNA against SBV via the quantitative detection of SBV (i.e. DNA copies). (**A**)

270   Control experiment without dsRNA application was conducted in two colonies: one was

271   artificially infected with SBV, and the increase in SBV in adult honeybees (Adult bee – SBV)

272   and larvae (Larva – SBV) was evaluated, and the other colony was maintained in a healthy

273   condition without SBV infection (Adult bee – no SBV). (**B**) In another colony, dsRNA was

274   supplied to the hive by spraying 3 days before SBV infection, and the SBV in adult honeybees

275   and larvae was quantified 2, 4, 7, and 10 days after infection.

276

Field application of dsRNA for the prevention and treatment of SBV

279   The preventive administration of dsRNA resulted in the absence of clinical symptoms

280   of SBV infection in all 78 *A. cerana* colonies that received dsRNA via spraying or oral

15

281   administration. The colonies that received doses of 10 mg or 20 mg of dsRNA developed

282   resistance against SBV (Table 3). Meanwhile, clinical symptoms of SBV disease were

283   observed in 3 of the 7 control colonies (42%), which were not administered dsRNA (Fig 3).

284

285   **Table 3. Field application of double-stranded RNA (dsRNA) for the prevention of**

286   **sacbrood virus (SBV)**

Groups	Application route (conc.)	Apiary No.	Colony No.	No. of hives with clinical symptoms
dsRNA treatment	Spray (10 mg)	5	9	0
	Oral (10 mg)	5	9	0
	Oral (20 mg)	7	60	0
	Subtotal	17	78	0 (0%)
Negative control	No treatment	4	7	3 (42%)

287

288   **Fig 3. Colonies of *Apis cerana* after sacbrood virus (SBV) infection and double-stranded**

289   **RNA (dsRNA) treatment.** After receiving dsRNA five times (1-week intervals), the SBV-

290   infected colonies showed the healthy condition, and there were no visible symptoms of the

291   disease in the hives (**A**). Meanwhile, the colonies without dsRNA treatment showed clinical

292   signs of SBV disease (**B**).

293

294   The quantitative detection of SBV in 10 colonies belonging to three randomly selected

295   apiaries showed the significant difference of SBV DNA per honeybee before and after period

16

296   of dsRNA treatment ($p = 0.000042$), SBV relative DNA per bee ranged from $3.20 \times 10^2 \pm 1.92$

297     $\times 10^1$ to $7.79 \times 10^3 \pm 1.13 \times 10^1$ copies (Fig 4). However, no symptoms of SBV infection were

298   observed in all the 78 colonies (Fig 3).

299

300   **Fig 4. Quantitative detection of sacbrood virus (SBV) in double-stranded RNA (dsRNA)**

301   **treatment apiaries.** Ten colonies (from three apiaries, apiary C, D, and G) out of the 78 dsRNA

302   treatment colonies belonging to 17 apiaries were randomly selected for quantitative detection

303   of SBV before and after the 5th dsRNA administration. Significant differences in SBV DNA in

304   the colonies were observed ($n = 10$, $p = 0.000042$) before and after treatment. Meanwhile, the

305   control colonies ($n = 7$) showed no significant differences ($p = 0.077$).

306

307   As for large-scale dsRNA application, the colonies with positive SBV detections by

308   qRT-PCR but no clinical signs of disease were used as the prevention group, with application

309   intervals of 2 or 4 weeks (Table 4). All the colonies exhibited healthy conditions during the

310   period of inspection. However, in the group in which dsRNA was administered to colonies

311   with SBV disease for treatment and without confining the queens to stop spawning during the

312   treatment period, dsRNA was not effective or was only partially effective against SBV,

313   depending on the application interval. dsRNA was administered to these colonies every 4

314   weeks, 2 weeks, or 1 week, which can be maintained for 2 months, longer than 2 months, or 8

17

315   months before the colony collapse occurred (Table 4).

