

## Effect of Cinnamaldehyde Addition on Zebrafish (*Danio rerio*) Eggs Against *Streptococcus agalactiae* Infection

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### ABSTRACT

The effect of cinnamaldehyde (CA) towards *Streptococcus agalactiae* on zebrafish egg was investigated. CA and *S. agalactiae* were tested by short-term effects assay. Fresh zebrafish egg, CA and *S. agalactiae* were observed in different groups, single and mix treatment. The result showed that the larval survival rate (%) of single CA concentration (2.5 and 5  $\mu\text{g mL}^{-1}$ ) were not significantly different compared to the control, with the value  $94\pm 2.8\%$  and  $93\pm 3.3\%$  respectively. *S. agalactiae* with a single concentration ( $10^9$  and  $10^{10}$  CFU  $\text{mL}^{-1}$ ) was significantly different in comparison to the control with the value  $73\pm 2.1\%$  and  $6\pm 2.5\%$ . Mix groups, larval survival rate (%) value were significantly different with the best combination value was  $17\pm 4.7\%$  at 5  $\mu\text{g mL}^{-1}$  of CA and  $10^9$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*. CA may potential for zebrafish egg shelter against *S. agalactiae* infection. Mixture (CA and *S. agalactiae*) treatments were not recommended because the low value was shown compared to the single treatments. This possibility may occur because of the debris formation of two substances which leads to the poor environment and causes to low fish larval survival rate (%) value. For further study, the water quality assay is needed.

**Keywords:** Cinnamaldehyde; *Streptococcus agalactiae*; Zebrafish egg.

### INTRODUCTION

Disease outbreak is one of the major problems in aquaculture and may affect a limiting factor for the economics of the fish

farmers (Bagum et al., 2013). For the early life stages, fish egg quality is one of the important factors for successful mass production of fish fry. When the fish egg quality is poor may decrease the potential for survival hatched larvae (KjØrsvik, 1990). The limited fish egg survival causes by a physical-chemical condition in hatcheries such as low water temperature, low dissolved oxygen, low water circulation, high concentrate and high density of egg (Rahman et al., 2017). Furthermore, the presence of pathogens such as viral, parasite, and bacteria also affected the significant economic losses in aquaculture industry (Situmorang et al., 2014).

*Streptococcus* sp. is not an obligate fish pathogen, because it can be found in the water environment without problems in the fish health (Chang and Plumb, 1996). Many *Streptococcus* species are a pathogen (illness-causing) to fish which found naturally in the environment and may become endemic to the aquaculture farm. Example of *Streptococcus* strain that has been reported disease-causing in fish includes *S. agalactiae* which isolated from tilapia world-wide (Mian et al., 2009). *S. agalactiae* is a gram-positive bacterium infected many kinds of freshwater and seawater fish species (Baecck et al., 2006; Garcia et al., 2008; Olivares-Fuster et al., 2008; Mian et al., 2009; Ye et al., 2011; Amal et al., 2012; Azad et al., 2012; Bowater et al., 2012; Chen et al., 2012). The clinical signs of *S. agalactiae* infection such as septicemia

infection, exophthalmia, corneal opacity, melanosis, swimming abnormalities, swelling and haemorrhage in the internal organs (Ye et al., 2011; Chen et al., 2012) but less information about *S. agalactiae* on fish eggs. For control the bacterial disease problem, the antibiotics commonly used in aquaculture which reported that the development and spread of antibiotic resistance, leading to a new problem as like as ineffective treatment for some diseases (Defoirdt et al., 2011). To overcome this problem, the development of natural product usage was chosen as an alternative method.

Cinnamon belongs to traditional herbal medicine to treat diabetes, rheumatism, aching joints and respiratory problems (Satya et al., 2012). Cinnamaldehyde is one of abundance chemical compound of cinnamon essential oil found in *Cinnamomum* as antibacterial effects (Nabavi et al., 2015). Cinnamaldehyde (CA) was a volatile essential oil which eradicates pathogens non-specifically (Rieger and Jessica, 2014). Cinnamaldehyde has many kinds of medicinal properties like antipyretic, astringent, antimicrobial activity (Ali et al., 2005), anti-inflammatory (Youn et al., 2008), antibacterial and cytotoxic effect (Chang et al., 2001; Fang et al., 2004; Lee et al., 2002).

