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Occurrence of *Streptococcus* spp. on Farmed Nile tilapia (*Oreochromis niloticus* L.) in Lubao, Pampanga, Philippines

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Abstract The prevalence and colony count of *Streptococcus* spp. in most of the organs of Nile tilapia were higher during the dry season than to the rainy season. Significantly difference was observed in the seasonal prevalence of *Streptococcus* spp. on the skin, foregut and bacterial count on the skin. The exposure of the cultured tilapia in high temperature ($\geq$31 ◦C) was considered as the main driving factor in the proliferation of the bacterium which observed in the results for prevalence and colony count. The identified risk factors on the occurrence of the bacterium included high stocking rate ($\geq$ 9 pcs./m^2), scale of operation (1 to 5 ha), high water temperature ($\geq$31 ◦C), pH above 8, low dissolved oxygen (<5 mg/L), and unsuitable sediment pH (below 7 and above 8) and phosphorus (80 to 100 mg/L). To prevent the occurrence or build-up of the bacterium in the pond system, the identified risk factors associated with operational/cultural practices should be addressed; risk factors related to water and sediment quality should be improved or maintained in ideal concentration. In the end, good husbandry is still the best way to prevent the entry and proliferation of pathogenic microorganism in aquaculture facilities.

Keywords: Bacteria, Prevalence, Risk factors, Sediment quality, Water quality

Introduction

Aquaculture has grown up in business around the world which contributed to the fish production. In 2016, the world production was 110.2 million tons that had an annual growth of 5.8% during the period 2011-2016 (Food and Agriculture Organization, 2018). In the Philippines, the fastest growing aquaculture is directed to tilapia aquaculture that marked the country to be the top tilapia-producing countries. In 2018, tilapia production has amounted of 321,080 MT (Philippine Statistics Authority, 2019).

Pampanga province has received a major center of production in polyculture system which associatied with the shrimp and tilapia and/or crabs

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(Naylor and Burke, 2005). Forty percent of tilapia production in Central Luzon are supplied by Pampanga (Bureau of Agricultural Statistics, 2006).

The success of aquaculture is impeded by the disease incidence (Suanyuk *et al.,* 2008). The impact of bacteria contamination on the tilapia industry is well-known among the population and health authorities due to their wide geographic distribution and causing the serious problems (El-Sayed, 2006). According to Food and Agriculture Organization (2004), bacterial infection in fishes is presented to grow fast with annual increased an approximately 12%. Diseases of tilapia cause economic losses in aquaculture with an approximate cost of US $150 million annually (Shoemaker *et al.,* 2001). There are many cases of fishkill caused by different bacterial species which reported in many countries including the Philippines (El-Sayed, 2006). The most common species of bacteria present in pond-cultured tilapia are *Streptococcus*, *Aeromonas*, *Pseudomonas*, *Vibrio*, *Staphylococcus*, *Mycobacterium*, *Edwardsiella* and *Flexibacter* (El-Sayed, 2006).

Animal production systems have intensified, interacted to disease agents and other factors such as environment, nutrition and genetics to become complex. This complex interplays among a variety of factors to delicate the balance while the goal of increasingly efficient production is sought. In this the type of system that is even slightly changed in the above factors to provide the stress causing the expression of disease (Cameron, 2002).

The study was conducted to document the seasonal prevalence and count the bacterium, *Streptococcus* spp. in the various organs (skin, kidney, liver, foregut, brain) of tilapia in pond water and sediment in Lubao, Pampanga, Philippines and to identify the possible risk factors associated in the occurrence of the bacterium.

Materials and methods

Collection of samples

Tilapia samples reared in 20 grow-out farms in Lubao, Pampanga, Philippines from August 2017 to March 2018 were randomly collected using a cast net. Composite pond water samples in each farm were collected using sterile Kemmerer water sampler. For sediment samples, composite samples in each farm were taken from a depth of 5 cm using a sterile grab sampler (Boyd *et al*., 2002). The fish were stored in aerated plastic bag provided with pond water. The collected water and sediment samples were stored in sterilized polyethylene bottles (PE) and plastic cups, respectively and were kept in ice chest for 4 to 5 hours. All samples were transported to the Water and Soil

Quality Laboratory and Fish Pathology Laboratory of the Freshwater Aquaculture Center (FAC) of the Central Luzon State University (CLSU) for analysis.

Survey of farm practices and management

The tilapia in farm operators were investigated and surveyed using a pre-tested questionnaire which designed by FAC-CLSU. The questionnaire encompassed the aspects of tilapia farming (*e.g.* technical information, feeds and feeding, water management, *etc.*). The location of the farm was used handheld GPS equipment (Garmin, USA). Coordinates were taken in fishpond dikes and projection was made inside the perimeter of the pond to facilitate faster information gathering.

