Available online on 15.04.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Research Article

Antibiotic susceptibility of *Streptococcus agalactiae* isolated from tilapia pond soil in Lubao, Pampanga, Philippines

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ABSTRACT

The main objective of this study was to isolate, quantify and assess the antibiotic susceptibility of *Streptoccoccus agalactiae* from tilapia pond soil in Lubao, Pampanga, Philippines. Composite soil samples were collected from five tilapia grow-out farms near the Pampanga River. Two series of 10-fold dilutions (10^{-1} and 10^{-2}) of soil samples were made in sterile distilled water. One hundred microliters of the diluted samples were plated into Edward Medium Modified. Blue to colorless colonies were counted and expressed as CFU/g. A total of five colonies from five farms were used in the antimicrobial susceptibility testing using the Kirby Bauer disc diffusion method.

Highest count of *S. agalactiae* in tilapia pond soil was recorded in Farm 3 and Farm 5 (2.1 x 10^5 CFU/g) followed by Farm 4 (1.6 x 10^5 CFU/g) and lowest in Farm 1 and Farm 2 (4.2 x 10^4 CFU/g). There was no big difference in log10 transformed CFU/g of *S. agalactiae* among the five collection farms because they share the same cultural and management practices and even the source of water.

The eight antibiotics used in the study were penicillin, gentamicin and ampicillin (10 µg), amoxicillin and nalidixic acid (20 µg), and chloramphenicol, tetracycline and vancomycin (30 µg). All *S. agalactiae* isolates were susceptible to gentamicin, nalidixic acid, chloramphenicol and tetracycline, thus, these antibiotics could be recommended in treating tilapia infected with this bacterium. The isolates were intermediate to susceptible to penicillin, ampicillin, amoxicillin and vancomycin.

Keywords: Antibiotics, antibiotic susceptibility, *Streptococcus agalactiae*, Nile tilapia

Article Info: Received 27 Feb 2019; Review Completed 02 April 2019; Accepted 09 April 2019; Available online 15 April 2019

Cite this article as:

Reyes AT, Fajardo LJ, Abando AKY, Antibiotic susceptibility of *Streptococcus agalactiae* isolated from tilapia pond soil in Lubao, Pampanga, Philippines, Journal of Drug Delivery and Therapeutics. 2019; 9(2-s):370-375

http://dx.doi.org/10.22270/jddt.v9i2-s.2530

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INTRODUCTION

Sediments of a body of water serve as sources or sinks of both organic and inorganic matter. Aquaculture sediment can affect the quality of the pond[1] and the final products from a rearing operation[2]. The aquaculture pond sediment differs from other sediment environments because there is a continuous input of organic matter. Thus, it is likely that the bacterial community composition also differs in other sediment environments[2].

Sediments have higher bacterial load as compared to freshwater and marine water environments. In a study conducted by Atlas and Bartha (1998), they provided an estimate of 3.0 x 10^6 to 5.0 x 10^8 bacterial populations per gram of sediment[3]. The high bacterial load in sediment could be due to a number of factors such as proximity to toilet facilities, animal farm wastes and refuse dump sites[4]. Abundance of microorganisms in sediments also relies on the availability of nutrients and multitude of environmental factors[5]. A number of authors have reported that the most abundant bacterial phyla in tilapia pond sediments were Proteobacteria and Bacteroides[6,7]. Pathogenic groups of bacteria namely *Escherichia coli, Staphylococcus*, *Enterococcus*, *Klebsiella*, *Proteus*, *Pseudomonas, Serratia*, *Aeromonas* and *Streptococcus* were also reported in sediments[5,8].

Streptococcus is gram-positive, non-acid fast, non-motile, oxidase-positive and catalase-negative coccus. Currently, over 50 species are recognized in this genus. *S. agalactiae* is the major species in farmed tilapia. *S. iniae* also causes mortality but to a lesser extent. Bunch and Bejerano (1997) acknowledged that *Streptococcus* spp. are widely spread in aquaculture environments[9]. Infection caused by *Streptococcus* spp. in fishes leads to pathological signs such as hemorrhage, exophthalmia, corneal opacity and dark body coloration, with nodular or abscess formation on the trunk and peduncle muscles. *Streptococcus* infections in fish can cause high mortality rates (> 50%) over a period of 3 to 7 days. Some outbreaks, however, are more chronic in nature and mortalities may extend over a period of several weeks, with only a few fish dying each day[10].

