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SCIENTIFIC NEWS

The size of householder pig population in Vietnam

EFFECT OF FUT1 GENE ON CARCASS PERFORMANCE AND MEAT QUALITY OF LARGE WHITE PIGS

Do Duc Luc[1], Ha Xuan Bo[1], Nguyen Hoang Thinh[1], Tran Xuan Manh[2], Nguyen Van Hung[2], Frédéric Farnir[3] and Vu Dinh Ton[1]*

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ABSTRACT

This study was carried out on 24 Yorkshire pigs (13 intact males and 11 females) to determine the effect of FUT1 genotype on carcass traits and meat quality. The genotypes were identified using PCR-RFLP method. The carcass traits were slaughter live weight, hot carcass weight, killing out percentage, dressing weight, dressing percentage and carcass length. The meat quality was assessed through measurements of pH (45 minutes and 24 hours *post mortem*), color C.I.E (L*, a* and b*), drip loss percentage (DL), cooking loss percentage (CL) and Warner-Bratzler shear force (SF) at 24 hours *post mortem*. The results show that FUT1 genotypes did not affect carcass traits and meat quality (P>0.05). These results suggest that selection of pigs resistant to diarrhea based on FUT1 at early growth stage should be without effects on carcass performance and meat quality.

Key words: *FUT1, swine, carcass traits, organoleptic meat quality.*

1. INTRODUCTION

Porcine post-weaning diarrhoea (PWD) is an enteric disease of piglets after weaning. PWD has an important impact on economic losses due to a reduction of the ADG and an increase in the mortality rate as well as the high costs of veterinary medication (Fairbrother *et al*., 2005). To date, no satisfactory method for controlling post-weaning diarrhoea is available (Coddens *et al*., 2008). In 1997, Vogeli and his colleagues identified the alpha (1, 2) fucosyltransferase gene (FUT1) as a candidate gene for controlling the expression of ETEC (Entero-Toxigenic *Escherichia Coli*) F18 (one of the adhesins produced by porcine ETEC strains) receptor (Vogeli *et al*., 1997).

FUT1 gene is located on pig chromosome 6 and harbors a biallelic (alleles A and G) polymorphism associated with a resistance to ETEC infections: the animals with AA genotype are resistant to ETEC E18 while those with AG and GG are sensitive (Meijerink *et al*., 1997). Production and reproduction performance of pigs are also affected by FUT1 gene (Jiang *et al*., 2005; Bao *et al*., 2011; Zhu *et al*., 2014; Do Duc Luc *et al*., 2016). The selection of pigs resistant to PWD based on FUT1 genotype could impact other economic traits. To our knowledge, there are no published studies on the effect of FUT1 gene on carcass traits and meat quality. The aim of this study is to find the relationship between FUT1 genotype, carcass traits and meat quality of Large White pigs.

2. MATERIALS AND METHODS

2.1. Animals and data collection

The study was carried out at Dabaco Nucleus Breeding Pig Company, Bac Ninh province, Vietnam (40km North from Ha Noi) in May 2017. A total of 24 Large White pigs (13 intact males and 11 females) were selected to investigate the effect of FUT1 gene on carcass performance and meat quality.

Pigs were slaughtered at the age of 5.5 month at the slaughter house of Dabaco Company. The carcass traits were measured immediately after slaughter. Before slaughter, the live weights of the animals were recorded (SLW, kg). Hot carcass with head, feet and leaf fat (HCW, kg) was weighed after slaughter. The killing out percentage (KOP, %) was calculated as a ratio of hot carcass to live weight. While dressing weight

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(DCW, kg) is a HCW without head, 4 feet, feet and leaf fat. Dressing percentage (DCP, %) were a ratio between DCW and SLW. Carcass length (CL, cm) was measured on carcass from *tuber ischiadicum* to *atlas vertebra*.

Samples were taken from left half-carcass of 24 abovementioned pigs from the 1st to 4th last ribs immediately after slaughter. At the laboratory of Department of Animal Breeding and Genetics, Vietnam National University of Agriculture (VNUA), a sample of 2.5-cm-thick was weighed and put individually in a tight plastic bag at 4°C until 24 hours *post mortem* for meat quality analysis. The meat quality was assessed through measurements of pH, color C.I.E (L*, a* and b*), drip loss percentage (DL), cooking loss percentage (CL) and Warner-Bratzler shear force (SF). The first measure was conducted on *longissimus dorsi* muscle between the 1st-4th last ribs at 45 minutes *post mortem* for determining pH value (pH45). The meat quality traits were determined in the laboratory at 24 hours *post mortem* according to the method described by Leroy *et al.* (2008). The pH, meat color and shear force were measured using a portable pH-meter (Testo 230 with an electrode type 03 pH, Germany), a colorimeter (Minolta CR-410, Japan) with settings of illuminant D65 and 2° standard observer and a Shear force Warner Bratzler 2000D (USA) respectively.

2.2. Determination of the FUT1 genotypes

The FUT1 genotype of pigs was identified at birth. The piglet tails were docked at birth, then stored in keeping sample boxes and transported to the laboratory the Faculty of Animal Science, Vietnam National University of Agriculture. Genomic DNA was isolated from the porcine tail sample following standard procedures (Sambrook *et al*., 1989). PCR-RFLP based on the G→A mutation at nucleotide 307 of the FUT1 gene was used to genotype the pigs (Meijerink *et al*., 1997).

2.3. Statistical analysis

The fixed effects of the FUT1 genotypes (AA, AG and GG), of the gender (females and intact males) and interaction between two fixed factors on the study traits were included in the statistical model. The data were analyzed using ANOVA procedure of SAS software (SAS, 1989) to identify significant sources of variation.

3. RESULTS

In our study, three genotypes (AA, AG and GG) were found at FUT1 locus. The interaction between two fixed factors (Genotype and Gender) was not observed for all study traits (P>0.05). Therefore, the interaction effect was not presented in this section.

3.1. Effect of FUT1 gene on carcass performance and meat quality of Large White pigs

The effects of FUT1 gene on carcass performance and meat quality of Large White pigs are shown in Tables 1 and 2 respectively. FUT1 genotype did not affect any of the carcass and meat quality traits (P>0.05). Although no significant difference was found between genotypes, the dressing percentage tended to be higher and carcass lengths to be longer for pigs carrying AA compared to those with AG and GG genotypes (Table 1). Additionally, the shear force tended to be lower (Table 2)

Table 1. Carcass performance of Large White pigs according to FUT1 genotype

Variable	**AA** (n=3)	**AG** (n=10)	**GG** (n=11)
	Mean±SD	**Mean±SD**	**Mean±SD**
Slaughter live weight (kg)	97.33±14.15	96.7±18.77	98.64±5.68
Hot carcass weight (kg)	71.7±9.27	70.17±12.84	71.65±5.73
Killing out percentage (%)	73.79±1.44	72.77±4.67	72.66±4.08
Dressing carcass weight (kg)	63.42±7.67	61.38±11.38	62.39±5.45
Dressing carcass percentage (%)	65.34±2.01	63.68±5.2	63.27±4.33
Carcass length (cm)	99±7.55	91.8±12.18	94.55±1.86

Table 2. Meat quality of Large White pigs according to FUT1 genotype

Variable	AA (n=3)	AG (n=10)	GG (n=11)
	Mean±SD	Mean±SD	Mean±SD
pH 45 min	6.52±0.15	6.43±0.41	6.45±0.21
pH 24 h	5.39±0.09	5.48±0.24	5.52±0.26
Drip loss, %	0.96±0.84	3.2±1.89	1.78±1.61
Cooking loss,%	31.53±3.52	30.42±3.91	30.68±3.62
L*	58.5±3.43	58.34±2.97	57.57±2.42
a*	12.79±0.64	13.23±1.62	13.37±0.81
b*	7.19±1.75	6.59±1.69	14.94±26.46
Shear force (N)	39.66±4.21	40.65±8.72	43.62±9.68

3.2. Effect of gender on carcass performance and meat quality of Large White pigs

The effects of gender on carcass performance and meat quality of Large White pigs are shown in Tables 3 and 4 respectively. At slaughter, the males were heavier than the females (P<0.05). Consequently, hot carcass weight of male was higher than female (P<0.05). Gender affected pH at 24 hour post mortem, L* and a* (P<0.05).

Table 3. Carcass performance of Large White pigs

Variable	Male(n=13)	Female(n=11)
	Mean±SD	Mean±SD
Slaughter live wt (kg)	102.8^{a}±11.8	91.64^{b}±12.27
Hot carcass wt (kg)	74.57^{a}±7.28	66.87^{b}±10.04
Killing out percentage	72.84±4.98	72.85±2.69
Dressing carcass wt (kg)	64.91±6.55	58.77±9.23
Dressing carcass (%)	63.46±5.23	63.99±3.44
Carcass length (cm)	95.38±6.02	92.27±10.57

Within rows, means followed by different letters are significantly different (P<0.05)

Table 4. Meat quality of Large White pigs

Variable	Male(n=13)	Female(n=11)
	Mean±SD	Mean±SD
pH 45min	6.36±0.37	6.57±0.1
pH 24h	5.61^{a}±0.21	5.34^{b}±0.18
Drip loss (%)	2.42±1.84	2.09±1.86
Cooking loss (%)	31.6±2.74	29.59±4.26
L*	56.69^{b}±2.78	59.56^{a}±1.55
a*	13.98^{a}±1.04	12.3^{b}±0.57
b*	6.05±1.41	15.74±26.18
Shear force (N)	42.66±10.29	40.97±6.57

4. DISCUSSION

4.1. Effect of FUT1 gene on carcass performance and meat quality of Large White pigs

To our knowledge, there are no published studies on the effect of FUT1 gene on carcass traits and meat quality of Large White pigs. In our study, SNP in FUT1 had no effect on carcass traits and meat quality. This result is consistent with study of (Binder *et al*., 2005) on Pietrain and Landrace. However, In the study of Jiang *et al*. (2005)Restriction Fragment Length</keyword><keyword>Quantitative Trait Loci</keyword><keyword>Swine/anatomy & histology/*genetics</keyword></keywords><dates><year>2005</year><pub-dates><date>Jul</date></pub-dates></dates><isbn>0253-9772 (Print on the hybrid finishing pigs Large White x Meishan, the authors found that the meat pH and color of AA genotype pigs were higher than those of AG. According to the pork classification of Warner *et al*. (1997), the organoleptic meat quality traits in our study were in the range of normal meat (pH<6 and, drip loss <5%) except L* (L*<50). The SNP in FUT1 had no effect on carcass traits and meat quality of Large White pigs. These results suggest that selection of pigs resistant to diarrhea based on FUT1 at early growth stage of pigs should be without effects on carcass performance and meat quality traits. Note however that, in our study, the sample sizes were small for all three genotypes. In particular, there were only 3 pigs with the AA genotype. Another larger experiment should be setup to confirm the absence of effect of this locus on the study traits.

3.2. Effect of gender on carcass performance and meat quality traits of Large White pigs

HCW, KOP and CL were not different between intact male and gilts in agreement with previous research (Latorre *et al*., 2003; Piao *et al*., 2004). Significant difference between males and gilts for the pH at 24 hours *post mortem* (pH4d) was in agreement with Larzul *et al*. (1997) and (Alonso *et al*., 2009) but in contrast with Piao *et al*. (2004); Võ Trọng Thành *et al*. (2017). The pH values of *longissimus dorsi* muscle in the present study were consistent with (Kaić *et al*., 2009) and were in the acceptable range.

An effect of gender on L* and a* was found. However, previous studies (Furman *et al*., 2007; Latorre *et al*., 2004) showed no effect of gender on these two traits. The shear force did not affect by gender, which confirm previous observations by

(Latorre *et al*., 2003). Results in current study disagree with those of Lampe *et al*. (2006), who reported that barrows had more tender meat than gilts.

5. CONCLUSION

The tested SNP polymorphism in FUT1 does not seem to have an effect on carcass performance and meat quality of Large White pigs. These results suggest that selection of pigs resistant to diarrhoea based on FUT1 at early growth stage of pigs should not affect strongly carcass performance and meat quality traits.

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GENETIC POLYMORPHISM OF 5′- FLANKING REGION OF THYROGLOBULIN GENE IN SEVEN VIETNAMESE YELLOW CATTLE POPULATIONS

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ABSTRACT

The single nucleotide polymorphism in 5′- flanking region of thyroglobulin gene (TG5) has been reported to be associated with level in intramuscular fat (IMF) content or marbling in beef cattle. This study was conducted to assess the TG5 polymorphism in 462 Vietnamese yellow cattle from seven distinct geographical populations using PCR-RFLP method. The 548 base pairs fragment of TG5 gen was amplified by PCR reaction and digested by *BstY*I restriction enzyme. The results revealed two alleles (C and T) and two genotypes (CC and CT) across the seven cattle populations studied. The frequency of C and T allele were 99.5 and 0.5%, respectively, CC and CT genotypes were 99 and 1%, respectively, across the seven cattle populations studied. The Chi-square test showed that the difference in *BstY*I allelic frequency between seven different geographical populations was not significant (P>0.05).

Keywords: *Yellow cattle, genetic polymorphism, thyroglobulin gene.*

1. INTRODUCTION

Thyroglobulin gene (TG) is considered as a functional and positional candidate gene for fat deposition cattle (Thaller *et al*., 2003). TG encodes thyroglobulin, a glycoprotein hormone that is synthesized in thyroid follicular cell, signal for fat cells development (Ailhaud *et al*., 1992). TG was mapped to the centromeric region of bovine chromosome 14. A polymorphism in the 5′- flanking region of the TG gene (TG5) was associated with variation in marbling in several cattle breeds from different countries (Barendse *et al*., 2004; Cacas *et al*., 2005; Rincker *et al*., 2006; Van Eenennaam *et al*., 2007). This variation is widely used in marker-assisted selection (MAS) programs to improve the predictability of marbling level and meat quality in beef cattle.

Vietnamese Yellow cattle is only a native breed across the country but subdivides into seven local populations (Ha Giang, Lang Son, Thanh Hoa, Nghe An, U Dau Riu, Phu Yen, Ba Ria Vung Tau) with diverse genetic diversity (Pham *et al*., 2013). The popularity of each population is based on cultural interest of local people and its adaptability to local conditions. In general, Vietnamese Yellow cattle are small in size and have low performance in meat and milk production, but they can adapt well with harsh conditions and have a good reproduction capacity (Ly *et al*., 1999). However, the number of Vietnamese Yellow cattle is decreased recently due to a cross-breeding with exotic breeds to improve the productivity, draught power and milk production.

The study on molecular genetics of Vietnamese Yellow cattle are limited, particularly no study has been done on the TG5 polymorphism so far. The present study aim to genotype TG5 and to compute TG5 allelic frequency in seven Vietnamese Yellow cattle populations.

2. METERIALS AND METHODS

2.1. Samples collection and DNA extraction

Tissue samples were collected from 462 unrelated cattle animals from seven local populations located across the country: Ha Giang (HG); Lang Son (LS); Thanh Hoa (TH), Nghe An (NA), Phu Yen (PY), Ba Ria Vung Tau (BRVT) and URiu (UR) (Fig1). Tissue samples were kept in ethanol and genomic DNA was then extracted using Bioneer kit (Korea). DNA quality and quantity were checked by agarose gel (0.8%) and UV spectrophotometer (Nano drop machine).

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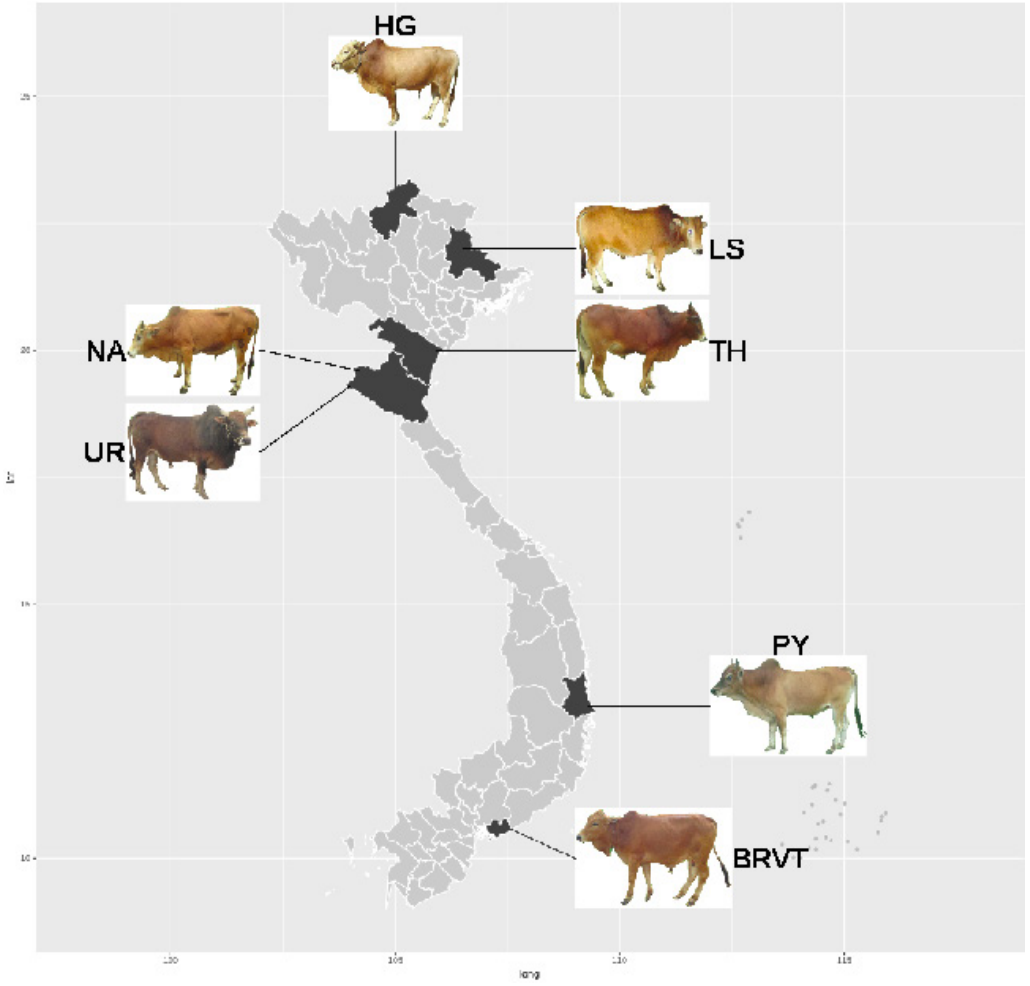

Fig 1. Geographic localizations of seven Vietnamese Yellow cattle populations

2.2. PCR-RFLP genotyping for TG5 gene

The fragment of 548 base pairs (bp) of 5′ promoter region of thyroglobulin gene was amplified using a following primer pair (forward 5′-GGGGATGACTACGAGTATGACTG-3′ and reverse 5′-GTGAAAATCTTGTGGAGGCTGTA-3′) described by De *et al*. (2004). The PCR reaction was carried out in a reaction volume of 25µl containing 50-100ng genomic DNA, 10 pmol each primer, 1.5mM MgCl2, 200µM dNTP, and 1.0U Taq polymerase (Thermor scientific). PCR was carried out with a PTC 100 apparatus (MJ Research, MA, USA). Amplification cycling conditions were 95^{0}C for 5 min; followed by 35 cycles at 94^{0}C for 35s, 63^{0}C for 45s, and 72^{0}C for 35s; with a final extension at 72^{0}C for 10 min. For the PCR–RFLP analysis, 15 µl each PCR amplified product was digested with 5UI *BstY*I enzyme (Thermor scientific) at 37^{0}C for overnight followed by 20 min of inactivation at 65^{0}C. The digestion products were separated on 2% agarose gel in TBE buffer by electrophoresing at 90 volts, 35 min. Gel was stained with ethidium bromide and visualized under UV light.

2.3. Statistical analysis

Allele and genotype frequency were calculated by direct counting. A chi square test was used to evaluate the significance of differences in allele and genotype frequency among studied populations by R software.

3. RESULTS AND DISCUSSION

A 548bp fragment of 5′ region of TG was amplified. The PCR products contain one common and one polymorphic cut site for restriction *BstY*I enzyme. After enzyme digestion gave a common band of 75bp, and three polymorphic bands of 178, 295 and 473bp. The T allele presented two bands of 473 and 75bp, allele C presented three bands of 295, 178 and 75bp (Fig 2).

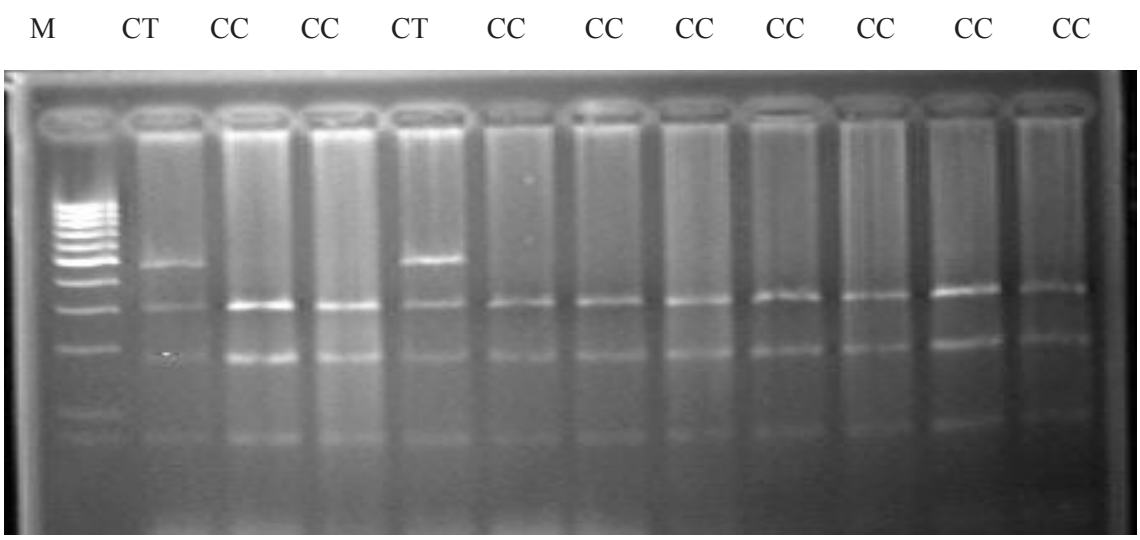

Fig 2: PCR-RFLP of TG5 gene polymorphism using *BstY*I restriction enzyme.

M: molecular weight standard 100 bp ladder (Fermentas).

PCR-RFLP genotyping in 462 animals identified two different genotypes CC (457 animals) and CT (5 animals), and no animal with TT genotype was identified. The numbers of genotype and fragment size corresponding to different genotypes were showed in Table 1.

Table 1. The number of animals with genotype and fragment size corresponding to different genotype after digestion a PCR product by *BstY*I restriction enzyme

Genotype	No. of cattle	Fragment size after digestion a PCR product by *BstY*I restriction enzyme
C/C	457	295 bp, 178 bp, 75 bp
C/T	5	473 bp, 295 bp, 178 bp, 75 bp
T/T	0	473 bp, 75 bp

The genotypic frequencies of CC and CT genotype were 99% and 1% respectively across all populations. The allelic frequencies of C and T alleles were ranged from 98.5 to 100% and 0.0 to 1.5%, respectively among the population investigated. The frequency of T allele was found to be very lower than that of C allele in all seven populations. The C allele was similar for Lang Son, Nghe An and Phu Yen populations. Overall, the allelic frequencies of C and T allele were 99.5 and 0.5% respectively across seven populations (Table 2). The Chi-square test showed that the difference in TG allele frequency between seven populations was not significant (P>0.05).

The T allele in TG5 gene has been proposed by Barendse *et al*. (2004); Casas *et al*. (2005); Bonilla *et al*. (2010) and Anton *et al*. (2011), as the favorable allele because the homozygous TT cattle tend to be higher in marbling score than heterozygous CT and homozygous CC cattle.

The results obtained in this study are similar to Smith *at al*. (2009) who reported genotypic frequencies for TG5 in Brahman steer to be 99.2, 0.78 and 0.0% for genotypes CC, CT

and TT, respectively but different from Rincker *at al.* (2006) who reported genotypic frequencies for TG5 in Simmental steer to be 26.8% for CC, 54.3% for CT and 18.9% for TT.

In fact, TT genotypes of TG5 gene were known to have a low frequency in *Bos indicus* such as Marina *at al*. (2009) reported that the genotypic frequencies of CC genotype were fixed (100%) in *Bos indicus* breed (Nelore breed) and the genotypic frequencies to be 96.2% for CC, 3.8 % for CT and 0.0% for TT in Rubia Gallega X Nelore breeds (1/2 *Bos indicus* 1/2 *Bos taurus*). In addition, the occurrence of T allele increased numerically with the higher *Bos taurus* influence on each genetic group: 15.8% in Brangus Three-way cross (9/16 *Bos taurus* 7/16 *Bos indicus*), 22% in Canchim (5/8 *Bos taurus* 3/8 *Bos indicus*) and 33.3% in Braunvieh Three-way cross (3/4 *Bos taurus* 1/4 *Bos indicus*). Van Eenennaam *et al*. (2007) also reported that the occurrence of T allele in *Bos taurus* breeds was higher than in *Bos indicus* breeds. Base on phenotype analysis, Ly *et al.* (1999) suggested that Vietnamese yellow cattle are crossbred between *Bos taurus* and *Bos indicus*. Using mtDNA analysis, Berthouly *et al.* (2010) found two distinct genetic lineages of *Bos taurus* and *Bos indicus* in Ha Giang cattle population. Le *et al.* (2018) sequence analysis of mtDNA and SRY gene on Y chromosome in Yellow cattle indicated that all obtained mtDNA haplotypes belong to *Bos indicus* genetic lineage.

Table 2. Genotype and allele frequencies of TG5 gene

Populations	No. of Animals	Genotype frequency %			Allele frequency, %	
		CC	CT	TT	C	T
Ha Giang	66	96.9	3.1	0	98.5	1.5
Lang Son	66	100	0	0	100	0.0
Thanh Hoa	66	98.5	1.5	0	99.3	0.7
Nghe An	66	100	0	0	100	0.0
Uriu	66	98.5	1.5	0	99.3	0.7
Phu Yen	66	100	0	0	100	0.0
Ba Ria Vung Tau	66	98.5	1.5	0	99.3	0.7

4. CONCLUSION

The TG5 polymorphisms were not statistically significant between studied populations. The frequencies of T allele were very low and no animal with TT genotype was identified in Vietnamese Yellow cattle populations. The results in this study also further confirmed that the frequency T allele occurred very low in *Bos indicus* and tropically adapted cattle populations.

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REPRODUCTIVE PERFORMANCE OF PUREBRED AND CROSSBRED YORKSHIRE SOWS

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ABSTRACT

This study aimed to evaluate the reproductive performance of three groups of sow: pure Yorkshire (Y), ♂Landrace × ♀Yorkshire (LY) and ♂Duroc × ♀LY (DLY). A total of 15 multiparous sows (3rd to 5th parity) were equally divided and artificially inseminated with Duroc boar semen. Their offspring were weekly weighed until weaning at 28 days of age to calculate growth rate. Significant effects ($P<0.05$) of breeds was observed with the crossbred LY and DLY having higher litter size at birth and at weaning compared to purebred Y. Growth rate (194.30-202.90 g/day) and relative growth rate (17.79-20.14%) of the piglets were non-significant different ($P>0.05$). The weight loss of crossbred LY sows was 3.83%, significantly lower than that of crossbred DLY (4.61%) and purebred Y (4.64%) sows. A significant adverse correlation was observed between the weight loss percentage of sows and the growth rate of piglets at 21 ($r=-0.57$, $P<0.05$) and 28 days of age ($r=-0.63$, $P<0.01$). The findings of this study indicate that crossbred LY and DLY as basis sows would be beneficial in pig production.

Key words: *Pure Yorkshire sow, crossbred, reproductive performance, growth rate, weight loss percentage.*

1. INTRODUCTION

The important goal of pig industry is minimize cost of production and maximize return on investment. Most of producers use breeding program which focused on genetic improvement in economic traits. Generally, the objective of a breed selection is to improve crossbred commercial performance and most genetic improvement programs are based on the positive relationship between purebred performance of the nucleus herd and their crossbred performance of their commercial herd (Abell *et al*., 2016). The improvement of quantitative traits can be achieved either by selection in purebred or crossbred sows and crossing is the most effective way to increase the number of pig per sow per year. By crossing, the number of newborn and weaned pigs increased while the number of still born piglets decreased compared to pure breeds (Lukač, 2013).

Production traits, such as growth rate, feed efficiency, carcass and meat quality tended to have high genetic correlations between purebreds and crossbreds (Zhang *et al*., 2017; Godinho *et al*., 2018). The crossbred sows of Landrace and Large White had higher reproductive performances (total piglets born, number of piglets born alive, average birth weight and average weaning weight) than purebred sows and had less stillbirth piglets than purebred sows (Thiengpimol *et al*., 2017). However, other results have shown that there was low or moderate genetic correlations between crossbred and purebred reproductive performances, indicating that selection for longevity or lifetime performance of the sows at the nucleus level may not result in an improvement at the crossbred level (Nakavisut *et al*., 2005; Abell *et al*., 2016).

Genetic evaluation in most of improvement programs has been predominantly based on reproductive performance. The most common reproductive traits used in breed selection were litter size and litter weight. The survival and growth of these piglets until weaning depend on sow productivity and equally important as reproductive traits (Skrzypczak *et al*., 2007; Hong *et al*., 2017; Nguyen Tien Thanh *et al*., 2018; Nguyen Tuyet Giang *et al*., 2018). In a meta-analysis, Douglas *et al.* (2014) made a detection of possible interactions of 23 studies and five production data sets to quantify the association between various factors and their interactions on sow performance. Piglet weight

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at birth was associated with sow weight at the end of gestation while the weaning weight was correlate with both initial and final weight of the sow during gestation.

In Vietnam, most of pigs raired on the farm population consist of two or more breeds, and it created a certain conditions for genetic improvement, meaning that the offspring's performance would be better than the average of the parents' performance. However, genetic evaluation using crossbred information has been limited due to the lack of recording systems. Therefore, critical traits of reproductive performance and litter productivity should be monitored. The aim of this study was to compare the reproduction performances of purebred and crossbred of Yorkshire sows under Vietnam conditions. The hypothesis tested was that reproductive performance would improve in crossbred sows compared to purebred sows.

2. MATERIALS AND METHODS

2.1. Animals

The study was conducted over the three-month period, from December 2018 to March 2019, at a pig farm located in Vung Liem disctrict, Vinh Long province, Vietnam. A total of 15 multiparous sows between 3rd to 5th parity was used in this study. The sows were segregated based on breeds which were equally devided to three breed variants: purebred Yorkshire (Y), crossbred ♂Landrace × ♀Yorkshire (LY) and crossbred ♂Duroc × (♀LY/DLY). Heat was diagnosed by observing the standing reflex tolerance when applying pressure on the spine. All sows were artificially inseminated with Duroc's semen.

2.2. Management

Sows were moved to farrowing rooms a week before the estimated farrowing day, the sows were moved into a farrowing house equipped with individual farrowing pens (3.5×4.0m) where they remained during the lactation period. Each pen had two feeders and two nipple drinkers for sows and piglets. Cross-fostering at birth was not applied and piglets were weaned at 28 days of age. The sows were fed commercial mixed feed (3,000 Kcal/kg ME, 16.0% CP). On the 5th to 7th day after birth, piglets received pre-starter diet (3,250 Kcal/kg ME; 20.5% CP). General handling procedures were performed during lactation and all animals had *ad libitum* access to water and feed. Sows were offered feed two times daily, at 7h30 and 15h30. Feeders were filled in the afternoon to provide adequate diet during the night and early morning. After weaning, all sows were returned to their gestation house, received a common diet and mated on their post-weaning oestrus.

2.3. Measurement

The assessment of reproductive performance was carried out for each sow involved in the study. In this study, the most important reproductive traits of sows such as the number of live born piglets, litter size and weaning of piglets were evaluated. For the analysis of growth performance, individual birth weight, mortality rate, average daily weight gain, body weight at 21 and 28 days of age of the piglets were recorded. Individually weighed of sows were recorded at 24h-post farrowing and at weaning (28 days) to calculate weight loss and percentage of weight loss as introduced by Le Thi Men (2015).

2.4. Data analysis

For analysis, the evaluated data of reproductive performance of sows and growth rate of piglets in relation to breed was exported to Excel sheet and analysed further using the general linear model (GLM) procedure of Minitab Software, version 16.0. Differences between the means were determined by ANOVA. The relationships between sow performance traits and piglet growth parameters were investigated with Pearson's correlation. The level of significance was set at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1. Reproductive performance of sows and growth rate of piglets

The impact of pure Y and crossbreds (LY and DLY) is presented in Table 1. The results shows that litter size at birth and weekly during lactation was highly affected by breeds ($P<0.05$). The average number of litter size was the highest in two ways and three way crossing (11.80

and 12.40 pigs, respectively), while the lowest on pure breeds (8.60 pigs). Differences in the number of piglets weaned at 21 and 28 days were also noted between breeds (P<0.05). The results indicated an improvement of reproductive traits by crossings and this finding is in general agreement with Lukač (2013). Using crossbred reproduction, maternal heterosis for litter size at birth increased compared to purebreds. However, the pre-weaning survival rate of litters was independent on pig breeds reared (P>0.05), highest from purebred Y (100.00%) and lowest from DLY (90.07%). It means that the mortality rates of piglets in this study is lower than the results in the literature of Alonso-Spilsbury *et al*. (2007) and Yuan *et al*. (2015) who noted that approximately 15-25% of piglets born do not survive to weaning.

Table 1 also shows the significant influence of the examined breeds on the litter weight at 21 and 28 days (P<0.05), but not the litter weight at birth (P>0.05). However, it can be noted that the litters born by purebred Y (13.84kg) were characterized as the lowest litter birth weight compared to litters born by crossbred LY (15.02kg) and DLY (16.66kg) Significant difference among breeds were observed in the individual birth weight of piglet (P<0.05). The birth weight per piglet in the farm on average was above 1.0kg in all breeds, higher in purebred Y groups (1.62kg) compared to other two crossbred groups (1.28kg from LY and 1.37kg from DLY), could be due to less number of piglets per litter. The piglet weight at weaning was non-significantly different among breed groups (P>0.05), averaged from 6.81 to 7.06 kg per piglet (Table 1). There was also a non-significant difference on the daily weight gain of piglets in different breeds (P>0.05), although piglet from crossbred LY showed highest performance of 202.90 g/day. These results are in agreement with Thapa *et al.* (2012) who reported that the weaning weight was negatively correlated to the litter size.

Table 1. Traits of the sow performance and piglet growth

Traits	Times	Breeds (n = 5/breed)			SE	P
		Y	LY	DLY		
Litter size (kg)	Birth	8.60^{a}	11.80^{b}	12.40^{b}	0.79	0.011
	21 days	8.60^{a}	10.80^{b}	11.00^{b}	0.41	0.002
	28 days	8.60^{a}	10.80^{b}	11.00^{b}	0.41	0.002
Survival rate (%)	21 days	100.00	92.64	90.07	3.59	0.169
	28 days	100.00	92.64	90.07	3.59	0.169
Total weight of litter (kg)	Birth	13.84	15.02	16.66	0.91	0.129
	21 days	49.47^{a}	62.33^{b}	62.62^{b}	2.11	0.001
	28 days	60.56^{a}	75.20^{b}	74.80^{b}	2.52	0.002
Individual weight of piglet (kg)	Birth	1.62^{a}	1.28^{b}	1.37^{ab}	0.09	0.049
	21 days	5.77	5.77	5.70	0.09	0.823
	28 days	7.06	6.96	6.81	0.09	0.179
Average daily gain (g/day)	Brith - 21 days	197.60	213.80	206.20	4.43	0.070
	Brith - 28 days	194.30	202.90	194.30	3.17	0.129

Note: Values with the same letters in the same row are not significantly different (P>0.05)

3.2. Loss of weight in sow during lactation period

The optimum body weight and body condition of the sows during gestation and lactation periods are important to improve reproductive efficiency, litter performance and sow longevity. The decreased feed intake during the lactation period results in excessive loss of body weight and difficulties to maintain milk production and growth of litter (Kim *et al*., 2016).

In this study, there was no significant difference in the weight of sows at 24h after farrowing and at weaning but the weight loss and weight loss percentages were significantly different (P<0.05) among three breed groups (Table 2). At weaning, the LY sows had the lowest significantly weight loss percentages, followed by DLY sows and Y sows (Table 2). This finding is consistent with the results of Le Thi Men (2015) conducted in crossbred LY and YL sows. Despite the suggestion that body condition of sows at service may not be as critical to the reproductive success, weight loss percentage of more than 10.0% before service reduced subsequent reproductive performance (Thaker and Bilkei, 2005). This effect was more pronounced in primiparous sows as they continue to generate their growth than milk production compared to multiparous sows, leading to the delayed return to oestrus, reduced conception rate and embryonic survival (Lavery *et al*., 2018).

Table 2. Lactation weight loss of the sows

Traits	Breeds (n = 5/breed)			SE	P
	Y	LY	DLY		
Weight at 24h post-farrowing (kg)	223.02	211.14	229.81	7.84	0.273
Weight at weaning (kg)	212.71	203.00	219.20	7.44	0.335
Weight loss (kg)	10.31^{ab}	8.14^{a}	10.61^{b}	0.62	0.031
Percentage of weight loss (%)	4.64^{a}	3.83^{b}	4.61^{ab}	0.21	0.032

3.3. Correlations between sow performance and piglet growth

The complete data set of all sows and piglets were used to investigate the relationship between reproductive performance and litter growth (Table 3). However, only biologically interesting and statistical significant correlations related to the objective of this study are discussed. Overall, number born alive (NBA) was positively correlated to the number of piglet at 21 (D21LS) and 28 days of age (D28LS) (r=0.92, P<0.001) as well as the total weight of litter from birth to weaning (r=0.62-0.91, P<0.01). Additionally, number of born alive and piglet survival at 21 and 28 days (D21SR and D28SR, respectively) are highly correlated (r=-0.89, P<0.001). The litter birth weight (LBWT) was positively correlated to the total weight of litter at day 21st and 28th (D21LW and D28LW, respectively) (r=0.59-0.62, P<0.05) but negatively correlated to piglet survival at 21 and 28 days (r=-0.55, P<0.05). The litter weight at 21 days of age had a positively correlation on the growth rate at day 21st (D21ADG) (r=0.55, P<0.05) but no effects on the pre-weaning daily gain (P>0.05). Interestingly, weight loss percentage (WLP) of the sows over lactation responded in an inverse relationship with the growth rate of piglets at 21 (D21ADG) (r=-0.57, P<0.05) and 28 days of age (D28ADG) (r=-0.63, P<0.01).

Litter size, litter weight and average daily gain of piglets may correspond with the change in mammary gland to increase the milk production and thus result in the reduction of sow body condition. In addition, sows that had insufficient nutrient intake may cause body composition loss for milk production maintenance (Kim *et al*., 1999). According Hong *et al*. (2016), sows fed dry feeding method made milk containing higher content of total solid, fat and casein than sows fed liquid feeding method which in turn increased litter weight at 21 day of lactation and litter weight gain during lactation period. However, sows consumed dry feed had higher body weight loss than liquid feeding treatment.

Litter size, directly related to productivity, has been considered as the most important traits in genetic selection. Large litter size resulted in more weaned and finished pigs compared with the smaller litters (Beaulieu *et al*., 2010). Despite technological advances in intensive pig production, both maternal genetic factors (including litter size), piglet factors (newborn vigour, acidosis, teat seeking ability and hypothermia) as well as technology changes and

husbandry skills may influence the survival trait of the piglets during lactation stage (Alonso-Spilsbury *et al*., 2007; Yuan *et al*., 2015). To Landrace breed, there was a negative correlation between maternal genetic effects on proportion survived from birth until 3 weeks of age. However, the correlations were not significant in the Yorkshire breed (Lund *et al*., 2002). Other studies has found that piglet birth weight and influence litter postnatal development. The increased litter size achieved in recent decades has been unfavorably correlated with piglet birth weight, survival rate and litter postnatal development, mainly during early life (Beaulieu *et al*., 2010; Zotti *et al*., 2016).

Table 4. Pearson's correlation coefficients between sow performance traits and piglet growth parameters

Traits	NBA	D21LS	D28LS	D21SR	D28SR	LBWT	D21LW	D28LW	D21ADG	D28ADG	WLP
NBA	1.00	0.92^{***}	0.92^{***}	-0.89^{***}	-0.89^{***}	0.62^{**}	0.88^{***}	0.91^{***}	0.29^{NS}	0.29^{NS}	-0.13^{NS}
D21LS		1.00	0.92^{***}	-0.59^{*}	-0.64^{*}	0.59^{*}	0.97^{***}	0.98^{***}	0.41^{NS}	0.18^{NS}	-0.10^{NS}
D28LS			1.00	-0.64^{**}	-0.64^{**}	0.59^{*}	0.97^{***}	0.98^{***}	0.41^{NS}	0.18^{NS}	-0.10^{NS}
D21SR				1.00	0.92^{***}	-0.55^{*}	-0.59^{*}	-0.64^{*}	-0.34^{NS}	-0.36^{NS}	0.15^{NS}
D28SR					1.00	-0.55^{*}	-0.59^{*}	-0.64^{**}	-0.34^{NS}	-0.36^{NS}	0.15^{NS}
LBWT						1.00	0.59^{*}	0.62^{**}	-0.18^{NS}	-0.26^{NS}	0.22^{NS}
D21LW							1.00	0.98^{***}	0.55^{*}	0.26^{NS}	-0.17^{NS}
D28LW								1.00	0.48^{NS}	0.30^{NS}	-0.17^{NS}
D21ADG									1.00	0.78^{***}	-0.57^{*}
D28ADG										1.00	-0.63^{**}
WLP											1.00

*Note: NS is P>0.05, * is P<0.05, ** is P<0.01, *** is P<0.001.*

5. CONCLUSION

In summary, results from this study shows the differences in reproductive performance between purebred and crossbred and provide information for livestock breeding companies when developing a selection program. The crossbred Yorkshire sows had higher performance of liter size at birth and at weaning compared to purebred Yorkshire sows. Additionally, to maximize the reproductive potential of female pigs and improve herd production, producers are recommended to closely monitor female traits. However, due to the high standard errors associated with the estimates of genetic correlations between purebred and crossbred performance, increasing the amount of data collection is required to validate this conclusion.

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EVALUATION OF GENETIC DIVERSITY OF H'MONG BOBTAIL BASED ON MITOCHONDRIAL D-LOOP SEQUENCE

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ABSTRACT

The H'Mong Bobtail, similar to Phu Quoc Ridgeback, Bac Ha and Lai, is a dog breed endemic to Vietnam. This breed possesses invaluable traits such as loyalty, intelligence and excellence in hunting; it has very high economic value and is usually kept as pets or trained for specialized professions. Consequently the breed population quickly increased, and directed breeding for certain morphological traits leaded to loss of genetic diversity. Previous researches showed that mtDNA is one of important molecular marker for evaluation of genetic diversity and phylogenetic studies of many dog breeds worldwide. This study evaluated the genetic diversity of Hmong Bobtail population based on mtDNA HV1 sequence and managed to identify 15 different haplotypes (belong to haplogroup A, B and C) on 31 sampled individuals. Remarkably, this study identified five novel haplotype (19.4% of the sampled individuals) never been published in previous researches. Most haplotypes took roughly equal proportions in the sampled individuals except A73 (29%). Basic genetic indexes such as haplotype diversity (Hd: 0.9); nucleotide diversity (Pi: 0.013) indicated high genetic diversity of H'Mong Bobtail population. This study's results will supplement the genetic database which is usable for further researches on Hmong Bobtail population's mtDNA.

Keywords: *H'Mong Bobtail, HV1, haplotype, haplogroup.*

1. INTRODUCTION

Domestic dog (*Canis familiaris*) is the first animal domesticated by human. Domestic dogs originated from wild wolf, were domesticated by ancient hunters and since have followed human to all over the world through history (Morey, 2006). Aside from supporting human in various daily activities, domestic dogs also serve as models for various studies in human genetic diseases, in disease diagnostic and treatment. Recent developments especially the successful sequencing of whole dog genome (Lindblad-Toh *et al*., 2005) has facilitated various molecular and genetic researches in dog populations worldwide. Moreover, with powerful assistance from molecular biotechnology, various molecular marker such as SNP (Single nucleotide polymorphism), SSR (short tandem repeat), Y chromosome, mtDNA can be used to evaluate genetic diversity, to rebuild phylogenetic tree, to clarify the origin, evolution and migration of domestic dogs and other animals over the world (Fan and Chu, 2007; Forster *et al*., 2000; Wang *et al*., 2016).

The complete sequencing of domestic dog mtDNA genome opened a new promising direction for better understanding dog mtDNA (Kim *et al*., 1998). Notably, dog mtDNA possesses a highly polymorphic Hypervariable Region 1 (HV1) on the Control Region (CR) with the greatest number of mutations in dog genome; this region is used in genetic analysis, haplotype identification and forensic analysis of highly degenerated DNA samples (Savolainen *et al*., 1997).

One of the pioneer researches on evolutionary genetics and origin of most of domestic dog breeds based on mtDNA HV1 region divided worldwide dog population into six different haplogroups (A, B, C, D, E, F). Results showed that haplogroups A, B, C occurs in 97.4% of dog population. On the contrary, D, E, F are rare groups make up of less than 3% of worldwide individuals, amongst them haplogroups E, F only make up of 1-2%. The study also pointed out that morphological differences between dog breeds do not result from separated domestication in different

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geographic areas but are due to migration and hybridization of different breeds (Savolainen *et al*., 2002; Pang *et al*., 2009). This study laid the foundation for further researches on genetic diversity and origins of different dog breeds (Savolainen *et al*., 2004; Klütsch *et al*., 2011; Oskarsson *et al*., 2012; van Asch *et al*., 2013).

In Vietnam, researches of genetic diversity based on analysis of mtDNA HV1 region mostly focus on Phu Quoc Ridgeback and other domestic dogs in various geographic regions in Vietnam; preliminary results showed high diversity of Vietnam's domestic dogs (Quan Quốc Đăng *et al*., 2016; Thai *et al*., 2016a; Thai *et al*., 2016b). However, these studies only investigated the Phu Quoc Ridgeback and other individuals in some narrow geographic areas; meanwhile in Vietnam there are still other endemic breeds such as H'Mong Bobtail, Bac Ha and Lai. Amongst them, the H'Mong Bobtail is one of the most ancient hunting dog which still preserves many wild traits; this breed is also genetically conserved due to being isolated from other breed by being kept in captivity in mountainous areas of northern Vietnam. This study carried out preliminary investigation and evaluation of genetic diversity on thirty-one sampled H'Mong Bobtail individuals to clarify the genetic structure of the breed population, to supplement the database of HV1 region for evaluation of genetic relationship between the H'Mong Bobtail and other Vietnamese breeds, and to make a contribution in conservation effort for this endemic breed.

2. MATERIALS AND METHODS

2.1. Sample collection and total DNA extraction

Hair samples were collected from thirty-one H'Mong Bobtail originated from mountainous areas of northern Vietnam and chosen randomly at breeding sites in Hanoi. The samples were named as CH1 to CH31. Sampled DNA was isolated follow the protocol of The ISOLATE II Genomic DNA Kit (BIOLINE) commercial kit. DNA purity and concentration was tested by spectrophotometry.

2.2. Amplification and sequencing

HV1 region sequence was amplified by primers 15412F: CCACTATCAGCACCCAAAG and 16625R: AGACTACGAGACCAAATGC (Gundry *et al*., 2007). PCR reactions were carried out in a total volume of 25µl of MyTaq Mix (1X); forward (15412F) and reverse primer (16225R) with final concentration of 0.2µM; genomic DNA with final concentration of 10-20 ng/µL. Amplification condition was as follows: preparing for denaturation at 95°C for 5 min (1 cycle); denaturation at 94°C for 2 min, primer binding at 58°C for 1 min, elongation at 72°C for 1 min (35 cycles); final elongation at 72°C for 10 min (1 cycle). PCR products were checked by electrophoresis on 2% agarose gel and ethidium bromide staining; satisfied products were purified and sequenced by Sanger method using 15412F forward primer (CCACTATCAGCACCCAAAG) and 16114R reverse primer (CCTGAAACCATTGACTGAATAG) (Gundry *et al*., 2007).

2.3. Data analysis

DNA sequences were modified by SeaView software and compared against reference sequence (GenBank accession entry: U96639.2) (Kim *et al*., 1998). Polymorphic sites were conventionally numbered (Pereira *et al*., 2004). Haplotypes of HV1 region 582bp sequences were identified by standardized tools (Thai *et al*., 2017). Relationships between haplotypes were constructed based on medium - joining network by Network5.01 software (Bandelt *et al*., 1999). Genetic diversity indexes such as haplotype diversity (Hd) and nucleotide diversity (Pi) were calculated by Arlequin software (Excoffier and Lischer, 2010).

3. RESULTS AND DISCUSSION

3.1. Haplotype identification

HV1 DNA sequences of 31 H'Mong Bobtail individuals were registered on GenBank with access number of MN206805-MN206835. Haplotypes of them were identified by standardized tools (Thai *et al*., 2017). Fifteen haplotypes from A, B and C haplogroups were identified, amongst them were five novel haplotypes (AN3, AN4, AN5, AN6, BN) never published in previous researches (Table 1). There were no haplotype in haplogroup D or E. This result was compatible with previous studies which claimed that haplogroup D only occurs in Europe and haplogroup D only occurs in several breeds such as Pungsan, Jindo and Shiba (Pang *et al*., 2009).

Table 1. Identified haplotypes on 31 sampled H'Mong Bobtail individuals

Haplogroup	Haplotype	Sample codename	Number/proportion (%)
A	A22	CH30	1 (0.03 %)
	A29	CH9, CH17, CH29	3 (0.1 %)
	A44	CH14, CH24, CH28	3 (0.1 %)
	A65	CH3	1 (0.03 %)
	A73	CH8, CH11, CH12, CH13, CH18, CH19, CH20, CH21, CH27	9 (0.29 %)
	A121	CH1, CH10, CH16	3 (0.1 %)
	A131	CH26	1 (0.03 %)
	A226	CH23	1 (0.03 %)
	AN3	CH2	1 (0.03 %)
	AN4	CH5	1 (0.03 %)
	AN5	CH6, CH22	2 (0.06 %)
	AN6	CH25	1 (0.03 %)
B	B5	CH4	1 (0.03 %)
	BN	CH15	1 (0.03 %)
C	C2	CH7, CH31	2 (0.06 %)

Among 15 identified haplotypes, lowest proportion of ones in haplogroup A was 3% (A22, A65, A131, A226, AN3, AN4 AN6) and highest was 29% (A73). Remaining haplotypes such as A29, A44, A121 made up of 10%, haplotypes AN5 and C2 occurred in 6% of the individuals. Two B haplotypes (B5, BN) occurred in H'Mong Bobtail individuals at 3% in both types. There was only one C haplotype (C2) at 6% (Table 1). Most identified haplotypes had similar proportions except the non-common A73 (occurred in low proportion in most of dog breeds worldwide) in this study had a very higher proportion (29%) than other identified haplotypes. This result implied that individuals with haplotype A73 were selected for and breeded based on certain morphological criteria, lead to inbreeding between close relatives and increased A73's frequency in the population.

3.2. Diversity of mtDNA HV1 region in H'Mong Bobtails

The results showed that, on investigated sequence in HV1 region of H'Mong Bobtails mtDNA there were 34 polymorphic sites, 33 transition sites, 2 tranversion sites and one indel sites (Table 2). Al haplotypes had one transition at position 15814 (C15814T) and one tranversion at position 15639 (T15939 A, G). Haplotypes B5 and BN had one common indel at position 15938 (G15938-). Haplotype AN3 had one novel tranversion at position 15531 (C15531A) which had never been published before.

Distribution of haplotypes in haplogroups, of haplotypes in one group and relationship between haplotypes were presented in median – joining network (Fig 1).

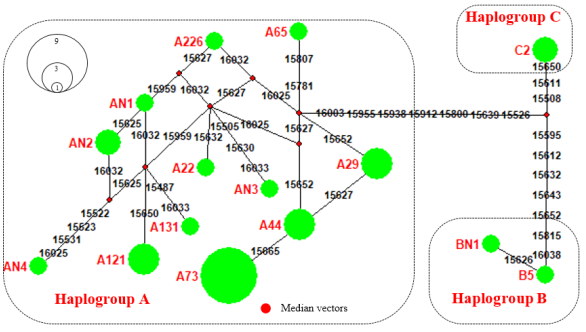

Fig 1. Median – joining network built on polymorphic sites of HV1 region sequence, *presented the relationship between 15 haplotypes of three haplogroups (A, B, C) identified in H'Mong Bobtail population. Haplotype node sizes are propotional to number of individuals with corresponding haplotypes. Red nodes (median vectors) represented unidentified intermediate haplotypes. Numbers on straight lines between haplotypes represent mutation sites between two haplotyped corresponding to the sites on reference sequence.*

Table 2. Polymorphic sites in HV1 region 582 bp sequence of H'Mong Bobtail individuals. Column numbers: nucleotide positions. Symbols: (.) nucleotide identical to reference sequence; (-) indel position. RE: reference sequence of Kim *et al.*, (1998). Hap: Haplotype

Group	Haplotype	16487	15505	15508	15522	15523	15526	15531	15595	15611	15612	15625	15626	15627	15630	15632	15633	15639	15643	15650	15651	15652	15655	15657	15658	15659	15660	15661	15662	15663	15664	15665	15666	15678	15680	15681	15681	15691	15693	15695	15695	15709	16023	16033	16038
	RE	C	T	C	T	T	C	C	C	T	T	T	A	A	C	C	T	A	T	G	T	C	C	T	C	T	C	C	T	T	T	T	A	C	C	T	C	G	C	C	A	T	A	A	C
1	A22	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
2	A29	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	A44	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	A65	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	A73	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6	A121	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
7	A131	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	A226	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9	AN4	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	AN5	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11	AN6	.	C	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12	AN3	.	.	.	C	C	.	A	.	.	.	C	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13	B5	.	.	.	T	.	T	T	.	C	.	.	.	.	.	.	T	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	T	.
14	BN	.	.	.	.	.	T	.	.	C	.	.	G	.	.	.	T	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
15	C2	.	.	T	T	.	T	.	.	.	C	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.

Haplotype median – joining network in Fig 1 showed high diversity among haplotypes of various groups (A, B, C) and among haplotypes in one group. Amongst haplogroup A, B and C, group A had highest diversity. A haplotypes made up 80% of total identified haplotypes (12/15 haplotypes), much higher than B (13.3%, 2/15) and C types (6.67%, 1/15). Remarkably, the study identified novel haplotype never published before at very high proportion of 33.3% (5/15 haplotypes).

Constructed H'Mong Bobtail network is not star-like (founder haplotype at the center, surrounding haplotypes are only one mutation site different from founder one) foe all three haplogroup (A, B, C), which means H'Mong Bobtail population was highly diverged especially in haplogroup A, the A haplotypes were highly separated except the pairs of (AN4 and AN5) and (A44 and A73) were only one mutation different from each others. The remaining types were pairwise from two replacement mutations (AN4 and A226) to seven replacement mutations (AN3 and A65) from each others. Haplogroup B was less diverged, B5 and BN were one replacement muutation from each others, and haplogroup C only had one haplotype (C2). These results implied that the sampled H'Mong Bobtail population may originate from various mother lines, there may be at least fifteen maternal lines took part in forming the current H'Mong Bobtail population.

The individual numbers of haplogroup A, B, C made up of 87.1, 6.67 and 6.67% of

the population, respectively (Table 3). Such proportion is compatible to previous studies claimed that most of domestic dog breeds belongs to haplogroup A, B and C, while group D, E and F are rare ones, make up of less than 3% worldwide population (Pang *et al*., 2009).

