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Effect of UV light exposure on the ampicillin susceptibility and Pathogenecity of *Enterococcus faecalis* to Nile tilapia (*Oreochromis niloticus*)

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Abstract
The main objective of this study was to expose wild type *Enterococcus faecalis* via ultraviolet (UV) irradiation and to compare its antibiotic susceptibility and pathogenicity to its wild counterpart. During the 24 hours and 48 hours of incubation, the zone of inhibition (ZOI) of wild type and UV-treated *E. faecalis* showed significant difference among the ampicillin dosages examined (0, 5, 10, 20, 40, 60, 80, 100, 200 and 400 µg/20 µL) except at 80 µg/20 µL for 24 and 48 hours, and 200 and 400 µg/20 µL for 48 hours. As a general trend, the wild type *E. faecalis* had higher ZOI as compared to the UV-treated *E. faecalis* at the majority of ampicillin dosages. Both the wild and UV-treated bacterium was classified as intermediate at 5 µg/20 µL and susceptible in the rest of ampicillin dosages according to the classification of Clinical Laboratory Standard Institute. In general, there was no difference in the minimum inhibitory concentration (MIC) of wild and UV-treated *E. faecalis*. Ampicillin with a breakpoint of 5 µg/20 µL was comparable with the results obtained in Kirby-Baeur method in which both isolates were classified as susceptible for as low as 5 µg/20 µL. It was also proven that the commensal *E. faecalis* can cause diseases or even death (40% mortality) to cultured tilapia at the density of 10^8 cells/mL. The gross signs of the enterococossis have appeared as early as day 2 post-inoculation (PI) and began to cause death after day 5 PI.

Keywords: Nile tilapia, *Enterococcus faecalis*, antibiotics, ampicillin, pathogenicity

Introduction
Aquaculture is growing rapidly in the many regions of the world, and aquaculture products constitute an important food supply with increasing economic important [1]. The industry covers a wide range of species and methods, from a traditional system, in which a fish is reared in small ponds for domestic consumption, to intensive industrial scale production system [1].

Disease outbreaks are considered to be a significant constraint to the development of the aquaculture sector, with a global estimate of disease losses of 7 billion US$ per year. The aquatic environment is more supportive to pathogenic bacteria independently of their host than the terrestrial environment and consequently, pathogen can reach high densities, which then ingested together with the feed or when fishes take up water [2]. As a result, cultured fishes suffer from highly unpredictable survival rates because of bacterial diseases [2].

Antibiotics are used in aquaculture in attempts to control bacterial diseases [2]. The wide range and frequent use of antimicrobial agents in aquaculture has resulted in the emergence of reservoirs of antimicrobial-resistant bacteria in fish and other aquatic animal, as well as in the aquatic environment [3]. A high incidence of bacteria resistant to the antimicrobials used in aquaculture, including multiple resistant bacteria, has been found in fish farms and the surrounding aquatic environments [4-5].

Enterococci bacteria are commensal bacteria that typically colonize the gastrointestinal tract of most animals and occasionally colonize the oral cavity and vaginal tract [6]. Enterococci are intrinsically resistant to many antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon acquisition containing genetic sequences that confer resistance in other bacteria [7]. Enterococci have been isolated from different aquatic habitats, such as wastewater, pristine water and aquaculture ponds [7].

Among commercially important fish species, enterococci have been reported worldwide in yellowtail (*Seriola* spp.), eels (*Anguilla japonica*), menhaden (*Brevoortia patronus*), striped mullet (*Mugil cephalus*) and striped bass (*Morone saxatilis*) [8]. Among the dozens of different enterococcal species, *Enterococcus faecalis* is the main cause of human enterococcal infections [6]. This is linked to the ruggedness of bacterium, which allows the organisms to survive many host defenses, compounded by the acquisition of a variety of variable virulence traits by horizontal genes transfer from other organisms [9].

DNA damaging agents such as UV irradiation may result to changes in genome [10]. Bacteria generally possess molecular mechanisms, such as photoreactivation and dark repair systems to restore its DNA lesions. Light-induced reactions have been analyzed already with respect to the survival of the organisms [11]. Meanwhile, bacteria use at least three different dark-activated repair mechanisms [12]. All mechanisms are regulated by the expression of recA gene, the key gene in this system [12]. The gene product recA is a multifunctional protein involve in a number of cellular process, including homologous recombination, DNA repair, SOS response and coordination of cell division [13].