Experimental studies demonstrated that *Streptococcus* spp. could be transmitted via a number of routes such as intraperitoneal injection, immersion, oral, gill and nare inoculation and cohabilitation with infected fish[11,12,13]. However, under natural environments, *Streptococcus* spp. could be transmitted horizontally via a number of pathways that include direct contact between healthy fish and diseased or dead fish as well as direct contact with the bacteria-contaminated water and sediment[14,15,16,17,18,19,20]. The ability of *Streptoccus* spp. to survive in water and sediment for long periods could make the fish more susceptible to these bacteria[21].

Komar (2008) has emphasized that bacterial diseases in fish could be managed by two major complementary strategies namely reactive and proactive. Reactive strategies mainly focus on treatment, whereas proactive strategies focus on prevention. One common example of reactive strategy is through antibiotic therapy. The main issue associated with the practice is antibiotic resistance[22]. In aquaculture and mariculture, the development of antibiotic resistance has been recorded in *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *E. icttaluri*, *Vibrio anguillarum*, *V. salmonicida*, *Pasteurella piscida* and *Yersinia ruckeri*[23]. *Streptococcus* isolated from cultured tilapia showed various forms of resistance to gentamicin, ofloxacin, nitrofurantoin, amoxicillin, tetracycline, cotrimoxazole, nalidixic acid and augmentin[24]. The use of antibiotics is indeed possible when it is conducted responsibly. The choice of antibiotic used should be determined based on antibiotic sensitivity and regulatory status[23].

Objectives of the Study

This study was conducted in order to isolate and quantify *S. agalactiae* from tilapia pond sediment in Lubao, Pampanga, Philippines and to evaluate the antibiotic susceptibility of the *S. agalactiae* isolates.

MATERIAL AND METHODS

Collection of Soil Samples

Soil samples were collected from five tilapia grow-out farms in Lubao, Pampanga, Philippines that are located near Pamapanga river and practice intensive farming. Composite surface soil were collected around the pond perimeter using improvised soil borer. Five centimeter depth was excavated for the collection of soil. The collected samples were placed in disinfected plastic cup and were transported in the laboratory for immediate analysis.

Surveying and GPS Reading

The tilapia grow-out farms were surveyed using a pre-tested questionnaire designed by the Freshwater Aquaculture Center in Central Luzon State University Philippines. The questionnaire encompasses some aspects of tilapia farming (e.g. technical information, feeds and feeding, water management, etc.). The exact location of the farm was known using handheld GPS equipment.

Isolation and Quantification of *Streptococcus*

Two series of 10-fold dilutions (10^{-1} and 10^{-2}) of pond soil was made in distilled water. One hundred microliters (100 µl) of the diluted sample was plated into Edward Medium Modified. After 18 to 24 h of incubation at 35 to 37 °C, blue to colorless colonies were counted and expressed as CFU/g using the formula:

$$\text{CFU/g} = \frac{\text{average no. of colonies x dilution factor}}{\text{volume plated (mL)}}$$

$$\text{CFU/g} = \frac{\sum C}{[(1 \times n1) + (0.1 \times n2)]\,(d \times Vp)}$$

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

In every collection site, one colony of *S. agalactiae* was selcted and inoculated in test tube containing Trypticase Soy Agar (TSA). A total of five colonies were used fo gram-staining, catalase test and antibiotic susceptibility testing.

Gram Staining

The isolate was streaked on TSA plate and incubated at 37 ˚C for 18 to 24 hours. A smear was prepared by mixing a small amount of growth with a drop of distilled water. The smear was air-dried and fixed by heat. The glass slide was labeled properly. The dried smear was stained with crystal violet for 1 minute and was rinsed thoroughly with tap water. Afterwards, the smear was covered with Gram's iodine for 1 to 2 minutes and was washed with tap water. The smear was decolorized by dripping 95% ethanol and was washed immediately. Then, the smear was counterstained with safranin for 45 seconds and was washed by tap water. The slide was examined under microscope. Gram-positive bacterium should be colored blue while Gram-negative bacterium should be colored red. Cell size, shape and arrangement were also noted.