The use of fish as an animal model in the research is such an interesting method. Fish models have been used for many research as replacement of mammals for ethical action in pharmacology, biology and genetic study (Bolis et al., 2001; Silva et al., 2008). Zebrafish is a common model fish which has a small size, easy to culture, growing fast and high fecundity because a female zebrafish can produce about 10,000 eggs per spawning (Dahm and Geisler, 2006). Beside that zebrafish adults and embryos were usually used in toxicology, eco-toxicology

and experimental animal model in various substances from pollutants, pathogens (Benard et al., 2012) to active medical substances (Moşneang et al., 2015). The aim of this study was to observe the influence of cinnamaldehyde as an antibacterial agent against pathogenic bacteria (*S. agalactiae*) by immersion method on zebrafish (*Danio rerio*) eggs as a freshwater model fish.

## MATERIALS AND METHODS

### Materials Preparation.

Zebrafish (*Danio rerio*) eggs, Cinnamaldehyde (CA) (Sigma-Aldrich, China), Tryptic Soy Broth (TSB) (Merck), *S. agalactiae*, 6-well plate (Alpha Plus, Taiwan), Embryo Medium 1x (EM 1x) and OD<sub>600</sub> spectrophotometer (Nano-300 Micro-Spectrophotometer) were obtained from Laboratory of Marine Molecular Biotechnology, Department of Biological Science and Technology, National Pingtung University of Science and Technology, Taiwan. The eggs age were used in this experiment were at the day of mating fish and were incubated around 2 hours after spawning then were checked under a microscope then collected the fertile eggs and removed the infertile eggs.

### Short-Term Effects of Cinnamaldehyde and Pathogenic Bacteria Assay.

Zebrafish fertile eggs were moved to the 6-well plates (50 eggs per well) and were incubated at 28°C in the incubator. CA was mixed with DMSO before then followed by 8 mL at concentrations 10 µg mL<sup>-1</sup>, 7.5 µg mL<sup>-1</sup>, 5 µg mL<sup>-1</sup>, 2.5 µg mL<sup>-1</sup>, and 0 µg mL<sup>-1</sup> was the control. The solution was used in this study was EM 1x sterile. Each set of treatment was performed in triplicate which each set of treatment was conducted by different spawning. The samples were soaked by CA around 24 hours and rinsed with EM 1x sterile. The eggs were incubated

for 3 days and the number of survival fish larvae were counted with a daily observation which removed the egg mortality and changed the solution with EM1x sterile in every day. The optimum concentration of CA which produced no achieved 50% of mortality eggs was used for the further assay.

*Streptococcus agalactiae* as a pathogenic bacteria were a culture in TSB medium around 12 hours then measured by OD<sub>600</sub> spectrophotometer to obtain the density of bacteria. After that cell pellet was collected by centrifugation at 4,000 rpm, 20 min, 28°C and was prepared followed by 8 mL at serially diluted from 10<sup>1</sup> to 10<sup>10</sup> CFU mL<sup>-1</sup> and no bacteria was the control. Zebrafish fertile eggs were placed to the 6-well plates (50 eggs per well) with EM 1x sterile solution

and were incubated in the incubator at 28°C. Each set of treatment was performed in triplicate which each set of treatment was conducted by different spawning. The samples were soaked with *S. agalactiae* about 24 hours and rinsed with EM 1x sterile. The eggs were incubated for 3 days and the number of survival fish larvae were calculated with a daily observation which discarded the egg mortality and rinsed with EM1x sterile in every day. The maximum concentration of bacterial dilution which produced to achieve 50% of egg mortality was used for the further assay.

