PRELIMINARY RESULTS OF *IN VITRO* GREENHOUSE GASES PRODUCTION AND FEED DIGESTION AFFECTED BY DIFFERENT PROBIOTIC ADDITIONS WITH RUMEN FLUID OF BEEF CATTLE AS AN INOCCULUM SOURCE

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ABSTRACT

This study aimed to evaluate the gas, CH_4, CO_2 and organic matter digestibility affected by 4 different probiotics. It included two *in vitro* experiments, which were arranged in 2 similar completely randomized designs with 5 treatments and 3 replications. Five treatments of Exp1 were non-probiotic supplementation (NP) the others were 0.25% of Vime-Subtyl, Vime-Bacilac, Biotic and Calphovit supplementation (DM basis) to Para grass (*Brachiaria mutica*) as a basal substrate. In Exp 2, the same percentage and kinds of probiotic supplementation of Exp 1 were done, however the main substrate were 80 (%DM) Para grass and 20 (%DM) concentrate feed including broken rice and soybean extraction meal and crude protein level in the substrate was fixed of 14.0 % (DM).

The results show that in Exp 1, CH_4 production at 72h of Vime-Bacilac and Calphovit (04.3 and 93.5 ml/g DOM, respectively) was significantly lower value compared to the others ($P<0.05$). OM digestibility (%) of Control (49.8) was significantly lowest ($P<0.05$) than the others. The results of Exp 2 indicates that CH_4 production at 24 and 72h of Calphovit (88.7 and 117 ml/g DOM, respectively) was the lowest compared to the others ($P<0.05$). The conclusion is that *in vitro* CH_4 production was different by probiotic sources. The probiotics that reduce methane production well from high to low were Calphovit, Biotic, Vime-Subtyl and Vime-Bacilac.

Key words: *yeast culture, biotic, Calphovit, Vime Subtyl, Vime Bacilac, climate change*

INTRODUCTION

Methane (CH_4) emission from ruminants particularly cattle is one of the major sources of atmospheric methane which is a greenhouse gas causing global warming (Martin et al., 2010). Apart from the contribution of ruminant methane emissions to anthropogenic greenhouse gases, methane represents a significant energy loss to ruminants. Therefore, the mitigation of CH_4 emissions from cattle are not only beneficial for the environment, but also beneficial for producers when feed energy-use efficiency is increased (Meale et al., 2012). Probiotics are microbial feed additives that influence rumen fermentation directly resulting in improved animal productivity (Iqbal et al., 2008). Relating to the lactic acid bacteria (LAB) such as *Lactobacillus, Streptococcus equinu, etc.,* Natasha et al. (2019) hypothesized that LAB could influence ruminal methanogenesisin three possible ways: (i) use of LAB or their metabolites to shift the rumen fermentation so that there is a corresponding decrease in CH_4 production, (ii) use of LAB or their metabolites to directly inhibit rumen methanogens and (iii) use of LAB or their metabolites to inhibit specific rumen bacteria that produce H_2 or methyl-containing compounds that are the substrates for methanogenesis. Probiotics such as yeast cultures are used to stimulate bacterial activity in the rumen. The probiotics have been shown to stabilize rumen pH, increase propionate levels and decrease the amount of acetate, methane and ammonia production (Tewodros and Mebrate, 2019). Addition of probiotic reduced methane production *in vitro* (Mutsvangwa et al., 1992). Therefore the objective of the present study was to determine the effects of probiotic on *in vitro* methane production and OM digestibility.

MATERIALS AND METHODS

Location and time

The experiments were done at the Department of Animal science, College of Agriculture of Can Tho University from April to December of 2019.

