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Assessment of the immunomodulatory effect of commercially available green barley extract on juvenile Nile Tilapia (*Oreochromis niloticus* Linn.*)* assessed *in vivo*

Anna Danica C. Tameta
Colegio de San Juan de Letran

Abstract: Aquaculture is an in-demand industry that constantly needs to maintain and sustain animals to meet the production demand. Although there are antibiotics available to possibly increase the health status particularly in fish, the use of plant extracts as dietary additive or supplements may be used as a cost effective and probably cheaper way of maintaining fish health. In this study, the possible effect of commercially available green barley as a supplement on the immune system of Nile tilapia was assessed by quantitative melano-macrophage center analysis. Results show that there is a significant decrease in the area occupied by MMC upon addition of 500mg green barley extract on fish feeds compared to the control ($p=0.013$). The results suggest that green barley extract at 500mg dose may have immunomodulatory capacity and may be used to increase health in Nile tilapia juveniles.

Keywords: *Nile Tilapia, green barley*

Introduction

One of the fastest growing food-producing sector is aquaculture (Harikrishnan et al 2011). Globally, one of the most commonly cultured fish is tilapia because they breed easily and could adapt to different environmental conditions (Verdegem et al., 1997). They also feed low in the food chain and highly resistant to stress and diseases. Among the tilapia species, Nile tilapia (*Oreochromisniloticus*) is one of the most important cultured fish and highly consumed fish in the world (Binuramesh et al., 2005), and one of the major producers of Nile tilapia is the Philippines.

Like any other ecosystem, the aquatic environment is constantly exposed to stressful conditions. Stress is a vital normal response of all living organisms

Correspondence: Anna Danica Tameta, Colegio de San Juan de Letran, 151 Muralla Street Intramuros, Manila, Philiipines.
Email: *ad.tameta@gmail.com*

(Iwama et al., 2004). It is a mechanism that evolved because of natural selection pressures to allow organisms to survive at different environments (Pickering, 1992). Stress begins with a stimulus recognized by the brain as a stressor, followed by the activation of stress response which can be physiologic fight/ flight/fright (Dhabhar, 2002). The response of an individual may depend on the type and duration of stress (Vosyliene & Kazlauskiene, 1999).

Fish culture conditions are rarely optimal (Robertson et al., 1987). Among the known stressors in aquaculture are physical disturbance, overcrowding, poor water quality, inadequate nutrition, prophylactic treatment, intense social interactions (Robertson et al., 1987; Pickering, 1992) evolutionary selection pressures (Dhabhar, 2002) and other environmental factors which can be naturally occurring or manmade (Bly et al., 1997). The nature of response to energy restructuring caused by stress can either be adaptive towards recovery and homeostasis or maladaptive leading to compromised performance (Iwama et al.2004), immunosuppression and increased susceptibility to diseases (Robertson et al. 1987; Schreck, et al.2001; Kubilay & Ulukoy 2002; Sala-Rabanal et al. 2003) and induced mortality (Pickering 1992).

The use of herbal medicines has recently received attention with their potential effect in the immune system particularly immunoenhancement. In aquaculture for example, there is a need to develop new possible therapeutics that could not give rise to the phenomenon of drug resistance (Pichietti et al, 2013).

The use of green barley as a supplement is not new. As proof, there has been a recent trend as evidenced by a wide variety of green barley products available commercially particularly in the Philippines. There have been many claims regarding the effect of green barley in humans, however, the scientific evidence to prove this claim is scarce. Most of the available literature is on the immune effect of β-glucans present in barley and other cereals.

β-glucans of oat and barley are known in lowering risk of cardiovascular diseases and mediating different immune responses, particularly in the production of cytokines. Barley β-glucans also show to be able to enhance phagocytic activities and productions of reactive oxygen species (ROS) against bacterial infections by *Staphylococcus aureus, Streptococcus pneumoniae, and Salmonella entericaserovars* (Fan et al, 2012)

In aquaculture in the United States, salmonid diets were traditionally using wheat as the carbohydrate source. Other cereal grains may also be suitable alternatives for inclusion in rainbow trout and other fish diets. Although barley has a potential to stimulate the immune system, the exogenous glucans in feeds added with barley may not be of high immunostimulation potential due to the inability of the exogenous glucans to activate intestinal macrophages (Sealey et al, 2008).