Table 3. Haplotype proportion per haplgroup and basic genetic diversity indexes

n	nH	n(ABC) (%)	nA (%)	nB (%)	nC (%)	Hd±SD)	Pi±SD
31	15	30 (100)	27 (87.1)	2 (6.67)	2 (6.67)	0.9±0.040	0.013±0.006

n: individual number; nH: haplotype number; Hd: haplotype diversity; Pi: nucleotide diversity

Beside above data, this study evaluated several important genetic diversity presented in Table 3 such as nucleotide and haplotype diversity. Nucleotide diversity in H'Mong Bobtail population was Pi=0.013, which means probability of randomly picking two different nucleotide at the same arbitrary on two HV1 sequences in H'Mong Bobtail population was 1.3%; it was a high ratio considering that the researched HV1 sequence in this study was only 582bp. Haplotype diversity of Hd=0.9 means probability of randomly picking two different H'Mong Bobtail individuals had two different haplotype was 90%, this ratio was also high considering that most H'Mong Bobtails are bred in captivity where inbreeding should occur at very high frequency when breeding for certain morphological criteria.

4. CONCLUSION

Fifteen haplotypes belong to three different haplogroups (A, B, C) were identified based on analysis of mtDNA HV1 region of 31 sampled H'Mong Bobtail individuals. Amongst 15 identified haplotypes there were five novel ones: AN3, AN4, AN5, AN6 and BN. Genetic diversity indexes showed that H'Mong Bobtail population are genetically highly diversified (Hd=0.9; Pi=0.013). This study results will supplement the database useful for further researches on genetic diversity and origin of other domestic dog breeds in Vietnam.

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COMPREHENSIVE EVALUATION ON SOME MORPHOLOGY INDICATORS IN PHU QUOC RIDGEBACK DOG (*CANIS FAMILIARIS*) OF VIETNAM

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ABSTRACT

Phu Quoc ridgeback is one of the unique dog breeds in Vietnam. However, Fédération Cynologique Internationale (FCI) has not yet recognized it as standard breed due to unspecified morphological and genetic information. Phu Quoc ridgeback dog is even assumed to be the descendant of Thai ridgeback dog. There is a certain demand for studies to point out specific morphological and/or genetic traits of this dog breed to use as a foundation for comparison with other breeds. It would serve as a guideline to form particular characteristics that aim toward a standard breed recognition by FCI for this dog. In this study, 175 matured (at least 19 months old) Phu Quoc Ridgeback dogs were sampled at Phu Quoc and Ho Chi Minh City. Difference in morphology was statistically estimated by t-test and linear regression. The results showed that measured size parameters (body height at withers and weight) in sampled and observed population were different from the morphological standard defined by the Vietnam Kennel Association (VKA). Moreover, Phu Quoc Ridgeback dog is entirely different from Thai and African Rhodesian ridgeback ones based on recorded height and weight parameters.

Keywords: *Body height at withers, body length, regression, t-test.*

1. INTRODUCTION

Phu Quoc is an island located at southwestern Vietnam and also a district of Kien Giang Province. The Phu Quoc Ridgeback dog is a native animal breed of this island with a distinctive morphological trait of a ridge-like pattern on the coat at the back of the dog. Fernand Doceul, an official in French colonial government in southern Vietnam, was the first person who brought the ridgeback breed from Phu Quoc to Paris in 1886. The first hypothesis described on Phu Quoc Ridgeback dog's origin was proposed by Emile Oustalet (Oustalet, 1981), a biologist and the director of the Department of Birds and Mammals at Muséum National d'Histoire Naturelle in 1873-1905 (Hellmayr, 1906). Later, Henri de Bylandt described *Phu Quoc Hunting dog (Lévrier Phu Quoc)* in his book *Les Races de Chien* (first published in 1897, reprinted in 1994, described 316 worldwide dog breeds, printed in English, French and German) (Bylandt, 1897). Nodaway, Phu Quoc ridgeback is one of the three ridgeback breeds in the world; however, the Fédération Cynologique Internationale (FCI) only recognized the two other breeds which are the African ridgeback (Rhodesian Ridgeback) and the Thai ridgeback [(FCI), 1996, 2004]. Since 2012 studies on genetic and morphological diversity of Phu Quoc ridgeback in Vietnam have been published, including (Quan *et al*., 2016b; Thai *et al*., 2016; Tran *et al*., 2016; Thai *et al*., submitted) that were studied on mitochondrial genetic diversity of Phu Quoc ridgeback; while (Quan *et al*., 2016a, 2017) studied on Phu Quoc ridgeback's morphology at Phu Quoc Island and the relationship between body size ratio and the occurrence of haplogroup E (one rare haplogroup in dogs) in this breed. However, some individual dogs born in Phu Quoc were sampled in previous studies; the result did not represent sufficient morphological diversity to identify and differentiate the Phu Quoc ridgeback from the African and Thai ridgeback. The result was also not enough to evaluate the differences in morphology and body size among

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Phu Quoc ridgeback population. This study applied several conventional basic methods in morphological identification on Phu Quoc ridgeback sampled from Phu Quoc Island and Ho Chi Minh City, where Phu Quoc ridgeback breeding kennels were mostly located in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials and samples

In this study, 175 Phu Quoc dog individuals with and without the back ridge (including 96 males and 79 females) were sampled. Sampled individuals must be at least 18 months old - the age at which the dogs are sexually matured and body size parameters no longer change (Morey, 1992). Samples were collected at Phu Quoc Island and at Ho Chi Minh City with adjacent areas, where the Phu Quoc ridgeback individuals were mostly transferred to and trading for commercial breeding and conservation. Among 175 sampled individuals, 31 of them were located at Phu Quoc's breeding kennels, and 144 of them were at natural and semi-natural breeding kennels in Ho Chi Minh City and adjacent areas. All sampled individuals had both paternal and maternal origins from Phu Quoc Island. Thirty sampled individuals did not have the back ridge while the remaining 146 ones had.

2.2. Body size measurement

This study applied measurement methods used in published researches of Dingo (Crowther *et al*., 2014; Smith *et al*., 2018), New Guinea Signing (Koler-Matznick *et al*., 2003), Italiano Bracco (Cecchi *et al*., 2016), Italian Cane Corso (Marelli *et al*., 2003) (Italy), Nigeria Indigenous (Bukar-Kolo *et al*., 2016) and Spanish Sigh Hound (González *et al*., 2014) (Spain) breeds.

Body size measurement taken in this study was: body weight (BW) by digital weight-scale, body height at withers (BHW), body length (BL), chest circumference (ChC), waist circumference (WC), muzzle length (ML), ear length (EL) and tail length (TL) (Sutter *et al*., 2008) using straight ruler and measuring tape (German *et al*., 2006).

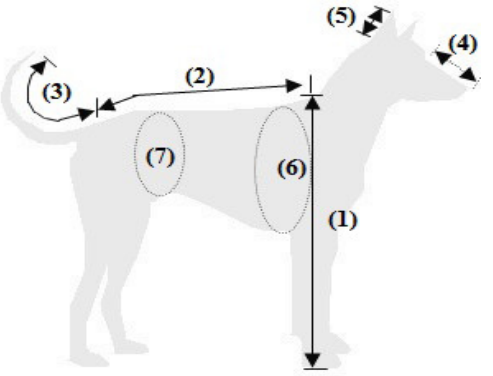

Fig 1. Morphological measurements of Phu Quoc dog. *Sampled individuals were kept at stacked stance during measurement. (1) Body height at withers (BHW) is the height of the withers above ground level and was measured by a straight ruler. (2) Body length (BL) is the length of backline from withers to tail base, measured by measuring tape. (3) Tail length (TL) is the length from tail base to the tail end, measured by measuring tape. (4) Muzzle length (ML) is the length from the furthest point of the upper jaw along the nasal bridge to the juncture between nasal bone and cranium, measured by measuring tape. (5) Ear length (EL) is the length from the ear tip to the middle point of the borderline between ear and skull, measured by measuring tape. (6) Chest circumference (ChC) is the largest circumference of the chest, measured by measuring tape. (7) Waist circumference (WC) is the smallest waist circumference at the abdomen right next to the hind legs, measured by measuring tape. (8) Body weight (BW) is the weight at normal condition before a meal.*

2.3. Statistical analysis

Descriptive statistics and body size criteria were presented in average values with corresponding standard errors. ANOVA and t-test analysis was used for the differences in body size between sexes and sampled populations at a confidence interval of 95%. The t-test was used to analyze two samples with non-identical variances. Regression analysis was used to evaluate the correlation between size parameters to predict the possibility of changes in body size during natural selection and artificial selection. The study made use of XLSTAT program, developed by Addinsoft for non-annual database market since 1993.

Results of descriptive statistics of all Phu Quoc dog individuals were recorded in tables.

Body weight was measured in k) and other size parameters were measured in cm.

The body scores and morphology of Thai ridgeback dog and Rhodesian ridgeback dog collected and investigated from Fédération Cynologique Internationale (FCI).

3. RESULTS

3.1. Average body size of Phu Quoc Ridgeback dog

PCR and PCR-RFLP product tests for RNF4 and RBP4 gene polymorphism are shown in Fig 1 and 2. PCR products of RNF4 and RBP4 were separated on a 2% agarose gel to confirm a 927 and 550bp product (Fig 1). Banding patterns of RNF4 and RBP4 were consistent with what were previously reported by Niu *et al.* (2009) and Rothschild *et al.* (2000). Two allelic specific patterns of RNF4 gene were obtained after SacII digestion, including an uncut 937bp fragment for allele T and two fragments of 545bp and 392bp for allele C; and consequently resulting in three genotypes, TT (937bp), CC (545 and 392bp) and TC (937, 545 and 392bp). For RBP4, two different alleles were also identified under this study: allele A with 190, 154 and 136bp fragments and allele B with 190, 136 and 125bp fragments. Consequently, three genotypes were observed: AA with 190, 154 and 136bp fragments; BB with 190, 136 and 125bp fragments and AB with 190, 154, 136 and 125bp were observed (Fig 2).

Table 1. Morphological size on ridged and non-ridged Phu Quoc ridgeback dog sampled at different locations

Ridged Phu Quoc dogs at Phu Quoc Island, Kien Giang				
	BW (kg)	**BL** (cm)	**BHW** (cm)	**ML** (cm)
Male	19.7 ± 3.4	53.2 ± 4.6	48.9 ± 5.2	10.4 ± 0.7
Female	18.1 ± 2.1	50.3 ± 2.7	46.4 ± 2.0	10.1 ± 0.6
	ChC (cm)	**WC** (cm)	**EL** (cm)	**TL** (cm)
Male	57.6 ± 5.7	47.1 ± 5.2	9.8 ± 0.7	29.3 ± 3.0
Female	53.5 ± 5.6	44.4 ± 5.3	9.7 ± 0.6	26.8 ± 1.7
Ridged Phu Quoc dogs at Ho Chi Minh City and adjacent areas in southern Vietnam				
	BW (kg)	**BL** (cm)	**BHW** (cm)	**ML** (cm)
Male	20.1 ± 2.2	50.0 ± 6.2	45.9 ± 6.4	10.2 ± 0.6
Female	20.1 ± 2.0	49.4 ± 6.2	44.6 ± 6.4	10.3 ± 0.5
	ChC (cm)	**WC** (cm)	**EL** (cm)	**TL** (cm)
Male	56.4 ± 8.8	45.8 ± 7.4	9.9 ± 0.8	29.9 ± 3.6
Female	54.4 ± 8.1	44.2 ± 7.3	9.8 ± 0.8	28.2 ± 2.2
Non-ridged Phu Quoc dogs at Ho Chi Minh City and adjacent areas in southern Vietnam				
	BW (kg)	**BL** (cm)	**BHW** (cm)	**ML** (cm)
Male	18.6 ± 1.7	51.3 ± 8.0	45.3 ± 6.6	10.5 ± 0.5
Female	18.4 ± 2.1	53.1 ± 9.6	47.2 ± 8.2	10.2 ± 0.6
	ChC (cm)	**WC** (cm)	**EL** (cm)	**TL** (cm)
Male	54.9 ± 8.3	45.6 ± 6.9	9.7 ± 0.9	29.9 ± 2.6
Female	60.6 ± 7.5	49.5 ± 4.9	9.7 ± 0.8	29.9 ± 2.4

Body height at withers (BHW) is one important criterion to define dog breeds according to FCI's regulations, researches on dog phenotype used this criterion to evaluate featured morphological traits, phylogenetic origins (Hubbard, 1948; Olsen, 1974; Sechi *et al.*, 2016; Jagatheesan *et al.*, 2017) and conservation characteristics via breeding expressed by phenotype (Sutter *et al.*, 2008). BHW is also a manifestation of the dog breed's nature, instinct and behaviour during its

coexistence and evolution process together with human (Gwatkin, 1934; Benecke, 1987; Morey, 1992; Vilà *et al*., 1997; Brewer *et al*., 2001; Favier *et al*., 2001; Clutton-Brock, 2016). Aside from tail length (28.2±5.3cm in female and 29.6±11.2cm in male) (Table 2), there was no difference in observed phenotype between different sexes in Phu Quoc dog.

Table 2. Results of t-test on basic morphological size between sexes in Phu Quoc dog

	BW	BL	BHW	ML	ChC	WC	EL	TL
t	-0.743	-0.698	-1.056	-0.360	-0.832	-0.853	-0.385	-3.371
tC	1.974	1.974	1.974	1.974	1.974	1.974	1.974	1.974
P<0.05	0.458	0.486	0.292	0.719	0.395	0.407	0.700	0.000*

**: significantly different at P<0.05*

3.2. Observed phenotypes of ridged Phu Quoc dogs at Phu Quoc and Ho Chi Minh City

The BWH and BL measurements of male ridged individuals in Phu Quoc were 48.9 and 53.2cm, respectively. Corresponding values in Ho Chi Minh City were 45.9 and 50.0cm, respectively (Table 1). Statistical results showed differences between BHW and BL in these two groups. Ridged individuals in Phu Quoc Island were 3cm higher in BHW than Ho Chi Minh City and 3.2cm longer in BL (P<0.05).

Female ridged individuals in two areas were not different in BHW and BL but had differences in body weight and tail length. Individuals in Ho Chi Minh City were 2kg heavier than in Phu Quoc Island (20.1 and 18.1kg, respectively; P<0.05) and the tail length was 1.2cm longer (28.0 and 26.8cm, respectively; P<0.05).

Table 3. Results of t-test on basic morphological size between Phu Quoc ridgeback dog in Phu Quoc Island and at Ho Chi Minh City, divided by sex

		BW	BL	BHW	ML	ChC	WC	EL	TL
Male	t	-0.461	2.358	2.038	1.035	0.650	0.814	-0.843	-0.824
	tC	2.077	2.024	2.030	2.069	2.015	2.201	2.032	2.030
	P<0.05	0.649	0.023*	0.049*	0.311	0.518	0.420	0.405	0.415
Female	t	-3.158	0.752	1.703	-1.456	-0.514	0.147	-0.835	-2.349
	tC	2.085	2.007	1.999	2.093	2.039	2.048	2.048	2.059
	P<0.05	0.005*	0.456	0.094	0.162	0.611	0.884	0.410	0.026*

3.3. Observed phenotypes of ridged and non-ridged Phu Quoc dogs

Ridged Phu Quoc dogs were 1.4kg heavier than non-ridged ones (19.9 and 18.5kg respectively; P<0.05). Tail length (TL) of ridged individuals was 1.1cm shorter than non-ridged ones (29.9 and 28.8cm respectively; P<0.05).

Table 4. The t-test on basic morphological size between ridged and non-ridged in total Phu Quoc dog

	BW	BL	BHW	ML	ChC	WC	EL	TL
t	-3.566	1.101	0.162	1.148	1.092	1.520	-0.965	2.232
tC	2.004	2.024	2.019	2.013	2.014	2.010	2.015	2.004
P<0.05	0.000*	0.277	0.871	0.257	0.280	0.135	0.344	0.029*

3.3. Growth correlation between body height and length in ridged Phu Quoc ridgeback dog at Phu Quoc and Ho Chi Minh City

BHW and BL are common criteria in the identification of dog breeds since they can be easily measured, do not depend on the nutritional condition and is proportional to other size parameters (Frynta *et al*., 2012). Allometry

is the ratio between body sizes during animal growth. Statistical results showed differences in size between sexes in Phu Quoc ridgeback dog population sampled in Phu Quoc; male individuals had greater body length (male 53.2cm and female 50.3cm), higher BHW (male 48.9cm and female 46.3cm). Meanwhile, males and females in Ho Chi Minh City were not significantly different in BL (50.33 and 50.17cm, respectively) and BHW (45.74 and 45.11cm, respectively).

Table 5. Average BHW and BL of Phu Quoc dogs in different sampling areas, measured in cm

	Phu Quoc		HCM City and adjacent area	
Sex	Female	Male	Female	Male
BL	50.285[a]	53.222[ac]	50.169	50.333[c]
BWH	46.357[b]	48.888[bd]	45.107	45.743[d]

a, b, c, d: significantly different at P<0.05

In observed male individuals, BL and BHW were different between Phu Quoc and Ho Chi Minh City (Phu Quoc: 53.2 and 48.8cm respectively, Ho Chi Minh City: 50.3 and 45.7cm respectively; P<0.05).

3.4. Allometry is the growth coefficient of an animal body

Distinct phenotype resulted in different allometry in Phu Quoc ridgeback dogs at Phu Quoc Island and Ho Chi Minh City (Fig 2 and 3). The results showed that Phu Quoc ridgeback dog populations in Phu Quoc and other regions tended to differentiate from each other. The allometric equation was built to predict the evolution of population phenotype over time. There was no observed difference between ridged and non-ridged dogs beside body weight, the elements which can be easily affected by environment, nutritional demands and preferences of breeders and buyers. Tail length also had differences however this trait probably is affected by external environments and requires further observations.

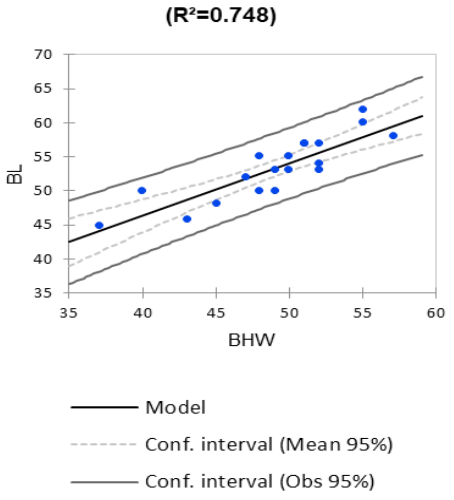

Fig 2. Graph of the regression function on the correlation between BHW and BL of Phu Quoc ridgeback dog in Phu Quoc Island. The equation estimated is: BL=15.41+0.77*BHW

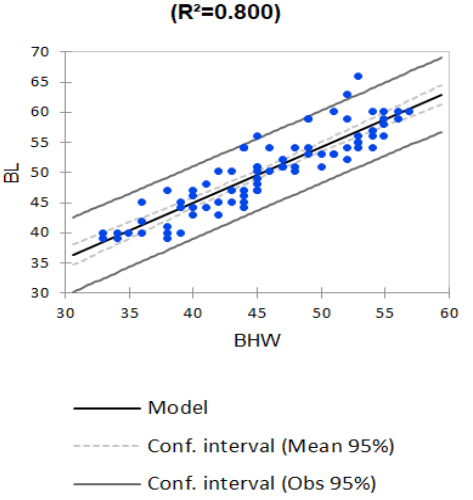

Fig 3. Graph of the regression function on the correlation between BHW and BL of Phu Quoc ridgeback dog at HCM City and adjacent area. The equation estimated is: BL=8.20+0.92*BHW

3.5. Diagnostic Phu Quoc ridgeback dog BWH and BW with Thai ridgeback dog and Rhodesian ridgeback dog

When comparing the research data from Phu Quoc ridgeback dog with database of dog breeds provided by Fédération Cynologique Internationale (FCI) (*http://www.fci.be/en/*), the body height at withers (BHW) and body weight (BW) measurements of Phu Quoc ridgeback dog were sharply different from related ridgeback breeds, especially the Thai ridgeback Height and weight were not significantly different between

sexes in Phu Quoc ridgeback dog while in Thai ridgeback the male individuals were clearly heavier and larger than females. Moreover, Phu Quoc dog's average weight was 19.6kg while in Thai ridgeback the female weight was 20.4kg and male weight was 22.7kg; Phu Quoc's average BHW was 45.9cm, different from Thai ridgeback (female 53.5 and male 58.5cm (Berkel, 1998; FCI, 2004) (*http://www.fci.be/en/nomenclature/THAI-RIDGEBACK-DOG-338.html*). BW and BHW of Phu Quoc ridgeback dog were also significantly lower than African ridgeback (female 32.0kg and 63.5cm; male 36.5kg and 66.0cm) [(FCI), 1996].

In addition, when compared with VKA's published criteria on Phu Quoc ridgeback dog breed in Vietnam, the study results are quite different, for example smaller BHW and heavier BW in both genders than previously published criteria (Fig 4, Table 6).

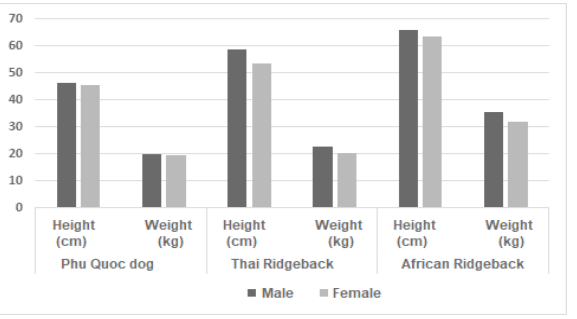

Fig 4. Height (BHW) and Weight (BW) of Phu Quoc ridgeback dog in comparison with Thai ridgeback dog and African ridgeback dog

Table 6. Three ridgeback dog breeds with body scores: body height at withers (BHW) and body weight

Sex	Rhodesian ridgeback dog[1]		Thai ridgeback dog[1]		Phu Quoc ridgeback dog[2]		Phu Quoc ridgeback dog[3]	
	BHW (cm)	**BW** (kg)	**BHW** (cm)	**BW** (kg)	**BHW** (cm)	**BW** (kg)	**BHW** (cm)	**BW** (kg)
Male	36.5	66.0	58.5	20.7	50 - 55	15 - 20	45.9	19.6
Female	32.0	63.5	53.5	22.4	48 - 52	12 - 18	45.3	19.5

[1]*The data collected from FCI morphology description and certification for breed dogs.*
[2]*The data collected from VKA* morphology description for Phu Quoc ridgeback dog (Vietnamese language)*
[3]*The results collected in this study*
**Vietnam Kennels Acsociation*

4. DISCUSSION

Phu Quoc ridgeback has a smaller body size and less weight than Thai and African counterparts, such differences are related to behaviour, environment and human domestication process (Vilà *et al*., 1997). Most publications on origin and genetics of ridgeback breeds mainly refer to African and Thai ridgebacks and consider Phu Quoc ridgeback as a subset of the Thai breed (Hofmeyer, 1963; Tshamala and Moens, 2000; Hillbertz, 2005; Hillbertz and Andersson, 2006; Hillbertz, 2007; Alvarez and Akey, 2012), but they show no evidence in morphology or genetics. Importantly, the recent published researches in Vietnam by Tran *et al*. (2016); Thai *et al*. (2016); Thai *et al*., submitted), by investigating the mDNA of Phu Quoc ridgeback dog, show that there have been no genetic relationship between Thai ridgeback dog and Phu Quoc ridgeback dog yet. The results of this study further reinforce the genetic differences expressed by some universal phenotypic indicators that are often quantified as characteristics of dog breeds in the world nowadays: body height at withers (BHW) and body weight (BW).

Both sexes of Phu Quoc ridgeback dog matured individuals are not different much in the average value of recorded parameters. Generally, Phu Quoc dogs have less BHW and body weight than Thai ridgebacks (Table 6 and Fig 2) although deviations in morphology between sampling locations, sexes and between ridged/non-ridged groups do occur. There is also apparent dissimilarity in general morphology and coat colour between the two breeds (unpublished data).

Locally, there is no difference in common phenotypic traits (body height and length, chest and waist circumference, ear length) between ridged and non-ridged Phu Quoc ridgeback dog which reinforced the scientific basis for the

argument that the back ridge originates from a mutation due to lack of nutrients from food and environment (Hillbertz and Andersson, 2006). There are observed differences between Phu Quoc ridgeback dog in Phu Quoc Island and in Ho Chi Minh City plus adjacent areas. Body height and length are significantly different between sexes of the Phu Quoc individuals while no significant difference was observed between sexes in Ho Chi Minh City and adjacent areas. Artificial selection based on human preferences probably facilitates the homogenization of phenotypes in different sexes, similar to the changes in morphology and size of several other breeds (Marelli *et al*., 2003; Sutter *et al*., 2008; Shearin and Ostrander, 2010; Bukar-Kolo *et al*., 2016; Sechi *et al*., 2016).

There is a significant difference in body weight between female ridged individuals in Phu Quoc and Ho Chi Minh City with its adjacent areas. (18.1±2.1 and 20.1±2.0kg). Body weight is a criterion for the diagnosis of excessive or lack of weight in sheltered dog and recently is used as an important reference for studies in dog obesity (Greer *et al*., 2007; Bland *et al*., 2010). The difference in body weight between sampled populations occurs only in ridged females; other size parameters have no difference. This probably due to excessive nutrients supply for reproduction in artificial breeding; additional researches on hormone content are required for a more detailed and accurate evaluation on body weight.

Body weight between ridged and non-ridged dogs (19.9±5.5 and 18.5±3.5kg, respectively) is also significantly different why other size parameters are not (Table 1-4). The reason probably is higher nutrient supply for ridged individuals that suit human preferences while non-ridged individuals are not regarded as genuine breed and receive less supply. Researches show that ridged trait is dominant in relation to non-ridged trait; therefore phenotype ratio of these traits is three ridged: one non-ridged according to Mendelian inheritance (Hillbertz and Andersson, 2006).

Tail length is also different between sexes and between sampled locations, probably related to sex roles and authority structure in dog packs. However additional researches are needed as tail length and morphology are also related to mental behaviour (Leaver and Reimchen, 2008).

Phu Quoc ridgeback dog has not been acknowledged as a separate dog breed yet; one of the main reasons is the lack of published researches on representative genetic traits and phenotypes of its populations in Vietnam. Previous descriptions of the breed are unsuitable as reference for breed criteria since they were only based on a too small number of individuals (Oustalet, 1981; Bylandt, 1897) to represent the whole Phu Quoc ridgeback population in Vietnam. The Vietnam Kennels Association (VKA), assigned to develop domestic dog breeds in Vietnam by FCI, published a description of the phenotypic criteria of Phu Quoc Ridge dog but it was not quoted from any previous research literature. The sizes range of this breed dog (male: BHW=50-55cm, BW=15-20kg; female: BHW=48-52cm, BW=12-18kg) has unknown confidence intervals which will be difficult to quantify further studies (Neyman, 1937; Morey *et al*., 2016) (Table 6). Furthermore, nowadays, because the geographic distribution of modern domestic dog breeds is highly overlapped, their traits had been evolving; the evolution process has to be evaluated positive and negative impact on dog breeding and the artificial selection based on human habit (Shearin and Ostrander, 2010; Crowther *et al*., 2014; Teng *et al*., 2016).

Addition, VKA regulated the BHW of this breed dog is 50-55cm in males and 48-52cm in females. However, it is different with results in this study that BHW in males is 39-51cm (confidence interval of 95%). Clearly, there is a significant discrepancy between regulated criteria and realistic observed value. Body weight of Phu Quoc ridgeback dog according VKA were 15-20kg in males and 13-18kg in females, so large range for defined an exact phenotype value. Meanwhile, our studies recorded BW values of this breed dog were 17.3-21.9kg in males and 17.3-21.7kg in females (Table 7).

The VKA regulates that the ratio between BHW and body length should be 1:1; it is complicated to achieve such ratio in Phu Quoc dog (www.vka.vn, in Vietnamese language). In one published study on Phu Quoc ridgeback

dog at semi-natural conservation site at Kien Giang, we emphasized the probable relationship between haplotype E on Phu Quoc ridgeback dog with BHW:BL ratio, in which the closer to that ratio is, the higher chance of being haplotype E is, maximum probability of being haplotype E can reach 47% (Quan *et al*., 2017). However, haplotype E is an ancient genetic line and has very low proportion, according to Thai Ke Quan (2016) it only makes up 15% of the total breed population; such value is already very high considering that haplotype E in some dog breeds only reached 2-3% (Thai *et al*., 2016). Therefore, the VKA standarded ratio 1:1 mean all this breed dogs has haplotype E, it is not impossible (my opinon). Furthermore, the 1:1 ratio was determined based on old images of the very first Phu Quoc dogs which sent to France before, it cannot represent the average ratio of Phu Quoc dogs in Vietnam nowadays.

Table 7. General observed morphological parameters in Phu Quoc dogs

	BW (kg)	**BL** (cm)	**BHW** (cm)	**ML** (cm)
Male	19.6±2.3	50.6±6.4	45.9±6.3	10.3±0.6
Female	19.5±2.2	50.2±6.5	45.3±6.2	10.2±0.5
	ChC (cm)	**WC** (cm)	**EL** (cm)	**TL** (cm)
Male	55.9±8.0	45.6±6.9	9.8±0.8	28.9±3.0
Female	55.3±7.9	45.1±6.8	9.8±0.8	28.1±2.3

There are differences between observed data (Table 6) and VKA's criteria on Phu Quoc ridgeback morphology, such differences requires further studies to standardize the essential morphological criteria (body weight, body height), the body size criteria should represent the real value of Phu Quoc ridgeback in Vietnam rather than only the standard regulations in morphology and size competitions.

Within the sampled population, morphological parameters are generally consistent on sampling locations, sexes and ridged/non-ridged groups. Ridged and non-ridged groups have no difference in body length and BHW, only have a difference in body weight and tail length. Female individuals between two sampling locations are different in body weight, and that result may be related to nutrition requirements in breeding and reproduction: if these processes are in high demand, females can gain more weight to satisfy the requirements for reproduction. Two important body size parameters that are BHW and body length are different between sexes and between sampling locations.

The regression coefficient of the sampled population at Ho Chi Minh City (0.800) is higher than in Phu Quoc (0.748), which means individuals at Ho Chi Minh City have a stronger linear correlation in BHW and body length. In other words, individuals at Ho Chi Minh City are under stronger artificial selection which leads to indifference between sexes in this area. On the contrary, individuals in Phu Quoc are under stronger natural selection leads to differences in these parameters between sexes.

A sound understanding of the taxonomy of threatened taxa is essential for setting conservation priorities and the development of species management strategies (Mace, 2004). A poor understanding of species taxonomy can hamper biodiversity conservation efforts by preventing the identification of unique evolutionary units, particularly if the species of potential conservation concern possesses morphological traits that are similar to those of closely related species (Daugherty *et al*., 1990). This is particularly true in canis where separate lineages easily hybridize and produce fertile offspring (Roy *et al*., 1994). Without the taxonomic tools to identify unique evolutionary lineages, it may not be possible to make accurate population estimates of species, identify threatened taxa or develop management strategies to enhance the conservation status of threatened taxa (Bacon and Bailey, 2006).

5. CONCLUSION

Today, some dog breeds have phenotypic changes with their original ancestors due to human needs and geographical isolation such as case studies on Dingo dogs (Australia) or Fonni dogs (Italy). The phenotypic indicators regularly need to be evaluated and collected to serve as a basis for the implementation of species conservation processes. Dog breeds phenotype change very quickly because of human habit and favourist. Our research will

provide additional data for previous genetic and phenotypic studies in Vietnam about Phu Quoc ridgeback dogs. In particular, it will provide a database to register the Phu Quoc ridgeback dog to FCI (until today, VKA has not done it). The next step for the conservation of Phu Quoc ridgeback dog is defining the traits differentiate them from hybrids, allowing natural selection and recognizing of the natural variation and the effect of environment and human selection on this breed.

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BIO-PROCESSING OF AGRO INDUSTRIAL WASTE INTO ENRICHED MICROBIAL PROTEIN AND USING THEM AS FEED SUPPLEMENTS TO POULTRY

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ABSTRACT

Proteins are the important dietary constituents which regulates the normal growth. Deficiency of protein in food is a big challenge. Single-Cell Protein (SCP) is a potential alternate to solve the global protein deficiency problem. The microbial proteins are popularly known as single cell protein which is dried cells of microorganisms. Several microbes like bacteria, algae, yeast and fungi are used for the production of SCP, wherein yeast occupies the unique place in this field. In poultry industry, the feed cost alone contributes to 75% of the total production cost. Agro industries generate huge amount of various fermentable and non-fermentable sugars which can be utilized by microorganisms to produce SCP. In India, Bengal gram is an essential pulse crop which is generally called as 'chick pea'. Chick pea occupies about 38 per cent of area under pulses and contributes about 50 per cent of the total pulse production of India. It is used for human consumption as well as for animal feeding. Chick pea straw is an excellent fodder for cattle. In Tamil Nadu, majority of poultry farms are concentrated in Namakkal, Coimbatore, Tiruppur, Karur, Dharmapuri, Salem and Hosur districts. Supplementation of synthetic origin feed supplements not only induces a number of invisible side effects in chicken but also results in many deleterious health effects in humans who are consuming these chicken. SCP, a viable supplement rich in many essential nutrients can be used as an alternative for these synthetic supplements. Bengal gram (*Cicer arientinum*) husk can be used for the production of SCP by yeast. Yeast will be isolated from the rice flour and identified by 16s RNA sequencing. *Cicer arientinum* husk powder was used as substrate and the various parameters pertaining to SCP production by SSF was optimized. After incubation, the microbial biomass was harvested. Purification of protein was performed by dialysis and chromatography. The bioconversion of protein was then estimated by Lowry method and molecular analysis by SDS PAGE. The bulk produced SCP was then used for conducting growth trial in broiler chicken. The biologically active compound in yeast was identified by various analytical methods like HPLC, NMR ($C^{13}H^{1}$) FTIR, XRD and GC-MS.

Keywords: *Microbial protein, Bioconversion, Dry biomass, Bio Molecular analysis, Animal feed.*

1. INTRODUCTION

Food waste is one of the main problems in both the developing and the developed countries (Huang *et al*., 2015). Wastes, particularly from the food industry, have significant amounts of two different sugars like fermentable and non-fermentable sugars which are utilized to manufacture by-products using microorganisms and produce the single-cell protein (SCP) for animal feed (Meireles *et al*., 2017). The recycle of food waste for feed production is an expansively investigated research area, for both purpose of health and developing the bio-based economy (Manu *et al*., 2017).

The rising world scarcity of protein is becoming a most important difficulty of humankind. Single cell protein describes the production of protein from biomass, originating from diverse microbial sources (Roth, 1980). Large-scale processes for SCP production have remarkable features which includes elevated productivity, quick growth rate of microorganisms and the independence of the various seasonal factors (Parajo *et al*., 1995).

In India, the poultry sector is valued at about Rs. 80,000 crores (2015-16) generally divided into two sub-sectors – one with a well-organized commercial sector with about 80% in total market share (say, Rs. 64,000 crores) and the other one is unorganized with about 20% in total

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market share of Rs.16,000 crores. The poultry sector plays an important role in additional income generation and family nutrition to the poorest of the poor (National Action Plan for Egg and Poultry, 2022). This study addresses the production of SCP which can be used as feed supplement in poultry field and more over which is cost effective and also a very good potential substitute for the synthetic origin feed supplements. Reutilization technique is an important step to provide the clean green environment. In Tamil Nadu, Erode district has more number of dal processing units which produces huge amount of husk as a waste. Part of this waste is used as cattle feed and mostly used as firewood for hotels and restaurants. From this cheaply available substrate, production of valuable microbial protein by bioconversion technique will help the people to enhance their socio-economic status.

Yeasts have advantages such as their larger size (easier to harvest), lower nucleic acid content, high lysine content and ability to grow at acidic pH. However, the most important advantage is familiarity and acceptability because of the long history of its use in traditional fermentations. Disadvantages include lower growth rates, lower protein content (45-65%) and lower methionine content than in bacteria (Richmond, 2004). Many raw materials are considered as substrate for the production of single cell protein (Nasseri *et al*., 2011). Both conventional (starch, molasses, fruit and vegetable wastes) and unconventional substrates (petroleum by-products, natural gas, ethanol, methanol and lignocellulosic biomass) are used for the SCP production (Bekatorou *et al*., 2006).

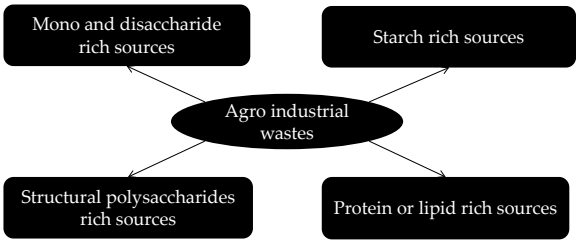

Fig 1. Categorization of agro industrial wastes

Monosaccharides and disaccharides rich sources need minimal pre-treatment, which provide these wastes discrete technical and economical benefit over other waste types, because it needs a simpler bioreactor, and does not require any sophisticated pretreatment processes. Structural polysaccharides rich wastes are obtainable in vast quantities all over the world; so, using these wastes does not have any competition with other industries which are used as resource for making of further value-added products. In contrast, competition more monosaccharides, disaccharides and starch rich sources are better, because those wastes are not so copious and are more simply used. If other waste types contain partial local accessibility, resourceful and economically reasonable pretreatment process can be used for hydrolysis. Structural polysaccharides rich wastes are mostly used for SCP production. In common, the following measures are very important for choosing the most appropriate waste product for SCP production viz., (i) easy availability of the particular waste product; (ii) costs of the pretreatment of waste product previous to using it in fermentation; (iii) low costs of waste product transportation; (iv) SCP concentrations in the ending microbial biomass after fermentation (Spalvins *et al*., 2018).

2. METHODOLOGY

2.1. Sample collection and processing

The waste of *Cicer arientinum* (Bengal gram) husk was collected from the various Dal processing industries in and around Erode district, Tamil Nadu. Proximate analysis of substrate was determined by Kjeldahl method. Ash, crude fiber and fat will be determined by using the standard methods as out lined in AOAC. Yeast was isolated from rice dough (3 days old). Samples were inoculated on the medium YPDcm agar plates and incubated for 48hrs. After incubation primary identification will be done by microscopy method. Then species identification will be performed by PCR amplification and sequencing.

2.2. Microbial protein production, estimation and purification

Bengal gram husk waste was used as substrate for submerged fermentation using substrate for production of SCP. Fermentation media was optimized according to Anbuselvi

et al. (2014). Protein extraction from yeast dry cell biomass was done as per Evelyn Sattlegger Jyothsna *et al*. (2011). After extraction, partial purification will be performed by Dialysis and chromatography technique.

2.3. Identification of bioactive compounds by analytical methods

The crude solvent extract is used for purification by HPLC and then compound identified by NMR, XRD, FTIR and GC-MS according to the procedure of Manikandaprabhu *et al*. (2015). Free radical scavenging activity DPPH (1,1-diphenyl-2-picryl hydrazyl) was performed to evaluate radical scavenging nature of extract of yeast (Kekuda *et al*., 2010).

2.4. Immunomodulatory activity

Cytokines plays a significant role in the growth of immune response, the yeast strain effect on the production of pro-inflammatory cytokines IFN-α and IFN-γ, and the anti-inflammatory cytokine IL-10 were evaluated. Lymphocyte proliferation assay was performed according to Ren *et al*. (2015).

2.5. *In vivo* analysis

The produced SCP was fed to broiler chicks at different inclusion levels and growth trial was carried over for 5 weeks duration.

3. RESULTS AND CONCLUSION

Cicer arientinum (Bengal gram) husk were collected from Dal industries. The proximate analysis showed the content of the substrate. Isolated yeast which was identified by 16hrs RNA was deposited in NCBI. By using the submerged fermentation, SCP was produced after the optimization. Produced microbial protein were estimated and purified by chromatography. Identification of bioactive compounds by analytical methods XRD, FTIR showed the capping agent, C^{13}NMR, H^{1}NMR which predicts the structure of compound and then confirmed by GC-MS. Identified compound showed good immunomodulatory and antioxidant activity. *In vivo* analysis showed significant growth in chicks which was confirmed by growth trial analysis of 5 weeks duration.

Cicer arientinum (Bengal gram) husk were collected from Dal industries and made powder which stored for further analysis.

Fig 2. *Cicer arientinum* (Bengal gram) husk

Fig 3. *Cicer arientinum* (Bengal gram) husk in powdered form

Fig 4. Yeast under microscope

Table 1. Proximate analysis of Bengal gram husk

Contents	Percentage (%)
Moisture	9.06
Crude protein	4.75
Sand and silica	0.18
Total ash	3.68
Crude fibre	45.56
Fat /oil	1.89
Carbohydrate	46.01

The isolate exhibited good growth on Rose Bengal Agar. The isolated yeast colonies were identified based on microscopic morphology and sequencing.

In vivo analysis shows significant improvement in growth of chicks which was confirmed by growth trial analysis of 5 weeks duration.

4. CONCLUSION

In conclusion, SCP shows incredibly attractive features as a nutrient supplement for

poultry. Microbes are used in the production of single cell proteins which gives numerous advantages over the conventional method. Microbes contain shorter generation time, allow easy transformation, utilize a lot of substrates, have no requirements in arable land or any particular season to grow and have the possibility of sustainable production in any part of the world. The cell yield varies according to the substrate and type of microorganism.

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BACILLUS SUBTILIS SUPPLEMENTATION DID NOT AFFECT BODY WEIGHT AND SOME DIMENSIONS IN 35-63 DAY-OLD NOI BROILERS

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ABSTRACT

This study was carried out to analyze the effect of different *Bacillus subtilis* supplementations on body weight and some dimensions of 35-63 day Noi broilers. A total of 96 Vietnamese native Noi chickens (48 heads for each gender) were allotted into four treatments with three replicates (four heads/replicate). Treatments included (i) T1: commercial feed GF1324 *ad libitum* (17% crude protein and 3,000 kcal/kg ME) produced by GreenFeed Vietnam Joint Stock Company, namely GF1324, and free clean water, (ii) T2: GF1324 and free *Bacillus subtilis* water (0.5g *Bacillus subtilis* $\geq 10^{11}$ spores/g mixed with 5L clean water) given every Monday from 6:00-18:00, (iii) T3: GF1324 and *Bacillus subtilis* water provided every Monday and Wednesday from 6:00-18:00, and (iv) T4: GF1324 and *Bacillus subtilis* water supplied every Monday, Wednesday and Friday from 6:00-18:00. The recorded parameters were not statistically significant different between treatments (P>0.05). This means that the addition of *Bacillus subtilis* with low and intermittent levels did not change the body weight and dimensions in chickens positively.

Keywords: *Bacillus subtilis, body weight, dimensions, Noi broilers.*

1. INTRODUCTION

Indigenous chickens are indispensable in livestock structure. Currently, there are many native chicken breeds, mainly coming from the Northern provinces of Vietnam. However, the purity of native breeds needs to be verified because crossing among poultry breeds is practiced simple and easy compared to other animal species. This is to satisfy people's needs and purposes (fighting, pets, food, etc), i.e. for fighting mission, selected chickens must have long legs and strong muscle tissues. Therefore, their morphological and productive characteristics are very diverse. Dorji and Sunar (2014) implied that commercial breeds (i.e. Hyline Brown) are better in performance than the local hens. On the other hand, this might lead to dilution of poultry genetic resource.

In the South of Vietnam, especially in the Southwest region, Noi is a famous native chicken breed chosen for different production purposes. Noi is often reared mainly in small scale at householders for improving quality of meals and increasing little daily income because of their good natural disease resistance and tasty meat quality. Therefore, they are also known as a backyard chicken breed that is playing an important role in animal production system in the Mekong delta.

For most chickens raised in backyard systems, vaccinating is often skipped or not done while diseases are not understood and treated effectively. Therefore, supplying probiotics into water can be considered as the easiest ways for householders to boost immune system and productivity of backyard chickens. Actually probiotics could protect broilers against pathogens by colonization in the gastrointestinal tract (Nisbet *et al*., 1993; Hejlicek *et al*., 1995; Pascual *et al*., 1999) and stimulation of systemic immune responses (Muir *et al*., 1998; Que´re´and Girard, 1999). Recently, although there have been many studies on Noi chickens, no one on their morphological characteristics, as well as the effects of probiotic supplementation on their morphological dimensions was investigated. It is also the goal of our study.

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2. MATERIALS AND METHODS

This study was conducted from December 2018 to April 2019 at the Can Tho Center for Breeds of Seed, Livestock and Fish, located at O Mon district, Can Tho city, Vietnam. It is cooperating with Can Tho University in several research activities.

A total of 96 Noi chickens (48 males and 48 females) at 35 days old with similar body weight for each gender were allotted to four treatments as below:

T1: GF1324 + clean water

T2: GF1324 + *Bacillus subtilis* water every Monday

T3: GF1324 + *Bacillus subtilis* water every Monday and Wednesday

T4: GF1324 + *Bacillus subtilis* water every Monday, Wednesday and Friday

Table 1. Nutrient component of the experimental feed

Items	GF1324
Crude protein (min), %	17
Humidity (max), %	14
Crude fiber (max), 5	5
Methionine and cysteine (min), %	0.7
Metabolism energy (min), kcal/kg	3.000
Calcium, %	0.8-1.2
Phosphorus, %	0.6-1.0
Total lysine (min), %	1

** Main ingredients: soybean cake, fish meal, rice bran, broken rice, corn, wheat bran, cassava, amino acids, vitamins and minerals (Source: GreenFeed Vietnam Join Stock Company)*

Bacillus subtilis water was prepared 30 minutes before drinking by mixing 0.5g *Bacillus subtilis* ($\geq 10^{11}$ spores/g) in five litters of clean water. *Bacillus subtilis* water was freely supplied from 6:00-18:00 on the experimental days. During the experiment, vitamin C was supplied three days per week.

All broilers were raised on commercial layer cages. Each cage contained four individuals (males or females). They were given *ad libitum* GF1324 commercial feed produced by GreenFeed Vietnam Joint Stock Company (Table 1).

At the beginning of the experiment and the end of every week, body weight (BW, g/head) and some dimensions such as beak length (BL, mm), skull length (SL, mm), skull width (SW, mm), neck length (NL, mm), back length (BaL, mm), wings length (WL, mm), thigh length (TL, mm), shank length (ShL, mm), keel length (KL, mm), breast diameter (BD, mm), and thigh diameter (TD, mm) (Francesch *et al*., 2011; Ukwu *et al*., 2014; Yunusa and Adeoti, 2014; FAO, 2012; Molenaar *et al*., 2008) were recorded.

The collected data were analyzed GML methods to compare mean among treatments by using R program (version 3.4.2).

3. RESULTS AND DISCUSSION

The obtained results indicated that no significant difference in body weight and measured dimensions were found among treatments at various weeks of age. Khoa *et al*. (2019) implied that it could be due to (i) somes standard deviations were large and variances of mean were not significant different, partly due to smallness of sampling size and number of replications; (ii) domestication and/or uniformity of Noi chickens were not high; and/or (iii) *Bacillus subtilis* was supplied at a low concentration. Thus their development is not strong enough in the digestive system to be able to positively affect the digestion and absorption of nutrients according to the competition mechanism and the interaction of beneficial bacteria.

Although significant difference was not found, BW (one of important economic traits in meat poultry production industry) in all three treatments supplied *Bacillus sublitis* water were always higher than T1 (control) at time points, especially at 56 days old. Additionally, according to Dorji and Sunar (2014) morphometric characterization provides baseline information for initiating population's improvement.

All they are in a strong agreement with

biological mechanisms explained in previous reports that *(i)* supplementing probiotics positively improve BW and/or ADG (Song *et al*., 2014; Jin *et al*., 1997; Zulkifli *et al*., 200; Kalavathy *et al*., 2003; Apata, 2008; Ashayerizadeh *et al*., 2009) because it enhanced digestion, absorption and availability of nutrition accompanying with positive effects on intestine activity and increasing digestive enzymes (Endens *et al*., 2003), *(ii)* inactivated probiotics, disrupted by a high-pressure homogenizer, have positive effects on the production performance of broiler chickens when used at certain concentrations (Huang *et al*., 2004); while *(iii)* some other evidence indicated that probiotic supplementation did not improve feed intake (Mansoub, 2010; Jin *et al*., 1998; Murry *et al*., 2006), gave inconsistent results, maybe because of type of diet ingredients which can affect probiotic's growth or metabolites of chicken (Timmerman *et al*., 2006), or was not significantly different in BW and FCR among treatment groups (Yousefi and Karkoodi, 2007).

Besides that, within each treatment, significant difference in ShL in T1 at 35 days old (P=0.011), BW (P=0,048) and ShL (P=0,032) in T1, SL in T3 at 42 days old, BW (P=0.046) and ShL (P=0,012) in T1, BL (P=0,037) in T3 at 49 days old, ShL (P=0,007) in T1 and SL in T4 (P=0.038) at 56 days old, ShL (P=0.017) in T1, BW (P=0.018) and BL (0.050) in T4 at 63 days old, whereas males always showed higher these traits than females, were found between sexes. This was normal biological development in animal and no evidence on effect of *Bacillus sublitis* on traits/sexes were demonstrated. Furthermore, although NL has no significant difference between genders, NL of males was different from that of females at 49 days old. Partly it may be one of important characteristics for males where long NL is flexible and quickly moving to peck the neck of their competitors in fighting games (Endo *et al*., 2015).

At 56 days old, BaL, KL, ShL, TL and BD of Guineafowl were 127.4, 57.3, 62.8, 57.9, and 187.4, respectively (Vy *et al*., 2016). At 140 days old, WL, KL, BD, TL, ShL, and BaL of Quy Phi chicken were 172.6, 154.7, 154.6, 173.3, 71.5, and 184.2, respectively (Thinh *et al*., 2018). These dimensions in Guineafowl and Quy Phi chicken were different from those in Noi chicken. This may be due to difference among breeds and/or measuring methods among studies.

It was indicated that fighting chickens were heavier than meat type because they have larger pectoral muscle and gastrocnemius as well as longer tarsal length than meat type (Vasinopas and Meckvichai, 2015). This is due to fighting chickens performed more frequently for flapping and jumping (Endo *et al*., 2013).

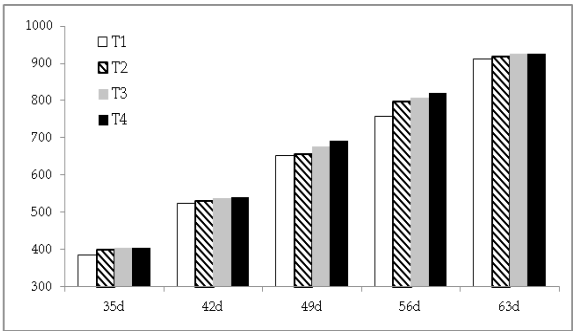

Fig 1. Body weight at different weeks of age

4. CONCLUSION

The results showed that there was no statistically significant difference in the body weight and dimensions among treatments supplementing different levels of *Bacillus subtilis* in chicken Noi at 35-63 days of age. It may be because of small amount of *Bacillus subtilis* supplementation and non-continuous experiment. It needs to be noted for further studies to investigate the effect of *Bacillus subtilis* supplementation on chicken economic characteristics in general and Noi chicken in particular.

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Table 2. Body weight and dimensions at 35 days old

Traits	T1			P	T2			P	T3			P	T4			P	P
	Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		
BW	385.62±42.69	398.75±43.44	372.50±39.34	0.135	398.35±48.81	400.75±43.56	395.73±56.03	0.812	403.75±47.63	401.08±46.40	406.42±50.75	0.791	404.67±56.02	403.50±58.01	405.83±56.52	0.921	0.687
BL	23.65±0.99	23.79±1.20	23.51±0.75	0.494	23.67±0.92	23.56±0.94	23.78±0.92	0.579	23.98±1.04	23.80±0.96	24.16±1.12	0.412	24.16±0.90	24.32±0.92	24.01±0.89	0.417	0.534
SL	24.86±0.74	24.93±0.79	24.78±0.71	0.648	25.33±1.02	25.24±0.92	25.43±1.16	0.673	25.07±1.13	25.22±1.05	24.93±1.23	0.541	25.39±1.04	25.12±0.96	25.65±1.08	0.226	0.508
SW	24.17±1.09	24.23±1.35	24.10±0.81	0.789	24.45±1.08	24.29±1.16	24.62±1.01	0.474	24.09±1.28	24.24±1.35	23.94±1.24	0.575	24.40±1.13	24.54±1.37	24.26±0.90	0.546	0.771
NL	87.79±1.35	87.92±1.24	87.67±1.50	0.660	85.74±1.54	86.00±1.70	85.45±1.37	0.410	86.92±1.32	86.83±0.94	87.00±1.65	0.764	88.13±1.54	88.33±1.56	87.92±1.56	0.520	0.849
BaL	129.71±5.22	130.67±5.63	128.75±4.83	0.380	133.39±10.07	131.10±10.89	135.91±8.89	0.260	132.83±7.38	132.33±7.25	133.33±7.78	0.748	133.54±5.90	133.75±6.44	133.33±5.60	0.867	0.447
WL	317.92±19.28	320.42±19.24	315.42±19.82	0.537	311.30±16.53	312.92±16.44	309.54±17.24	0.636	307.92±14.21	302.92±8.38	312.92±17.25	0.085	306.96±24.88	305.17±32.40	308.75±15.40	0.733	0.522
TL	130.13±6.57	131.42±7.94	128.83±4.86	0.347	129.04±8.45	128.60±7.94	129.54±9.34	0.792	132.96±6.26	133.83±5.72	132.10±6.90	0.506	132.88±6.68	131.67±6.51	134.10±6.91	0.387	0.590
ShL	32.58±1.50	33.33±1.37	31.83±1.27	0.011	29.43±3.17	29.75±3.17	29.10±3.18	0.624	32.79±2.96	33.33±2.31	32.25±3.52	0.382	32.42±2.48	32.33±1.97	32.50±3.00	0.874	0.717
KL	84.38±4.77	84.67±5.76	84.08±3.78	0.772	82.70±6.36	82.75±6.48	82.64±6.55	0.967	84.00±6.03	84.50±5.23	83.50±6.93	0.694	85.29±5.74	85.83±5.64	84.75±6.05	0.654	0.992
BD	183.87±8.33	185.67±8.78	182.08±7.82	0.302	188.09±9.52	186.50±7.43	189.82±11.50	0.416	188.33±8.43	187.92±7.82	188.75±9.32	0.815	189±9.15	189.33±9.10	188.67±9.60	0.863	0.611
TD	57.79±1.28	57.67±1.37	57.92±1.24	0.644	54.30±2.49	54.92±2.23	53.64±2.70	0.227	56.17±2.08	56.42±1.56	55.92±2.54	0.567	57.67±1.46	58.00±1.65	57.33±1.23	0.274	0.582

BW: body weight (g/head); BL: beak length (mm); SL: skull length (mm); SW: skull width (mm); NL: neck length (mm); BaL: back length (mm); WL: wings length (mm); TL: thigh length (mm); ShL: shank length (mm); KL: keel length (mm); BD: breast diameter (mm); TD: thigh diameter (mm).