Feed samples, probiotics and chemical analysis

The feeds used in the experiments (Exp) were broken rice, soybean extraction meal and Para grass. Para grass samples were chopped to 1-2 cm length to dry at 55°C in an oven for 48 hours. Soybean extraction meal after oil extraction of the soybean and broken rice were bought from the feed shop at Binh Thuy district of Can Tho city. All the feed samples were finely ground to pass a 1mm sieve, prior to analyze for chemical composition,and used in both two experiments. The probiotic sources in powder form (the commercial products) included ***Vime-Subtyl*** (*Bacillus Subtilis* 10^8-10^{10} CFU/kg), ***Vime-Bacilac*** (*Bacillus subtilis*10^9 - 10^{10} CFU/kg,*Lactobacillus spp*10^6-10^9 CFU/kg and *Saccharomyces cerevisiae* 106 - 109 CFU/kg) and ***Calphovit*** (*Bacillus subtilis* 109 -1010 CFU/kg, *Pendiococcus spp* 10^6 -10^9 CFU/kg), which were produced by the Vemedim company. While ***Biotic*** (*Lactobacillus acidophilus* 2x10^{10} CFU/kg, *Bacillus subtillis* 2x10^{10} CFU/kg, *Saccharomyces cerevisiae* 2x10^{10} CFU/kg and *Aspergillus oryzae* 2x10^{10} CFU/kg) was the product of the Bio-pharmachemie. The amount of probiotics used for the experiments following the instructions of the company.

Experimental design

The present study included two *in vitro* experiments, which were arranged in 2 similar completely randomized designs with 5 treatments and 3 replications. Five treatments of Exp1 were non-probiotic supplementation (NP) the others were 0.25% of Vime-Subtyl, Vime-Bacilac, Biotic and Caphovit supplementation (DM basis) to Para grass (*Brachiaria mutica*) as a basal substrate. In Exp 2, the same kinds and percentage of probiotic supplementation of the Exp 1 were done, however the main substrate were 80% Para grass and 20% (DM basis) concentrate feed, which included broken rice and soybean extraction meal, and crude protein level in the substrate was fixed of 14.0% (DM).

Materials and*in vitro*gas production technique

Representative samples of substrates (0.2 gDM of the substrate) were put into the incubation 50-ml syringes. Buffer solution and cattle rumen fluid were added, prior to filling each bottle with carbon dioxide following the method described by Menke and Steingass (1988). Then, the syringes were put in the water bath at 39^{o}C for incubation.

Measurements taken

Chemical compositions of the substrate ingredients, which were analysed for dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash according to the standard methods of AOAC (1990), while neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed following procedures suggested by Van Soest et al. (1991).

Total gas, CH_4 and CO_2 production. Gas, CH_4 and CO_2 volumes over time (0, 3, 6, 9, 12, 24, 48 and 72 hours) were recorded and collected, while the CH_4 and CO_2 concentrations were measured by the Biogas 5000 Geotechnical Instruments (UK) Ltd, England.

Organic matter digestibility (OMD) at 24, 48 and 72 hours. Unfermented solids at 72 hours was determined by filtering through two layers of cloth and drying at 105°C for 24 hours and ashing for 5 hours to measure the dry matter digestibility (DMD) and organic matter digestibility (OMD), respectively.

Statistical analysis

The experiment data were calculated by Excel software and statistically analyzed by using ANOVA with the general linear model (GLM) following the complete randomized design and the Tukey test was used for a comparison of two treatments (Minitab, 2010).

RESULTS AND DISCUSSION

Chemical composition of feeds

The chemical composition of feeds of the study was presented in Table 1.

Table 1. Chemical composition (%) of feeds used in the experiment

Feed	DM	OM	CP	EE	CF	NFE	NDF	ADF	Ash
Para grass	94.3	88.6	10.1	3.65	30.4	44.7	62.5	31.2	11.4
Broken rice	86.1	99.1	9.32	1.47	0.93	87.4	3.90	3.32	0.90
Soybean extraction meal	89.6	93.8	43.7	2.93	7.00	40.2	8.45	2.65	6.20

DM: dry matter, OM: organic matter, CP: crude protein, EE: Ether extract, CF: crude fiber, NFE: nitrogen free extract, NDF: neutral detergent fiber, ADF: acid detergent fiber

Exp 1

Gas production (ml) over incubation times

The Fig. 1 describedthe gas production of the NP, Vime-Subtyl, Vime-Bacilac, Biotic and Calphovit treatments over incubation times. It indicated that there was not clearly different in the lines of gas production among the treatments over incubation times in case of only the Para grass substrate used, however at 72 h the gas production of the Vime-Subtyl was higher than the others.

