Immune Boosting Activity of Aqueous Lemongrass (Cymbopogon citratus) Leaf Extract on Native Chickens (Gallus gallus domesticus) Challenged with Newcastle Disease

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Abstract – Newcastle Disease is a highly infectious disease which can cause up to a 100% mortality in native chickens and no treatment has been yet found for it. Lemongrass (Cymbopogon citratus) is a plant that has alkaloid, saponin, tannin, and flavonoid to combat Newcastle disease. This study aims to measure and compare the antibody titre gain of Philippine native chickens treated with different dilutions of C. citratus aqueous extract. Fofty-four two-month-old chickens were infected with live virus-concentrated solution. Blood was collected from all the samples prior to infection and after the treatment Samples were grouped into four treatment groups A, B, C, and D, according to the dilution factor. Samples from the treatment group A were given with 1 mL undiluted C. citratus extract, treatment group B with 5-fold dilution, treatment C with 10-fold dilution, and treatment D with 20-fold dilution. Antibody titre gain was determined by hemagglutination inhibition. Result show that there is a significant increase with the antibody titre gain for unvaccinated and vaccinated groups. Unvaccinated samples do not require a treatment diluted in a specific volume of distilled water while vaccinated samples require a treatment diluted in 9 mL distilled water for an optimal antibody increase when infected with ND. Treated samples have an increase in the average live weight with treatments B and C exhibiting a significant increase. Survival rate of the chickens is also high with 85.21%.

Introduction. – Poultry is the most progressive animal enterprise today and has been a significant contributor to the country's agriculture sector (Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development 2011). According to the Philippine Statistics Authority (PSA), it accounted for 15.72 percent of the total agricultural production from April to June 2016.

The chicken industry constitutes a large part in the poultry sector. As of July 2016, the countrys chicken inventory has approximately 17.5 million, of which 44.2% are native chickens (Philippines Statistics Authority or PSA 2016a). Native chickens belong to a population of chickens with no extensive information about the breeds. These are the local backyard chickens raised by farmers often showing large phenotypic diversity (Cabarles et al. 2012).

The raising of native chickens is an integral part of the farming systems of Filipinos as they are the main source of eggs and meat for backyard farmers. According to PCAARRD (2016), native chicken meat has always been preferred over that of commercial broilers because of its unique taste, distinct flavor and texture, presence of functional compounds, and lower fat content. Also, the study of Sokoowicz et al. (2016) has found that native chicken meat contains less fat and has a healthier ratio than that of broilers. However, two main problems entail the native chicken industry: the unstable supply of slaughter, and a high chicken mortality rate of 40% caused by poultry diseases (PCAARRD 2016).

The chicken production from April to June 2016 slightly declined by 0.02 percent. The drop was attributed to the low disposition of native chickens due to the incidence of

Newcastle Disease that prevailed in Luzon (PSA 2016b). Linden (2016) reported that 41,000 birds died in Luzon because of Newcastle disease (ND). Barnes (2016) also reported that 109,255 chickens died of ND infection in the northern region as of February 29. In La Union, the same virus also infected 25,000 chickens (Alhambra 2016).

According to the Department of Agriculture (2016), one contributing factor of the spread of Newcastle disease virus was the change of weather that weakened the immune system of chickens. The spreading worsened due to contact of chickens in cockfights allowing the virus to spread from one poultry farm to another (Reano 2016).

Newcastle disease (ND) is a highly infectious disease in domestic poultry and wild birds. ND is caused by a single-strand, non-segmented, negative-sense RNA virus known as Avian paramyxovirus 1 (APMV-1) and is regarded as one of the most important avian diseases (Catolli et al. 2011; Chollom et al. 2012). Birds infected with ND experience respiratory and nervous attacks. Effects further include paralysis of wings and legs, twisting of the head and neck, rapid drop of egg production and sudden death (Mississippi State University 2014). Humans can also be affected by ND. Effects include mild conjunctivitis, influenza-like symptoms, and laryngitis (Badr date unknown).