316

317   **Table 4. Extended field application of double-stranded RNA (dsRNA) for the prevention**

318   **and treatment of sacbrood virus (SBV)**

Group	Before administration	Apiaries (Hives)	Administration interval (weeks)	After administration	Results	Efficacy
Prevention	Healthy; no clinical symptoms	1 (33)	4	Healthy	Healthy & splitting	Effective
		16 (134)	2	Healthy	Healthy & splitting	Effective
Treatment	Clinical symptoms	4 (18)	4	Pulled out larvae	CCD* within 2 months	Not effective
		11 (78)	2	Pulled out larvae	Remaining colony after 2 months, but finally CCD	Not effective
		1 (6)	1	Less pulled out larvae	Keeping colony until 8 months	Partially effective
	Total	33 (269)				

319   *Colony collapse disorder (CCD)

320

Discussion

322   dsRNA for use against SBV was produced and tested at three levels: *in vitro* reared

323   larvae, under controlled conditions, and for large-scale field application. The capacity of

324   dsRNA to inhibit SBV was demonstrated as it was efficiently used as a gene therapy for the

325   prevention and treatment of SBV. In addition, dsRNA was demonstrated to be safe for

326   application in honeybees.

18

327   Viral diseases significantly increase the loss of honeybee colonies worldwide, with

328   more than 20 viruses that infect honeybees being identified [27]. However, to our knowledge,

329   there are no specific chemotherapies for the prevention and treatment of these viral diseases.

330   Traditional methods for the management of viral diseases are good practice for beekeeping and

331   help with the early diagnosis of diseases and selection of honeybee strains that are resistant to

332   viruses [28]. When RNAi was demonstrated to effectively inhibit Israel acute paralysis virus

333   (IAPV) and SBV [7, 23], a gene therapy relying on dsRNA for field treatment of IAPV was

334   developed in 2010 [22]. However, the high costs of the *in vitro* production of dsRNA leads to

335   difficulties in large-scale production. A method for the *in vivo* production of dsRNA against

336   SBV was introduced in 2016 [24]. Although the cost was lower compared with that of *in vitro*

337   production (224 USD per mg dsRNA), it was still highly expensive for field application, which

338   requires around 20 mg for one dose. This cost-related restriction was addressed in the present

339   study. The large-scale production of dsRNA in coordination with Genolution, Inc. (Seoul,

340   South Korea) decreased commercial costs to around 10 USD for 60 mg, which was enough for

341   three doses of field application.

342   Although SBV causes the death of infected larvae [2], the clinical signs in infected

343   adult honeybees are unclear [29, 30]. Therefore, during the field application of dsRNA for the

344   disease prevention in infected colonies, the queens were confined for the first 3 weeks so that

345   the number of larvae in the colony did not increase any further. This mitigated the replication

19

346   of SBV and would be helpful for dsRNA treatment in the infected colonies. After five

347   administrations of dsRNA, the presence of SBV decreased below the level required to generate

348   the disease in the infected colonies ($\leq 7.79 \times 10^3 \pm 1.13 \times 10^1$ copies/bee) [31]. However, these

349   results were obtained after 2 months of treatment, and further investigation to assess how long

350   the administration of dsRNA could maintain SBV at a safe level, as well as the time required

351   for SBV to reach the DNA replication level that causes the associated disease after terminating

352   the use of dsRNA, is required.

353   The dsRNA present in the cells of honeybee could be processed into small interfering

354   RNAs (siRNA). Such siRNA are then incorporated with the RNA induced silencing complex

355   (RISC) [32]. The siRNA in RISC becomes a guided strand via complementary binding to target

356   RNA and the RISC complex with Argonaute protein, a major component, causing the

357   degradation of targeted RNA [33, 34]. Therefore, supplying the anti-SBV dsRNA to the

358   honeybee prior to SBV exposure could provide protection from SBV infection. The

359   effectiveness of dsRNA in preventing SBV disease was demonstrated following the

360   administration of single dose of 10 mg 3 days before artificial infection with SBV, which

361   prevented disease outbreak. The detection of SBV indicated that the virus remained at a high

362   level 7 days following infection before decreasing considerably from days 7 to 10 and being

363   completely eliminated after 3 weeks (data not shown). Furthermore, the five dsRNA

364   administrations (20 mg/dose) at 1-, 2-, and 4-week intervals indicated 100% efficacy in

20

365 protecting colonies from SBV disease outbreaks. However, the present study did not assess

366 how long dsRNA can remain in honeybees to maintain protection following each

367 administration. Understanding how long the dsRNA could confer protection could facilitate its

368 large-scale application.