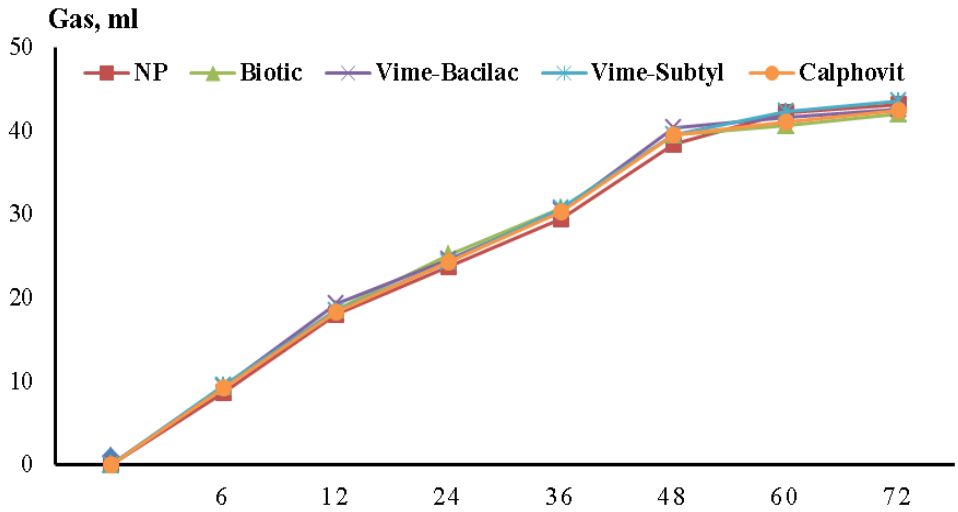

Fig. 1. Gas production of different treatments over incubation time in the Exp 1

In vitro gas, CH_4 and CO_2 production, and OM digestibility at 24 hours of Exp 1

Gas, CH_4 and CO_2 production, and OM digestibility at 24 hours of Exp 1 were presented in Table 2.

Table 2. Gas, CH_4 and CO_2 production, and OM digestibility at 24 h of Exp 1

Item	Treatments					SE	P
	NP	Vime-Subtyl	Vime-Bacilac	Biotic	Calphovit		
Gas, ml	29.5^{b}	30.7^{a}	30.5^{a}	30.8^{a}	30.3^{a}	0.130	0.010
CH_4, ml	5.13^{bc}	5.38^{a}	5.35^{a}	5.22^{b}	5.28^{b}	0.020	0.001
CO_2, ml	18.1	18.7	18.8	18.8	18.4	0.090	0.081
OMD, %	33.7^{e}	37.2^{a}	36.7^{b}	36.0^{c}	35.2^{d}	0.030	0.001
Gas, ml/g OM	168^{b}	175^{a}	174^{a}	175^{a}	173^{a}	0.760	0.001
CH_4, ml/g OM	29.2^{c}	30.6^{a}	30.4^{b}	31.4^{b}	30.0^{b}	0.760	0.001
CO_2, ml/g OM	103^{b}	107^{a}	106^{a}	107^{a}	105^{ab}	0.522	0.001
Gas, ml/g DOM	498^{a}	476^{b}	466^{c}	472^{b}	490^{ab}	2.00	0.001
CH_4, ml/g DOM	86.6^{a}	82.8^{c}	82.2^{c}	83.1^{b}	85.2^{ab}	0.349	0.001
CO_2, ml/g DOM	306^{a}	292^{bc}	285^{c}	297^{b}	297^{b}	1.42	0.001

OMD: organic matter digestibility. a, b, c, d, e Means with different letters within the same rows were significantly different at the 5% level (P<0.05).

Table 2 showed that *in vitro* gas, CH_4, CO_2 production (ml) at 24 hours of Exp 1 were significantly different (P<0.05) among the treatments. The gas production (ml) for the NP treatment (29.5) was lower than that of the Vime-Subtyl (30.7), Vime-Bacilac (30.5), Biotic (30.8) and Calphovit treatments (30.3). Similarly CH_4 production was higher for the probiotic supplementation treatments. While the *in vitro* CO_2 production (ml) of different treatments was similar (P>0.05). The gas production, CH_4 and CO_2 production (ml/g OM) were also higher for the probiotic supplementation treatments. Organic matter digestibility (OMD) significantly increased (P<0.05) when probiotics added to the treatments, with the highest values for the Vime-Subtyl treatment (37.2%) and the lowest for the NP treatment (33.7%). When the calculation of the gas, CH_4 and CO_2 values based on the DOM (digested organic matter), they were significantly lower (P<0.05) for the Vime-Subtyl, Vime-Bacilac and Biotic treatments compared to the NP treatment.