Quantification of Streptococcus

Eight hundred (800) tilapia, 40 pond sediment and 40 pond water samples were subjected to dilution in order to quantify the bacterium. The surface of the organs (skin, kidney, liver, brain and foregut) was disinfected first with 70% ethyl alcohol. Two series of 10-fold dilutions (10^{-1} and 10^{-2}) of tissue homogenates of tilapia organs, pond water and pond sediment were separately made in Phosphate Buffered Saline (PBS). The weight and volume of the sample was 1 g for the tilapia organs and sediment, and 1 mL for the pond water. One hundred microliters (100 µL) of the diluted samples were plated onto a selective medium, Edwards Medium (Murray *et al.*, 2003). The plates were incubated for 18 to 24 h at 35 to 37 ℃. The colonies of *Streptococcus* appeared bluish to colorless in the selective medium. The number of *Streptococcus* spp. was expressed in CFU/g or CFU/mL.

The seasonal prevalence of *Streptococcus* spp. in the different tilapia organs, pond water and sediment was computed using the formula below:

$$\%\, Prevalence = \frac{No. of\ positive\ samples}{Tot.\ no. of\ samples}\ x\ 100$$

Seasonal prevalence of *Streptococcus* was statistically compared using Independent sample T-test.

Streptococcus counts were transformed to log values which were then used in data analysis. Seasonal *Streptococcus* counts were statistically compared using Independent sample T-test.

Analysis of water and sediment samples

Temperature, dissolved oxygen (DO), pH and total dissolved solids (TDS) readings were determined on-site using Yellow Springs Instrument (YSI) multi-parameter equipment (YSI, Ohio). The stored water samples in PE bottles were used for analysis of alkalinity, phosphorus, total ammonia nitrogen (TAN) and nitrite following the methods prescribed in the Manual of Aquatic Ecology of the College of Fisheries-CLSU.

Sediment samples were air-dried (up to 0.3 to 0.4% moisture content) and immediately pulverized using mortar and pestle. Parameters such as pH, percent organic matter and available phosphorus were analyzed using the prescribed methods of the Philippine Council for Agriculture and Resources Research (1980).

Relative risk

The line listing of generated data on water and quality, and management practices were concerned in EpiInfo for the computation of relative risk (RR) or the risk factors associated to the occurrence of *Streptococcus*. RR having value of >1 indicated a positive association (the exposed group has higher incidence than the non-exposed group) while RR value of < 1 indicated negative association (the unexposed group has higher incidence) (Cameron, 2002).

Results

Operational and cultural practices of the Nile tilapia grow-out farms

Based on the project of the Central Luzon State University (CLSU) entitled "Development of Comprehensive Geo-referenced Database for Ecological Risk Analyses of Tilapia Pond Culture in Pampanga", the municipality of Lubao, Pampanga, Philippines is composed of 44 barangays. Thirty-eight (38) of these barangays have existing tilapia grow-out ponds. In this present study, a total of 20 tilapia grow-out farms in the said municipality served as the collection sites for fish, water and sediment during the rainy and dry seasons of 2017 and 2018. These 20 tilapia grow-out farms covered the 17 barangays of Lubao, Pampanga (Baruya/San Rafael, De La Paz, Prado Siongco, Remedios, San Agustin, San Jose Gumi, San Matias, San Nicolas 2nd, San Pablo 1st, San Pablo 2nd, San Roque, Sta. Catalina, Sta. Cruz, Sta. Lucia, Sta. Monica, Sta. Rita, Sta. Teresa 2nd) with existing fishponds. Seventy-five percent (75.00%) of these farms are privately owned while the remaining fraction (25.00%) is rented. Near half (40.00%) of the farms had flood history. The productive area of the 20 tilapia farms ranges from 1.8 to 10.4 ha with 1 to 5 fish pond units per farm. Majority (75.00%) of these farms have two production cycles per year; some have one to two cycles per year (25.00%). The major source of water in ponds is the nearby Pampanga River, followed by water pump and/or combination of river and water pump. The strain of tilapia used by the operators is either size 22 FaST (Freshwater Aquaculture Center Selected Tilapia) which is a product of the Freshwater Aquaculture Center in CLSU or size 22 GenoMar produced by a Norwegian-based company. Stocking rate ranges from 5 to 10 pcs./m^2 and this falls under the umbrella of semi-intensive (4 to 8 pcs./m^2) to intensive (>8 pcs./m^2) level of management All of the farms practice intensive farming with full feeding or dependency on commercial feeds. Because river is the main water source, majority of the tilapia farms seldom (90.00%) practice exchange of water. The stocked tilapia fingerlings are reared for at least 150 days. Some of the problems which are encountered during the operation are fish mortality especially during the summer months, poor water quality, occurrence of parasitic, bacterial and fungal infection, absence of water quality equipment and the likes. Some of the remedies that have been made to solve or lessen the experienced problems are water exchange, application of salt to the water to eradicate unwanted microorganisms, application of lime and fertilizer to improve water quality, cessation of feeding during very high (40 to 42 ℃) or low (7 to 10 ℃) water temperature, reduction of stocking density and early or emergency harvest during mass mortality. Of the farms included in the study, only two tried to use probiotics during the incidence of poor water quality and/or occurrence of diseases. According to them, the use of probiotics is very costly even for medium or large-scale tilapia operators.

