Enhanced resistance against *Vibrio harveyi* infection by carvacrol and its association with the induction of heat shock protein 72 in gnotobiotic *Artemia franciscana*

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Received: 14 August 2016 / Revised: 5 February 2017 / Accepted: 7 February 2017 / Published online: 16 March 2017
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Abstract Induction of HSP72 is a natural response of stressed organisms that protects against many insults including bacterial diseases in farm (aquatic) animals. It would therefore be of great health benefit to search for natural compounds that are clinically safe yet able to induce HSP72 in animals. The phenolic compound carvacrol, an approved food component, had been shown in in vitro study to act as a co-inducer of HSP72, enhancing HSP72 production only in combination with a bona fide stress compared to the compound alone. However, in vitro model systems do not completely represent an in vivo physiology. Here, using the well-established gnotobiotic *Artemia* model system, we determined whether carvacrol could induce HSP72 in vivo, whether this putative effect could generate resistance in *Artemia* against biotic/abiotic stress and also unraveled the mechanism behind the possible HSP72-inducing effect of carvacrol. The gnotobiotic system is crucial for such studies because it avoids the interference of any extraneous factors on host-compound interaction. Here, carvacrol was shown to be a potent HSP72

inducer. Induction of HSP72 was associated with the generation of resistance in *Artemia* larvae against subsequent lethal heat stress or pathogenic *Vibrio harveyi*. Our results also provided new insight on the mode of HSP72 inducing action of carvacrol, in which the initial generation of reactive molecule H₂O₂ by the compound plays a key role. Overall results add new information about the bioactivity of carvacrol and advance our knowledge of this compound as potential prophylactic agent for controlling *Vibrio* infection in aquaculture animals.

Keywords Gnotobiotic *Artemia* · Carvacrol · Heat shock protein · *Vibrio harveyi* · Stress

Introduction

The 70-kDa heat shock proteins (HSP70s) are a group of highly conserved proteins of which expression is constitutive or inducible under different conditions. Functionally, HSP70s are molecular chaperones that play key roles in many vital cellular functions under both normal and stressful conditions, and those include assisting in the folding of nascent proteins, translocation of these proteins between cell organelles, assembly and disassembly of multi-subunit complexes, refolding or degradation of denatured proteins due to stresses, dissolution of pathological protein aggregates, and other processes enhancing the survival of normal and diseased cells (see review Roberts et al. 2010). Additionally, the stress-inducible HSP72 is also implicated in the generation of protective immune responses against many diseases, as demonstrated in a wide variety of experimental models in vitro and in vivo (Chen and Cao 2010; Johnson and Fleshner 2006; Tsan and Gao 2009). Because of its multifaceted beneficial roles, the inducible form of HSP70 has gained considerable attention as a potential agent for the

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prevention and treatment of diseases in human as well as in farmed terrestrial and aquatic animals (Nagai et al. 2010; Roberts et al. 2010; Westerheide and Morimoto 2005).

So far, several strategies have been employed for enhancing the availability of HSP72 within aquaculture animals, and these include exposure of the animals to nonlethal thermal shock, preconditioning stress, and transfection of HSP72 genes (Roberts et al. 2010). However, the main constraints in the application of these techniques in aquatic farming system are the high application cost, nonuser friendly, and risk associated with increased temperature on the animals. Because of these issues, there is currently a strong impetus in identifying natural compounds that possess the property of inducing HSP70 within animals in a noninvasive manner and that are easy for application in farm animals (Roberts et al. 2010).

The phenolic compound carvacrol is a major component of essential oils obtained from the herbs oregano and thyme (Skoula et al. 1999; Fig. 1). It is recognized as a generally recognized as safe (GRAS) compound (FDA 2011) for use as feed additive and food preservative in the food industry (Shoji and Nakashims 2004). So far, carvacrol has been studied mainly for its antibacterial effects (Burt 2004), and from that perspective, it has been described to increase the expression of HSP60 but not HSP70 in prokaryotes (Burt et al. 2007). However, in an in vitro study in eukaryotic (mammalian) cells, carvacrol was shown to co-induce cellular HSP70 expression (Wieten et al. 2010). In contrast to an HSP inducer, which upregulates HSP expression by itself, a co-inducer increases HSP levels only in combination with a bona fide stress signal (Ohtsuka et al. 2005). It is important to mention that in vitro model systems (e.g., cell culture) do not completely represent in vivo physiology. The results obtained from in vitro studies therefore may not provide sufficient or realistic information to precisely understand the biological property (e.g., HSP72 induction) of the candidate compound (Astashkina et al. 2012). The fact that carvacrol is a GRAS compound, determining the HSP72-inducing activity of this compound under in vivo condition, could be of great relevance for applying this compound as anti-infective agent in farmed aquatic animals.

