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The Effects of Oral Vaccination of *Streptococcus agalactiae* on Stimulating Gut-associated Lymphoid Tissues (GALTs) in Tilapia (*Oreochromis* spp.)

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ABSTRACT

Vaccination of fish by intraperitoneal (i.p.) injection and bath immersion against bacterial infections has been proven to be a commercial success. However, those routes of vaccination are not economical in practice due to several reasons such as high labour cost, highly time consuming, and causing stress to the fish. Meanwhile, oral vaccination is considered as the best route to vaccinate the fish due to less stress to the fish, ability to treat large batch at one time, and easy and practical to administer booster vaccination. In this study, effect of oral vaccination with various regimes in stimulating gut-associated lymphoid tissues (GALTs) against *Streptococcus agalactiae* infection was observed. In this vaccination experiments, four groups of fish with four replicates consisting of 15 tilapias each were used; four groups per treatment received antigen incorporated vaccine in different regimes. Group 1 was fed with vaccine once per week, Group 2 was fed three consecutive days per week, and Group 3 was fed five consecutive days per week, while Group 4 (control) was fed with standard commercial feed. Booster dose was administered at day-14 after the first administration, and humanely killed at day-28 post-booster vaccination. Ten fish from each group were collected for gut sampling and subjected for histological analysis using Olympus FIVE Image Analyzer. Aggregations of GALTs were observed in lamina propria of the gut. The sizes of GALTs were measured and the numbers of lymphoid cells were also counted. The diameter of GALTs showed no significant ($p>0.05$) difference between Groups 1 to Group 2 and Group 2 to Group 3, but a significant difference ($p<0.05$) was observed between Groups 1 and 3. In terms of the numbers of lymphoid cells, no significant differences ($p>0.05$) were found between Group 1 to Group 2 and Group 2 to Group 3; however, a significant difference ($p<0.05$) was observed between Groups 1 and Group 3. As a conclusion, the frequencies of administration play a role in stimulating the size of GALT which is correlated with the number of aggregated lymphoid cells in the gastrointestinal tract of tilapia.

Keywords: *Streptococcus agalactiae,* oral, gut associated lymphoid tissue (GALT)

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INTRODUCTION

Among fresh water fish, tilapia is the most cultured food fish in the world (Anon, 2004). Wide aquaculture systems are used in cultivating tilapia, either in ponds or tanks or cage-cultured. However, tilapia has been found to be susceptible to bacterial disease, particularly streptococcal disease. Moreover, the infection is usually transmitted from fish to fish, in which the bacteria is released from dead and dying fish that is considered as the most important source of infection (Kitao, 1993).

Teleost fish also possesses primary and secondary lymphoid organs; however, there are major structural and morphological differences between fish and mammalian immune systems (Salinas *et al*., 2007). The thymus is divided into a cortex and medulla that are composed of epithelial cell and thymocytes. Kidney is a major lymphoid organ and the foremost part lacks excretory tissue, frequently referred to as the head kidney (Press and Evensen, 1999). The head kidney is a predominantly lympho-myeloid compartment, which is also an important haematopoietic organ (Fange, 1986), and it has morphological similarity with bone marrow in higher vertebrates (Meseguer *et al*., 1995). The mucosa-associated lymphoid tissues (MALT) in teleost include skin, gills and gut-associated lymphoid tissues (GALT) (Salinas *et al*., 2007). Their layer of mucus and an array of non-specific immune defences are exposed to the external environment and form initial barrier to invasion by pathogens (Dalmo *et al*., 1997). In teleosts, GALT is obtained as individual leukocytes or as a lymphoid accumulation, including macrophages, lymphocytes, granulocytes, and plasma cells (Nakamura *et al*., 2000). Intra-epithelial leukocytes are present in the gut but most leucocytes are found in the lamina propria of the gut folds and luminal to the stratum compactum (McMillan and Secombes, 1997). Endo *et al*. (2002) found that self-fed tilapia had significantly lower blood cortisol, higher antibody production and a higher number of blood lymphocytes. Allison *et al*. (1979) found that sinking pellets were better utilized than unpelleted feed by blue tilapia. This report describes the stimulation of the GALT following oral exposures to killed whole-cell *Streptococcus agalactiae* that was incorporated into the feed.

MATERIALS AND METHODS

Fish Samples

A total of 240 tilapias (*Oreochromis* spp.) were selected from Aquaculture Extension Centre (AEC) in Jitra, Kedah, before they were transferred and conditioned at the National Fish Health Research Centre (NaFisH), Batu Maung, Penang. The mean weight was 150±10 g. Prior to experimentation, all the fish were screened for *S. agalactiae* to ensure that they were free of streptococcosis.