Catalase Test

The isolate was streaked on TSA plate and was incubated at 30 ˚C for 18 to 24 hours. A loopful of the bacterium was transferred to a clean slide. One to two drops of freshly prepared 3% hydrogen peroxide (H_2O_2) were dropped into the slide. Bubble formation indicates presence of catalase enzyme.

Antibiotics Susceptibility Testing

About 2 to 3 colonies of *S. agalactiae* was suspended in Trypticase Soy Broth (TSB). The bacterial suspension was incubated for 1 to 2 hours at 37 °C and then adjusted to 0.5 McFarland turbidity standards. The adjusted suspension was streaked in TSA plate using a sterilized cotton swab The antibiotics discs (amoxicillin = 20 µg; chloramphenicol = 30 µg; penicillin = 10 µg; tetracycline = 30 µg; gentamicin = 10 µg; vancomycin = 30 µg; ampicilin = 10 µg; nalidixic acid = 20 µg) were placed on the surface of the inoculated plate using sterile forceps. The plates were incubated at 37 °C for 24 hrs. The zone of inhibition was measured using a ruler. The susceptible, intermediate and resistant categories of *S. agalactiae* to antibiotics were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute (2012).

Statistical Analysis

Statistical difference in zone of inhibitions among antibiotic discs was compared using One Way Analysis of Variance. Comparison of means was done using Tukey's Test.

RESULTS AND DISCUSSION

Surveying and GPS Reading

The five tilapia farms that served as collection sites for pond soil samples were located in Brgy. Bancal Pugad, Lubao, Pampanga, Philippines. This barangay is located in one of the tributaries of Pampanga River. The river served as the main source of water for the grow-out culture of tilapia which was operated in a medium (3 to 6 ha) to large scale (>7 ha) production. The farm owners performed drying and liming of pond bottom, believing that this combination was enough to prepare their ponds. Each pond was stocked with >9 tilapia/m^2 and the fish were totally dependent on commercial diet. Water exchange was done at least once a week. The interviewed fish farmers already experienced problems related on fish diseases, fish kills and extreme environmental conditions. The usual remedies performed in case of disease/fish kill occurrences were application of salt and lime, water exchange and early harvest (Table 1).

Isolation and Quantification of *Streptococcus agalactiae* in Tilapia Pond Soil

S. agalactiae from tilapia pond soil was isolated using a selective and differential medium known as Edward Medium Modified (EMM). This medium contains crystal violet and thallium salts which are responsible for the selective isolation of streptococci and inhibition of other types of bacteria.

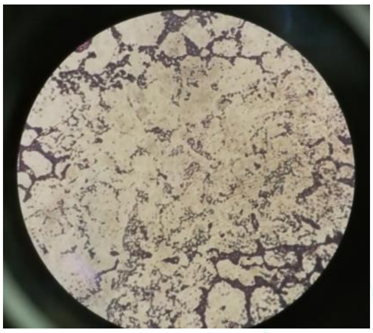

Figure 1: Gram staining. Gram-positive colonies in pair or chains.

The esculin in the medium is accountable for the differentiation of esculin-positive streptococci (group D streptococci) from esculin-negative streptococci such as *S. agalactiae*[25]. *S. agalactiae* colonies appear blue to colorless in EMM.

Representative colony from the five collection sites was subjected to gram staining and catalase test. All of the five colonies were Gram-positive with round cells (cocci) in pair or chain (Figure 1) when observed under the microscope. The colonies were negative to catalase test because there was no formation of bubbles when it was dipped in hydrogen peroxide (Figure 2). Gram-positive reaction and negative to catalase test were some of the phenotypic traits of *S. agalactiae*[26].