A major handicap in studying the HSP-inducing activity of a compound in an in vivo system is the difficulty to eliminate (or distinguish the effect of) the microbial communities that is naturally present in the system (Baruah et al. 2014, 2015a). In fact, under germ-associated experimental conditions, the

microbial communities might metabolize the compound of interest, thereby making it difficult to better comprehend the activity of the compound. Additionally, the standing microbial communities are also a good source of the endotoxin lipopolysaccharide (LPS), which has been shown to induce HSPs in different animal model systems (Chiu et al. 2007; Kojima et al. 2004). This indicates that under germ-associated conditions, the physiological responses of the host (in terms of HSP production) due to exposure to the tested compound could simply reflect the influence of the various microbial communities (LPS or other cellular component) rather than a genuine biological effect of the compound. Hence, the selection of an appropriate animal model system that allows to better understand the HSP72-inducing activity of a compound is therefore of high importance.

The brine shrimp *Artemia franciscana*, an aquatic invertebrate that can be reared under gnotobiotic conditions (allowing full control over the host-associated microbial communities, Marques et al. 2006), represents an exceptional experimental system for carrying out such studies as it allows to distinguish direct effects on the host [by (pre) exposing axenic *Artemia* larvae to the compound for a certain duration] from indirect effects. It also eliminates any possible microbial interference during the exposure period and hence facilitates the interpretation of the results in terms of a cause-effect relationship (Baruah et al. 2014). In this study, using the highly controlled gnotobiotic *Artemia* model system, we aimed to investigate whether carvacrol could manipulate the production of HSP72 in vivo and whether this putative effect could contribute to the induction of resistance in *Artemia* against pathogenic *Vibrio harveyi*, an opportunistic bacterium that causes significant mortalities in the farmed aquatic animals, including *Artemia* (Austin and Zhang 2006). Furthermore, we also unraveled the mechanism behind the possible HSP72-inducing effect of carvacrol.

Materials and methods

Bacterial strain and growth conditions

Vibrio harveyi BB120, stored in 4.3 M glycerol at -80°C , was grown at 28°C for 24 h on Marine Agar (Difco Laboratories, Detroit, MI, USA) and then to log phase in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) by incubation at 28°C under constant agitation. Bacterial cell numbers were determined spectrophotometrically at 550 nm as previously described (Baruah et al. 2009).

Reagents

Carvacrol purchased from Sigma-Aldrich (98% Diegem, Belgium; Fig. 1) was used for the experiment. An amount of

Fig. 1 Chemical structure of carvacrol

20 mg (20.5 µl) of carvacrol was dissolved in 9.98 ml of sterile distilled water using a vortex mixer to obtain a volume of 10 ml with concentration of 2 g/l (13.3 mM). Superoxide dismutase (SOD; 2000–6000 units/mg protein) and catalase (2000–5000 units/mg protein) were obtained from Sigma. Catalase was dissolved in sterile distilled water at 0.5 g/l as a stock solution. All the stock solutions were prepared fresh for each experiment.