The main objective of this study was to expose wild type *E. faecalis* via ultraviolet (UV) irradiation. The specific objectives of this study were as follows:

1. To establish 90% killing curve that was used as basis for mutagenesis experiment;
2. To expose wild type *E. faecalis* using the duration of UV exposure that gives 90% kill from the establishment of killing curve;
3. To compare the antibiotic susceptibility of wild and UV-treated *E. faecalis* using ampicillin;
4. To compare the minimum inhibitory concentration (MIC) of wild and UV-treated *E. faecalis*; and
5. To assess the pathogenicity of wild and UV-treated *E. faecalis* to Nile tilapia.

2. Materials and Methods

2.1. Source and maintenance of *Enterococcus faecalis*

Pure culture of *E. faecalis* was obtained from the Fish Pathology Laboratory of the College of Fisheries in Central Luzon State University Philippines. The identity of isolate was confirmed by 16s rRNA sequencing. The isolate was maintained in Trypticase Soy Agar (TSA) with mineral oil and stocked under room temperature.

2.2. Establishment of killing curve

E. faecalis was streaked on TSA plate and it was incubated at 30 ^{0}C for 18 to 24 hours. A loopful of bacterium was transferred into 10 mL Trypticase Soy Broth (TSB) for overnight incubation. Three milliliter from the overnight culture was sub-cultured to 30 mL TSB. The cell population was estimated using the McFarland standards. UV lamp was turned on at least 30 minutes prior to use. The culture was centrifuged and the cell pellet was resuspended in appropriate volume (~40 mL) of 0.1 M $MgSO_4$ to obtain a cell density of 1 x 10^8 cells/mL. The resuspended cell pellet was incubated for 3 hours. Five milliliter (5 mL) of the cell suspension was transferred to sterile plates. The plate was placed at a distance of 24 to 30 inches from UV lamp. The plate was opened to expose the cell suspension for 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 seconds. The exposure was strictly conducted in the absence of light to prevent photoreactivation. In every exposure, the cells were subjected into a series of ten-fold dilutions up to 10^{-6} of the cell suspension. Around 0.1 mL from 10^{-3} to 10^{-6} dilutions was spread on TSA plates and the plates were incubated at 30 ^{o} C for 18 to 24 hours. Colonies were counted after the prescribed incubation time. Percent survival was plotted against period of exposure to determine exposure period that gives 90% kill [14].

2.3. Mutagenesis

A loopful of 18 to 24 hours *E. faecalis* was transferred into 10 mL TSB for overnight incubation. Three milliliter from the overnight culture was sub-cultured to 30 mL TSB. The culture was centrifuged and the cell pellet was resuspended in appropriate volume (~40 mL) of 0.1 M $MgSO_4$ to obtain a cell density of 1 x 10^8 cells/mL. The resuspended cell pellet was incubated for 3 hours. Five milliliter (5 mL) of the aliquots was exposed to UV light for the period of 90% kill. Five milliliter (5 mL) from treated cell suspensions was separately centrifuged. The cell was washed with Physiological Saline Solution (PSS) and it was resuspended in 10 mL TSB for 2 hours' incubation at 30 ˚C. Harvested cells were washed once using PSS and resuspended in 5 mL TSB and incubated at 30 ˚C for 30 minutes. A series of 10-fold dilutions were prepared and 0.1 mL of 10^{-3} to 10^{-6} dilutions was spread on TSA plates. The plates were incubated at 30 ˚C for 24 hours. The number of bacterial colonies in treated cell suspensions was counted. A representative colony was used for antibiotic susceptibility testing [14]. The viable colonies were counted and expressed as CFU/mL using the formula shown in equation 1 and 2.

$$\frac{CFU}{mL} = \frac{average\ number\ of\ colonies\ x\ dilution\ factor}{volume\ plated\ (mL)} \quad (1)$$

$$\frac{CFU}{mL} = \frac{\sum C}{[(1\ x\ n1) + (0.1\ x\ n2)]\ (d\ x\ Vp)} \quad (2)$$

Whereas

C = colony counts
n_1= no. of plates in 1st dilution counted
n_2= no. of plates in 2nd dilution counted
d = dilution from which the 1st counts were obtained
V_p= volume plated

2.4. Preparation of filter paper discs

Approximately 6 mm holes were made in Whatman filter paper No. 3 using a puncher. The filter paper discs were autoclaved at 15 lbs pressure for 30 minutes.

2.5. Preparation of antibiotic stock solution

Powdered form of ampicillin was purchased in drugstores. In order to obtain a stock solution of 20 µg/µl, a known weight of the antibiotics was dissolved in sterile distilled water. The stock solution was diluted at the time of disc preparation to obtain the working solution of 10 mL. The concentrations of antibiotics solutions that were evaluated are presented in Table 1. Using a micropipette, a fixed volume of 20 µl was loaded on each disc one by one.