369

370 # Conclusions

371   The low production cost of dsRNA in the present study enabled the use of dsRNA as

372 a specific drug for the prevention and treatment of SBV for beekeeping. The application of

373 dsRNA was safe for honeybees, and the usefulness of dsRNA for preventing SBV outbreaks

374 as well as for the treatment of SBV infection was demonstrated. However, further investigation

375 is important to understand the duration of protection after each administration, the adequate

376 administration frequency, and the optimal season for application.

377

378 # Acknowledgments

381 # References

382   1. Ghosh RC, Ball BV, Willcocks MM, Carter MJ. The nucleotide sequence of sacbrood virus of the
383   honeybee: an insect picornavirus. J Gen Virol. 1999;80:1541–9. doi: 10.1099/0022-1317-80-6-1541.
384   2. Bailey L. Recent research on honey bee viruses. Bee World 1975;56:55–64. doi:
385   10.1080/0005772X.1975.11097544.

21

386   3. Bailey L, Fernando EFW. Effects of sacbrood virus on adult honey-bees. Ann Appl Biol. 1972;72:27–
387   35. doi: 10.1111/j.1744-7348.1972.tb01268.x.

388   4. Bailey L, Gibbs AJ, Woods RD. Sacbrood virus of the larval honey bee (*Apis mellifera* Linnaeus).
389   Virology. 1964;23:425–9. doi: 10.1016/0042-6822(64)90266-1.

390   5. Ahn AJ, Ahn KS, Suh GH, Noh JH, Kim YH, Yoo MS, et al. Efficacy of silver ions against sacbrood
391   virus infection in the Eastern honey bee *Apis cerana*. J Vet Sci 2015;16:289–95. doi:
392   10.4142/jvs.2015.16.3.289.

393   6. Choi YS, Lee MY, Hong IP, Kim NS, Kim HK, Lee KG, et al. Occurrence of sacbrood virus in Korean
394   Apiaries from *Apis cerana* (Hymenoptera: Apidae). Korean J Apic. 2010;25:187–91.

395   7. Liu X, Zhang Y, Yan X, Han R. Prevention of Chinese sacbrood virus infection in *Apis cerana* using
396   RNA interference. Curr Microbiol. 2010;61:422–8. doi: 10.1007/s00284-010-9633-2.

397   8. Ma M. New insights of sacbrood virus. Virol Sin. 2014;29:410–3. doi: 10.1007/s12250-014-3540-9.

398   9. Shan L, Liuhao W, Jun G, Yujie T, Yanping C, Jie W, et al. Chinese sacbrood virus infection in Asian
399   honey bees (*Apis cerana cerana*) and host immune responses to the virus infection. J Invertebr Pathol.
400   2017;150:63–9. doi: 10.1016/j.jip.2017.09.006.

401   10. Charpentier G, Vidau C, Ferdy JB, Tabart J, Vetillard A. Lethal and sub-lethal effects of thymol on
402   honeybee (*Apis mellifera*) larvae reared *in vitro*. Pest Manag Sci 2014;70:140–7. doi: 10.1002/ps.3539.

403   11. Vung NN, Kim I, Lee MY, Kim HK, Kim DW, Choi YS. Controlling sacbrood virus disease in *Apis*
404   *cerana* colonies with biological methods in Korea. Korean J Apic. 2018;33:283–95. doi:
405   10.17519/apiculture.2018.11.33.4.283.