In vitro gas, CH_4, CO_2 production and OM digestibility at 72 hours

The accumulated gas, CH_4 and CO_2 production and OM digestibility at 72 h in Exp 1 were showed in Table 3.

The accumulated gas, CH_4 and CO_2 production values (ml) at 72h of Exp 1 were significantly different (P<0.05) among the treatments with the highest value for the Vime-Bacilac treatment being 43.6, 7.88 and 26.3, respectively.The results of gas production were similar to those at 72 h reported by Huynh Hoang Thi (2013) whenreplacing Para grass to *Operculia turpethum* and *Psophocarpus scandens* being from 38.5 to 38.9 ml. Organic matter digestibility (OMD) values were significantly different (P<0.05) among the treatments with the higher values for the probiotic supplementation treatments. Arcos-Garcia et al. (2000) stated that probiotics stimulate the activity and growth of rumen cellulolytic bacteria and Whitley et al. (2009) concluded that there were significant improvement in DM, CP, NDF and ADF digestion in goats fed diets added probiotics than in the control group. The gas, CH_4 and

CO_2 production (ml/gOM and ml/gDOM) were significantly different (P<0.05) among the treatments and they were reduced in the probiotic supplementation treatments, particularly for the Calphovit and Biotic treatment. El-Waziry (2007) also reported that adding probiotics increased fiber digestibility, and protein synthesis of microorganisms (Uyeno et al., 2015).

Table 3. Gas, CH_4 and CO_2 production, and OM digestibility at 72 h of Exp 1

Item	Treatments					SE	P
	Cont.	*Vime-Subtyl*	*Vime-Bacilac*	*Biotic*	*Calphovit*		
Gas, ml	43.1^{b}	42.6^{c}	43.6^{a}	42.1^{d}	42.4^{c}	0.05	0.001
CH_4, ml	7.77^{a}	7.77^{a}	7.88^{a}	7.77^{a}	7.52^{b}	0.03	0.001
CO_2, ml	25.9^{b}	25.6^{c}	26.3^{d}	25.5^{c}	25.1^{a}	0.06	0.001
OMD, %	49.8^{e}	53.0^{a}	52.7^{b}	51.6^{c}	50.8^{d}	0.02	0.001
Gas, ml/g OM	245^{b}	248^{a}	242^{c}	239^{d}	241^{c}	0.27	0.001
CH_4, ml/g OM	44.2^{a}	44.8^{a}	44.2^{a}	44.2^{a}	42.8^{b}	0.16	0.001
CO_2, ml/g OM	148^{b}	149^{a}	146^{c}	145^{c}	143^{d}	0.34	0.001
Gas, ml/g DOM	469^{a}	414^{a}	401^{a}	408^{a}	417^{a}	16.5	0.001
CH_4, ml/g DOM	97.3^{a}	97.0^{a}	94.3^{b}	96.2^{a}	93.5^{b}	0.35	0.001
CO_2, ml/g DOM	325^{a}	323^{a}	311^{c}	316^{b}	312^{bc}	0.80	0.001

OMD: organic matter digestibility. $^{a, b, c, d, e}$ *Means with different letters within the same rows were significantly different at the 5% level (P<0.05).*