Chickens are the most susceptible to clinical disease among most avian species susceptible to infection with the virus. It may cause outbreaks with up to 100% mortality and is considered a major threat to both commercial and village poultry flocks (Chollom et al. 2012).

There is still no specific treatment for the disease (Butcher et al. 2015). Vaccines are recommended to prevent the occurrence of ND among healthy chickens. However, Newcastle disease remains unabated despite various vaccination programs (Chollom et al. 2012). Also, another way of transmission aside from direct contact is through contamination of poultry vaccines (Badr date unknown).
Lemongrass (Cymbopogon citratus) or locally known as tanglad, contains phytochemicals such as alkaloids, flavonoids, saponins, and tannins found in plants are considered as antiviral agents (Nambiar and Matela 2010). Alkaloids bind with RNA and can cause chain terminations, thus inhibiting the synthesis of virions (Mcdaniel date unknown); saponins induce the production of SOCS2 (Lee et al. 2012); tannins inhibit the virus from attaching to the host cell (Cheng et al. 2004); and flavonoids inhibit the neuraminidase activity of the virus disabling the removal of siallic acid on the surface of the host cell, thus not allowing infection of other cells (Hanh et al. 2014).

Native chickens play an essential role to the economy of the country. Since it is free range, it is perceived as chemical and antibiotic free. There is a global trend from recent years showing the shift of consumers interest into organic and naturally produced products, which makes native chicken more preferable compared to commercial chickens (PCAARRD 2016). It is also a cheap source of animal protein and provides extra income to families in rural areas (Cabarles et al. 2012). The sustained use of native chickens in the traditional poultry production system implies the need to consider the value of native chickens (Padhi 2016). Given the recent ND outbreaks, there is a need for a treatment when the virus breaches the immunity of chickens given by vaccines.

This study aims to determine the immune boosting activity of C. citratus aqueous leaf extract on native chickens challenged with Newcastle disease. Its objective is To determine whether C. citratus aqueous leaf extract increases antibody titre, live weight, and survival rate of native chickens challenged with Newcastle disease, specifically, to measure and compare the antibody titre, live weight gain, and survival rate of chickens treated with different concentrations C. citratus aqueous leaf extract.

There has been no treatment found against ND. If the bird begins to show symptoms, vaccines are ineffective. Since the diagnosis for this disease is poor, there is a 100 percent mortality rate once infected, which can therefore affect the poultry economy (Foster and Smith 2016). Thus, it provides the need for a treatment to boost the immunity of the chickens against the disease.

Methods. –

Collection and Identification of Plant Material. Six kilograms of C. citratus were obtained from the Central Philippine University - College of Agriculture, Resources and Environmental Science (CPU - CARES) farm in Leon, Iloilo. The plant samples were cut at the stem using a sickle. The collected plant materials were certified by Dr. Jaime Cabarles, dean of CPU - CARES. The samples were then washed with distilled water.

Preparation of Stock Solution. Preparation of the aqueous extract was done according to the methodology of Hindumathy (2011). C. citratus aqueous extract was obtained by juicing one kilogram of C. citratus leaves with 1 L of distilled water using a juicer provided by CPU - CARES.

Preparation of Serial Dilutions. From the stock solutions, serial dilutions were prepared to obtain the test solutions of different concentrations of 1 mL extract/1 mL, 1 mL extract/5 mL, 1 mL extract/10 mL, and 1 mL extract/20 mL respectively.

Experimental Birds and Cultural Management. This study utilized 54 two-month-old native Philippine chickens of unspecified breeds. Chickens were kept in premade

cages with the dimensions 2 ft 2 ft 45 cm and enclosed in a 3 m 7 m sized knotted nylon mesh netting. Samples were divided into nine groups including the control group. They were given one week upon transfer for acclimatization to their new environment to prevent possible stress. Samples from four of the groups were already vaccinated against ND. The control group was separated from the treated group to avoid contaminations. Birds were fed with 58 g of CPU Feeds Chick Grower daily. Each of the samples was also given water in their corresponding waterer.