Table 3. Body weight and dimensions at 42 days old

Traits	T1			P	T2			P	T3			P	T4			P	P
	Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		
BW	522.92±68.89	550.42±71.17	495.42±56.63	0.048	528.96±71.82	536.67±66.10	520.54±79.95	0.602	537.29±81.76	557.50±74.70	517.08±86.64	0.234	540.00±78.96	541.25±85.02	538.75±76.19	0.940	0.622
BL	26.43±0.38	26.50±0.36	26.40±0.40	0.407	26.16±0.66	26.25±0.72	26.07±0.60	0.524	26.60±0.67	26.73±0.53	26.47±0.78	0.354	26.60±0.46	26.70±0.44	26.50±0.47	0.297	0.983
SL	26.58±0.90	26.74±0.83	26.42±0.97	0.394	26.84±0.81	26.99±0.79	26.68±0.84	0.366	26.96±0.97	27.35±0.57	26.58±1.16	0.052	27.11±0.77	26.93±0.85	27.29±0.66	0.264	0.159
SW	26.58±0.79	26.58±0.96	26.60±0.63	0.956	26.08±0.80	26.20±0.93	25.96±0.65	0.481	26.33±1.38	26.39±1.40	26.27±1.41	0.830	26.61±0.91	26.45±1.16	26.78±0.56	0.387	0.791
NL	100.42±4.64	100.42±3.96	100.42±5.42	1.000	92.83±6.00	94.59±5.82	90.91±5.84	0.146	97.71±4.66	97.50±3.99	97.92±5.42	0.832	101.88±5.28	102.08±4.98	101.67±5.77	0.852	0.524
BaL	155.92±6.41	156.67±7.14	155.17±5.80	0.578	156.26±10.46	153.58±12.36	159.18±7.39	0.207	155.08±8.61	132.33±7.25	133.33±7.78	0.748	158.29±6.85	158.75±6.94	157.83±7.03	0.751	0.447
WL	353.12±17.50	355.00±17.45	351.25±18.10	0.611	344.13±14.67	345.83±16.07	342.27±13.48	0.573	340.42±13.67	335.42±9.64	345.42±15.59	0.072	341.25±26.01	338.75±34.38	343.75±14.79	0.648	0.515
TL	137.67±5.52	138.42±6.75	136.92±4.12	0.518	134.52±8.54	133.83±8.42	135.27±9.01	0.696	138.38±5.32	139.08±4.78	137.67±5.94	0.526	140.08±5.28	139.17±5.60	141.00±5.01	0.407	0.703
ShL	36.13±1.65	36.83±1.47	35.42±1.56	0.032	33.43±2.21	33.67±2.27	33.18±2.23	0.611	35.92±2.69	36.25±2.42	35.58±3.00	0.555	35.75±2.37	35.33±2.06	36.17±2.66	0.400	0.384
KL	92.17±5.67	92.50±7.10	91.83±4.10	0.781	90.30±6.00	90.50±6.23	90.09±6.04	0.875	91.33±6.04	92.08±5.80	90.58±6.44	0.555	93.17±6.28	94.00±6.30	92.33±6.44	0.528	0.981
BD	203.54±9.50	205.42±10.33	201.67±8.62	0.345	206.30±11.40	204.58±9.40	208.18±13.47	0.463	206.04±9.21	205.42±8.91	206.67±9.85	0.747	208.54±9.72	208.75±10.03	208.33±9.85	0.919	0.649
TD	69.13±4.27	68.33±4.05	69.92±4.50	0.375	61.09±3.72	62.00±3.52	60.09±3.83	0.226	63.58±3.55	63.75±3.57	63.42±3.68	0.824	67.50±3.89	68.50±4.32	66.50±3.29	0.215	0.342

Table 4. Body weight and dimensions at 49 days old

Traits	T1			P	T2			P	T3			P	T4			P	P
	Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		
BW	651.67±84.84	685.83±84.04	617.50±73.75	0.046	656.43±84.86	661.50±75.01	650.91±97.92	0.773	676.25±107.12	681.25±106.00	671.25±112.70	0.825	693.13±119.43	718.25±134.12	668.00±102.27	0.313	0.679
BL	28.46±1.05	28.61±0.84	28.32±1.24	0.504	28.00±1.19	28.05±1.33	27.94±1.07	0.831	28.22±1.28	28.76±1.24	27.69±1.12	0.037	29.09±0.77	29.29±0.65	28.89±0.86	0.207	0.438
SL	27.29±0.89	27.47±0.94	27.12±0.84	0.344	27.76±1.16	27.72±0.95	27.80±1.40	0.880	27.88±0.91	28.16±0.95	27.60±0.82	0.134	28.01±1.01	28.40±1.25	27.75±0.58	0.113	0.596
SW	27.64±0.74	27.79±0.78	27.48±0.71	0.329	27.69±1.20	27.52±1.20	27.87±1.24	0.494	27.65±1.13	27.75±0.90	27.54±1.35	0.666	27.90±1.20	27.78±1.48	28.02±0.89	0.629	0.672
NL	114.88±5.93	115.83±5.15	113.92±6.71	0.441	107.04±6.00	109.00±6.47	104.91±4.85	0.103	112.25±5.82	112.00±5.64	112.50±6.23	0.839	117.13±6.83	118.33±7.18	115.92±6.54	0.398	0.643
BaL	165.33±6.03	166.17±6.83	164.50±5.28	0.511	166.91±13.16	163.67±15.44	170.45±9.61	0.225	164.42±10.59	162.92±11.35	165.92±10.03	0.500	168.54±7.13	169.25±7.05	167.83±7.46	0.637	0.385
WL	385.17±22.99	388.17±21.79	382.17±24.70	0.535	374.26±20.34	375.33±22.59	373.09±18.60	0.799	369.00±19.11	363.25±14.72	374.75±21.78	0.144	373.67±22.18	374.58±24.12	372.75±21.10	0.845	0.516
TL	154.79±7.08	156.17±8.70	153.42±5.02	0.353	152.35±10.53	151.42±10.33	153.36±11.15	0.668	155.75±6.87	156.25±6.84	155.25±7.16	0.730	158.13±7.59	157.00±7.68	159.25±7.66	0.480	0.678
ShL	39.88±1.78	40.75±1.76	38.99±1.35	0.012	36.74±2.73	36.92±2.81	36.55±2.77	0.753	39.75±3.31	40.17±2.79	39.33±3.85	0.550	39.75±2.98	39.33±1.97	40.17±3.79	0.506	0.443
KL	108.63±6.71	108.75±8.00	108.50±5.49	0.930	106.00±8.06	106.50±73.99	105.45±8.49	0.764	107.71±7.56	108.42±6.80	107.00±8.50	0.656	109.54±7.37	110.75±6.77	108.33±8.04	0.434	0.968
BD	221.54±13.24	224.75±15.14	218.33±10.73	0.244	224.39±13.40	222.50±10.95	226.45±15.95	0.492	223.25±11.23	222.58±10.16	223.92±12.62	0.778	227.50±12.64	227.33±11.75	227.67±14.00	0.950	0.550
TD	86.00±4.51	85.42±4.62	86.58±4.52	0.538	75.57±4.98	76.42±5.35	74.64±4.61	0.404	78.88±4.74	79.08±4.89	78.67±4.79	0.835	84.67±5.15	85.92±5.87	83.42±4.19	0.242	0.582

Table 5. Body weight and dimensions at 56 days old

Traits	T1			P	T2			P	T3			P	T4			P	P
	Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		
BW	757.17±97.92	730.58±95.25	783.75±97.12	0.190	796.52±122.05	800.83±115.48	791.82±134.37	0.864	808.08±109.56	812.00±115.60	804.17±108.16	0.865	819.25±137.81	870.00±150.39	768.5±107.12	0.070	0.154
BL	30.31±0.96	30.03±0.85	30.59±1.01	0.157	30.31±1.30	30.42±1.25	30.19±1.40	0.680	30.05±1.20	30.26±1.15	29.84±1.26	0.411	30.67±1.18	31.06±1.22	31.29±1.06	0.115	0.248
SL	28.36±0.72	28.55±0.75	28.17±0.65	0.192	28.75±1.21	28.85±0.93	28.65±1.49	0.705	29.29±0.77	29.23±0.69	29.35±0.87	0.709	28.83±1.05	29.27±1.25	28.39±0.58	0.038	0.321
SW	28.76±0.78	28.86±0.83	28.66±0.76	0.541	28.93±1.34	28.90±1.24	28.97±1.51	0.902	28.83±1.24	28.79±0.93	28.88±1.54	0.852	28.96±1.22	28.86±1.65	29.05±0.63	0.716	0.949
NL	137.08±4.87	137.92±4.50	136.25±5.28	0.414	126.52±11.09	128.75±10.03	124.09±12.15	0.326	134.25±6.19	134.17±5.97	134.33±6.67	0.949	139.79±6.83	140.42±6.20	139.17±7.64	0.664	0.744
BaL	171.13±7.71	172.00±8.65	170.25±6.92	0.590	172.87±14.38	169.50±16.60	176.55±11.10	0.250	171.58±10.35	170.17±10.97	173.00±9.95	0.514	174.54±8.60	174.42±8.64	174.67±8.95	0.945	0.523
WL	420.00±23.96	421.25±22.37	418.75±26.38	0.805	408.13±22.80	409.50±23.92	406.64±22.57	0.771	402.83±20.30	395.83±14.28	409.83±23.47	0.091	408.25±22.45	409.75±22.84	406.75±22.97	0.751	0.483
TL	165.04±7.70	166.83±9.53	163.25±5.10	0.263	160.83±10.84	159.92±10.69	161.82±11.44	0.684	165.33±7.67	166.00±7.78	164.67±7.83	0.680	167.50±7.56	166.08±7.56	168.92±7.62	0.371	0.553
ShL	44.33±2.22	45.50±2.02	43.17±1.80	0.007	40.57±2.94	40.83±3.16	40.27±2.80	0.658	43.88±3.70	44.33±3.11	43.42±4.29	0.556	43.92±3.81	43.33±2.71	44.50±4.72	0.465	0.316
KL	115.60±8.99	115.75±10.88	115.42±7.12	0.930	113.30±9.79	113.00±9.94	113.64±10.09	0.880	114.46±9.76	115.50±9.03	113.42±10.73	0.612	117.38±9.72	118.17±9.54	116.58±10.25	0.699	0.964
BD	238.79±15.56	242.33±17.31	235.25±13.40	0.274	242.09±15.77	239.58±12.14	244.82±19.22	0.439	240.50±13.98	239.58±12.83	241.42±15.57	0.756	245.21±15.73	245.83±15.43	244.58±16.68	0.851	0.568
TD	92.08±4.80	91.33±5.14	92.83±4.53	0.456	80.74±6.22	81.82±6.56	79.45±5.85	0.355	84.63±5.30	84.67±4.48	84.58±6.22	0.970	90.96±5.23	91.83±6.09	90.08±4.29	0.425	0.601

Table 6. Body weight and dimensions at 63 days old

Traits	T1			P	T2			P	T3			P	T4			P	P
	Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		Mean (n=24)	Male (n=12)	Female (n=12)		
BW	912.92±115.52	937.08±101.45	888.75±127.82	0.316	917.70±151.64	950.83±151.64	881.55±150.13	0.284	926.46±142.44	943.08±138.84	909.83±150.14	0.579	925.83±171.98	1006.67±158.43	845.00±149.95	0.018	0.403
BL	31.58±0.94	31.38±0.82	31.79±1.04	0.290	31.89±1.40	32.16±1.32	31.59±1.48	0.334	31.75±1.31	31.77±1.51	31.72±1.15	0.920	32.16±1.38	32.70±1.34	31.61±1.24	0.050	0.192
SL	29.41±1.05	29.55±1.15	29.26±.97	0.513	29.55±1.20	29.77±0.99	29.31±1.42	0.378	30.09±1.01	29.93±0.82	30.25±1.19	0.448	29.87±1.03	29.86±1.24	29.28±0.69	0.170	0.482
SW	29.64±0.73	29.76±0.65	29.51±0.82	0.420	30.09±0.92	30.24±0.79	29.93±1.05	0.433	29.85±1.30	30.04±0.94	29.65±1.61	0.470	29.94±1.39	29.93±1.88	29.94±0.69	0.980	0.933
NL	139.08±5.57	140.00±5.64	138.17±5.59	0.432	129.61±9.99	131.67±10.94	127.36±8.80	0.313	135.83±6.37	135.83±5.57	135.83±7.33	1.000	141.67±7.76	142.50±7.83	140.83±7.93	0.610	0.815
BaL	181.88±9.51	182.92±10.54	180.83±8.68	0.603	185.30±17.14	181.08±18.96	189.91±14.35	0.225	181.88±12.22	180.17±11.97	183.58±12.75	0.506	187.29±9.29	188.42±9.16	186.17±9.68	0.564	0.372
WL	446.04±25.54	448.75±23.85	443.33±27.91	0.614	433.91±21.84	436.25±22.47	431.36±21.92	0.604	427.29±20.27	420.83±15.64	433.75±22.88	0.121	433.42±23.29	435.17±24.18	431.67±23.29	0.721	0.458
TL	178.38±11.11	181.17±13.30	175.58±8.00	0.226	174.30±12.24	172.92±11.56	175.82±13.33	0.582	178.79±8.83	179.83±8.54	177.75±9.37	0.575	180.71±10.48	178.92±10.07	182.50±11.01	0.414	0.414
ShL	47.54±2.38	48.67±2.23	46.42±2.02	0.017	43.35±3.58	43.67±3.58	43.00±3.71	0.666	47.04±3.98	47.50±3.37	46.58±4.62	0.584	47.00±3.72	46.58±2.81	47.42±4.54	0.594	0.501
KL	122.71±7.95	123.17±9.74	122.25±6.06	0.785	118.39±9.56	119.08±1.17	117.64±9.28	0.726	120.63±9.67	121.75±8.87	119.50±10.67	0.580	123.54±9.96	124.08±10.29	123.00±10.03	0.796	0.995
BD	252.25±14.25	254.67±15.30	249.83±13.33	0.418	254.65±16.55	252.58±12.17	256.91±2.71	0.544	254.42±13.77	253.92±12.35	254.92±15.59	0.863	259.63±14.15	260.00±14.19	259.25±14.73	0.900	0.765
TD	93.88±4.72	93.42±5.52	94.33±4.23	0.645	82.87±6.02	84.42±6.43	81.18±5.31	0.205	87.08±6.09	86.58±5.78	87.58±6.60	0.697	93.83±4.80	94.08±5.77	93.58±3.82	0.805	0.521

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EFFECT OF SUPPLEMENTS OF YEAST *(SACCHAROMYCES CEREVISIAE)*, RICE DISTILLERS' BY-PRODUCT AND FERMENTED CASSAVA ROOT ON METHANE PRODUCTION IN AN IN VITRO RUMEN INCUBATION OF ENSILED CASSAVA ROOT, UREA AND CASSAVA LEAF MEAL

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ABSTRACT

An *in vitro* rumen incubation was carried out to determine effects on methane production of supplementing ensiled cassava root, urea and cassava leaf meal with rice distillers' byproduct, fermented cassava root and yeast (*Saccharomyces cerevisiae*). The four treatments in a completely randomized design were: CTL: No supplement; RDB: 4% (in DM) rice distillers' byproduct; FCR: 4% (in DM) urea-fermented cassava root; Yeast: 1% (in DM) commercial yeast. The quantity of substrate in each fermentation bottle was 12g DM to which was added 240ml of rumen fluid (from slaughtered cattle) and 960ml of buffer solution. The incubations were done using one-liter water bottles with gas collection by water displacement. Measurements of total gas production and methane percentage in the gas were made at intervals of 0-3, 3-6, 6-12, 12-18 and 18-24h. The hourly rate of gas production increased to a maximum in the 6-12h of incubation interval and then decreased linearly. In contrast, the proportion of methane in the gas increased linearly with incubation interval from the beginning until the final 18-24h interval. Total gas production was highest for the fermented cassava root additive, followed by the rice distillers' byproduct with lowest values for the control and yeast treatments. The methane content of the gas was highest for the control treatment, followed by the fermented cassava root and yeast with the lowest value for rice distillers' byproduct, for which the overall reduction in methane was of the order of 25%. Methane production per unit DM digested showed a similar trend as the methane percentage in the gas. It is suggested that the benefits from brewers' grains and rice distillers' byproduct, in reducing methane production in the rumen fermentation, both *in vitro* and *in vivo*, are the indirect effects of these additives increasing the proportions of propionic acid in the rumen VFA.

Keywords: *β-glucan, greenhouse gas, hydrolysis, propionate, urea.*

1. INTRODUCTION

Agriculture is an important source of greenhouse gas mainly through emissions of methane from enteric fermentation in ruminants and decomposition of manure (Gerber *et al.*, 2013). Ruminants are estimated to produce up to 95 million tons of CH_4 annually, mainly from enteric fermentation and to a lesser extend from decomposition of manure (O'Mara, 2011; Patra, 2014). Strategies to reduce these emissions should first address the need to increase productivity of ruminant livestock; secondly, the feeding systems that lead to increased ruminant productivity are those that lead to increased proportions of propionic acid; and thirdly, the escape of protein from the rumen contributes amino acids directly to the animal through enzymatic digestion of protein in the intestines. The additional advantage of this process is that fibrous feed particles that escape the rumen attached to the protein will still be fermented to useful end products but in the cecum-colon section of the ruminant digestive tract in which the fermentation process does not produce methane (Demeyer, 1991).

In countries located in temperate latitudes, cereal crops such as maize and barley are the choice of feeds for intensifying ruminant production. Maize is grown in tropical

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latitudes but yields do not compete with those in temperate climates. By contrast, cassava (*Manihotesculenta Crantz*) is a crop that originated in the tropics (in the Caribbean) and is now grown in over 90 countries world-wide (Lebot, 2009). Of importance in a warming world is that cassava is potentially highly resilient to future climatic changes and according to Jarvis *et al.* (2012) "could provide Africa with options for adaptation whilst other major food staples face challenges". Regarding the use of cassava for cattle production, these developments have been the driving force for a series of researches directed at optimizing the use of both the pulp and the fresh cassava root as the basis of intensive systems of livestock production, especially the fattening of local cattle (Phanthavong *et al*., 2014; 2015; 2016; 2017; Inthapanya *et al*., 2016). An additional advantage of cassava over cereal crops such as maize is that the foliage has proved to be a valuable source of bypass protein such that the cassava plant becomes a source both of highly digestive carbohydrate (from the root) as well as protein (from the foliage). The only additional features needed are a source of fermentable nitrogen (available locally as fertilizer grade urea) and minerals.

The development of cattle feeding systems based on cassava has stimulated an important outcome, namely how to manage the potential toxicity linked with the presence throughout the plant of cyanogenic glucosides that give rise to hydrocyanic acid when exposed to favorable conditions (eg: appropriate enzymes) in the plant itself or within the digestive of animals that consume it. Recent research, much of it in the laboratory of the senior author of this paper, has shown that the potentially toxic cyanogenic glucosides in cassava can be neutralized by supplementing the animal diet with small quantities of locally available by-products from fermentation industries, specifically from the production of beer which gives rice to "brewer' grains"; and the artisan distillation of fermented rice to make an alcoholic wine, which produces a byproduct known in Lao PDR as "Quilao", in Vietnam as "Hem" and in Cambodia as "Bar Ran". Brewers' grains have been shown to aid directly in the detoxification of HCN in cases where forage of a "bitter" (high HCN potential variety) was fed (Binh *et al*., 2017). Both brewers' grains and rice distillers' byproduct, fed at less than 5% of the diet, have resulted in reduced production of rumen methane and improved growth rates in cattle fed ensiled cassava pulp-urea of ensiled cassava root-urea as basal diet (Keopaseuth *et al*., 2016; Sengsouly and Preston, 2016; Inthapanya *et al*., 2017).

Access to brewers' grains is limited to farmers living in close proximity to the beer factory. Rice distillers' byproduct is more widely available in rural areas, but supplies are limited. For these reasons, research to identify alternatives to both brewers' grains and rice distillers' byproduct are considered to be of high priority. As both brewers' gains and rice distillers' byproduct are products of fermentation by yeast (specifically *Saccharomyces cerevisiae*), it was decided to evaluate: (i) a commercial source of yeast commonly available in local markets; and (ii) cassava root enriched by yeast fermentation with additional sources of nitrogen (urea) and phosphorus (di-ammonium phosphate – DAP). The purpose of the present study was to determine effects on CH_4 production in an *in vitro* rumen fermentation when yeast, rice distillers' byproduct, or fermented cassava root, were added in small amounts (4% DM basis) to a basal substrate of ensiled cassava root supplemented with urea and cassava leaf meal.

2. MATERIALS AND METHODS

2.1. Location and duration

The experiment was conducted in the laboratory of the Department of Animal Science, Faculty of Agriculture and Forest Resources, Souphanouvong Uni., LuangPrabang, Lao PDR.

2.2. Treatments and experimental design

The experimental design was completely randomized (CRD), 4 treatments and each with 5 replicates. The treatments were:

CTL: Ensiled cassava root

RBD: CTL + rice distillers' byproduct at 4% of DM

FCR: CTL+ fermented cassava root at 4% of DM

Yeast: CTL+ yeast at 1% of DM

All the treatments included urea (2% of root DM), cassava leaf meal from a bitter variety (25% of substrate DM) and S-rich minerals (1% of DM substrate).

2.3. *In vitro* rumen fermentation system

The in vitro rumen fermentation system (Diagram 1) was the same as that used by Sangkhom *et al.* (2011). The water bottles (capacity 1.5liters) were used for the fermentation and collection of the gas. These were connected by plastic tube (id 4mm) to a similar bottle which received the gas (the bottom of which had been removed) and which was suspended in a larger bottle (3liters capacity) partially filled with water, so as to collect the gas by water displacement. The bottle that was suspended in water was calibrated at 50ml intervals to indicate the volume of gas.

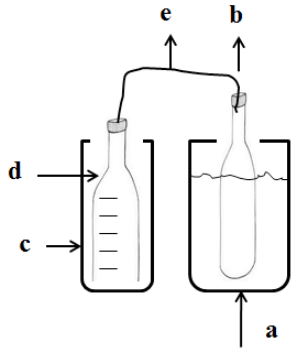

Fig 1. A schematic view of apparatus to measure in an in vitro rumen fermentation

a) Water bath
b) Fermentation bottle (1.5liters)
c) Water storage reservoir (3 liters)
d) Gas collection bottle (1.5liters)
e) Plastic tube (id: 4mm)

2.4. Experimental procedure

The cassava roots and leaves were collected from the Souphanouvong University farm; the roots were chopped into pieces around 1-2cm of length, ground in a liquidizer, and then stored in a plastic bag for ensiling over 7 days. Cassava leaves were chopped into small pieces around 1-2cm in length, then dried in the oven at 80ºC for 24h before grinding. The rice distillers' byproduct was collected from a farmer accustomed to produce "rice wine alcohol".

Buffer solution was prepared before incubation by mixing 0.04g $CaCl_2$, 9.30g; $NaHPO_4.12H_2O$, 0.47g; NaCl, 0.57g; KCl, 0.12g; $MgSO_4.7H_2O$, 9.80g; $NaHCO_3$, 0.25g of Cysteine and distil-water up to one litter together (Tilly and Terry, 1963).

The procedure for producing "fermented cassava root" was as follows: the roots were chopped and steamed for 30 minutes, allowed to cool for 15mintutes and then mixed with di-ammonium phosphate (DAP) at 0.8, 3% of yeast (*Saccharomyces cerevisiae*) and 2% of urea (all on DM basis) prior to being fermented in a closed plastic bag for 7 days. Representative samples (12g DM) of the substrates was put in the incubation bottle to which was added 0.96l of buffer solution and 240ml of rumen fluid (obtained from a slaughtered cow) prior to filling each bottle with carbon dioxide. The bottles were incubated at 38ºC in a water bath for 24h.

2.5. Data collection and measurements

The gas volume was recorded over intervals of 0-3, 3-6, 6-12, 12-18 and 18-24h. The methane concentration in the gas collected over each interval was measured with a Crowcon infra-red analyzer (Crowcon Instruments Ltd, UK). At the end of the incubation, the remaining substrate was filtered through cloth and the solid residue dried at 100°C to determine the DM digested.

2.6. Chemical analyses

Samples were analyzed for DM, ash, CP and CF according to AOAC (1990) methods. The solubility of the protein in diet ingredients was determined by extraction with M NaCl (Whitelaw and Preston, 1963).

2.7. Statistical analysis

The data were analyzed by the general linear model option of the ANOVA program in the Minitab software (version 16.0). In the model the sources of variation were: treatments, and error. Tukey's pair-wise comparisons was used to determine the differences between treatments when the P value of F test <0.05. The statistical model used was: $Y_{ij} = \mu + T_j + e_{ij.}$ *Where: Y_{ij} was the dependent variable, μ was the overall mean, T_j was the effect of treatment, and e_{ij} was random error.*

3. RESULTS AND DISCUSSION

3.1. Chemical composition

Table 1. Chemical composition of substrate components

Items	DM, %	CP	CF	Ash	Soluble CP % of total CP
		As % of DM			
Ensiled cassava root	32.1	2.51	1.12	0.88	9.22
Fermented cassava root	39.9	10.4	1.13	0.87	11.6
Bitter cassava leaf meal	90.3	19.0	15.2	6.14	31.3
Rice distillers' by-product	7.46	24.2	2.29	4.45	37.9
Yeast		45.0			32.4

The values for DM, CP and solubility of the protein in the rice distillers' by-product (Table 1) were similar to previous studies (Luu Huu Manh *et al*., 2009; Sangkhom *et al*., 2017) and the cassava root after fermentation with yeast, urea and DAP had a similar level of CP as reported for the same procedure by Vanhnasin *et al*. (2016) and Manivanh *et al.* (2016).

3.2. Gas production

Gas production was higher for fermented cassava root than rice distillers' by-product and yeast or control treatment (Table 2), whereas the CH_4 content of the gas was differed among treatments with highest values for the control treatment, followed by the fermented cassava root, yeast and rice distillers' by-product, respectively.

Table 2. Means for gas production, methane, digestibility and methane per unit substrate digested

Items	CTL	FCR	Yeast	RDB	SEM	P
Gas production, ml: 0-3hr	360^{a}	420^{b}	370^{a}	380^{a}	11.7	0.012
3-6hr	510^{a}	700^{b}	520^{a}	580^{a}	36.7	0.008
6-12hr	760^{a}	1070^{b}	820^{a}	950^{c}	36.7	<0.001
12-18hr	640^{a}	940^{b}	690^{ac}	720^{c}	20.6	<0.001
18-24hr	470^{a}	630^{b}	480^{a}	600^{b}	19.0	<0.001
Methane,%: 0-3hr	8.4^{a}	7.8^{a}	6.4^{b}	6.4^{b}	0.28	0.001
3-6hr	11.4^{a}	10^{b}	8.4^{c}	7.6^{c}	0.35	<0.001
6-12hr	18.6^{a}	17.6^{a}	15^{c}	12.6^{b}	0.70	<0.001
12-18hr	22^{a}	21.2^{a}	19.6^{c}	17^{b}	0.39	<0.001
18-24hr	24.2^{a}	23.6^{a}	21.2^{c}	19^{b}	0.37	<0.001
Total gas, ml	2740^{a}	3760^{b}	2880^{a}	3230^{c}	62.7	<0.001
Total methane, ml	484^{b}	637^{c}	427^{a}	426^{a}	11.5	<0.001
Methane, % total gas	17.7^{d}	16.9^{c}	14.8^{b}	13.2^{a}	0.227	<0.001
DM digestibility, %	59.2^{a}	69.4^{b}	61.1^{a}	65.2	0.83	<0.001
CH4, ml/ g DM digested	68.4^{c}	76.5^{d}	58.2^{b}	54.4^{b}	1.76	<0.001

Where: Values on the same row with different superscripts differ (P<0.05)

3.3. Effect of incubation interval

The rate of gas production increased to a maximum in the interval 6-12h of incubation then decreased linearly. In contrast, the proportion of methane in the gas increased linearly with incubation interval (Table 2).

Table 3. Mean values for percent methane in the gas, and gas production per hour, during successive intervals of the fermentation

Items	0-3	3-6	6-12	12-18	18-24	SEM	P
Methane, %	7.25	9.35	16.0	20.0	22	0.203	<0.0001
Gas, ml/h	128	193	150	125	91	3.8	<0.0001

3.4. Effect of additives

Total gas production was highest for fermented cassava root, followed by RDB with lowest values for the yeast and control treatments (Table 3). The CH_4 content of the gas differed among treatments with highest values for the control treatment, followed by the fermented cassava root, yeast and RDB. On the RDB treatment the overall reduction in percent methane was of the order of 25%. CH_4

production per unit DM digestibility showed the same trend as the CH_4 percent in the gas (Table 3), respectively.

The increases in CH_4 production in the gas with duration of fermentation time, indicative of the transition to a secondary fermentation of the VFA to methane, supports earlier findings using a similar *in vitro* incubation system but with different substrates (Le Thuy Binh Phuong *et al.,* 2011; Outhen *et al.,* 2011; Sangkhom *et al*., 2011; Thanh *et al*., 2011).

The beneficial effect of the small quantity (4% of substrate DM) of rice distillers' by-product in reducing CH_4 production from rumen fermentation is similar to the response reported by Sangkhom and Preston (2016) when brewers' grains and rice distillers' by-product were added to *in vitro* incubations of ensiled and fermented cassava roots. Addition of yeast at 1% of the substrate also reduced CH_4 production but was slightly less effective than the rice distillers' by-product. By contrast, an attempt to simulate some of the features of RDB by fermenting cassava root with yeast, urea and di-ammonium phosphate had no effect on CH_4 production in the *in vitro* fermentation. It has been postulated that the benefits of yeast-based additives in improving human health and growth rates of animals are related to the β-glucan present in the yeast cell wall and their effect in stimulating the immune system (Dritz *et al.,* 1995; Hanh *et al*., 2006; Novak and Vetvicka, 2002; Waszkiewicz-Robak, 2013).

In ruminant systems it is probable that their effects are modulated through effects on microbial ecosystems in the rumen and/or lower down the digestive tract. Thus alleviation of hydrocyanic acid toxicity, in cattle fed cassava foliage from a variety rich in HCN precursors, has been suggested as being due to the β-glucans in brewers' grains supporting biofilm-based fermentations in the rumen that favored detoxification of the HCN (Inthapanya *et al.,* 2017). A shift in the microbial fermentation towards propionate at the expense of acetate production increases the overall yield of metabolisable energy. It also increases availability of glucogenic substrate which is often critically low in some feeds (Preston and Leng, 1987), H_2 produced when acetate is the end product of organic matter fermentation is converted to CH_4 and thus reduces overall metabolisable energy. Improvements in growth rate and feed conversion in cattle supplemented with brewers' grains or rice distillers' product are thus to be expected from the combined benefits of increased availability of both glucose precursors from propionic acid and increased total metabolisable energy as a result of decreased CH_4 production in the rumen (Sangkhom and Preston, 2016; Sengsouly and Preston, 2016; Binh *et al.,* 2017). Brewers' grains and rice distillers' by-product is the presence of yeast which has been subjected to heating (100˚C) under acid conditions during the process of distilling off the ethanol as is standard practice in production of beer from barley (brewers' grains) and from polished/ broken rice (rice "wine"). It is then assumed that it is the β-glucan derived from the cell walls of the yeast (and of the barley), which modifies microbial activities in the rumen biofilms (Leng, 2014) favouring higher proportions of propionic acid in the rumen VFA. β-glucans are present in the cell wall of barley (Havrlentová and Kraic, 2006) and yeast (Waszkiewicz-Robak, 2013). An important issue is the need, or otherwise, to isolate the β-glucan which, as described by Nguyen Thi Thuy and Nguyen Cong Ha (2016), required high pressure homogenization to break the yeast cell wall followed by acid, then alkaline hydrolysis. The results from our experiment, in which there were no effects on CH_4 by supplementing the substrate with fermented cassava root, partially support the hypothesis that breakage of the yeast/barley cell walls, followed by acid hydrolysis, are necessary first steps in facilitating the action of the β-glucan. The positive effect of the commercial yeast "starter" in decreasing CH_4 production implies that some of the β-glucan in this product may have been in the free-state. However, the greater impact of the rice distillers' by-product in reducing CH_4 production would probably have been facilitated by the degree of hydrolysis of the yeast cells that were likely to have occurred in the process of distilling the ethanol. The positive effect of rice distillers' by-product in reducing CH_4 production *in vitro* mirrors the results obtained

in vivo when local cattle were fed basal diets of ensiled or fermented cassava root supplemented with urea and fresh cassava foliage (Sengsouly *et al.*, 2016; Sangkhom *et al.*, 2016). In both these experiments the reduction in CH_4 emissions were directly linked with improved growth rates and better feed conversion.

4. CONCLUSIONS

Obtained results indicated that (i) the rate of gas production increased to a maximum in the 6-12h incubation interval and then decreased linearly. In contrast, the proportion of methane in the gas increased linearly with incubation interval from the beginning until the final 18-24h interval; (ii) Total gas production was highest for the fermented cassava root additive, followed by the rice distillers' by-product with lowest values for the control and yeast treatments; (iii) The CH_4 content of the gas was highest for the control treatment, followed by the fermented cassava root and yeast with the lowest value for rice distillers' by-product, for which the overall reduction in CH_4 was of the order of 25%; and (iv) CH_4 production per unit DM digested showed the same trend as the CH_4 percentage in the gas.

It is suggested that the reported benefits from brewers' grains and rice distillers' by-product in reducing CH_4 production in the rumen fermentations, both *in vitro* and *in vivo*, are the indirect effects of these additives increasing the proportions of propionic acid in the rumen VFA.

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THE EFFECT OF DRIED CASSAVA FOLIAGE WITH ENSILED CASSAVA ROOT ON GROWTH PERFORMANCE OF LOCAL YELLOW CATTLE IN LAO PDR

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ABSTRACT

This study was carrying out to evaluate the efficacies of using ensiled cassava root incorporates with different levels of dry cassava foliage that used as a source of bypass protein for fattening local yellow cattle in Lao PDR, nine local "Yellow" cattle with initial live weight of 90-105kg live weight (LW) were used in this study. The experimental design was arranged in completely randomize design (CRD) with three replications of each treatment. The factors were different levels of dried cassava foliage (CTL, 25 and 50% of total diet DM). The experiment was carried out 14 days for adaptation to the pens and diets. Ensiled cassava root was offered as a basal diet supplemented with rice straw and mineral block, and adding dried cassava foliage by different levels. The results indicated that the increase of dried cassava foliage levels were displayed the effects in feed conversion rate (FCR) ($P<0.05$) and when increased the level of dried cassava foliage at 50% of total diet DM incorporated with the diet DM found the cattle were presented significant growing rate as high as 0.3 kg/d of ADG ($P<0.05$).

Keywords: *Local Yellow cattle, dried cassava foliage, growing, diets.*

1. INTRODUCTION

The livestock in Lao PDR is shown to be an important component of smallholder farms. In many areas of upland and lowland, the sales of livestock account are more than 50% of cash income. Over 95% of livestock in Laos is commonly produced by smallholders. Cattle are regarded as one ruminants of livestock that is important for smallholder and it is mainly related to the Chinese Yellow Cattle, adapted to the environment as well, the yellow cattle is small, agile, hardy, good reproductive rates, and it has few calving problems, the body weight of yellow cattle is up to 350kg for males, 250kg for females (Werner *et al.,* 2002). The raising native cattle in Laos are found throughout the country which it can enhance livelihood as provide several productions such as meat, milk, traditional ceremonies, draught power, transportation and manure (Phanthavong and Wanapat, 2004). The cattle population have increased significantly from year 2002 to 2007 at the rate of 1.9 % per annum. For the barriers and efficacies of cattle production, the feed sources for cattle are mainly native grasses, legumes and tree leaves that are available in the natural grassland and forests but it is available only in rainy season and limited qualities, especially in the cropping season (Sangkhom *et al.,* 2012).

Cassava production is widely cultivated in several areas of Laos and mainly produced in order root, the total yield is about 2.4 million tonnes of cassava root production annually and about 75,465 ha of total areas have used for producing cassava. The cassava production is mainly cultivated by both cassava starch companies and farmers (normally farmers produce cassava root for trading to factory as fresh and dry forms and some excess amount they keep for feed and food). Recently, the production of cassava root that plants by farmers is very low price (Ministry of Agriculture and Forestry, 2016), so it is an alternative feed that shows considerable potential for use as a feedstuff to feed several kinds of animals in Lao PDR.

One of important part of cassava is the cassava foliage that has been shown to be excellent

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sources of protein, as a direct supplement or in concentrate mixtures. Supplements are required to correct the deficiencies of essential nutrients; thereby increasing basal feed intake and hence animal production as well (Tham *et al.*, 2008). After harvest ofcassava root, the leaves are left in the field which has been shown as a residue but it is still a good quality protein source that is available for many kinds of animals particularly for the cattle in the dry season. The cassava foliage can be made as a sun-dried cassava foliage that is used as a supplement feed for ruminants and it also can be fed for reduce the nematode egg counts in buffaloes (Sath *et al.*, 2008).

In Asia, the rice straw is regarded as the crop residue that widely has found in several regions, it is the main feed source for ruminants in both rainy and dry season when natural grasses are in short supply. The characterization of rice straw is contained high fiber level (39-53% ADF) and nutrient deficiencies, especially protein (2-4% crude protein), vitamins, minerals and soluble carbohydrates. Around 98% of silica is presented in rice straw so the digestibility of ruminants is low, the performance of which ranging 41-59% (McAllister *et al.*, 1994; Sangkhom *et al.*, 2012).

The aim of the experiment was to evaluate the efficacies of using ensiled cassava root incorporates with different levels of dried cassava foliage as a feed for local Yellow cattle in Lao PDR.

2. MATERIALS AND METHODS

2.1. Location and duration

The experiment was conducted in the demonstrative station (Km13 station), Faculty of Agriculture and Forestry, Champasack University, Lao PDR. It is far from the city center about 13 kilometers and around 250 meters of above sea level.

2.2. Animals and housing

Nine local "Yellow" male cattle were used with initial live weight of 90-105kg LW. They were confined in individual pens, made from wood and bamboo with the size of each pen 1.5*2m. Vaccination was done against epidemic diseases and drenching against internal parasites before the commencement of the experiment.

2.3. Treatments and experimental design

The experiment was determined for 84 days and the experimental design was a "production function" arrangement with 3 levels (0, 25 and 50%) of supplementation with dried cassava foliage in total diet DM. The basal diet was ensiled cassava root, dried cassava foliage was used as the source of bypass protein and fiber. The allocation of treatments to pens was accorded to a completely randomized design (CRD) with three replications of each treatment. The treatments are as follows:

Table 1. The feed formula as treatments (kg)

Feed ingredients	CTL	DCF25	DCF50
Ensiled cassava root	0.90	0.90	0.90
Urea	0.09	0.09	0.09
Dried cassava foliage	0.00	0.75	1.50
Rice straw *(ad libitum)*	2.01	1.26	0.51
Total	3.00	3.00	3.00

The basal diet was ensiled cassava root and rice straw was fed *ad-libitum*, mineral block, and water was available in the experiment.

2.4. Feeding and management

Animals were adapted gradually to the experimental feeds for two weeks prior to starting the experiment. Cassava root was bought from cassava farmers and ensiled for five days, then stored in closed plastic bags to maintain the ensiled condition (pH <4). Cassava foliage and rice straw were purchased from farmers in the area. Urea was purchased from the market. A mineral mixture containing salt (NaCl) 40%, Sulphur 5% and Lime (calcium carbonate) 35% were processed as a mineral block and was provided *ad-libitum*, and water was supplied *ad-libitum*.

Fig 1. Dried cassava foliage

Fig 2. Ensiled cassava root

2.5. Data collection and measurements

The cattle were weighed before feeding in the morning at the beginning of the experiment and at 14-day intervals. Feeds offered, and residues were recorded daily; samples of each were collected every 14 days and stored at -18°C. At the end of the experiment, the samples were bulked on an individual animal basis prior to analysis.

2.6. Chemical analysis

Feed samples were analyzed for dry matter (DM), ash and nitrogen following the procedures of AOAC (1990).

2.7. Statistical analysis

Live weight gain was determined from the linear regression of live weight (Y) on days in the experiment (X). The recorded data were analyzed by the general linear model option in the ANOVA program of the Minitab software (MINITAB, 2000). Sources of variation in the model were treatments and error. As there appeared to be an improvement in growth response to the levels of dried cassava foliage, the data analyses were run of the three months of the experiment.

3. RESULTS

3.1. Chemical composition of feeds

Crude protein, ash and dry matter of ensiled cassava root, dried cassava foliage and rice straw (Table 2) were similar value to the studies of Sangkhom and Preston (2016), and Inthapanya *et al.* (2016).

Table 2. Chemical compositions of experimental diets (DM)

Feed	DM	Ash	CP
	%	As in DM basis, %	
Ensiled cassava root	35.60	0.81	2.07
Dried cassava foliage	89.50	6.12	22.90
Rice straw	89.40	13.10	3.50

3.2. Feed intake

The intake of ensiled cassava root and rice straw which it was not indicated significant difference, but when added 25 and 50% in total diet DM were significantly increased feed conversion rate (FCR) of cattle (P<0.05, Table 3).

Table 3. Mean values for intake of diet components

Feed	CTL 0%	DCF 25%	DCF 50%	*SEM*	P
Rice straw (DM) (kg)	49.22	46.98	45.02	2.62	0.56
Ensiled cassava root (DM) (kg)	44.63	44.14	46.49	2.46	0.78
Dry cassava leaf (DM) (kg)	0.00^{c}	46.85^{b}	98.14^{a}	1.33	0.01
Urea (kg)	0.56	0.54	0.54	0.02	0.68
Total feed intake (kg)	94.42^{c}	138.51^{b}	190.19^{a}	6.16	0.01
Feed conversion rate (FCR) (kg)	9.09^{b}	13.44^{a}	14.91^{a}	0.70	0.03

3.3. Growth rate

The supplementation of 50% dried cassava foliage in total diet DM of cattle was able to improve the live weight gain of local yellow cattle as high as 0.3 kg of ADG (P<0.05), but when added 25% dried cassava foliage in the diet was not shown any significant difference when compared with control (Table 4).

Table 4. Mean values for growth performance

Growth rate	CTL	DCF 25%	DCF 50%	SEM	P
Initial weight (kg)	119.50	114.40	112.60	4.90	0.61
Final weight (kg)	129.90	123.70	125.40	4.77	0.66
Average daily gain (ADG) (kg/d)	0.25^{a}	0.25^{a}	0.30^{b}	5.78	0.01

4. DISCUSSION

The chemical compositions of ensiled cassava root, dried cassava foliage and rice straw (Table 1) were crude protein and ash which were similar with the studies of Sengsouly and Preston (2016). Moreover, Tilahun *et al.* (2013) was also analyzed the chemical composition of dried cassava foliage found the equality value as about 21.9% of crude protein, except Ash was showed higher as about 12.3%. The chemical compositions were found in the range of those reported in https://www.feedipedia.org.

The positive effects on adding dried cassava foliage for fattening local "Yellow" cattle was indicated that the supplementation of 50% dried cassava foliage of total diet DM had the potential benefits as displaying in the FCR was 14.9. Many reports were evaluated the utilization of dried cassava foliage for fattening cattle such as Phuong *et al.* (2012) was studied on effects of NPN source as cassava leaves on growth performance and methane emissions in cattle found similar amount of FCR as about 16.2 while when compared with Tham *et al.* (2008) was showed higher FCR as about 21.8, the reasons may due to the supplementation of molasses by spraying to the rice straw, which Khalili *et al.* (1993) was evaluated the efficacies of molasses with grass hay as a supplementation feed which molasses can be improved the dry matter intake of cattle.

Cassava leave has investigated to incorporate with the diet of cattle to reduce methane emission from cattle production, which methane is considered as large amount of gas that cause to global warming, Sina and Preston (2017) was reported the effects of cassava leaf processing and variety of cassava leaf on methane production in an in vitro rumen incubation, the results indicated that methane production was lower when the substrate contained bitter cassava leaf rather than from sweet cassava variety and fresh rather than sun-dried cassava leaves. In addition, Phongphanith, Preston and Leng (2016) was displayed the evidences by using cassava leaf meal (*Manihot esculenta* Crantz) with biochar in an *in vitro* rumen incubation which methane emission was decreased when cassava leaf meal replaced water spinach leaf meal as the major protein source, and when biochar was added to the substrate.

5. CONCLUSION

The experiment with local Yellow cattle fed ensiled cassava root and rice straw, and added 50% dried cassava foliage of total diet DM there were positive indications ($P<0.05$) that, cattle were significantly shown growing rate and feed conversion rate.

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NUTRITIONAL VALUE OF MICROALGAE ISOLATED FROM VIETNAM

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ABSTRACT

Recently algae have been known as a potential source of food, energy, medicines, cosmetics or used for wastewater treatment. In this study, we evaluated nutritional value of the five microalgal species (*Cyclotella* sp., *Scenedesmus protuberans, Scenedesmus acuminatus v. biseratus, Pediastrum duplex* and *Coelastrum microporum*) isolated from Vietnam via protein content and the development of zooplankton (*Daphnia magna*) which feed on the algal isolates during 21 experimental days. The results showed that protein content in these algae was relatively high, ranged from 37% to 48% dry weight, highest in *C. microporum* and lowest in *Cyclotella* sp. Besides, *D. magna* fed *C. microporum* and *S. acuminatus v. biseratus* had a better survival and more reproductive than those fed the other three algae. Our finding offers a high potential on protein exploitation out of microalgae from Vietnam for aquaculture, livestock and human beings. Further investigations on amino acid proportion and other compounds from Vietnam microalgae are suggested.

Keywords: *Microalgae; zooplankton; nutritional value; protein content; development.*

1. INTRODUCTION

In recent years, reduction in food security and resource depletion have been extremely serious issues worldwide, especially in the developing countries, hence many scientists are looking for alternatives to traditional foodstuffs. There have been numerous investigations revealing that microalgae have enormous potentials in providing nutrition for not only livestocks but also humans due to their high nutrient value, fast growth and wide distribution (Kent *et al*., 2015). For example, Henrikson (1994) demonstrated that comparing to soybeans, powdered milk, animal meat and fish, the microalga *Sprirulina* (*Arthorspira*) has higher protein content, particularly it contains 65% protein and fatty acid, 20% caborhydrates, 7% mineral and 5% fat. Besides, the alga also have high amount of useful secondary metabolites (e.g. carotene β, vitamin E, B12, B6) and other essential minerals (e.g. iron, calcium, magnesium). Similarly, Lopez *et al.* (2010) recorded the protein content in the five distinct microalgae *Porphyridium cruentum*, *Scenedesmus almeriensis*, *Arthrospira platensis*, *Muriellopsis* sp. and *Synechocystis aquatilis* which ranged from 15-62% dry weight. Moreover, some algae have been proven to contain various healthy substances (e.g. antibiotics, antiflammatories, disease prevention; Bloor and England, 1989; Robertson *et al*., 2015; Ku *et al*., 2013). Vietnam is located in the tropical area which has a high diversity of species including algae. However, there has been no report on the potential nutrition value of algae from Vietnam (Dao and Luu, 2015).

Beside the chemical analysis, biological assay could help to evaluate the nutrient in algae used as the sole food for zooplankton. Lampert (1977) fed the *Daphnia pulex* with nine different species of microlalgae and found that the shape, size and nutrient quality of the microalgae strongly influenced on the food consumption of the animal. The food consume of zooplankton would closely relate to the nutrient uptake and energy availability for all processes in the animals such as activity maintenance, growth, development, reproduction among others. The aim of this investigation is to assess the potential of using the algae originated from Vietnam as the nutrition resource through (i) determining nitrogen content in the five distinct microalgae species (*Cyclotella* sp., *Scenedesmus protuberans*, *Scenedesmus acuminatus v. biseratus*, *Pediastrum*

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duplex and *Coelastrum microporum*) after successfully isolating, culturing and harvesting their biomass and (ii) recording the development and reproduction of micro-crustacean (*Daphnia magna*) fed by these algae over the period of 21 experimental days.

2. MATERIALS AND METHODS

2.1. Total protein determination

Algae samples were collected at two site along Nhieu Loc – Thi Nghe canal (Location No.1 - Thi Nghe bridge, ward 19, Binh Thanh district, Ho Chi Minh city and Location No.2 - Bridge No. 8, ward 12, district 3, Ho Chi Minh city) by towing a standard net (mesh-size 20 μm) horizontally near the water surface. The clear plastic bottles were utilized to contain the samples during approximately 2 hours prior to morphological identification and isolation under the microscopic observation in the laboratory (Sournia, 1978). After isolating successfully, almost algal strains (*S. protuberans, S. acuminatus v. biseratus, P. duplex* and *C. microporum*) were grown in the artificial medium called Z8 (Kotai, 1972) at 27±2^{0}C, a light intensity of about 3,000Lux, and a 12h light : 12h dark cycle (APHA, 2012), excluding *Cyclotella* sp. was cultured in the Z8 medium with a salinity of 3‰. Dry biomass of five distinct algal strains was collected individually by filtering through the glass fiber filters (particle retention rate: 1.6μm; Whatman) and drying in the SS Memmert drying stove at the temperature of 60^{0}C during 4 hours. Kjehdahl method was utilized for nitrogen determination of the dry mass of the algae (APHA, 2012). The total protein of the algae is estimated by multiplying the determined nitrogen content by a nitrogen-to-protein conversion factor (f = 5.95, Lopez, 2010).

2.2. Evaluation on the nutritional value of microalgae by feeding micro-crustacean *Daphnia magna*

Daphnia magna was obtained from MicroBio Test (Belgium) and maintained in the ISO medium in the laboratory under the temperature of 25±1^{o}C, a light intensity of 1,000 Lux and a 14h light : 10h dark cycle (APHA, 2012). The experiment for assessment the nutritional value of microalgae was conducted on the micro-crustacean *D. magna* according to Gustaffson and Hansson (2004) with minor adjustment. Prior to the experiment, 50 mother Daphnia were collected, cultured in 1l glass beaker containing 800ml ISO medium and fed by the green alga *Chlorella* sp. After that, neonates (<24h old) were randomly collected and used for the experiment. In each treatment, 5 neonates were raised in the 100 mL glass beaker containing 80mL ISO medium, and five replicates (n=5) were prepared. The *Daphnia* was fed ad libitum with each of the microalga (*Cyclotella sp., S. protuberans, S. acuminatus v. biseratus, P. duplex* or *C. microporum*). The medium and food were renewed three times a week. The survival and reproduction of *Daphnia* were daily recorded over the period of 21 days.

Table 1. Nitrogen content in the isolated algae
(Mean±SD, mg N/g dry biomass)

Species	Nitrogen content
S. protuberans	68.6±9.41
S. acuminatus v. biseratus	79.4±9.45
P. duplex	74.2±1.81
C. microporum	79.9±2.33
Cyclotella sp.	62.9±3.47

3. RESULTS

3.1. Total nitrogen and protein in the microalgal isolates

The nitrogen content in dry biomass of algae was showed in Table 1. Among the five isolates, the nitrogen content was highest in the algal species *C. microporum* (~79.9mg N/g dry mass) and *S. acuminatus v. biseratus* (79.4mg N/g dry mass). This value was lowest in the diatom *Cyclotella* sp., 62.9mg N/g dry mass (Table 1).

The total protein content in the microalgal isolates was estimated and presented in Table 2. Comparing to the proportion of protein in other nutrient food (22% in fish, 26% in animal meat, 3.4% in powdered milk, 13% in fresh egg and 2.7% in rice, USDA (2018)) and other algal species, our results demonstrated that protein content in five common algae from Vietnam was relatively high, ranged from 37% (*Cyclotella* sp.) to 48% (*C. microporum*) dry weight.

Table 2. Protein content (%) in the algae in this study and several previous investigations

Species	Protein	Sources
S. protuberans	40.8	This study
S. acuminatus v. biseratus	47.3	This study
P. duplex	44.2	This study
C. microporum	47.5	This study
Cyclotella sp.	37.5	This study
Chlorella vulgaris	33-46	Seyfabadi (2010)
Porphyridium cruentum	33-35	Lopez (2010)
Scenedesmus almeriensis	35-37	Lopez (2010)
Muriellopsis sp.	15-20	Lopez (2010)
Synechocystis aquatilis	15-20	Lopez (2010)
Arthrospira platensis	55-62	Lopez (2010)
Dunalliella sp.	64	Thomas *et al*. (1984)
Chlorella sp.	15-23	Brown and Jeffrey (1992)
Thalassiosira oceanica	12-38	Knuckey *et al*. (2002)
Thalassiosira pseudonana	12-38	Knuckey *et al*. (2002)
Arthrospira massartii	41-72	Dao and Luu (2015)

3.2. Evaluation on the nutritional value of microalgae by feeding micro-crustacean *Daphnia magna*

Although all *D. magna* lived well during the first six-day of experiment, there was a significant difference in the number of living organisms between the treatments afterwards. Particularly, the survival proportion of organisms fed by *S. protuberans*, *S. acuminatus v. biseratus* and *C. microporum* was 4, 92 and 96%, respectively, whereas all *Daphnia* fed by *P. duplex* and *Cyclotella* sp. died by the end of the test (Fig 1).

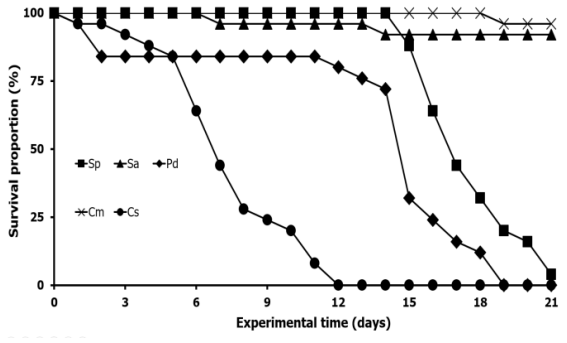

Fig 1. The survivorship of D. magna fed by five distinct algae

Sp, Sa, Pd, Cm and Cs was treatment that *D. magna* was fed by *S. protuberans*, *S. acuminatus v. biseratus*, *P. duplex*, *C. microporum* and *Cyclotella* sp., respectively

Additionally, reproductive performance among animals fed different algae was not similar. Over the period of incubation, the number of total neonates of mother *Daphnia* fed by *S. protuberans*, *S. acuminatus v. Biseratus*, *P. duplex*, *C. microporum* and *Cyclotella* sp. were 247, 881, 100, 1127 and 4, respectively (Fig 2). These results revealed that among the algae used as food, *C. microporum* (protein content was 47.5%) seemed to be the best food for micro-crustacean *D. magna* because both the survivor proportion and reproductive performance of the organisms fed by this alga were highest. In contrast, animals fed by *Cyclotella* sp. (protein content was 37.5%) died completely after the first 12 days and only 4 offsprings were laid over the experimental period. Moreover, *S. acuminatus v. biseratus* (protein content was 47.3%), *S. protuberans* (protein content was 40.8%) and *P. duplex* (protein content was 44.2%) made test organisms lived well in the first days of experiment, however, afterwards these algae sticked on the tails of animal resulting in immobility and mortality.

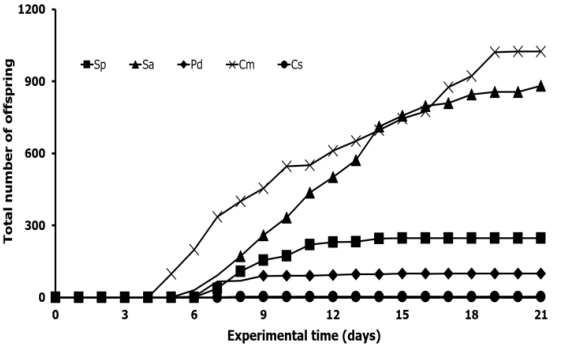

Fig 2. Accumulative neonates of *D. magna* fed by five distict algae during 21 experimental days (Abbreviation as in Fig 1).