Analysis of water and sediment quality of Nile tilapia ponds

The pond water and sediment quality parameters that were analyzed in this study and their corresponding recommended readings for Nile tilapia culture varied between seasons (Table 1). Majority of the water quality parameters are within the optimum reading except for the total dissolved solids (TDS), dissolved oxygen (DO), nitrite (NO_2) and phosphorus (P) in both seasons. The sediment pH in both seasons was slightly higher as compared to the recommended concentration.

The mean water and sediment parameters in the 20 collection sites during the dry and rainy season were statistically compared with one another (Table 1). Water parameters such as temperature, TDS, alkalinity and NO_2 were significantly higher during the dry season ($p \leq 0.05$); the rest of the water parameters (pH, DO, TAN and P) in the compared seasons were not statistically significant ($p > 0.05$).

Table 1. Seasonal comparison of the analyzed water and sediment parameters and their recommended concentration for Nile tilapia culture

	Dry season	Rainy season	Recommended
Water parameters			
Temperature (°C)	33.71 ± 0.93^{a}	30.22 ± 0.86^{b}	20 to 35
Total dissolved solids (mg/L)	$3{,}850.71 \pm 1{,}947.96^{a}$	$2{,}2878.80 \pm 968.42^{b}$	≤ 400
pH	8.71 ± 0.93^{a}	8.97 ± 0.39^{a}	6.5 to 9
Dissolved oxygen (mg/L)	3.47 ± 2.05^{a}	3.77 ± 0.93^{a}	≥ 5
Alkalinity (mg/L)	139.30 ± 28.84^{a}	93.56 ± 12.94^{b}	50 to 200
Total ammonia nitrogen (mg/L)	1.99 ± 0.69^{a}	1.79 ± 0.53^{a}	≤ 5
Nitrite (mg/L)	0.11 ± 0.07^{a}	0.04 ± 0.04^{b}	≤ 0.02
Phosphorus (mg/L)	0.26 ± 0.12^{a}	0.28 ± 0.16^{a}	0.005 to 0.2
Sediment parameters			
pH	8.16 ± 0.22^{a}	8.12 ± 0.15^{a}	7 to 8
Organic matter (%)	5.44 ± 0.71^{a}	5.39 ± 0.70^{a}	≤ 5.8
Phosphorus (mg/L)	91.34 ± 24.59^{a}	89.18 ± 20.95^{a}	80 to 100

Means (±SD) not sharing a common superscript along column are significantly different ($p \leq 0.05$)

Streptococcus spp. count in Nile tilapia pond water and pond sediment

The farm level *Streptococcus* spp. count of water in this study ranged from 2.76 to 3.94 log10 CFU/mL during the dry season and from 2.70 to 3.66 log10 CFU/mL during the rainy season. Meanwhile, the farm level bacterial count in the sediment ranged from 2.70 to 5.31 log10 CFU/g in the dry season and 2.55 to 4.35 CFU/g in the rainy season. The pond sediment as compared to pond water had higher count of *Streptococcus* spp. in both seasons.

The bacterial counts in the pond water and pond sediment of the two seasons were separately pooled and statistically compared with one another. The pond water bacterial count during the dry season (3.47±0.23 log10 CFU/mL) was significantly higher as compared to the rainy season (3.28±0.22log10 CFU/mL ($p \leq 0.05$). Also, the pond sediment during the dry season (4.28±0.96 log10 CFU/g) had significantly higher bacterial count compared to the rainy season (3.48±0.51 log10 CFU/g) (Figure 1).

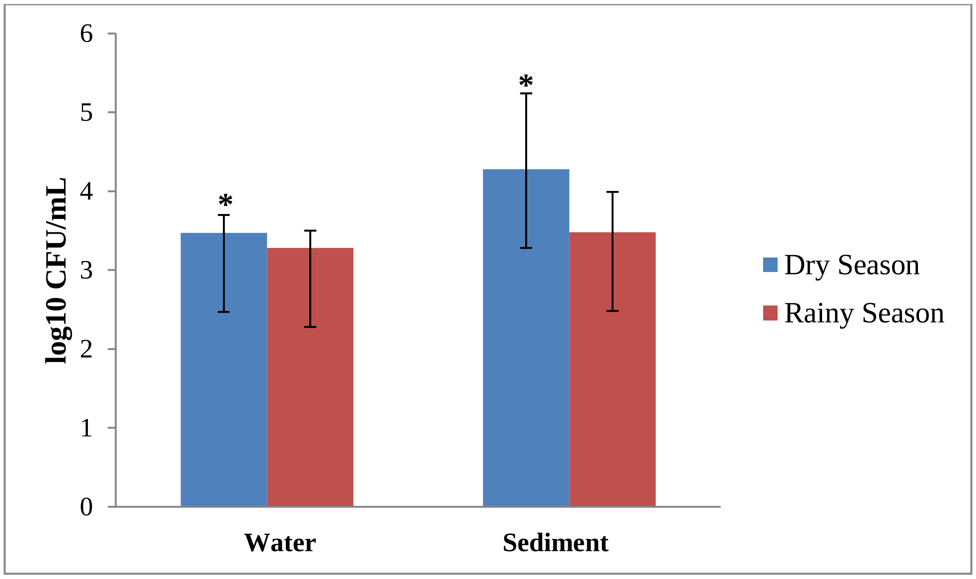

Figure 1. Seasonal count of *Streptococcus* spp. in the pond water (CFU/mL) and pond sediment (CFU/g) (*statistical significance at $p \leq 0.05$)