Weighing of Samples. Live weight of samples were measured days before infection and after treatment. Samples were each taken from their cages and set on top of the digital weighing machine.

Virus Infection. Preparation of Virus-concentrated Solution. The LaSota vaccine, bought from a local livestock supply store, was diluted with a vaccine solvent specifically for freeze-dried avian vaccines provided by CPU - CARES. The ratio of the vaccine to the solvent was modified by the Department of Agriculture (DA) so that the final solution would be more concentrated with live virus.

Infection of Samples. The final solution was injected to either the thigh or the breast part of the chicken samples. Cottons were also soaked with the solution and was taped at the top of the cages to contaminate the air with the virus. This was done under the supervision of Dr. Jaime Cabarles Jr. of CPU - CARES and Dr. Jonic Natividad of DA.

Treatment. Infected samples were drenched using 1 mL syringes with aqueous extracts from the C. citratus plant every afternoon. Groups from vaccinated samples vaccinated against ND were labeled as Group VA (vaccinated, group A) , Group VB (vaccinated, group B), Group VC (vaccinated, group C), and Group VD(vaccinated, group D). Groups from the unvaccinated samples were labeled as Group UA (unvaccinated, group A), Group UB (unvaccinated, group B), Group UC (unvaccinated, group C), and Group UD (unvaccinated, group D). Samples from groups VA and UA were administered with aqueous extract dosed at 1 mL extract, groups VB and UB with 1 mL extract/5 mL, groups VC and UC with 1 mL extract/10 mL, and groups VD and UD with 1 mL extract/20 mL. Survival rate and live weight gain were measured 14 days after the start of treatment. Survival rate was computed by treatment group using the equation (total number of chickens - number of deaths)total number of chickens) x 100 . With regards to the side effects of the phytochemicals on chickens, the study of Raza et al. (2015) showed that plants containing alkaloids, flavonoids, saponins, and tannins that were tested against the ND virus in vitro and in vivo exhibited positive results and did not show any adverse reactions on chickens.

Blood Collection and Serology. Blood for serum samples was collected through either the brachial or jugular vein of the samples. Collection was done five days before infection and 14 days after treatment, following the method of Orajaka and Ezema (2004) with modification on the number of days before infection. The chicken was held horizontally on its back, holding the legs and under the back to support the chicken and pull its wing towards the holder. The wing vein is the bifurcating form (V-shaped) that ran between the biceps and the triceps muscles and with tendon of the pronator muscle running across it. Small feathers that obscured the vein were plucked. Seventy percent by volume alcohol was swabbed around the bleeding site for disinfection. A 23 mm gauge needle was inserted not too deeply under the tendon and directed into the wing vein towards the blood flow, keeping clear of the ulnar nerve. The plunger of the syringe was gently pulled once the tip of the needle was in the vein. If blood did not flow, the end of the needle was repositioned slightly, and if haematoma formed, the other wing was used. After bleeding, pressure was applied to the vein for a few seconds to prevent further bleeding. The needle was removed and the cap was placed on its end to prevent leakage (FAO 2005). The collection of blood was performed quickly and gently to avoid vein damage. Minimizing the blood loss lessens trauma to the chickens. The collection of blood was conducted under the supervision of Dr. Jaime Cabarles Jr. of CPU - CARES and Dr. Jonic Natividad, certified veterinarian of DA - R6. ND virus antibody titres in the sera of inoculated hens were examined through hemagglutination inhibition (HI) assays carried out by the Department of Agriculture - Region 6 Regional Animal Disease Diagnostic Laboratory.

Safety and Disposal. To guarantee the safety of this experimental study, biosafety level (BSL) was be considered. According to USDA, Newcastle disease virus has a biosafety level 2 (BSL-2). This means that Newcastle disease virus is an agent that can be associated with some human diseases such as mild conjunctivitis. The guidelines of U.S. Department of Health and Human Services (2009) was followed in the containment of infected samples, and the protocol of The Ministry of Health and Welfare Notification No. 1997-22 was used to transfer blood samples for HI testing.