The author was able to do a preliminary study on the effect of green barley on tilapia using *in vitro* techniques and the result showed that the barley may potentially be immunoenhancing or immunosuppressing depending on the concentration and the age of Nile tilapia. In addition, this preliminary study should be followed by confirmatory experiments. Thus, the proposed study will assess the effect of commercially available green barley mix on the fish immune system using *in vivo* techniques. Specifically, the effect of varying dosage of commercially available green barley extracts on body weight, organ to body weight ratio, formation of melanomacrophage centers and microbial susceptibility will be assessed. The result of this study is significant to aquaculture industry who is in constant search of new cost-effective therapeutic methods to increase production. In addition, fish studies may also be used as animal models to assess the effect of a certain treatment to humans.

Materials and Method

Test Animal

Mixed-sex Nile Tilapia juveniles were obtained from SEAFDEC Binangonan. Fish were maintained in glass aquaria with aeration and fed to satiation at a laboratory in Colegio de San Juan de Letran until experimentation.

Green barley preparation

Commercially available green barley powdered juice drink was used to test its effect on several immune parameters *in vivo*. Two concentrations were prepared based on the manufacturer's suggested concentration (250mg and 500mg) taken daily based on the body weight of tilapia juveniles.

Experimental design

Three experimental tanks were used in the experiment. One served as a control aquarium while the two remaining tanks were used in the low and high concentration experiments. For each aquarium, thirty mixed-sex fish were reared for 30 days.

Aquaria were cleaned and replaced with water as often as needed and fish were fed daily with commercial floating pellets at 5% body weight ration. To be able to add the treatment, the desired concentration of green barley were mixed with tilapia feeds and were coated with gelatin. All fish were dissected after the 30-day treatment period.

Preparation of mucus sample

At the end of the treatment period, fish were taken to obtain the mucus and spleen. Mucus samples were obtained by placing the ice-immobilized fish in an

autoclaved jar with cover and shaking it to allow mucus to be secreted. Secretions were collected and pooled by three fish of the same treatment. For each pool, 500ul of mucus were diluted with 249.5 ml of distilled water. Diluted samples were sent in a microbial analysis laboratory (FAST Lab) for quantitation of microbial population. The results of this assay may assess the susceptibility of the fish to bacterial infection.

Preparation of spleen

The method for the preparation of spleen was adopted from Zelikoff et al.(1996). Fish were taken and each of their spleens were aseptically collected. The ventral portion of the fish was sprayed with 70% ethyl alcohol prior to dissection, and all dissecting equipments were flame-sterilized to minimize contamination. Each fish was dissected by cutting the fish open midventrally from the anus to the posterior margin of the operculum. Spleen will be excised and fixed in 10% Neutral Buffered Formalin. Fixed spleen samples will be processed for routine histological procedure with H&E stain at the Philippine Kidney Dialysis Foundation in Quezon City.

Quantitative histological analysis of MMCs

One cross section (3um) of the middle portion of each spleen were obtained and melanomacrophage centers were quantified based on the procedure of Bucke*et al* (1992) with several modifications. The histological section from each spleen were viewed with amicroscope at 400x magnification.Three random fields of view from each spleen were analyzed and the areas were calculated using ImageJ. The area occupied by MMC was reported as percentage of the total area per field of view. Scattered individually stained macrophages werebe measured. Spleen samples from fish that died before each sampling date were discarded.

Statistical Analysis

Area occupied by melanomacrophage centers and bacterial counts were analyzed per treatment using ANOVA. All tests were performed at p=0.05 significant level.

Results and Discussion

Aquaculture strives to maintain profitability and sustainability in the era where fishmeal and fish oil's demand is increasing. In the trend where plant-based feedstuffs are usually being incorporated into the meal, it is important to understand how plant components may alter fish growth and health. Information on the biologically active compounds present in plants is necessary to avoid negative impact on production (Sealey et al., 2008).

Fish possess a competent immune system which enables them to cope with disease-causing pathogens such as bacteria, viruses and parasites (Tort et al., 2003). In order to survive, fishes are equipped with both innate and adaptive immunity. Innate immunity provides rapid although incomplete anti microbial host defense until a more specific adaptive immunity takes place (Fearon and Locksley, 1996).