Prevalence of Streptococcus spp. in Nile tilapia samples during the dry and rainy seasons

The 20 tilapia grow-out farms that served as collection sites were found to harbor *Streptococcus* spp. all year round using the selective Edwards Medium. The prevalence of *Streptococcus* spp. on tilapia skin ranged from 80.00 to 100.00% during the dry season and 50.00 to 100.00% during the rainy season. In the kidney, the prevalence of the bacterium ranged from 70.00 to 100.00% in both the dry and rainy seasons. The prevalence of the bacterium in the kidney was 60.00 to 100.00% during the dry season and 70.00 to 100.00% during the rainy season. The brain of the sampled tilapia was also found positive to the bacterium with prevalence of 45.00 to 100.00% and 50.00 to 100.00% during the dry season and rainy season, respectively. The bacterium was also isolated in the foregut with prevalence of 90 to 100% during the dry season and 80.00% to 100.00% in the rainy season.

The prevalence of *Streptococcus* spp. in the 20 grow-out farms in each season from four sampling dates was pooled and the result for each season was statistically compared. Except in the liver (89.75±9.80%), the seasonal prevalence of the bacterium on the skin (93.50±7.45%), kidney (93.25±7.99%), brain (85.25±13.52%) and foregut (97.25±4.44%) was higher during the dry season as compared with the other season (skin = 85.50±13.56%, kidney = 91.00±8.37%, liver = 92.25±7.69%, brain = 80.75±14.44%, foregut =

90.00±8.43%). Significant difference was observed in the seasonal prevalence of the bacterium on the skin and foregut ($p \leq 0.05$) (Figure 2).

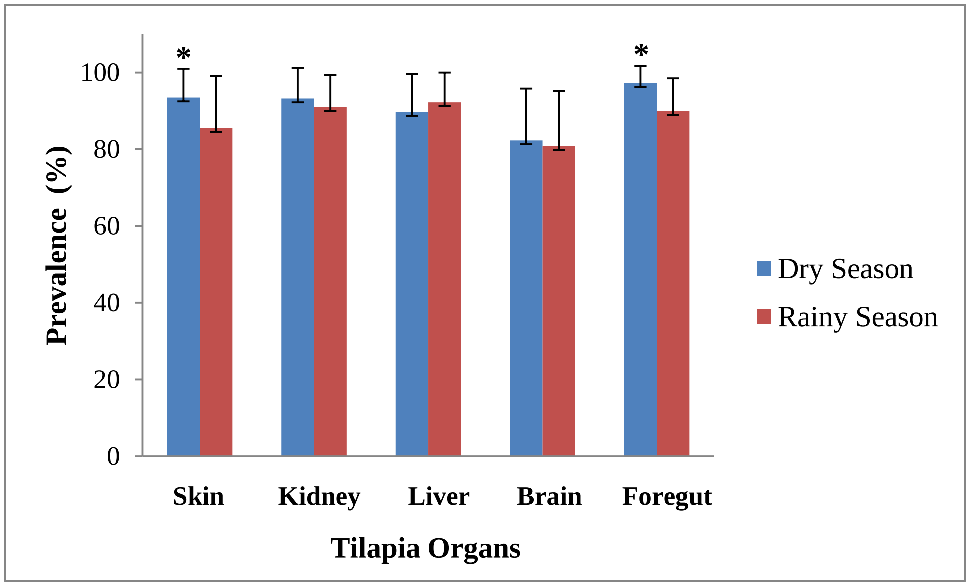

Figure 2. Seasonal prevalence of *Streptococcus* spp. (%) in the various organs of Nile tilapia (*statistical significance at $p \leq 0.05$)

Streptococcus spp. count in Nile tilapia samples during the dry and rainy seasons

The population of *Streptococcus* spp. on tilapia skin ranged from 2.85 to 4.46 log10 CFU/g during the dry season and 1.75 to 4.97 log10 CFU/g during the rainy season. In the kidney, the concentration of the bacterium ranged from 2.66 to 4.87 log10 CFU/g in the dry season and 2.74 to 4.76 log10 CFU/g in the rainy season. The count of the bacterium in the kidney was 2.13 to 4.57 log10 CFU/g in the dry season and 2.81 to 4.35 log10 CFU/g in the rainy season. The brain of the samples was also infected with *Streptococcus* reaching 1.59 to 4.26 log10 CFU/g and 1.65 to 4.55 log10 CFU/g during the dry and rainy seasons, respectively. The bacterium was also isolated in the foregut with counts of 3.52 to 4.97 log10 CFU/g during the dry season and 2.65 to 4.56 log10 CFU/g during the rainy season.