Hatching of germ-free *Artemia* larvae

All experiments were performed with high-quality hatching cysts of *Artemia franciscana* (EG® Type, batch 21,452, INVE Aquaculture, Dendermonde, Belgium). Approximately 1.5 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21,452, INVE Aquaculture, Dendermonde, Belgium), were hydrated in 89 ml of distilled water for 1 h. Sterile cysts and larvae were obtained via decapsulation using 3.3 ml NaOH (8.0 M) and 50 ml NaOCl (6.7 M). During the reaction, 0.2-µm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all tools were autoclaved at 121°C for 20 min. The decapsulation was stopped after about 2 min by adding 50 ml Na₂S₂O₃ at 10 g/l. The aeration was then terminated and the decapsulated cysts were washed with sterile artificial seawater containing 35 g/l of Instant Ocean® synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were suspended in 1-l glass bottles containing sterile seawater and further incubated for 28 h with constant illumination of approximately 27 µE/m²/s. After 28 h of incubation, the sterility of the hatched *Artemia* larvae was verified by spread plating (100 µl) as well as by adding (500 µl) hatching water on Marine Agar and Marine Broth, respectively, followed by incubating at 28°C for 5 days (Baruah et al. 2009). All these manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and larvae. Experiments started with nonsterile larvae were discarded.

In vivo pre-pretreatment of *Artemia*

In total, five separate tests were carried out. In the first and second tests, the putative protective effect of carvacrol was determined. To this end, a dose-response relationship of carvacrol was determined under two different challenge conditions as described previously (Baruah et al. 2012, 2015b). Briefly, hatched *Artemia* larvae at developmental stage II were collected, counted volumetrically by first counting a subsample of exactly 1 ml and multiplying that number by the total measured volume (ml) of hatched larvae. Thereafter, the larvae were transferred to 250-ml sterile glass bottles containing sterile seawater. The larvae were pretreated with increasing concentrations of carvacrol (16.6, 33.2, 66.4, 99.6, 132.8, 166.4 µM) for 2 h at 28°C. The larvae were rinsed repeatedly

with sterile artificial seawater to wash away the compound and then allowed to recover for 2 h at 28°C. Following recovery period, groups of 20 larvae from the 250-ml glass bottles were transferred to sterile 40-ml glass tubes that contained 10 ml of sterile artificial seawater (35 g/l salinity). Subsequently, it was verified whether such pretreatment can protect the larvae against subsequent challenge with pathogenic *V. harveyi* at 10⁷ cells/ml (Baruah et al. 2010) or against a lethal heat stress at 41°C for 20 min (Baruah et al. 2012). The survival of *Artemia* larvae was scored manually at an indicated period after challenge as described previously (Baruah et al. 2015b). In both the tests, non-pretreated larvae that were not challenged with *Vibrio*/lethal heat stress or challenged were maintained as controls. Five replicates were maintained for each treatment and control.

In the subsequent test, the cytotoxic effect of carvacrol was determined in the axenic *Artemia* larvae as previously described (Baruah et al. 2015b). The axenic larvae were pretreated with increasing concentrations of carvacrol (16.6, 33.2, 66.4, 99.6, 132.8, 166.4 µM) as described above. They were rinsed repeatedly with sterile artificial seawater to wash away the compound and then allowed to recover for 2 h at 28°C. *Artemia* larvae that did not receive carvacrol pretreatment served as control. Five replicates were maintained for each treatment and control. Toxicity bioassay was performed in sterile 40-ml glass tubes with 20 larvae in 10 ml of sterile artificial seawater per tube. The toxicity of the compound was determined after 48 h of recovery period by scoring the number of survivors as previously described (Baruah et al. 2015b).

In the third and fourth tests, the mode of action of carvacrol was determined. The germ-free larvae were pretreated with an optimized dose of carvacrol (dose which gave maximum protection to challenged larvae in the dose-response assay), a mixture of antioxidant enzymes catalase (10 mg/l) and SOD (75 units), or a combination of carvacrol and antioxidant enzymes mixture in a similar fashion as described above. The *Artemia* larvae in the control group did not undergo any pretreatment. Groups of 20 larvae were counted, distributed into sterile 40-ml glass tubes, and then challenged with *V. harveyi* or lethal heat stress as described in the dose-response study. Controls were maintained as described in the figure legend. Survival of the larvae was scored after 48 h of challenge.

