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Dry green leaves of Indian almond (*Terminalia catappa*) to prevent streptococcal infection in juveniles of the Nile tilapia (*Oreochromis niloticus*)

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Abstract

Two concentrations of dry leaves in powder (5 and 10 % in feed) of *Terminalia catappa* (Combretaceae) were embedded in a fish feed to prevent and limit the impact of *Streptococcus agalactiae* in juveniles of *Oreochromis niloticus*. Fish were fed with this enriched feed for 15 days before challenge disease and until 21 days after challenge. The cumulative incidence of disease and mortality were significantly reduced in treated fish compared to the controls. The relative percent survival (RPS) were 78% and 62%, respectively for 10 and 5% of leaves powder added in the feed. No negative impact on condition factor, weight gain and specific growth rate were observed in fish fed with dry green leaves of *T. catappa*.

Introduction

Different species of streptococcus are responsible of disease in tilapia in Asian countries, however, epidemiological studies have shown that *Streptococcus agalactiae* is the dominant bacteria involved in streptococcal disease (Amal et al., 2008; Zhang et al., 2013; Jantrakajorn et al., 2014). This is also the case in Indonesia (Lusiastuti et al., 2014), even if *S. iniae* and *Lactococcus garviae* were also found (Anshary et al., 2014) in tilapia floating cages. Antibiotics are commonly used against streptococcal disease (Shoemaker and Klesius, 1997) but antimicrobial resistance is already observed in *S. agalactiae* (Faria et al., 2014; Lukkana et al., 2016). Furthermore,

small scale fish farmers often do not master the use of antibiotics. This may contribute to the promotion of antibiotic resistance within bacterial communities and increase the presence of residual antibiotic within the flesh of the fish. Both are serious concerns for public health, and misuse (and abuse) of antibiotics is considered a dangerous and unsustainable practice in aquaculture. Disease prevention using vaccines is a key approach to fighting diseases; however, a commercial vaccine for both biotype 1 and 2 of *S. agalactiae* is still not available and vaccine costs may not be affordable for small-scale fish farmers. For all of these considerations, alterna-

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tives to antibiotics and new strategies for health management are strongly required for small scale fish farming. Herbal therapy has huge potential for the development of alternatives to antibiotics and sustainable pharmacopeia for aquaculture (Citarasu, 2010). In Indonesia, the Indian almond (*Terminalia catappa*, Combretaceae) is amongst the most used plants by fish farmers particularly for improving water quality but also as a therapeutic compound (Caruso et al., 2013). Several experimental studies reported the use of leaves as being effective against parasites, bacteria and fungi of fish (Chansue, 2007; Chansue and Assawawongkasem, 2008). Generally, Indonesian fish farmers put the red leaves directly into rearing water, however, administration of natural compounds by bath cannot be adapted for fish farming because of the large quantity of raw plants or their dry powders required to reach an efficacious concentration. Administration of herbal compounds through feed can be a more appropriate way to realise fish treatments in ponds or cages. The aims of this trial were to test the efficacy of dry leaves of *Terminalia catappa* embedded in feed to reduce incidence and mortality induced by experimental infection of *S. agalactiae* on fingerlings of *Oreochromis niloticus*.

Material and Methods

Fingerling of *Oreochromis niloticus*, both male and female (13.4 ± 0.33 g) were purchased from a local hatchery. Following three weeks of quarantine, fish were randomly split in three groups (A, B, C in triplicate), into 9 tanks of 40 L (n = 20 fish). Water parameters during the experiment were as follows: temperature 27.1-31.9 °C; pH 6.79-8.75; DO 3.92-10.7 ppm; NO₂- 0.00-1.04 ppm; NO₃- 0.05-3.89 ppm. Ten percent of water was renewed daily during the whole experi-