The tilapias were randomly assigned to sixteen 200-L tanks. Light cycle was held constantly at 12 h light per day. Feeding was *ad libitum* with local commercial feed. The water was continuously aerated while its temperature was checked on a daily basis. The water temperature, pH and dissolved oxygen were measured using HQ40d Meter (Hach Company, Loveland, CO). Meanwhile, ammonia, sulphate and nitrites were determined daily using a DR 2800 Portable Spectrophotometer (Hach Company, Loveland, CO).

Preparation of the Antigen

S. agalactiae, isolated from outbreaks of streptococcosis in tilapia, was cultured on blood agar at 37°C before the colonies were further sub-cultured into the BBL Brain Heart Infusion broth (BHIB; BD, USA) and incubated in a shaker incubator at 37°C for 18 h. Following the incubation, the bacterial concentrations were determined using the standard plate count technique. The bacteria were then killed by introducing 0.5% buffered formalin and incubated overnight at 4°C. After that, the bacteria were washed five times with Phosphate-Buffered Saline (PBS). Finally, the formalin-killed bacteria (FKB) were added homogenously into the feed mixture prior to pellet preparation.

Experimental Design

At the start of the experiment, the tilapias of Group 1 were fed with the FKB-incorporated feed at the rate of one day per week, whereas Group 2 was fed three consecutive days per week, Group 3 was fed five consecutive days per week and Group 4 served as the control that was fed with normal commercial pellet. Booster doses were applied two weeks after the primary oral exposure for the respective groups.

All the fish from each group were killed on day-28 post-booster vaccination before the entire gut was separated and fixed into 10% buffered formalin. The portion of the gut, that was located at 10 cm from the stomach, was sampled, routinely processed and stained with haematoxylin-eosin (HE) for histological examination.

Serology

The ELISA was performed as previously described by Shelby *et al*. (2001) and Grabowski *et al*. (2004) with slight modification by analyzing the mucus immunoglobulin M (IgM). The mucus was collected at weeks 0, 1, 2, 3, 4, 5, and 6 from all the fish. The body mucus was collected from anaesthetized fish via swabbing on one side of the fish 10 times from head to tail with sterile cotton bud and placed in micro-centrifuge tubes containing 0.9 mL of PBS supplemented with 0.02% (w/v) sodium azide. Briefly, mucus IgM levels were detected using 100 L of goat anti-tilapia immunoglobulin serum, and diluted at 1: 5000. Then, 100 L of conjugated rabbit anti-goat IgM-horseradish peroxidase (Nordic, the Netherlands), which were diluted at 1: 5000, were added. After the final three-wash step with PBST, bound conjugate was detected using 100 L of TMB One Solution substrate (Promega, USA), before stop reaction with 0.2 mol/L sulphuric acid, and the plates were read at 450 nm wavelengths (Anthos Zenyth 340st, Austria).

GALT Determination

The selected tissue samples were embedded in paraffin and sectioned at 4 m for slide preparation. Sections were allowed to dry overnight at 40°C. All the samples were processed and subjected to haematoxylin and eosin (HE) staining method. A total of 10 microscopic fields were examined for the presence of GALT. Once identified, the size of GALT and the number of lymphoid cells in each GALT were determined using FIVE Image Analyzer (Olympus, Japan).

Statistical Analysis

The relationship between the size and number of GALTs data were analyzed using a single linear regression (SLR). In highlight the significance of the results, the one-way analysis of variance was employed further using Tukey HSD in Statistix 9 (Analytical Software, USA). The results were considered as significant at $p<0.05$.

RESULTS AND DISCUSSION

The water quality parameters (mean±SD) were 4.97±0.3 mg/L dissolved oxygen, 32.6±0.8°C, 7.47±0.1 pH, 2.37 mg/L ammonia and 0.023 mg/L nitrate concentrations. These parameters were within the normal range of water quality (El-Sayed, 2006). All the guts of tilapias, exposed to oral vaccination, showed the presence of aggregations of lymphoid cells both within the epithelium and the lamina propria. Nonetheless, there was no observation of lymphoid cell aggregation in the gut of tilapias of the unexposed Group 4.

Table 1 shows the average size of GALT and the average number of lymphoid cells in the GALT of tilapias exposed to the killed *S. agalactiae* incorporated in feed. The GALT was observed in the exposed tilapias of Groups 1, 2 and 3 (*Figs. 2, 3,* and *4*), but not in the unexposed tilapias of Group 4 (*Fig. 5*). All the vaccinated groups were found to be significantly different when compared to the unexposed tilapias of Group 4. In the vaccinated tilapia, no significant difference ($p>0.05$) was observed in the size of

TABLE 1
Average size of the GALT and the number of lymphoid cells in tilapias, following different frequencies of oral exposures to killed *S. agalactiae*

Group	GALT	
	Diameter (µm)	No. of lymphoid cells
1	112.6 ± 0.05^{a}	458 ± 5^{x}
2	140.0 ± 0.5^{a}	763 ± 5^{x}
3	205.0 ± 0.5^{b}	1098 ± 5^{y}
4	0^{c}	0^{z}

^{a,b,c,x,y,z} Values with different superscripts are significantly ($p<0.05$) different within the column.
Group 1: orally exposed once in a week.
Group 2: orally exposed for three continuously days.
Group 3: orally for five continuously days.
Group 4: unexposed control.