Dead samples were removed from their cages and then collectively burned in an excavation. After the conduct of the study, all remaining samples were also incinerated. The excavation was then covered back with soil after the burning process.

Statistical Analysis. Data for initial and final antibody titre and live weight were analyzed using paired-sample t-test. Mean antibody and live weight difference of vaccinated samples and vaccinated samples were compared using independent-sample t-test. Vaccinated and unvaccinated treatment groups were compared separately using one-way ANOVA in IBM SPSS Statistics version 22

Table 1: The mean antibody titre gained sample groups.

Sample		Mean Antibody Titre Gain
Treated	Vaccinated	167.67
	Unvaccinated	74.67
	Overall	121.17
Control	85.33	

software.

Results. – This study aimed to determine the antiviral activity of C. citratus aqueous leaf extract by measuring and comparing the antibody titre of chickens treated with a) undiluted 1 mL, b) 5-fold dilution, c) 10-fold dilution, and d) 20-fold dilution of C. citratus aqueous extract. Thirty two-month-old Philippine native chickens were infected with Newcastle disease. Six chickens served as control and the remaining 24 chickens were infected and treated. From the treated group, 24 unvaccinated samples were each divided into four subgroups, A, B, C, and D. Subgroups were then given with undiluted 1 mL, 5-fold dilution, 10-fold dilution, and 20-fold dilution of C. citratus aqueous extract respectively. Final and initial live weight and blood of the samples were measured and collected before infection and after treatment of C. citratus extract.

Antibody titre. The treated group, which comprises the vaccinated and the unvaccinated samples, has a greater mean antibody titre gain of 117.74, compared to the control group that has 85.33 antibody titre mean. The vaccinated group has a 171.11 mean antibody titre gain, which is higher than the unvaccinated groups with 68.00 mean antibody titre gain. Both are greater than the control group.See Table 1

The average antibody titre of the treated sample increased. Vaccinated samples with 20-fold dilution of C. citratus aqueous extract had the highest antibody titre gain against Newcastle disease. Samples given with 1 mL undiluted C. citratus aqueous extract had the lowest antibody titre. The average antibody titres of vaccinated samples increase as the dilution factor increases. Unlike in vaccinated samples, unvaccinated samples given with undiluted 1 mL C. citratus aqueous leaf extract had the highest average antibody titre among all the other treatments in the unvaccinated group. Moreover, samples treated with 20-fold dilution of C. citratus aqueous had the lowest antibody titre in the unvaccinated group. The average antibody titres of unvaccinated samples decrease as the dilution factor increases. See Fig 1.

The treatment group C had the most significant p-value among all the vaccinated treatment groups. This implies that it had the most significant mean antibody titre gain. It is closely followed by treatment group B, and then C. On the other hand, the mean antibody titre gain of treatment group A was not significant.See Table 2

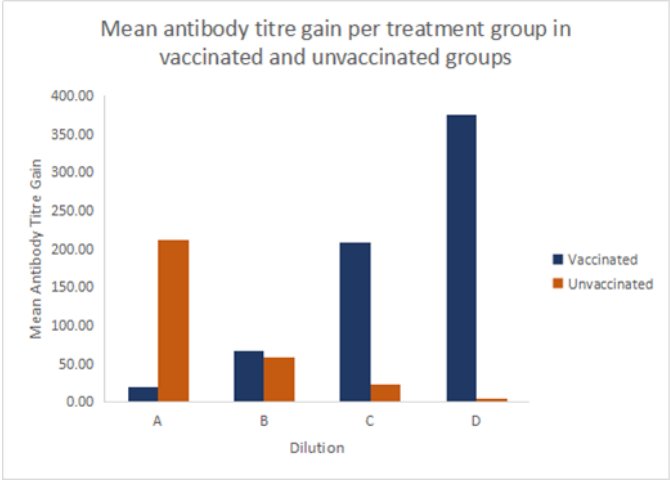

Fig. 1: Mean antibody titre gain per treatment group in vaccinated and unvaccinated groups