Survival rate percentage and egg mortality percentage were slightly modified according to Yisa et al. (2014) following the formula:

$$\text{Egg mortality (\%)} = \frac{\text{Total of fish eggs dead}}{\text{Total fish eggs}} \times 100\%$$

$$\text{Survival rate (\%)} = \frac{\text{Total of survival fish larvae from hatching out eggs}}{\text{Total fish eggs}} \times 100\%$$

### Zebrafish Larvae Survival Rate

The 50 fertile eggs each were prepared in the 6-well plates followed 8 mL for 9 groups of treatment (2.5 µg mL<sup>-1</sup> of CA, 5 µg mL<sup>-1</sup> of CA, 10<sup>9</sup> CFU mL<sup>-1</sup> of *S. agalactiae*, 10<sup>10</sup> CFU mL<sup>-1</sup> of *S. agalactiae*, 2.5 µg mL<sup>-1</sup> of CA and 10<sup>9</sup> CFU mL<sup>-1</sup> of *S. agalactiae*, 2.5 µg mL<sup>-1</sup> of CA and 10<sup>10</sup> CFU mL<sup>-1</sup> of *S. agalactiae*, 5 µg mL<sup>-1</sup> of CA and 10<sup>9</sup> CFU mL<sup>-1</sup> of *S. agalactiae*, 5 µg mL<sup>-1</sup> of CA and 10<sup>10</sup> CFU mL<sup>-1</sup> of *S. agalactiae*), and EM1x as a negative control. Each group were soaked by different groups about 24 hours then rinsed with EM 1x sterile. The eggs were incubated around 3 days at 28°C with a daily observation which refused the egg mortality and rinsed every day with EM1x sterile. The number of the survival rate of larvae at the last day was calculated to obtain the survival rate percentage (%) of larvae.

### Statistical Analysis

The statistical analysis was determined by one-way analysis of variance (ANOVA) in SigmaPlot 12.5 software followed by Dunnett's test. Data were expressed as a mean ± standard error of the mean (SEM) which statistically significant differences required that p<0.05.

## RESULTS AND DISCUSSION

### Short-Term Effects Assay

The result of short-term effects of CA or *S. agalactiae* values were used for the first step to determine which concentration would be used for the further step. CA or *S. agalactiae* at different concentrations in zebrafish eggs confirmed that the different mortality number was observed at all treatments in CA (Table 1A) or *S. Agalactiae*

(Table 1B). Inappropriate to the result of this study, death percentages values of both treatment groups indicated that the higher concentrations were tested in different concentrations of CA or *S. agalactiae* caused increasing the egg mortality. Commonly, the LC<sub>50</sub> value was used for measuring the acute toxicity of compound based on the effects of a single dose or repeated exposure over a short time. For fish and aquatic organisms, the LC<sub>50</sub> value was determined based on the concentration of the compound in water for exposure periods of 24 to 96 hours. Lethal concentration (LC<sub>50</sub>) was a value which statistical estimation of the dosage necessary to kill 50% of the most population on the test species under started condition (National Research Council, 1993). Based on the result, for CA 2.5 µg mL<sup>-1</sup> and 5 µg mL<sup>-1</sup> were chosen for further step with dead value was 4±0.67% and 15±2.52% respectively. 10<sup>9</sup> CFU mL<sup>-1</sup>, 10<sup>10</sup> CFU mL<sup>-1</sup> were chosen for *S. agalactiae* were 11±0.58% and 100±0% LC<sub>50</sub> value. For *S. agalactiae* 10<sup>9</sup> CFU mL<sup>-1</sup>, it also chose for the next step although the result was below 50% of fish eggs mortality. It was a consideration because the value of 10<sup>10</sup> CFU mL<sup>-1</sup> and 10<sup>9</sup> CFU mL<sup>-1</sup> were obviously different and was needed 10<sup>9</sup> CFU mL<sup>-1</sup> for comparison.

**Table 1A. Cinnamaldehyde Short-term Assay at 24 hours Exposure over a Period of 3 Days**

Concentration (µg mL <sup>-1</sup> )	Survival Rate (%)	Egg Mortality (%)
0	100±0.68	2±0.67
2.5*	98±0.68	4±0.67
5*	87±2.58	15±2.52
7.5	55±1.36	46±1.33
10	27±4.92	73±4.82

Value describes the means ± SE (n=3) with (\*) represents as selected concentrations with survival rate above 50%.