Table 1: Computed volume of stock solution in each concentration of working solution

Concentration of Stock Solution (µg/µl)	Volume of Stock Solution (mL)	Concentration of Working Solution (µg/20 µl)	Volume of Working Solution (mL)
20	0.00	0	10
20	0.13	5	10
20	0.25	10	10
20	0.50	20	10
20	1.00	40	10
20	1.50	60	10
20	2.00	80	10
20	2.50	100	10
20	5.00	200	10
20	10.00	400	10

2.6. Drying and impregnation of discs

The antibiotic discs were allowed to dry in a clean incubator at 37 °C for 4 hours. Meanwhile, about 2 to 3 colonies of 18 to 24-hour bacterium were suspended in TSB. The tube was incubated at 37 °C for 1 to 2 hours. The bacterial suspension was adjusted to 0.5 McFarland turbidity standards and was evenly spread in TSA plates using a sterile cotton swab. After the inoculum has dried, the prepared antibiotic discs were placed on the surface of the inoculated plate using sterile forceps. The plates with discs were incubated at 37 °C and were observed after 18, 24 and 48 hours of incubation. The diameter of the zone of inhibition was measured in millimeters using ruler. The susceptible, intermediate and resistant categories were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute [15].

2.7. Determination of minimum inhibitory concentration

One milliliter of the working antibiotics solution (0, 5, 10, 20, 40, 60, 80, 100, 200 and 400 µg) and 9 mL of TSA were poured in a sterilized plate. Around 3 to 4 colonies of wild and UV-treated *E. faecalis* was cultured in 3 mL TSB tube. The tube was incubated at 37 °C for 18 to 24 hours until it achieved the turbidity of 0.5 McFarland standards. The standardized inoculum was diluted in sterile distilled water (1:10) to obtain a concentration of 10^6 CFU/mL. From the diluted inoculum, 0.1 mL was streaked on the surface of prepared TSA plates. The plates were incubated at 37 °C for 18 to 24 hours. The MIC was taken as the lowest concentration that inhibits the growth of the bacterium.

2.8. Challenge test

Glass aquaria with water capacity of 50 L and dimension of 60 cm x 30 cm x 42 cm were used in pathogenicity test. Each aquarium was stocked with 10 pieces (25 to 30 g) acclimatized tilapia.

A 0.1 mL of UV-treated *E. faecalis* with approximate concentration of 10^8 cells/mL was injected intraperitoneally to the experimental fish. The same procedure was applied for wild *E. faecalis*. Tilapia in control group was injected with the same volume of sterile distilled water. The fish was fed twice a day at 5% body weight. Clinical signs and morbidity were recorded daily for a period of four weeks. The set-up was terminated when 100% morbidity or mortality had occurred among the challenged group. Dead or moribund fish was necropsied, and smears from kidney was serially diluted (10^{-3} and 10^{-4} dilutions) and spread aseptically on TSA plates. The plates were incubated at 37 ˚C for 18 to 24 hours. Mortalities were valid if the recovered colonies in TSA have the same morphological characteristics with *E. faecalis*. Simple test such as catalase test and gram staining was done for further identification of recovered bacteria. Meanwhile, water quality parameters such as temperature, pH and dissolved oxygen were only assessed during the pathogenicity set-up.

2.9. Statistical analysis

Trendline analysis in Microsoft Excel was used in determining 90% kill in the establishment of killing curve for mutagenesis experiment. T-test analysis was used in determining significant differences in the zone of inhibition between wild and UV-treated *E. faecalis.* Analysis of variance (ANOVA) was used in determining significant differences in water quality parameters during pathogenicity assay and Tukey's test was used for the post hoc comparison of means.

3. Results and Discussion

3.1 Establishment of killing curve for the mutagenesis experiment

Initial experiment was done, applying the liquid-plate irradiation protocol, to determine the appropriate duration for UV irradiation that will result to 90% kill in the bacterium. The UV light used on the establishment of killing curve has a wavelength of 254 nm according to the manufacturer. Table 2 shows the pronounced reduction in bacterial count expressed in colony forming unit per millilitre (CFU/mL) against various durations of UV light exposure. The initial viable cell count of *E. faecalis* used in killing curve experiment was 9.42 x 10^9 CFU/mL. Highest percent kill was obtained at 45 seconds of exposure to UV light followed by 40, 35, 30, 25, 20, 15, 10, and 5 with a corresponding viable colony count of 1.2 x 10^9, 8.81 x 10^8, 8.45 x 10^8, 4.48 x 10^8, 2.8 x 10^8, 1.34 x 10^8, 7.8 x 10^7, 3.6 x 10^7 CFU/mL, respectively. Ninety percent kill was obtained at 8.54 seconds and was computed using the trendline analysis in Microsoft Excel (Figure 1). The mutagenesis experiment was repeated using 8.54 seconds as the duration of UV light exposure.