4. DISCUSSION

Interestingly, the results on protein content in these algae were agreed with the findings in the previous investigation. Dao and Luu (2015) recorded the protein content in 10 *Arthorspira massartii* strains isolated from Sai Gon river

fluctuating from 41 to 72% (Table 2). Last not least, to the best of our knowledge, the current study firstly offers the information on protein content in the five microalgae *Cyclotella sp., S. protuberans, S. acuminatus v. biseratus, P. duplex* and *C. microporum.*

This study also revealed that beside the protein content, the survivor and reproduction of animals could be depended on many features of alga utilized as food (e.g. morphology and size). Previously, Burns (1968) demonstrated that at different stages in the life cycle, *D. magna* had different food size limitation. It means that there was a relationship between the maximum size of alga and the body size of organism. This correlation was showed through the formula: y = 22x + 4.87, where x is the body length of *D. magna* (mm) and y is the maximum size of alga, that could be digested by *D. magna* (μm) (Burns, 1968). The average body size of *D. magna* at two distinct stages, offspring (<24 hours old) and adult (10 days old) was measured by using the microscope (Olympus BX 51) coupled with a digital camera (DP71), was 0.79 and 3.20mm (data not showed), respectively. Therefore, the maximum size of algae that could be digested by *D. magna* neonates and adults in our study was 22 and 75μm, respectively. Similarly, the average size of 5 test algae were also measured, *C. microporum* (16.98μm) < *S. acuminatus v. biseratus* (17.04μm) < *S. protuberans* (24.85μm) < *Cyclotella* sp. (37.51μm) < *P. duplex* (108.23μm; data not showed). Although almost the test algae had suitable size cell for digestion of *D. magna* adults (excluding *P. duplex*), only *C. microporum* and *S. acuminatus v. biseratus* were appropriate to use as food for neonates. Therefore, organisms fed by *C. microporum* and *S. acuminatus v. biseratus* had significantly higher survivorship and reproduction than other algae.

Lampert (1977) showed that the smaller the cell size of alga was, the higher the digestive ability of micro-crustacean was. Furthermore, morphology of algae also impacted the digestive ability of micro-crustacean. For example, they could digest effectively the big filamentous algae but it was extremely difficult for them to digest the small algae having stability of colonies. Hence, comparing to the study of Lampert (1977) the results of this investigation were in agreement. *Daphnia* fed on and grew better with the smaller the cell size of algae (C. *microporum* < *S. acuminatus v. biseratus* < *S. protuberans* < *Cyclotella* sp. < *P. duplex*). However, although the cell size of *Cyclotella* sp. was smaller than that of *P. duplex*, organisms fed by these algae still died, that might be explained as that *Cyclotella* sp. (our isolate) is a brackish alga and not be a favourite food of *D. magna*. This assumption needs further investigations to clarify.

5. CONCLUSION

This study indicated that protein content in five distinct algae from Vietnam (*Cyclotella* sp., *S. protuberans, S. acuminatus v. biseratus, P. duplex* and *C. microporum*) was relatively high, ranged from 37% to 48% dry weight. Specially, *C. microporum* and *S. acuminatus v. biseratus* were proven to be potential food sources for aquaculture, livestock and human beings as *D. magna* fed by them had high survivorship and reproductive performance. However, in order to evaluate exactly, further investigations on amino acid proportion and other compounds (including both toxic and nutrient components) from Vietnam microalgae are highly suggested.

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EFFECT OF RICE DISTILLERS' BYPRODUCT AND BIOCHAR AS ADDITIVES WITH A FORAGE-BASED DIET FOR GROWING AND FEED CONVERSION OF NATIVE MOO LATH PIGS

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ABSTRACT

The objective of the research was to evaluate the effect of biochar on the growth rates of native Moo Lath pigs fed a high forage diet, and to compare it with rice distillers' by-product already shown to be effective as a prebiotic in diets of Moo Lath pigs. Native Moo Lath pigs (n=20; initial live weight 15.8±1.3kg) were housed in individual concrete pens (1.2*0.6m). They were fed a basal diet of ensiled banana pseudo stem and ensiled taro foliage supplemented with broken rice and soybean meal. There were four dietary treatments arranged in a completely randomized design (CRD) with 5 replications. The treatments (% in diet DM) were (i) no additive (CTL), (ii) 4% rice distillers' by-product (RDB), (iii) 1% biochar (BIO) and (iv) the combination of RDB and BIO (RDB+BIO). Growth rate tended to be better (P=0.089) and feed conversion was improved (P=0.048) for both additives, fed separately or together, when compared with the control diet. The improvements in weight gain were 20.1 and 22.9% for biochar and RDB added separately and 22.9% for the combined additives. For feed conversion the relative degrees of improvement were 10.6, 12.2 and 9.30%. There were no benefits from combining both additives compared with feeding each one separately. Whilst considerable research needs to be done, the possibility is that biochar and distillers' by-products bind toxins from the feed which are either excreted in the faces or degraded by some organisms in the animal's gut microbiome.

Keywords: *Banana pseudo-stem, native pig, prebiotic, taro foliage.*

1. INTRODUCTION

Rice distillers' by-product (RDB) is the residue derived from yeast fermentation of rice to make rice wine usually at household level. Rural smallholder farmers in Vietnam and Laos have successfully used RDB as a protein sources for pigs (Manh *et al*., 2009; Taysayavong and Preston, 2010; Manivanh *et al*., 2012). Recently it has been hypothesized that RDB fed in small quantities (4% of the diet) also acts as a prebiotic safeguarding cattle from potential toxicity caused by hydrocyanic acid in cassava foliage (Binh *et al*., 2017), increasing N retention in growing pigs (Sivilai and Preston, 2017) and improving growth and feed conversion in pregnant-lactating gilts and in the growth rate of their piglets to weaning (Sivilai *et al*., 2018).

Biochar is the residue from the carbonization of fibrous biomass at high temperatures (500-1000°C) in a downdraft gasifier (Rodriguez and Preston, 2010; Orosco *et al*., 2018) or in an updraft gasifier stove (Philavong *et al*., 2017). It has been extensively researched as a means of sequestering carbon in soils (Lehman, 2007) with associated beneficial effects on crop and plant growth (Lehman and Joseph, 2015; Preston, 2015).

Feeding biochar to animals is a recent development arising from the finding that 1% of biochar in a cassava-based diet enhanced growth rate of cattle (Leng *et al*., 2012a; Sengsouly and Preston, 2016) and reduced the release of methane in an *in vitro* rumen incubation (Leng *et al*., 2012b). The potential of feeding biochar to fish was indicated by the superior growth rates in striped catfish fed 1% biochar in their diet and the associated improvement in water quality in the fish tank (Lan *et al*., 2016).

The objective of the research described in this paper was to evaluate the effect of biochar on the growth rates of indigenous Moo Lath pigs

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fed a high forage diet, and to compare it with rice distillers' by-product already shown to be effective as a prebiotic in diets of Moo Lath pigs (Silivai and Preston, 2017; Sivilai *et al*., 2018).

2. MATERIALS AND METHODS

2.1. Location and duration

The experiment was conducted in the farm of the Department of Livestock and Fishery, Faculty of Agriculture, National University of Laos, Vientiane capital, Lao PDR. The temperature during the experiment ranged from 24 to 35°C. The experiment was carried out for four months from 27 Nov. 2017 to 27 Feb. 2018.

2.2. Experimental design

Twenty native Moo Lath pigs with 15.8±1.3kg initial live weight with 2 months of age were housed in individual concrete pens (1.2*0.6m). They were fed a basal diet of ensiled banana (*Musa* spp**)** pseudo stem and ensiled taro (*Colocasia esculenta***)** foliage supplemented with broken rice and soybean meal. There were four dietary treatments arranged in a completely randomized design with 5 replications. The treatments (% in diet DM) were no additive as control diets (CTL), rice distillers' by-product (RDB) at 4% added in diets, biochar (BIO) at 1% added in diets and combination of RDB and BIO (RDB+BIO) added in the diets.

2.3. Feeding and management

The pigs were vaccinated against hog cholera and de-wormed with Ivermectin (1ml/20kg LW) and adapted to the new feeds for 12 days prior to collection of data. The daily allowances were prepared immediately prior to feeding at 7:30am and 4:30pm; water was freely accessed through nipple tap drinkers in each pen.

Fig 1. The biochar was the residue from rice husks used as fuel in a gasifier stove

The rice distillers' by-product (RDB) was bought from traditional rice wine producers, mostly smallholder farmers, in the area surrounding Vientiane city. It was stored in closed containers to maintain the quality and avoid mould growth.

The biochar was made from rice husk combusted in a gasifier stove (Fig 1). The water retention capacity (volume of water retained per unit weight of dry biochar) was 4.74 which is similar to the value (5.6) reported for biochar produced from rice husks in a down-draft gasifier (Orosco *et al*., 2018).

Taro foliage (leaves+petioles) were collected from ponds in the local village where waste water was stored. The banana pseudo stems were obtained from farmers' gardens after harvesting the fruit. Each foliage was chopped into small particles of 1-2cm and wilted under shade for 24 h prior to being stored in closed plastic bags in which they were preserved for 14 days before being fed to the pigs.

2.4. Data collection and proximate analysis

The pigs were weighed every 14 days during the 90-day experiment. Average daily weight gain was determined from the linear regression of live weight (Y) against days in the experiment (X). Feeds offered and refused were recorded daily. Representative samples were stored at -18°C until the end the experiment when they

were bulked on an individual animal basis for determination of DM, crude protein, crude fiber and ash according to procedures (AOAC, 1990).

2.5. Statistical analysis

The data of DM feed intake, live weight change and feed conversion were analysed by the general linear model option in the ANOVA program of the Minitab software (Minitab, 2016). Source of variation in the model were treatments and error.

$Y_{ij} = \mu + T_i + e_{ij}$, *where:* Y_{ij} *is experimental observation value,* μ *is overall mean,* T_i*: effect of treatments (i = 1-4), and* e_{ij}*: experimental error (random error).*

3. RESULTS

3.1. Composition and proximate analysis of the diets

Approximately 55% of the protein in the diets came from the taro silage with soybean meal accounting for 26%. When RDB was added, it represented about 6% of the total protein supply (Tables 1 and 2). The value of 4.73 for water retention capacity of the biochar (weight of water adsorbed per unit weight of biochar) was less than that (5.60) reported for rice husk carbonized in a continuous-flow downdraft gasifier (Orosco *et al.*, 2018).

Table 1. Proximate composition of diet ingredients

Diets	DM, %	% in DM			OM	pH	WC
		CP	CF	Ash			
Ensiled banana pseudo stem	10.4	3.6	35.0	2.3	93.7	4.5	-
Ensiled taro foliage	12.8	15.2	17.3	2.5	97.5	3.9	-
Broken rice	85.2	7.8	2.8	2.4	97.6	-	-
Soybean meal	86.3	48.6	5.2	7.9	92.1	-	-
Rice distillers' by-product	8.7	23.4	3.6	13.6	96.4	3.6	-
Biochar	93.7	-	-	39.6	60.4	9.35	4.73
Mineral mixture	92.5	-	-	85.3	14.7	-	-

 # Mixture of $CaCO_3$ ***30%, $CaHPO_4$ 30% and NaCl 40%;*** *DM: dry matter; CP: crude protein; CF: crude fiber; organic matter; WC:* ***Water-retention capacity***

Table 2. Composition of experimental diets

Diets	Treatments, as % in DM basis			
	CTL	RDB	BIO	RDB+BIO
Ensiled banana pseudo stem	10.0	10.0	10.0	10.0
Ensiled taro foliage	54.1	50.1	51.1	48.9
Broken rice	20.0	20.0	20.0	20.0
Soybean meal	8.0	8.0	9.8	8.0
Soybean oil	6.4	6.4	6.6	6.6
Rice distillers' by-product	0.0	4.0	0.0	4.0
Biochar	0.0	0.0	1.0	1.0
Mineral mixture	1.5	1.5	1.5	1.5
Calculated chemical composition, as % DM basis				
Dry matter	33.3	33.1	35.4	33.9
Crude protein	14.0	14.4	14.5	14.2
Crude fiber	13.8	13.3	13.4	13.1
Ash	4.0	4.0	4.4	4.4
Organic matter	96.0	96.0	95.6	95.6
pH	4.3	3.8	4.6	4.4
ME, Kcal/Kg	3,002.1	3,012.3	3,005.8	3,001.2

Mixture of $CaCO_3$ 30%, $CaHPO_4$ 30% and NaCl 40%; CTL: control diet, RDB: rice distillers' by-product; BIO: biochar

3.2. Dry matter intake, growth rate and feed conversion

There was no effect of the additives on feed intake (Table 3; Fig 2). Growth rate tended to be better (P=0.089) and feed conversion was improved (P=0.048) for both additives, fed separately or together, when compared with the control diet. The improvements in weight gain were 20.1 and 22.9% for biochar and RDB added separately and 22.9% for the combined additives. For feed conversion the relative degrees of improvement were 10.6, 12.2 and 9.30%. There were no benefits from combining both additives compared with feeding each one separately.

Table 3. Live weight, feed intake and conversion for Moo Lath pigs fed rice distillers' by-product, biochar or both

Live weight	CTL	BIO	RDB	BIO+RDB	SEM	*P*
Initial, kg	15.9	15.8	15.6	16.0	0.619	0.692
Final, kg	36.5	40.5	40.1	41.6	1.52	0.423
Daily gain, g	179	215	220	220	9.51	0.089
DM intake, g/d	787	850	859	874	41.7	0.580
DM conversion (kg/kg)	4.43[b]	3.96[a]	3.89[a]	4.02[a]	0.13	0.048

Mean values without common superscript are different at P<0.05

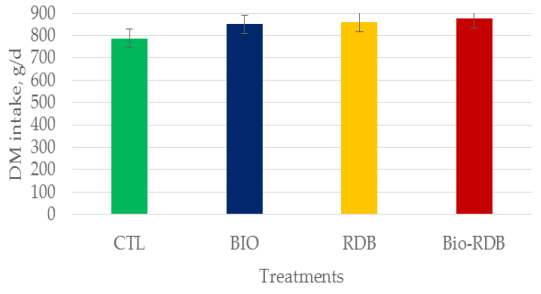

Fig 2. Effect of additives (DM basis) of rice distiller's by-products (4%) and biochar (1%) on DM intake

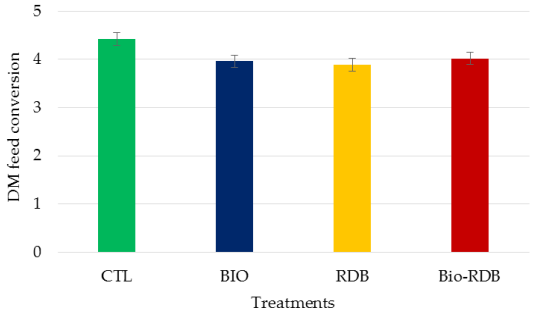

Fig 4. Effect of additives of rice distiller's by-product (4%) and biochar (1%) on DM feed conversion

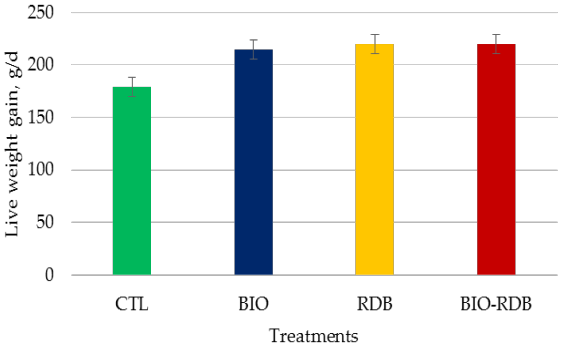

Fig 3. Effect of additives of rice distiller's by-product (4%) and biochar (1%) on live weight gain

4. DISCUSSION

Dietary composition of ensiled taro foliage combined with ensiled banana pseudo stem was used as forages basal diet for Moo lath pigs. It confirmed in previously results that suitable dietary composition was applied with the trial comparing to supplementation of small amount of rice distillers' by-product. The positive effect of rice distillers' by-product on growth rates and feed conversion of the Moo Lath pigs agrees with previous results in our laboratory where: (i) 4% (as DM) of rice distillers by-product improved N retention in growing Moo Lath pigs by 36% and the biological value of the nitrogen by 18% (Sivilai and Preston, 2017): and (ii) 4% (as

DM) of rice distillers by-product increased the litter weight of weaned pigs from Moo Lath gilts by 67% and the overall feed conversion (feed consumed in pregnancy and lactation/weight of weaned piglets) by 64% (Sivilai *et al*., 2018).

To our knowledge, the feeding of biochar to pigs has not previously been reported. The degree of response observed in this experiment with growing pigs (20-23% and 11-14% for growth and feed conversion) is similar to the 15 and 18% improvements in growth and feed conversion reported for biochar fed to cattle (Sengsouly and Preston, 2016) and the 27 and 13% improvements in growth and feed conversion when biochar was fed to goats (Silivong and Preston, 2016).

Distillers' by-products contain the remains of the yeast that produce the alcohol. The cell wall of *S. cerevisiae* contains a scaffold of β-glucans attached to highly glycosylated mannoproteins (Shetty *et al*., 2006) that can bind numerous compounds, microorganisms or provide habitat for biofilm formation. Biochar is relatively inert but also has sites for microorganisms, sorption of chemicals and is known to provide habitat for biofilm formation (Leng, 2017). Therefore, both additives have numerous sites for absorption and binding of compounds and microorganisms and potentially also provide habitat for biofilm attachment.

Biochar and rice distillers' by-product each increase pig growth rate and efficiency of feed utilization but the effects were not additive when they were combined. It is possible that both additives are controlling or preventing reactions that removed whatever caused the lowered production in their absence. It is possible that the feed used in the present study had become contaminated with mould that produces a variety of mycotoxins. The silage component although high in moisture, with a low pH can contain moulds on the herbage before and after harvesting and even in the ensiling process before acidification causes the fermentative process to cease. Leng (2017) hypothesized that given time the rumen microbiome has an enormous capacity to degrade many phytotoxins and mycotoxins provided a specific habitat is available together with the toxin. Similarly, the caecum/colon of the pig may have the same capacity. Whilst considerable research needs to be done, the possibility here is that biochar and distillers' by-products bind toxins from the feed which are either excreted in the faces or degraded by some organisms in the animal's gut microbiome. This concept is supported by the report of Prasai *et al* (2017) that: "supplementation of feed fed to hens with biochar, zeolite or bentonite improved egg yield and feed conversion ratio, with these additives potentially acting as detoxifiers or inhibiting growth of microbial pathogens, slowing digestion or altering the gut anatomy and microbiota to improve feed conversion ratio". Leng (2017) argued a similar case for the detoxification of mimosine and fluoroacetate in ruminants that had never been exposed to these in their feed.

The related issue is the extent to which the soil ameliorating properties of biochar will be observed when the excreta of biochar-fed animals is returned to the soil as fertilizer. This expectation is conditional on the animals being fully integrated in the farming system.

5. CONCLUSIONS

In pigs, growth rate tended to be better and feed conversion was improved for both additives, fed separately or together, when compared with the control diet. The improvements in weight gain were 20.1 and 22.9% for biochar and RDB added separately and 22.9% for the combined additives.

For feed conversion the relative degrees of improvement were 10.6, 12.2 and 9.30%. There were no benefits from combining both additives compared with feeding each one separately.

It is postulated that biochar and distillers' by-product recommended to bind toxins from the feed which are either excreted in the faces or degraded by some organisms in the animal's gut microbiome.

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EFFECT OF EXTRACTED FENUGREEK SUPPLEMENTATION IN DAIRY DIET ON FEED INTAKE, MILK YIELD, METHANE PRODUCTION AND ECONOMIC BENEFIT

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ABSTRACT

The experiment is carried out at Nong Lam University research farm of Ho Chi Minh city, Vietnam to investigate, whether addition of saponin from fenugreek have a long history of human use as foods tuffs, cosmestic ingredients, medicines, detergents and feed additives with effects on feed intake, milk yield and quality of cows and methane production. For this aspect, a fenugreek extract containing some saponins (Norponin Cotyl®) is included into diets either without or added with 25 or 50 g/cow. Fifteen dairy cows (420±50kg BW, 1-6 months post calving, parity of 1-4, with average yield of 14 (12.0-16.0) liters per day, and body condition score 2.5-5.0) are randomly allocated in one of three experimental groups: Control (C), 25 g Norponin Cotyl® (SP25) and 50 g Norponin Cotyl® (SP50), respectively. The trial last 63 days and divided in three periods of change-over design. The feed intake is determined by weighing of feed supply and residual at the beginning and at the end of each day during the respective period. Parameters recorded are feed intake, milk yield as well as milk quality in both control group and treatments groups. That trial shows a highest milk yield on 50g saponin supplemented diet compared with control and 25 g saponin supplementation, but also significant difference for the milk quality by adding Norponin Cotyl® in diets. The positive performance data also resulted in better economic benefits with 106.3% for group SP25 and 110% for group SP50.

Keywords: *Fenugreek extract, saponin, sotolons, methane production, feed conversion, milk yield and quality.*

1. INTRODUCTION

Normally, 95 to 99% of dairy farms in Vietnam can be classified as small-holder farms under mixed crop-livestock farming systems. Crop residues, shrubs, and tree fodders are locally available feed in large amounts and are important in small-holder farms to alleviate shortages of feed and increase the efficiency of the production systems in the tropics (Leng, 1993). Concentrate supplementation for lactating dairy cows has been practiced by many small-holder farmers in the tropics by giving 0.5kg concentrates per 1kg of milk (ratio of 1:2), as a normal rule, without taking into account the nature of the roughage used and the actual nutrient requirements. In some areas of Vietnam, concentrate use was found to be even higher than 1:1 concentrate to milk yield, which could possibly result in rumen acidosis, especially when effective sources of fibre are not available. Although, high level of fed concentrates usually achieve greater levels of milk production, is not often that they have been economically efficient. Feed costs are about 70% of total operating costs, the largest being expenditure on concentrates up to 80% (Duong Nguyen Khang *et al*., 2019; Nguyen Thanh Hai *et al*., 2019) resulting in increasing production costs. Therefore, strategic supplementation of local feed resources at lower ratios of concentrate to milk yield, from 1:2 to 1:3 or lower should be advantageous. There were many reports shown that malt husk produced from beer processing factory could be used as the best by-product in diet for dairy cows.

Saponin is a substrate which is easy to extract from many plants, and therefore is recommended for use as a dietary supplement to improve rumen ecology and rumen fermentations (Budan *et al*., 2012). Some studies

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reported that saponin could increase palatability and feed intake (Kandals *et al*., 1976, Kakani *et al*., 2012). Addition of saponin from fenugreek have a long history of human use (ABMPC, 2011) as foodstuffs, cosmestic ingredients, medicines, detergents and feed additives with effects on feed intake, milk yield and milk quality of cows (Shah and Mir, 2004**).** But there were few studies on how to optimize the feeding of malt husk within supplementation of saponin product in dairy diets for milk production. Therefore, the objectives of this study were to examine the level of saponin supplementation on production of lactating dairy cows and methane mitigation. This fenugreek extract product called Norponin Cotyl ®, expressed as innovative products developed by Nor-Feed could be study.

2. MATERIALS AND METHODS

2.1. Location and duration

This experiment is conducted in Dairy, Research and Technology Transfer farm, Nong Lam university of Ho Chi Minh City, from February to April 2019. During this experiment, the mean daily temperature was 29^0C ($26-32^0$C) and mean relative humidity was 88%.

2.2. Experimental design, diet and treatments

The experimental design is a 5x3 Change over design with 21 days for each period. The 63 days milking was divided in 3 periods, 21 days for each period to account for the cow's changing nutritional requirements, respectively.

The treatments are no supplementation (control) and two levels of Norponin Cotyl® product of Nor-Feed (Table 1), as follows:

Table 1. Experimental design

Treatment	Diet	Dosage	Cows
C	Basal diet	-	5
SP25	Basal diet+Norponin Cotyl®	25g/cow	5
SP50	Basal diet+Norponin Cotyl®	50g/cow	5

C: Control; SP25: 25g of Norponin Cotyl®/head/day; and SP50: 50g of Norponin Cotyl®/head/day

2.3. Animals and housing

Fifteen crossbred dairy cows (more than 75% Holstein-Friesian) in lactations 1-4 and body condition score 2.5-4.5 were used. The cows are in mid-lactation (10-100 days) and with an average live weight of 420±50kg. Cows were housed in individual cages according to a completely randomized block design with five replicates of the treatments, and are fed *ad libitum* malt husk. Mombasa grass (*Panicum maximum)* is fed around 10 kg per day. A supplement of 0.5 kg concentrate (18% CP) per 1 kg milk yield per head per day was offered two times a day during milking. A supplement of 0.5kg palm kernel per head per day was done two times a day during milking. All feedstuffs are mixed with the concentrate before feeding, according to treatment. The concentrate was offered in two equal portions during milking times. The diets were fed for 21 days during each experimental diet 7 days adaptation and last 14 days for data collection. Clean fresh water and mineral blocks were available ad libitum. Cows are milked by bucket milking machine twice a day (7.00h and 17.00h). After milking, the cows are confined together in the barn.

2.4. Samplings and analysis

2.4.1. Weight monitoring

Feed intake and feed chemical composition: All feeds are weighed before feeding and supplied separately to the cows and feed refusals were weighed each morning for calculation of daily feed intake. Feed nutritional composition is analyzed (Table 2).

Milk yield and composition: Daily milk yield, feed intake, feed conversion rate per milk were measured during 63 days, 21 days per each period. Daily milk yield in the morning and in the afternoon of each individual cow was recorded. Milk samples were taken from morning and afternoon milking (morning milk to afternoon milk = 60:40) at the last days of each period. Milk samples were collected from two consecutive a.m. and p.m. milkings of each cow and milk composition was analyzed (fat, protein, lactose, total solids not fat) using Milko scan.

Methane production: At the end of each experiment period, a sample of mixed eructed and respired gas from each animal was analyzed carbon dioxide and methane concentrations

using the Gasmet equipment (GASMET 4030; Gasmet Technologies Oy, Pulttitie 8A, FI-00880 Helsinki, Finland), based on the approach suggested by Madsen *et al*. (2008). The dairy cows were held for few hours in a closed chamber before taking the measurements of carbon dioxide and methane concentrations then calculation the ratio of carbon dioxide and methane.

Economic result: Feed costs per kg milk were calculated using current market prices in VND.

Table 2. Nutritional composition of raw materials used in the experiment (Mean±SE)

Feed stuff	DM, %	% of dry matter			
		CP	EE	CF	Ash
Mombasa grass	23.85 ± 0.13	13.55 ± 2.42	2.55 ± 0.11	17.32 ± 0.14	8.51 ± 0.09
Malt husk	91.82 ± 0.04	17.55 ± 0.19	5.64 ± 0.24	11.82 ± 0.32	12.72 ± 0.02
Palm kernel	91.24 ± 0.32	16.25 ± 0.51	6.84 ± 0.02	4.22 ± 0.05	6.42 ± 0.09
Cassava pulp	28.78 ± 0.23	4.02 ± 0.58	1.25 ± 0.23	10.72 ± 0.27	5.26 ± 0.10
Concentrate	90.25 ± 0.03	17.04 ± 1.23	9.72 ± 0.39	6.65 ± 0.06	9.82 ± 1.32

3.4.2. Statistical analysis

All data were subjected to Analysis of variance (ANOVA) according to a Change over design using the General Linear Models (GLM) of the Minitab for Windows (Minitab 16.0, 2018). The Tukey Test for paired comparisons is used to separate means when the differences are significant at the 5% level ($P<0.05$). The statistical model is: $Y_{ijk} = m + T_i + C_j + R_k + e_{ijk}$.

Where, Y_{ijk} is the criteria under study, in treatment i, column j, row k; m is over all sample mean; T_i is effect of treatment i, C_j is effect of column j; R_k is effect row k and e_{ijk} is error

3. RESULTS

3.1. Feed intake and milk yield

Feed intake and milk yield in experimental period is shown in Table 3.

Table 3. Milk yield and feed intake of experimental period of 63 days

Parameter	Control	SP25	SP50	SEM	P
Number of animals, n	5	5	5		
Daily feed intake, kg/cow/day	17.02^{b}	18.15^{ab}	18.23^{a}	0.20	0.001
Relative to control on intake	100	106.64	107.11	-	-
Daily milk yield, kg	14.19^{b}	15.35^{ab}	15.58^{a}	0.24	0.001
Relative to control on milk yield	100	108.17	109.80	-	-
Feed conversion rate for milk	1.23	1.21	1.24	0.02	0.574
Relative to control on FCR for milk	100	98.37	100.81	-	-

When the mean temperature is high in the dry season, daily feed intake with 25 and 50g Norponin Cotyl® supplemented diets is significantly higher than control by 6.64% and 7.11% (Table 3, Fig 1). Dietary nutrient concentrations in three treatments are enough high, the protein and energy intakes met recommended daily intakes for the lactating period (McDonald *et al*., 1995).

Mean daily milk yields (Table 3, Fig 2) is significantly different ($P<0.001$, R^2 _ 0.87) among treatments. Norponin Cotyl® diets improves milk yield with 8.17% and 9.80% compared with control treatment, probably because Norponin Cotyl® sotolons and saponins enhance palatability which helps to increase feed intake and nutritive digestibility (Begum *et al*, 2016), but also phyto-oestrogens (Diosgenin and Isoflavons) can lead to increase prolactin level (Tabares *et al*., 2014) and by the way increase milk quantity performances on dairy cows.

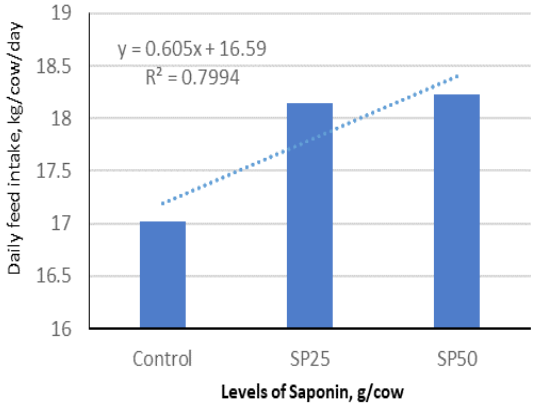

Fig 1. Effect of SP on feed intake of dairy cows fed cassava pulp and malt husk as basal diet

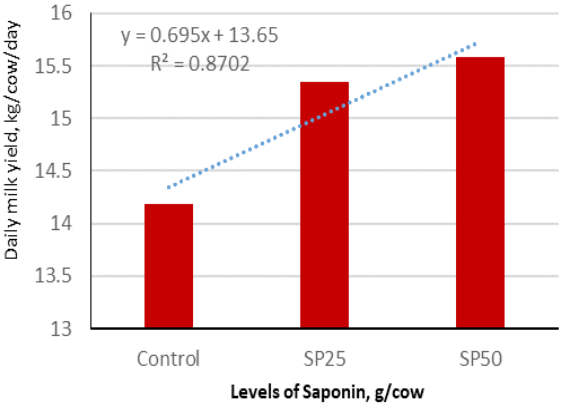

Fig 2. Effect of SP on milk yield of dairy cows fed cassava pulp and malt husk as basal diet

Mean feed conversion rate (Table 3) were similar among treatments (P=0.574). The higher feed conversion rate is with the supplementation 0.50 g/cow/day of Norponin Cotyl® with 0.81% compared with control treatment.

Concentrate feed with the supplementation of Norponin Cotyl® significantly shows the highest values of feed intake, milk yield as well as FCR, compared to other feeding groups (Table 3). FCR in the group SP25 is lower than the FCR in the group SP50. The reason for the lower FCR in the group SP25 is due to the obvious similar to feed intake as compared to that in the group SP50 with 18.15 kg/cow/day and 18.23 kg/cow/day, respectively. However, only a lightly increase of milk yield is observed in these groups (15.58 kg/cow/day for group SP50 and 15.35 kg/cow/day for group SP25).

3.2. Milk quality

Mean solid non-fat content (Table 4, Fig 3) is significantly different (P=0.006, R^2=0.99) and fat contents (Table 4, Fig 4) is different (P=0.036, R^2 = 0.84) among treatments, probably because Norponin Cotyl® sotolons and saponins enhance palatability which helps to increase feed intake, improve nutritive digestibility and milk quality (Shar and Mir, 2004).

Table 4. Effect of Norponin cotyl on milk quality

Parameters	Norponin cotyl, g/head/day			SEM	P
	0	25	50		
Solid non fat, %	8.73^{b}	8.87^{ab}	9.03^{a}	0.048	0.006
Protein, %	3.39^{b}	3.45^{ab}	3.50^{a}	0.027	0.049
Fat, %	3.46^{b}	3.52^{ab}	3.93^{a}	0.011	0.036
Lactose, %	4.69^{b}	4.76^{ab}	4.85^{a}	0.025	0.006

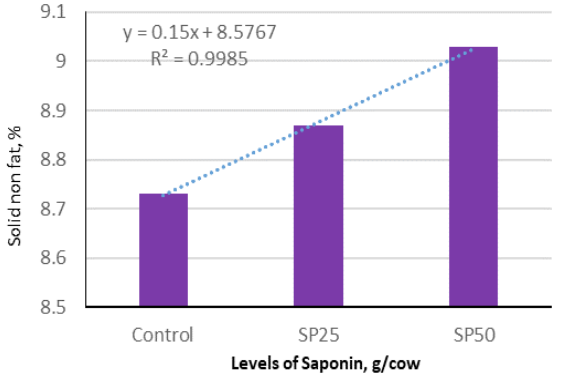

Fig 3. Effect of SP on solid non-fat content of dairy cows fed cassava pulp and malt husk as basal diet

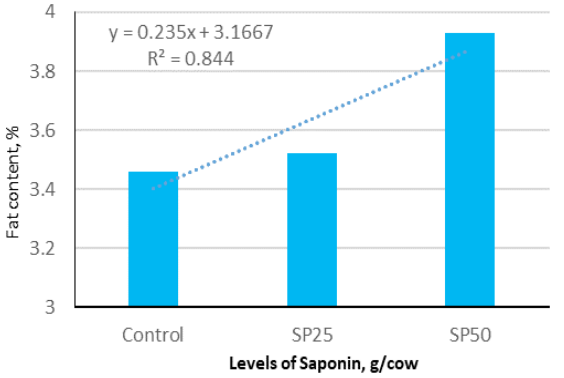

Fig 4. Effect of SP on fat content of dairy cows fed cassava pulp and malt husk as basal diet

The ratio of methane to carbon dioxide in eructed gas was reduced by feeding increasing levels of Norponin Cotyl® (Table 5). There are two possible explanations for the reduction in methane with increasing levels of Norponin cotyl in the diet. The explanation is that the increasing levels of saponin content in the ration would have a direct effect in reducing rumen methanogenisis decreasing rumen protozoa as reported in an earlier paper show that *Enterolobium ciclocarpum* leaves associated with the *Enterolobium ciclocarpum* saponins had an impact on rumen methanogenisis (Duong Nguyen Khang and Bui Xuan An, 1994; Rodriguez and Fondevila, 2012). Replacing the Norponin cotyl led to an overall decrease in the rumen protozoa number and this was directly related to the methane:carbon dioxide ratio. The other explanation is that a similar relationship between methane production and solubility of the dietary protein was reported by Silivong and Preston (2016); Nguyen Thanh Duy *et al*. (2016); Nguyen Thanh Duy and Duong Nguyen Khang (2016) and was attributed to the shift in metabolic disposal of hydrogen from methane to acetate when the balance of fermentative digestion was changed as in the case of feeds escaping from the rumen to be fermented in the cecum-colon (Leng, 2014).

3.4. Economic performances

Economic performances of Norponin Cotyl® supplementation is shown in table 6. Net economic benefit is higher when Norponin Cotyl® is added. The both groups SP25 and SP50 showed the higher economic income which resulted in the better income over feed cost with the benefit of 108.89% and 111.97% compared to control group, respectively. Milk quality economic improvement is not calculated in these studies because dairies in Vietnam do not pay any extra value for this parameter.

Table 6. Effect of Norponin Cotyl on economic benefit

Items	Control	SP25	SP50	SEM	P
Total milk, kg	14.19^{b}	15.35^{ab}	15.58^{a}	0.24	0.001
Relative to control	100	108.17	109.80	-	-
Milk sell per day (14,000 VND per kg milk)	228,022	246,406	252,136	3,964	0.001
Relative to control	100	108.06	110.58	-	-
Feed and Norponin Cotyl cost per day (VND)	71,476	75,947	76,849	935	0.001
Income per day (VND)	156,546	170,459	175,287	-	-
Relative to control	100	108.89	111.97	-	-

4. CONCLUSION

Norponin Cotyl® can be use for improving the milk yield and quality of cows, and reducing Methane production, resulting in higher economic benefit and can thus be recommended for milk producers.

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EFFECTS OF WATER SPINACH AND BIOCHAR ON DIGESTIBILITY, N-RETENTION, RUMEN CHARACTERISTICS AND GROWTH PERFORMANCE OF GOAT FED *BAUHINIA ACUMINATA* AND MOLASSES AS THE BASAL DIET

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ABSTRACT

A study was arranged in a 2*2 factorial with 4 replications to determine the effects of water spinach and biochar on enteric methane emissions, feed intake, digestibility, nitrogen retention and growth performance in local goats fed *Bauhinia acuminata* and molasses as basal diet. Two experimental factors were (i) with or without water spinach as well as (ii) with or without biochar. Therefore, sixteen goats with an initial body live-weight 11.65±3.95kg at 5-6 months of age were set up in individual pens. The live weight was recorded at the beginning and at the end of the experiment and at intervals of 10 days during the experiment (9 periods) before feeding in the morning. Representative samples of each component were analyzed for DM, N and ash. Digestibility and N retention were recorded at four time points of after 20, 40, 60 and 80 days. To measure eructed gases on the last day of the experiment, goats were placed in a plastic-covered cage for 10 minutes. As results, daily live weight gain, feed conversion, DM digestibility and N retention were improved by feeding water spinach and by supplying with biochar ($P<0.05$). The higher value of rumen ammonia in goats fed water spinach reflected the greater solubility of the crude protein in the water spinach. Supplementation of water spinach led to a reduction in the methane/carbon dioxide ratio in the eructed breath of the goats but this criterion was not affected by biochar ($P=0.641$)

Keywords: *Carbon dioxide, climate change, methane, N-balance, protein solubility, rumen ammonia.*

1. INTRODUCTION

Livestock are the most important source of protein food and family cash income of farmers in Laos, and also give manure for cropping in the rural areas. Most of the production from livestock such as goats, cattle, pigs and poultry comes from smallholders using traditional management systems. The main feed resources for ruminants are native grasses, legumes and tree leaves that are available in the natural grassland and forests (Phengsavanh, 2003). The conventional feeding system for goats in Lao PDR is based mainly on the use of natural grasses. However, in the dry season, natural pasture decreases in nutritive value and improved grasses cannot grow. Therefore, it is important to find an alternative feeding system because purchased supplements are too expensive for poor farmers. On the other hand, there are many trees and shrubs available. Some authors emphasized that in tropical countries one of the most appropriate ways to improve feed supplies for ruminants is through utilization of tree and shrub foliages (Leng, 1997; Preston and Leng, 2009). Actually, water spinach (*Ipomoea aquatica*) supplementation of low quality tree foliage (from Fig, Jujube and Mango trees) increased the DM and crude protein intake of goats, and improved the apparent digestibility and N retention (Daovy *et al*., 2007). Additionally, the live weight gain of goats fed Mimosa foliage was increased by supplementing with fresh water spinach at 27% of the total DM intake (Thu Hong *et al*., 2011) while goats fed a sole diet of cassava foliage also responded with increased DM digestibility and N retention when fresh water spinach was provided as a supplement (Pathoummalangsy and Preston, 2006). The legume tree *Bauhinia acuminata* is widely distributed in the LuangPrabang province

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and it has been observed that the foliage is readily consumed by goats. As is the case with most foliages from legume trees, it contains many secondary plant compounds including tannins (Queiroz *et al*., 2012). It was therefore hypothesized that the growth performance of goats fed *Bauhinia acuminata* foliage as the basal diet would be improved by supplementing it with water spinach.

The negative feature of livestock is that they contribute some 18% of the greenhouse gases that are causing global warming (Steinfeld *et al*., 2006). Enteric methane from fermentative digestion is the main source of these emissions. Thus when new or modified feeding systems are being researched the effects of these changes on enteric methane emissions should be monitored, in view of the need to reduce methane emissions so as to meet future targets for mitigating global warming.

A positive approach to the problem of how to reduce methane emissions from live stock has been to incorporate a low level (1%) of biochar in the diet (Sangkhom *et al*., 2012; Leng *et al*., 2012a, b, c). Biochar is the product of incomplete carbonization of fibrous biomass at high temperatures (Lehmann and Joseph, 2009). It is a highly porous material which gives it valuable properties as a support mechanism for biofilms that may facilitate the adsorption of consortia of micro-organisms and nutrients (Leng, 2014).

In the research reported here, it was hypothesized that: (i) the performance of growing goats fed *Bauhinia acuminata* as the basal diet would be improved by supplementation with water spinach as a rapidly fermentable protein source; and (ii) that incorporation of a low level of biochar in the diet might reduce enteric methane emissions.

2. MATERIALS AND METHODS

2.1. Location of the study area

The experiment was conducted in the Animal Science Farm and Laboratory of the Faculty of Agriculture and Forest Resource, Souphanouvong University, LuangPrabang province, Lao PDR. The site is located 7.5km from LuangPrabang town to the South-west, at an altitude of 385m above sea level.

2.2. Treatments and experimental design

The experimental plan was a 2*2 factorial arrangement in a Randomized Completely Block Design (RCBD) with 4 treatments and four replications. The factors applied to a basal diet of fresh Bauhinia foliage were (i) with or without water spinach (WS and No-WS) and (ii) with or without biochar (BC and No-BC). Individual treatments were (i) BA = *Bauhinia acuminata* ad libitum; (ii) BABC = *Bauhinia acuminata* ad libitum + 1% biochar on DM intake; (iii) BAWS = 70% *Bauhinia acuminata* and 30% water spinach on DM basis; and (iv) BAWSBC = 70% *Bauhinia acuminata* and 30% water spinach + 1% biochar.

2.3. Animals and management

Sixteen local weaned goats with initial average body weight of 11.65±3.95kg and 5-6 months of age were used. They included 4 males (non-castrated) and 12 females. These animals were purchased from Phoukhoun District LaungPrabang Province. They were housed individual pens and made from local material such as: bamboo (dimensions of width 1 m, length 1 m and height 0.9 m) and designed to collect separately feces and urine. They were vaccinated against Pasteurellosis, foot and mouth disease and treated with Ivermectin (1ml/20 kg live weight) to control internal and external parasites. They were adapted to the pens and the feeds for 7 days before starting the experiment. The experiment lasted 97 days, including the adaptation period.

2.5. Feed and management

Molasses diluted with fresh water by ratio of 1:9 (1kg of molasses and 9l of fresh water) was used as the carrier for the biochar and was given *ad libitum* on all diets. Foliages of *Bauhinia acuminata* and water spinach were collected daily from natural stands in the University campus. They were hung in bunches above the feed trough. Fresh feeds were offered twice daily at 07:30 and 16:00h.

2.6. Data collection

Live weight was recorded in the morning before feeding at the beginning and at the end of the experiment and at intervals of 10 days during the experiment. Quantities of feed offered and refused were recorded daily. Every 10 days, samples were taken for analysis of DM and N. Samples of *Bauhinia acuminata* foliage offered and residues were separated into stem and leaves (containing attached petioles). Representative samples of each component were analyzed for DM, N and ash.

Samples of rumen fluid were taken by stomach tube 2h after morning feeding on the last day of the experiment. The pH value was measured immediately with a portable digital pH meter. A drop of concentrated sulphuric acid was added prior to determination of ammonia by steam distillation. Digestibility and N retention were recorded four times, over 5 days periods at 20 day intervals (after 20, 40, 60 and 80 days). In each collection period, samples of feeds offered and refused were taken daily and bulked for the 5 days of each period. Urine was collected in buckets containing 20ml of a solution of sulphuric acid (10% sulphuric acid concentrate + 90% distilled water). Feces were collected daily and stored in the refrigerator at 4-8ºC and at the end of each period. Sub-samples were mixed together and ground with a coffee grinder.

Samples of eructed gases were measured on the last day of the experiment, in the morning 2h after feeding. The goats were placed in a plastic-covered cage and after a period of 10 minutes for equilibration with the surrounding air, the concentrations of methane and carbon dioxide were recorded over a 10 minute period, using a GASMET 4030 meter (Gasmet Technologies Oy, Pulttitie 8A, FI-00880 Helsinki, Finland).

2.7. Chemical analyses

The sub-samples of feces and of feeds offered and refused were analysed for DM, N and ash according to AOAC (1990) methods. Urine was analysed for nitrogen (AOAC 1990). Solubility of the protein in the leaves was determined by shaking 3g of dry leaf meal in 100 ml of M NaCl for 3h then filtering through Whatman No.4 filter paper, and determining the N content of the filtrate (Whitelaw *et al*., 1963).

2.8. Statistical analyses

The data were statistically analyzed as a Randomize Complete Block Design (RCBD) by variance analysis (ANOVA) using the general linear model (GLM) procedure of Minitab software version 16.0 (Minitab, 2014). The treatment least square means showing significant at difference at the probability level of $P<0.05$ were compared Turkey's pair wise comparison procedure.

The model for growth study was: $Y_{ijk} = \mu + B_k + P_i + A_j + P_i*A_j + e_{ijk}$. *Where, Y_{ijk} is dependent variables;* μ is overall mean; B_k *is block live weight effect, P_i is effect of with or without water spinach supplementation; A_j is effect of with or without biochar supplementation; P_i*A_j is effect of interaction between with or without water spinach * with or without biochar; and e_{ijk} is random error*.

The model for digestibility study was: $Y_{ijk} = \mu + T_i + P_j + A_k + e_{ijk.}$ *Where, Y_{ijk} is dependent variables; μ is overall mean; T_i is effect of treatments (i=1-4); P_j is effect of columns (j=1-4); A_k is effect of rows (k=1-4) and e_{ijk} is random error*.

The relationship between N intake and N retention was developed by regression analyses. The best model was selected based on adjusted R^2 while the methane to carbon dioxide ratios were used to calculate the reduction of methane production according to the formula proposed by Madsen *et al*., 2010: Ratio CH_4/CO_2 = (a-b)/(c-d) (Where: "a" is methane concentration in mixed eructed gas plus air; "b" is methane in the air in the goat shed; "c" is carbon dioxide concentration in mixed eructed gas plus air; and "d" the carbon dioxide in goat shed air).

3. RESULTS AND DISCUSSION

3.1. Chemical composition

The concentrations of crude protein (CP) and ash and the solubility of the protein were lower, and of DM were higher, in *Bauhinia acuminata* than in water spinach (Table 1).

Table 1. Chemical composition of dietary ingredients (% in DM, except DM which is on fresh basis)

Items	DM	N*6.25	Ash	Protein solubility, %	Tannin, %	NDF	ADF
Bauhinia leaves	40.0	15.0	21.2	23.7	1.1	43.7	32.4
Bauhinia stem	38.1	12.3	4.29	-		42.7	31.5
Water spinach	8.16	18.3	9.74	69.4		42.3	33.3
Molasses	80.4	5.4	10.5				
Biochar	-	-	38.3	-		-	-

3.2. Feed intake, growth rate and feed conversion

DM intake expressed as a percentage of live weight was not affected by supplementation with biochar or water spinach (Table 2).

Daily live weight gain and feed conversion were improved by feeding water spinach and by supplementation with biochar (Table 3). There was a close relationship between live weight gain and feed conversion ratio (Fig 1).

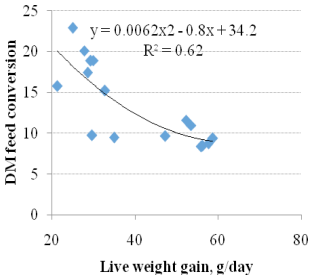

Fig 1. Relationship between live weight gain and feed conversion in goats fed Bauhinia acuminata foliage and molasses supplemented or not with water spinach and biochar

Table 2. Mean values of feed intake by goats fed Bauhinia acuminata supplemented with water spinach (WS) **or biochar (BC) or not supplemented**

Items	PS		P	BCS		P	SEM	Interaction	
	WS	No-WS		BC	No-BC			SEM	P
Molasses	213	224	0.004	218	219	0.67	2.52	14.4	0.369
Bauhinia	149	229	<0.001	164	214	<0.001	3.07	35.69	0.719
Water spinach	161	0	<0.001	82.0	79.2	0.025	0.87	6.25	0.683
Biochar	2.47	2.85	<0.001	5.31	0	<0.001	0.03	0.4 1	0.371
Total	526	455	<0.001	469	512	<0.001	3.77	47.67	0.558
Per kg LW	32.9	33.1	0.21	32.8	33.2	0.016	0.13	0.6	0.923
N*6.25, % in DM	12.5	10.3		11.5	11.5				

BC: biochar, BCS: biochar source, d: day, g: gram, Kg: kilogram, LW: live weight, P: probability value, PS: protein source, SEM: standard error of the mean with df_{error}: 9, WS: water spinach.

Table 3. Mean values for live weight, live weight change, feed DM intake and DM feed conversion for goats fed a basal diet of Bauhinia acuminata foliage and molasses

Items	PS		P	BCS		P	SEM	Interaction	
	WS	No-WS		BC	No-BC			SEM	P
In wt, kg	12.9	12.2	0.598	11.8	13.3	0.263	0.87	1.22	0.571
Fin wt, kg	18.6	15.3	0.019	16.5	17.4	0.495	0.87	1.22	0.382
LWG, g/d	51.4	28.7	<0.001	43.7	36.5	0.047	2.32	3.28	0.543
DMI, g/d	526	455	<0.001	469	512	<0.001	3.77	47.67	0.558
FCR, g/g	10.7	16.2	0.014	11.4	15.5	0.055	1.34	1.90	0.580

BC: biochar, BCS: biochar source, DMI: DM intake, FCR: DM feed conversion, Fin wt: final weight, In wt: initial weight, Kg: kilogram, LWG: live weight gain, P: probability value, PS: protein source, SEM: standard error of the mean with df_{error}: 9, WS: water spinach.

3.3. Apparent digestibility and N retention

Supplementing the basal diet of *Bauhinia acuminate* and molasses with water spinach increased the apparent digestibility of DM, OM and crude protein, but there were no differences due to biochar (Table 4). Daily N retention, N retention as percent of N intake and of N digested were all improved by supplementation with water spinach. There was a tendency for biochar to improve daily N retention (*P*=0.082) and this effect was significant when N retention was expressed as a percent of N intake or of N digested.

Table 4. Mean values of apparent digestibility and N balance in goats fed *Bauhinia acuminata* and molasses supplemented with water spinach (WS) and biochar (BC) or not supplemented (No-WS; No-BC)

Items		PS		P	BCS		P	SEM	Interaction	
		WS	No-WS		BC	No-BC			SEM	P
Apparent digestibility, %	DM	72.0	64.5	<0.001	69.1	67.5	0.100	0.68	1.68	0843
	OM	75.0	67.4	<0.001	71.5	70.9	0.579	0.74	2.10	0.591
	N*6.25	69.2	43.8	<0.001	55.0	57.9	0.294	1.98	6.48	0.502
N balance, g/day	Intake	11.1	7.0	<0.001	8.8	9.3	0.296	0.32	1.04	0.502
	Feces	3.2	2.3	<0.001	2.5	3.0	0.021	0.16	0.53	0.583
	Urine	1.8	1.4	<0.001	1.4	1.8	0.007	0.09	0.29	0.371
	Retention	6.1	3.4	<0.001	4.9	4.5	0.082	0.16	0.41	0.719
N retention as	% N intake	55.0	50.0	0.008	56.2	48.8	<0.001	1.32	3.73	0.324
	%N digested	76.8	72.1	0.009	77.1	71.7	0.003	1.25	3.76	0.440

BC: biochar, BCS: biochar source, DM: dry matter, Kg: kilogram, N: nitrogen, OM: organic matter, P: probability value, PS: protein source, SEM: standard error of the mean with df_{error}: 9, WS: water spinach

3.4. Rumen ammonia, pH and methane to carbon dioxide ratio

Rumen pH did not differ among the diets (Table 5). Rumen ammonia, which was high on all diets, was increased by supplementation with water spinach but was not affected by biochar.

Table 5. Mean values of rumen pH and ammonia, and ratio of methane to carbon dioxide in eructed breath of goats fed *Bauhinia acuminata* and molasses supplemented with water spinach (WS) and biochar (BC) or not supplemented (No-WS; No-BC)

Items	PS		P	BCS		P	SEM	Interaction	
	WS	No-WS		BC	No-BC			SEM	P
Rumen pH	7.08	7.03	0.465	7.04	7.06	0.756	0.05	0.063	0.727
NH_3, mg/liter	397	298	<0.001	347	347	0.999	9.70	13.71	0.935
CH_4:CO_2	0.0211	0.0292	0.043	0.0243	0.0260	0.641	0.003	0.0036	0.800

BC: biochar,BCS: biochar source, CH_4: methane, CO_2: carbon dioxide, NH_3: ammonia, pH: percentage of hydrogen ion, P: probability value, PS: protein source, SEM: standard error of the mean with df_{error}: 9, WS: water spinach.

The ratio of methane to carbon dioxide in eructed breath of the goats was lower in the eructed breath of the goats supplemented with water spinach and not affected by supplementation with biochar.

The results of this experiment agree with the findings of: (i) Kongmanila *et al*. (2011) that supplementation with water spinach increased the digestibility of Mango foliage growing goats; and (ii) Phongpanith *et al*. (2013) who reported that water spinach supplementation increased the digestibility and N retention of goats fed Muntingia foliage. Presumably the high solubility of the protein in water spinach

furnished amino acids and peptides required by micro-organisms for efficient rumen digestion, and that these were limited in the *Bauhina acuminata* foliage because of its low protein solubility.

In contrast with the report by Leng *et al*. (2014) that biochar reduced methane emission from cattle fed urea-treated rice straw, in the present experiment with goats fed tree foliage there was no effect of biochar on methane emissions. However, in agreement with these authors, the biochar had a positive effect on growth performance.

4. CONCLUSIONS

Goats fed *Bauhinia acuminata* foliage and molasses, supplemented with water spinach, grew faster and had better DM feed conversion, and higher apparent digestibility of DM and crude protein, and higher N retention, than goats fed only *Bauhinia acuminata* foliage and molasses.

The higher value of rumen ammonia in goats fed water spinach reflected the greater solubility of the crude protein in the water spinach.

Supplementing the basal diet of *Bauhinia acuminata* and molasses with water spinach led to a reduction in the methane/carbon dioxide ratio in the eructed breath of the goats.

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IDENTIFY OF ANIMAL NUTRIENTS REQUIREMENT IN FEED FORMULATION TO FIT WITH DIVERSIFIED ENVIRONMENTAL AND REARING CONDITIONS

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ABSTRACT

Livestock production has been obtained rapidly progressiveness for recent times. To gain that successfulness, in area of animal nutrition, several studies have been conducted to found "new" requirement of nutrients need to be concerned to enhance performances and health of the food animals. On the other hand, development of the world in terms of population and social development has also born much more concerned issues such as climate change, greenhouse effect, animal welfare, or antibiotics banned in livestock feed. From the point of views of animal/feed producers, to deal with all or almost those above concerns, it needs to understand and needs to add more new appropriate criteria into set of animal nutrients requirement as solving the feed formulation problem. The more comprehensive and appropriate nutrients requirement is set into a kind of feed produced, the much more benefits of that feed could bring to animal, farm owner and also, the community involved.

Keywords: *Animal nutrients requirement; feed quality.*

1. INTRODUCTION

It could be said that the history of feed industry has been developed for more than 200 years with started operation of water-powered grist mills (Bursiek *et al*., 2005). In manner of documentation, in 1944, the National Research Council (NRC) of United States firstly developed a series of feeding standards for livestock and poultry that are updated routinely and are the recognized benchmarks for feed formulation today (Bursiek *et al*., 2005).