406   12. Saleh MC, van Rij RP, Hekele A, Gillis A, Foley E, O'Farrell PH, et al. The endocytic pathway mediates
407   cell entry of dsRNA to induce RNAi silencing. Nat Cell Biol. 2006;8:793–802. doi: 10.1038/ncb1439.

408   13. Wynant N, Santos D, Broeck JV. Biological mechanisms determining the success of RNA interference
409   in insects. Int Rev Cell Mol Biol. 2014;312:139–67. doi: 10.1016/B978-0-12-800178-3.00005-1.

410   14. Wynant N, Santos D, Van Wielendaele P, Vanden Broeck J. Scavenger receptor-mediated endocytosis
411   facilitates RNA interference in the desert locust, *Schistocerca gregaria*. Insect Mol Biol. 2014;23:320–
412   9. doi: 10.1111/imb.12083.

413   15. Siomi H, Siomi M. On the road to reading the RNA-interference code. Nature. 2009;457:396–404. doi:
414   10.1038/nature07754.

415   16. Brutscher LM, Flenniken ML. RNAi and antiviral defense in the honey bee. J Immunol Res
416   2015;2015:941897. doi: 10.1155/2015/941897.

417   17. Yan S, Ren B, Zeng B, Shen J. Improving RNAi efficiency for pest control in crop species.
418   BioTechniques. 2020;68:283–90. doi: 10.2144/btn-2019-0171.

419   18. Powell ME, Bradish HM, Gatehouse JA, Fitches EC. Systemic RNAi in the small hive beetle *Aethina*
420   *tumida* Murray (Coleoptera: Nitidulidae), a serious pest of the European honey bee *Apis mellifera*. Pest
421   Manag Sci. 2017;73:53–63. doi: 10.1002/ps.4365.

422   19. Huang ZY, Bian G, Xi Z, Xie X. Genes important for survival or reproduction in *Varroa destructor*
423   identified by RNAi. Insect Sci 2019;26:68–75. doi: 10.1111/1744-7917.12513.

22

424   20. Chen YE, Evans JD. RNAi in treating honey bee diseases. Bee Culture 2012;140:27–9.

425   21. Desai SD, Eu YJ, Whyard S, Currie RW. Reduction in deformed wing virus infection in larval and adult
426   honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. Insect Mol Biol 2012;21:446–55.
427   doi: 10.1111/j.1365-2583.2012.01150.x.

428   22. Hunter W, Ellis J, van Engelsdorp D, Hayes J, Westervelt D, Glick E, et al. Large-scale field application
429   of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*,
430   Hymenoptera: Apidae). PLoS Pathog 2010;6:e1001160. doi: 10.1371/journal.ppat.1001160.

431   23. Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, et al. IAPV, a bee-affecting virus associated with
432   colony collapse disorder can be silenced by dsRNA ingestion. Insect Mol Biol. 2009;18:55–60. doi:
433   10.1111/j.1365-2583.2009.00847.x.

434   24. Zhang J, Zhang Y, Han R. The high-throughput production of dsRNA against sacbrood virus for use in
435   the honey bee *Apis cerana* (Hymenoptera: Apidae). Virus Genes. 2016;52:698–705. doi:
436   10.1007/s11262-016-1346-6.

437   25. Bland JM, Altman DG. Survival probabilities (the Kaplan-Meier method). BMJ. 1998;317(7172):1572.
438   doi: 10.1136/bmj.317.7172.1572.

439   26. Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations, Journal of the American
440   Statistical Association. 1958;53(282):457-81. doi: 10.1080/01621459.1958.10501452.

441   27. Ullah A, Gajger IT, Majoros A, Dar SA, Khan S, Kalimullah, et al. Viral impacts on honey bee
442   populations: A review. Saudi J Biol Sci 2021;28:523–30. doi: 10.1016%2Fj.sjbs.2020.10.037.

443   28. Chen Y. Viruses and Viral Diseases of the Honey Bee, *Apis mellifera*. In: Liu T, Kang L, editors. Recent
444   Advances in Entomological Research. Berlin, Heidelberg: Springer; 2011. p. 105–20.