Table 2: Viable colony count and percent kill of *E. faecalis* at different duration of exposure to UV light

Duration of Exposure (seconds)	Colony Count			CFU/mL	% Kill
	10^{-4}	10^{-5}	10^{-6}		
0	1341	1140	942	9.42×10^{9}	--
5	--	--	120	1.20×10^{9}	87.2611
10	--	66	31	8.81×10^{8}	90.6389
15	94	44	49	8.45×10^{8}	91.0249
20	130	69	48	4.48×10^{8}	94.9045
25	165	32	28	2.80×10^{8}	97.0276
30	121	134	34	1.34×10^{8}	98.5775
35	--	78	105	7.80×10^{7}	99.1719
40	--	36	56	3.60×10^{7}	99.6178
45	90	57	70	9.00×10^{6}	99.9045

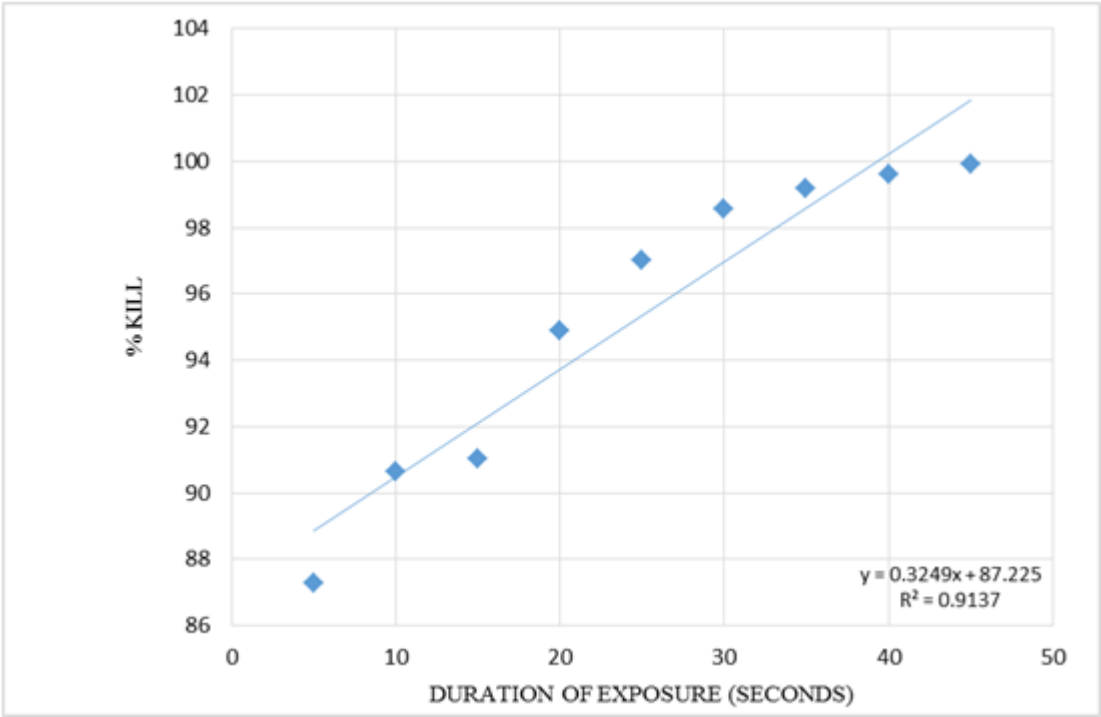

Fig 1: Scattered plot for the establishment of 90% killing curve of *E. faecalis* using the trendline analysis in MS Excel

According to Kalisvaart [16] the spectrum of the UV radiation is divided into four sub-regions: UV-A (315 to 400 nm), UV-B (280 to 315 nm), UV-C (200 to 280 nm) and vacuum UV (100 to 200 nm). Genomes, proteins and enzymes with unsaturated bonds are known to absorb UV-C and UV-B, which may result to significant damage to the organisms [16]. In this experiment, the UV light that was used to establish the killing curve has a wavelength of 254 nm which is categorized as UV-C. UV light with the wavelengths from 100 to 400 nm is enough to cause damage to DNA, cell membranes and cytoplasmic proteins [17].