In first period of the developing history, those nutrient requirements were considered quite simply in terms of number of nutrients as well as of quantitative identification of those nutrients. It could be seen for example, as in the document titled "Nutrient Requirements of Poultry" published by NRC in 1960 or "Swine: Standards and Guildlines for the Breeding, Cares and Management of Laboratory Animals" in 1971 which are still available in internet website (www.nap.edu) till to now (annex 1).

Huge achievements in nutrition science has been developed for years of 20th century and nowadays has educated and forced scientists and practicers to concern on much, much more of animal requirements of nutrients to let their livestock could accomplish of a great performance in effort of supplying enough food for an increasing human population. In addition, the development of a civilized social is also a pressure on livestock producers. Feed industry has to be practiced in a way that could both satisfy animal high nutrient requirements and keep safe, clean environment and supply to consumers absolute safe food for their health and their palatability.

2. GENERAL TARGETS OF A FEED FORMULA

Before talking on what are inside a feed formula, it needs to identify what that formula of feed used for. Depends on targets of animal production or another way, depends on the wish of livestock producer, the feed produced need to fulfill those targets. In this paper, it is just focus on food animal's production but not on companion animals.

To food animals or sometimes called agricultural animals or just livestock, main targets aimed to be gained as using a specify kind of feed are: highest production rate based on live weight gained or egg or milk production plus an efficiency feed conversion ratio (FCR); and the health status of the stocks and also price of feed needed to be very reasonable to animal producers.

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By those above wished, the feed producers have to set a feed formula with so enough parameters (specification or nutrients standard) that make the feed product could satisfy not only animal's nutrient requirements but also, quite important, to satisfy the owner of animal stock.

3. COMMON PARAMETERS IN A DIET SPECIFICATION

To satisfy the need of animal as well as of animal producer, a "good" feed formula needs to be set of appropriate standards. In general, those standards could be listed as in Table 2.

Table 2. Categorized nutrients in a set of standards of feed formula

Group	Nutrients	Purpose
Main functions(1)	Energy, crude protein, amino acid, lipid, macro and micro minerals	To let animals take necessary materials to build its body
Nutritive value (2)	Fiber, starch	Implement of nutritive value of the feed, especially on digestion and energy value of feed
Metabolism(3)	vitamins, macro and micro minerals	
Feed additives(4)		Enhances of digestive value of feed to animal; health status of animal; physical qualities of the feed in form of mash/pelleted/extruded; Satisfy wish of farm owner

Look at more details on those groups of standard; it could see a lot of changes have been occurred for just last more than 50 years.

To make a comparison, look at the Table 1 (next page) in "Nutrient Requirements of Poultry" published by NRC in year 1964.

This Table 1 did not show or mention about energy requirement even under form of gross energy (GE); it also just suggested level of crude protein for different type of chicken but not any about amino acid; and then showed recommendations of vitamins and minerals. Of course, phosphorus was listed in total value but not available phosphorus as applying at our present times.

Meanwhile, during recent 20 years, thanks to discovers and achievements of science, even a college student who majors in animal nutrition could now know a lot about those changed ones.

Table 1. Nutrient Requirement of chicken[1] (In percentage of Amount per Pound of Feed)

	Starting chickens 0-8 weeks	Growing chickens 8-18 weeks	Laying hens	Breeding hens
Total protein, per cent	20	16	15	15
Vitamins				
Vitamin A activity (U.S.P. Units)[2]	1200	1200	2000	2000
Vitamin D (I.C.U.)[3]	90	90	225	225
Vitamin E	See text			
Vitamin K_1, mg	0.24	?	?	?
Thiamine, mg	0.8	?	?	?
Riboflavin, mg	1.3	0.8	1.0	1.7
Pantothenic acid, mg	4.2	4.2	2.1	4.2
Niacin, mg	12	5.0	?	?
Pyridoxine, mg	1.3	?	1.3	1.3
Biotin, mg	0.04	?	?	?
Choline, mg	600	?	?	?
Folacin, mf	0.25	?	0.11	0.16
Vitamin B12, mg	0.004	?	?	0.002
Minerals				
Calcium, percent	1.0	1.0	2.25	2.25
Phosphorus, per cent[6]	0.6	0.6	0.6	0.6
Sodium, per cent[7]	0.15	0.15	0.15	0.15

	Starting chickens 0-8 weeks	Growing chickens 8-18 weeks	Laying hens	Breeding hens
Potassium, per cent	0.2	0.16	?	?
Manganese, mg	15	?	?	15
Iodine, mg	0.5	0.2	0.2	0.5
Magnesium, mg	220	?	?	?
Iron, mg	9.0	?	?	?
Copper, mg	0.9	?	?	?
Zinc, mg	20	?	?	?

Notes:
1. These figures are estimates of requirements and include no margins of safety. Underlined figure are tentative: See text, page 2
2. May be vitamin A or pro-vitamin A
3. See text, page 3
4. See text, page 4
5. This amount of calcium need not be incorporated in the mixed feed, in as much as calcium supplements fed free choice are considered as part of the ration
6. At least 0.45% of the total feed of starting chickens should be inorganic phosphorus. All of the phosphorus of non-plant feed ingredients is considered to be organic. Approximately 30% of the phosphorus of plant products is non-phytin phosphorus and may be considered as part of the inorganic phosphorus required. A portion of the phosphorus requirement of growing chickens and laying and breeding hens must also be supplied in inorganic form. For birds in these categories the requirement for inorganic phosphorus is lower and not as well defined as for starting chickens.
7. Equivalent to 0.37 per cent of sodium chloride.

- Energy requirement of swine now commonly described by net energy (NE) while the one of poultry was metabolisable energy (ME);

- Crude protein now is just a parameter for reference. It should concern about not only total amino acids but also digestive amino acids value in either Standard Ileal Digestibility (SID) to swine or Total Digestibility (TD) to poultry. In addition, not only four common amino acids (lysine, methionine + cysteine, threonine, tryptophan) are considered as practiced before 10 years ago but most of ten essential amino acids (lysine, methionine + cysteine, threonine, tryptophan, valin, leucin, isoleucine, arginine, histidin, and phenylalanine) are now listed in specification of feed for both swine and chicken. From those ones, a new concept related to protein has been described as **ideal protein** to let people could produce a feed formula which is much more balance of amino acids for animal and could reduce waste nitrogen excreted to the environment.

- Lipid in terms of crude lipid (fat or oil) now is still listed in a formula but it commonly add at least another parameter related as linoleic acids and/or other essential fatty acids that need for animal and human health.

- Similar to fiber, it is now considered not only crude fiber but also neutral detergent fiber (NDF), acid detergent fiber (ADF) then fermented fiber or non-starch polysaccharides (NSP). Including of those concepts is application of fiber (NSP) enzyme to enhance digestion of animals hence, again implement on nutritive value of the feed formula.

- For each single of vitamin or mineral, it seems not much change in total number of vitamin and mineral that have been discovered useful for animal. However, in detail they have known more deeply in function and/or nature characteristics of some specific minerals such as phosphorus with new concept of available phosphorus and now digestive phosphorus. With sodium, chloride, and potassium, now they know that those minerals work not only as independent element in the body, but they combine together to form a so-called electrolytes balance that affect much on physiology of several internal system in the body. Therefore, a quite new parameter called electrolytes balance or dissolve electrolytes balance (dEB) has been already added into feed formula since few decades.

In fact, scientists have discovered useful

function of some other minerals compare to those listed in Table 1 hence, they added requirement of selenium and sometime also of chromium into standard of feed formula.

- The last group of Table 2 is in fact, not nutrients. However, many kinds of feed additive preparations are now accepted in a feed formula. Some sub-group of those ones are considered as must have in a feed formula. For example, since threats of microorganisms and/or mycotoxin, a feed formula now must contain mycotoxin binder to prevent risks of mycotoxin; contains probiotics/organic acids/phytogenics/prebiotics preparation in single or combination to enhance animal health as well as to ensure a more safety animal product for human health due to ban or limitation of using antibiotics growth promotant (AGPs) as in few decades ago.

4. UPDATED PARAMETERS TO A MODERN FEED FORMULA AND DIFFERENTIATIONS IN VIETNAM

Based on information above, it could be seen a feed formula now should be like those in Table 3.

Table 3. Composition of a feed formula for swine or poultry

Ingredients	Min	Max	Nutrients	Min	Max
Energy sources			Dry matter, %	X	
Corn grain			NE swine, Kcal/kg	X	
Rice bran			ME poultry, Kcal/kg	X	
Others ...			Crude protein, %	(1)	(1)
Fat/oil			(Total amino acids, %)	(2)	
Protein sources			SID amino acids, %	(3)	
Soybean oil meals			(TD amino acids), %	(4)	
Milk source (piglets)			Lipid, %	X	(5)
Other oilseed meals			Linoleic acid, %	(6)	
Animal sources (rare)			Crude fiber, %		(7)
Mineral sources			NDF, %		
Salt			ADF, %		
Limestone			Fermented fiber, %		
DCP/MCP			Ash, %		(8)
Others ...			Calcium, %	X	X
Small amount ing.			Total phosphorus, %	(9)	(9)
Vit. Min. Premix			Avai. phosphorus, %	X	
Others ...			Sodium, %	X	X
Feed additives			Chloride, %	X	X
Mycotoxin binder	X		dEB, meq/kg	X	X
Enzyme preparations	X				
Probiotics	X				
Organic acids	X				
Phytogenics	X				
(Coccidiostat)	(X)				
(Antibiotics)	(X)				
Pigments (chicken)	(X)				
Others					

Notes: X means the parameter is must be identified by a certain value. This requirement is based on recommendation from scientific literature but not necessarily required by the Vietnamese regular; the one put in pair of parentheses means it somehow does not match between requirement of literature and of the regular.

To feed additives, optimum dosage would be applied depends on the manufacturer and depends on user's strategy. To the nutrient standard, some of those ones are identified by the regular as in Table 4 and Annex 1 as announced in The Circulars No. 66/2011/TT-BNNPTNT. Since that time till to now, many things have been changed and in some cases, it causes a contrast between scientific understanding and law acceptance.

Table 4. List of technical parameters need to be announced in standards of livestock feed

(Follow of Circulars No. 66/2011/TT-BNNPTNT, dated on 10/10/2011 by Minister of Ministry of Agriculture and Rural Development)

(for complete feed and concentrate feed)

Parameter	Unit	Announced form
Moisture	%	Max.
Crude Protein	%	Min. (1)
Metabolisable energy (ME)*	kcal/kg	Min.
Crude fiber	%	Max.
Calcium	%	Between
Total phosphorus	%	Between
Total Lysine	%	Min.
Total Methionine + Cystine	%	Min.
Total Threonine	%	Min.
Ash	%	Max.
Insoluble ash (Chlohydric acid)	%	Max.
Chemical, antibiotics (original or international name and certain content - if yes)	mg/kg	Max.

For example, note number (1) must be announced in form of minnimum level while to scientific mind and/or follow many developed countries, it should be in form of maximum level to be sure that a kind certain feed would not contain so many of nitrogen that could excrete and causes pollution for water resources. Similar to note number (9), The Circulars allows it is announced in a range while commonly, it should be announced in form of maximum level with same reason of note number (1). In contrast, to note number (2), (3), (4), the Circulars requires simple amount of amino acids to be announced, just three amino acids: lysine, methionine + cystin, and threonin and with form of total amino acids while a real high quality feed for livestock needs to be calculated how to fulfill all of ten essential amino acids and under the form of digestible value but not total.

Some other factors could implement to choosing of appropriate nutrient standards for a feed formula in Vietnam is belonged in many Vietnamese customers' perception. Many customers believe that a broiler with dark yellow leg skin means meat from this one would be better than broiler with pale yellow leg skin even the customers of course, would not eat leg skin of chicken. In another hand, many people believe that an dark brown eggshell means that egg is better in quality compared to an egg with a light brown eggshell. This belief plus some other thinking has made no any white chicken egg is produced in Vietnam for a long time till to now.

Beside "strange" belief or wish on characteristics of the animal products from consumers, many animal farm owners have also "strange" perception on characteristics. For example, they think that chicken like bright color objects rather than dark or pale color ones hence, they require all kinds of chicken feed pellet have got an strong yellow color outside each of pellet piece. Or, they could require a feed pellet for duck has to should a quite darker color compared to feed for pig, perhaps they think that dark color would fit with soil ground where ducks are common scavenged.

5. CONCLUSION

To have a premium quality feed product with reasonable price, it depends on several elements. One important element in first step is the one who responsible for this task has to identify what nutrient and/or including of kind of feed additives needs to be incorporated into the feed formula with appropriate level. It means that he/she has to pass a well-educated program in animal nutrition, feed ingredients and an comprehensive knowledge about the market, customers' psycho as well as related rules from government. It also means that a such person could reach that level only as he/she can combine knowledge gained from the college and real experience accumulated through much practicing by him/herself.

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EFFECTS OF PINEAPPLE BRAN ON GROWTH PERFORMANCE AND CARCASS QUALITY OF MUSCOVY DUCKS

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ABSTRACT

A study was conducted to investigate the effect of pineapple bran (PB) in Muscovy ducks diets on productive performance and carcass quality. A total of 256 one-day old mixed-sex Muscovy ducklings were randomly allotted to 4 dietary treatments in a Completely Randomized Design. There were 4 cages (replications) in each treatment with 16 ducks/cage. The four dietary treatments were 0 (Control), 10, 20 and 30% PB, respectively. Feed and water were provided ad libitum for 8 weeks period. The results showed that feed intake, weight gain, feed efficiency and feed cost per kilogram of weight gain of ducks fed 0, 10, 20 and 30% PB were significantly different among treatments ($P<0.05$), while no significant differences were found in mortality of Muscovy ducks ($P>0.05$). However, ducks fed 10% PB had no significant differences in feed intake, weight gain, feed efficiency and feed cost per kilogram of weight gain when compared to the control diet. As dietary PB levels increased from 10 to 30%, the average feed efficiency and feed cost per kilogram of weight gain of ducks were increased. In addition, carcass percentage and abdominal fat data also showed no significant differences ($P>0.05$). The results suggest that pineapple bran can be included in Muscovy ducks diet by 10% without showing any detrimental effects on growth performance and carcass quality.

Keywords: *Pineapple bran, Muscovy duck, growth performance, carcass quality.*

1. INTRODUCTION

The poultry industry in Thailand, chicken and duck production, has been growing rapidly recently. Furthermore, the quality of feed, production efficiency, genetics, management and disease prevention have continuously improved. However, the feed cost is a major proportion of the cost of production. Feed is approximately 60-70% of the total cost of production. This is due to the high expense of ingredients which are used as the primary source of energy and protein. Also, some of the materials used in the production of renewable energy results in higher production costs for farmers. Therefore, it is necessary to consider alternative feedstuffs, such as by-products from industry or agricultural waste, to reduce animal feed costs.

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Pineapple (*Ananas comosus* from the Bromeliaceae family) originates from South America. It is enhance in vitamins, fiber, many other nutrients and antioxidants. It is grown in tropical aria countries in Latin America, Africa and Asia, such as Brazil, Costa Rica, China, Philippines, Indonesia and Thailand. In Thailand, pineapple plantations are found in Prachuapkhirikhan, Ratchaburi, Rayong, Phetchaburi, Phitsanulok and Kanchanaburi Province with a total land use of 86,454 hectares (540,339 rai) and a yearly production of 2,123,177 metric tons in 2017 (FAO, 2019).

Pineapple bran (PB) is a by-product from pineapple processing factories. Pineapple fruits are juicy, thus the liquids must be extract from the fruits. PB (more aptly described as pulp) consists of the skin and often the core of the pineapple, and accounts for an estimated 40-50% of the total pineapple weight. Because of its rather high fibre content, the bran is more suitable for ruminants than for monogastric animals (Otogaki *et al.*, 1961; Maliwan *et al.*, 2012). Pineapple bran is prepared for use as animal feed by sun-drying

or oven-drying. However, PB provided as the sole food source does not cover the animal's requirements because it is low in protein and neutral detergent fiber (NDF) (Costa *et al*., 2007). PB is not beneficial to poultry. It was found to adversely affect growth and feed conversion in chicks even at low levels (Ross, 1966; Devendra, 1988). Another experiment found that 15% PB in chicken diets depressed the feed conversion ratio and decreased weight by 20% (Hutagalung *et al*., 1973). A metabolizable energy (ME) value of 1,864 kcal/kg dry matter (DM) was proposed (Hutagalung *et al*., 1973; Heuzé *et al*., 2012). According to composition values, PB contains 85.30% DM, 4.69% crude protein (CP), 22.74% crude fiber (CF), 2.23% ether extract (EE), 3.28% ash, 67.06% nitrogen free extract (NFE), 0.19% calcium and 0.07% phosphorus on a DM basis (Chawanitnadakorn,1987), while Lima *et al*. (2012) reported the composition of pineapple bran as 85.58% DM, 4.60% CP, 36.96% NDF, 11.91% acid detergent fiber (ADF), 13.17% CF, 0.58% EE, 4.41% ash and gross energy 3,701 kcal/kg on a DM basis. The compositions of pineapple bran are 5.84% CP, 23.51% CF, 2.53% EE, 2.44% ash, 65.68% NFE, 0.16% calcium and 0.07% phosphorus on a DM basis (Maliwan, 2008). PB can be included in poultry feed up to about 9–10% without showing any detrimental effects on growth performance (Maliwan, 2008; Maliwan *et al*., 2012). However, very limited information on the using of PB in diet is available in Muscovy ducks. Thus, the aim of this study was to evaluate the effect of PB in Muscovy ducks diet on productive performance and carcass quality very limited information meat ducks.

2. MATERIALS AND METHODS

2.1. Animals

A total of 256 three-day old mixed-sex Muscovy ducklings with initial body weights (BW) of 160.94 ± 3.54 g were used to study the effects of PB in Muscovy ducks rations on productive performance and carcass quality. All ducklings were randomly allotted to 16 pens. Each pen (n = 16) was fed 1 of 4 diets and there were 4 replicate pens per diet. All ducks received starter and finisher diets in mash form from 0–3 and 3–8 weeks, respectively. The ducks were reared in an open-sided, naturally ventilated house, with a daily photoperiod of 24 h of light. The ducks were housed in floor pens (1 m × 2.5 m) on rice husks, and each pen had 1 tray feeder and 1 manual drinker. At 7 days of age, the tray feeder was replaced with a round-bottomed feeder. All ducks were inoculated with Duck Plague vaccine at 21 days of age and with inactivated Fowl Cholera vaccine at 49 days of age. Feed and water were provided *ad libitum* throughout the experimental period (0-8 weeks). For each pen, body weight and feed consumption were recorded weekly. These values were used to calculate BW gain, feed intake (FI), feed conversion ratio (FCR), feed cost per kilogram of weight gain and productive index of the Muscovy ducks for the periods of 0–3, 3–8 and 0–8 weeks. Mortality was recorded daily to calculate survival rate. At the end of the experiment, 2 ducks (male and female) were randomly selected with equal weight from each pen, were fasted overnight with access to water, and killed by cervical dislocation their carcasses being weighed individually. Weights of the abdominal fat pads and gizzards were recorded and calculated relative to the final body weight.

2.2. Experimental design

The experiment was a Completely Randomized Design (CRD) with 4 dietary treatments and 4 replicate pens. There were 16 birds per each pen (total of 256 birds), such that the mean initial body weight did not differ among dietary treatments. The four dietary treatments were 0 (control), 10, 20 and 30% PB, respectively. All experimental diets were formulated to similar levels of AME_n and CP (Table 1). The formulation of diets and calculation of nutrient requirements for ducks were based on NRC (1994).

Table 1: Composition and nutrient contents of the experimental Muscovy ducks rations
(air dried basis)

Experimental diets	0–3 week of age				3–8 week of age			
	Level of Pineapple Bran in diet				Level of Pineapple Bran in diet			
	0% PB	10% PB	20% PB	30% PB	0% PB	10% PB	20% PB	30% PB
Ingredients (%)								
Pineapple bran	0.000	10.000	20.000	30.000	0.000	10.000	20.000	30.000
Corn	22.640	19.507	16.380	13.000	26.922	24.650	22.369	20.110
Broken rice	30.200	21.190	12.300	3.425	31.200	22.000	12.810	3.650
Rice bran	9.500	7.000	4.500	2.200	16.250	13.580	10.910	8.180
Soybean meal	29.800	31.470	33.140	34.785	18.950	20.557	22.162	23.776
Fish meal	5.000	5.000	5.000	5.000	4.000	4.000	4.000	4.000
Palm oil	0.000	2.890	5.780	8.670	0.000	2.500	5.000	7.500
Dicalcium phosphate	1.850	1.850	1.850	1.850	1.500	1.500	1.500	1.500
Oyster shell (%)	0.250	0.250	0.250	0.250	0.340	0.340	0.340	0.340
Salt	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300
Premix [1]	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250
DL- methionine	0.210	0.230	0.250	0.270	0.204	0.239	0.275	0.310
L-lysine					0.084	0.084	0.084	0.084
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
Calculated nutrient (%)								
AME_n (kcal/kg)	3,001	3,007	3,013	3,016	3,096	3,090	3,084	3,078
Crude protein	22.767	22.771	22.773	22.774	18.149	18.150	18.151	18.152
Crude fiber	2.852	4.676	6.498	8.325	2.662	4.492	6.322	8.150
Calcium	1.091	1.107	1.123	1.139	0.925	0.941	0.956	0.972
Available phosphorus	0.524	0.523	0.522	0.521	0.461	0.459	0.458	0.456
Methionine	0.504	0.504	0.504	0.505	0.477	0.493	0.509	0.524
Methionine + Cystine	0.873	0.857	0.841	0.826	0.800	0.800	0.800	0.800
Lysine	1.089	1.090	1.092	1.094	0.930	0.930	0.931	0.931
Threonine	0.828	0.823	0.818	0.813	0.656	0.651	0.646	0.641
Tryptophan	0.290	0.286	0.283	0.279	0.227	0.223	0.219	0.215
Feed cost (baht/kg)	12.76	12.77	12.77	12.78	12.06	12.01	11.97	11.92

[1] Premix contents: Vitamin (g/kg): Vitamin AD_3 1.5; Vitamin E_{50} 6; Vitamin K 0.15; Vitamin B_{12} 4; Biotin 0.03; Choline Chloride 260; Folic acid 0.11; Niacin 7; Pantothenic acid 2; Pyridoxine 0.7; Riboflavin 0.72; Thiamine 0.36 Mineral (g/kg): $MnSO_4$ 16.493; $CuSO_4$ $5H_2O$ 3.142; $FeSO_4$ $4H_2O$ 32.038; $ZnSO_4$ H_2O 10.98; KI 0.046; $NaSeO_4$ 0.036

2.3. Statistical analysis

Data were analyzed by ANOVA using SPSS version 13.0 (SPSS, 2004). When the treatment effect was significant, the differences among treatment means were considered statistically significant at P<0.05 by Duncan's New Multiple Range Test (DMRT).

3. RESULTS AND DISCUSSION

All data were computed and analyzed for 3 peroids (0-3 weeks, 3-8 weeks and the overall period 0–8 weeks of age) (Table 2). The period 0–3 weeks of age, there were significant differences among treatments (P<0.05) in FI, BW gain and FCR. As dietary PB levels increased from 0 to 30, the average FI and BW gain were decreased. However, the FCR was poorer with increasing dietary PB levels. During 3–6 weeks, FI and FCR showed significant differences (P<0.05) but there was no significant change on the BW gain. For the overall period 0–8 weeks of age, the FI, BW gain, FCR and feed cost per kilogram of weight gain of Muscovy ducks fed 0, 10, 20 and 30% PB were significantly different among treatments

(P<0.05) but no change occurring in survival rate. Muscovy ducks fed 10% PB was similar FI, BW gain, FCR and feed cost per kilogram of weight gain compared to the control diet. In addition, carcass percentage, gizzard and abdominal fat data also showed no significant differences (P>0.05). This result suggests that pineapple bran can be included in Muscovy ducks diets up to 10% without showing any detrimental effects on growth performance and carcass quality and this is similar level of PB in the broiler diet from the data of Maliwan (2012). In poultry, crude fibre is known for its negative effects as well as its beneficial physiological functions (Mateos *et al.* 2012). However, the optimum dietary crude fiber level in diet for meat duck from 1 to 21 days of age is 7.52% (Han *et al.* 2017).

Table 2. Effect of pineapple bran on growth performance and carcass quality of Muscovy ducks at 0–3, 3–8 and 0–8 weeks of age

Attributes	Level of Pineapple Bran in diet				SEM	P-value
	0% PB	10% PB	20% PB	30% PB		
0–3 weeks of age						
Feed intake (g/bird)	1,837^{a}	1,800^{a}	1,729^{ab}	1,647^{b}	42.43	0.0370
Weight gain (g/bird)	946^{a}	880^{b}	841^{b}	701^{c}	13.04	<0.0001
FCR: feed/gain	1.94^{b}	2.04^{b}	2.06^{b}	2.35^{a}	0.04	<0.0001
3–8 weeks of age						
Feed intake (g/bird)	5,509^{b}	5,847^{ab}	6,171^{a}	6,075^{a}	115.41	0.0074
Weight gain (g/bird)	1,544	1,541	1,585	1,464	47.15	0.3687
FCR: feed/gain	3.56^{b}	3.80^{ab}	3.91^{ab}	4.15^{a}	0.10	0.0076
0–8 weeks of age						
Feed intake (g/bird)	7,346^{b}	7,647^{ab}	7,900^{a}	7,722^{ab}	119.92	0.0424
Weight gain (g/bird)	2,490^{a}	2,421^{a}	2,426^{a}	2,165^{b}	47.24	0.0019
FCR: feed/gain	2.95^{c}	3.16^{bc}	3.26^{b}	3.56^{a}	0.06	<0.0001
Feed cost/weight gain (Baht/kg)	36.10^{c}	38.52^{bc}	39.62^{b}	43.18^{a}	0.67	<0.0001
Survival (%)	98.44	100.00	100.00	98.44	1.10	0.5885
Carcass characteristics (%)						
Hot carcass	88.84	87.60	87.14	87.84	1.33	0.0806
Dressing carcass	77.28	76.55	75.93	75.51	1.68	0.6292
Gizzard	2.26	2.09	2.25	2.32	0.12	0.2943
Abdominal fat	0.71	1.15	1.12	0.77	0.15	0.2792

$^{a, b, c}$ *: Means within columns not sharing a common superscripts are significantly different (P<0.05)*

4. CONCLUSION

Pineapple bran is a by-product of pineapple processing factories and can be used as an alternative feedstuff. We concluded that the optimum PB level in diet for Muscovy ducks from 0 to 8 weeks of age is 10% without showing any detrimental effects on either growth performance or carcass quality.

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USING EFFICIENCY OF LYSINE AND METABOLIZABLE ENERGY OF H'MONG BROILERS FROM 0 TO 28 DAY OF AGE

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ABSTRACT

An experiment was conducted to evaluate the interactive influences of dietary ME and lysine on growth of H'mong broilers from 0 to 28 day of age. Its study was in a 3x3 two-factorial design, with the first factor being 3 levels of ME (3,000; 3,100; and 3,200 kcal/kg of feed) and the second one being 3 levels of lysine (1.1, 1.0 and 0.9%), and 4 replicates with 6 broilers for each. Two hundreds and sixteen broilers were placed into 36 bamboo floor pens (6 birds/pen). The diets were formatted from corn yellow, soybean meal, soybean oil, DCP, shell, mineral and vitamin premixes, salt, and some of synthetic essential amino acids. Chicks were fed *ad libitum* and provided water all of time. The feeding trial was lasted for 4 weeks. The result indicated that the ME 3,000 kcal/kg of feed and lysine 1.1% diet resulted in the most efficient FCR.

Keywords: *Metabolizable energy, lysine, and H'mong broiler*.

1. INTRODUCTION

Local H'mong chickens have black skin, bone, and meat (Dao and Duc, 2011) and their meat contains high lysine and methionine giving it good taste when consumed (Hong *et al.*, 2007). As other chicken varieties, growth and development of H'mong broilers depend on several factors, especially metabolizable energy (ME) and protein of diets, which play a very important role. While D'Mello (2003) indicated that when CP content increases from 140 to 280 g/kg of diet, there is a marked and progressive reduction in the efficiency of utilization of the first-limiting amino acid (AA), lysine. Supplementation of protein for feed using efficiencies on birds' growth belongs to better balanced essential AAs in the diets (Ngoan *et al.*, 2004). Moreover, the animal body only synthesizes proteins from patterns of balanced AAs and if unlimited AAs are provided, the diets will have unbalanced AAs. Birds' growth performance has maintained when essential AAs were supplied in the low-protein diets (Aletor *et al.*, 2000). Besides, other studies have indicated that low-protein diets but adequate AAs still supported best for broilers' feed intake and growth ability (Yamazaki *et al.*, 1998; Aletor *et al.*, 2000).

Lysine is used as a reference AA for calculating ideal ratios, because it is an limited essential AA. Therefore, ideal AA ratios, with lysine as the reference AA, are being increasingly used throughout the world for diet formulation for poultry today (NRC, 1994; Baker, 1997; Emmert and Baker, 1997; Mack *et al.*, 1999; Baker *et al.*, 2002). Mack *et al.* (1999) also found that the lysine requirement for maximal feed efficiency was substantially higher than that required for maximal weight gain. Hence, the lysine content of the diets was calculated according to high levels to investigate the growth performance of H'mong broiler breed in this study.

Supplementation of synthetic essential AAs in the low-protein diets has also restricted enviromental polutions by nitrogen (Corzo *et al.*, 2005). Thus, the purpose of this study was to determine effects of ME and lysine on growth of H'mong broilers from 0 to 4 weeks of age.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

Two hundreds and sixteen H'mong chicks of 0-day-old were allocated into nine treatments in a 3*3 factorials, 4 replicates, and 6 birds for each experimental unit. The first factor consisted of 3 levels of ME (3,000; 3,100; 3,200 kcal/kg of feed) and the second factor was 3 levels of lysine (0.9, 1.0, 1.1%). Methionine + cystine, threonine, and tryptophan followed the AA ideal profile of Baker (1997). All birds were raised in bamboo floor cages, continuous lighting, natural ventilation, and 27-30^{o}C of ambient temperature. The diets were offered *ad libitum* and water was freely available throughout the four-week-trial.

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Table 1. Chemical compositions of feedstuffs

Feedstuffs	DM (%)	Chemical compositions of feedstuffs (% of feed)									
		CP	ME (kcal/kg)	EE	CF	Lys	Met+cys	Thr	Trp	Ca	P
YC	86.90	7.59	3,236	3.31	4.84	0.21	0.28	0.25	0.05	0.63	0.29
SBM	87.50	42.74	2,512	2.35	7.35	2.23	1.14	1.51	0.52	0.63	0.67
SBO	-	-	8,227	-	-	-	-	-	-	-	-
DCP	-	-	-	-	-	-	-	-	-	24.11	19.29
Sell	-	-	-	-	-	-	-	-	-	29.22	-

Table 2. Diets and their nutrient values

Feedstuffs (kg)	ME 3,000 kcal			ME 3,100 kcal			ME 3,200 kcal		
	Lysine 0.9%	Lysine 1%	Lysine 1.1%	Lysine 0.9%	Lysine 1%	Lysine 1.1%	Lysine 0.9%	Lysine 1%	Lysine 1.1%
YC	56.18	55.81	56.06	54.49	53.25	53.52	54.17	53.40	52.99
SBM	38.45	38.6	38.03	37.97	38.9	38.36	36.56	36.99	37.00
SBO	2.48	2.58	2.65	4.5	4.71	4.77	6.24	6.42	6.57
L-Lysine-HCl	-	0.03	0.14	-	0.03	0.14	-	0.10	0.17
DL-Methionine	0.06	0.13	0.20	0.06	0.13	0.20	0.09	0.15	0.22
L-Threonine	-	-	0.03	-	-	0.03	-	-	0.05
DCP	2.00	2.02	2.02	2.15	2.15	2.15	2.11	2.11	2.17
Sell	0.10	0.10	0.14	0.10	0.10	0.10	0.10	0.10	0.10
Premix	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Total	100	100	100	100	100	100	100	100	100
Nutrient value (%)									
ME (kcal/kg of feed)	2,987	2,987	2,987	3,087	3,087	3,087	3,185	3,185	3,185
CP	20.76	20.89	20.88	20.42	20.82	20.83	19.83	20.11	20.77
EE	5.24	5.33	5.40	7.20	7.38	7.44	8.89	9.05	9.19
CF	5.55	5.54	5.51	5.43	5.44	5.41	5.31	5.30	5.28
Ca	1.10	1.11	1.12	1.13	1.12	1.12	1.11	1.10	1.11
P	0.81	0.81	0.81	0.83	0.83	0.83	0.81	0.81	0.82
Lysine	0.90	1.00	1.10	0.90	1.00	1.10	0.90	1.00	1.10
Met+cys	0.65	0.72	0.79	0.65	0.72	0.79	0.65	0.72	0.79
Threonine	0.72	0.72	0.74	0.71	0.72	0.74	0.68	0.69	0.74
Tryptophan	0.22	0.23	0.20	0.22	0.23	0.22	0.22	0.22	0.22

2.2. Feed analyses and calculations

The feedstuffs were analyzed for dry matter (DM), crude protein (CP), crude fibre (CF), ether extract (EE), ash, calcium, and phosphorus by standard Weende methods (AOAC, 1990) at the College of Agriculture and Applied Biology-Can Tho University. ME of soybean oil (SBO) was calculated by Ketels and DeGroote (1989) $ME=8.227-10.318^{[-1,168(\text{ratio unsaturated fatty acid: saturated fatty acid})]}$. AA contents were analyzed in Laboratory department of Animal nutrition - Institute of Agricultural Science for Southern Viet Nam. ME content of the feedstuffs was calculated from chemical analysis data using the equation of Kinh (2003).

Yellow corn (YC): ME (kcal/kg of feed) = 19.0 +37.5CP + 78.5EE + 11.2CF + 37.7 NFE.

Soybean meal (SBM): ME (kcal/kg of feed) = -2.7 + 35.1CP + 96.7EE - 4.2CF + 28.6NFE.

2.3. Data collection

Birds were weighed to evaluate bodyweight gain (BWG) and feed was weighed to calculate their feed consumption every week. Weight,

feed intake (FI), and feed conversion ratio (FCR) (g of feed/g of BWG) were measured for each week.

2.4. Data analysis

Using ANOVA with the General Linear Model procedure in Minitab 13.2 (2000).

3. RESULTS AND DISCUSSION

3.1. Effects of dietary ME on BWG, FI, and FCR of broilers

Table 3 shows that daily feed, ME, CP, and lysine intake; daily BWG; and FCR were significantly different among broilers in diets of three different dietary ME levels. The results of ME intake daily of birds decreased when the dietary ME was over 3,000 kcal/kg of feed. This finding is similar to report by Lung and Man (2001) that birds' FI was a negative correlation with the ME level of diets.

Summers (2000) demonstrated that dietary energy concentration strongly influenced in FI, or the daily FI of birds negatively correlated with ME in the diets. Birds also decreased FI after obtaining adequate energy for their requirement (Liem, 2003). The amount of FI of H'mong broilers was equivalent to that of FI of 0-4 week-old broilers imported from Egypt at 10-34 g/bird/day in the researching result of Dat *et al.* (2008); at 10-34 g/bird/day in the study of Tam (2008).

H'mong broilers consumed higher feed in treatments containing 3,000 kcal/kg of feed but lower ME compared to others. Previously, it was found that broilers still have good ability to control its FI based on desire requirement to normalize FI and to regulate its FI to supply for the lacks of dietary ME changes (Leeson *et al.*, 1996). Moreover, Loi (2009) found that ME consumed by local Ac broilers in high-ME-diets was higher than that of low ME diets because of the difference between ME of diets. However, ME consumed by H'mong broilers was 45.04-46.88 kcal/bird/day, which was lower than that consumed by broilers imported from Egypt at 53.7-55.5 kcal/bird/day (Tam, 2008).

Dozier *et al.* (2006) pointed that caloric intake did not differ as dietary available ME increased because the broilers were able to adjust feed consumption abilities to achieve the similarity of caloric intake when provided diets varying in available ME. Therefore, caloric consumption per unit of BWG decreased as available dietary ME increased, but caloric intake per unit of total white meat was not affected by the dietary ME. In the current research, H'mong broilers adjusted the amount of FI in the diets varying ME to balance energy consumption, but broilers' feed consumption was only regulated relatively.

In addition, CP consumption reduced when progressively increasing ME of diets. This was because CP concentrations of diets were similar; the broilers decreasingly ingested resulting in reduced CP consumption. H'mong's daily CP intake was lower than that of broilers imported from Egypt at 3.9-4.02 g/bird (Tam, 2008). Moreover, because there was difference of feed consumption among treatments, the amount of lysine was differently consumed by H'mong broilers.

Therefore, the birds' BWG tended to reduce as they were provided diets above 3,000 kcal/kg of feed. The BWG of H'mong was closely positive correlated to its consumed lysine (r=0.842) meaning that when decreasing lysine consumption, broilers obtained lower BWG. As the dietary ME also contained over 3,000 kcal/kg of feed, FI of broilers was limited. This resulted in decreasing some of the consumed nutrients in the diet, especially lysine contents.

H'mong broilers had higher ratio of dietary lysine to ME of the diet containing 3,000 kcal/kg of feed attained, as compared to that in the diets containing 3,100 and 3,200 kcal/kg of feed. Similarly, the findings of Kerr *et al.* (1999); Labadan *et al.* (2001); and Mbajiorgu *et al.* (2011) found that the ratio of dietary lysine to energy did not have significant effects on feed intake, nitrogen digestiblity, and ME intake, but the high-ratio diets supported for optimum of growth rate and FCR. Conversely, as H'mong chicken breed is low growth, they were raised by the diets over 3,000 kcal ME/kg of feed resulted in decreasing FI and BWG.

The current BWG of H'mong at 4 weeks was equivalent to the investigating results of Van *et al.* (2006). However, it was lower than the findings of Van (2005) and Hong *et al.* (2007). FCR of

broilers differed signicantly and FCR increased when ME of diets was above 3,000 kcal/kg of feed. Birds' FCR of this research was lower than that of Van (2005); Quyen and Son (2008). This implied that if diets are balanced well, especially lysine, feed using efficency will be better.

Table 3. Effects of ME on BWG, FI, and FCR of birds

Terms	ME (kcal/kg feed)			SEM	P
	3,000	3,100	3,200		
FI daily, g/bird	15.08[a]	15.19[a]	14.64[b]	0.09	0.001
ME intake,kcal/bird	45.04[b]	46.88[a]	46.63[a]	0.27	0.001
CP intake, g/bird	3.14[a]	3.14[a]	2.94[b]	0.02	0.001
Lysine intake, g/bird	0.152[a]	0.153[a]	0.147[b]	0.001	0.001
L/ME ratio, g/bird	3.370	3.261	3.161	-	-
Initial BW, g/bird	28.37	28.40	28.37	0.14	0.98
Final BW, g/bird	193[a]	190[b]	182[c]	0.67	0.001
BWG daily, g/bird	5.90[a]	5.77[b]	5.48[c]	0.03	0.001
FCR	2.44[b]	2.50[a]	2.49[a]	0.01	0.001

Means within each row with the same superscript letter are not significantly different (P>0.05)

3.2. Effects of dietary lysine on BWG, FI, and FCR of broilers

In Table 4, it is shown that feed, ME, and CP intake were not significantly different among trials. This indicated that increasing of dietary lysine 0.9-1.1% did not change feed, ME, and CP intake. Lysine is a basal unit of protein and adding lysine to balance diets will also supply dietary CP. Hence, when supplying lysine into the dietary with fixed CP, FI was not influenced. The result also showed a low correlation ratio between lysine intake and feed consumption (r=0.258). The study was likely in line with the report of Araújo *et al.* (2005) that when supplying 0.95, 1.05 and 1.15% lysine into the diets, the feed consumption of broilers did not change.

Table 4: Effects of dietary lysine on BWG, nutrient intake, and FCR of birds

Terms	Levels of lysine (%)			SEM	P
	0.9	1.0	1.1		
FI daily (g/bird)	15.03	14.86	15.01	0.09	0.32
ME intake (kcal/bird)	46.39	45.83	46.33	0.27	0.30
CP intake (g/bird)	3.06	3.06	3.10	0.02	0.18
Lysine intake (g/bird)	0.135[c]	0.150[b]	0.167[a]	0.001	0.001
L/ME intake (g/Mcal)	2.918	3.275	3.599	-	-
Initial BW (g/bird)	28.31	28.46	28.37	0.14	0.75
Final BW (g/bird)	181[c]	186[b]	198[a]	0.67	0.001
BWG daily (g/bird)	5.45[c]	5.64[b]	6.05[a]	0.03	0.001
FCR	2.60[a]	2.50[b]	2.34[c]	0.01	0.001

However, lysine intake, BWG, and FCR of H'mong were significantly different and the amount of lysine intake also correlated closely with H'mong's BWG (r=0.842). Moreover, increasing the amount of dietary lysine resulted in higher lysine/ME ratio and hence improving BWG.

Besides, as lysine is an AA to calculate other essential AAs following an ideal AA pattern, a little change of lysine content only influenced broilers' BWG. This was similar to the results of Baker *et al.* (2002) that the broilers' diet containing 1.27% lysine had more BWG than that of 0.84%

lysine. Also, Kidd *et al.* (1997) supplied lysine to the broilers' diet at 105% recieved better BWG. Conversely, if broilers were raised by diets being lack of lysine, the birds' BWG reduced 45% compared with birds which were raised by diets with normal lysine levels (Tesseraud *et al.*, 1996).

Moreover, the correlation between consumed lysine and FCR was -0.81 and this indicated that the ratio was closely negative correlation. This finding confirmed the results of Araújo *et al.* (2005) and Kidd *et al.* (1997), which pointed out that increased dietary lysine resulted in improved feed consumption efficiency of broilers. FCR of 14 old-day broilers was also better in excessive AA diets (Corzo *et al.*, 2004; Bartov and Plavnik, 1998) and supplement of AAs in high levels will ensure to provide AAs adequately. As the level of dietary lysine increased 0.1% compared with the basal diet, BWG of birds was added more 0.19 g/bird/day, but FCR decreased 0.1 kg of feed over kg of BWG.

3.3. Effects of dietary ME and lysine on BWG, FI, and FCR of broilers

In Table 5, it is shown that feed, ME, CP, and lysine intake did not differ significantly. In contrast, the final bodyweight of birds was significantly different. This pointed out that there was an interaction between dietary ME and lysine. H'mong's BWG of the current study was higher than that of Noi birds of Quyen (2008), similar to that observed by Van *et al.* (2006), and lower than that result of Van (2005).

Table 5. Effects of ME and lysine of diets on BWG, nutrient intake, and FCR of birds

Terms	ME 3,000 kcal/kg of feed interacting with 3 levels of lysine			ME 3,100 kcal/kg of feed interacting with 3 levels of lysine			ME 3,200 kcal/kg of feed interacting with 3 levels of lysine			SEM	P
	0.9%	1.0%	1.1%	0.9%	1.0%	1.1%	0.9%	1.0%	1.1%		
FI daily (g/bird)	15.05	15.11	15.07	15.25	15.00	15.31	14.79	14.46	14.66	0.15	0.67
ME intake (kcal/bird)	44.97	45.13	45.02	47.08	46.31	47.27	47.12	46.07	46.69	0.47	0.66
CP intake (g/bird)	3.13	3.16	3.15	3.11	3.13	3.19	2.93	2.91	2.97	0.03	0.69
Lysine intake (g/bird)	0.136	0.153	0.167	0.137	0.152	0.170	0.133	0.146	0.163	0.02	0.56
Lys/ME intake (g/Mcal)	3.013	3.381	3.716	2.915	3.272	3.596	2.826	3.171	3.485	-	-
Initial BW (g/bird)	28.33	28.35	28.43	28.33	28.55	28.33	28.28	28.48	28.35	0.24	0.98
Final BW (g/bird)	182^{ef}	190^{c}	208^{a}	183^{de}	189^{cd}	198^{b}	177^{f}	180^{ef}	188^{cd}	1.16	0.001
BWG daily (g/bird)	5.50^{ef}	5.77^{c}	6.41^{a}	5.54de	5.73^{cd}	6.04^{b}	5.32f	5.42^{ef}	5.70^{cd}	0.04	0.001
FCR	2.61^{a}	2.51^{b}	2.21^{d}	2.61^{a}	2.49^{b}	2.40^{c}	2.59^{a}	2.49^{b}	2.41^{c}	0.02	0.001

The study also showed that diets containing three levels of ME interacting with 3 levels of lysine made significant difference of birds' FCR. With value of P<0.01 and delimitated analyzing results of treatment pairs, it was demonstrated that the main impact factor was the interaction between ME and lysine. FCR of H'mong of this study was lower than the research findings of Quyen and Son (2008) that FCR of Noi broilers was 2.96-4.42 when birds were raised by the diets mainly based on ME and crude protein.

4. CONCLUSION

In conclusion, 0-4 week-age H'mong broilers should be raised by the diet containing ME 3,000 kcal/kg of feed and lysine 1.1% for better obtaining BWG and FCR.

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LIVESTOCK DEVELOPMENT IN VIETNAM IN THE DECADE: OPPORTUNITIES AND CHALLENGES

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ABSTRACT

Vietnam is home of about 97.5 million inhabitants and is still an agriculture-based country. In agriculture, livestock plays an important role in term of GDP contribution and its sustainability. In the decade (2010-2018), livestock production quickly has been increased in population, product production and consumption. In general, annual increases are found in animal population, particularly 11% for chickens and 3% for pigs; in also product production such as 4% for live weight meat, 10% for egg, and 25.6% for milk; and in livestock product per capita as 2.7% for meat, 7.5% for egg and 22.9% for milk. Livestock sector is not only changing in its production but also in production systems that shifting from semi-intensive to intensive and industrialized systems. However, livestock production has been faced in disease problems such as FMD, African Swine Fever, etc., environmental pollution and price uncertainty due to market constraints. In recent, ASF is spreading in many parts of the country. In the other hand, livestock product diversity does not pay much attention so that it can limit supply chains in the country and the world market. In conclusion, development and sustainability of livestock production are needed an attention not only from the government but also scientists and producers.

Keyword: *Disease problem, livestock development, market, sustainability.*

1. INTRODUCTION

Vietnam is located in the centre of South East Asia, comprising the eastern boundary of the Indochinese Peninsula, with a natural area of about 333,000 km^2. It is divided into 8 agri-ecological zones (from north to south): North Eastern; North West; Red River Detla; North Central Coast; South Central Coast; Central Highland; South East; and Mekong River Delta.

In the past 9 years, Vietnam's population has grown by around 1.1% per year, increasing from 88.47 million in 2010 to 97.26 million in 2019**.** In 2010, the proportion of urban population reached 30.5% and increased 35.7% in 2018 (GSO, 2018). Also in this period, the gross national income per capita increased from US$ 1,310 (2010) to US$2,342 (2017), changing Vietnam from a low- income country to a lower-middle-income country (World Bank, 2017).

Food demand has also increased rapidly as population and incomes have increased, and food consumption patterns have shifted. The past decade has seen very high levels of growth in the consumption of meat, particularly pork, milk, and eggs-growth rates higher than those experienced by any country in the region (Jaffee *et al*., 2016).

The production livestock sector in Vietnam plays an important role in agriculture. It accounts for 28% of agricultural gross value added (Dinh Xuan Tung, 2017) and is one of the fastest growing agricultural subsectors. Over about 10 years, the livestock production in Vietnam has undergone major changes, and the livestock population has been growing while the number of households raising animals has declined (Dinh Xuan Tung, 2017). With increasing animal population resulted in some problems such as environmental pollution, disease and unfluctual price.

2. LIVESTOCK DEVELOPMENT

2.1. Pig production

Pig production accounts for 60% of the value of Vietnam's livestock industry. Pig population is 7th biggest in the world after China, US, Germany, Spain, Brazil and Russia. According to GSO (2018), in 2018 pig population is 28.15 million heads and annually increases 0.36% during 2010-2018 period; however, preliminary product increases 3.26% per year (3.03 vs 3.82 million tons of slaughtered live weight).

Pig production systems have been changing from small scale (small household farmers) to large scale (industrial farm) system. In 2014,

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70% of pig number and 60% pig meat produced by the household farm (Dinh Xuan Tung, 2017). At present, number of livestock farms including small and large scales estimates around 3 million; however, the household farm number reduced 5-6% per year in 2012-2017 period.

In the next 10 years, the picture of pig industry is shaped in the direction: First, the number of large scale farms increases. By 2027, the product production in large farms is estimated to account for over 70%. Second, small-scale farm is sharply reduced (5-7% per year), equivalent to the production raised in small households about 30%. Thirdly, pig raising is mainly a "game" of enterprises and "professional" breeders. Along with that are the prospects: Chain linkage cooperation develops strongly between producers, slaughter houses and distributors. Estimated to 2027, 100% of farms have output contracts; the state will strictly manage livestock and slaughtering, food safety, environment; applying livestock registration nationwide and strongly developing food chain with traceability.

2.2. Chicken production

Chicken production is 2nd biggest livestock production in the country after pig production. In the past 8 years, the chicken population has increased faster than average. While the populations of cattle and buffalo slightly decreased by 0.25 and 2.06% per year, respectively, the chicken population in contrast increased gradually at a rate of 5.65% per year in the same period (Table 1). However, annually chicken meat production significantly increased by 10.7% and egg production increased at 10.5% (Table 2). Consequently, chicken meat consumption per capita relatively accounted for 15.4% in 2010 and increased 20.6% of total meat product in 2018 (Table 3).

Chicken production systems have been changing. In 2013, smallholders (1-50 birds) accounted for 89.6%, semi-industrial (50-99 birds) 7.2% and industrial farms (>100 birds) only 3.25% (GSO, 2013). However, proportions of industrial chicken (meat type and laying) in total chicken population have been significantly decreased. In 2010, meat type chicken accounted for 46% and laying accounted for 50% of total, and 8 years later, those proportions of 26 and 43.3%, respectively (GSO, 2010; 2018).

2.3. Cattle and buffalo production

In general, number of cattle decreased by 0.25% in 2010-2018 period. However, it fluctuated from 5.92 million in 2010 to 5.16 million in 2013, and increased again in 2015-2018. However, number of buffalo gradually decreased at a rate of 2.06% per year (Table 1). Cattle and buffalo mainly located in North Mountainous region and Central Coastal region (Table 3).

In contrast, according to GSO (2018), dairy population dramatically increased by 16.1% per year (128.58 thousand in 2010 and 294.38 thousand dairy in 2018), and milk production by 25.6% at the same period (Table 2). About 84% of dairy herds were located in 6 provinces (Hanoi, Son La, Nghe An, Lam Dong, HCMC and Long An).

Table 1. Livestock population during 2010 – 2018 (10^6 head)

Livestock	Year				Change (%/year)
	2010	2015	2017	2018	
Buffalo	2.91	2.52	2.49	2.43	- 2.06
Cattle	5.92	5.37	5.65	5.80	-0.25
Pig	27.37	27.75	27.41	28.15	0.36
Chicken	218.2	259.3	295.2	316.9	5.65

Table 2. Livestock distribution in agri-ecological zones in 2018

Zone	Livestock (%)				
	Buffalo	Cattle	Pig	Poultry	No. AU*/ km^2
NE and NW	56	18	25	21	64.2
RRD	5	9	25	25	212.2
Central Coast	32	41	18	21	63.3
Central Highland	4	13	7	5	32.9
South East	2	7	12	11	95.7
MRD	1	13	12	17	70.1

Cattle and buffalo: 1; sheep: 0.2; goat: 0.15; hens and duck: 0.01; swine: 0.4 and piglet: 0.03. Country mean=89.7 AU/km^2

Table 3. Preliminary product production during 2010-2018

Products	Year			Increase (%/year)
	2010	2015	2018	
Meat (10^3tons)	4,036.9	4,806.6	5,340.5	4.0
- Pig	3,027.3	3,491.6	3,816.3	3,3
- Chicken	451.4	700.8	839.6	10,7
- Ruminants	363.1	385.1	426.5	2.2
Eggs (billion)	6.3	8.9	11.6	10.5
Milk (10^3tons)	306.7	723.2	936.0	25.6

Table 4. Preliminary livestock product per capita per year during 2010-2018

Items	2010	2015	2017	2018
Total meat (kg)	46.2	52.1	54.2	56.2
Pork meat	75.5	72.7	71.8	71.5
Poultry meat	15.4	19.0	19.9	20.6
Ruminant meat	9.1	8.3	8.3	7.9
Eggs (unit)	72.5	96.2	110.8	115.8
Fresh milk (kg)	3.5	7.8	9.2	9.9

3. CHALLENGES IN LIVESTOCK DEVELOPMENT

3.1. Disease

According to the Department of Animal Health (DAH) under the Ministry of Agriculture and Rural Development (MARD), Viet Nam has managed to stamp out most diseases among cattle and poultry. Currently, no outbreaks of bird flu, blue ear or foot-and-mouth disease are reported in the country (Viet Nam News, 2018). However, the DAH urged localities to watch out, warning that the diseases can occur anytime with negligence in epidemic prevention, particularly as the risk of transmission of African swine fever (ASF) from China to Viet Nam is high. In last February, the DAH confirmed that ASF has been detected on farms in Viet Nam.

3.2. Feed industry

The consumption of feed has grown in line with the development of livestock sector around 11% per year and about 20 million tons of animal feed to be consumed in 2018 (VFA, 2018). However, 85% of feed ingredients were imported with cost of 3.9 billion USD (Department of Livestock, MARD, 2019).

3.3. Environment pollution

According to MARD (2015), Vietnam generates an estimated 80 million tons of animal waste per year. Pigs account for 30%, poultry 29%, cattle 23%, and buffalos and other animals 18%. About 80% of the manure is generated by smallholder farms, and the remainder comes from commercial farms. Around 36% of total animal manure is estimated to be discharged directly into the environment, with the rate ranging from 16% in intensive farms to 40% in smallholder farms (Tung, 2017). Therefore, animal waste affects the environment and human health in many aspects including the pollution of surface water, underground water, gas and soil conditions. However, there have been few studies on pollution due to livestock farming in Vietnam so far.

3.4. Market demand and constraints

Viet Nam, a large market with 95 million consumers, has imported a large amount of pork, beef from other countries such as Spain, Australia, etc. It found that in 2011-2017, Vietnam's beef production increased by 6.3%, while consumption rose by 28.6%, which shows that demand is higher than supply. According to Department of Animal Husbandry under MARD (2018), Viet Nam exported animal products such as pork, milk products, salted eggs, and feedstuff to China, Hong Kong, etc. with commercial value of 1 billion USD. Fluctual price of animal products is main constraint.

4. CONCLUSION

In the past decades, livestock has been one of the fastest growing agricultural sub-sectors in Vietnam. Livestock production has rapidly responded to the growing demand for meat, milk, and eggs during the last decades. However, disease problems, environmental pollution and price uncertainty probably are main constraints for livestock development strategy.

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ALTERNATIVES TO ANTIBIOTIC GROWTH PROMOTERS AN "OMIC" APPROACH

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ABSTRACT

The poultry industry is currently facing very many challenges such as the increasing cost of feed ingredients, ban of antibiotic as growth promoters, the antimicrobial resistance and consumers' awareness of residues in meat and meat products. Antibiotic growth promoters are generally used in feeds at sub-therapeutic doses because they suppress pathogenic bacteria and result in improvements in nutrient absorption by birds. Owing to its cheap cost, the frequency of antibiotic use in animal feeds has increased. Unfortunately, this has raised the concern that using antibiotics in animal feeds has increased the ability of pathogenic bacteria to develop resistance to antibiotics. At this juncture, identification of alternatives to antibiotics that mimic their effects has become inevitable. The search for new antimicrobial agents has received a boost in recent years, with natural products playing an important role in this field. In fact, several methods have been proposed to investigate the antibacterial activities of natural products. The recent advances in nutrigenomic studies has brought about a number of new research tools (transcriptomics, proteomics and metabolomics), which are important in animal nutrition research. This review presents an overview on the application of nutrigenomics tools (omics technologies) in elucidating the modes of action of bioactive natural products and understanding about the biological processes and pathways behind different complex traits of chickens.