GALT between Groups 1 and 2, but significantly ($p<0.05$) larger size was observed in Group 3 when it was compared to Groups 1 and 2.

Similarly, in vaccinated tilapias, there were no significant differences ($p>0.05$) in the number of lymphoid cells between Groups 1 and 2, but ($p<0.05$) more numbers of lymphoid cells were significantly observed in Group 3 than Groups 1 and 2. Therefore, the frequency of antigen administration seemed to play an important role in the stimulation the size of GALT and the number of lymphoid cells within the GALT.

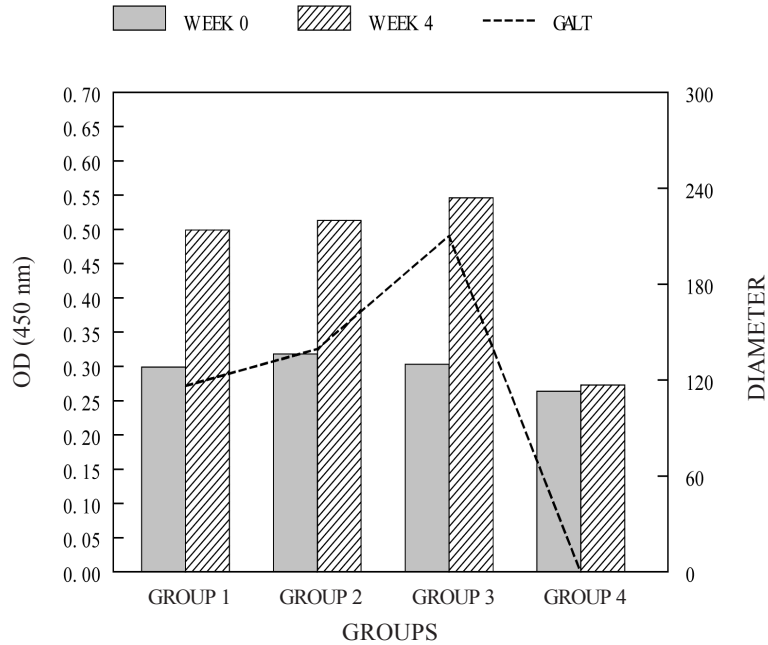

Fig. 1: Mucus antibody responses correlated to GALTs diameter in the exposed and unexposed tilapias

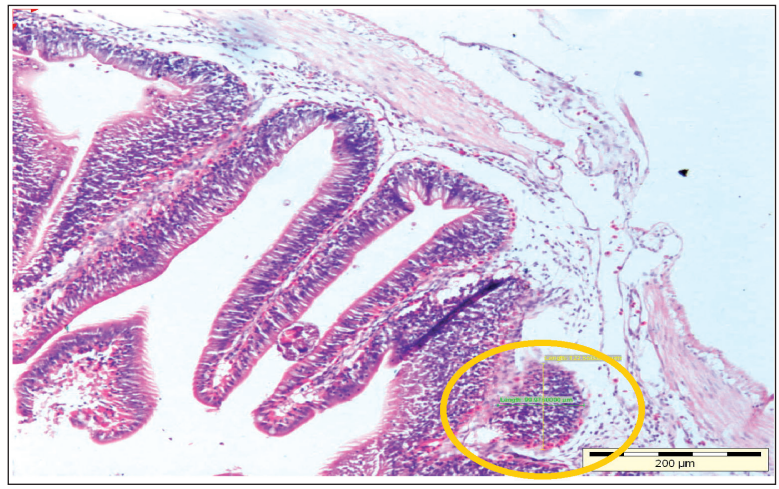

Fig. 2: A cross-section of the gut of fish from Group 1. The aggregation of lymphoid cells or GALT formed in the lamina propria is marked in yellow circle (HE × 200)