The 1og10 transformed *Streptococcus* spp. counts in the 20 grow-out farms in each season were combined and the result for each season was statistically compared. Except for the bacterial count in the liver, the counts in the other parts were considered higher during the dry season (skin = 3.92±0.45 log10 CFU/g, kidney = 4.05±0.52 C log10 CFU/g, liver = 3.72±0.56 log10

CFU/g, brain = 3.47±0.65 log10 CFU/g, foregut = 4.29±0.45 log10 CFU/g) as compared to the rainy season (skin = 3.47±0.82 log10 CFU/g, kidney = 4.01±0.64 log10 CFU/g, liver = 3.82±0.43 log10 CFU/g, brain = 3.30±0.81 log10 CFU/g, foregut = 3.89±0.51 log10 CFU/g); significant difference was observed on tilapia skin which was higher during the dry season than in the rainy season ($p \leq 0.05$) (Figure 3).

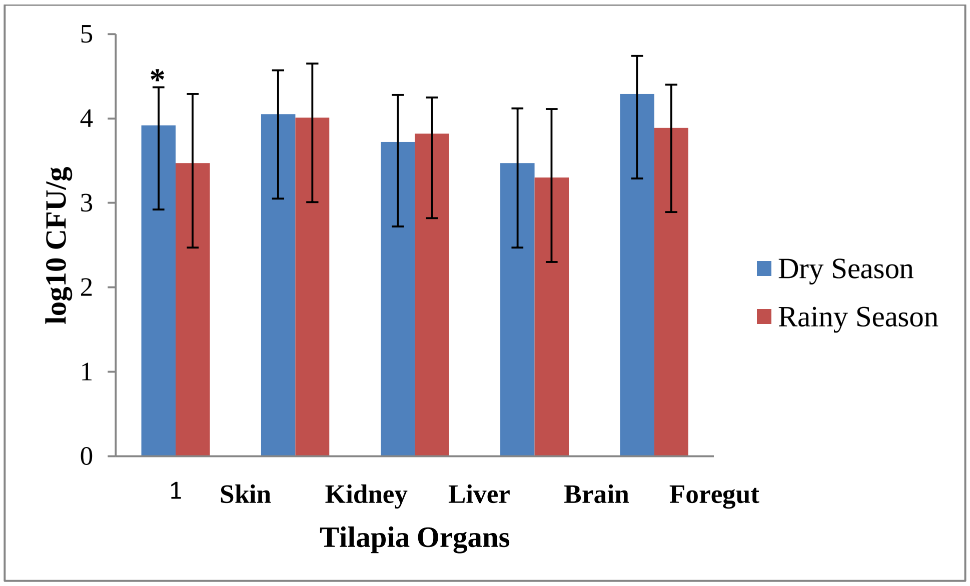

Figure 3. Seasonal count of *Streptococcus* spp. (CFU/g) in the various organs of Nile tilapia (asterisk indicates statistical significance at $p \leq 0.05$)

Identification of risk factors associated with the occurrence of Streptococcus spp.

The computed relative risk (RR) in each risk factor in the two seasons is provided in Table 1. RR having value of >1 indicates positive association (the exposed group has higher incidence than the unexposed group) while RR value of < 1 indicates negative association (the unexposed group has higher incidence than the exposed group). Tilapia that encountered the risk factors such as unfavorable operational/cultural practices, and not optimum water and sediment quality were included in the exposed group. For tilapia that had no exposure to the above mentioned risk factors, they were included in the unexposed group.

High stocking rate of tilapia ($\geq$9 pcs./m^2) in ponds yielded RR of >1 in both seasons. Medium to large scale operation (1-5 ha) in rainy season was considered a risk factor because the computed RR was >1. Water parameters such as temperature (33.71±0.93 ◦C), pH (8.71±0.93), DO (3.47±2.05 mg/L) and P (0.26±0.12 mg/L) were considered as risk factors in the occurrence of the bacterium during the dry season. Meanwhile, water parameters such as DO (3.77±0.93 mg/L) and NO_2 (0.04±0.04 mg/L) were the identified risk factors during the rainy season. Sediment parameters such as pH (8.12±0.15) and P (89.18±20.95 mg/L) recorded RR of >1, thus, these parameters were possible risk factors associated with the occurrence of the bacterium in the rainy season (Table 2).

Table 2. Possible seasonal risk factors associated with the occurrence of *Streptococcus* spp. in tilapia grow-out ponds in Lubao, Pampanga through the computation of relative risk (RR)

Possible risk factors	RR values	
	Dry season	**Rainy season**
Operational/cultural practices		
Scale of operation	0.87	1.02
Pond preparation	n/a	n/a
Source of water	n/a	n/a
Water exchange	0.93	0.98
Level of management (rate of stocking)	1.55	1.07
Feeding	n/a	n/a
Water parameters		
Temperature	1.18	n/a
Total dissolved solids	n/a	n/a
pH	1.07	n/a
Dissolved oxygen	1.11	1.01
Alkalinity	n/a	n/a
Total ammonia nitrogen	n/a	n/a
Nitrite	0.97	1.07
Phosphorus	1.03	0.98
Sediment parameters		
pH	0.97	1.07
Organic matter	0.92	1.00
Phosphorus	0.95	0.98

Note: n/a computation of relative risk was not possible because of either zero incidence rate in the exposed group or zero incidence rate in the unexposed group.
RR value of $\geq$1 is considered as risk factor.