Keywords: *Nutrigenomics, proteomics, metabolomics, feed efficiency.*

1. INTRODUCTION

New technologies that permit simultaneous monitoring of many hundreds, or thousands, of macro–and small molecules promise to allow functional monitoring of multiple (or perhaps all) key cellular pathways simultaneously. The new "global" methods of measuring families of cellular molecules, such as RNA, proteins, and intermediary metabolites have been termed "- omic" technologies, based on their ability to characterize all, or most, members of a family of molecules in a single analysis (Fig 1). With these new tools, we can now obtain complete assessments of the functional activity of biochemical pathways, and of the structural genetic (sequence) differences among individuals and species, that were previously unattainable. The terms 'Ome' and 'Omics' are derivations of the suffix -ome, which has been appended to a variety of previously existing biological terms to create names for fields of endeavor like genome, proteome, transcriptome and metabolome that are either speculative or have some tangible meaning in particular contexts.

Omics has become the new mantra of molecular biology. 'Omic' technologies include genomics, transcriptomics (gene expression profiling), proteomics and metabolomics. The recent availability of masses of omic data is responsible for the major growth spurt of systems biology. Pharmaceutical companies and others need to make sense out of all this omic information in order to take the next step in overcoming their innovation deficits. Systems biology provides the methods, computational capabilities, and inter-disciplinary expertise to facilitate this jump. The technology platform of genomics, transcriptomics, proteomics and metabolomics are high-throughput technologies. They increase substantially the number of proteins/genes that can be detected simultaneously and have the potential to relate complex mixtures to complex effects in the form of gene/protein expression profiles.

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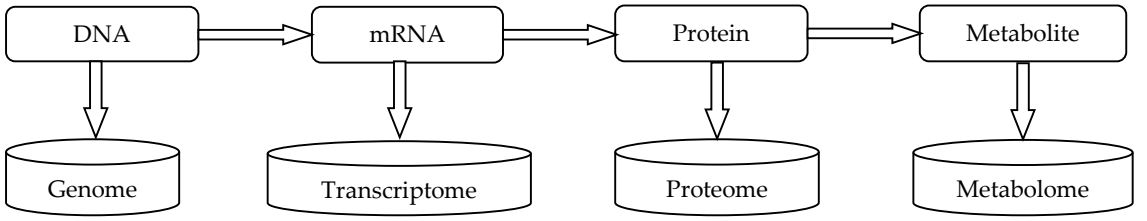


Fig 1. The Central Dogma and the interacting "ome" includes the study of genome, proteome, transcriptome and metabolome

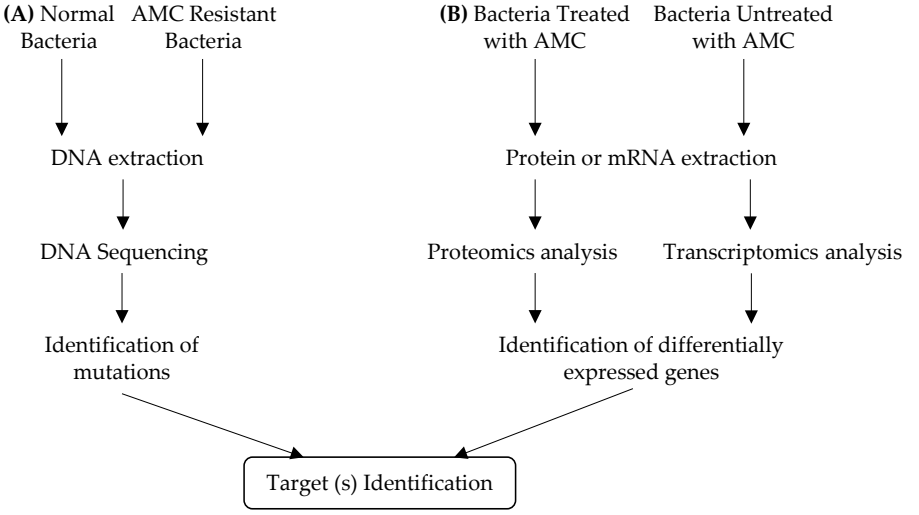
The new omics technologies seem set to fulfill their huge expectations and in combination they might prove extremely valuable in functional gene analyses. Genomic and transcriptomic studies are mostly conducted by DNA microarray technologies. Proteomics and metabolomics have no standardized procedures yet, but usually, proteome analysis is done by two-dimensional gel electrophoresis and Liquid Chromatography-mass spectrometry, while metabolome analysis is conducted through Gas chromatography-mass spectrometry, Liquid chromatography-mass spectrometry and Liquid chromatography-Nuclear magnetic resonance. Usually, these technologies are applied in a "differential display" mode, i.e. by comparing two situations (e.g. diseased versus healthy) in order to reduce the complexity in data by examining only differences (Corthésy-Theulaz *et al*., 2005). Transcriptomics provides the tool for deciphering gene expression networks, and proteomics links these networks to protein products. The third crucial partner is metabolomics, which defines the metabolic network(s) linked to gene expression. NMR and mass spectrometry enable the broad screen analysis of the metabolome and its transformation pathways, transcending classical targeted metabolic studies. It is, however, necessary to further standardize and automate the methods of especially proteomics and metabolomics in order to make efficient and reproducible high-throughput analyses. Omics will not only have an impact on our understanding of biological processes, but the prospect of more accurately diagnosing and treating disease will soon become a reality (Loughlin, 2007).

2. ANTIBIOTIC GROWTH PROMOTERS (AGPS)

Chicken meat represents an important source of animal proteins and fats, as well as a source of a whole range of organic and inorganic nutrients. Demand for processed chicken meat has been growing by 15-20% per annum. This growth in production of chicken meat is due to increasing demand that arises with increase in number of inhabitants and their increasing purchasing power. Moreover, the popularity with regard to chicken meat is because; it is cheaper than other type of meat.

Sub-therapeutic level of antibiotics used as growth promoters in broiler chicken over 5 decades to limit the proliferation of pathogenic bacteria in the gut and thereby to increase the feed efficiency which results in presence of antibiotic residues in poultry products and there by antibiotic resistance in humans necessitate looking for an alternative to antibiotic growth promoters. The alarming spread of bacterial resistance to antibiotics is one of the most serious challenges to global public health, as drug resistance has been found for all classes of antibiotics used in clinical practice (Arias and Murray, 2015). The resistance of bacteria against antibiotics is a growing worldwide concern in the field of animal husbandry, and more importantly in human medicine. Nutrition has a crucial function in animal performance as well as in the maintenance of optimal animal health and welfare status. Specialty feed ingredients used in feed are pivotal contributors to ensuring adequate nutrition and optimal animal welfare. The effects of novel feed additives – such as growth promotion, nutrient quality preservation, mycotoxin mitigation and pathogen prevention–contribute

to a preventive approach that reduces the need for antimicrobials. The rapid advancement of gene sequencing technologies have recently made it possible to investigate a number of related questions regarding antibiotics, such as the prevalence and transmission of antibiotic resistance, as well as the mode of action of antibiotics and feed additives. It is important to understand the cellular mode of action of AGPs in order to develop suitable alternatives and optimize animal nutrition. By using RNA sequencing, it has been confirmed that beyond their antimicrobial effect, AGPs interact with the host tissue and modulate the anti-inflammatory response. A more sustainable method of growth promotion would, for example, modulate the same anti-inflammatory response without contributing to antibiotic resistance



(A) Genomic Approach; **(B)** Proteomic & Transcriptomic Approach; **(C)** Metabolomic Approach

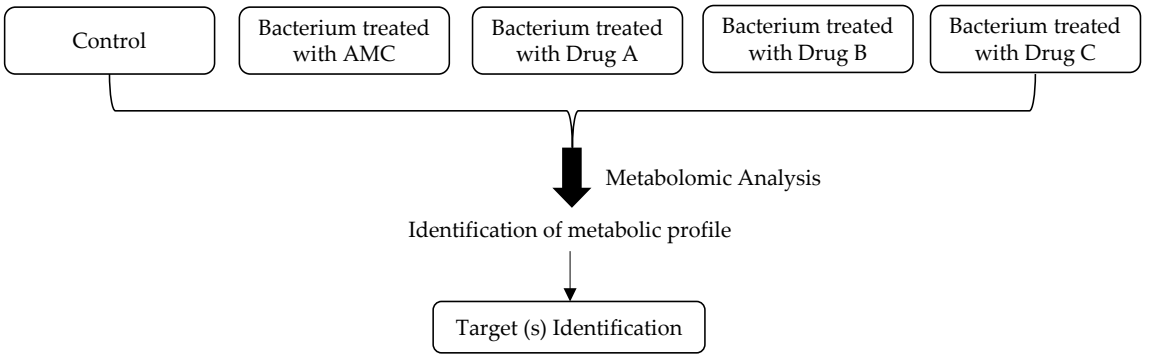


Fig 2. Overview of Omics Technologies Vs Mode of Action of Antimicrobials (AMC)

Among the numerous alternatives like probiotics, prebiotics, acidifiers, the plant origin compound attracts more interest than else. These plant extracts have antimicrobial action, immune enhancement, anti-stress property, antioxidant and gut microflora manipulation, nutrigenomics effects, digestibility enhancer, stress lowering effect, cholesterol-lowering effect, etc (Prabakar *et al*., 2016). With their wide range of activities, these phytobiotics will emerge as a new group of feed additives for better growth rather than simply as another alternate to in-feed antibiotics in food production industry. Among many feed

additives plant leaves, roots, flowers and whole plants are used for the production of phytobiotic products. Products may comprise dried form of whole plants or their parts or extracts of the plants which have shown potential benefits for broiler chicken (Park *et al*., 2014). Traditional knowledge regarding the use of medicinal plants has driven the identification of plant-derived products with different chemical structures (Radulovic *et al*., 2013). The biggest challenge in identification and isolation of antimicrobial phytochemicals now, is the characterization of their mechanism of action (MOA), which is essential to support their use as lead molecules for drug-development programs (Swinney and Anthony, 2011).

Recent advances in omics technologies have provided crucial insights into the MOA of antimicrobial compounds. An overview of application of omics technologies in the assay of mechanism of action of natural antimicrobial compounds (AMC) is presented in Fig 2.

3. RECENT ADVANCES IN ANTIBIOTICS RESEARCH

The recent advances in genetic sequencing technologies have resulted in the identification of a range of genes related to essential processes for bacterial survival, virulence, and mutagenesis. These genes and their products are therefore considered as potential new antimicrobial targets (Roemer and Boone, 2013). This knowledge is of utmost importance to the development of new screening platforms for identification and selection of novel compounds targeting specific essential genes based on protein interactions and mutant libraries (Zhang *et al*., 2015; Morita *et al*., 2016). Several studies support that the MOA of an antibiotic is related to the interaction of multiple pathways. Thus, techniques for the analysis of gene expression on a large scale (transcriptional analysis) have been widely applied to evaluate the mechanisms action of an antibiotic, including those derived from medicinal plants.

Microarrays have been extensively used to elucidate the mechanism of action of several phytochemicals. To highlight some of them, licochalcone A isolated from *Glycyrrhiza inflata* (Fabaceae), shows different pharmacological activities, including the inhibition of *S. aureus* biofilm formation and planktonic cells. The transcriptional profile of this pathogen treated with licochalcone A was evaluated by a microarray assay. Significant alteration in expression of several genes was observed after licochalcone A treatment, especially those related to autolysis, cell wall, pathogenic factors, protein synthesis, and capsule synthesis (Shen *et al*., 2015). The transcriptional changes induced by sub-inhibitory doses of pentacyclic triterpenoids (such as α-amyrin, betulinic acid, and betulinaldehyde) in a methicillin-resistant *S. aureus* strain were analyzed by microarray, showing that they act by targeting pathways such as cell division (FtsZ), ABC transporters (OPP-1C), fatty acid (fabz), and peptidoglycan (fmhB, PBP2) biosynthesis, DNA replication (ccrA), two-component regulatory system, and β-lactam resistance (mecR1). The proposed MOA was by inhibition of cell growth by destabilization of the bacterial cell membrane combined with the cessation of protein and fatty acids synthesis.

RNA-Sequential studies have also been performed to evaluate the MOA of some plant-derived products. The molecular pathways involved in the action of ursolic acid (a pentacyclic triterpenoid) and resveratrol (a polyphenol found in red wine) against the biofilm produced by methicillin-resistant *S. aureus* was analyzed by this method. The anti-biofilm action of ursolic acid was found to be related to a reduction in the expression of genes involved in amino acid metabolism and adhesin expression. Conversely, resveratrol was found to act through the inhibition of pathways related to quorum sensing, surface proteins, and capsular polysaccharides (Qin *et al*., 2014).

The second molecular (proteomic and Transcriptomic) approach has not been adopted extensively in the study of the antibacterial MOAs of plant-derived compounds. In a recent study, the effects of tea polyphenols (TP; a mix of polyphenols extracted from green tea) on protein expression in *P.aeruginosa* and *Serratia marcescens* were investigated. The results revealed that the metabolic disorder caused by TP treatment was associated with an increase

in the amount of membrane proteins, such as chaperones and key enzymes involved in the control of membrane component biosynthesis pathways. These data supported a possible MOA for TP: increased membrane permeability leading to release of cellular components.

Metabolomic approach refers to the study of the global profile of metabolites present in a biological system under certain conditions and time. "Metabolites" refers to a range of end products of cellular processes that belong to different classes, viz., organic acids, amino acids, fatty acids, sugars, sugar alcohols, steroids, nucleic acid bases, etc. Metabolomic approaches have been widely adopted to study the responses of microorganisms to various environmental stressors such as heavy metals, temperature, and organic compounds, and they are suitable tools for the study of metabolism disorders in microorganisms treated with antibiotics (Aliferis and Jabaji, 2011). On the contrary, the application of metabolomic approaches for investigating the mechanisms of action of plant-derived compound is still largely unexplored.

4. ALTERNATIVES TO ANTIBIOTICS-NUTRITIONAL PERSPECTIVE

As of now, our understanding of the mechanisms of action of sub -therapeutic antibiotics can be summarized as follows; (i) inhibition of sub -clinical infections, (ii) reduction of growth-depressing microbial metabolites, (iii) reduction of microbial use of nutrients, and (iv) enhanced uptake and use of nutrients through the thinner intestinal wall associated with antibiotic-fed animals. All these mechanisms share the common postulate that intestinal bacteria, whether commensal or pathogenic, depress animal growth, either directly or indirectly, through their metabolic activities (Collier *et al.*, 2003). It has already been established that imprudent use of feed grade antibiotics is neither desired nor acceptable in animal production due to the development of antibiotic resistance and its possible ramifications on human health.

If the use of antibiotics in animal feed is considered to be unacceptable, then it is imperative to find alternatives which will serve the same purpose of improving animal performance and maintaining the health status of the animal. As discussed earlier, there are a number of alternatives that are commonly used in place of antibiotics, viz., probiotics, prebiotics (non-digestible oligosaccharides), organic acids, enzymes and, modifiers of microbial activity (herbs). These alternatives are effective in part or entirely in mimicking the mechanisms of antibiotics. It may be possible to use these alternatives in combination to achieve the same effect that antibiotics have. With the advancement in research, it is possible to discover many other potential compounds which can replace antibiotics in feed (Verstegen and Williams, 2002).

5. ANTIBIOTIC ALTERNATIVES IN FEED EFFICIENCY OF BROILERS: OMICS APPROACH

Feed efficiency (FE) represents one of the most important and complex traits in poultry production, since up to 70% of total production cost are attributed to feed. Many efforts were made to understand the molecular aspects in different tissues of broiler chickens which may exert a huge effect on the overall expression of FE phenotype.

In chickens, muscle being one of the main metabolic organs, the bioenergetics processes within the muscle can deeply influence FE in broilers. As mitochondria are responsible for producing around 90% of the energy pool for cells, studies have been conducted to evaluate whether the expression of different FE phenotypes would be associated with differences or inefficiencies in muscle mitochondria structure and functionality. Gene level studies showed differences in the expression of genes involved in mitochondria biogenesis [peroxisome proliferator-activated receptor-γ (*PPAR-γ*), PPAR-γ coactivator-1α (*PGC-1α*) and inducible nitric oxide synthase (*iNOS*)] and energy metabolism [avian adenine nucleotide translocator (*avANT*), cytochrome oxidase III (*COX III*), and avian uncoupling protein (*avUPC*)] in breast muscle of birds revealing either high or low FE (Ojano-Dirain *et al.*, 2007). Regarding the physiological aspects, the activity of mitochondria complexes I, II, III, and IV has been reported to be higher in breast

muscle of high FE birds compared to low ones. Recently, the up-regulation of genes associated with electron transport chain (ETC) complex I, as well as the greater predicted activity of complex I, III, IV and V (Kong *et al*., 2011) in breast muscle of high FE birds, seem to confirm an overall increased activity of mitochondrial complexes in the high FE phenotype.

When breast muscle global mRNA expression was assessed using a microarray-based approach (Kong *et al*., 2011), high FE birds were characterized by an up-regulation of genes either involved in anabolic processes (protein packaging and scaffolding activity, purine and pyrimidine biosynthesis, prevention or delay of apoptosis and modulation of gene transcription), or related to major signal transduction and cascade mechanisms pathways [Protein kinase-A (PKA), c-Jun NH(2)-terminal protein kinase (Jnk), retinoic acid and retinoid X receptor (RAR-RXR)] or in sensing the energy status and regulating energy production in the cell [Adenosine monophosphate AMP-activated protein kinase (AMPK) and protein kinase AMP-activated non-catalytic subunit gamma 2 (PRKAγ2)]. At the same time, high FE birds showed down-regulation of genes associated with cytoskeletal organization, as well as cyto-architecture and integrity-related genes, major histocompatibility complex cell recognition, stress-related heat shock proteins and several platelet derived growth factor genes. Of late, the biological basis of the differences between high and low FE chickens investigated through mRNA-seq and pathways analysis identified a total of 1,059 differentially expressed genes between high and low FE chickens. High FE birds had a greater expression of genes related to muscle development, hypertrophy and remodeling, as well as a decreased expression of protein degradation and atrophy-related genes. These results, associated with the predicted activation of growth hormone and insulin-like growth factor-I/phosphatidylinositol 3-kinase/protein kinase B (IGFs/PI3K/Akt) signaling pathways, might explain the higher breast yield observed in high FE birds.

Adipose tissue plays a central role in energy homeostasis being a metabolically active organ with endocrine and regulatory functions. Low FE chickens showed higher lipid accumulation, which was likely determined by the up-regulation of genes involved in lipid synthesis, as well as down-regulation of genes enhancing triglyceride hydrolysis and cholesterol transport from adipose tissue. The gut is also considered as one of the most important tissues able to influence the expression of different FE phenotypes due to its function in nutrient digestion and absorption, as well as for its immunological role. Ojano-Dirain *et al*. (2007) found a higher level of oxidized proteins in duodenal mucosa homogenate and duodenal mitochondria of low FE birds. On the other hand, higher mRNA expression of *PPAR-γ* and *iNOS* was observed in the duodenum of high FE birds, whereas no significant difference was reported for *PGC-1α*, *avANT* and *COXIII*.

6. CONCLUSIONS

The "omic" technology platform has the potential to relate complex mixtures to complex effects in the form of gene/protein expression profiles. By their nature, these technologies reveal unexplored properties of biological systems. Advances in "omics" technologies have the potential to revolutionize our approach to development of novel non-antibiotic (natural) growth promoters (NAGP). However, the promise of rapid advances in NAGPs "from the lab desk to the bird desk" has not manifested as of yet. In reality great progress has been made, however understanding processes such as antibiotic response and resistance requires systematic insight into dynamic differences in gene regulation, interaction and function. Although each of the analytical platforms provides very useful outputs, they are able to describe only a part of the entire biological picture if considered singularly. Each protein, regardless its role and form, expresses a function that assumes significance only in the context of all the other functions and activities also being expressed in the same cell. Therefore, the next step should be focussed to integrate all the information obtained by the different omics platforms using appropriate bioinformatics and statistical tools. This relatively new approach would provide a holistic overview of the

entire biological system rather than its single component alone.

In summary, the new omics technologies seem set to fulfill huge expectations. However, it is necessary to further standardize and automate the methods of especially proteomics and metabolomics in order to make efficient and reproducible high-throughput analyses.

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STRATEGIES IN DEVELOPMENT OF VACCINE AGAINST SWINE ETEC

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) remains a leading cause in pigs, especially for piglets and post-weaning one. Vaccination is considered an effective, economic, and practical prevention approach against swine ETEC. However, developing ETEC vaccines has encountered technical challenges, mainly because of virulence factor heterogeneity and diversity, poor immunity of key virulent toxins and/or adhesive factors, and tolerance tendency in intestines for orally administrated antigens. Combination and/or fusion of key ETEC's virulent toxins and/or adhesive factors using protein engeneering for multivalent vaccine development not only helps in reducing multible injections, facilitating broadly effective protection, but also enhancing poor immunity of key virulent toxins and/or adhesive factors. Besides, targeting immune cells in gut (M cells) not only reduces immunotolerance but also lowers required antigen amount to mount a proper, protective mucosal immunity. By exploiting DNA manipulation, we deveploped fusion antigen comprising of STb fused LTB, F18 fused F4 in order to increase immunity of STb and F18, repesctively, as well as generating a recombinant barker yeast harboring M-cell targeting peptide fused F18 on its surface for immunological evaluation and protection against ETEC on animals. The preliminary data laid a ground work for a swine's budget, broadly effective oral vaccine development subsequently

Keywords: *ETEC, F4, F18, LTB, oral vaccine, STb, vaccine development.*

1. OVERVIEW

1.1. Postweaning diarrhea associated Enterotoxigenic *Escherichia coli*

Intestine, which digests and absorbs nutrients, is an important organ of the gastro intestinal tract. However, it contacts frequently with a large number of pathogenic microorganisms. Therein, enterotoxigenic *E. coli* (ETEC) is a leading cause of postweaning diarrhea in pigs (PWD). The disease affects piglets during two week after wearing, causing of sudden death or diarrhea and growth retardation in surviving piglets (Fairbrother *et al*., 2007). In addition to ETEC, there are the others causes such as the sow's pregnant condition, times of reproduction, a great deal of food and its quality.

Enterotoxigenic *Escherichia coli* causes increased secretion of water, electrolytes, water absorption, leading to dehydration, diarrhea and death. Morbidity associated with this disease may reach 50% of piglets. Mortality can be up to 30% (Rhouma *et al*., 2017). PWD is cause of economic losses to the global pig industry globally, loss of 20,000 dollars/pig herd each year.

1.2. Toxins and adhensives

Enterotoxigenic *Escherichia coli* are characterized by the production of toxins and adhesives (Nagy and Fekete, 2005). Toxins isolate mainly is LT (31.9%), STa (38.1%) and STb (59.1%), these toxins affect the intestinal epithelium of pigs, cause mild to severe diarrhea and lead to death. (Fairbrother *et al*, 2007). The adhesives are include: F4, F5, F6, F18, F41, and high rate of them found are F4 (45.1%) and F18 (33.9%). The adhesion of F4 and F18 depends on F4R and F18R -specific receptors (Nagy and Fekete, 2005). Usually, PWD is associated with both F4 and F18, while preweaning diarrhea is only associated with F4 (Fairbrother *et al*., 2007).

1.3. Adhesives

The first step in infected process is adhering fimbriae to its ligand on the intestinal epithelium, allowing ETEC to survive and cause diarrhea. Therefore, this is an essential step in the pathogenesis process.

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Fimbriae F4 is polyadhesin, includes the major subunit FaeG, and minor subunits FaeF, FaeH, FaeC, and FaeI (Van *et al*., 2000). FaeG is two roles by both structure and adhesion function (Kraehenbuh and Neutra, 2000). There are three different F4 variants: F4ab, F4ac and F4ad. F4ac is the most important variant found in PWD. F4ad strongly adhere with glycolipids, F4ab and F4ac bind well to glycoproteins. Studies have demonstrated that F4 fimbriae have carbohydrate binding specificities (Grange *et al*., 2002). There is cross-reactivity between these variations using F4 vaccine.

Fimbriae F18 is fibrillae (1 to 2mm fibers) basing on a major structural protein FedA, and minor subunits FedB, FedC, FedE, FedF. FedF is role in binding to receptor, which is highly conserved in different strains from different countries (Smeds *et al*., 2001; Tiels *et al*., 2005). F18 exists in two types of antigen variants: F18ab and F18ac, F18ac is often associated to ETEC, causing PWD, while F18ab is associated with edema (Byun *et al*., 2013). There is no cross- reaction between F18ab and F18ac using vaccine (Bertschinger *et al*., 2000).

1.4. Toxins

After binding to intestinal epithelial, ETEC begin secreting heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). Moreover, there is a small amount of toxins EAST1 (Zhang *et al*., 2006). These toxins increase the secretion of water, electrolytes and limit the absorption of water in intestinal epithelial cells. There is no damage or morphological changes in intestinal tissue morphology due to the effects of these toxins. No damage or morphological changes in intestinal tissue cause from the effects of these toxins.

LT toxin is a protein consisting of a bioactive A subunit and B five subunits. The A subunit is enzyme linking with B subunits by non-covalent interaction. B subunits bind to mono sialotetrahexosyl ganglio side (GM1) on intestinal surface (Dubreuil *et al*., 2016). Therefore, LT toxicity depends on the binding of B subunits to GM1. Lately, subunit B has been shown to be associated with blood group regulatory factors (Vasile *et al*., 2014). Therewith, LT can be a role in supporting bacterial adhesion with GM1.

ST toxin consists of two variants, STa and STb. Therein, STb is the major toxin in PWD (Harada *et al*., 2005). STb receptors are acidic glycosphingolipid (Rousset *et al*., 1998). STb is a rapid action toxin. The reaction occurs after 30 minutes and reaches the threshold after 3 hours (Hitotsubashi *et al*., 1992). The anti-STb antibodies can neutralize STb toxicity STb but not toxins STa (Hitotsubashi, 1992).

2. TREATMENT AND PREVENTION

2.1. Treatment

The current popular treatment is antibiotics (Table 1). According to recent studies, ETEC isolated from PWD is resistant to many antibiotics, including colistin - the ultimate solution for diseases caused by multidrug-resistant gram infections (Table 2) (Kempf *et al*., 2013).

Reporting of colistin resistance in *E. coli* and detection of horizontal transfer mechanism of colistin resistance requires us have to quickly develop next-generation antibiotics or to develop ways to prevent: vaccine (Harada *et al*., 2005; Nordmann and Poirel, 2016).

Table 1. Examples of resistance rates to different antibiotics of E. coli strains isolated from healthy and diseased pig in different countries (%) (Aarestrup *et al*., 2008)

Antibiotic	USA	Brazil	Korea	China	Spain	Belgium	Germany	France	Poland
Fluoroquinolones	0	30	64.9	64	14	39	8	6	30
Ceftiofur	22	-	-	-	4	1	1	1	-
SXT	23	62	75.7	90	-	71	51	66	78.8
Neomycin	66	32.8	-	9.4	20	2	-	11	-
Apramycin	30	-	-	-	13	13	10	3	-
Gentamycin	48	39	77	57	20	46	12	6	45

Table 2. Examples of colistin resistance in E. coli isolate from healthy and diseased pigs in different countries

Country	Origin of the isolates	% resistance/ non wild type strains
France	faeces, healthy pigs	0.5%
Sweden	healthy pigs	0%
Denmark	healthy pigs	0%
Belgium	pigs with diarrhea	9.6%
Croatia	pigs with diarrhea	3%
Brazil	pigs with diarrhea	28.1%
UK	Slaughterhouse, healthy pigs	34.1%
China	pigs with diarrhea	33.3%

2.2. Prevention, a limit for F18 vaccine development

There are three vaccine types used for PWD. Firstly, intramuscular injection is used to stimulate systemic immunity (Van, 1999). Secondly, oral vaccine of live attenuated or live wild-type non-enterotoxigenic *E. coli* strains carrying the fimbrial adhesins is currently exploited (Fairbrother *et al*., 2017). Thirdly, purified fimbriae, instead of the whole bacteria which are administrated orally (Verdonck *et al*., 2004). These types have succeeded in creating an immune response against F4, and are now commercially available in Canada and Europe.

Vaccine against F18 has been developed but cannot mount a sufficient protection for piglets from PWD. F18 uses FedF subunit as adhensive moiety. However, FedF is a minor subunit having weak interaction with FedA, easily lysed in intestinal environment when it exists alone (Tiels *et al*., 2007; Tiels *et al*., 2008). That's the reason why it's hard to create anti-FedF antibodies. Meanwhile, anti-F18 antibodies which have only anti-FedA cannot prevent ETEC adhesion to intestinal epithelium. When ETEC's F18 clings to intestinal epithelium, it is less affected by anti-F18 antibodies. Recently, there is an oral vaccine for FedF carried by F4, but it cannot create an immune response to FedF. Because the immune response of F4 is too strong that affects the creation of anti-FedF antibodies (Tiels *et al*., 2008).

2.3. Vaccine strategies

Developing ETEC vaccines has encountered technical challenges, mainly because of virulence factor heterogeneity and diversity, poor immunity of key virulent toxins and/or adhesive factors, and tolerance tendency in intestines for orally administrated antigens.

Combination and/or fusion of key ETEC's virulent toxins and/or adhesive factors using protein engineering for multivalent vaccine development not only helps in reducing multiple injections, facilitating broadly effective protection, but also enhancing poor immunity of key virulent toxins and/or adhesive factors like F18. Besides, targeting immune cells in gut (M cells) not only reduces immunotolerance but also lowers required antigen amount to mount a proper, protective mucosal immunity.

The enhancement of immunity can be achieved by transporting antigens to immune competent cells, M-cell. In addition to guiding to the target, it also helps to limit immune tolerance and reduce antigen amount. M cells with special structures are responsible for collecting and transporting antigens (Kraehenbuh and Neutra, 2000). The number of M cells in gastrointestinal tract is very low (5-10%), thus requiring means to target M cells via receptors on their cell surface. Ligands receptors on M cells are discovered (Kim and Jang, 2014).

Table 3. M-cell-binding ligands and M-cell-specific molecules

Ligand type	Ligand	Receptor on M cell
Lectin	Galectin-9	N-Glycans
	AAL	α-L-Fucose
Monoclonal antibodies	NKM 16-2-4	α1,2 Fucose
	3G7-H9	Glycoprotein 2
Protein is derived from microorganisms	FimH	Glycoprotein 2
	Hsp60	PrPC
Peptide	Co1	C5a
	C-terminus of *Clostridium perfringens* enterotoxin	Claudin-4

With the advancement of recombinant protein technology, peptide and protein-based ligands are preferable in order to fuse key ETEC's virulent toxins and/or adhesive factors with M cell targeting peptide/protein (Fig 1).

The second technical challenge is to deliver vaccine to its required destination. Carrier system such as liposome, virus-like particles requires sophisticated technical facilities and long-term investment, thus increasing a higher cost for production (Davitt *et al.*, 2015). To overcome these difficulties delivery system based on Generally Recognized As Safe (GRAS) organisms like lactic-acid bacteria or barker yeast has been extensively exploited. Thanks to recombinant protein technology, these organisms, notably *Lactobacillus* spp. or *Saccharomyces cerevisiae*, are modified to be able of expressing foreign proteins on their surface. By expressing M cell targeting peptide and/or antigen(s) on the surface combining with antigen(s) in cytoplasm, these cells can be use directly as oral vaccine (Fig 1). *Lactobacillus* per se requires a long time growth as well as rich culturing media thereby increasing production cost. In sharp contrast, barker yeast *S. cerevisiae* also requires a basic medium to grow with a shorter culturing time than *Lactobacillus*. Besides, barker yeast is also well-known for its nutrient rich composition. Thus, the organism is the most suitable antigen delivery system for oral vaccine development, especially for farmed pig.

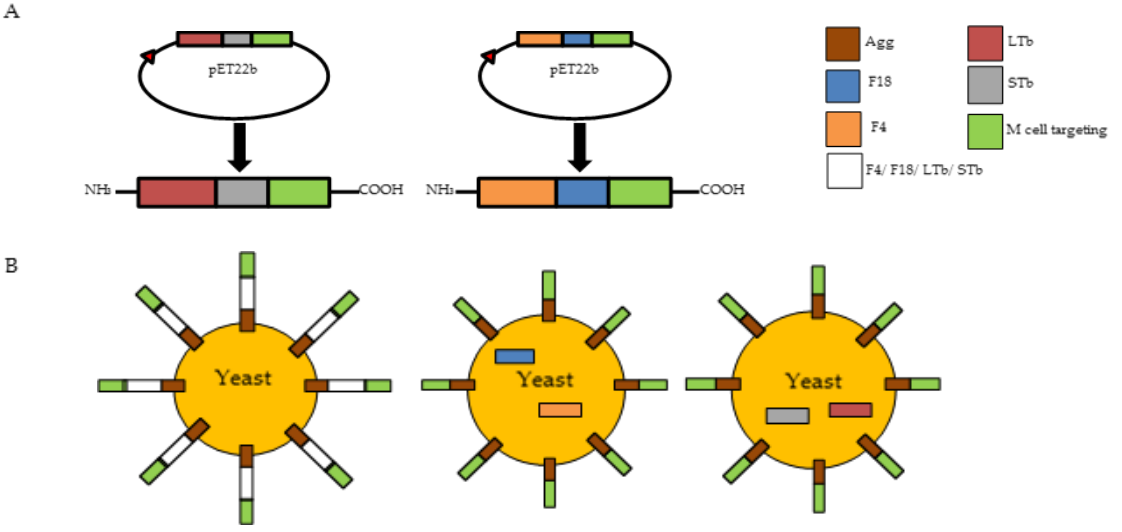

Fig 1. Strategies for broadly effective oral vaccine development

A. fused key ETEC's virulent toxins and/or adhesive factors with M cell targeting peptide/protein by recombinant protein technology; B. M cell targeting peptide and/or antigen(s) expressing on yeast surface combining with antigen(s) expressing on the surface and/or in cytoplasm used as oral vaccine

3. CONCLUSION

Despite current scientific and technological advances, pig industry still faces PWD, the causative of significant economic losses. Antibiotics have contributed significantly to reducing the losses, but this leads to an increment in antibiotic resistant ETEC. Therefore, development of preventive approach is unmet needed. Current vaccines only provide protection from ETEC's F4, while F18 remains ineffective. By exploiting recombinant protein technique on barker yeast *S. cerevisiae* in combination with M cell targeting approach, we could provide a comprehensive solution for an affordable oral ETEC vaccine with broad spectrum of protection for pig.

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DETECTION OF PORCINE PARVOVIRUS (PPV) IN PIGS IN CENTRAL PROVINCES OF VIET NAM

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ABSTRACT

In order to detect and type of porcine parvovirus (PPV), PCR assay was developed and applied on porcine lung samples in this study. Each of target genes of PPV1, PPV2 and PPV3 was amplified using the specific primers. Total of 146 lung/blood samples collected from several slaughter houses belonged to seven provinces of central part of Vietnam were tested, which are Quang Binh, Quang Tri, Thua Thien Hue, Quang Nam, Da Nang, Quang Ngai and Binh Dinh. Overall prevalence of singular PPV1, PPV2 and PPV3 were 52.7% (77/146), 56.2% (82/146) and 5.5% (8/146), respectively. The results also showed that the PPV1, PPV2 and PPV3 were co-circulated while the PPV1/PPV2 co-infection was predominant of 34.2% (50/146). The rates of co-infection of PPV1/PPV3 and PPV2/PPV3 were similarly lower with 4.1 and 4.8%, respectively. Only 3.4% (5/146) of pigs was positive with three genotypes PPV1/PPV2/PPV3. The prevalence of PPV genotypes was consistent with sequencing results. It proved that convenient PCR assay could be used as a differential diagnostic tool for monitoring and control of PPVs in the field. The result of this study confirmed the circulation of the PPVs infection in pigs in central Vietnam.

Keywords: *Detection, PCR, prevalence, PPV genotype, Vietnam.*

1. INTRODUCTION

Porcine parvovirus (PPV) is a member of the genus *Parvovirus* and the family of *Parvoviridae* (Wilhelm *et al*., 2006). PPV is a small non-enveloped, round shape, 18-26nm in diameter virus, that causes porcine reproductive failure in swine (Mengeling *et al*., 2000). The linear, negative, single-stranded DNA genome of PPV has a number of unique characteristics with a molecular size of about 5-6.3kb (Molitor *et al*., 1983). PPV's genome is characterized by a hairpin structure at two 5'-3' ends (Bergeron *et al*., 1996; Tattersal *et al*., 2006) and two open reading frames (ORF) coding for non-structural protein (NSP) and viral coat and capsid protein (VP). PPV is stable to environmental factors, living in a pH range of 3.0-8.0 and is heat resistant for hours at 80°C (Choi *et al*., 1987).

PPVs can be presented in pigs of all ages. The effects of disease are mainly in sows. Clinical signs of PPV-infected sows are characterized by miscarriage, premature birth, and fetal wood rejuvenation with many different sizes, embryonic death and impaired fertility. This disease was characterized by stillbirth, mummified fetuses, embryonic death, and infertility (SMEDI).

Worldwide, PPV was discovered for the first time in Munich, Germany in 1965 in animal cell culture when cultivating classic fever viruses in pigs (Mayr and Mahnel, 1964). Currently, several complete genomic sequences of PPPV strain are available in Genbank, and seven PPV subtypes (from 1 to 7) have been circulated around the world and are the main cause of reproductive failure in pigs (Lau *et al*., 2008; Cheung *et al*., 2010; Huang *et al*., 2010; Xiao *et al*., 2013a; Ni *et al*., 2014; Cui *et al*., 2016; Chen *et al*., 2018).

In Vietnam, the SMEDI syndrome caused by PPV has been interest since the early 1990s. The disease causes great losses in breeding sows. Currently, PPV disease in Vietnam is effectively controlled by inactivated PPV vaccine. Survey on seroprevalence of PPVs in Long An province revealed that PPV is an important factor affecting pig's fertility decline (Huynh *et al*., 2000). The circulation of PPV1, 2 and 3 was confirmed from different slaughterhouses in Bac Giang, Hoa Binh, Hanoi, Ha Tinh provinces. The results also showed the relatively high prevalence of identical

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PPV genotypes and co-infection of two and three genotypes of PPV in those locations (Cuong, 2018). In this study, the genotype prevalence and co-infection of PPVs were first investigated in seven provinces belong to central Vietnam.

2. MATERIALS AND METHODS

2.1. Samples

A total of 157 lung and blood samples were collected from commercial slaughterhouses in seven provinces of central Vietnam from May 2018 to April 2019. The numbers and types of samples distributed from each provinces were recorded as below (Table 1).

Table 1. Sample information collected in 7 provinces

Province	Type of sample	Number of sample
Quang Binh	Blood	17
Quang Tri	Lung	22
Thua Thien Hue	Lung	28
Da Nang	Lung, blood	14
Quang Nam	Lung, blood	16
Quang Ngai	Lung	24
Binh Dinh	Lung	25
Total		146

2.2. Methods

Total DNA was extracted and purified according to the methods of Sambrook and Russell (2001) with some modifications. DNA was diluted in TE buffer, and stored at 4°C for analysis.

The primer pairs were designed by Primer software 3 based on reference sequences listed below. Information on primer sequence, annealing temperature (Ta) and product size were shown in Table 2.

Table 2. Primer information

Genotype	Sequence (5′-3′)	Ta (°C)	Size of PCR product (bp)	Reference
PPV1	F: GGGAGGGCTTGGTTAGAATCTC R: ACCACACCCCCCATGCGTTAGC	54	194	*MF447833*
PPV2	F: AGATTCTTGCAGGCCGTAGA R: CCAAGGGTCAGCACCTTTTA	50	222	*KM926355*
PPV3	F: GCAGTCTGCGCTTAACTT R: CTGCTTCATCCACTGGTC	60	392	*KY586145*

A total of 20µl PCR reaction contained 10µl Dream Taq Master Mix (Thermo Scientific), 10µmol primer pairs (forward and reverse), and 100ng total DNA.

The thermal cycle for a PCR reaction was carried out at an initial denaturation temperature of 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 seconds, annealing at 54/50/60°C for 25 seconds, 72°C for 30 seconds, final synthesis at 72°C for 5 minutes and holding at 14°C.

PCR products were checked on agarose gel electrophoresis and spectrophotometer at 260/280nm. Amplicons were purified and sent to Macrogen (Korea) for sequencing. Sequence analysis was carried out with BioEdit version 7.0.9.0 (Hall, 1999). Obtained nucleotide sequences were identified with the Basic Local Alignment Search Tool (BLAST, NCBI) (Altschul *et al*., 1990).

3. RESULTS AND DISCUSSION

3.1. Detection PPV genotypes

At OD 260/280 ratio from 1.84 to 2.16, the quantity and quality of total DNA were sufficient for subsequent study. The PCR products for genotyping of PPV1, PPV2 and PPV3 are shown in Fig 1, 3 and 5, respectively. In Fig 1, single clear band was observed in wells 3-8, corresponding to positive control (+) with molecular size of 194 bp. That means those samples were positive of PPV1 genotype. No DNA band appeared at negative control (-) and negative samples (well 1-2). These results have been futher checked by DNA sequencing.

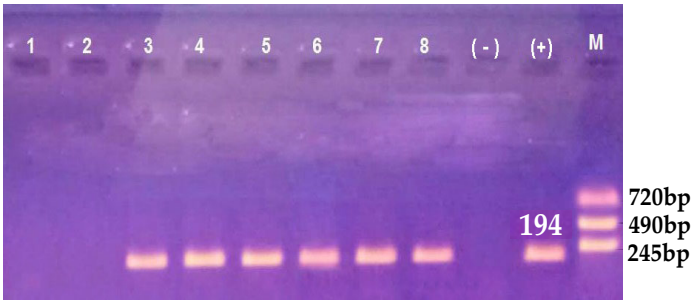

Fig 1. Agarose gel electrophoresis of PPV1 genotyping PCR product (194bp)

M: DNA marker; 1-2: negative samples; 4-8: positive samples; (-): negative control; (+): positive control with DNA band of 194 bp

Porcine parvovirus strain NADL2 capsid protein gene, complete cds
Sequence ID: KF049424.1 Length: 2263 Number of Matches: 1

Range 1: 688 to 884 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
359 bits(194)	2e-95	196/197(99%)	0/197(0%)	Plus/Plus

```
Query  1    GGGGGAGGGCTTGGTTAGAATCTCTGCACACGCATCAAGACTCATACATCTAAATATGCC  60
            ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  688  GGGGGAGGGCTTGGTTAGAATCACTGCACACGCATCAAGACTCATACATCTAAATATGCC  747

Query  61   AGAACACGAAACATACAAAAGAATACATGTACTAAATTCAGAATCAGGGGTGGCGGGACA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  748  AGAACACGAAACATACAAAAGAATACATGTACTAAATTCAGAATCAGGGGTGGCGGGACA  807

Query  121  AATGGTACAAGACGATGCACACACACAAATGGTAACACCTTGGTCACTAATAGATGCTAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  808  AATGGTACAAGACGATGCACACACACAAATGGTAACACCTTGGTCACTAATAGATGCTAA  867

Query  181  CGCATGGGGAGTGTGGT  194
            |||||||||||||||||
Sbjct  868  CGCATGGGGAGTGTGGT  884
```

Fig 2. BLAST result revealed the positive sample with 99% nucleotide identity compared with reference PPV1 strain (*KF049424*)

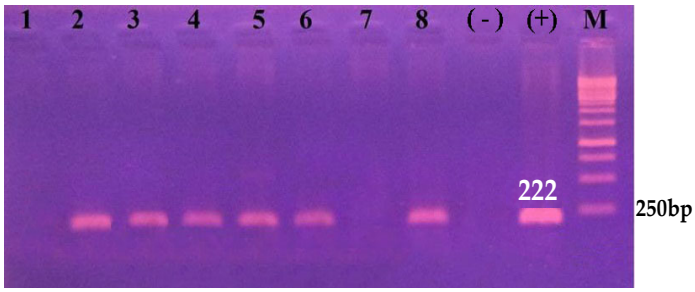

Fig 3. Agarose gel electrophoresis of PPV2 genotyping PCR product (222bp).

M: DNA marker 250bp; 1, 7: negative samples; 2-6, 8: positive samples; (-): negative control; (+): positive control with DNA band of 222bp

Porcine parvovirus 2 isolate SERUM-SMU non-structural protein and structural protein genes, complete cds
Sequence ID: MG345019.1 Length: 5438 Number of Matches: 1

Range 1: 828 to 1035 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
357 bits(193)	8e-95	204/209(98%)	1/209(0%)	Plus/Plus

```
Query  17   TAGATTCTTGCAGGCCGTAGACTGGCTGGTAGACAATGGCTGCTGCACCGAGGCCAAGTG  76
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  828  TAGATTCTTGCAGGCCGTAGACTGGCTGGTAGAGAATGGCTGCTGCACCGAGGCCAAGTG  887

Query  77   GATCGAGATGGACAAGATGGGATATCGTTCTTTCATGTCCACACTCTCAAGGGGTGCTGC  136
            |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct  888  GATCGAGATGGACAAGATGGGATATCGTTCTTTCATGTCCAC-CTCTCAAGGGGTGCTGC  946

Query  137  AGGTGAAGAACGCTCTCAACCTTGCACGCAGGGAACTCGTAGCAGGGAACAAACTGTTGG  196
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  947  AAGTGAAGAACGCTCTTAACCTTGCACGCAGGGAACTCGTAGCAGGGAACAAACTGTTGG  1006

Query  197  AGAGCATAGTAAAAGGTGCTGACCCTTGG  222
            |||||||||||||||||||||||||||||
Sbjct  1007 AGAGCATAGTAAAAGGTGCTGACCCCTGG  1035
```

Fig 4. BLAST result revealed the positive sample with 98% nucleotide identity compared with reference PPV2 strain (*MG345019*)

The expected band represented for PPV1 genotype was purified and sequenced. Obtained nucleotide sequence of 194bp in length was identified by BioEdit software. As shown in Fig 2, there was 99% nucleotide identity between gene sequences obtained from PPV1 positive samples and the reference sequence *KF049424* representing the PPV1 strain (NADL2) isolated in Germany in 2013. Sequencing result confirmed positive PPV1 samples from PCR result.

Similar results were obtained with PPV2/PPV3 detection and genotyping. As pressented in Fig 3 and 5, single clear bands withexpected molecular size of 222 bp and 392 bp were detected in positive samples of PPV2 and PPV3, respectively.

The expected bands represented for PPV2/PPV3 genotypes were purified and sequenced. Obtained nucleotide sequences of 222 bp and 392 bp in length were identified as PPV2 and PPV3 by BioEdit software, respectively. As shown in Fig 4 and 6, the BLAST analyses also revealed the high nucleotide identities (98%) between PPV2/PPV3 sequences and reference strains (*MG345019/MG345026*). Sequencing results were consisted with PCR tests for PPV2/PPV3 positive samples.

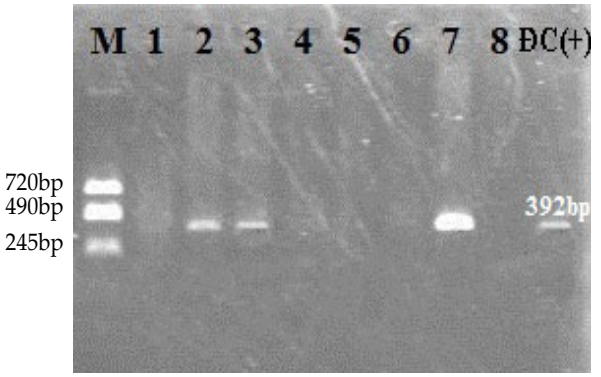

Fig 5. Agarose gel electrophoresis of PPV3 genotyping PCR product (392bp); M: DNA marker; 1, 4- 6: negative samples; 2, 3, 7: positive samples; (-): negative control; (+): positive control with DNA band of 392bp

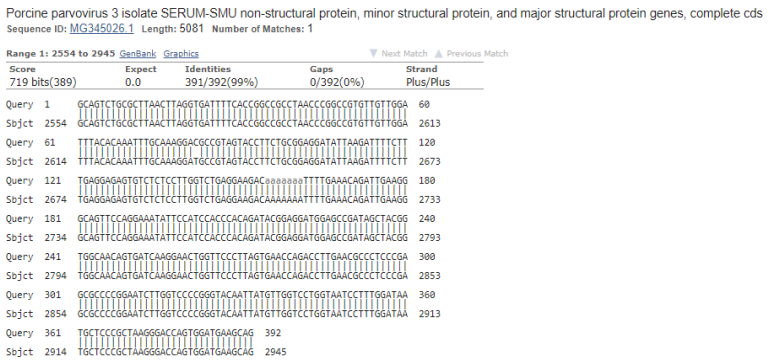

Fig 6. BLAST result revealed the positive sample with 98% nucleotide identity compared with reference PPV3

3.2. Prevalence of PPV genotypes

The prevalence of PPV genotypes in seven provinces in Central Vietnam was summarized in Table 3. Out of 146 samples, 103 samples (70.6%) were positive for at least one PPV genotype. Overall, the prevalence of PPV2 is the highest with 56.2% (82/146), followed by PPV1 at 52.7% (77/146), while the prevalence of PPV3 genotype was only 5.5% (8/146). Considering to the geographic location, samples from Quang Tri, Hue and Quang Ngai were positive with all three PPV genotypes. Quang Binh and Quang Nam occurred only PPV2 genotype, in which 100% (17/17) pigs collected from Quang Binh was positive to PPV2. PPV2 was appeared at all provinces, except Da Nang. The results also showed that there was no occurrence of PPV3 genotype in four provinces such as Quang Binh, Da Nang, Quang Nam and Binh Dinh.

Table 3. Prevalence of PPV genotypes in seven provinces

Location	n	Prevalence of PPV genotype (%)		
		PPV1	PPV2	PPV3
Quang Binh	17	0	100 (17)	0 (0)
Quang Tri	22	81.82 (18)	45.45 (10)	13.6 (3)
TT Hue	28	85.7 (24)	71.4 (20)	10.7 (3)
Da Nang	14	50.0 (7)	0 (0)	0 (0)
Quang Nam	16	0	37.5 (6)	0 (0)
Quang Ngai	24	62.0 (15)	75 (18)	8.33 (2)
Binh Dinh	25	52.0 (13)	44 (11)	0 (0)
Total	146	52.7 (77)	56.2 (82)	5.5 (8)

The investigation on PPV serotyping in Long An province showed that the positivity was 69% in sow (Nguyen Huynh *et al*., 2000). This result indicated the relatively high of occurrence of PPVs in pigs in southern Vietnam.

The overall prevalence of PPV1 genotype observed in our study (52.7%) was similar to that of commercial pig herds in Thailand (53.0%) (Saekhow and Ikeda, 2015). The prevalence of PPV1 detected in Japan and Germany were 67 and 61%, respectively, which was not much different in comparison to that of our study (Saekhow *et al*., 2016; Streck *et al*., 2012) Whereas, study of Sun *et al*. (2015) and Oppressing *et al*. (2014) showed the lower circulation of PPV1 in China and North America with 5.56 and 8.93%, respectively.

In our study, PPV2 was predominant with 56.2% (82/146). Similarly, high PPV2 prevalence of 78% and 83% were confirmed in swine herds in Germany (Streck *et al*., 2012), in Thailand (Saekhow and Ikeda, 2015). Lower positive rates were reported in North America with 36.8%) and in Poland with 19% (Opriessnig *et al*., 2014; Cui *et al*., 2016).

For PPV3, the circulation of genotype in pigs from Asia countries was relatively high, such as, 73% in Thailand (Saekhow and Ikeda, 2015) and 45.11% in China (Sun *et al*., 2015), whereas PPV3 appeared at lower rate in Europe, for example 20% in Germany, 7.7% in Poland (Streck *et al*., 2012; Cui *et al*., 2016), 19.1% in Slovakia (Sliz *et al*., 2015). The detection rate of PPV3 genotypes in this study was much lower at 5.5%.

The result of co-infection of PPV genotype in pig from central provinces was presented in Table 4. As shown in table 4, no co-infection of PPV genotypes detected in three provinces Quang Binh, Da Nang and Quang Nam. Only co-infection of PPV1/PPV2 was observed in Binh Dinh with 28% (7/25). Overall, the highest co-infection rate of PPV1/PPV2 was 34.2% (50/146). The rates of infection of PPV1/PPV3 and PPV2/PPV3 were similarly lower with and 4.1 and 4.8%, respectively. Only 3.4% (5/146) was positive with three genotypes PPV1/PPV2/PPV3.

Table 4. Co-infection of PPV genotypes in pigs

Location	n	PPV co-infection (%)			
		PPV1+2	PPV1+3	PPV2+3	PPV1+2+3
Quang Binh	17	0 (0)	0 (0)	0 (0)	0 (0)
Quang Tri	22	40.91 (9)	13.6 (3)	9.1 (2)	9.09 (2)
TT Hue	28	67.9 (19)	7.14 (2)	10.71 (3)	7.14 (2)
Da Nang	14	0 (0)	0 (0)	0 (0)	0 (0)
Quang Nam	16	0 (0)	0 (0)	0 (0)	0 (0)
Quang Ngai	24	62.5 (15)	4.2 (1)	8.33 (2)	4.17 (1)
Binh Dinh	25	28 (7)	0 (0)	0 (0)	0 (0)
Total	146	34.2 (50)	4.1 (6)	4.8 (7)	3.4 (5)

In the study of Cadar *et al*. (2013), co-infection rate of two or more PPV genotypes were quite high, in which, co-infection of PPV2/PPV3 was dominant with 79% (31/39) in swine and 95% (169/177) in wild pigs. PPV2 appearing in co-infected samples of other PPV genotypes was most common with 80.2% (65/81) observed in six commercial pig farms in Poland (Cui *et al*., 2016). In some Asian countries, such as Japan and Thailand, co-infection rate of PPV genotype 1, 2, 3 and 4 in pig herds in Japan were 93, 78, 53 and 22%, respectively (Saekhow *et al*., 2016). In Thailand, the prevalence of co-infections from three genotypes was 61% and co-infection with four PPV genotypes was also quite high at 28% (Saekhow and Ikeda, 2015). Taken together, results on PPV co-infection showed that co-infection rate of two and three PPV genotypes observed in our study was lower than that of other studies.

This is the first report on prevalence and co-infection of PPV1-3 genotypes in central provinces of Vietnam. This result is also proved that convenient PCR assay could be used as a differential diagnostic tool for monitoring and controlling PPVs in the field.

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PURIFICATION AND CHARACTERIZATION OF ENDOGLUCANASE FROM *BACILLUS SUBTILIS* S20

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ABSTRACT

The study on Purification and characterization of endoglucanase from *Bacillus subtilis* S2O was carried out to establish a purification procedure for endoglucanase and determine its characters. Broth incubated with *Bacillus subtilis* S2O at 38°C in an anaerobic condition for 5 days was collected and then centrifuged to remove bacterial cells and substrate. Endoglucanase was precipitated by ammonium sulfate and purified by ion exchange chromatography on Uno Sphere Q gel. The fraction of 70 and 80% saturation of ammonium sulfate exhibited the highest specific activity, 105 and 119 (U/mg), respectively. These fractions were further purified in next step. The F1 fraction, eluted by 0.12M NaCl on Uno Sphere Q gel, had the highest specific activity of 720 U/mg and 0.24mg protein in total. The yield and purity level were 24.3% and 23, respectively. The purified endoglucanase can be two isozymes with molecular weight of 79.6 and 74kDa. The purified enzyme had optimum temperature at 60°C and optimal pH 8. Endoglucanase activity was activated by K^+ ion up to 148% when compare to the control treatment (100%) and was inhibited by Mn^{2+}, Fe^{3+} and Cu^{2+}. Endoglucanase exhibited highest activity against CMC. However, it also had ability to hydrolyze crystalline forms of cellulose such as avicel and filter paper but it was too low.