ment. Leaves of *T. catappa* were obtained from trees of the Indonesian Medicinal and Aromatic Crops Research Institute (BALITTRO) of Cimangu, Bogor. Green leaves were washed and dried in an oven at 50 °C, ground and sieved at 425µm. Experimental feeds were prepared in the same way: a commercial fish-feed (32% protein, 6% lipid, 11% ash) was dampened with water and *T. catappa*'s powder was added at a ratio of 1/10 (100 g/1 kg of aliment). Tapioca was added to the mix at 3% as a binder. This mixture was re-pelleted in pellets of 3 mm diameter, and dried in an oven until dry. The control feed was prepared as referred above, without leaves powder. Group A was fed only with medicated feed at 3.3% of fish biomass/day; Group B received a mixture of 50% medicated and 50% of control feed (0% of dry powder of *T. catappa* leaves) at rate of 3.15% of fish biomass/day. Group C was fed only with control feed at 3% of biomass/day. Fish were fed two times per day, 15 days before challenge and then for the following 21 days. Specific Growth Rate (SGR %) was determined at the end of the first feeding period (15 days) and after experimental infection at the end of experimental observation (21 days), using the following equation:

$$\text{SGR} = [(\ln\text{FBW} - \ln\text{IBW}) / \text{number of feeding days}] \times 100$$

The Condition factor was set as: $\text{FC} = \text{W}/\text{TL}^3 \times 100$ A strain of non-haemolytic *S. agalactiae* biotype 2, isolated from a diseased *Oreochromis niloticus* and kept in Brain Heart Infusion (BHI) broth, with 50% of glycerol at -80 °C, was used for this trial. The bacterial strain was serially cultured three times on BHI agar (24 h at 32°C) and again in BHI broth on the day before bacterial challenge. After 24 h of growth, BHI broth was

centrifuged (5000 rpm for 20 min at 10°C) and the resulting pellet was suspended in sterile PBS to obtain a bacterial concentration of 1.2×10^8 CFU/mL.

After 15 days of feeding, the fish from the different replicates of the same group were pooled, anaesthetised using clove oil at 50 µL/L and then intraperitoneally injected, with 0.2 mL of bacterial solution in PBS and a 2.4×10^7 CFU of *S. agalactiae*. In order to make negative (non-infected) controls from each group, three fish from each replicate were injected with 0.2 mL of sterile PBS. Following injections, 9 to 12 fish of each group were placed in triplicates in glass aquaria (30 L), equipped with an individual mechanic filtration system and air stone. Fifty percent of water was exchanged every day in each aquarium. Fish feeding continued unaltered for 21 days. Mortality and symptoms of streptococcal disease such as: -cataract; blind eyes and whirling behaviour, and reddish anus - were recorded daily. The presence of *S. agalactiae* infection was confirmed by autopsies on 15% of the dead fish, from which bacterial isolation was performed on BHI agar from eyes, brain and kidney samples. Streptococcus infection was identified according phenotypic characters (Zilberg et al., 2010).

Cumulative Incidence rate of disease was calculated according to the formula:

Incidence rate = $\frac{\text{New case of infected fish}}{\text{initial number of fish} - (\text{diseased fish} + \text{dead fish})}$

Chi square test was used to analyse the cumulative incidence of disease and the cumulative mortality, whilst Kaplan Meyer survival analysis were used to analyse the dynamics of incidence of disease.

The Relative Percent Survival (RPS) between treatments and control was calculated as follows:

$$\text{RPS} = (1 - (\% \text{ mortality in treated group}) / (\% \text{ mortality in control group})) \times 100$$

Differences between groups for weight, CF and SGR were analysed using One Way ANOVA or Kruskal-Wallis One Way Analysis of Variance on Ranks depending if either normality (Shapiro-Wilk test) or the variance test (Brown-Forsythe) were respected. Multiple differences were evaluated by the Holm-Sidak test with $\alpha = 0.05$.

Results

No differences were found in the analysed parameters among the replicates of each group, thus the data from replicates were pooled. A relatively high mortality occurred in all groups during the first period of experimentation (15 days). This was attributed to the aggressiveness of fish and it was not significantly different among groups A, B, C (respectively 18.3, 25.0 and 23.3%, $\chi^2 p=0,658$). No significant difference in fish weight between groups was observed after 15 days of the feeding period ($p=0.636$) nor after 21 days post- infection ($p=0.851$). After the first 15 days of feeding, the condition factor of group B was significantly higher than in group A ($p=0.008$); however at 21 days post challenge, the condition factor of surviving fish was not significantly different between the three treatments ($p=0.547$). No difference of SGR was observed across groups before challenge ($p=0.937$); differences between fish SGR before and after the challenge were significant only for group B ($p=0.02$). Also 21 days following challenge SGR was not statistically different ($p=0.982$) across groups (Figure 1).