Induced mutation is a product of treatment by physical or chemical agent that changes that genetic material. Examples of chemical mutagens are ethyl methane sulphate (EMS), methyl methane sulfonate (MMS), diethylsulfate (DES), nitrosoguanidine (NTG, NG, MNNG) and nitrous acid [18]. UV light can also induce mutation by causing DNA lesions that block DNA replication [19]. In this study, wild type *E. faecalis* was exposed to UV light at 8.54 seconds that resulted to 16.67% presumed mutagenesis. Prior to the experiment, the latter bacterium was exposed to magenesium sulfate ($MgSO_4$) which is widely used in genetic experiment and has the tendency to increase the rate of mutation since the Mg^{2+} is a cofactor for a lot of DNA repair and replication enzymes which are high in demand for cells with UV damaged DNA.

Mutation rates in bacteria can be increased by stress-induced reversible activation of some gene functions, which results in a transient mutator phenotype and the SOS response is being the paradigm process [20]. In other literature, they mentioned that mutations arise in stressed bacteria only as an accidental result of repairing of DNA lesions [21]. Mismatch repair system is said to be one of the responsible for the mutagenesis of damaged DNA and consequently the majority of these mutations are arise during DNA synthesis [21].

The greatest absorption of DNA and RNA of UV radiation is at 260 nm. The purine and pyramidine bases of nucleic acids absorb the UV radiation strongly in which it causes damage to DNA. Pyramidine dimers, in which two adjacent pyrimidine bases (cytosine and thymine) on the same strand of the DNA become covalently bonded to another one. This greatly inhibits DNA polymerase activity or greatly increases the probability of DNA polymerase misreading of sequence and may resulted to mutation. By definition, mutation is a heritable change in the genetic material. Therefore, if damaged DNA can be corrected before the cells divide (~ 30 minutes), no mutation will occur. Cells

have variety of DNA repair processes to correct mistakes and repair damage. Some type of damage particularly the large-scale damage may cause lesions which interfere with replication. The SOS system initiates a number of DNA repair. The SOS systems allows DNA repair to occur without a template which may cause error in incorporation of base pairs hence mutations occur. The SOS systems are greatly discussed by Madigan *et al*. [22] in which the SOS system is regulated by two proteins, LexA and RecA. LexA is a repressor that normally prevents the expression of the SOS system. The RecA protein, which normally functions in genetic recombination, is activated by the presence of damaged DNA, particularly in single-stranded DNA that results when replication is delayed. The activated form of RecA stimulates the LexA to inactivate itself by self-cleavage that leads to the derepression of the SOS system and results in the coordinated expression of a number of proteins that take part in DNA repair [22].

In the study of McKinney and Pruden [23], they observed that the gram-positive bacteria were more resistant than in gram-negative in UV disinfection. This suggests that the thicker peptidoglycan layer of gram-positive organisms may offer some protection against UV inactivation [23]. They also found out that the UV is inadequate in its potential to damage antibiotic resistance genes in bacteria. Gram-positive bacteria that were used in their experiment possessed smaller total genome sizes, which also have reduced their susceptibility to UV because of fewer potential pyrimidine dimer targets [23].

3.2. Ampicillin susceptibility testing

Widespread use of antibiotics in aquaculture as prophylactic and therapeutic agents to bacterial diseases has been associated with the emergence of antibiotic resistance in bacterial pathogen and the alteration of the microbiota of aquaculture environment [24, 25]. According to Lukasova and Sustackova [26], *Enterococcus* spp. is a good indicator of antimicrobial resistance in animals, human, and the environment including soil, manure, and water samples. They are known for the capability to acquire resistance determinants by rapid adaptation to environmental conditions. Resistance to antimicrobial drugs can arise either from new mutations in the bacterial genome or through the acquisition of genes encoding antibiotic resistance. These genetic changes consequently alter the defensive function of the bacteria by changing the target of the drugs, by detoxifying or ejecting the antimicrobial, or by routing metabolic pathways around the disrupted point [27].

In the ampicillin profile against *E. faecalis*, both wild and UV-treated exhibited a statistically significant difference at 95% confidence in all antibiotic dosages tested in 18 hours of incubation. Furthermore, in 24 hours and 48 hours of incubation, the zone of inhibition (ZOI) of wild type and UV-treated bacterium showed significant difference among dosages examined except at 80 µg/20 µL for 24 and 48 hours, and 200 and 400 µg/20 µL for 48 hours. Highest ZOI was observed at 400 µg/20 µL (18 hours = 32.50±1.05; 48 hours = 29.67±0.61) in the wild type bacterium. As a general trend, the wild type bacterium has higher ZOI as compared to the UV-treated bacterium at the majority of dosages examined. UV mutagenesis in *E. faecalis* has resulted to decrease in the ZOI to ampicillin (Table 3).