Live Weight. The treated group has a mean live weight gain of 78.93 g. This result is higher than the control which has a mean live weight gain of 47.33 g. The mean live weight gain of the unvaccinated samples has the highest mean live weight gain of 95.77 g, followed by the vaccinated with 62.08 g, while the control has gained the least weight.See Table 3

Among the treatment groups in vaccinated samples, samples treated with 1 mL undiluted C. citratus aqueous leaf extract had the highest mean live weight gain of 85.75 g, while samples treated with 10-fold dilution of C. citratus aqueous extract had the lowest with 31.50 g. Samples treated with 10-fold dilution C. citratus aqueous extract had the highest mean live weight gain among all the treatment groups in unvaccinated samples with 112.00 g. Meanwhile, samples treated with 5-fold dilution of C. citratus aqueous extract had the lowest among all the treatment groups in unvaccinated samples with 75.83 g of mean live weight gain.See Fig 2.

Survival Rate. The treated group had an overall survival rate of 81.25%. The unvaccinated group had a higher survival rate compared to the unvaccinated group. The control group has a 100% survival rate, higher compared to the survival rates of vaccinated and unvaccinated treatment groups. The vaccinated samples had the lowest average survival rate of 79.17%.See Table 4

Vaccinated chickens treated with 10-fold dilution of C. citratus aqueous extract is observed to have the highest survival rate among vaccinated treatment groups with a 100%, which means that none of the samples died. On the other hand, vaccinated samples from treated with undiluted 1 mL C. citratus aqueous extract and samples treated with 20-fold dilution of C. citratus aqueous extract have the lowest survival rate among vaccinated samples, with a rate of of 66.6%, indicating two deaths. The lowest survival rate in unvaccinated samples also

Table 2: Mean antibody titre gain per treatment group in vaccinated and unvaccinated groups

Vaccinated Treatment Groups	P-value
A	0.278
B	0.001
C	0.000
D	0.020

come from the samples from treated with 20-fold dilution of C. citratus aqueous extract. Unvaccinated chickens from treated with 5-fold dilution of C. citratus aqueous extract and treated with 10-fold dilution of C. citratus aqueous extract had zero deaths indicating a 100% survival rate.See Fig 3. Vaccinated chickens treated with 10-fold dilution of C. citratus aqueous extract is observed to have the highest survival rate among vaccinated treatment groups with a 100%, which means that none of the samples died. On the other hand, vaccinated samples from treated with undiluted 1 mL C. citratus aqueous extract and samples treated with 20-fold dilution of C. citratus aqueous extract have the lowest survival rate among vaccinated samples, with a rate of of 66.6%, indicating two deaths. The lowest survival rate in unvaccinated samples also come from the samples from treated with 20-fold dilution of C. citratus aqueous extract. Unvaccinated chickens from treated with 5-fold dilution of C. citratus aqueous extract and treated with 10-fold dilution of C. citratus aqueous extract had zero deaths indicating a 100% survival rate.

Possible causes of deaths were considered with the guidance of Dr. Jaime Cabarles, Jr. Five out of eight deaths were speculated to be due to bacterial infection and environmental condition. Two were due to stress, possibly from mishandling.See Table 5

Discussion. – The initial mean antibody titres in overall treated group comprised by the vaccinated and vaccinated samples significantly increased (p¡0.001) after given the treatment of C. citratus aqueous leaf extract, indicating the efficacy of C. citratus aqueous extract in boosting immunology of native chickens. Additionally, mean antibody titre gain of vaccinated samples is not significantly different (p¿0.05) from the mean antibody titre of unvaccinated samples. This implies that vaccination does not contribute to the effectiveness of C. citratus in increasing the antibody titre of native chickens infected with ND. Vaccination enhances the bodys immunity against diseases by using antigens to trigger the immune systems production of antibodies. The induced immune response enables the immune cells to quickly recognize and react to a specific disease-causing organism (Pharmaceutical Research and Manufacturers of America

Table 3: Mean antibody titre gain per treatment group in vaccinated and unvaccinated groups