**Table 1B. *Streptococcus agalactiae* Short-term Assay at 24 Hours Exposure over a Period of 3 Days**

Concentration (CFU mL <sup>-1</sup> )	Survival Rate (%)	Egg Mortality (%)
Control	100±0	0±0
10 <sup>1</sup>	94±1.15	3±0.58
10 <sup>2</sup>	87±2.52	6±1.26
10 <sup>3</sup>	89±1.68	5±0.84
10 <sup>4</sup>	87±1.02	6±0.51
10 <sup>5</sup>	84±1.15	8±0.58
10 <sup>6</sup>	84±1.15	8±0.58
10 <sup>7</sup>	85±1.68	7±0.84
10 <sup>8</sup>	90±1.15	5±0.58
10 <sup>9**</sup>	78±1.15	11±0.58
10 <sup>10*</sup>	0±0	100±0

Value describes the means ± SE (n=3) with (\*) represents as selected concentration for further step with survival rate below 50% and (\*\*) represents lower value.

CA was known as a bioactive compound which had many advantages, especially with the antimicrobial agent. In the right dosage, CA was an optimal action against pathogen but when the dosage was too high they will be a toxin which killed not only the pathogen but also the host. In this case, that phenomenon would not accept it. The CA concentrations were chosen which have safe concentration. This assay was applied to *S. agalactiae* as a pathogen which commonly found in hot climate, with associated with different freshwater, estuary and marine fish species (Evans et al., 2002). The capable of causing infection or called by infective dose was needed to assay how strong the bacteria can kill the fish eggs. For this study the high density of bacteria might occur because fish eggshell contains by chorion which damage by bacterial colonization excessively in the fish egg surface result in hypoxia, lactic acid accumulation and the

death of fish eggs with the eggshell was penetrated or produce toxic metabolites. The fish eggshell is mainly composed by glycol-proteins which are chemo-attractive for bacterial colonization (Hansen and Olafsen, 1989). Yoshimizu et al. (1980) reported that naturally, in the salmonid eggs the population of bacteria around  $10^3$ - $10^6$  bacteria  $g^{-1}$ . Yeasmin et al. (2015), reported that embryos and hatchlings mass mortalities found when the bacterial count more than  $1,800 \times 10^2$  colonies  $mL^{-1}$ .

#### Zebrafish Larvae Survival Percentage.

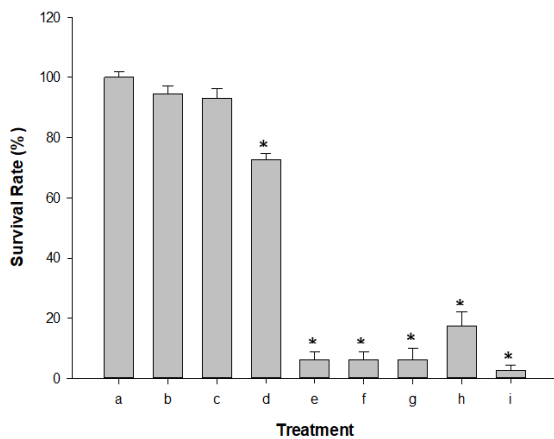
The survival rate of zebrafish larvae for each treatment compared to the control was given to Figure 1. The survival rate in almost all treatments shown the different significant compared to the control ( $P < 0.05$ ). The observed of each treatment  $10^9$  CFU  $mL^{-1}$  of *S. agalactiae*,  $10^{10}$  CFU  $mL^{-1}$  of *S. agalactiae*,  $2.5 \mu g mL^{-1}$  of CA and  $10^9$  CFU  $mL^{-1}$  of *S. agalactiae*,  $2.5 \mu g mL^{-1}$  of CA and  $10^{10}$  CFU  $mL^{-1}$  of *S. agalactiae*,  $5 \mu g mL^{-1}$  of CA and  $10^9$  CFU  $mL^{-1}$  of *S. agalactiae*,  $5 \mu g mL^{-1}$  of CA and  $10^{10}$  CFU  $mL^{-1}$  of *S. agalactiae* showed the significant different in comparison to the control was meant that those treatments appeared the decrement of survival rate of zebrafish larvae than control. Conversely,  $2.5 \mu g mL^{-1}$  of CA,  $5 \mu g mL^{-1}$  of CA did not show the significantly different compared to the control.