Figure 2. Catalase test. *S. agalaciae* was negative to catalase test as indicated by drops of hydrogen peroxide without the formation of bubbles. Drop with bubbles was inoculated with a known bacterium which is positive to catalase test.

Highest count of *S. agalactiae* in tilapia pond soil was recorded in Farm 3 and Farm 5 (2.1 x 10^5 CFU/g) followed by Farm 4 (1.6 x 10^5 CFU/g) and lowest in Farm 1 and Farm 2 (4.2 x 10^4 CFU/g) (Table 2). There was no big difference in log10 transformed CFU/g of *S. agalactiae* among the five collection farms because they share the same cultural and management practices and even the source of water. According to the study of Reyes, Fajardo and Bullanday (2018), the candidate risk factors associated to the occurrence of *S. agalactiae* in tilapia pond water in Lubao, Pampanga were high stocking density, full feeding, unsafe source of water and incomplete pond preparation practices[27]. This study revealed that *S. agalactiae* was more abundant in soil (4.2 x 10^4 to 2.1 x 10^5 CFU/g) than in water (250 to 270 CFU/mL). The presence of *S. agalactiae* in tilapia pond water could be attributed to the higher count recorded in the soil. This only proves that the pond preparation practices implemented in the five farms were not enough to eradicate pathogenic bacteria in the soil.

Table 1. Summary of the farm management practices of the five collection sites.

Farm	Farm Location/ Address	Scale of Operation	Pond Preparation	Water Source	Water Exchange	Level of Management	Feeding	Problems Incurred	Preventive/ Control Measures
1	Bancal Pugad N 14°54.757' E 120° 34.560'	Large-scale	Drying and liming	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, liming, early harvest
2	Bancal Pugad N 14°54.583' E 120° 34.945'	Medium-scale	Drying	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, early harvest
3	Bancal Pugad N 14°57.247' E 120° 36.597'	Large-scale	Drying	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Water exchange, liming
4	Bancal Pugad N 14°58.145' E 120° 35.695'	Medium-scale	Drying	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, liming, early harvest
5	Bancal Pugad N 14°54.935' E 120° 35.047'	Medium-scale	Drying and liming	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, early harvest

Table 2: Colony forming unit (CFU) of *S. agalactiae* per gram of pond soil in the five collection sites.

FARM	NUMBER OF S. agalactiae COLONIES		CFU/g	Log10 CFU/g
	10^{-1} Dilution	10^{-2} Dilution		
1	228	136	4.2 x 10^{4}	4.62
	264	28		
	280	148		
2	384	37	4.0 x 10^{4}	4.60
	330	205		
	245	27		
3	420	160	2.1 x 10^{5}	5.32
	512	220		
	548	244		
4	630	78	1.6 x 10^{5}	5.20
	583	153		
	512	243		
5	868	216	2.1 x 10^{5}	5.32
	424	192		
	848	228		

Antimicrobial Susceptibility Testing

For antibiotics with dose of 10 µg, gentamicin was the most effective against *S. agalactiae* isolates with zone of inhibition from 26.80 to 31.40 mm. *S. agalactiae* isolate from Farm 1 was considered to be the most susceptible to gentamicin (31.40±0.55 mm), penicillin (33.20±1.79) and ampicillin (25.60±1.14 mm) (Table 3).

At 20 µg dose, nalidixic acid (22.40 to 25.60 mm) had wider zone of inhibition as compared to amoxicillin (16.00 to 25.40 mm). Farm 1 isolate (25.60±0.55 mm) was the most susceptible to nalidixic acid. Highest zone of inhibition in amoxicillin was observed in Farm 2 isolate (25.40±2.07 mm) and it was significant as compared in Farms 3, 4 and 5 (Table 3).

Chloramphenicol (27.40 to 33.20 mm) at 30 µg dose was more effective than vancomycin (18.20 to 23.80 mm) and tetracycline (22.20 to 28.00 mm) of the same dosages based upon the range of zone of inhibition. Farm 1 isolate recorded the highest zone of inhibition for tetracycline (28.00±1.87 mm) and chloramphenicol (33.20±1.79 mm). Meanwhile, vancomycin was most effective to Farm 4 isolate (23.80±1.79 mm) (Table 3).