Protein extraction and HSP72 detection

Following pretreatment, live *Artemia* larvae (0.1 g) from all groups of the first and third tests were collected separately on 50-µm sieves, rinsed in distilled water, immediately frozen in liquid nitrogen and stored at −80°C for Hsp70 analysis. *Artemia* samples were homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM MgCl₂, 5 mM NaH₂PO₄, 40 mM HEPES, pH 7.4) (Clegg

et al. 2000), supplemented with protease inhibitor cocktail (Sigma-Aldrich®, USA) as recommended by the manufacturer. Subsequent to centrifugation at 2200×g for 1 min at 4°C, supernatant protein concentrations were determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard. Supernatant samples were then combined with loading buffer, vortexed, heated at 95°C for 5 min, and electrophoresed in 10% SDS-PAGE gel, with each lane receiving equivalent amounts of protein. HeLa (heat shocked) cells (Enzo Life Sciences, USA) (6 µg) were loaded on to one well to serve as a positive control. Gels were either stained with Coomassie Biosafe (BioRad Laboratories) or transferred to polyvinylidene fluoride membrane (BioRad Immun-Blot™ PVDF) for antibody probing. Membrane was incubated with blocking buffer [50 ml of 1× phosphate-buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature and then with mouse monoclonal anti-Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO), which recognizes both constitutive and inducible Hsp70 (Baruah et al. 2012), at the recommended dilution of 1:5000. Horseradish peroxidase-conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, CO). The membrane was then treated with enhanced chemiluminescence reagent (GE healthcare, UK), and the signals were detected by a ChemiDoc MP Imaging System (BioRad, Belgium).

Fluorescence measurement of hydrogen peroxide production in the water

The optimum dose of carvacrol and/or a mixture of antioxidant enzymes (catalase, 10 mg/l and SOD, 75 U) was added to 30 ml of sterile seawater in glass bottles and incubated at 28°C for 2 h. A control was maintained without the addition of compound/enzyme mixture in the distilled water. Water samples were taken after 2 h of incubation and immediately analyzed for hydrogen peroxide (H₂O₂) release/production using the Amplex® Red Hydrogen Peroxide Assay Kit (Invitrogen, Belgium) as described previously (Baruah et al. 2014).

Statistical analysis

Survival data were arcsin transformed to satisfy normality and homoscedasticity requirements as necessary. The data were then subjected to one-way analysis of variances (ANOVA) followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences version 20.0. *P* values ≤0.05 were considered significant.

Results

Impact of carvacrol on the survival of *Artemia* larvae challenged with *V. harveyi*

In a first experiment, we investigated whether carvacrol could confer protection prophylactically against *V. harveyi* to the host *Artemia*. To this end, germ-free *Artemia* larvae were pretreated with varying concentrations of carvacrol under indicated conditions, and then the larvae were challenged with *V. harveyi*. *Artemia* larvae that received carvacrol pretreatment in the range of 16.6 to 166.4 µM exhibited a significant increase in their survival compared to the control (Fig. 2). However, maximum survival (sixfold higher than the control) was observed at a concentration of 66.4 µM. Pretreatment of larvae with carvacrol at a concentration higher than 66.4 µM did not further improve the larval survival. In contrast, the survival tended to decrease with further increase in the concentration.

Impact of carvacrol on the survival of *Artemia* larvae challenged with lethal heat stress

Next, we tested how the *Artemia* larvae pretreated with different carvacrol concentrations would respond to a temperature shock that is lethal for *Artemia* grown at standard temperature. We found that, on exposure to a typically lethal stress (41°C for 20 min), *Artemia* larvae that were pretreated with carvacrol at 66.4 µM concentration had significantly higher survival than the control (Fig. 3). It is worth noting that the increase in the thermal resistance was not displayed by the *Artemia* larvae that were pretreated with carvacrol at concentration lower than 66.4 µM. In addition, no marked improvement in the survival was also observed in the groups that were pretreated with carvacrol at a concentration higher than 66.4 µM. These data from the thermal challenge test reported here, together with the data from the above bacterial challenge test, suggested a carvacrol concentration of 66.4 µM as the optimum dose and were therefore chosen for carrying out subsequent experiments.