Exp 2

Gas production (ml) over incubation time

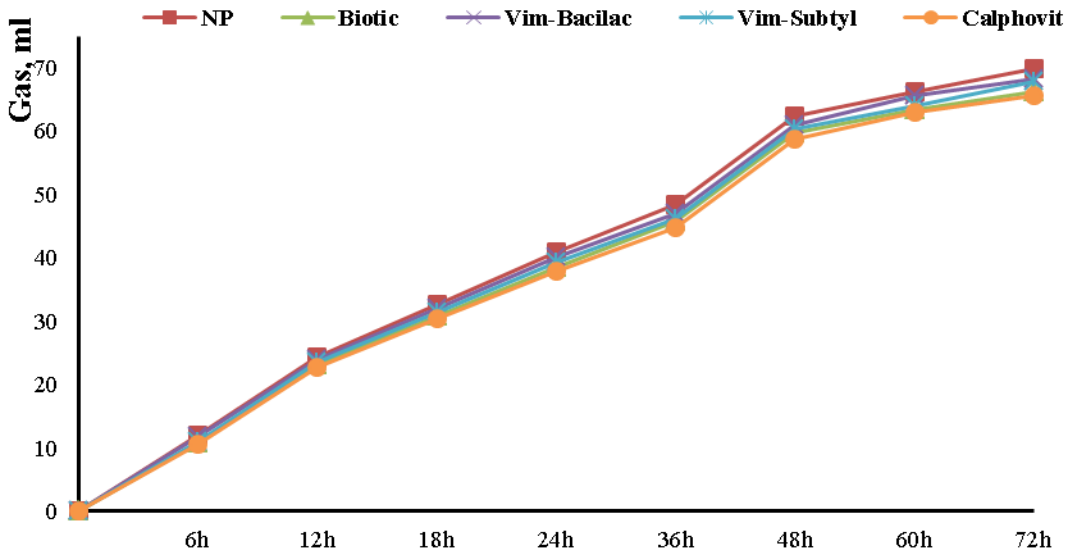

Fig. 2. Gas production of different treatments over incubation time in the Exp 2

The Fig. 2 presented the gas production of the NP, Vime-Subtyl, Vime-Bacilac, Biotic and Caphovit treaments over incubation time. It indicated that in case of Para grass and concentrate feed used as the main subtrate, the gas production was clearly showed the higher line for the NP treatment, while Calphovit treatment was lower during incubation times. This could be explained that when concentrate added, the gas would be produced more than that of

the Exp 1. Thus the difference of the gas volume among the treatment was clearly recorded and analyzed.

In vitro gas, CH_4, CO_2 production and OM digestibility at 24 hours

Gas, CH_4 and CO_2 production (ml) and OM digestibility (OMD) of different treatments at 24h were presented in Table 4.

Table 4. Gas, CH_4 and CO_2 production (ml) and OM digestibility (OMD) at 24 h of Exp 2

Item	Treatments					SE	P
	NP	Vime-Subtyl	Vime-Bacilac	Biotic	Calphovit		
Gas, ml	41.0^{a}	40.1^{b}	39.4^{c}	38.5^{d}	38.0^{e}	0.02	0.001
CH_4, ml	9.50^{a}	9.01^{b}	8.4^{d}	8.61^{c}	8.38^{d}	0.02	0.001
CO_2, ml	29.9^{a}	27.6^{b}	28.1^{b}	27.6^{b}	27.4^{b}	0.15	0.001
OMD, %	47.8^{e}	49.1^{d}	50.8^{c}	52.1^{b}	53.4^{a}	0.10	0.001
Gas, ml/g OM	231^{a}	226^{b}	223^{c}	217^{d}	214^{e}	0.09	0.001
CH_4, ml/g OM	53.6^{a}	50.9^{b}	47.5^{d}	48.6^{c}	47.3^{d}	0.12	0.001
CO_2, ml/g OM	143^{a}	133^{bc}	135^{b}	131^{c}	131^{c}	0.69	0.001
Gas, ml/g DOM	485^{a}	461^{b}	438^{c}	417^{d}	402^{e}	0.83	0.001
CH_4, ml/g DOM	112^{a}	104^{b}	93.5^{c}	93.3^{c}	88.7^{d}	0.29	0.001
CO_2, ml/g DOM	299^{a}	271^{b}	266^{b}	251^{c}	245^{c}	1.41	0.001

OMD: organic matter digestibility. $^{a, b, c,d,e}$ means with different letters within the same rows were significantly different at the 5% level ($P<0.05$).

In case of Exp 2 with the substrate including Para grass and concentrate feed (Table 4), the gas, CH_4 and CO_2 production (ml) at 24h were significantly different among the treatments and the highest values were for the NP treatment. However, the OMD (%) was significantly higher ($P<0.05$) for the probiotic supplementation treatments compare to the NP treatment, and the highest value for the Calphovit treatment. The greenhouse gas emission (ml/gOM and ml/g DOM) weregradually reduced for the NP, Vime-Subtyl, Vime-Bacilac, Biotic and Caphovit treaments.

***In vitro* total gas, CH_4, CO_2 production and OM digestibility at 72 h**

Gas, CH_4 and CO_2 production (ml) and OM digestibility (OMD) of different treatments at 72 hin Exp 2 were presented in Table 5.