Discussion

Other authors have stated that temperatures above 31℃ are known to predispose the occurrence of *Streptococcus* spp. in tilapia (Evans *et al.,* 2006;

Amal *et al.,* 2008; Mian *et al.,* 2009). The average water temperature during the dry and rainy seasons observed in this study was 33.71±0.93 ˚C and 30.22±0.86 ˚C, respectively, thus, the proliferation of the bacterium was more conducive during the dry season. It was also found in this study that the bacterial count in pond water and sediment during the dry season was significantly higher as compared to the rainy season which explains the significantly higher counts on the skin and foregut. In fish, the intestine constantly interacts with the external environment and is expected to be colonized with a diverse population of microbes (Ki and Rotstein, 2008). This organ provides niches for adherence, colonization, and proliferation of probiotic and pathogenic species that affect many physiological and immunological functions of the host (Li *et al.,* 2018). According to Conway (1996), microorganisms can colonize the intestine when they persist for a long time because they have a multiplication rate higher than the expulsion rate. Meanwhile, the intestine provides a favorable ecological niche for this bacterium (Horsley, 1977). The overgrowth of *Streptococcus* spp. may occur following a breach of intestinal microfloral barrier, which results from deficiencies in the host immune defense system or damage to the intestinal mucosal barrier (Berg and Fuller, 1992; Ringo and Birkbeck, 1999). In addition, water temperature and bacterial count was directly and significantly correlated with one another. It was also proven in the study of Rodkhum *et al*. (2011) that Nile tilapia reared in high water temperature condition was more susceptible to *S. agalactiae* via water exposure route; this was supported by higher recorded mortality at 33 ˚C as compared to 30 ˚C and 25 ˚C. Clinical signs of bacterial infection on fish maintained at 25 ˚C were not observed (Rodkhum *et al.,* 2011).

The high prevalence of *Streptococcus* spp. in the majority of tilapia organs during the dry season might lead to the activation of the adaptive immunity of the fish. Regardless of the influence of stress or pathogen, the adaptive system tends to be activated at high temperature. As the recorded average temperature during the dry season was still conducive to Nile tilapia, there is no decrease in the non-specific and specific immune responses of the fish (Le Morvan *et al*., 1998; Ndong *et al.,* 2007). The adaptive arm of the fish immune system is inducible, pathogen-specific, and generally results in immunological memory by cellular (B cells and T cells) and humoral (antibodies) components (Uribe *et al.,* 2011; Zhu *et al.,* 2013). T lymphocytes recognize antigens presented by cells expressing MHC I and II and, upon recognition, induce specific cytotoxicity or release cytokines that act on other lymphocytes and innate immune cells to direct a specific response against a pathogen, respectively (Nakanishi *et al.,* 2015). B lymphocytes secrete antibodies upon antigen recognition/activation, which then perform a number of functions, including opsonization, neutralization, agglutination, and complement activation (Nakanishi *et al.*, 2015).

It has been suggested that *Streptococcus* sp. can withstand the bactericidal activity of blood macrophages and multiply in the blood of tilapia (Eldar *et al.*, 1994; Evans *et al.*, 2002; Hernandez *et al.*, 2009; Jantrakajorn *et al.*, 2014; Zlotkin *et al*., 2003; Zamri-Saad *et al.*, 2010). Many streptococcal species can prevent phagocytosis and opsonisation through surface antigens or an extracellular polysaccharide capsule (Fuller *et al.*, 2001; Lindahl *et al.*, 2005; Locke *et al.*, 2007). Failure of initial phagocytosis and killing of the bacteria by the host immune response would thus enhance their survival and systemic proliferation. As macrophages may also act as a carrier for the pathogens, their dissemination to other organs and tissues would potentially be facilitated (Bowater *et al.*, 2012; Evans *et al.*, 2001; Nguyen *et al.*, 2002; Zlotkin *et al.*, 2003) and this may help to explain the widespread presence of *Streptococcus* spp. among all the examined organs of the Nile tilapia samples in this present study.

In the study of Pradeep *et al.* (2007), the highest prevalence of *Streptococcus* spp. in Nile tilapia was in the liver (*S. agalactiae*, 75% and *S. iniae*, 50%) which was also observed in this study. During the rainy season, the liver posted the highest prevalence of the bacterium (92.25±7.69%). The presence of the pathogens was also commonly detected in other organs such as the gills (*S. agalactiae*, 70% and *S. iniae*, 30%) and kidney (*S. agalactiae*, 65% and *S. iniae*, 35%) (Pradeep *et al.*, 2007). As the *Streptococcus* spp. was detected in the organ that is involved in the defense of fish from pathogens, such as the kidney, it is possible that the reported mortalities in the early developmental phases in farmed tilapia due to streptococcosis could be due to their compromised defense mechanisms. Experimental infection of *S. agalactiae* in red tilapia (*Oreochromis* spp.) has revealed that this pathogen can pass though the gastrointestinal epithelium and can accumulate and localize in the kidney as one of the preliminary organs of infection (Iregui *et al.*, 2015). Although blood could be used as a non-lethal and easy source of pathogen detection, the results of Pradeep *et al.* (2007) showed that *Streptococcus* spp. are localized more in the organs than in the blood. Other experimental studies on the pathogenicity of *Streptococcus* spp. in tilapia have recommended that brain, eye and kidney tissues are the main target organs (Abuseliana *et al*., 2011; Rodkhum *et al.*, 2011).