Both the wild and UV-treated bacterium was classified as intermediate at 5 µg/20 µL and susceptible in the rest of ampicillin dosages according to the classification of Clinical Laboratory Standard Institute [15]. If ampicillin will be utilized as treatment for this bacterium, the minimum recommended rate is 10 µg/20 µL to have a better result. No significant changes in the classification of both isolates being assessed even there was consistent reduction in the ZOI (Table 4).

In veterinary, one of the uses of ampicillin is for the treatment of multidrug-resistant *E. faecalis* and *E. faecium* according to the report of Magdesian [28]. Ampicillin is a semi-synthetic type of beta-lactam antibiotics. Beta-lactams antibiotics are cell wall synthesis inhibitors. Since the cell wall and its synthesis mechanisms are unique in to bacteria, the beta-lactam antibiotics are highly selective and non-toxic to host cells. Since the benzylpenicillin are active against gram-positive bacteria because the gram-negative are impermeable to the antibiotic, chemical modification broadens the actions of the beta-lactams group of antibiotics. Ampicillin for instances extends its activity against gram-negative group of bacteria. Structural differences allow the ampicillin to be transported inside the gram-negative outer membrane where they inhibit cell wall synthesis. Despite of chemical modification, ampicillin just like other beta-lactams antibiotics is sensitive to beta lactamase, an enzyme produced by a number of penicillin-resistant bacteria [22].

Table 3: Zone of inhibition of wild and UV-treated *E. faecalis* against ampicillin at various dosages and hours of incubation

Antibiotic Dosages (µg/20 µL)	18 hours		24 hours		48 hours	
	Wild Isolate	UV-treated Isolate	Wild Isolate	UV-treated Isolate	Wild Isolate	UV-treated Isolate
0	6.00±0.00^{a}	6.00±0.00^{a}	6.00±0.00^{a}	6.00±0.00^{a}	6.00±0.00^{a}	6.00±0.00^{a}
5	18.50±1.10^{a}	16.67±2.04^{b}	18.50±0.84^{a}	17.00±2.28^{b}	18.50±0.95^{a}	16.00±2.53^{b}
10	23.50±0.27^{a}	20.08±0.49^{b}	23.25±0.69^{a}	20.25±0.42^{b}	22.83±0.93^{a}	19.75±0.52^{b}
20	24.33±0.27^{a}	22.92±1.20^{b}	24.75±0.99^{a}	23.00±0.84^{b}	25.50±0.63^{a}	22.83±1.13^{b}
40	26.75±0.69^{a}	25.08±0.66^{b}	26.92±0.49^{a}	25.25±0.76^{b}	26.67±0.75^{a}	25.33±0.75^{b}
60	28.08±1.36^{a}	26.42±0.80^{b}	29.75±0.61^{a}	26.50±0.84^{b}	28.00±1.45^{a}	26.25±0.42^{b}
80	30.33±0.52^{a}	27.00±1.10^{b}	28.33±3.72^{a}	27.33±1.17^{a}	28.08±1.63^{a}	27.08±0.66^{a}
100	31.00±1.26^{a}	27.58±0.74^{b}	30.67±1.03^{a}	27.92±0.58^{b}	28.92±0.58^{a}	27.33±0.61^{b}
200	31.67±0.52^{a}	28.58±0.86^{b}	32.17±1.17^{a}	28.75±1.78^{b}	28.50±0.45^{a}	29.08±1.69^{a}
400	32.50±1.05^{a}	29.58±0.20^{b}	32.00±0.63^{a}	29.67±0.61^{b}	30.58±0.92^{a}	29.58±1.02^{a}

Note: Statistical comparison was made between wild and UV-treated *E. faecalis* at various dosages and hours of incubation; different superscript was statistically significant at $p< 0.05$

Table 4: CLSI classification of wild and UV-treated *E. faecalis* at different dosages of ampicillin and hours of incubation

Antibiotic Dosages (µg/20 µL)	18 hours		24 hours		48 hours	
	Wild Isolate	UV-treated Isolate	Wild Isolate	UV-treated Isolate	Wild Isolate	UV-treated Isolate
0	--	--	--	--	--	--
5	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate
10	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
20	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
40	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
60	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
80	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
100	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
200	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
400	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

3.3. Minimum inhibitory concentration of ampicillin

MIC is considered to be the "gold standard" for determining the susceptibility of organisms to antimicrobials among all antimicrobial sensitivity testing (AST). MIC's are used in diagnostic laboratories to confirm the unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of AST. The MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after incubation [29].

The antimicrobial activity of the various dosages of ampicillin against the wild and UV-treated *E. faecalis* was presented in Table 5. In general, there was no difference in the MIC of wild and UV-treated *E. faecalis*. Ampicillin with a breakpoint of 5 µg/20 µL was comparable with the results obtained in Kirby-Baeur method in which both isolates were classified as susceptible for as low as 5 µg/20 µL. Starting at 10 µg/20 µL dose of ampicillin, the bacterium can be totally inhibited.