Table 5 showed that the gas, CH_4 and CO_2 production (ml) at 72h of Exp 2 decreased when probiotic was added to the substrate and they were gradually reduced for the NP, Vime-Subtyl, Vime-Bacilac, Biotic and Calphovit treatments. In contrast, the OMD (%) gradually increased for the above treatments. The gas, CH_4 and CO_2 production values (ml/g OM and ml/g DMD) were significantly different among the treatments with a gradual reduction for the NP, Vime-Subtyl, Vime-Bacilac, Biotic and Calphovit treatments. In general these values were much higher than those the Exp 1. It was thought that yeast culture (probiotic) reduces methane production in four ways: (i) by increasing butyrate or propionate production (Lila et al., 2004); (ii) by reducing the amount of protozoan (Newbold et al., 1998); (iii) by promoting acetogenesis (Chaucheyras et al. 1995); and (4) by improving animal productivity (Bruno et al., 2005). The probiotics have been shown to stabilize rumen pH, increase propionate levels and decrease the amount of acetate, methane and ammonia production (Tewodros and

Mebrate, 2019). Addition of *Sacchromyces cerevisiae* reduced methane production *in vitro* (Mutsvangwa et al., 1992). According to Eun et al. (2003), brewer yeast culture enriched the activity of bacteria which convert H_2 to acetate and thus can reduce CH_4 production by 25% in a continuous culture of ruminal microorganisms. In general, the Exp 2 showed that addition of probiotics was shown to increase organic matter digestibility with the highest values of Calphovit treatment (69.9%) and the lowest values of NP treatment (57.1%) and the *in vitro* greenhouse gas production was clearly reduced.

Table 5. Gas, CH_4 and CO_2 production, OM digestibility at 72 hours in Exp 2

Item	Treatments					SE	P
	NP	***Vime-Subtyl***	***Vime-Bacilac***	***Biotic***	***Calphovit***		
Gas, ml	69.9^{a}	68.2^{b}	67.8^{c}	66.2^{d}	65.6^{e}	0.10	0.001
CH_4, ml	16.1^{a}	15.2^{b}	14.7^{c}	14.4^{d}	14.5^{d}	0.20	0.001
CO_2, ml	46.0^{a}	43.4^{c}	44.6^{b}	42.7^{c}	43.2^{c}	1.60	0.001
OMD, %	57.1^{e}	63.9^{d}	66.7^{c}	68.0^{b}	69.9^{a}	2.0	0.001
Gas, ml/g OM	394^{a}	385^{b}	383^{c}	374^{d}	370^{e}	7.70	0.001
CH_4, ml/g OM	90.8^{a}	85.6^{b}	83.0^{c}	81.3^{d}	81.9^{d}	1.56	0.001
CO_2, ml/g OM	260^{a}	245^{c}	252^{b}	241^{c}	244^{c}	9.04	0.001
Gas, ml/g DOM	691^{a}	603^{b}	574^{c}	549^{d}	530^{e}	31.2	0.001
CH_4, ml/g DOM	159a	134^{b}	124^{c}	120^{d}	117^{e}	2.56	0.001
CO_2, ml/g DOM	455^{a}	384^{b}	378^{b}	355^{c}	349^{c}	14.5	0.001

OMD: organic matter digestibility. $^{a, b, c, d, e}$ *Means with different letters within the same rows were significantly different at the 5% level (P<0.05).*

CONCLUSION AND RECOMMENDATION

It was concluded that:

Adding probiotics to the substrate could improve the *in vitro* organic matter digestibility and greenhouse gas production, and more reduction of CH_4 and CO_2 was for the Calphovit and Biotic.

It was also found that supplementing concentrate to the substrate of Para grass would induce to increase the greenhouse gas production andfurther investigations in *in vitro, in vivo* and performance studies on probiotics to confirm the results should be implemented for future applications.

ACKOWLEDGEMENT

This research is funded in part by the Can Tho University Improvement Project VN14-P6, supported by a Japanese ODA loan. The Authors also thank to the JIRCAS project and Dept of Animal Sciences of College of Agriculrure, Can Tho University for facilitating the equipments using and Laboratory works of the experiments.

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Received date: 10/9/2020

Submitted date: 18/9/2020

Acceptance date: 21/10/2020

Opponent: Dr. Chu Manh Thang