Streptococcus spp. are considered to be neurotropic in tilapia due to the symptoms generated from the central nervous system, such as aberrant fish behavior and swimming patterns (Evans *et al.*, 2000). Lymphocytic infiltration of brain is usually reported in clinical streptococcosis of tilapia (Chang and

Plumb, 1996; Suanyuk *et al.*, 2010) and it is one of the most infected organs by *Streptococcus* spp. (Klesius *et al.*, 2008). In the current study, the brain tissue from red tilapia was confirmed to contain *Streptococcus* spp. during the dry (85.25±13.52%) and rainy (80.75±14.44%) season suggesting that these pathogens may use immunologically privileged sites, such as the brain, as the immune function in these organs is weak or reduced (Simpson, 2006).

Based on the findings of Iregui *et al.* (2004), invasion of *S. agalactiae* in tilapia through skin is of low frequency, with little effect on the subcutaneous tissue and muscles. The finding of their experiment was similar to the results obtained in this study because of lesser counts of *Streptococcus* spp. on the skin of the fish during the two seasons. The skin of fish is coated by mucus, which is continuously secreted by goblet cells and contains antibodies as well as lysozyme. The cells of the epidermis are the next barrier followed by the scales (Sicherer, 2001). Fish are often more vulnerable in areas not covered by mucus or scales, such as the gills; macrophages may therefore be found on gill surfaces (Sicherer, 2001). The *Streptococcus* spp. count within the intestine of the fish was rather dense and highest during the dry season, indicating that the intestine provides a favorable ecological niche for this bacterium (Horsley, 1977). The study of Bowater *et al*. (2012) demonstrated that macrophages can possibly act as a vehicle for *S. agalactiae*, allowing it to cross the blood-brain barrier and gain access to the central nervous system, thereby becoming disseminated throughout the organism's organ systems. This might be the reason why the bacterium was isolated from all the organs of the fish in this study. The kidney and spleen are the major sites of phagocytic cells and this can be the reason of the high count in the kidney of the fish in both seasons (Romano *et al.*, 1997). In *S. agalactiae* - infected Nile tilapia after 2 to 4 days post inoculation (PI), the recovered count of the bacterium was 7.70±0.70 log10 CFU/g in the brain, 7.58±0.50 log10 CFU/g in the kidney and 6.79±0.40 log10 CFU/g in the liver (Rodkhum *et al.*, 2011).

Three groups of possible risk factors in the occurrence of *Streptococcus* spp. were considered, namely operational/cultural practices, water quality and sediment quality. According to the study of Reyes and Reyes (2019), the following operational/cultural practices were recommended in Lubao, Pampanga for a successful tilapia business: medium-scale of operation (3 to 5 ha); complete pond preparation that includes draining, elimination of predators and nuisance weeds, harrowing, drying, repairing of dikes, water inlet and outlet canals, screening of water canals, liming, initial filling of water and leveling of pond bottom, and fertilization; safe source of water such as deep well; frequent water exchange (2 to 3 times a week); moderate stocking rate (4 to 8 pcs./m^2); and feeding based on the average body weight of the fish. The recommended water and sediment quality that should be maintained in order to prevent the occurrence of *Streptococus* spp. are as follows: temperature not exceeding 31 ◦C; TDS below 400 mg/L; water pH not exceeding 8; DO not lower than 5 mg/L; alkalinity in between 50 to 200 mg/L; NO_2 below 0.02 mg/L; water P within 0.005 to 0.2; sediment pH from 7 to 8; organic matter below 5.8%; and sediment P below 80 mg/L (Yanong and Francis-Floyd, 2002; Reyes and Reyes, 2019).

To prevent the occurrence or build-up of the bacterium in the pond system, the identified risk factors associated with operational/cultural practices should be changed (*e.g.* reduction of stocking rate to 4 to 8 pcs./m^2, use of ponds less than 5 ha). Risk factors related to water and sediment quality should be improved or maintained in ideal concentration as discussed above (*e.g.* pH should be buffered using lime, provision of paddle aerators to support the oxygen requirement of the fish, feeding based on fish average body weight to lessen accumulation of NO_2, P and organic matter in the water and sediment).