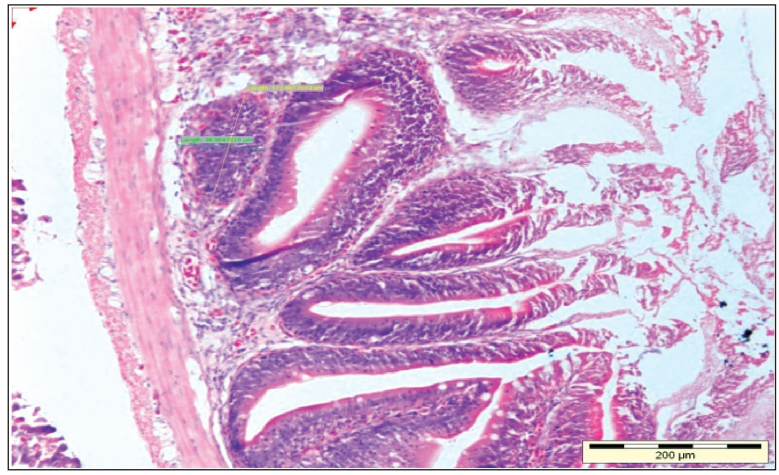

Fig. 3: A cross-section of the gut of fish from Group 2. The aggregation of lymphoid cells or GALT formed in the lamina propria is marked in yellow circle (HE × 200)

Gut is one of the organs of the mucosal immune system organ and it is the site where there is only a thin barrier between internal and external milieu (MacDonald and Miller, 2005). Consequently, this makes gut very sensitive to foreign antigens such as bacteria and reacted by stimulating the GALT by producing antigen-specific antibodies. The GALT consists of leukocytes, macrophages, lymphocytes, granulocytes, and plasma cells (Nakamura *et al*., 2000). Previous studies carried out on GALT indicate its importance to the function of the local immune system (Rombout *et al*., 1986). Meanwhile, an administration of antigens into the gut can lead to increasing of the numbers of intraepithelial leukocytes (Davina *et al*., 1982) and induce the production of specific antibodies in the mucosa and bile (Hart *et al*., 1987). *Fig. 1*

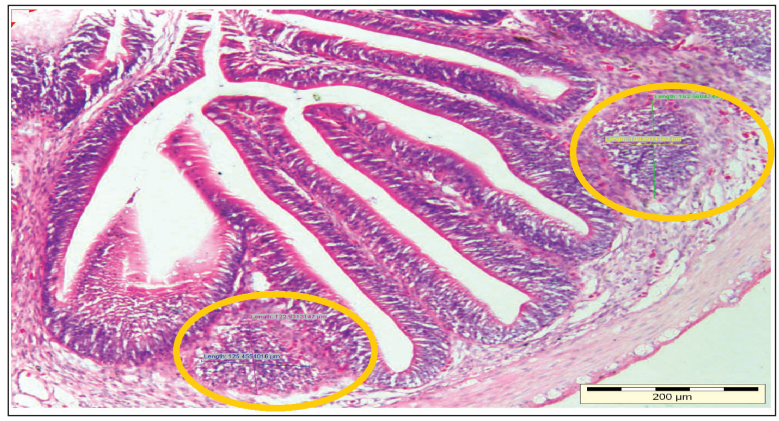

Fig. 4: A cross-section of the gut of fish from Group 3. The aggregation of lymphoid cells or GALT formed in the lamina propria is marked in yellow circles (HE × 200)

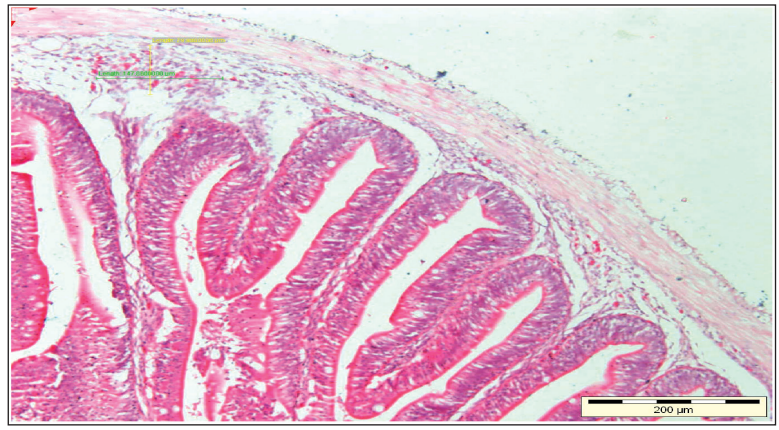

Fig. 5: A cross-section of the gut of fish from Group 4. No aggregation of lymphoid cells or GALT was formed in the lamina propria (HE × 200)

shows the correlation between mucus antibody responses and average size of GALTs in the fish exposed to the killed *S. agalactiae*. The graph shows a linear correlation between the average size of GALT and the mucus antibody responses level, indicating an oral exposure to the killed *S. agalactiae* that was incorporated in the feed could have also stimulated response in skin mucus.

This study has revealed that exposure at the rate of once a week to killed *S. agalactiae* incorporated in feed was sufficient enough to stimulate the GALT and skin mucus antibody responses. However, the data showed that frequent exposures could stimulate better GALT responses, as observed in the tilapias of Groups 2 and 3. This is an early indication that oral exposures to killed antigen that was incorporated in feed may be an alternative vaccination procedure against infection by *S. agalactiae*.

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