Table 3: Zone of inhibition of the antibiotics used against the five *S. agalactiae* farm isolates.

ANTIBIOTIC	FARM 1	FARM 2	FARM 3	FARM 4	FARM 5
Gentamicin(10µg)	31.40±0.55^{ab}	26.80±0.84^{c}	29.40±1.34^{b}	32.40±.55^{a}	26.80±1.64^{c}
Penicillin(10µg)	33.20±1.79^{a}	14.60±3.21^{b}	15.60±0.89^{b}	17.60±2.88^{b}	15.60±1.95^{b}
Ampicillin(10µg)	25.60±1.14^{a}	15.20±2.59^{c}	20.60±0.89^{b}	19.60±0.55^{b}	21.00±2.00^{b}
Nalidixic(20µg)	25.60±0.55^{a}	25.20±0.84^{a}	24.00±1.87^{ab}	22.40±.55^{b}	24.80±1.48^{a}
Amoxicillin(20µg)	24.00±0.71^{a}	25.40±2.07^{a}	16.00±1.22^{b}	17.40±1.95^{b}	16.20±2.77^{b}
Vancomycin(30µg)	20.40±0.55^{ab}	20.60±1.14^{ab}	21.20±3.63^{ab}	23.80±1.79^{a}	18.20±1.48^{b}
Tetracycline(30µg)	28.00±1.87^{a}	25.20±1.92^{ab}	25.80±1.30^{a}	26.80±2.17^{a}	22.20±1.30^{b}
Chloramphenicol(30µg)	33.20±1.79^{a}	27.40±1.14^{b}	30.00±5.05^{ab}	28.80±3.63^{ab}	28.60±.55^{ab}

Note: Different superscript was significant at $p<0.05$

The isolates were classified whether resistant, intermediate or susceptible to the various antibiotics on the basis of critical points recommended by the Clinical and Laboratory Standards Institute (2012)[28]. All five isolates were susceptible to gentamicin, nalidixic acid, chloramphenicol and tetracycline (Figure 3). The isolates were intermediate to susceptible to penicillin, ampicillin, amoxicillin and vancomycin (Table 4). In the study conducted by Reyes et al. (2018), *S. agalactiae* isolates from pond water were found resistant to penicillin and ampicillin at 10 µg dose, amoxicillin at 20 µg dose and vancomycin at 30 µg dose, and susceptible to tetracyline at 30 µg dose and chloramphenicol at 30 µg dose[27].

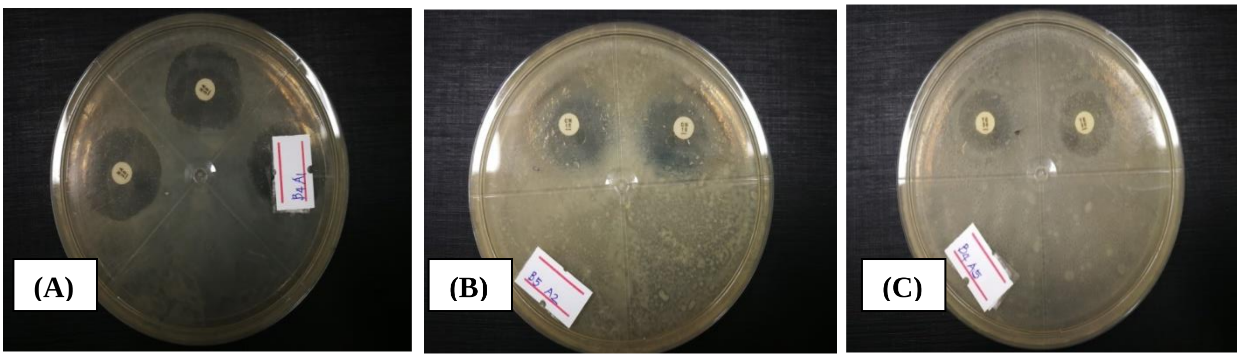

Figure 3. *S. agalactiae isolates* susceptible to nalidixic acid (A), chloramphenicol (B) and tetracycline (C)