Tilapia aquaculture in Pampanga is dependent on Pampanga River, river tributaries and irrigation canals as main sources of water. However, the river has been reported to be already highly polluted and contaminated based upon physical, chemical and biological analysis of its water and sediment (Atayde and Reyes, 2018 unpublished; Reyes and Labasan, 2018). The water had very low DO (2.66 to 3.43 mg/L) and very high P (0.30 to 0.60 mg/L). High level of P in water coupled with low DO is good indication of eutrophication (Reyes and Labasan, 2018). A number of pathogenic bacteria in fishes (*A. hydrophila* and *P. aeruginosa*) and humans (*Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*) were also isolated from the water and sediment of Pampanga River in the study made by Atayde and Reyes in 2018 (unpublished). The total bacterial count (TBC) and total coliform count (TCC) observed in the water was 1.93×10^7 to 2.72×10^8 CFU/mL and 1.45×10^5 to 3.30×10^6 CFU/mL, respectively, while in sediment, the TBC was 3.62×10^9 to 4.38×10^9 CFU/g and the TCC was 1.19×10^5 to 1.06×10^6 CFU/g (Atayde and Reyes, 2018 unpublished). Although the RR value of water source was not amenable to computation in this present study, still, the water from the river and river system is unsafe for use in tilapia aquaculture in Pampanga. Basically, the tilapia operators are pumping river water to their pond system containing high level of P, deficient in DO along with pathogenic organisms. Based on interview, the immediate remedy in case of fish mortality which was water exchange could only aggravate the problem because of the unsafe source of water from the river. Improper pond preparation practices such as absence of pond drying and liming, and intensive stocking could be considered as additional risk factors for the deterioration of the quality of water in ponds. The interviewed farmers failed to follow the complete set of activities involved in pond preparation (*e.g.* draining, elimination of predators and nuisance weeds, harrowing, drying, repairing of dikes, water inlet and outlet canals, screening of water canals, liming, initial filling of water and leveling of pond bottom, and fertilization). Pond preparation should be done properly in order to provide the fish an environment which is free of pests and predators, and a pond bottom suitable for growth of natural food. Most of the tilapia growers in Pampanga practice an intensified stocking rate in order to replenish loss of stocks due to mortality. As the density of susceptible individual increases, so does the probability that an infectious disease epidemic will occur (Anderson and May, 1992). Not surprisingly, this increase in fish density led to *Streptococcus* spp. disease outbreaks. Increase in stocking rate beyond the biological capacity of the pond system could necessitate the total dependency in commercial feeds. Excess feed input and accumulated waste in the system could deteriorate both the water and sediment leading to high NO_2 and P. NO_2 was identified as risk factor during the rainy season and this was supported by slightly higher NO_2 (0.04 ± 0.04 mg/L) concentration. Roughly, 25% of the P in aquafeeds is harvested in aquaculture biomass and the remainder is in uneaten feed and feces that are decomposed resulting to the release of phosphate, or metabolic excretions of culture animals (Boyd, 2007). However, almost 80% of the unrecovered P in harvested fish is found in the pond bottom. To lessen the effect of water and sediment pollution due to intensive feeding, the fish should be fed based upon their average body weight.

Aside from high temperature or drastic temperature fluctuations, other environmental stressors related to outbreaks of streptococcosis include pH above 8 and low concentration of DO (Chang and Plumb, 1996; Bunch and Bajerano, 1997; Bowser *et al.*, 1998; Yanong and Francis-Floyd, 2002; Mian *et al.*, 2009). In this present study, the recorded average pond water pH in both seasons was beyond 8 due to accumulation of nutrients from feeds coupled with high temperature; also, average DO readings in both seasons were below the recommended concentration for tilapia farming. Oxygen is the main limiting factor for fish health; it is claimed that the metabolism, growth and disease resistance are depressed when the DO falls below 1 mg/L for extended periods (Popma and Masser, 1999), which predispose tilapia to streptococcosis and other diseases. In addition, Evans *et al*. (2013) demonstrated that long periods of low DO level in the water increased a stress response in the fish which lead to impaired immune response resulting in decreased resistance against *S. agalactiae* in experimental Nile tilapia. Generally, excessive feed inputs to support high fish density have resulted to the elevation of nutrients such as phosphorus in ponds and this phenomenon might trigger the overgrowth of algae in water. The subsequent death of algae will lead to the demand for oxygen which is used in the microbial decomposition of the algae. It is reported that sublethal DO levels cause hypersecretion of catecholamines and corticosteroids in fish with changing in blood glucose level (Mazeaud *et al*., 1977; Wedemeyer and McLeay, 1981). Blood glucose is detected and considered to be reliable indicator of stress responses in fish (Thomas and Robertson, 1991; Rotllant and Tort, 1997). Hyperglycemia resulted to change the liver glycogenolysis which increased conversion of reserved glycogen to glucose (Mazeaud and Mazeaud, 1981). This concerns a severe energy demand to the depletion of reserve glycogen on the stressed fish. The severe energy demand causes an energy crisis resulting in the impairment of resistance to pathogens (Wedemeyer, 1976; Screck, 1981). The DO of the water during the two seasons was identified as risk factors by the below optimum average DO recorded during the dry (3.47 ± 2.05 mg/L) and rainy season 3.77 ± 0.93 (mg/L).

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