Toxicity of carvacrol to axenic *Artemia* larvae

Since the survival of *Vibrio*- or thermal-challenged *Artemia* larvae pretreated with carvacrol at concentration higher than 66.4 appeared to decrease, we next investigated whether it was due to the toxic effect of carvacrol following methodology as described in Materials and Methods. As shown in Fig. 4, *Artemia* larvae pretreated with carvacrol in the range of 16.6 µM to 66.4 µM did not exhibit significant difference in the survival compared with the control. However, at concentrations higher than 99.6 µM, the survival of the larvae significantly decreased. This result indicated that carvacrol

Fig. 2 Effect of carvacrol pretreatment on the survival of *Artemia* after 48 h of challenge with *V. harveyi*. The larvae were pretreated with carvacrol at different doses (16.6, 33.2, 66.4, 99.6, 132.8, 166.4 μM) for 2 h, rinsed to wash away the compound, and then allowed to recover for 2 h. The larvae were subsequently challenged with *V. harveyi* at 10^7 cells/ml of rearing water. Non-pretreated larvae that

were either challenged with *V. harveyi* (positive control) and those not challenged with *V. harveyi* (negative control) served as controls. Error bars represent the standard error of five replicates. Bars with same alphabet letters are not different significantly ($P > 0.05$, Duncan's new multiple range test)

appeared to exert toxic effect on the experimental animal under the present experimental conditions.

Impact of carvacrol on HSP72 production in *Artemia* larvae

Next, we investigated whether carvacrol could induce HSP72 in vivo. To address this, germ-free *Artemia* were pretreated

with different doses of carvacrol and analyzed for HSP72 as described in the “Materials and Methods.” Interestingly, we found that there was a constitutive production of HSC70 in the control *Artemia*. However, carvacrol pretreatment at concentration of 66.4 μM significantly increased HSP72 production as compared with the control. Maximum induction of HSP72 was observed to occur in *Artemia* that were pretreated with a dose of 166.4 μM. However, the increase in the HSP72

Fig. 3 Effect of carvacrol pretreatment on the survival of *Artemia* after 24 h of challenge with a lethal heat stress. The larvae were pretreated with carvacrol at different doses (16.6, 33.2, 66.4, 99.6, 132.8, 166.4 μM) for 2 h, rinsed to wash away the compound, and then allowed to recover for 2 h. The larvae were subsequently challenged with a lethal heat stress at

41°C for 20 min. Non-pretreated larvae that were either challenged with lethal heat stress (positive control) and those not challenged with lethal heat stress (negative control) served as controls. Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly ($P > 0.05$, Duncan's new multiple range test)

Fig. 4 Toxicity of carvacrol to *Artemia* larvae. The larvae were pretreated with different doses of carvacrol as described in Fig. 2. Non-pretreated larvae served as control. Survival was scored 48 h after the recovery period. Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly ($P > 0.05$, Duncan's new multiple range test)

induction level in the 166.4 μM pretreated group did not differ significantly from that of the groups pretreated with 99.6 and 132.8 μM (Fig. 5). This result suggests that carvacrol is an effective inducer of HSP72.

Having observed this, we next sought to investigate the mechanism of action of carvacrol in inducing HSP72 within *Artemia*. As reactive oxygen species (e.g., hydrogen peroxide, H_2O_2) generated from the phenolic compounds were linked with HSP72 induction within *Artemia* (Baruah et al. 2014, 2015b), we hypothesized that carvacrol induces HSP72 by mechanism of generating ROS and that eliminating these

reactive molecules by using ROS scavenging enzymes neutralizes the carvacrol-mediated induction of HSP72. We found that upon pretreatment with an optimized dose of carvacrol (66.4 μM), a significant increase in the production level of HSP72 was observed in the pretreated group as compared with the control (control vs. carvacrol; Fig. 6). However, co-pretreatment of the *Artemia* larvae with carvacrol and antioxidant enzyme mixture resulted in a significant twofold decrease in the HSP72 production level as compared to the carvacrol-pretreated *Artemia* (carvacrol vs. carvacrol + antioxidant enzymes; Fig. 6b). Pretreatment of *Artemia* larvae

Fig. 5 Induction of HSP72 protein in *Artemia* larvae. *Artemia* larvae were pretreated with different doses of carvacrol as described in Fig. 2. Non-pretreated larvae served as control. **a** Protein extracted from different experimental groups was resolved on an SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane and probed with an

antibody to *Artemia* HSP72. Five microgram of *Artemia* protein was loaded in each lane. Molecular mass standards (M) in kilodaltons are shown on the left. The level of HSP72 in HeLa cells was regarded as 1. Cropped blot image was shown. **b** Quantitative analysis of HSP72 levels in *Artemia* larvae is presented relative to HSP72 production in HeLa cells