Colony forming units were counted based on pour plate method using mucus samples of individual fish. As part of the primary immune response, it is assumed that more mucus secretions are present when the fish is at a stressful condition. Upon exposure to stress, it is also hypothesized that higher levels of microorganisms, some of which may be pathogenic, is present on the fish skin. However, results of this experiment show that all fish coming from different aquaria has an estimate of >6500 CFU per ml which suggests that addition of treatment extract on fish feeds did not induce any significant stress on the fish.

Melanomacrophage centers were observed in most of the spleen samples. The area occupied by the MMC per field of view was measured and found to vary between treatment groups and between individual fish. Comparing the addition of 250mg and 500mg green barley extract with that the control, significant difference was only observed between control group (without extract) and at higher concentration (500mg) at p=0.013. From this result, it can be suggested that the concentration of 500mg is enough to modulate the immune system of mature Nile tilapia.

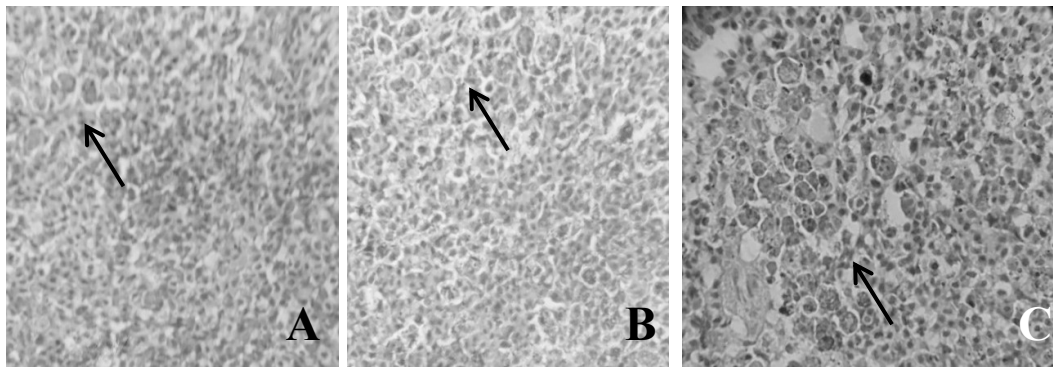

Figure 1. Representative pictures showing MMC formation in fish from the control group (A) and groups fed with addition of green barley extract at 250 mg/5% body ration feeds (B) and 500mg (C).

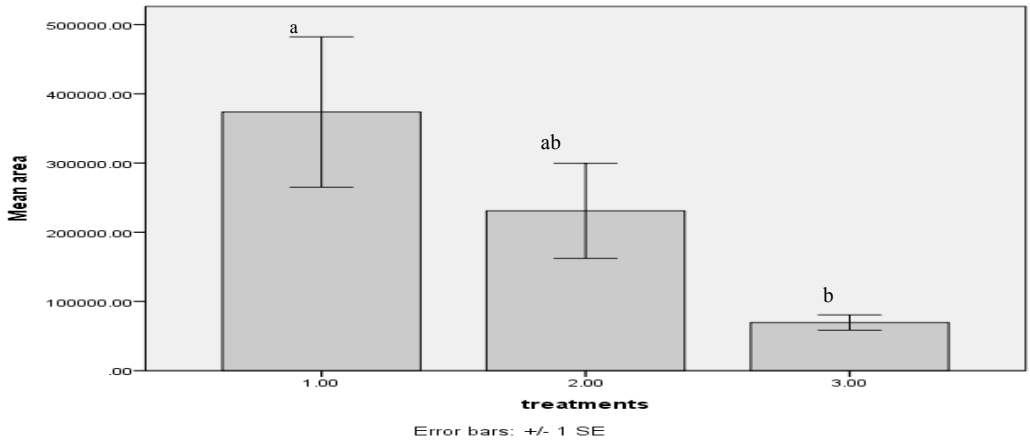

Figure 2. Graph showing the mean area occupied by MMC per treatment group. Significant difference was observed between 1 (control) and 3 (500mg) at p= 0-013

The result shows a possible immunostimulatory effect at higher doses particularly in mature Nile tilapia. Most probably, the effect is due to its β-glucancontent which has the ability to increase the bactericidal capability of the macrophages through intracellular ROS production (Fan et al, 2012). Positive effects on disease resistance were observed when barley is added to trout diet. (Sealey 2008). Further studies regarding the optimum concentration and immunostimulatory mechanisms must be done to fully understand the effect of green barley in fish immune system.

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