Group	Mean Live Weight Gain (g)	
Treated	Vaccinated	62.08
	Unvaccinated	95.77
	Overall	78.93
Control		47.33

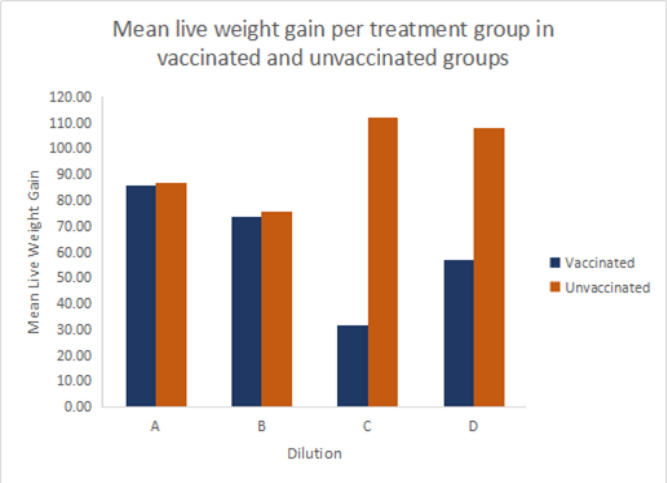

Fig. 2: Mean live weight gain per treatment group in vaccinated and unvaccinated groups

Table 4: Mean survival rate of sample groups

Group	Survival Rate	
Treated	Vaccinated	79.17
	Unvaccinated	87.50
	Overall	81.25
Control		100.00

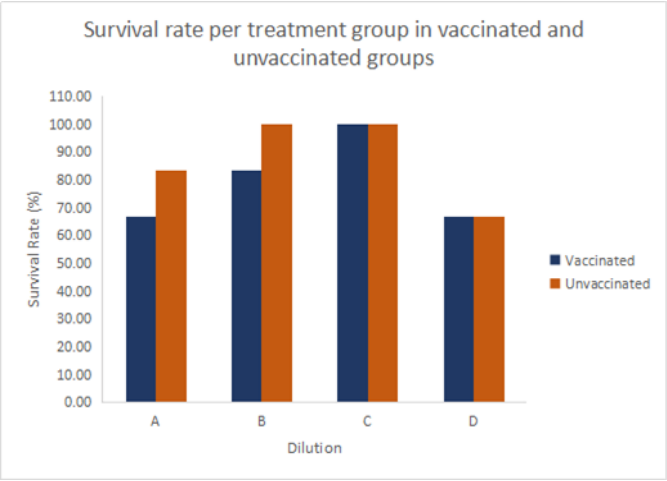

Fig. 3: The survival rate per treatment group in vaccinated and unvaccinated groups

Table 5: Possible causes of death of samples that died throughout the duration of the study

Sampl	Possible cause of death
VA5	stress after blood collection
VA6	unknown
VB4	coryza
VD2	extreme heat
VD3	stress
UA2	coryza
UD1	coryza
UD2	extreme heat

2013). However, failures in vaccination attenuation, vaccine regimes or administration can cause vaccine-related failures. Host-related failures are caused by genetics, immune status, age, health or nutritional status of the host (Wiedermann 2016). The vaccine loses its effectiveness due to these failures, thus the lack of the vaccine-induced antibody titre (Siegrist n.d.).