Keywords: *Bacillus subtilis* S2O, *characterization, endoglucanase, purification, substrate specificity.*

1. INTRODUCTION

Cellulose is unarguably the most abundant natural organic substance on the earth and is the predominant component throughout the plant kingdom; therefore it could be manipulated for the benefit of mankind. The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria also have been isolated and characterized. These enzymes cleaving the β-1,4-glycosidic bond of cellulose belong to the large family of glycosyl hydrolases. Cellulase is a multicomponent enzyme, consists of three major components: endoglucanase, exoglucanase and β-glulcosidase. Currently, cellulase is widely used in controlling industrial slime (Wiatr, 1990), research for generation of protoplast (Liu and Zhu, 2000) and for creation of antibacterial chitooligosacchrides (Tsai *et al*., 2000). In specific cases, for example in medical uses, enzymes must be purified and characterized to ensure safe and effective uses. Besides that, cellulases from different origins have different specificities and mode of actions. Therefore, purification is an important step to study and apply cellulases. Among bacteria, *Bacillus* spp. can produce numbers of extracellular cellulases.

2. MATERIALS AND METHODS

Bacillus subtilis S2O isolate obtained from the Biochemistry Lab, Biotechnology R&D institute, Can Tho University.

Culture medium: liquid medium (Ryckeboer, 2003), modified by Bui Thi Thien Thanh (2010).

Ion exchange chromatography: Uno Sphere Q gel was packed into a column (1.5x15cm). The column was then equilibrated with 2 column volume of Tris-HCl buffer (20mM, pH 8.75). The protein was loaded through the column at a flow rate 1ml/min. This step allows the small particles in the solution to be washed out. After loading, the unbound proteins were washed away with the same buffer in the reverse flow direction.

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The bound protein was eluted by linear gradient of 0-1M NaCl in Tris-HCl buffer (20mM, pH 8.75) at the flow rate of 1 ml/min.

Determination of protein concentration: Protein concentration was determined by the method of Bradford (1976). The protein concentration was calculated based on the standard curve of BSA (bovine serum albumin) with concentrations in a range from 30-300 µg/ml.

Determination of enzyme activity: Endoglucanase activity was determined by Nelson method (1944). The enzyme activity was calculated based on the standard of glucose with the range of glucose concentration from 0-1mM.

3. RESULT AND DISCUSSION

3.1. Purification of endoglucanase by fractional precipitation with ammonium sulfate

As showed in Table 1, the crude extract began to precipitate at 60% saturation of ammonium sulfate. The fraction of 60% saturation of AS had the highest total protein compared with other fractions. However, total activity of this fraction was too low (17U). Fraction of 90% saturation of AS had no endoglucanase activity. Only two fractions 70% and 80% saturation AS had endoglucanase activity of 218 and 140U, respectively.

Table 1. Purification of endoglucanase by fractional precipitaion with ammonium sulfate

Sample	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Crude extract	1,000	22.6	708	31.3
30%	0	0	0	0
40%	0	0	0	0
50%	0	0	0	0
60%	24	3.97	16.9	4.29
70%	20	2.09	218	105
80%	17	1.17	140	119
90%	34	1.21	0	0

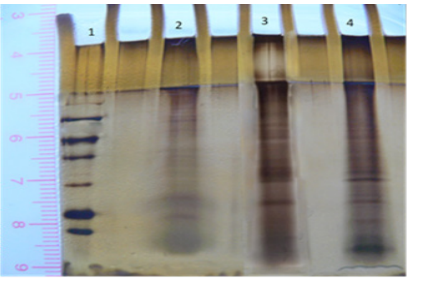

Fig 1. SDS-PAGE of AS precipitate fractions

Lane 1: standard protein, lane 2: crude extract, lane 3: fraction of 70%, lane 4: fraction of 80%

As indicated on Fig 1, there were many bands on lanes of these fractions with molecular weight lower than 25kDa. This result revealed that these fractions still contained many kinds of protein and was not purified. Therefore, these fractions would be applied to next purification step to further remove contaminants and get higher purity.

3.2. Purification of endoglucanase by ion exchange chromatography

From Fig 2, it was concluded that most of contaminant was excluded after ion exchange chromatography on Uno Sphere Q gel through fractions UB, F2, F3, F4 and F5. These fractions explained for 0.48mg protein in total, however, it exhibited no activity.

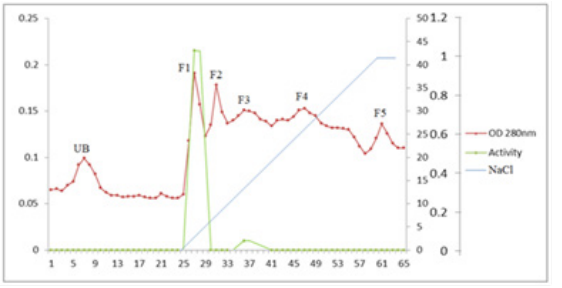

Fig 1. Chromatogram on Uno Sphere Q gel

After applying to ion exchange chromatography on Uno Sphere Q gel, the fraction 70 and 80% ammonium sulfate were separated into 6 fractions (Table 2). Among these fractions, only fraction F1 had significant activity of 43 U/ml. This fraction was eluted by 0.12M NaCl and had total protein, specific activity of 0.24mg, 720 U/mg, respectively.

Table 2. Result of chromatography on Uno Sphere Q gel

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	NaCl (M)
UB	3.2	0	0	0	0
F1	4	0.24	172	720	0.12
F2	3.2	0.13	0	0	0.22
F3	3.2	0.08	5.6	72.7	0.42
F4	3.2	0.19	0	0	0.84
F5	3.2	0.07	0	0	1

Table 3. Summary of the purification procedure

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity (fold)
Crude extract	22.6	708	31.3	100	1
AS 70-80%	3.26	358	110	50.5	3.51
Unosphere Q (F1)	0.24	172	720	24.3	23

The recovery and purification fold were 24.3% and 23, respectively (Table 3). Fig 3 (a and b) revealed that the purified endoglucanase may include two kinds of endoglucanase with molecular weight (MW) of 88.5, 79.6 or 74kDa. However, Bacilli endoglucanase have MW ranging from 35-82kDa (Park *et al*., 1991; Han *et al*., 1995; Kim *et al*., 1995 and Mawadza *et al*., 2000). From the above, therefore, it was concluded that the purified endoglucanase had MW of 79.6 and 74kDa. These MW was higher than some other endoglucanases obtained from *Bacillus* spp. such as 54kDa from CH43 and HR68 (Mawadza *et al*., 2000) or 32.5kDa from *Bacillus subtilis* YJ1 (Yin *et al*., 2010) and was similar with that of *Bacillus pumilus* (67 kDa) (Christakopoulos *et al*., 1999). The two endoglucanases could be the isozyme of each other because they have the same band's shape and density.

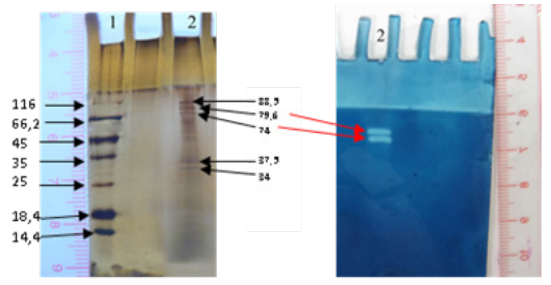

Fig 3. SDS-PAGE of endoglucanase

(a) Silver stain, (b) Activity stain. Lane 1: standard, lane 2: purified endoglucanas

3.3. Effect of temperature on activity of purified endoglucanase

As indicated in Fig 4, the purified endoglucanase showed activity from 30 to 80°C. The optimal temperature of it was found to be 60°C. This result was similar to that of *Bacillus subtilis* YJ1 (Yin *et al*., 2010). The optimum temperature of the enzyme was lower than that of some other Bacillus strains [65°C (CH43) and 70°C (RH68)] but higher than *Bacillus cereus* (55°C) (Yan *et al*., 2011). The purified enzyme retained about 40% of its activity at 70°C and it was almost completely inactivated at temperature higher than 80°C.

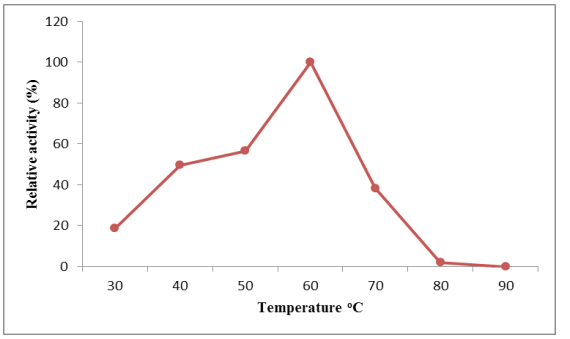

Fig 4. Effect of temperature on activity of purified endoglucanase

3.4. Effect of pH on activity of purified endoglucanase

The effect of pH on purified endoglucanase activity was clarified on Fig. 5. *Bacillus subtilis*

S2O endoglucanase exhibited high activity at pH from 7.5-8.5 and the highest activity at pH 8. A study of Yan *et al*. (2011), carried out on *Bacillus cereus*, reported the same result. This optimal pH was higher than pH 6-6.5 from *Bacillus subtilis* YJ1 (Yin *et al*., 2010). In acidic environment (pH<5) the purified endoglucanase had no activity. It was also inhibited at pH above 9.

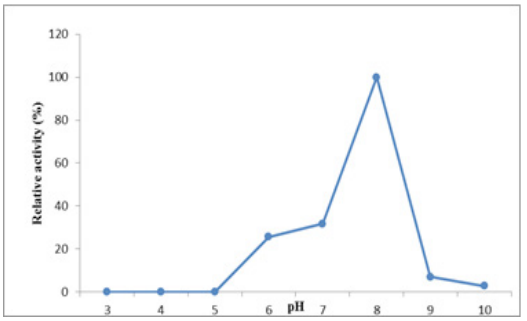

Fig 5. Effect of pH on activity of purified endoglucanase

3.5. Effect of metal ions on activity of purified endoglucanase

As indicated in Table 4, only K^+ ion activated the purified endoglucanase activity up to 148% when compare to control treatment (100%). The K^+ ion was also determined to have ability to increase endoglucanase activity from other strain such as *Bacillus subtilis* YJ1 (Yin *et al*., 2010). However, K^+ ion did not affect endoglucanase activity from some other Bacillus strain such as *Bacillus cereus* (Yan *et al*., 2011) or *Bacillus subtilis AU-1* (Chan and Au, 1987). In contrast to K^+, Mn^{2+}, Fe^{3+} and Cu^{2+} inhibited endoglucanase activity and the relative acitivity was 81.3%, 51.9% and 41.2%, respectively. The same result was reported by Mawadza *et al*. (2000), Vijayaraghavan and Vincent (2011) and Yan *et al*. (2011).

Table 4. Effect of metal ions on activity of purified endoglucanase

Ion	Relative activity (%)
None	100
Mn^{2+}	81.3
K^+	148
Fe^{3+}	51.9
Cu^{2+}	41.2

* *The counter ion of these metals is sulfate*

3.6. Substrate specificity of purified endoglucanase

The purified enzyme showed the highest activity against CMC. This result confirmed that the purified enzyme was an endoglucanase. Its activity against crystalline forms of cellulose such as avicel and filter paper was also observed (15.2 and 10.7%, respectively). Similar results were obtained with some other bacterial strains, Bacillus CH43, HR68 (Mawadza *et al*., 2000) and *Bacillus subtilis* YJ1 (Yin *et al*., 2010). The purified endoglucanase had no activity against cellulose acetate. This substrate, the acetate ester of cellulose, requires an esterase to cleave the ester bond in first step of hydrolysis process.

Table 5. Substrate specificity of purified endoglucanase

Substrate	Relative activity (%)
CMC	100
Avicel	15.2
Cellulose acetate	0
Filter paper	10.7

4. CONCLUSION

The purification of endoglucanase in this study was conducted to purity through two steps, first step, ammonium sulfate precipitate and second step, ion exchange chromatography on Uno Sphere Q gel with the yield and purity of 24.3% and 23, respectively. The purified endoglucanase had specific activity of 720 U/mg and and may consist of two isozymes with the molecular weight of 79.6 and 74kDa.

The purified endoglucanase exhibited the highest activity against CMC at 60^{o}C, pH 8 with the activation of K^+ ion. It also had activity against crystalline forms of cellulose such as avicel and filter paper.

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CONTAMINATION OF SALMONELLA IN RETAIL VEGETABLES IN THE MEKONG DELTA, VIETNAM

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ABSTRACT

From July 2017 to March 2018, a total of 358 retail vegetables were collected to clarify the contamination of *Salmonella* in the Mekong Delta, Vietnam. *Salmonella* was isolated from 58 (16.2%) of 358 samples. The isolation rate of *Salmonella* from retail vegetables in the rainy season was significantly higher than that in the dry season, 20.9% and 7.3% respectively. Among of *Salmonella* isolates, *Salmonella* Weltevreden was the most predominant serovar (29.5%) identified from retail vegetable in all of the wet markets, followed by *S*. Derby (8.2%), *S*. Lexington (3.3%), *S*. Worthington (3.3%). All *S*. Weltevreden isolates (100%) were susceptible to 9 antibiotics examined (ABPC, OTC, CP, NA, CEZ, SM, KM, GM, and OFLX). As peoples in Southeast Asian countries have a habit to eat raw vegetables, they could be infected with *Salmonella* via consumption of *Salmonella*-contaminated vegetables. Thus, retail vegetables were considered as the important vehicle of *Salmonella* transmission to humans in the Mekong Delta.

Keywords: *Mekong Delta, retail vegetables, Salmonella, wet markets*.

1. INTRODUCTION

Salmonella is recognized worldwide as an important foodborne and human zoonotic pathogen. The infectious route of *Salmonella* is mainly via oral infection, person to person transmission (Thompson *et al*., 2013). The consumption of *Salmonella*-contaminated food such as meat, vegetables, fruits, and drinking water is the available route to cause human salmonellosis. The raw vegetables are considered as an important source of foodborne pathogens, including *Salmonella* (Olaimat and Holley, 2012; Park *et al*., 2012). Losio *et al.* (2015) indicated that fresh leafy vegetables and "ready-to-eat" vegetables retailed in supermartkets or farm markets in Italy were contaminated with multiple pathogens including *Salmonella*, *Listeria*, *E. coli* O157:H7, thermotolerant *Campylobacter*, *Y. enterocolitica*, and norovirus. Consumption of fresh vegetables, juices were associated with 5.0% of foodborne outbreaks in the EU in 2012. *Salmonella* spp. and viruses were the most common causative agents (in total 48.7%) of reported cases for both pathogens (EFSA, 2014). As peoples in Southeast Asian countries have a habit to eat much raw vegetables, they could be infected with *Salmonella* via consumption of *Salmonella*-contaminated vegetables. However, few reports have been published regarding to the role of vegetables as a source of human *Salmonella* infection in these countries. In the present study, the contamination with *Salmonella* in retail vegetables was examined to know the role of retail vegetable as the vehicle of *Salmonella* transmission in the Mekong Delta, Vietnam.

2. MATERIALS AND METHODS

2.1. Sample collection

From July 2017 to March 2018, a total of 358 retail vegetables were collected in the wet markets of Can Tho city in the Mekong Delta, Vietnam. Among the total of 358 samples, 235 vegetable samples were collected in the rainy season (from April to November) and 123 samples were in the dry season (from December to March). Retail vegetable were purchased in wet markets and brought to the laboratory in the cold condition. The contents of the retail vegetable samples were shown in Table 1.

2.2. Isolation and identification of *Salmonella* in retail vegetables

About 25g of each vegetable sample was suspended in 225m*l* of EEM broth (Eiken, Tokyo, Japan) for pre-enrichment at 37°C for

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24h. After that, 1 ml of the pre-enrichment broth was transferred to 9 ml of Hajna tetrathionate broth (Eiken) for further incubation. A loopful of enrichment broth was then streaked on the selective media mannitol lysine crystal violet brilliant green agar (MLCB, Nissui, Tokyo, Japan) and deoxycholate hydrogen sulfide lactose agar (DHL, Nissui) to incubate at 37°C for 24h. After that, these suspected *Salmonella* colonies on these media were identified by biochemical characteristics with triple-sugar iron agar (TSI, Nissui), VP medium (Eiken) and lysine indol motility medium (LIM, Nissui). *Salmonella* isolates identified were serotyped according to the White–Kauffmann–Le Minor scheme (Popoff, 2001) with commercial O and H antisera (Denka Seiken, Tokyo, Japan).

2.3. Antimicrobial susceptibility of *S.* Weltevreden isolates

Salmonella Weltevreden isolates from retail vegetables were examined for the antimicrobial susceptibility. Disk diffusion method was carried out according to the Clinical Laboratory Standards Institute (CLSI) procedure M02-M07 (CLSI, 2016). A total of 9 antibiotic agents that are regularly used for treatment to animals and humans in Southeast Asian countries, were used in this study including ampicillin (ABPC, 10μg), oxytetracycline (OTC, 30μg), chloramphenicol (CP, 30μg), nalidixic acid (NA, 30μg), cefazolin (CEZ, 30μg), streptomycin (SM, 10μg), kanamycin (KM, 30μg), gentamycin (GM, 10μg), and ofloxacin (OFLX, 5μg). The antibiotic disks purchased from Becton Dickinson (BD, USA) were used in this study.

2.4. Data analysis

The data were tested by Chi-square (χ^2) and Fisher's exact test with 95% confidence intervals.

3. RESULTS

The isolation rate of *Salmonella* in retail vegetables was shown in Table 1. Of 358 vegetable samples, *Salmonella* was detected from 58 samples (16.2%). No significant difference in the prevalence of *Salmonella* among vegetable species was observed. However, a significant difference in the prevalence of *Salmonella* was observed between the rainy (20.9%) and the dry season (9.1%) (P<0.01).

Table 1. Contamination of *Salmonella* in retail vegetables in the Mekong Delta, Vietnam

Season	Vegetable species		No. of samples	No. of *Salmonella* positive samples (%)
	Common name	Nomenclature		
Rainy season	Mustard green	*Brassica juncea*	31	7 (22.6)
	Water spinach	*Ipomoea aquatica*	29	6 (20.7)
	Long coriander	*Eryngium foetium*	30	5 (16.7)
	Rice paddy herb	*Limnophila aromatica*	28	8 (28.6)
	Mint	*Mentha arvensis*	29	5 (17.2)
	Sweet basil	*Ocimum basilicum*	28	5 (17.9)
	Green leaf lettuce	*Lactuca sativa*	20	3 (15.0)
	Water dropwort	*Oenanthe javanica*	20	6 (30.0)
	Watercress	*Nasturium officinale*	20	4 (20.0)
		Subtotal	235	49 (20.9)[a)]
Dry season	Crisphead lettuce	*Lactuca sativa var. acephala*	60	5 (8.3)
	Cutting lettuce	*Lactuca sativa var. capitata*	63	4 (6.3)
		Subtotal	123	9 (7.3)
Total			358	58 (16.2)

[a)] *Rainy season > Dry season (P<0.01)*

The distribution of *Salmonella* serovars from retail vegetables was shown in Table 2. Of 61 *Salmonella* isolates from retail vegetables, 9 *Salmonella* serovars were identified. Among them, the most predominant serovar was *S.* Weltevreden (29.5%), followed by *S.* Derby (8.2%), *S.* Lexington (3.3%) and *S.* Worthington (3.3%). All 18 *S.* Weltevreden isolates (100%) originated

from retail vegetables showed susceptibility against 9 antibiotics examined in this study.

Table 2. Serovars of *Salmonella* isolated from retail vegetables in the Mekong Delta, Vietnam

Serovar	No. of isolates	%
S. Weltevreden	18	29.5
S. Derby	5	8.2
S. Lexington	2	3.3
S. Worthington	2	3.3
S. Bareilly	1	1.6
S. London	1	1.6
S. Saintpaul	1	1.6
S. Typhimurium	1	1.6
S. Virchow	1	1.6
Other serovars	29	47.5
Total	61	100

4. DISCUSSION

A few reports concerning about *Salmonella* contamination in vegetables have been published. Ni *et al.* (2018) reported that 4.5% of lettuce sold in markets was positive for *Salmonella* in Shanghai, China. Quiroz-Santiago *et al.* (2009) reported that *Salmonella* was isolated from 7% of lettuce and watercress in the supermarket in Mexico. In Malaysia, *Salmonella* was contaminated in 100% of water dropwort, 83.3% of Asiatic pennywort and 32% of water spinach in the wet markets (Salleh *et al.*, 2003; Najwa *et al.*, 2015). In the present study, retail vegetables in the wet markets in the Mekong Delta were contaminated with *Salmonella* at a relatively high rate (16.2%). Moreover, *Salmonella* was detected from all types of retail vegetables and all wet markets in this study. These results indicated retail vegetables sold in the wet markets were widely contaminated with *Salmonella* in the Mekong Delta, Vietnam.

S. Typhimurium or *S.* Enteritidis was the most common serovar isolated from vegetables in China and Mexico (Quiroz-Santiago *et al.*, 2009; Ni *et al.*, 2018). In contrast, *S.* Weltevreden was the predominant serovar (29.5%) in retail vegetables in the Mekong Delta. Lertworapreecha *et al.* (2013) isolated *S.* Weltevreden from vegetables at a high rate (20%) in the wet markets in Thailand. Vegetables could be contaminated with *Salmonella* from many sources including from the wastewater, human, wild animals, etc. distributing in the markets (Melloul *et al.*, 2001; Salleh *et al.*, 2003; Islam *et al.*, 2004; Taban and Halkman, 2011). Furthermore, the isolation rate of *Salmonella* in retail vegetables in the rainy season was higher than that in the dry season in this study. As our hypothesis, the differences between two seasons might be affected by environmental temperature and activities of wild animals in these markets. Further research should be done to clarify that reason.

Moreover, *S.* Weltevreden was reported as one of the most predominant serovars isolated from salmonellosis patients in Southeast Asian countries (Bangtrakulnonth *et al.*, 2004; Vo *et al.*, 2006; Ly *et al.*, 2010; Thong *et al.*, 2011; Tu *et al.*, 2017). Foodborne outbreaks due to *S.* Weltevreden were also reported in India (Antony *et al.*, 2009) and in Réunion Island, France (D'Ortenzio *et al.*, 2008). These results indicated that vegetables contaminated with *Salmonella*, especially *S.* Weltevreden could be emerging vehicles causing salmonellosis for human in this region.

All *S.* Weltevreden isolates in retail vegetables in the Mekong Delta show susceptibility to 9 antibiotics examined in this study. *S.* Weltevreden isolated from lettuce in Thailand was also sensitive to ampicillin, tetracycline, chloramphenicol, cefotaxime, and norfloxacin (Niyomcheda *et al.*, 2016). Lee *et al.* (2008) reported that *S.* Weltevreden isolated from vegetables in Malaysia showed the resistance to streptomycin (15%), followed by cephalothin (12%), ampicillin (9%), and gentamycin (9%). The difference of antimicrobial susceptibility might be due to the difference of sampling places as well as *S.* Weltevreden isolates distributed in those regions. Further investigation should be done to clearly determine the antimicrobial susceptibility of this serovar isolated from vegetables in the Mekong Delta.

5. CONCLUSION

As fresh raw vegetables are usually eaten in Southeast Asian countries, vegetables

are considered as the important source of *Salmonella* infection to humans in the Mekong Delta. Therefore, food hygiene should be paid more attention to prevent *Salmonella* infection, especially *S*. Weltevreden from vegetables to consumers in this region.

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DUCK COLIBACILLOSIS IN THE MEKONG DELTA: A LOOK AT EPIDEMIOLOGY

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ABSTRACT

An investigation on epidemiology of duck collbacillosis was carried out on 224 colibacillosis duck flocks from 1 city and 4 provinces in the Mekong delta. The study results showed that duck colibacillosis was popular in the Mekong delta and caused significant losses in duck production with quite high morbidity (9.09%) and mortality (5.86%) in population, death percentage of diseased ducks was very high up to 64.44%. The disease happened throughout the year, but number of outbreaks was lowest at the end of year (November and December). All duck ages were susceptible to colibacillosis, 1 to 30 day old ducklings were most frequently affected, but the biggest losses were reported in ducks at the ages of 31 to 60 days. Running ducks and ducks for eggs had higher morbidity and mortality than those of ducks for meat and captive reared ducks. Ducks raised in small scales (50-200 birds) or big scale (>5,000 birds) had higher morbidity and mortality than those of ducks raised in medium ones. Prevention methods such as disinfection, vaccination, antibiotic using and deworming showed to be important factors in reducing losses caused by colibacillosis in ducks. Examining the influence of preventive monofactor on duck colibacillosis status, deworming showed to be the most important factor in reducing the severity of colibacillosis status when it happened in ducks with the lowest morbidity and mortality (4.31 and 0.98%). The results also showed that flocks getting combination of disinfection, vaccination and deworming (D-V-Dw) had lowest morbidity and mortality (2.01 and 0.55%). These epidemiological data are useful for planning effective strategy in disease control and prevention.

Keywords: *Duck, colibacillosis, Mekong delta, morbidity, mortality.*

1. INTRODUCTION

Avian colibacillosis is a complicated disease with many localized and systemic infections caused by avian pathogenic *Escherichia coli* (APEC). Nowadays, there is general agreement that avian colibacillosis is one of the leading causes of mortality and morbidity associated with economic losses in the poultry production throughout the world (Sojka, 1965; Barnes *et al.*, 2008; Roshdy *et al.*, 2012). Economic losses can be due to decreased hatching rates, decreased egg production, mortality, lowered production, carcass condemnation at slaughter and costs associated with treatment and prophylaxis. Good understanding of its epidemiology will assist veterinarians and poultry raisers in tentative diagnosis and having an early response in reducing and eliminating this disease. Duck production is well developed in the Mekong delta (MD) of Viet Nam, accounting for 48.3 % of the poultry population in Vietnam (FAO, 2008). *E. coli* infection in ducks was recognized as a popular and important duck disease in the Mekong delta (MD) with 74.50% of ducks in Long An was infected (Nguyen Trong Phuoc, 1997) and mortality of *E. coli* infection ducks could be high as 40-50% (Nguyen Xuan Binh *et al.*, 2000). So this study was carried with aims to understand the epidemiological characteristics of duck colibacillosis from the diseased flocks in MD.

2. MATERIAL AND METHODS

Epidemiology of duck colibacillosis was studied by analyzing epidemiological information of 224 duck flocks which were diagnosed being affected by Escherichia infection by clinical signs, macro-lesions and laboratorial testing (Ho Thi Viet Thu *et al.*, 2019). These flocks were surveyed from Can Tho city, Hau Giang, Vinh Long, Dong Thap and Tra Vinh in whole year of 2017. Epidemiological data of these diseased flocks were collected by retrospective survey questionnaires and direct interviews from duck raisers. Data of colibacillosis duck flocks

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which were used for this study are information on number of outbreaks occurred by month in the year, ages, breeds of diseased ducks, duck population, number of diseased ducks and number of deaths in colibaccillosis flocks, mode of duck production, desinfection, vaccination, using or without using antibiotics or/and helminthics,...These information were used for analyzing some epidemiological characteristics of duck colibacillosis in the Mekong delta (MD) as below:

The seasonal characteristic was analyzed basing on the distribution of duck colibacillosis outbreaks by month in a year. Age of ducks affected was analyzed basing distribution of diseased flocks by periods of age (1-30, 31-60, 61-90 and older than 90 day old), morbidity and mortality of duck flock population, and mortality of diseased flocks in each period. So on, the influences of modes of duck raising, ducks breeds, size of production and preventive method application (disinfection, antibiotic using, vaccination, and deworming) also were analyzed by the same ways

The data obtained were analyzed by Minitab software 13.2 (Ryan *et al*., 2000), using Goodness to fit test for analyzing the flock distribution and Chi square test to assess significant differences in morbidity and mortality of colibacillosis ducks.

3. RESULTS

3.1. Distribution of duck colibacillosis outbreaks by month in the year

Duck colibacillosis were reported in all months of the year. The highest number of outbreaks was found in Mars (37 outbreaks) and the lowest one was discovered in November (4 outbreaks). Until now, there have had any report on seasonal characteristic of duck colibacillosis. Chicken colibacillosis is supposed to occur all year round, especially in the end of autumn and winter (Barnes *et al*., 2008). However, seasonal characteristic of a disease depends on many factors such as the regional weather and climate, as well as mode of animal raising. The result in Table 1 showed no significant difference between the numbers of ill flocks occurred by month (P=0.829). The seasonal variability in duck colibacillosis incidence was unobvious since the climate of MD ranges from temperate to tropical and often humid that is suitable for the survival of *E.coli* in the environment. The lower outbreak numbers were in November and December because the climate of this time is temperate and lacking rain. Hence, ducks were seldom suffered from rain stress when running in the rice fields or heat stress when being transported by narrow boats. More works should be done for full understand.

Table 1. Distribution of duck colibacillosis outbreaks by month in a year

City/Province	Number of outbreaks happened												Total
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
Can Tho	8	9	10	1	1	0	7	8	0	1	0	4	49
Hau Giang	7	0	4	2	14	8	0	0	0	4	2	1	42
Vinh Long	8	6	16	1	5	0	3	4	2	5	0	0	50
Dong Thap	2	3	7	8	8	0	7	3	0	1	2	2	43
Tra Vinh	1	0	0	0	0	0	0	17	7	15	0	0	40
Total	26	18	37	12	28	8	17	32	9	26	4	7	224
Percentage	11.61	8.04	16.52	5.36	12.50	3.57	7.56	14.29	4.02	11.61	1.79	3.13	100.0

3.2. Age of ducks affected

One hundred twenty one out of 224 colibacillosis flocks were younger than 30 day old comprising 54.02% of total colibacillosis flocks (Table2). Numbers of outbreaks were gradually lower at the older age ducks and the least number of diseased flocks was reported in ducks older than 90 days. This is similar to other research results of avian colibacillosis which claimed that all ages of birds are susceptible, but young ones are more frequently affected, and severity of disease is greater in young birds, including development embryos (Goren, 1978; Johnson et al., 2001; Montgomery et al., 1997).

Study result of Islam et al. (2004) showed that E. coli infected chicks died at 2 to 12 day old because of infecting E. coli by feces and layer oviduct. Montgomery et al. (1999) reported that E. coli infected embryos were often died at the end of incubation, especially newly hatched chicks. Yolk sac infections are usually following fecal contamination of the egg surface and most frequently observed towards the end of the egg incubating period. They often result in embryonic mortality or death of the young birds for up to 3 weeks following hatching (Dho-Moulin and Fairbrother, 1999). In this study, we found that the youngest affected ducks were one day old (with 15 flocks) and the oldest was 420 day old (with 1 flock). Colibacillosis can affect very young ducks at day old due to eggs infected E. coli from oviduct or egg shells, and very short incubation from 1 to 3 days (Barnes et al., 2008).

Table 2. Morbidity and mortality of colibacillosis ducks by ages

Days of age	No. of outbreaks	Population	No. of Diseased ducks	Morbidity (%)	No. of Deaths	Mortality (%)	Mortality of ill ducks (%)
<30	121	103,455	5,953	5.75[a]	2,603	2.52[a]	43.73[a]
31-60	62	58,097	10,052	17.30[b]	7,997	13.76[b]	79.56[b]
61-90	22	15,900	1,218	7.66[c]	414	2.60[ac]	33.99[c]
>90	19	25,895	1,258	4.86[d]	895	3.46[d]	71.14[d]
Total	224	203,347	18,481	9.09	11.909	5.86	64.44

Values in the same column with different letters are significant different (P<0.05)

There were 62 outbreaks in ducks from 31-60 day old, comprising 27.68% (62/224) of total flocks, this percentage was lower than that of under 30 day old duck flocks. However, morbidity and mortality of ducks at this age were highest (17.30% and 13.76%). It might be that ducks at this age frequently affected by colisepticemia and more susceptible to other pathogens due to maternal antibody reduction. Study results of Leibovitz (1972) also demonstrated that colibacillosis can occured in ducks at any age, but losses in newly hatched ducklings were lower. In addition, duck production in MD is mainly based on rice field scavenging and ducklings were sent to the field for running from 30 day old, being still young so they were easily suffered from environment stresses while their maternal antibody vanished and active immune had not fully reponsed so their healths were more severe by concurrent and or/secondary pathogens.

3.3. Influence of duck production mode on colibacillosis situation

Duck colibacillosis happened in both ways of raising, and there was 54.01% of diseased flocks (121/224) belonged to confined flocks. This can be explained that this condition was more frequently to occur in young ducklings which were captive in duck houses until 30 days old at least. But their morbidity and mortality were lower than those of scavenging ducks due to captive ducks were mostly younger than 30 day old, they expose to colibacillosis by infection from oviducts of layers or egg surfaces and the incidence of birds with omphalitis increased after hatching, then declines after about 6 days with occasional losses continuing up to 3 weeks (Barnes *et al.*, 2008). Number of colibacillosis duck flocks in scavenging mode were a little bit lower 103/224 (46.91%), but the morbidity and mortality were significant higher (P=0.000 and P=0.000).

Table 3. Morbidity and mortality of colibacillosis ducks by mode of raising

Production mode	No. of Flocks	Population	No. of Diseased ducks	Morbidity (%)	No. of Deaths	Mortality (%)	Mortality of ill ducks (%)
Confined	121	103,455	5,953	5.75[a]	2,603	2.52[a]	43.73[a]
Scavenging	103	99,892	12,528	12.54[b]	9,306	9.32[b]	74.28[b]
Total	224	203,347	18,481	9.09	11,909	5.86	64.44

3.4 Colbacillosis situation in duck breeds

In MD, Vit Co (in Vietnamese) is the most popular breed which have been raised for egg production and Super Meat breed for meat purpose, some householders raise Muscovy ducks for meat with small scales (often lower than 100 animals) for meat or day old ducklings used for next cycle of production. Results in Table 4 showed that morbidity and mortality of colibacillosis ducks in egg laying breed flocks were higher than those of meat breed flocks. This can be explained that egg laying ducks mostly were raised in big scale, about 2,000-5,000 birds; particularly, some flock populations were higher than 10,000 birds which were reared with very high densities in intensive farming systems and they were often transported for scavenging in the rice fields. These negatively affected duck health and increased risks of pathogen infection from other poultry flocks, wild birds and contaminated environment. Muscovy ducks were in small scales, they were confined in house, ponds or ditches, so farmers did not pay enough attention such as sanitation, vaccination and deworming. These facts could make the condition more severe so that morbidity and mortality of colibacillosis ducks in this breed (48.0 and 36.67%) were highest.

Tabe 4. Morbidity and mortality of colibacillosis ducks by breed

Production purpose	Population	No. of Diseased ducks	Morbidity (%)	No. of Deaths	Mortality (%)	Mortality of ill ducks (%)
Egg laying	120,435	12,992	10.79[a]	9,613	7.98[a]	73.99[a]
Meat	82,762	5,417	6.55[b]	2,241	2.71[b]	41.37[b]
Other (Muscovy)	150	72	48.00[c]	55	36.67[c]	76.39[c]
Total	203,347	18,481	9.09	11,909	5.86	64.44

3.5. Influence of duck production size on colbacillosis situation

Totally, there were 170 out of 224 duck colbacillosis flocks with population from 201-2,000 since this was most popular size of duck flocks for eggs or meat in MD, and the morbidity and mortality of this flock size were lowest (6.67% and 3.18%). While the morbidity and mortality of the smallest size (50-200) were highest (20.77% and 10.80%), followed by those (18.94% and 17.87%) of the highest size (>5,000). These consequences were risen from lack of care for ducks from small holder and stresses of ducks in high population flocks.

Table 5. Morbidity and mortality of colibacillosis ducks by population

Production size	No. of diseased flocks	Population	No. of Diseased ducks	Morbidity (%)	No. of Deaths	Mortality (%)	Mortality of ill ducks (%)
50-200	37	4,520	939	20.77[a]	488	10.80[a]	51.97[a]
201-2000	170	134,037	8,941	6.67[b]	4,269	3.18[b]	47.75[b]
2001-5000	14	39,790	3,867	9.72[c]	2,684	6.75[c]	69.41[c]
>5000	3	25,000	4,34	18.94[d]	4,468	17.87[d]	94.38[d]
Total	224	203,347	18,481	9.09	11,909	5.86	64.44

3.6 Influence of preventive method on colbacillosis situation

Results of surveys from retrospective survey questionnaires and direct interviewing from 224 colibacillosis duck flocks revealed that there were 63.84% (143/224) of flocks being applied disinfection, 40.63% (91/224) being used antibiotics for prevention purposes, 35.27% (79/224) being vaccinated, but only 8.04% (18/244) being used helminthics. Kinds of vaccines were used for ducks depending on their ages. In MD, ducks were mainly vaccinated by duck plague and avian influenza vaccines. Although, vaccination and deworming are necessary to perform when ducks get appropriate ages, but duck farmers did not pay

much care on disease prevention, especially on deworming. Colibacillosis in avian can be from primary or secondary infection, this condition often happens when their immune systems are suppressed by infectious or noninfectious factors. *E. coli* infection occurs when skins or mucosal barriers are compromised (e.g. unhealed navel, wounds, mucosal damage from viral, bacterial or parasitic infection, lack of normal flora, etc.), the mononuclear-phagocytic system is impaired (e.g., viral infections, toxins) exposure is overwhelming (e.g., environmental contamination, poor ventilation, contaminated water, malnutrition) and /or birds are exposed to abnormal stress of have an inappropriate response to stress (Barnes, 2008). Hence, the colibacillosis more often occurred in flocks being inadequately preventive and economic losses increase in cases of having more factors causing suppression of their immune responses. Examining the influence of preventive monofactor on duck colibacillosis status, deworming showed to be the most important factor in reducing the severity of colibacillosis status when it happened in ducks with the lowest morbidity and mortality (4.31 and 0.98%).

Table 6. Morbidity and mortality of colibacillosis ducks with being applied by preventive method

Preventive method	No. of Flocks	Population	No. of Diseased ducks	Morbidity (%)	No. of Deaths	Mortality (%)	Mortality of ill ducks (%)
Disinfection	143	121,554	13,718	11.29[a]	9,524	7.84[a]	69.43[a]
Antibiotics	91	104,118	13,651	13.11[b]	10,088	9.69[b]	73.90[d]
Vaccination	79	82,377	11,793	14.32[c]	1,748	2.12[c]	14.82[c]
Deworming	18	11,706	505	4.31[d]	115	0.98[d]	22.77[d]

Table 7. Morbidity and mortality of colibacillosis ducks being applied by combination of preventive methods

Preventive method	No. of Flocks	Population	No. of Diseased ducks	Morbidity (%)	No. of Deaths	Mortality (%)	Mortality of ill ducks (%)
D-A	62	42,926	3,457	8.05[a]	2,040	4.75[a]	59.01[a]
D-V	59	41,876	5,305	12.67[b]	3,783	9.03[b]	71.31[b]
D-Dw	18	12,006	285	2.37[c]	87	0.72[c]	30.53[c]
A-V	35	51,537	5,291	10.27[d]	4,157	8.07[d]	78,57[d]
A-Dw	3	1,736	98	5.65[e]	30	1.73[e]	30.61[c]
V-Dw	7	6,630	370	5.58[e]	89	1.34[e]	24.05[c]
D-A-V	27	36,640	3,396	9.27[f]	1,654	4.51[a]	48.70[c]
D-A-Dw	2	1,526	74	4.85[e]	19	1.25[e]	25.68[c]
D-V-Dw	8	6,750	136	2.01[g]	37	0.55[c]	27.21[c]
A-V-Dw	1	800	45	5.63[e]	8	1.00[e]	17.78[c]
S-A-V-D	2	920	24	2.61[c]	5	0.54[c]	20.83[c]
Tổng	224	203,347	18,481	9.98	11,909	5.86	64.4

Results in Table 7 showed that the combination of disinfection and using antibiotics was applied to most of flocks (62/224) followed by combination of sanitation and vaccine with 59/224 flocks being applied, and only 2 flocks got all of these methods. The results also showed that flocks getting combination of disinfection, vaccination and deworming (D-V-Dw) had lowest morbidity and mortality (2.01 and 0.55%)

4. CONCLUSION

Duck colibacillosis was popular in the Mekong delta and caused significant losses in duck production. Ducks need to take care by

all necessary preventive methods, especially deworming in order to prevent and reduce the severity of colibacillosis status. These epidemiological data are useful for planning effective strategy in disease control and prevention.

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IN VIVO EFFECT OF RECOMBINANT *BACILLUS SUBTILIS* EXPRESSED CHICKEN INTERFERON ALPHA (*B. SUBTILIS*-CHIFNA) IN PREVENTION OF INFECTIOUS BURSAL DESEASE IN CHICKENS

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ABSTRACT

The *in vivo* effect of recombinant *Bacillus subtilis* expressed chicken interferon alpha (*B. subtilis*-ChIFNα) in prevention of infectious bursal disease (IBD) was investigated in 3 week old chicks. Each chicken was given a dose of $0.5x10^{10}$ spores of *B. subtilis*-ChIFNα with 1 or 5 supplies by eye drop or oral administration. Challenges with dose of 10^4 ELD50 of virulent infectious bursal disease (vIBDV) per experimented chicken through oral route were carried out after 2 or 6 hours of *B. subtilis*-ChIFNα administration. The results showed that *B.subtilis*-ChIFNα could be used as a potential preventive and therapeutic antiviral agent against IBD, *B.subtilis*-IFNα administration to chickens by oral route at 6 hours before vIBDV infection gave the best effect in IBD prevention

Keywords: *Bacillus subtilis, chicken, interferon alpha, infectious bursal disease.*

1. INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious, viral infection of young chickens that has lymphoid tissue as its primary target with predilection for bursa of Fabricius. The period of greatest susceptibility to clinical disease is between 3 and 6 weeks of ages. Normally, susceptible chickens younger than 3 weeks do not develop clinical signs but have subclinical infection that are economically important as a result of severe, long-lasting immunosuppression due to destruction of immature lymphocytes in the bursa of Fabricius, thymus, and spleen.

For many years, infectious bursal disease (IBD) or Gumboro disease has constituted a serious problem for the poultry production. Direct losses are linked to high morbidity and specific mortality in clinical disease, and its indirect economic impact is also considerable from concurrent and/or secondary infections due to immunosuppression. Prevention of IBD necessitates hygiene measures and medical prophylaxis. The pathogens are very resistant in the environment, antibiotics have no effect on IBDV (infectious bursal disease virus) so immunization of chickens is principal method for controlling the disease. In spite of regular vaccinations, severe outbreaks still occur and exit as endemic in most Asian countries due to limitation of IBD vaccines. So an urgent need for new antiviral strategies to protect and treat against IBD is necessary.

Recombinant *Bacillus subtilis* expressed chicken interferon alpha (B. *subtilis*-ChIFNα), a product was produced by Faculty of Pharmacy in HCM city university of Medicine and Pharmacy showed potentially useful in the prevention of Newcastle diseases *in vitro* and *in vivo* (Nguyen Ngoc An *et al.*, 2010; Ho Thi Viet Thu *et al.*, 2012). In this study, we examined the efficiency of this product by dose, route and time point of administration in prevention of IBD *in vivo*.

2. MATERIALS AND METHODS

2.1. Materials

Biological components used for survey in this study included *(i) Bacillus subtilis* expressed chicken interferon alpha (*B. subtilis*-ChIFNα) (Faculty of Pharmacy, Ho Chi Minh City Medicine and Pharmacy University), *(ii) Bacillus subtilis* (Faculty of Pharmacy, Ho Chi Minh City Medicine and Pharmacy University), *(iii)* Standard ChIFNα (GenWay Biotech, USA), and (*iv*) Gumboro disease vaccine (NAVETCO).

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In addition, other necessary materials for this study were infectious bursal disease virus (vIBDV) identified by RT-PCR technique (NAVETCO), one hundred and eighty of 3-week old chickens without antibody against IBDV confirmed by ELISA test, and vaccines for prevention of Newcastle, pox, avian influenza and fowl cholera diseases, ELISA kit (Synbiotics, USA), PBS (phosphat buffured saline) solution and NaCl 0.85% solution.

2.2. Methods

Experiment preparation

One day old chickens were raised in experimented farm of Can Tho University. They were vaccinated with Newcastle disease, avian influenza and avian pox vaccines. When they got 3-week of age, they were bled for testing maternal antibodies against IBDV by ELISA test. Before carrying out the experiment, suspension of vIBDV was titrated to calculate embryo lethal dose 50% (ELD50) in 11 day old chicken embryos by Reed and Muench method (Reed and Muench, 1938).

All chickens were negative with maternal antibodies against IBDV by ELISA test at 3 weeks of age which were randomly designed into 2 experiments, all experiment chickens had uniform weights and raised under the same conditions.

Experiment design 1: Examination on virulence of IBDV

This experiment was used to test the virulence of IBDV which will be used in chick challenges in this study. Chicks were randomly designed with 2 treatments and 3 replications, each experiment contained 2 chickens. Each chicken in virus treatment was provided 0.3ml of NDV suspension containing 10^4 ELD50 by oral route and each chickens in PBS (Phosphate buffered saline) treatment was supplied 0.3ml of PBS. Design of Experiment 1 was presented in table 1.

Table 1. Experimental design for virulent examination of infectious bursal disease virus suspension

Treatments	Repeats	Total of chickens per treatment	Doses	Route
PBS	3	12	0.3ml/bird	Oral
Viral challenge	3	12	$4x10^4$ ELD50/0.3ml/bird	Oral

After carrying out the experiment 1, if the viruses caused typically clinical signs of IBD and deaths in chickens while others in the control group are healthy, the virus suspension was employed in experiment 2.

Experiment design 2: Examination on preventive efficiency of *B. subtilis*- ChIFNα by dose, time point and route of administration

The experiment was randomly designed with 3 factors (dose: 1 administration or 5 administrations in 5 consecutive days; administration time point: at 2 hours or 6 hours before chick challenging by vIBDV; administration route: oral or eye drop), including 24 treatments and 3 replications. Each experimental unit contained two 3 week old chickens. Totally, 156 experimental chickens were used in this experiment.

For each factor we observed on 3 biological products (*B.subtilis*-ChIFNα, standard ChIFNα, *B.subtilis*). Each chicken in *B.subtilis*-ChIFNα and *B.subtilis* treatments was given a dose of $0,5x10^{10}$ spore. Each chicken in treatment of standard ChIFNα was administrated 10^4 UI of standard ChIFNα. In vaccine treatment, each chick was dropped 1 dose of IBD vaccine at 6 hours before viral challenge. All experimented treatment was then challenged with 10^4 ELD50 of IBDV suspensions.

Experimented chickens were observed carefully during experiment time in order to make the record on numbers of ill and dead chickens and observe the signs and lesions of sick and dead chickens. The internal organs of dead chickens were collected for testing the presence of vIBDV by agar gel precipitation. The target parameters were morbidity and mortality of experimental chickens. Statistical differences were analyzed by Chi-square test of Minitab software version 13.2 (Ryan *et al.*, 2000).

Morbidity *(%) = (No. of diseased chickens - No. challenged chickens) x100*

Mortality *(%) = (No. of dead chickens - No. challenged chickens) x100*

3. RESULTS AND DISCUSSION

Virulence of infectious bursal disease virus

Results of experiment showed that 100% of experimented chickens (12/12) challenged by vIBDV were sick, and 50% of them died after short course. All of dead chickens showed typical signs and lesions caused by IBDV, such as depression, diarrhea with urates, ruffled feathers, hemorrhages in the pectoral and thigh muscles in most cases, and typical lesions in the bursa of Fabricius, proventriculus, thymus and kidneys, meanwhile 100% (12/12) chickens in PBS treatment was healthy. In fully susceptible flocks, the disease appears suddenly and there is high morbidity rate, usually approaching 100%, mortality may be nil but can be as high as 20 to 30% (Eterradossi and Saif, 2008). But in late 1980s, strains of very virulent infectious bursal disease virus (vvIBDV) became a problem in Europe, some of these isolates caused mortality rate up of 90% (Chettle *et al.*, 1989) to 100% (Van den Berg *et al.*, 1991) in 4 week old susceptible Leghorn chickens. Results in Table 2 suggested that the IBDV strain used in this experiment was quite virulent.

Table 2. Morbidity and mortality of experimented chickens after viral challenging

Treatment	No. experimented chickens	No. diseased chickens	Morbidity (%)	No. dead chickens	Mortality (%)
Viral challenge	12	12	100,00[a]	6	50.00[a]
PBS	12	0	0,00[b]	0	0.00[b]

The values in the same column which have different characters are significantly different (P≤0.05)

3.2. Efficiency of *B.subtilis*-ChIFNα in protection of chickens from IBD

In this study, we used standard ChIFN-α and *B. subtilis* as 2 controls in order to evaluate the pre-eminent efficiency of *B.subtilis*-ChIFNα when ChIFN-α was expressed by *B. subtilis*, IBD vaccine also was used for comparing the preventive effect of this product (which stimulate the specific immune response) with that of interferon (IFN) which is a type of non-specific antibody. Efficiencies of these biological products were evaluated by the morbidity and mortality of chickens after viral challenging. The overall results of the study were presented in table 3.

Table 3. Morbidity and mortality of experimented chickens by treatment

Treatment	No. experimented chickens	No. diseased chickens	Morbidity	No. dead chickens	Mortality
Vaccine	12	12	100,00[a]	1	8,33[a]
B. subtilis	48	18	37,50[b]	1	2,08[a]
Standard ChIFNα	48	16	33,33[b]	4	8,33[a]
B.subtilis-ChIFNα	48	11	22,92[b]	1	2,08[a]

The results in the table 3 showed that the lowest morbidity (22.92%) and mortality (2.08%) were reported from chickens in *B.subtilis*-ChIFNα treatment and the highest morbidity (100%) was in vaccine treatment. Chickens from both treatment of vaccine and standard ChIFNα had mortality of 8.33%, highest compared to that of other treatments. However, there was no significant difference reported from mortalities of all treatments, but the morbidities of *B.subtilis,* Standard ChIFNα and *B.subtilis*-ChIFNα treatments (37.50, 33.3 and 22.92%, respectively) were significant lower than that of vaccine treatment (100%) with P=0.00. This experimental result also revealed that vaccine could not help to reduce the morbidity but mortality of 8.33% lowered comparing to 50.00% (Table.2). *B. subtilis* gave a better effect in prevention of IBD than vaccine because it is a useful and beneficial probiotic that supports digestion, enzyme production, immune and digestive system health, and its actions can bring in short time after being administration to animal body while vaccine needs much more time to induce the immune response. *B. subtilis* is described as an antidiarrheal microorganism

and a potential probiotic on immune response and resistance of diseases (Mazza, 1994). Possible mechanisms of action include antimicrobial effect by synthesis of antimicrobial substances, antidiarrheal effect, immunostimulatory effect, competitive exclusion of pathogens, prevention of intestinal inflammation, and stimulation of growth of intestinal normal flora. The results also showed that experimented chickens were crucially protected from vIBDV challenge by ChIFNα and *B. subtilis*- ChIFNα. IFNs belong to the large class of glycoproteins known as cytokine. Among them, IFNα and IFNβ are the most important (Tizard, 2004). They have antiviral abilities by the inhibition of viral RNA and protein syntheses and by partial activation of *lymphocytes in response to viral infection (Nguyen Thi Chinh and Ngo Tien Hien, 2001; Tran Thu Hoa et al., 2001).* In birds, ChIFNα is a potentially antiviral agent. When chickens were supplied with high doses of ChIFNα through oral route, it could reduce viral load in chickens (Marcus *et al.,* 1999). ChIFNα also have abilities of preventing and treating several viral diseases in chickens such as H9N2 avian influenza (Meng *et al.,* 2011), infectious bronchitis (Pei *et al.,* 2001), inhibiting the tumors caused by Rous sarcoma virus (Plachy *et al.,* 1999). It was also proved effective in treating IBD and Newcastle disease in commercial layers (Mo *et al.,* 2001). *B.subtilis*-ChIFNα gave the best effect in IBD prevention. It can be explained that spores of *Bacillus subtilis* expressed chicken interferon alpha coud germinate into vegetative cells in chicken gastrointestinal tract, then multiply in numerous cells which help to increase the amount of IFN (Tran Thu Hoa *et al.,* 2001). In this study, we use spores of *B.subtilis* expressed ChIFNα which is a domestic product, it is much lower price and more applicable in condition of Vietnam compared to standard ChIFNα. Therefore, *B.subtilis*-ChIFNα is a better choice in IBD prevention and treatment.

3.3. The effects of 1 and 5 doses *B.subtilis*-ChIFNα in IBD prevention

In this study, we only concerned on morbidity because the mortalities of IBD chickens were very low that could not show any difference between the results of treatments.

Table 4. The morbidities of experimented chickens in treatments by doses of administration

Dose	Treatment	No. experimented chickens	No. diseased chickens	Morbidity (%)
1	B.subtilis	24	10	41.67
5	B.subtilis	24	8	33.33
1	Standard ChIFNα	24	9	37.5
5	Standard ChIFNα	24	7	29.17
1	*B.subtilis*-ChIFNα	24	6	25.00
5	*B.subtilis*-ChIFNα	24	5	20.83

The results in Table 4 showed that chickens in treatment which were administrated 5 doses of *B.subtilis*-ChIFN-α had mortality of 20.83% lower than that of chickens administrated by only 1 dose (25.00%), but the difference was not signification. The effects of increased administrations of other products (*B. subtilis* and Standard ChIFNα) were similar, 5 applications of these products could help to reduce mortalities, but no significant difference was found. The study results of Cantell and Pyhala (1973) also proved that the repeated injections did not affect the pattern of the circulating IFNα in any significant way. Other studies need should to be carried out in order to have reliable conclusion.

3.4. Efficiency of B.subtilis-ChIFNα in prevention of infectious bursal disease by time point of application

Chickens administrated by standard ChIFNα at 2 hours and 6 hours before viral challenge had the same mortality of 33.33%. The morbidity of chickens supplied with *B.subtilis*-ChIFNα at 6 hours before vIBD challenge was (20.83%) lowest comparing to those of others, and was significantly different with that (50.00%) of *B.subtilis* treatment at 2 hours before challenge (P=0.035). Generally, administration of products containing *B.subtilis* at 6 hours before infection could give better results, but no significant difference was reported in examining

the influence of 2 administration time points of individual product. *B.subtilis* is a probiotic which was often used in form of spore (Usahakova *et al*., 2016), it started to multifly at 6 hours after entering to the gastrointestinal tract and the peak number reach at 24 hours (Gabriella and Simon, 2002). Therefore, we cannot conclude that time point of *B.subtilis*-ChIFNα application does not influence its efficiency. Another study should be done with more time points of administration time such as 12 hours, 24hours before viral challenge in order to bring out their influences. In this experiment, *B.subtilis*-ChIFNα gave the best effect in IBD prevention. It can be explained that *Bacillus subtilis* expressed chicken interferon alpha germinated into vegetative cells in chicken gastrointestinal tract, then multiply and also form the spores which helped to increase the amount of IFN (Tran Thu Hoa *et al*., 2001).