In the present study, Fish egg hatching out and zebrafish larval survival rate were directly proportional. The deathly of fish eggs were bacteria. It has been proven that the eggs death majority from bacteria groups even mixed or single treatment. The appeared of high numbers of bacteria on egg surfaces can increase the mortality of developing embryos and some bacteria can digest egg shells which easier for bacteria penetrate into the embryos (Zawada et al., 2014). In the other hand, the hatchability of

fish eggs was influenced by many factors like biotic (eggs and larval density) (Guisande et al., 1998) and abiotic (temperature, DO, pH, light, and pesticides) (Kaur and Toor, 1980) factors. When the bacteria attached to the fish eggs surface and would be a stressor for fish eggs. In the natural environment, most bacteria live in a surface-attached form that will make a variety of physical stresses. To defend themselves from many detach factors like mechanical stresses (fluid flow or something like of scrapping), many bacteria did encapsulate-form in a sticky matrix called biofilm (Costerton et al., 1995). Based on the result prove that *S. agalactiae* has strong enough to kill fish eggs as like as *Flavobacterium* spp., *Pseudomonas* spp., *Aeromonas* spp., and *Vibrio* spp. which are easy to colonized and developed quickly after fertilization (Hansen and Olafsen, 1989).

The survival rate results of mixed treatment  $2.5 \mu g mL^{-1}$  of CA and  $10^9$  CFU  $mL^{-1}$  of *S. agalactiae*,  $2.5 \mu g mL^{-1}$  of CA and  $10^{10}$  CFU  $mL^{-1}$  of *S. agalactiae*,  $5 \mu g mL^{-1}$  of CA and  $10^9$  CFU  $mL^{-1}$  of *S. agalactiae*,  $5 \mu g mL^{-1}$  of CA and  $10^{10}$  CFU  $mL^{-1}$  of *S. agalactiae* shown  $6 \pm 3\%$ ,  $6 \pm 4\%$ ,  $17 \pm 5\%$ ,  $3 \pm 1\%$  respectively. Otto (2014), mentioned that bacteria which live in the environment often discovered attachment only by hydrophobic or electrostatic interaction. Based on observation, cinnamaldehyde was a common hydrophobic and simple to bind the bacteria in the fish eggs surface which blocked the fish eggs osmoregulation for developing embryos. Therefore, fish eggs might occur high levels of stress. The high number of survival rate in these groups were opposite by CA single group  $2.5 \mu g mL^{-1}$  of CA,  $5 \mu g mL^{-1}$  of CA shown  $94 \pm 3\%$ ,  $93 \pm 3\%$  respectively with control  $100 \pm 2\%$ . CA has been known as the most active antimicrobial component which played a strong inhibitory

agent in MIC assay against the tested bacteria (Naveed, 2013). Even though CA plays an important role to kill bacteria but in this case, it was suspected that CA killed the bacteria by binding to the active site of the bacteria. Then, the bacteria and CA precipitated in the environment and changed the water condition become poor and concentrate. When the fish eggs occurred in this situation the developing activity would be interrupted and killed the embryos.



**Figure 1.** Survival rate (%) of Zebrafish larvae after 24 hours exposure with various treatment groups (a: control; b: 2.5  $\mu\text{g mL}^{-1}$  of CA; c: 5  $\mu\text{g mL}^{-1}$  of CA; d:  $10^9$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*; e:  $10^{10}$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*; f: 2.5  $\mu\text{g mL}^{-1}$  of CA and  $10^9$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*; g: 2.5  $\mu\text{g mL}^{-1}$  of CA and  $10^{10}$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*; h: 5  $\mu\text{g mL}^{-1}$  of CA and  $10^9$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*; i: 5  $\mu\text{g mL}^{-1}$  of CA and  $10^{10}$  CFU  $\text{mL}^{-1}$  of *S. agalactiae*) over a period of 3 days. All treatment data were compared to the control and standard error of the mean (SEM) as the error bars with  $P < 0.05$ .

### CONCLUSIONS AND SUGGESTION

The single CA concentration (2.5 and 5  $\mu\text{g mL}^{-1}$ ) did not significantly influence larval survival rate (%) of zebrafish, a contrast to single *S. agalactiae* concentration ( $10^9$  and  $10^{10}$  CFU  $\text{mL}^{-1}$ ). The highest mixed group value was 5  $\mu\text{g mL}^{-1}$  of CA and  $10^9$  CFU  $\text{mL}^{-1}$

of *S. agalactiae*. Although the survival rate (%) tended to low, it was suspected due to the poor environment, especially water quality parameters. Nevertheless, for further research is needed to confirm this hypothesis.

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