Table 4. Classification of isolates on the basis of critical points recommended by the Clinical andLaboratory Standards Institute (2012)

ANTIBIOTICS/DOSAGES	CATEGORIES				
	FARM 1	FARM 2	FARM 3	FARM4	FARM 5
Penicillin (10 µg)	Susceptible	Intermediate	Intermediate	Intermediate	Intermediate
Gentamicin (10 µg)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Ampicillin (10 µg)	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
Amoxicillin (20 µg)	Susceptible	Susceptible	Intermediate	Intermediate	Intermediate
Nalidixic acid (20 µg)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Chloramphenicol (30 µg)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Tetracycline (30 µg)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Vancomycin (30 µg)	Intermediate	Susceptible	Susceptible	Susceptible	Intermediate

Note: Resistant = < 14 mm; Intermediate = 15 to 19 mm; Susceptible = > 20 mm

Chloramphenicol inhibits microbial protein synthesis. Tetracycline binds to the 30S ribosomal subunit of the bacterium and it interferes with the binding of aminoacyl-tRNA to the messenger RNA molecule/ribosome complex, thus, disrupting the bacterial protein synthesis[29]. Tetracycline can inhibit the protein synthesis in mitochondria when it binds with the 70S ribosomes[30]. Gntamicin is able to inhibit the protein synthesis in bacteria by binding to one of the ribosomal subunits[31]. Meanwhile, nalidixic acids are able to interfere with DNA replication and transcription in bacteria[32].

Multiple antibiotic resistances have been reported in fish pathogen and bacteria from aquaculture environment with a variety of drug or an uncertain antibiotic usage history[33,34,35]. Antibiotics percolated from the food and faeces may diffuse into the sediment and can be propelled by currents to the distant sites[36,37,38,39,40]. These residual antibiotics will remain in the sediment, exerting selective pressure, thereby altering the composition of the microflora of the sediment and selecting for antibiotic-resistant bacteria[41,42,43,44,45,46]. Furthermore, it has been shown that antibiotic resistance in sediment bacteria increases along heavy metal contamination[47].

There are number of ways by which microorganisms are resistant to antimicrobial agents. These includes: (1) Bacteria produce enzymes which destroy the antimicrobial agents before it reaches its targets e.g. Beta lactamase enzyme hydrolyses beta lactam drugs which develop resistance; (2) Impermeable cell for antimicrobial drugs e.g. Gram-negative bacteria may become resistant to Beta lactam antibiotics by developing permeability barrier; (3) Mutation e.g. Ribosome methylation of ribosomal RNA develop macrolide resistant; (4) Bacterial efflux pump that expels antimicrobial drugs from cell before it can reach its targets; and (5) Specific Metabolic pathways in the bacteria are genetically altered so that antibacterial agents cannot exert an effect[48,49]

CONCLUSIONS

At 20 µg dose, nalidixic acid (22.40 to 25.60 mm) had wider zone of inhibition as compared to amoxicillin (16.00 to 25.40 mm). Chloramphenicol (27.40 to 33.20 mm) at 30 µg dose was more effective than vancomycin (18.20 to 23.80 mm) and tetracycline (22.20 to 28.00 mm) of the same dosages based upon the range of zone of inhibition. All *S. agalactiae* isolates were susceptible to gentamicin (10 µg), nalidixic acid (20 µg), chloramphenicol (30 µg) and tetracycline (30 µg), thus, these antibiotics could be recommended in treating tilapia infected with *S. agalactiae*. The isolates were intermediate to susceptible to penicillin (10 µg), ampicillin (10 µg), amoxicillin (20 µg) and vancomycin (30 µg).

ACKNOWLEDGEMENT

The authors expressed their profound gratitude to Mr. Alfred Reyes, Mr. Ismael Geroza, Mr. Mark John Bullanday, Mr. Eric Jorduela, Mr. Francis Gerald Madrid and other members of Alpha Sigma Phi Delta Iota Chapter CLSU who helped in the collection and analysis of soil samples.

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