445   29. Anderson DL, Gibbs AJ. Transpuparial transmission of Kashmir bee virus and sacbrood virus in the
446   honey bee (*Apis mellifera*). Ann Appl Biol. 1989;114:1–7. doi: 10.1111/j.1744-7348.1989.tb06781.x.

447   30. Bailey L. The multiplication and spread of sacbrood virus of bees. Ann Appl Biol. 1969;63:483–91.
448   doi: 10.1111/j.1744-7348.1969.tb02844.x.

449   31. Gauthier L, Tentcheva D, Tournaire M, Dainat B, Cousserans F, Colin ME, et al. Viral load estimation
450   in asymptomatic honey bee colonies using the quantitative RT-PCR technique. Apidologie.
451   2007;38:426–35. doi: 10.1051/apido:2007026.

452   32. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs
453   mediate RNA interference in cultured mammalian cells. Nature. 2001;411(6836):494-8. doi:
454   10.1038/35078107.

455   33. Gu L, Knipple DC. Recent advances in RNA interference research in insects: Implications for future
456   insect pest management strategies. Crop Prot. 2013;45:36-40. doi: 10.1016/j.cropro.2012.10.004.

457   34. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic
458   and biochemical analyses of RNAi. Science. 2001;293(5532):1146-50. doi: 10.1126/science.1064023.
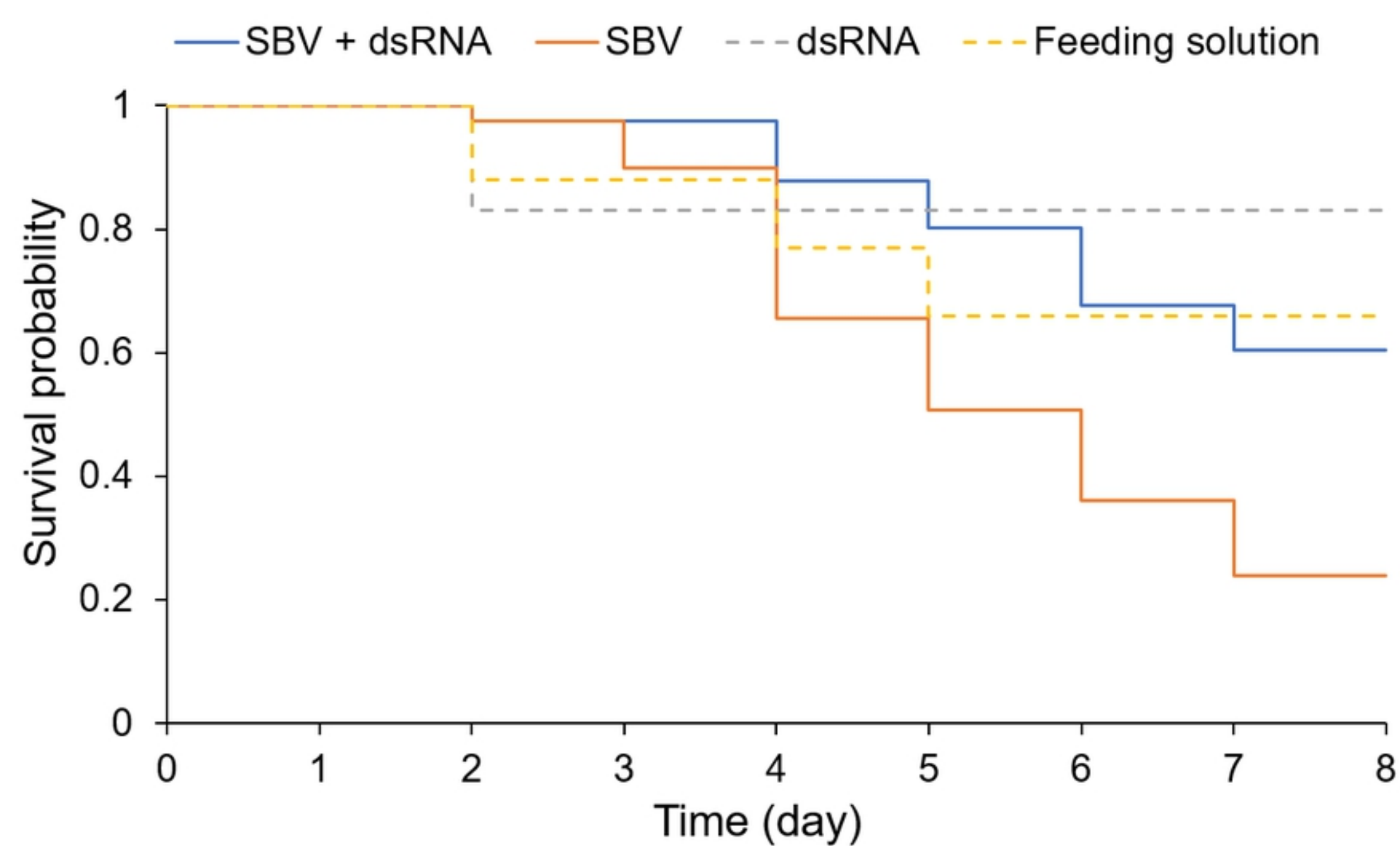
459