Based on clinical signs, the cumulative incidence of infection were 17.3%, 26.8% and 71.1% in group A, B, C, respectively, with significant differences between groups (Chi-square= 9,024 with 2 degrees of freedom $p=0.011$). Dynamics of disease incidence between group A, B and C were significantly different (Log-rank test 15.489, df 2, $p<0.001$). In particular, both groups treated with *T. catappa* were significantly different from the controls (Figure 2). Cumulative mortalities following the experimental infection were low: 5.4%, 9.1% and 24.2%, for group A, B and C, respectively, but with significant differences among groups (Chi-square= 6.161 with 2 degrees of freedom $p=0.046$). When compared to the control group (C), RPS was 78% for group A and 62% for group B. At the end of observation (day 21), the prevalence of infected fish in each group were 11.4% ($n = 35$), 13.3% ($n = 30$) and 28% ($n = 25$), respectively, for A, B and C, with no significant differences among groups (Chi-square= 3.243 with 2 d.f. $p=0.198$).

Discussion

Within the limits of data provided by this small trial using small numbers of fish, the Relative Percent Survival (RPS) was over 60% observed in treated fish reaching a threshold considered by Amend (1981) as effective protection. Therefore the green dry leaves of Indian almond can be considered, similarly as noted for other herbal substances, as a protective agent against streptococcal disease of tilapia, when added in the feed (Rattanachaikunsopon and Phumkha-chorn, 2009; Zilberg et al., 2010; Yılmaz et al., 2013). Antibacterial activity of *T. catappa* extracts has been reported and ethanolic extracts have a moderate antibacterial activity against *S. agalactiae* (Bolou et al., 2011; Caruso et al., 2016). Regarding *T. catappa*, the activity seems to be higher in dehydrated young green leaves (used in this trial) than in old red leaves (Nadirah et al., 2013) and its antimicrobial activity could also be related to the inhibition of bacterial quorum sensing (Taganna et al., 2011). The immunostim-

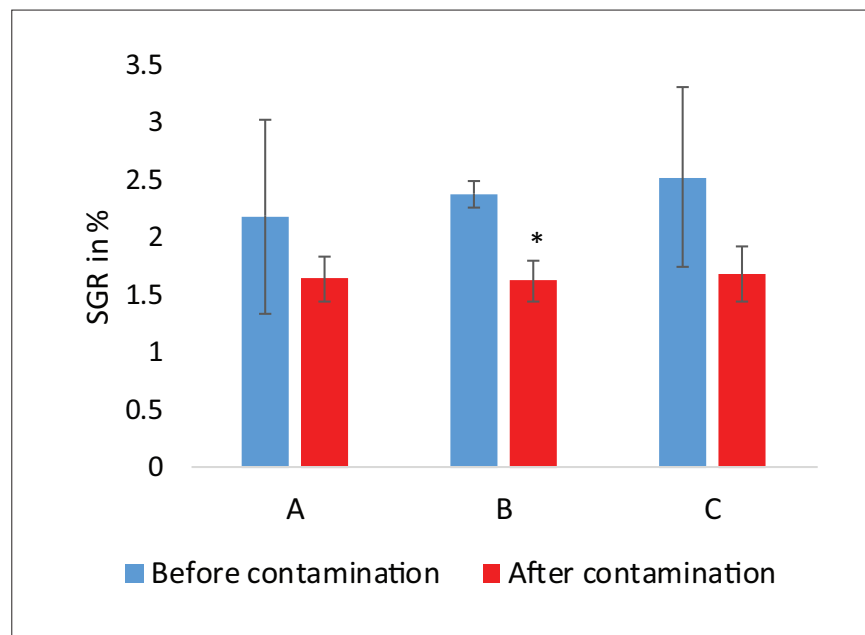


Figure 1. Specific growth rate of *Oreochromis niloticus* treated with *Terminalia catappa* dry green leaves (A 10%, B 5%, C 0%). Data are expressed as mean \pm SEM. Asterisk denotes significant differences between before and after challenge ($P<0.05$).