Fig. 6 Induction of HSP72 protein in *Artemia* larvae. *Artemia* larvae were pretreated with carvacrol, antioxidant enzymes, or in combination with both (carvacrol + antioxidant enzymes) as described in Fig. 2. Non-pretreated larvae were maintained as control. **a** Protein extracted from different experimental groups was resolved on an SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane and probed with an antibody to *Artemia* HSP72. Seven micrograms of *Artemia* protein was loaded in each lane. Molecular mass standards (M) in

kilodaltons are shown on the left. The level of HSP72 in HeLa cells was regarded as 1. Cropped blot image was shown. **b** Quantitative analysis of HSP72 levels in *Artemia* larvae is presented relative to HSP72 production in HeLa cells. Dual bands appeared in **a**, in accordance with our earlier studies (Baruah et al. 2012; Norouzitallab et al. 2015). The upper and lower bands in **a** are predicted to represent constitutive HSP70 (HSC70 or HSP73) and the stress-inducible HSP70 (HSP70 or HSP72)

with a mixture of antioxidant enzymes caused no significant effect on the induction of HSP72 as compared with the control.

Impact of carvacrol on the generation of ROS

In the subsequent experiment, we aimed to verify whether carvacrol generates ROS in the *Artemia* rearing water, and for that, we measured the level of an ROS member H_2O_2 in the water at 2 h after addition of the compound. From Fig. 7, it is evident that addition of carvacrol into the *Artemia* rearing medium led to the release of a significant amount of H_2O_2 in the medium. However, in the presence of a mixture of the antioxidant enzymes catalase and SOD, the H_2O_2 level in the carvacrol added group was significantly reduced (carvacrol vs. carvacrol + antioxidant enzyme mixture). Controls without carvacrol (with or without antioxidant enzymes mixture) had no detectable levels of H_2O_2 .

Impact of carvacrol in the presence or absence of an antioxidant enzyme mixture on the survival of *Artemia* larvae challenged with *V. harveyi* or lethal heat stress

To further confirm the contribution of carvacrol-generated H_2O_2 to the mediation of HSP72 induction, we conducted additional in vivo survival assays as described in the Fig. 8

legends. We assumed that scavenging of the carvacrol-generated H_2O_2 by the antioxidant enzymes mixture would neutralize the HSP72-inductive effect of the compound and, consequently, its protective effects toward challenge with *V. harveyi* or lethal heat stress. We found that *Artemia* larvae pretreated with carvacrol (66.4 μ M) were markedly protected upon challenge with *V. harveyi* or lethal heat stress, with survival augmenting by fivefold and threefold in the case of *V. harveyi* and lethal heat stress, respectively, as compared with the controls (Fig. 8). However, co-pretreatment with a mixture of the antioxidant enzymes partially neutralized the protective effect of the compound. The survival of challenged larvae that were only pretreated with the antioxidant enzyme mixture did not show a significant difference in survival compared with the non-pretreated challenged larvae (control; Fig. 8). Furthermore, to exclude the possibility that the decreased survival in the group pretreated with a combination of antioxidant enzyme mixture and carvacrol was due to a negative effect of the antioxidant enzymes, we tested the effect of the antioxidant enzyme mixture on the survival of axenic *Artemia* larvae and found that there was no apparent adverse effect (data not shown, see Baruah et al. 2015b). These results indicated that H_2O_2 generated by carvacrol in the *Artemia* rearing medium appeared to be, at least in part, involved in the induction of HSP72 within *Artemia* larvae.

Fig. 7 Production of H₂O₂ in the rearing water of *Artemia* larvae. **a** Carvacrol and/or a mixture of the antioxidant enzymes catalase and SOD at indicated doses were added to 30 ml of rearing water. After 2-h incubation, water samples were analyzed for H₂O₂ production. Error

bars represent the standard error of three replicates. Bars with the same alphabet letters are not different significantly ($P > 0.05$, Duncan's new multiple range test)