Resistance to the antibiotics is mediated by the permeability barrier and/or efflux system, intrinsic insensitive target enzymes, regulational, mutational and recombinational changes in the target enzymes genes and acquired drug-resistant genes [30]. The emergence of antibiotic resistance among fish pathogens undermines the effectiveness of the prophylactic use of antibiotics in aquaculture [31] and increases the possibilities for passage not only of these antibiotic-resistant bacteria but also their antibiotic resistance determinants to bacteria of terrestrial animals and human being, including the pathogen.

Table 5: MIC of ampicillin against wild and UV-treated *E. faecalis*

Bacterial Isolate	Working Concentrations (µg/20 µl)									
	0	5	10	20	40	60	80	100	200	400
Wild Type	+	*	-	-	-	-	-	-	-	-
UV-Treated	+	*	-	-	-	-	-	-	-	-

Note: * = breakpoint, + = positive growth, - = negative growth

3.4. Pathogenicity assay

The current trend in aquaculture development is towards the increased in intensification and commercialization of aquatic product. Like other farming sector, the likelihood of major disease problems increases as aquaculture activities intensify and expand [32]. In this study conducted, it was proven that the commensal *E. faecalis* can cause diseases or even death (40% mortality) to cultured tilapia at the density of 10^8 cells/mL. The *E. faecalis* pathogenesis consists of two mechanisms; these may cause pathologic changes such as the host inflammatory cascade or by direct damage as a result of secreted toxins or proteases [33]. Entorococcal cytolysin and two proteases, a zinc metalloprotease (gelatinase) and a serine protease are secreted factors that contribute to the severity of disease [34]. In addition to secreted proteins, *E. faecalis and E. faecium* can produce a toxic oxygen metabolite that results to cell or organ damage [35].

The gross signs of the enterococossis have appeared as early as day 2 post-inoculation (PI) and began to cause death after day 5 PI (Table 6). The infected groups (wild type and UV-treated) showed a pronounced gross clinical signs of bacterial diseases such as uni-eye exopthalmia and eye opacity, reddening on the base fin and nasal area, protruded belly and ulcerative lesions. Ulcerations of the body surfaces were reported by Austin and Austin [36] in tilapia as a characteristic of septicemia. Septicemia is a systemic disease cause by the invasion and multiplication of pathogenic microorganisms in the blood stream. Septicemia might be due to the inoculated bacterium which successfully invaded the circulatory system.

Behaviorally, the infected tilapia exhibited loss of appetite and swim erratically. Erratic swimming might cause by the inoculated bacterium and it affects the mechanical receptor of the fish. Like any other invertebrates, fishes possess an internal ear (labyrinth organ) including the semicircular canal which informs the animal regarding changes in mechanical coordination- speed and direction of motion [37].

Observed internally was the accumulation of fluid in the peritoneal cavity. Since the route of infection was by intraperitoneal injection, the kidney and other internal organs were infected by the bacterium. Kidney consists of millions of individual tubular units technically known as nephrons. The primary function of these cells is to regulate the normal body homeostasis by cleansing the circulatory system via the removal of blood waste metabolites and foreign substances. Therefore, if the kidney is badly affected by foreign substances such as bacteria, this may result to the abnormal functioning of nephrons and consequently leads to the accumulation of fluids in the peritoneal cavity [37].

Presence of hemorrhages was observed on the necropsied tilapia. According to Perera *et al*. [38], hemorrhaging of internal organs or other gross dermal and epidermal lesions was common in diseased fish infected with Streptococcal group.

According to the report of Plumb [39], the clinical signs and pathological manifestations of *E. faecalis* are similar to streptococcosis which exhibits exophthalmia, muscular hemorrhages, acute bronchitis, supurative inflammation in the eyes and necrosis of the spleen and kidney. Furthermore, in the study performed by Romalde *et al.* [40], they found out that the *Enterococcus* spp. strains isolated from diseased turbot showed host specificity when the bacteria were inoculated intraperitoneally, being turbot the only species among salmon, trout, seabream and mice which is susceptible to infection with a high degree of virulence (50% lethal dose of 10^4 per g of fish). All dead and moribund turbot shows hemorrhages in the skin specifically in the head and at the base fins. Some of the infected fish displays an obvious uni or bilateral exophthalmia. Internally, the livers were observed as pale and peritoneal cavities were filled with ascitic fluid. They reported that the mortalities began as early as day-3 PI and rapidly increased approximately after 2 days, and continued slowly for 3 to 4 days. The routes for horizontal transmission are mainly the fecal-oral route and through water bodies if body injuries exist. In addition, intragastric inoculation confirmed that the enterococcal infection may occur when the feeds and feces are contaminated with *Enterococcus* spp. The present study come up with the conclusion that the Nile tilapia is one of the specific hosts of Enterococci group among freshwater species and can cause diseases and mortalities while there's a possibility that none in other freshwater fishes [40].