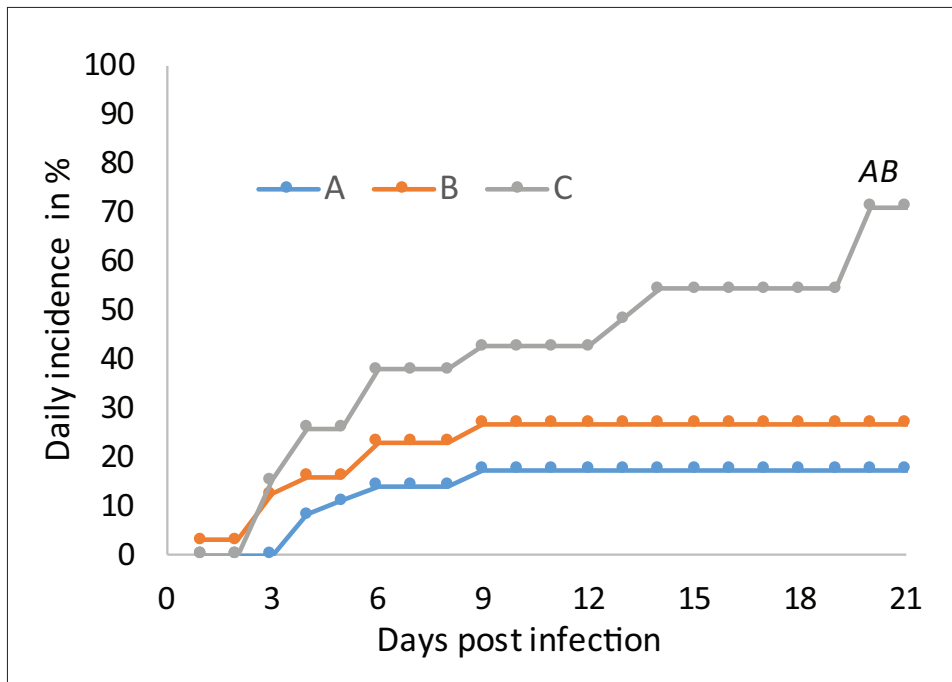


Figure 2. Daily incidence of disease in experimental groups of *Oreochromis niloticus* fed with different levels of *Terminalia catappa* dry green leaves (A 10%, B 5%, C 0%). Denominator was determined as: initial fish number – (infected fish + dead fish)

ulating activity on fish may also be another very important activity of plants (Van Hai, 2015) but to our knowledge, there is no report concerning Indian almond leaves used in tilapia or other fish species. Whatever the mechanism inducing the protective effects against disease, the reduction of incidence of diseases observed in our experiment is noteworthy. Indeed, epidemiologic parameters suggest the need to implement preventive care and to measure the effectiveness of methods undertaken to control acute and chronic diseases. Dry leaves of *T. catappa* appear to be able to significantly reduce the incidence of streptococcal disease, probably additionally reducing the disease relapse, as attested by the delayed infection observed in untreated fish. However, the low number of fish used in the challenge and the weak mortalities observed in this experimental infection may have influenced the positive outcome of this ex-

periment. Since the fingerlings used in this trial originate directly from a production pond, we cannot exclude that the fish had acquired some resistance against *S. agalactiae*. Additionally, it is possible that the bacterial strain used in this trial had a low pathogenicity, as virulence can be variable according to clinical isolate, genetic origin and the strain population (Pereira et al., 2010; Kayansamruaj et al., 2014). In order to avoid extensive territorial behaviour, further challenges should involve larger numbers of fish of a smaller size and the use of a bacterial strain which can induce disease by bath or cohabitation challenge. These routes are more suitable to test the efficacy of a product against natural infection. Results on the zootechnical performances suggested that dry green leaves of *T. catappa* added in feed are not detrimental for juveniles of *O. niloticus*. These juveniles were able to tolerate up to 10% of dry leaves

in the feed (3 g /1kg of body weight/day) for a period longer than 35 days, without modifications of the weight gains, SGR and Condition Factor, however further specific nutritional studies are required. Either way, the apparent harmlessness of the Indian almond on growth of tilapia enhances its suitability for preventive or therapeutic use against streptococcal disease in *O. niloticus*. The protective effect seems to be dose-dependent and the efficacious dose could comprise between 1.5-3 g of dry leaves /per kg of body weight/day.

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