Mean antibody titre gain of vaccinated treatment groups differ significantly from each other (p¡0.05). The treatment group given with the 20-fold dilution of C. citratus aqueous leaf extract had the most significantly different mean antibody titre gain. The vaccinated group given with 5-fold, 10- fold, and 20-fold exhibited a significant increase in mean antibody titre gained. This means that the lower the concentration the higher is the antiviral activity against ND which contradicts the results of Hindumathy (2011) which states that the higher the concentration, the higher is its inhibition of microbial growth. The difference can be because Hindumathys study measured the bacterial activity of lemongrass. Bacteria are different from viruses because they can survive on their own, while virus reproduce inside a host cell, and is bigger compared to virus (National Institute of Allergy and Infectious Diseases 2009). Among the 5-fold, 10-fold, and 20-fold, the group receiving the 10-fold treatment exhibits the most significant increase followed by 5-fold, then by the 20-fold. After reaching 10-fold, the antibody gain decreases. According to Arnason et al. (1995), the antiviral activity is expressed as the virus titer reduction at the maximal nontoxic dose (MNTD) of the test substance. Toxic doses of the plant extract are dilutions that can deteriorate and degenerate the monolayer which causes the inability to determine the viral titre. In this case, 1-fold solution is a toxic dose of the extract that causes no significant increase in the antibody and dilutions more than 10-fold approaches the toxic dose, thus weakening the antiviral activity of C. citratus against ND.

The mean antibody titre of unvaccinated treatment groups on the other hand, do not significantly differ (p¿0.05) from each other. This implies that unvaccinated samples do not require a treatment diluted in a specific volume of distilled water for their antibody to increase when infected with ND. The initial mean live weight of the treated samples has increased significantly (p¡0.05) after given treatment of C. citratus aqueous leaf extract. There is no significant difference between the mean initial and the final live weight of samples from the control group. This indicates that C. citratus contributed to the growth of the treated samples. This can be attributed to the significant increase in the antibody of the samples because one of the symptoms of ND infection is loss of appetite, which leads to weight loss, but then administration of C. citratus treatment could have induced antibody production and eventually resulted to a significant increase in the live weight of the sample (Grimes 2002). C. citratus increased the level of protection of the treated samples. The survival rate of treated samples is higher than the normal survival rate of native chickens in the Philippines (Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development 2016) which suggests that C. citratus aqueous extract is effective for the protection of chickens against Newcastle disease.

It was found that there were eight recorded deaths which all came from the treated group. Only one death occurred after infection and during the treatment period. The other four deaths occurred during the acclimatization period, and then the other three after the infection but before the treatment (See Table 3 in appendix A). Factors such as stress, vitamin deficiency, and bacterial diseases can be attributed to the deaths before the virus infection. Stress can weaken the immune system of the chickens that make them prone to bacterial, fungal, and other types of diseases. Malfunction in the metabolism can also be a result to stress (Antony 2013). Heat stress, specifically, is one of the most important stressor that affects poultry production (Lara and Rostagno 2013). The housing utilized for the study was enclosed with plastic to avoid the spread of the airborne virus (Figure 1 in appendix C). This caused the heat to be trapped inside and eventually weakened the chickens. Vitamins and minerals are very important components of a chickens diet (The Poultry site 2008). According to Natividad (2017), the chicken samples used were showing signs of vitamin deficiency before infection. This deficiency, according to Davis (2015), can change the antibody count. For the duration of the study, the infection of coryza was observed (Figure 2.2 in appendix C). Coryza is an infectious disease caused by the bacterium Haemophilus paragallinarum that is characterized by sneezing, swollen face, and mucus from the eyes (Luciana et al. 2016). Before infection, some samples were already infected with coryza which could have affected their vulnerability to the Newcastle disease virus.

Conclusion. – In conclusion, C. citratus aqueous leaf extract is effective in boosting the immunity of native chickens when challenged with Newcastle disease. In addition, it is more practical to not vaccinate samples because unvaccinated samples infected with ND, when treated with C. citratus, has an increase in antibody titre and

live weight just as much as when it is vaccinated, and saves expenses on vaccination, and do not require a treatment diluted in a specific volume of distilled water for their antibody to increase when infected with ND. Vaccinated samples on the other hand require a treatment diluted in 9 mL distilled water for an optimal antibody increase when infected with ND.

Recommendations. – The 10-fold dilution of aqueous C. citratus leaf extract is recommended as an immune booster to chickens that have been infected with ND. It is also recommended to conduct further related studies to utilize an equal number of samples among the overall treated group and the control group. External factors such as heat stress and bacterial infections were observed. It is recommended to conduct the study in a more controlled laboratory. Also, the use of isolated Newcastle disease virus is recommended.

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