Supporting information

S1 Fig. Detection and isolation of sacbrood virus (SBV) from infected honeybee larvae. (A) SBV in infected *Apis cerana* larvae was detected with qRT-PCR and confirmed by electrophoresis, with an expected band of 131 bp in length. SBV+ and SBV- are samples with positive and negative detection, respectively. "+" and "-" are the positive and negative controls with SBV recombinant DNA and without a DNA template, respectively. **(B)** Band of purified SBV from infected larvae in 40% sucrose gradient density (indicated by the arrow). **(C)** Viral particles of SBV, with a size of 27.8 ± 0.4 nm and a shape similar to that of other picornaviruses, observed and measured under transmission electron microscopy.

S2 Fig. Double-stranded RNA (dsRNA) of sacbrood virus VP1. The dsRNA (185 ng) from one of the tubes containing dsRNA (685 mg/tube) was loaded into 1% agarose gel for electrophoresis. The 596 bp band corresponds to dsRNA. "M" is 1 kb DNA marker.
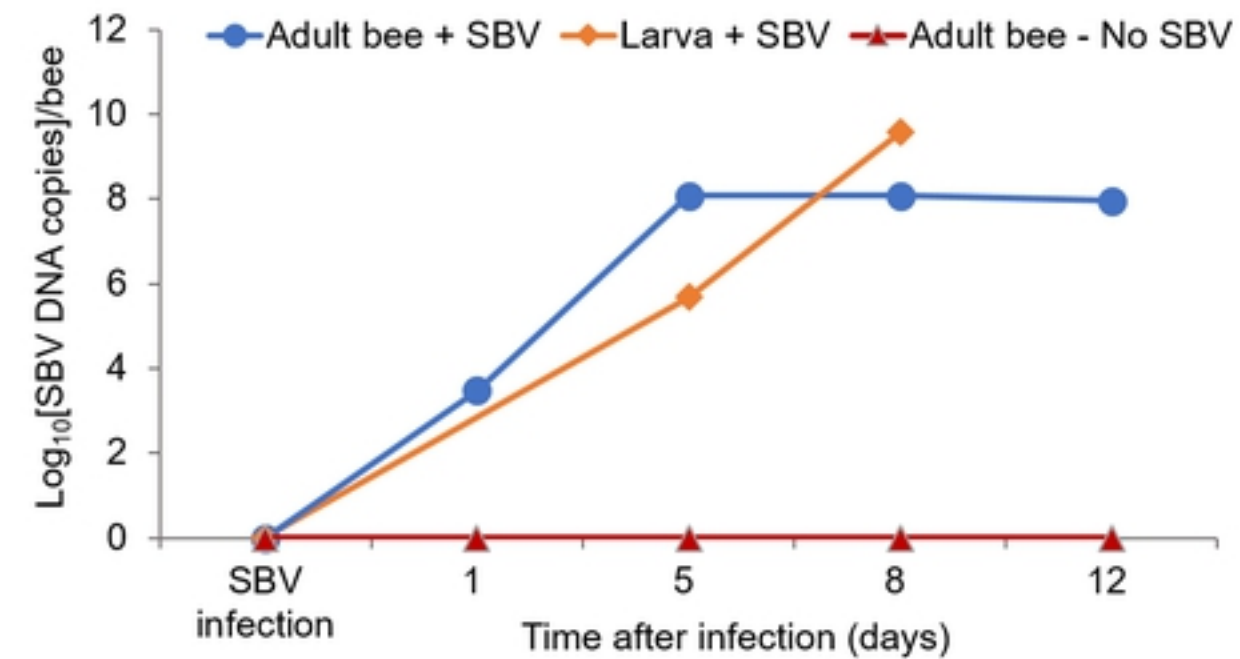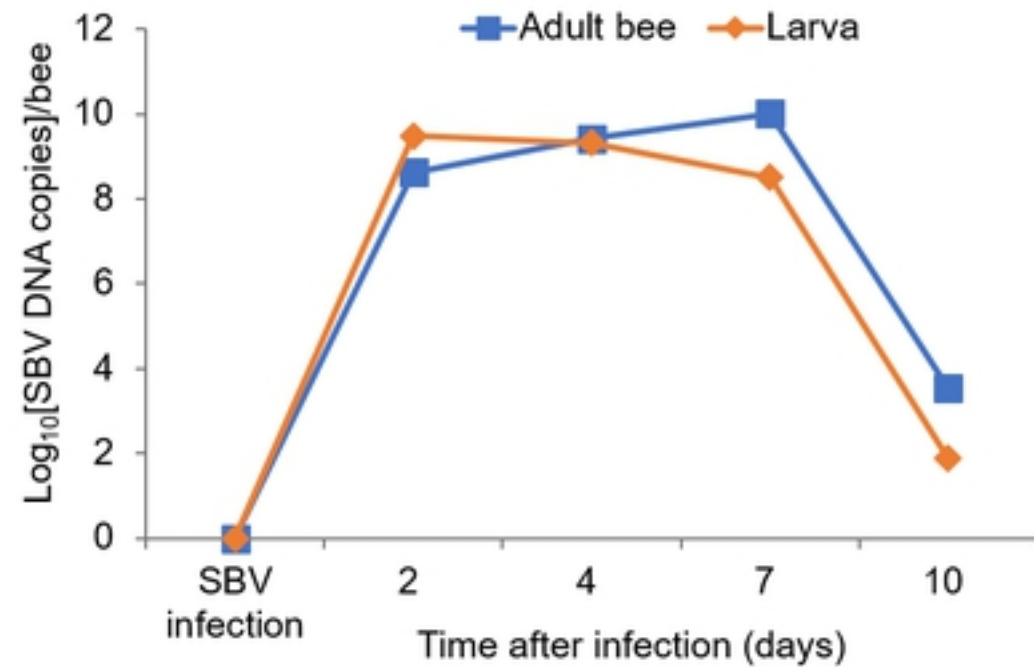
S3 Fig. Standard curves of sacbrood virus (SBV) DNA amplification. (A) Ten-fold serial dilution of SBV recombinant