Discussion

In recent years, the molecular chaperone HSP72 has become a new molecular target for development of antibacterial strategy for farmed aquatic animals, particularly given our findings that induction of HSP72 resulted in a significant reduction in the mortality of aquaculture animals mediated by bacteria (see review, Roberts et al. 2010). This motivated us to search for HSP72 inducers from natural sources, as has been done

previously by a number of researchers, including ourselves (Baruah et al. 2012, 2014; Nagai et al. 2010; Sung et al. 2012; Yan et al. 2004), with the defining characteristic feature of this study being that we focused on a natural compound carvacrol, which had already received approval as a safe food component (FAO/WHO 2001). In this way, carvacrol, if it is proven to be an inducer of HSP72, could potentially be employed immediately for use as a disease control agent in aquaculture animals. In the present study, we have shown that

Fig. 8 Survival (%) of *Artemia* larvae pretreated with carvacrol, antioxidant enzymes, or in combination with both. For the treatment groups, refer to Fig. 6 for explanations. The larvae were challenged with *V. harveyi* at 10^7 cells/ml of rearing water (**a**) or with a lethal heat stress at 41°C for 20 min (**b**). The survival was scored at 48 and 24 h of challenge with *V. harveyi* and lethal heat stress, respectively. Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly ($P > 0.05$, Duncan's new multiple range test)

carvacrol induces HSP72 within the host *Artemia*. This HSP72-inducing property of the compound was observed under controlled germ-free experimental conditions, in which there is no presence/interference of any stressing agent. Based on these observations, we can therefore conclude that carvacrol indeed acts as a potent inducer of HSP72, rather than as a coinducer as previously reported (Wieten et al. 2010). To our knowledge, this is the first direct observation in germ-free animal model of HSP72 induction in response to carvacrol exposure.

The inducible form of HSP70 seems to play an important role in defining the tolerance of organisms to several stressors. For instance, in our earlier studies, we have shown that induction of HSP72 in aquatic animals upon exposure to a classical HSP72 inducer, i.e., nonlethal heat shock or to plant-derived compounds significantly improved the resistance of the animal toward subsequent abiotic or pathogenic biotic stress (Baruah et al. 2012, 2014, 2015b; Boerrigter et al. 2014; Sung et al. 2012). Similar increased tolerance to extreme temperatures or to pathogens, following induction of HSP72, has also been demonstrated in other organisms from diverse phyla, including bacteria, coelenterates, molluscs, fish, shrimp, echinoderms, and humans (Baruah et al. 2013; Correia et al. 2013; Iwama et al. 1998; Roberts et al. 2010; Sanders 1993). This improved resistance was due to the fact that HSP72 plays a crucial function as molecular chaperone and is involved in protein biogenesis and protein homeostasis in the cells or it contributes to the generation of protective immune responses in the host (Baruah et al. 2013; Clegg et al. 2000). In this study, our results unequivocally showed that carvacrol induces protective effects against lethal heat stress and *V. harveyi* infection. However, it should be noted that these effects appeared to be concentration-dependent, with maximum protection at 66.4 μ M carvacrol. Interestingly, the concentration required for marked induction of HSP72 and for affording maximum protection to *Artemia* against thermal and *V. harveyi* challenges was the same. These results suggested that the protective effect seen with carvacrol is mediated, at least in part, by HSP72 induction. It is also noteworthy to mention that under our experimental condition, pretreatment with carvacrol beyond an optimum concentration caused negative effect on the *Artemia* larvae. A possible explanation for the observed negative effect of carvacrol pretreatment at a higher dose could be attributed to exposure to higher concentration of ROS, inflicting, e.g., cellular damage, but other toxic effects may occur as well (Baruah et al. 2014; Fittipaldi et al. 2014; Fridell et al. 2005).

It is important to mention that carvacrol is lipophilic and is sparingly soluble (forms small globules) in water (Chen et al. 2014). In this study, stock solution of carvacrol was prepared by dissolving the compound in water rather than in organic solvents (such as ethanol). The rationale for following such a protocol is that organic solvents cause a significant influence on the