The bacterium isolated from the kidney of dead and moribund tilapia was comparatively similar to the colonial characteristics of *E. faecalis* and results on gram-staining and Catalase test, thus, proving the assumption that the disease and mortality were caused by the injected bacterium. In the control group, 30% mortality was recorded. Control group was injected with Physiological Saline Solution (PSS) at the same volume received by the mentioned infected groups. Necropsy report showed negative signs of *E. faecalis* infection. The bacterium was not isolated from the diseased tilapia in the control. This may suggest that the cause of death was due to the heavily damaged vital organ during the injection of PSS.

The bacterium that was used in this present study was isolated from marketable size farmed tilapia in Minalin, Pampanga, Philippines where tilapia ponds were highly dependent in river and irrigation canal [41]. *E. faecalis* was recorded at 71.00% prevalence in 15 grow-out farms in Minalin, Pampanga. Other bacteria were also isolated and identified such as *Streptococcus* spp., *Staphylococcus aureus*, *S. epidermidis*, *Enterobacter aerogenes*, *Escherichia coli*, *Aeromonas hydrophila*, *Vibrio vulnificus*, *V. parahaemolyticus*, *Pseudomonas aeruginosa*, *Lactobacillus bulgaris* and *Providencia alcalifaciens* [41].

Table 6: Gross clinical signs of tilapia challenged with wild-type and UV-irradiated *E. faecalis*

Treatments	Mortality Rate (%)	Gross Clinical Signs
Control (Injected with PSS)	30	---
T1 (Wild type *E. faecalis*)	40	uni-eye exophthalmia; reddening of the base fin and nasal area; loss of appetite; erratic swimming; protruded belly; ulcerative lesions
T2 (UV-treated *E. faecalis*)	40	

3.5. Assessment of water quality parameters

Table 7 shows the comparison of means of water quality parameters throughout the 30-day pathogenicity assay. Failure to maintain the suitable condition of water parameters in aquaculture system may cause death to fish. Mortalities among experimental groups were not associated to poor water quality since readings were within the optimum ranges. No significant difference was observed when water temperature, pH and DO in all experimental groups were compared ($p>0.05$).

The mean temperature on the three experimental groups ranged from 24.95 to 25.02 ºC. Water temperature in Control and T2 was just below the permissible range for fish culture according to the reported water temperature (25 to 36 ºC) of Delong *et al.* [42]. Rainfall occurrence, shady environment and the place of set-up might cause the water temperature to fall in a minimal rate. The pH which is defined as the quantity of ions (H^+) dissolved in water ranged from 7.78 to 7.84 which was within the optimum range for freshwater aquaculture according to Delong *et al.* [42]. When the pH readings are below 4.5 or above 10, mortalities will likely to occur. Mean DO ranged around 5.05 to 5.23 ppm which falls under an optimum range in tilapia culture which is 5 to 7 mg/L according to Delong *et al.* [42].

Table 7: Mean water quality parameters (± SD) of challenged group during the pathogenicity assay

Treatments	Temperature (ºC)	pH	Dissolved Oxygen (ppm)
Control (Injected with PSS)	24.95±1.01 [a]	7.80±0.15 [a]	5.23±0.49 [a]
T1 (Wild type *E. faecalis*)	25.02±0.99 [a]	7.84±0.19 [a]	5.19±0.68 [a]
T2 (UV-treated *E. faecalis*)	24.95±0.96 [a]	7.78±0.21 [a]	5.05±0.54 [a]

Note: Mean±SD with the same superscript letters are not significantly different from each other at 95% confidence

4. Conclusion

As a general trend, the wild type bacterium has higher ZOI as compared to the UV-treated bacterium at the majority of dosages examined. UV mutagenesis in *E. faecalis* has resulted to decrease in the ZOI to ampicillin. Generally, no significant changes on the antibiotic susceptibility profile of wild and UV-treated *E. faecalis* based upon CLSI classification. Thicker peptidoglycan of Gram-positive bacteria may offer protection to UV radiation. Gram-positive bacteria have a smaller total genome sizes, which also have reduced their susceptibility to UV radiation because of fewer potential pyrimidine dimer targets and failed to damage or inactivate antibiotic resistance genes (ARGs). Other virulent genes that contributed to the pathogenesis of *E. faecalis* may not inactivated by UV treatment.

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