DNA, namely, from 10^{10} to 10^1 copies, was used for triplicate PCRs. **(B)** Correlation between the initial number of DNA copies ($\log_{10}$-transformed values) and the cycle threshold of amplification.
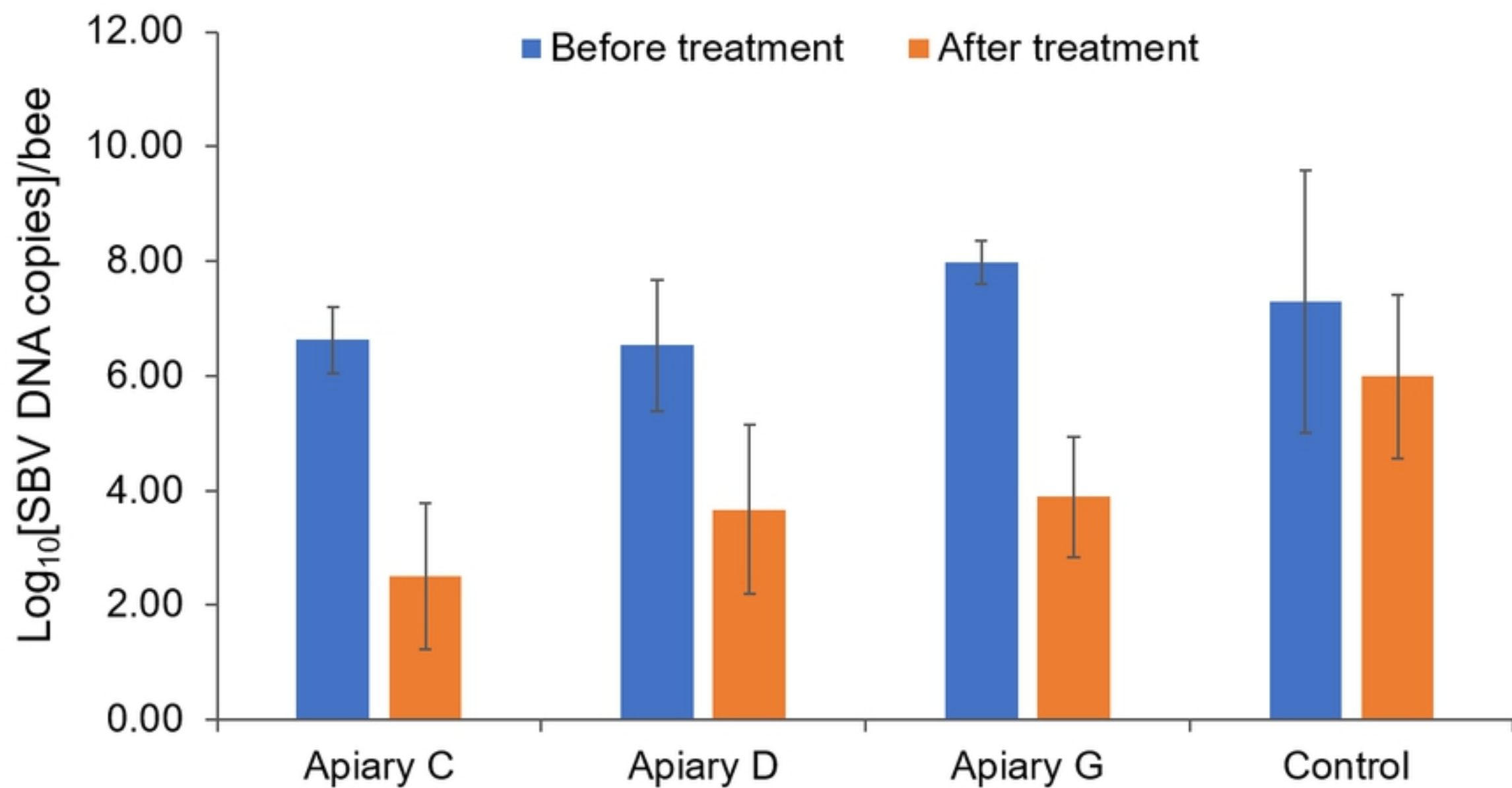
24

479

25

Figure

A

Log$_{10}$[SBV DNA copies]/bee vs. Time after infection (days)

Legend: Adult bee + SBV, Larva + SBV, Adult bee - No SBV

X-axis: SBV infection, 1, 5, 8, 12

B

Log$_{10}$[SBV DNA copies]/bee vs. Time after infection (days)

Legend: Adult bee, Larva

X-axis: SBV infection, 2, 4, 7, 10

Figure

Figure

Figure