stress responses of the host by causing HSP72 induction (Calabrese et al. 2000; Muralidharan et al. 2014) and by reducing the resistance of the host against bacterial challenge (Niu et al. 2014). As the main focus of this study was to determine the putative HSP72-inducing (and prophylactic) property of carvacrol, the compound was therefore dissolved in water to avoid the interfering effect of the organic solvents on the heat shock responses of the model organism. However, considering the limited water solubility of carvacrol, it can be argued that the concentrations of carvacrol that were shown to induce HSP72 in *Artemia* in reality caused the observed effect at doses relatively lower than the tested doses. To address the issue of the solubility of carvacrol and the concentrations used in this study, we conducted an additional dose-response/challenge test as described in Fig. 2. Here we used carvacrol, the stock solution of which was prepared by dissolving 20.5 μ l of the compound (20 mg) first in 150 μ l of ethanol (95%) and then dissolving the solution in 9.83 ml of sterile distilled water to obtain a final concentration of 2 g/l (Chen et al. 2014). Interestingly, the results showed a pattern of survival that is similar to the one described in the Fig. 2—increased protection against *V. harveyi* at 66.4 μ M and decreasing effect at higher concentrations (data not shown). Based on the results of these two dose-response tests, we can assume that the concentrations used when dissolved in water are correct. However, these are pure speculation and needs further verification by detailed measurements of the compound in the solution following standard analytical methods.

A number of phenolic compounds with different chemical structures were shown to generate reactive oxygen species (e.g., H_2O_2) within the host cells and/or in the rearing/culture medium (Akagawa et al. 2003; Baruah et al. 2014, 2015b; Liang et al. 2013). In our earlier studies, we have demonstrated that ROS molecule H_2O_2 plays a key role in modulating the induction of HSP72 (Baruah et al. 2015b; Fittipaldi et al. 2014). For instance, it was shown that an increased production of oxygen-free radicals in cultured human neuroblastoma cells upon exposure of the cells to a classical HSP inducer, i.e., heat stress, causes an increased induction of HSP (Omar and Pappolla 1993). In another study in the *Artemia* experimental model, we have shown that H_2O_2 generated by a plant-based product/phenolic compound pyrogallol act as regulatory mediator in the induction of HSP72 within the organism, and this induction contributed to the resistance effect of the animal toward pathogenic vibrios (Baruah et al. 2014, 2015b). Since few studies have indicated that carvacrol exhibited ROS-inducing features, albeit without unknown mechanism (Huang et al. 2010; Liang et al. 2013), we therefore raised the question whether the observed Hsp70-inducing effect of carvacrol was due to the generation of ROS by the compound. As expected, our data suggested that ROS molecule H_2O_2 generated at the initial stage from the compound contributes to the induction of HSP72 in *Artemia*. This suggestion was made on the basis of our observations that the induction of HSP72 as well as the

improvement in the resistance against *V. harveyi* or lethal heat stress in *Artemia* was partially nullified when the release of H_2O_2 by the compound was neutralized by the addition of a mixture of ROS scavenging enzymes SOD and catalase (see Figs. 6, 7 and 8). While we have not examined the contribution of other types of carvacrol-generated ROS molecules, such as superoxide anion in the induction of HSP72, we expect that these reactive molecules also account for the induction of HSP72, an assumption that needs further verification. In our study, we did not investigate the reaction mechanism underlying the ROS-inducing features of the compound. Future studies focusing on unraveling the reaction mechanism for carvacrol generating ROS molecules will provide better insight into the mode of action of carvacrol in inducing HSP72.

In conclusion, our results provide strong evidences that carvacrol is a potent inducer of HSP72 and that this compound-mediated HSP72 seems to be responsible for inducing resistance in *Artemia* larvae against subsequent stress mediated by lethal heat stress or pathogenic *V. harveyi*. This report also provides new insight on the mode of HSP72 inducing action of carvacrol, in which the initial generation of reactive molecule H_2O_2 by the compound plays a key role. Overall results advance our knowledge of this compound as potential prophylactic agent.

Acknowledgements The authors acknowledge financial support from the Research Foundation Flanders, FWO-Vlaanderen, Belgium [postdoc grant to Dr. Kartik Baruah, FWO13/PDO/005], GOA project entitled 'Host microbial interaction in aquatic production' (project number: 01G02212), and the Belgian Science Policy Office (BELSPO) project entitled 'AquaStress', project number: IUAPVII/64/Sorgeloos. We highly acknowledge the Special Research Fund of Ghent University (BOF12/BAS/042) for procuring the chemiluminescence detection system.

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