Table 5. Protection rates of experimented chickens by time of application of three biological products

Time of application before challenging	Treatments	No. chickens	No. sick chickens	Morbidity (%)
2 hours	*B.subtilis*	24	12	50.00^{a}
6 hours	*B.subtilis*	24	6	25.00^{ab}
2 hours	Standard ChIFNα	24	8	33.33^{ab}
6 hours	Standard ChIFNα	24	8	33.33^{ab}
2 hours	*B.subtilis*-ChIFNα	24	7	29.75^{ab}
6 hours	*B.subtilis*-ChIFNα	24	5	20.83^{b}

Efficiency of B.subtilis-ChIFNα on pretreatment of infectious bursal disease by route of application

Morbidities of experimented chickens in treatment by way of administration

Table 6. Morbidities of experimented chickens in treatment by route of administration

Routes of application	Treatments	No. chickens	No. sick chickens	Morbidity (%)
Oral	*B.subtilis*	24	8	33.33^{ab}
Eye drop	*B.subtilis*	24	10	41.67^{a}
Oral	Standard ChIFNα	24	10	41.67^{a}
Eye drop	Standard ChIFNα	24	6	25.00^{ab}
Oral	*B.subtilis*-ChIFNα	24	3	12.50^{b}
Eye drop	*B.subtilis*-ChIFNα	24	8	33.33^{ab}

The morbidity of chickens in standard ChIFNα treatment eye drop was (25.00%) lower than that by eye oral route (41.67%). Study results of Mo *et al.* (2001) demonstrated that eye drops administration of ChIFNα was more effective than oral administration or intramuscular injection. This could be explained that IFN is a glycoprotein so that it can be destroyed by enzymes in chicken intestines such as trypsin, chymotrypsin and papain (Nguyen Thi Chinh and Truong Thi Hoa, 2001; Mo *et al.*, 2001). Furthermore, when supplied via eye drop route, ChIFNα could have a certain effect on Harder's gland of the chicken eyes, an immune organ of chickens, therefore, the immune response of chickens increased so they had less risks of catching IBD. Chickens were supplied by both *B.subtilis* and *B.subtilis*-ChIFNα by oral route had lower morbidities (33.33% and 12.50%), compared with those of eye drop (41.67%, 33.33%, respectively). **Bacillus subtilis** is currently used as an oral probiotic (Mazza, 1994). Robustness of spores is thought to enable passage across the gastric barrier (Green *et al*., 1999), and small intestine is preferred tissues for them to germinate and multiply. The results in the Table 6 also showed that the lowest morbidity (12.50%) was in oral *B.subtilis*-ChIFNα treatment. This percentage was significant lower (P=0.045) than morbidities of chickens in Standard ChIFNα treatment with oral route (41.67%), and that of *B.subtilis* treatment with eye drop administration (41.67%). This can be explained that chickens were administrated by spores of *B.subtilis*-ChIFNα by oral route, these spores germinated and colonized in the small intestine (Mazza, 1994), and multiply that increase amount of ChIFNα, stimulated and enhanced the body's

immune system following germination of the spores (Hosoi and Kiuchi, 2004). Thus, oral application of *B.subtilis*-IFNα can help both anti-IBDV action and protection of digestive mucous membrane. This explains the higher efficiency of oral *B.subtilis*-IFNα administration. However, there was no significant difference between the morbidities of chickens supplied through oral and eye drop routes in individual product. In summary, *B.subtilis*-IFNα administration to chickens by oral route at 6 hours before vIBDV infection gave the best effect in IBD prevention.

3.5. Confirmation of IBD from diseased and dead experimented chickens

All of sick experiment chickens were examined for tentative diagnosis by clinical signs and macro-lesions. Totally, 69/69 sick birds showed typical symptoms of IBD, and 100% (13/13) dead experiment chickens were confirmed to be infected by vIBDV by macro-lesions and agar gel precipitation test.

4. CONCLUSION

The present study suggested that *B.subtilis*-ChIFNα could be used as a potential preventive and therapeutic antiviral agent against IBD, especially through oral administrations. Other studies should be done with more time points of administration and different doses in order to bring out their influences

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A SIMPLE METHOD FOR IGG PURIFICATION FROM DOG SERA: PROSPECT OF PRIMARY MATERIAL FOR USE IN THE IMMUNODIAGNOSIS AND TREATMENT THERAPY IN DOG DISEASES

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ABSTRACT

Dog IgG have been used in immunodiagnosis and treatment of diseases. We showed in this study a simple two-step procedure for purification of IgG from dog sera. In the first step, IgG was precipitated by 60% saturated ammonium sulfate. This precipitate then was used in the second step which was conducted with a protein G column affinity chromatography. Our results showed that the obtained IgG was 95.1% pure. This method can be easily up-scaled for IgG production for use in diagnosis and treatment therapy in dogs.

Keywords: *Dog IgG, protein G, purification, chromatography, protein precipitation*.

1. INTRODUCTION

Dogs (*Canis familaris*), considered the first domesticated mammal, have learned to live with humans for 13,000-17,000 years ago (Driscoll *et al*., 2009). There are many immunological diseases, such as immune-mediated hemolytic anemia, idiopathic thrombocytopenic purpura, chronic polyneuritis causing inflammation affecting the health of dogs. Immunoglobulins are required not only for the treatment of these diseases but also for strict disease monitoring systems in which pure IgG is needed (Pedersen, 1999). The purification of IgG is simple due to its electrical properties, high molecular weight and normal abundance in serum (Sadeghi *et al*., 2018). Polyclonal antibodies are widely used in countries around the world. Purified dog IgG antibodies do not only brings economic benefits but also helps develop pet care services, meeting the growing demand.

IgG antibodies are antigen specific binding proteins and produced by B lymphocytes after antigen exposure. Purification of IgG antibodies in animals has been conventionally made by affinity chromatography using different ligands (Huse *et al*., 2002). One of the most ligands used is protein G which has specific binding activity to Fc gamma portion of IgG in several animals including dogs. In this study, a simple method for IgG purification from dog sera was investigated using two-steps procedure: ammonium sulfate precipitation and Protein G affinity chromatography (Duong-Ly and Gabelli, 2014; Matulis, 2016). This procedure can be up-scaled and the purified IgG can be used for further applications.

2. MATERIALS AND METHODS

2.1. Sera

Dog sera were collected from healthy dogs raising in District 12, Ho Chi Minh City, and were kept in -20°C until use.

2.2. IgG Precipitation

100% saturated ammoniun sulfate solution was prepared and drop by drop added into the sera. For optimization of IgG precipitation, different final concentrations (30, 45 and 60%) of saturated ammonium sulfate solution were examinated (Duong-Ly and Gabelli, 2014; Hebert *et al*., 1973). IgG precipitates were washed twice by centrifugation at 6000 rpm in 15 min by using the same final concentrations of saturated ammonium sulfate solution. The obtained precipitates were overnight dialysed against PBS, pH 7.4 containing 0.05% NaN_3. IgG were then analysed by SDS-PAGE and kept in -20°C until use.

2.3. Affinity chromatography using protein G column

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The precipitated IgG was applied to the protein G column in which IgG was traped because of its Fc gamma affinity to protein G. A washing buffer containing 0.1M glycine and 0.3M NaCl was applied to elute the IgG which was then analysed by SDS-PAGE and kept in -20°C until use.

2.4. Protein quantification

The amount of IgG protein was determined by spectrophotometric method at 280 and 260nm (Aitken and Learmonth, 2002). Protein concentration is calculated according to the formula: C=[(1.55xA280)–(0.76xA260)]xD. In which: C - Concentration of protein (mg/ml); A280 and A260 - Absorbent at 280 and 260nm; D - Dilution of samples.

2.5. Protein electrophoresis

IgG and other proteins before and after purification steps were tested by SDS-PAGE. The samples were treated (denatured) or untreated (native) with beta-mercaptoethanol. Acrylamide gel was 12.5%. Protein in the gel was detected by Coomassie blue dye method. Electrophoresis images were scanned by HP image scanner and analyzed by Quantity-one software.

2.6. Western blotting

Purified dog IgG was tested by western blotting using commercial goat anti-dog IgG antibodies conjugated with HRP (Santa cruz, USA) at 1/5000 dilution. The specific binding was detected by luminol-peroxidase photoluminescence method; the image was recorded by a C-Digit Blot scanner (LICOR, USA) and processed by C-digit Imaging StudioLite software.

2.7. Statitical analysis

Data analysis was performed using Graphad Prism 5.0.

3. RESULTS AND DISCUSSION

3.1. IgG precipitation by ammonium sulfate precipitation

It was shown that the concentration of saturated ammonium sulfate which is used for precipitating animal serum IgG is commonly between 30 and 60% (Duong-Ly and Gabelli, 2014; Hebert *et al*., 1973). Our experiments used 30, 45 and 60% saturated ammonium sulfate for precipitation of dog IgG. By SDS-PAGE analysis, the results showed that IgG starts to be precipitated at 30% saturated ammonium sulfate (Fig. 1). In addition, spectrometer analysis showed that the amount of protein increased when higher of concentrations of saturated ammonium sulfate was used (Table 1). At 60% saturated ammonium sulfate, 67% of proteins in the sera was precipitated. We conclude that 60% saturated ammonium sulfate may be optimal for precipitation of dog IgG which was then used for further purification by affinity chromatography.

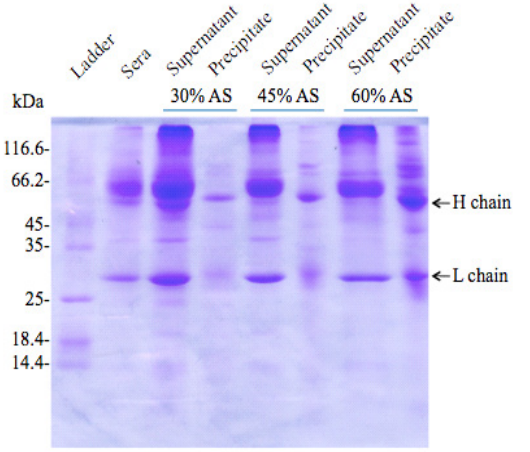

Fig 1. SDS-PAGE analysis of protein fractions from dog sera in different concentration of saturated ammonium sulfate (AS)

Table 1. Amount of protein in fractions separated by different concentrations of saturated ammonium sulfate

% of saturated ammonium sulfate		Protein amount (mg)	%
30%	Supernatant	64.8	84.4
	Precipitate	12.0	15.6
	Total	76.8	100
45%	Supernatant	31.5	51.2
	Precipitate	30.0	48.8
	Total	61.5	100
60%	Supernatant	19.5	33.0
	Precipitate	39.5	67.0
	Total	59.0	100

3.2. IgG purification by protein G affinity chromatography

IgG precipitated with 60% saturated ammonium sulfate showed above was applied to

protein G column in which the IgG was trapped because of its high affinity of Fc gamma with protein G. After the elution step, there are 2 peaks detected and shown on chromatogram (Fig 2A). Denatured SDS-PAGE analysis in the presence of beta-mercaptoethanol (+Met) shows that peak 2, but not peak 1, contains ~50kDa heavy (H) chain and ~25kDa light (L) chain of IgG (Fig 2B). In addition, native-PAGE analysis without mercaptoethanol (-Met) shows the appearance of the full length structure of IgG which have molecular weight of ~150kDa (Fig 2B).

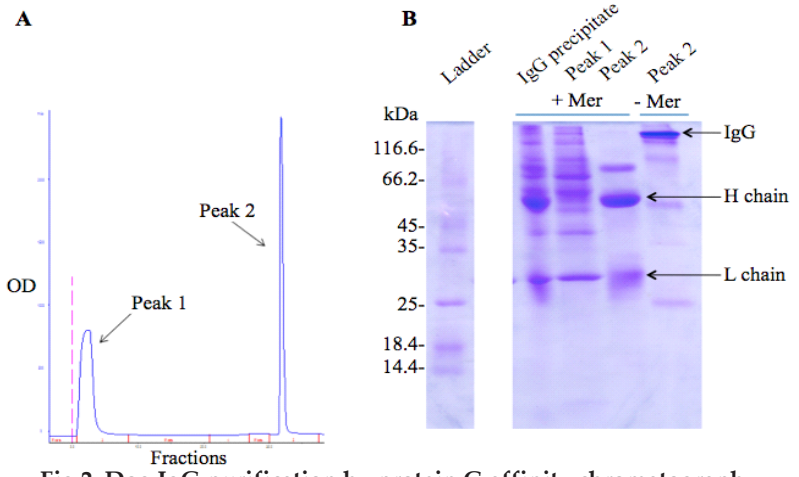

Fig 2: Dog IgG purification by protein G affinity chromatography.

(A: chromatogram. B: SDS-PAGE analysis of purified IgG. + Mer or – Mer indicates the presence or absence of beta-mercapthoethanol, respectively)

To confirm for IgG purification, a western blotting assay was conducted using goat anti-dog IgG antibodies conjugated with HRP enzyme (Fig 3). The results showed high quality of purified IgG obtained by our chomatography procedure using protein G as affinity ligand. Beside bands of heavy (H) chain and light (L) chain detected, we found another band which shows a molecular weight of ~75kDa and recognized by goat anti-dog IgG antibodies indicating for a possible H+L associate complex made from H and L chains. Imaging analysis of SDS-PAGE gel by Quantity One program showed that the obtained IgG was 95.1% pure and can be used for further investigation.

(Goat anti-dog IgG antibodies conjugated with HRP were used for detection of targeted dog IgG

Protein A was shown tightly bind to Fc-gamma portion of IgG from dog and has been used for IgG purification by affinity chromatography (Scott *et al*., 1997). In this study, protein G instead was used as binding ligand in the affinity column for capturing dog IgG. Our reults showed that this approach allows us to obtain dog IgG with high recovery efficiency and purity (Fig 2 and 3). In another experiment, we also used protein A column for dog IgG purification. The IgG obtained by protein A column was equivalent to that obtained by protein G column (data not shown).

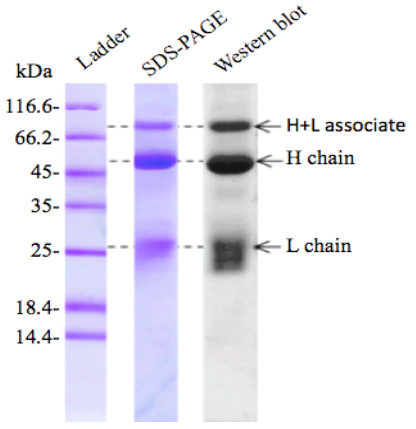

Fig 3. Regconition of purified IgG by western blotting assay

4. CONCLUSION

We showed in the study a simple method to get highly purified IgG from dog serum and can be easily up-scaled for commercial production. The obtained IgG can be used for developing products and for use in the diagnosis and treatment of diseases in dogs.

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LABORATORY ANIMALS MANIPULATION PERSONNEL TRAINING PROGRAM AND ITS APPLICATION: VASOCONTRACTILE RESPONSIVENESS ASSESSMENT

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ABSTRACT

Skilled laboratory animal manipulation technique could improve the animal welfare, and it increases the stability and success rate of animal studies. Therefore, in this article we will show the flowchart illustrating the personnel training program for laboratory animals (rat or mice) and how to use the isolated vascular tissue (aorta) to evaluate the vascular contractile responsiveness. For the laboratory animal manipulation training program, we set it into three stages: 1) Realizing the 3Rs principles (replacement, reduction, refinement) for laboratory animal use; 2) Preparing the proper macro- and micro-environment for laboratory animals; 3) Practicing the laboratory animal manipulation techniques (holding, ear numbering, gavage, anesthesia, sacrifice and tissue collection). After that, we will use the isolated tissue such as aorta (vascular tissue) for further study. In this article we will also show how to use Wire Myograph System to measure the vasocontractile or vasorelaxant responsiveness in the presence of endocrine-disrupting chemicals (EDCs) such as triclosan (antibacterial agent), nonlyphenol (surfactant), octylphenol (surfactant) and DEHP (plasticizer) and demonstrate its effects on vasoconstriction or vasorelaxation.

Keywords: *Laboratory animal manipulation; 3Rs principles for laboratory animal use; Isolated vasocontractile responsiveness; endocrine-disrupting chemicals.*

1. INTRODUCTION

Animal study is one of the important parts in biomedical research for in vivo study; however, many students and investigators have no idea how to manipulate that. Therefore, the Institutional Animal Care and Use Committee (IACUC) in the University have to hold the personnel training program regularly for discussing the principles of 3Rs (Replacement, Reduction and Refinement) in experimental animal use and for teaching the manipulated skills such as ear numbering, gavage, anesthesia, sacrifice and tissue isolation/collection in the research. Well trained students and investigators are necessary for improving animal welfare, increasing success rate and maintaining the stability of research results in animal study.

For the animal welfare and manipulation techniques training program, lectures and practice courses are required for the students and researchers. Additionally, texts, graphic illustration and video are good materials used in the training course. After that, an examination is asked to evaluate the learning effectiveness, and certification will be released to the person who passed the examination. Every three year, the certificated person will be re-educated in the training program, and get the extension of certification.

With the animal welfare training certification, the students or investigators are responsible for applying the application documents to IACUC to get the permit of animal study. During the experimental period, the students or investigators have to maintain a suitable macroenvironment which is the housing place with temperature, moisture and noise control, and a comfortable microenvironment which is the feeding cage with clean and sufficient food, water and bedding. Additionally, they need to check the animal situation and environmental condition twice every day. In the experimental designed timing, some of the laboratory animals will be sacrificed followed by the guideline of IACUC for further study, and the sacrificed animal body will be kept in refrigerator at -20 degree C. In the end of study, all the bodies will be collected and sent to the incinerator.

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For further study, in this article we will take the "evaluating the rat aortic vascular tone regulation-vasoconstriction and vasorelaxation" as an example. The Wire Myograph System used for measurement of aortic tension is useful for investigating the endothelial nitric oxide release, which is an index for predicting the risk of atherosclerosis, and it also proper to assess the smooth muscle contractile mechanism and response in physiological situation such as gender difference and in pathological situation such as exposure to hypertension, diabetes or environmental pollutants. In this case, we will show the results about the effects of endocrine-disrupting chemicals, one of the environmental pollutants, on aortic vasocontractile and vasorelaxant responsiveness.

2. MATERIALS AND METHODS

2.1. Animal research application to IACUC

Before ordering the experimental animals (we take "rats" as an example in this article), students or investigators whom getting animal welfare and manipulation techniques training program certification have to send the animal research application form to IACUC for evaluating the research process is proper or not. If the study is followed by the guideline of IACUC, the students or investigators will get the permit, which is a document with IACUC number, to order the laboratory rats.

2.2. Animals ordering, shipping, and housing

With the IACUC permit, it is legal to order laboratory rats from local or foreign companies. The shipping process should be temperature controlled at 25°C and supplied with food, bedding and gel containing water. After getting the rats, students or investigators need to check the gender of rats immediately to make sure the right order. And then, the rats are transferred from shipping cage into feeding cage with clean and sufficient food, water and bedding. After 1-2 weeks for adaption of housing environment, the rats are used for manipulation technique training program and further study.

2.3. Laboratory animals manipulation personnel training program

2.3.1. Holding and ear numbering

Because my dominant hand is my right hand (Fig 1), I use right hand to take the scissor and left hand to hold the rat. I use my thumb and index finger of left hand to control the forelimb of the rat, and push the rat down to the desk to restrict its movement. At the same time, I quickly use a scissor to make a triangle cut on the rat's ear to mark the rat. Since one feeding cage could have four rats, the rats could be marked by "No-Cut" (number 1), "Right-Ear Upper Cut" (number 2), "Left-Ear Upper Cut" (number 3) and "Right-Ear Lower Cut" (number 4).

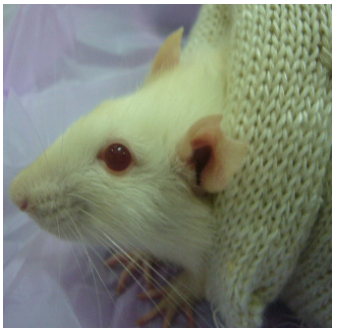

Fig 1. The picture of holding and ear numbering of rat

Fig 2. The picture of gavage of rat

Fig 3. The picture of intraperitoneal injection and anesthesia of rat

2.3.2. Gavage

Because my dominant hand is my right hand (Fig 2), I use right hand to take the gavage tubing (stainless steel) and left hand to hold the rat. I use my thumb and index finger of left hand to control the forelimb of the rat, and push the rat into my chest near Processus xiphoideus to restrict its movement. At the same time, I

quickly insert the gavage tubing through mouse, esophagus into the stomach. The insertion length is around 3/4 of the gavage tubing (80mm).

2.3.3. Intraperitoneal injection and anesthesia

Because my dominant hand is my right hand (Fig 3), I use right hand to take the syringe with Zoletil® 50 (Tiletamine + Zolezepam) at the dosage of 40 mg/kg body weight and left hand to hold the rat which is placed in the linen glove. The needle with the syringe containing Zoletil® 50 is injected intraperitoneally at the angel of 45 degree at 1/2 middle line of the abdomen. When the rat loses the eyelid reflex, it is a sign of deep anesthesia, which is a proper situation for sacrifice.

2.3.4. Sacrifice and tissue collection

During the deep anesthesia (Fig 4), the rat is removed the hair first, and then cut the skin, the abdominal muscle, and the sternum. After that, various tissues or organs (Fig 5) such as liver, kidney and spleen could be collected for further study.

Fig 4. The picture of sacrificing of rat

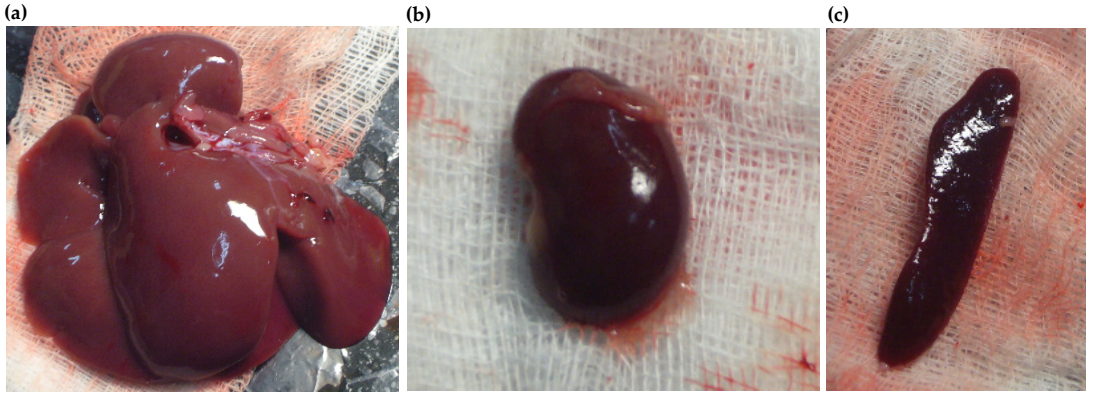

Fig 5. The picture of tissue collection of rat: (a) liver, (b) kidney, (c) spleen

2.3.5. Thoracic aorta isolation and vascular tone (vasoconstriction and vasorelaxation) assessment by Wire Myograph System

Thoracic aorta is isolated from 12 to 16-week old animals anesthetized with Zoletil (i.p. 40 mg/kg), and then peripheral tissues are cleaned, cut into slices of 2 mm in length for evaluating the contractile and relaxant response as shown Fig 6.

Briefly, aortic rings are carefully mounted on an isometric force transducer (XDFT05, Singa, Taiwan) with a tension of 1.8g, and placed in an organ chamber filled with Krebs solution (NaCl 99.01mM, KCl 4.69mM, CaCl2 1.87mM, MgSO4 1.20mM, K2HPO4 1.03mM, glucose 11.1mM) maintained at pH 7.4 and bubbled with 95% O2-5% CO2 as shown above. After an

equilibration of 90 min, 1µM of phenylephrine (PE) is added to the organ chamber for the assessment of contractile activity, and then 30µM of acetylcholine (ACh) is added to assess the endothelial integrity as shown Fig 7.

Rings with PE-induced tension <0.3g or ACh-induced relaxation <30% of pre-contractile tension are discarded. After the equilibration, vasocontractile response induced by phenylephrine (PE) and vasorelaxant response induced by acetylcholine (ACh) are evaluated in the absence and presence of endocrine-disrupting chemicals such as triclosan (antibacterial agent), nonlyphenol (surfactant), octylphenol (surfactant) and DEHP (plasticizer). All data are acquired and analyzed using the XctionView system (XctionView, Singa, Taiwan) (Fig 8).

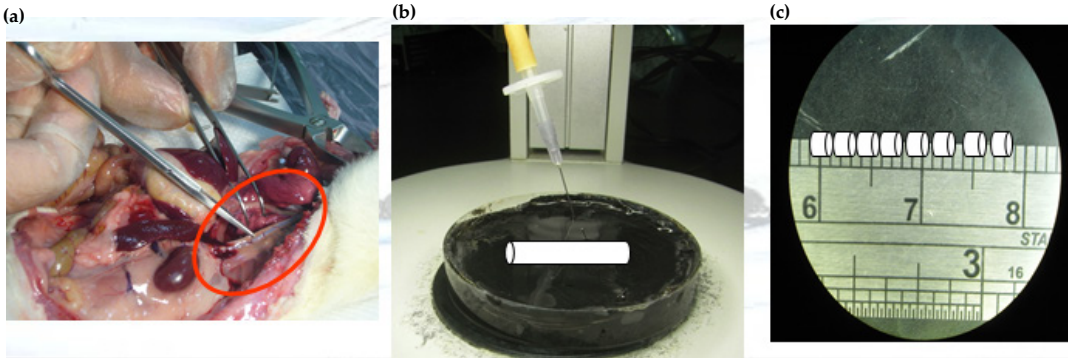

Fig 6. The picture of (a) thoracic aorta isolation, (b) peripheral tissues removal, (c) vascular slices of 2 mm in length preparation

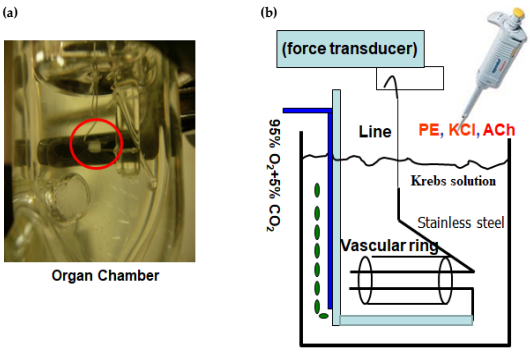

Fig 7. The picture of (a) aortic rings in organ chamber, (b) schematic diagram of Wire Myograph System

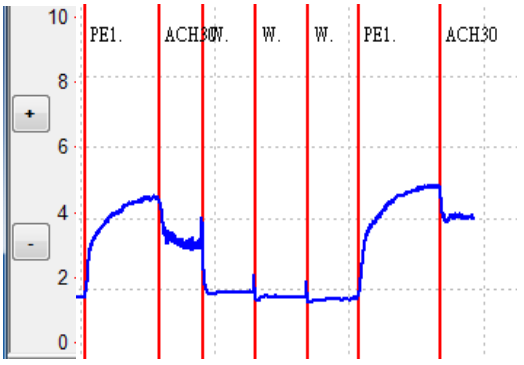

Fig 8. The original trace recording of vascular tension development by Wire Myograph System

3. RESULTS

3.1. Effects of tricolsan on phenylephrine (PE)-induced vasoconstriction

Triclosan (2,4,4'- trichloro-2'-hydroxy ether) is an widely used antibacterial agent, and it is used in toothpaste, shower gel, hand cream and mouthwash. In this study, we observed that triclosan dose-dependently (from 0.312 to 10.4mM) inhibited PE (alpha 1-adrenergic receptor agonist)-induced vasoconstriction as shown in Fig 9(a).

3.2. Effects of nonylphenol on phenylephrine-induced vasoconstriction

Nonylphenol (NP), the final degradation product of alkylphenol polyethoxylate used as non-ionic surfactants, migrates from contaminated water and polyvinyl-chloride food-packing films into human beings. In this study, we found that low concentration (1µM) of NP had little effects on PE-induced vasoconstriction; however, higher concentration (5-10µM) of NP markedly reduced vasocontractile response

mediated by PE. Results were summarized in Fig 9(b).

3.3. Effects of octylphenol on phenylephrine-induced vasoconstriction

Octylphenol (OP) is main observed in the production of PTOP (Para-tert-octylphenol) based resins used as tackifiers in tire manufacture. Additionally, because the structure of OP, one of the alkylphenols, is like the nonylphenol (NP) structure, NP, which is prohibited used in some products, is replaced by OP by some traders. In this study, we fig out that although middle concentration (1-5μM) of OP had little effects on PE-induced vasoconstriction; however, high concentration (10μM) of OP significantly reduced PE-induced vasocontractile response by around 40% as shown in Fig 9(c).

3.4. Effects of DEHP on phenylephrine-induced vasoconstriction

DEHP (di(2-ethylhexyl)phthalate) is one of the most commonly used plasticizers and it is also reported as an environmental hormone. Few years ago, there had a "plasticizer storm", which is DEHP was abused as a clouding agent in processed foods, in Taiwan. Although we observed the body weight development was reduced in DEHP (62.5 mg/kg/day)-treated rats for 24 weeks (data not shown), DEHP had little effect on PE-induced vasoconstriction as shown in Fig 9(d).

3.5. Effects of alkylphenols on acetylcholine-induced vasorelaxation

Acetylcholine (ACh)-mediated nitric oxide-cyclic GMP-dependent vasorelaxation pathway is well known in aortic rings, therefore, we investigate that whether alkylphenols such as nonylphenol or octylphenol exposure affect ACh-induced vasorelaxation in this study. We found that alkylphenols (nonylphenol and octylphenol) significantly affected the ACh-mediated vasorelaxant response; however, the effects of nonylphenol and octylphenol on vasorelaxation were not consistent [Fig 9(e)].

4. DISCUSSION

Although in vitro cell culture system is a simple model for mechanism investigation in various research fields, in vivo animal study is necessary for phenomenon demonstration in the complicated organism. Because of the animal welfare, we have to get the training courses about the 3Rs concept (Replacement, Reduction and Refinement) in laboratory animal use and manipulation program for techniques such as ear numbering, gavage, anesthesia, sacrifice and tissue isolation/collection in the research. Additionally, all of the processes of study need to follow the guideline of IACUC in University.

Good quality of experimental animal housing, keeping and operating could get the reliable and reproducible research results, and this will also reduce the laboratory animal use in the study, which is the goal of "Reduction" of 3Rs concept. Therefore, the laboratory animal manipulation personnel training program is essential to hold by IACUC in the University. Additionally, advanced instruments such as anesthesia machine, ultrasonic machine or In Vivo Imaging System (IVIS) are worth considering for the goal of "Refinement" of 3Rs concept.

Well-trained students or investigators and good environment including advanced instruments could make the study process smoothly and get some perspective conclusions like we shown in this article. We focus on the effects of endocrine-disrupting chemicals (EDCs) on vasocontractile response using laboratory rat's aorta as materials, and we found that: 1) parts of EDCs such as triclosan and alkylphenols (nonylphenol and octylphenol) significantly decreased the alpha-1 adrenergic receptor-mediated contractile response, and this phenomenon could be associated with hyporeactivity of vascular tissues to agonists in some pathophysiological condition; 2) vascular tissues seem not to be the target tissue for some of EDCs such as DEHP (di(2-ethylhexyl)phthalate), therefore, it did not affect vascular response to agonists; 3) nitric oxide-cyclic GMP pathway of vascular endothelium (endothelial cells) is also affected by alkylphenols (nonylphenol and octylphenol) exposure; however, nonylphenol decreased the ACh-induced response, but octylphenol increased the ACh-mediated response. This phenomenon is not observed in in vitro cell culture study. Therefore, we suggest that in vitro effects of EDCs cannot totally reflect the in vivo effect of them.

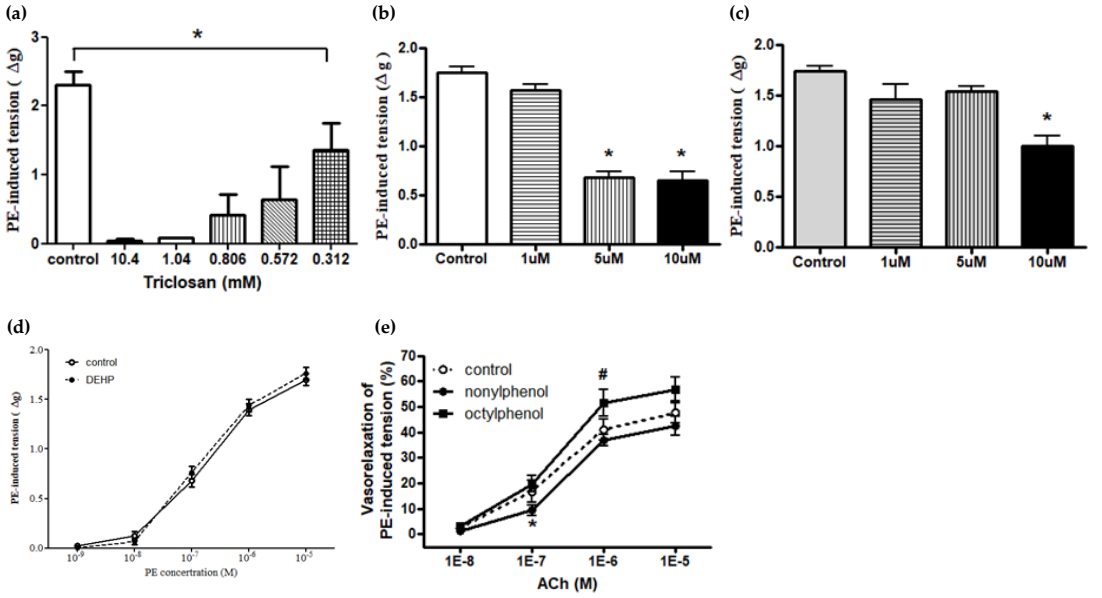

Fig 9. The graphs of (a) effects of triclosan, (b) effects of nonylphenol, (c) effects of octylphenol, (d) effects of DEHP, on PE-induced vasoconstriction and (e) effects of alkylphenols on acetylcholine-induced vasorelaxation

5. CONCLUSION

Biomedical research goes hand over fist and the animal study is going to play a more and more important role in various biomedical researches, which their results are not consistent with those from in vitro cell culture studies. Therefore, students or investigators with laboratory animal manipulation training certification will become more and more valuable, and this kind of technique ability is accepted internationally. This means well-trained students or investigators will get more cooperative opportunity from companies and universities all over the world. Therefore, a good laboratory animal manipulation personnel training program hold by IACUC is necessary in University.

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IMPROVEMENT OF MAIZE STOVER NUTRITION AS RUMINANT FEED WITH MANURE PLUS AND INORGANIC FERTILIZER

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ABSTRACT

Zea mays in Indonesia is used by farmer under crop-livestock integrated farming system (CLIFS). Integrated farming system hold special position as in this system nothing is wasted, the by-product of one system becomes the input for other. Crop yield for food and crop residues (stover and cornhusk) can be used for livestock feed, while farmyard manure as organic fertilizer can enhance agriculture productivity by intensifying nutrients that improve soil fertility, reducing the use of chemical fertilizers. The quality of farmyard manure could be enhanced by adding organic phosphorus (P-guano, P-phosphate rock) and organic nitrogen (N-legume) at initial phase of decomposition process, that was called as manure plus. Manure plus being locally available is cheaper souces of nutrient availability, and could be an alternative organic fertilizer to replace TSP and urea and reduce cost of crops production as well. Application of manure plus to improve maize stover and cornhusk nutrition as ruminant feed have been done since 2013 until 2018 in Central Java Indonesia will be presented in this paper.

Keywords: *Maize, manure, nitrogen, phosphorus, nutrient.*

1. INTRODUCTION

Zea mays is one of the key food crops in the world, serving as a staple food and main economic contributor for the people of the Asian continent. Sweet corn (*Zea mays* var. *saccharata*) is a type of speciality corn used as fresh or canned vegetable. It has sugar content greater than 25% during the milking stage (Singh and Reddy, 2011) with milky and sweet grain (Silva *et al*., 2007). Another variety of maize is sticky maize or waxy corn (*Zea mays ceratina* L.). It is a variety of maize with high amylopectin content 90% (Ramansyah *et al*., 2013). Sweet corn and sticky maize are utilized as raw materials for various uses also with a majority for food, while corn stover and cornhusk are used for ruminants feed in the tropics. Sweet corn and sticky mays are used by farmers under crop-livestock system (CLS) in Indonesia. The characteristic of CLS is crop yield for food and stover for ruminant feed, being the manure used as organic fertilizer which important to maintain the fertility of cultivated soils. The sweet corn can be harvested in 70-75 days after planting, while sticky maize in 75-80 days after planting. Both the cornhusk and stover of sweet corn and sticky maize have moisture content between 70-80% (Silva *et al*., 2007; Lukiwati *et al*., 2019).

Most of the soil in Indonesia that cultivated for crops production is deficient in P and N. An insufficiency of P and N in the medium from which the crops its mineral nutrition results in retarded growth and reduced development. Phosphorus and nitrogen deficiency can be corrected by applying P and N fertilizer to the soil at or before sowing (Lukiwati, 2007; Abdullah, 2009). Superphosphate (SP) and urea fertilizer is used to improve soil fertility in Indonesia. Superphosphate-36 contains 36% P_2O_5, with the P in a water-soluble form. However, the high cost of SP is now focusing attention on rock phosphate (RP) or guano fertilizer. Finely ground RP is an apatite mineral not readily soluble in water, and when added to acid soils, the solubilization of RP is increased. The availability of organic P is enhanced under acidic conditions in the soil. Therefore, an expensive P source (SP) can be replaced by RP when the application of the later is used in acid soil.

Reactive RP when directly applied at initial rates of between 80-360kg P_2O_5/ha, not only increased yields of corn, but resulted in similar yields compared to SP (Nassir, 2001). Rock

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phosphate and SP fertilizers increased maize grain yield, and dry matter (DM) yield of maize stover over those of control that did not receive P fertilizer. Phosphorus fertilizer could increase the plant growth, especially if the P nutrient is a major limiting factor to the plant production. Assumedly, the solubilization of RP on 300kg P_2O_5/ha increased and similar with SP on the same level. Therefore, an expensive P source (SP) can be replaced by PR when the application of the later on 300kg P_2O_5/ha (Lukiwati, 2002).

Most of the studies conducted on farmyard manure and organic-P and organic-N were done as separated fertilizer, while only a view work focused on the aspects of using organic-NP enriched manure to improve maize nutritive value of stover and cornhusk.

2. CROP LIVESTOCK INTEGRATED FARMING SYSTEM

Indonesia is considered as an agricultural country where agriculture has long been serving as the back bone of its economy. Predominant characteristics of Indonesian grass-root farming systems are family based, small holdings, small capital, subsistence crop and traditional (non-mechanized) management. Human and animal remain predominant power sources of farm work in Indonesia. Increasing inputs of chemicals and fertilizers in some agricultural production systems may pose a threat to the natural areas surrounding farmlands. Therefore, the solution is to continue increasing the production value while at the same time to minimize the environmental damage and to conserve the resources, as well as reduce poverty, hunger and malnutrition. Integrated farming system (IFS) might be one of the best approaches to be implemented in practice of sustainable agriculture production and management system (Syuaib, 2016). Crop-livestock integration (CLI) may represent a model of sustainable farming according to principles of nutrient recycling and efficient use of land and resources (Moraine *et al*., 2014). Integrated farming systems hold special position as in this system nothing is wasted, the by-product of one system becomes the input for other. Crop residues can be used for animal feed, while livestock and livestock by-product production and processing can enhance agricultural productivity by intensifying nutrients that improve soil fertility, reducing the use of chemical fertilizers (Lukiwati *et al*., 2018; Lukiwati *et al*., 2018; Lukiwati *et al*., 2019). Nutrient cycling and soil fertility can be improved at field and farm levels by animal-waste recycling and by including organic NP (Witjaksono *et al*., 2018).

Indonesia has no other option for achieving national food security than to manage its available and suitable sub-optimal lands for food production (Mariyono, 2014). The availability of land for agriculture in the moment and the future will be in sub-optimal land which has low pH and poor nutrients. The increasing pressure on land and the growing demand for livestock products in Indonesia makes it more essential to ensure the effective use of land and feed resources, including crop residues. An integrated crop-livestock farming system represents a key solution for increasing soil productivity, enhancing crop and livestock production, and safeguarding the environment through prudent and efficient resource use (Soelaiman and Maswar, 2014).

Indonesia has a higher density of cattle, buffalo, sheep and goats compared to other Southeast Asian countries. Livestock contribute 5-41% of farmer's income in the wet and dry agro-ecological zone and 60% in semi-arid zones. There is a tendency of farmers to consider livestock as household banks, easily liquidated when money is required (Nimwegen *et al*., 2009). Integrated farming systems hold special position as in this system; the byproduct of one system becomes the input for other. Food crop residues such as straws or other biomass provide a suitable fodder for cattle and other ruminants, while occasionally, the food crop provides supplementary grain feed for productive livestock. On the other hand, livestock waste in the form of manure and urine is the source of soil organic matter and fertilizer. Manure usually applied directly to the soil as a source of organic fertilizer to improve soil productivity and crop yields (Gupta *et al*., 2012). Recent research in various regions of the world has indicated that ICLS can enhance sustained crop and livestock production by efficiently using

agriculture system resources (Liu *et al*., 2012). Livestock are important in maximizing benefits because they turn crop residue into dollars while further improving the soil by depositing manure. Manured soils also were shown to be more porous and have lower bulk density (a measure of compaction) and higher aggregate stability than fertilized soils (Edmeades, 2003). Soil micro-organisms increase as organic matter increases, further improving the health of the soil and the crops grown on it (Coffey and Mumma, 2014).

Livestock production is a function of feed availability and quality. Livestock ruminants in general are fed on poor quality forages, and pasture produced on relatively infertile lands in the tropics. Cattle grazing P deficient areas typically develop symptoms of a phosphorosis which are reflected as retarded growth, poor reproductive performance, reduced milk yield, and bone abnormalities (Winks, 1990). Most of the studies conducted on farmyard manure and organic-P and organic-N were done as separated fertilizer, while only a view work focused on the aspects of using organic-NP enriched manure to improve maize nutritive value of stover and cornhusk.

3. PHOSPHORUS FERTILIZER

In commercially oriented livestock production, superphosphate fertilizer is applied to increase the forage production on non-productive land in Indonesia. However, the high cost of SP is now focusing attention on RP fertilizer. RP is a natural P source, it is relatively cheaper than SP and its locally available. There are RP deposits in Cirebon (West Java), Magelang/Pati (Central Java), and in Gresik (East Java). According to Kerridge and Ratcliff (1982), RP can also be used as a maintenance fertilizer for pasture species on acid soils in humid areas. RP contains P in a form that is not readily soluble in water but soluble in neutral ammonium citrate solution making it slowly available to plants. Water-insoluble P fertilizer do not all behaves in the same way as SP. Finely ground RP is an apatite mineral and when added to acid soils, the solubility of RP is increased so that below pH 5.5 it begins to make a significant contribution, and on really acid soils it can be a useful source of phosphate.

To alleviate the P deficiency in acid latosolic soil, superphosphate (SP) or rock phosphate (RP) are added. Phosphorus dynamics in the soils are complex, because they involve both chemical and biological processes and the long-term effects of sorption (fixation) and desorption (release) processes (Bationo and Kumar, 2002). Both types of P fertilizer (rock phosphate and superphosphate) are directly applied to the soil. Lukiwati (1999) reported that SP produced significantly higher maize grain yield and dry matter yield of stover than those that received application of rock phosphate. According to Jones (1990), the response obtained to applications of P fertilizers will be a function of many factors i.e., the initial availability of soil P, the pasture species used, the form of fertilizer applied, and the presence or absence of effective mycorrhizae fungi in the soil.

Rock phosphate as one of the phosphorus sources, its price is relatively cheaper than superphosphate and it is available in fast amount in Indonesia. However, RP belongs to slow available source of phosphorus. The residual effect of RP and SP fertilization was not significantly different on the maize grain yield and DM yield of maize stover (Lukiwati and Waluyanti, 2001).

Different management practice is adopted to increase and optimize the maize yields. For example, use of organic manures alongside inorganic fertilizers often lead to increased soil organic matter (SOM), soil structure, water holding capacity and improved nutrient cycling and helps to maintain soil nutrient status, cation exchange capacity (CEC) and soil's biological activity (Saha *et al*., 2008).

4. ORGANIC PHOSPHORUS ENRICHED MANURE

Manure enriched with RP show better agronomic efficiency than di-ammonium phosphate (DAP) when applied on equal P_2O_5 basis (Sekhar *et al*., 2008). In the first season planting of sweet corn, RP (66kg P/ha) enriched manure (20 t/ha) and inoculated with biodecomposer (EM4, stardec, startmic) depend on the treatment, and that was called

manure plus applied in the vertisol soil. The result showed that RP enriched manure with or without biodecomposer, in granular or non-granular form, resulted in similar on CP, P and Ca concentration of sweet corn stover (Table 1) (Lukiwati *et al*., 2015).

Table 1. Nutrient concentration of sweet corn stover in the first planting season (%) (Lukiwati *et al*., 2015)

Treatments	CP	P	Ca
Manure	5.70	0.56	0.19
Manure + EM4	6.69	0.48	0.30
Manure + Stardec	6.84	0.51	0.18
Manure+ StartMik	6.74	0.55	0.17
Granular manure+EM4	7.11	0.47	0.20
Ganular manure+Stardec	5.70	0.43	0.24
Granular manure+Startmik	6.44	0.39	0.25

Residual effect of manure plus inoculated with biodecomposer resulted in similar on crude protein concentration, and higher on P and Ca concentration of sweet corn stover compared to manure without biodecomposer. Residual effects of application rock phosphate enriched manure can be used for the next growing season of second planting (Lukiwati and Pujaningsih, 2015).

Table 2. Nutrient concentration of sweet corn stover in the second planting season (%) (Lukiwati and Pujaningsih, 2015)

Treatments	CP	P	Ca
Manure	8.72	0.24	0.27
Manure + EM4	8.73	0.33	0.36
Manure + Stardec	8.94	0.33	0.40
Manure+ StartMik	8.33	0.38	0.39
Granular manure+EM4	6.06	0.38	0.53
Ganular manure+Stardec	8.47	0.37	0.54
Granular manure+Startmik	8.64	0.43	0.45

Table 3. Calsium and phosphorus concentration of sweet corn stover in the third season of planting (%) (Lukiwati *et al*., 2017)

Treatments	Ca	P
Manure	0.17	0.37
Manure + EM4	0.24	0.42
Manure + Stardec	0.17	0.41
Manure+ StartMik	0.19	0.40
Granular manure+EM4	0.24	0.50
Ganular manure+Stardec	0.21	0.48
Granular manure+Startmik	0.24	0.44

Mineral concentration (Ca and P) of sweet corn stover at the third season of planting resulted in higher if inoculated with biodecomposer compared to without biodecomposer. Table 3 showed mineral concentration of sweet corn stover in the third season of planting (Lukiwati *et al*., 2017).

5. ORGANIC NITROGEN AND PHOSPHORUS ENRICHED MANURE

Nitrogen and phosphorus as a major plant nutrient and the primary factors limiting crops yield (Zaidi *et al*., 2009). Therefore, application of N and P fertilizers is essentially required to improve crop yield. Applying fertilizers, particularly in the inorganic form, in excess of plant rewuirement can increase the chances of fertilizer loss and environmental pollution. Organic manures, apart from improving physical and biological properties of soil, help in improving the efficiency of chemical fertilizers. Manure in general low in most of major nutrient, and organic nitrogen (N-legume) and organic phosphorus (rock phosphate, guano) can advantageously compensate the imbalance of manure on N and P nutrients (Ramilison, 2001. Application of organic fertilizers with the addition of inorganic fertilizers, increased soil fertility and crop production also saved the use of inorganic fertilizers (Saha *et al*., 2013). This is in line with the Regulation of the Minister of Agriculture No.40/2007 that the purpose of organic material return or the provision of organic fertilizers equipped with inorganic fertilizers is to improve soil conditions and fertility. Lukiwati *et al*., (2018) reported that organic-NP enriched manure which added 200kg N/ha (*Gliricidea sepium*) and organic phosphorus 66kg P/ha (rock phosphate, guano) and all plot was added 125 K/ha (KCl) resulted in similar on CP (crude protein) and Ca uptake of sweet corn stover (Table 4). Organic-N (N-legume) and organic-P (RP, guano) and organic-NP enriched manure resulted in similar on CP and Ca uptake of stover compared to inorganic fertilizer (TSP+SA). Application of organic NP enriched manure could be a promising organic fertilizer to replace inorganic fertilizer (TSP, SA) to produce sweet corn in vertisol soil.

Tabel 4. Calsium and CP uptake of sweet corn stover in the first season of planting (g/plot) (Lukiwati et al., 2018)

Treatments	CP uptake	Ca
TSP	380.50	16.02
Sulphate acid (SA)	631.62	25.11
TSP+SA	584.42	25.48
Manure	402.53	19.97
Manure+RP	445.84	20.11
Manure+guano	483.07	24.20
Manure+ *G. sepium*	422.10	27.70
Manure+RP+*G. sepium*	415.39	24.37
Manure+guano+*G.sepium*	486.75	29.32

Lukiwati *et al*. (2019) reported that fresh waxy corn (sticky maize) stover production and fresh cornhusk production significantly affected by the manure plus treatment (organic-NP enriched manure). Manure plus resulted in similar on fresh waxy corn stover production and fresh cornhusk production compared to inorganic-NP fertilizer. It was concluded that organic-NP enriched manure replaces NPK as inorganic fertilizer (Table 5).

Table 5. Fresh production of stover and corn-husk of waxy corn with manure plus and inorganic fertilization in the first season of planting (kg/plot) (Lukiwati et al., 2019)

Treatments	Fresh stover production	Fresh corn-husk production
SA+TSP+KCl	24.50	4.30
Manure	17.90	6.30
Manure+RP	19.30	5.60
Manure+guano	21.40	3.80
Manure+*G. sepium*	23.40	4.40
Manure+RP+*G. sepium*	25.50	4.00
Manure+guano+*G.sepium*	27.30	4.20

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WASTE TRANSFORMATION CLOSED LOOP FARMING: A CONCEPT

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ABSTRACT

"Transforming Biodegradable Waste, Integrating Plant and Animal Systems, Deindustrializing Agriculture, Reducing Carbon Emissions, Sequestering Carbon, De-commodifying Food and Restoring Biodiversity". This paper has seven major themes, as its subtitle indicates. Small farmers transform biodegradable waste at the highest possible levels, they closely integrate multiple plant and multiple animal systems, and they deindustrialize the production of food. These three strategies allow them to play a major role in reducing carbon emissions and in sequestering carbon. Furthermore, small farmers participate in social enterprises that share and integrate waste resources and waste technologies, that provide education and training, and that take care of all aspects of selling to consumers. With these basic elements in place, small farmers are able to decommodify the sale of food. Food is not just another commodity to be traded in the global marketplace. The market value of food should never be allowed to override broader issues relating to food safety, food security, food justice, food sovereignty, income inequality, the health of the environment and the biodiversity of our planet.

Keywords: *Poverty, pollution, food safety, food security, food justice, food sovereignty, income inequality, biodiversity.*

1. INTRODUCTION

Throughout my life, I have always seen waste as one of the most valuable resources anyone could ever possess. This might sound a bit unusual and startling to those who seldom think about waste, but most forms of waste are anything but waste.

Fig 1. Dense medium separators at CFF

Back in the '90's, I designed a series of dense medium separators to sort and recover non-ferrous metals and plastics from automobile and industrial shredder residue (Fig 1). Even though this waste had a non-ferrous metal content that averaged about 15%, year after year it was being discarded as worthless. Traditional separators were inaccurate, whereas my separators could distinguish particles that differed in specific gravity by only a few points to the third decimal place. One complete set of my separators could process the non-ferrous residue of as many as 500 automobiles per hour. On a single site, my clients were able to recover about 40 tons an hour of non-ferrous metals such as magnesium, aluminum, copper, brass, lead and so forth. At an average value per ton at that time of $1,000 US dollars, each of my clients had a revenue stream of about $40,000 US per hour. The largest recycling companies in Western Europe, - NV Galloo, CFF Recycling and GDE Guy Dauphin Environment - all bought this unique dense medium process. Since then, tens of billions of US dollars of metals have been rescued from landfill. The same separators can be used to separate electronic waste as well as household waste free of biodegradable waste.

In 1997, I set up a $25 million US dollar separation facility at Chaparral Steel in Midlothian, Texas. With this installation Chaparral Steel was able to earn a yearly profit of over $300 million US dollars, a profit greater than that generated by their steel mill. The non-ferrous waste they had been throwing away had a greater value than the ferrous scrap they were

[1] Empowering the Poor though Waste Transformation (EPWT), Vietnam (http://epwt.vn/en/home).
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refining into steel. Furthermore, the rubber, foam rubber and plastics isolated by my separators were free of PVC and could be used to power a cement kiln owned by the same steel company. This cement kiln did not have to burn fossil fuels to make cement.

2. POVERTY AND POLLUTION IN VIETNAM

After moving to Vietnam in 2006, I saw two things that immediately grabbed my attention: a lot of poverty and a lot of pollution. So I asked a simple question: could the transformation of biodegradable waste be the means that would enable poor farmers to acquire substantial wealth? I looked at every possible form of biodegradable waste, and nowhere could I find anything that could not be transformed into products of considerable value.

But to derive the highest economic return and the greatest environmental benefit in the transformation of biodegradable waste, the right technologies are needed. If technologies are expensive or sophisticated, small farmers will not be able to afford them or acquire the skill to operate them. Each technology has to be simple and low-cost, yet when brought together and properly integrated, something happens that goes far beyond any one technology viewed in isolation. Many natural cycles of waste transformation are needed, all deeply interconnected, all adding value to one another, all endlessly renewing themselves in a self-sustaining, cascading and looping manner. Here not a gram of waste is wasted, and biodegradable waste, in all of its many forms, becomes the greatest resource that a small farmer could ever possess.

But before we can tap into the bounty that biodegradable waste has to offer, there's a waste hierarchy that we should recognize and respect. Biodegradable solid waste can be situated into four different types in descending order of nutrient content (Fig 2). The first two types of waste are putrescent, and the last two types are non-putrescent.

2.1. Examples of type 1 waste

Fig 2. The four levels of waste transformation

Types of waste	Methods of transformation	Products
1. *high-grade putrescent*	*heat treatment and/or fermentation*	*Feed*
2. *low-grade putrescent*	*larvae and red worm bioconversion*	*larvae, worms, vermicompost*
3. *high-grade non-putrescent*	*composting*	*compost*
4. *low-grade non-putrescent*	*gasification*	*syngas and biochar*

A few examples of Type 1 waste: banana pseudostem, coffee pulp, spent coffee grounds, cashew apples, passion fruit peel, passion fruit seed, sugar beet pulp, cassava pulp, sweet potato vines, taro foliage, tofu wake, sisal waste, brewery and distillery waste, peanut press cake, coconut pulp, cacao pods, crustacean waste, fish byproducts, fish mortalities, hatchery waste, poultry carcass waste, poultry feathers, poultry viscera, slaughterhouse waste, food waste and bone waste. Type 1 waste also includes many different kinds of co-cropped biomass (such as perennial peanut) that can easily be transformed into a feed.

2.2. Heat treatment and/or fermentation at level 1

In the transformation to Type 1 waste into feed, one can heat-treat and/or ferment. Heat

treatment can be carried out at a profit using high-grade heat generated by combined heat and biochar gasifiers that situate at Level 4. One might ask: how is it possible to produce high-grade heat at a profit? The answer is simple: the high-quality biochar produced by this type of gasifier has a greater value than the biomass from which it is derived. With regard to fermentation, I found several thousand papers published the by Livestock Research for Rural Development (http://www.lrrd.org). These papers outline in great detail the many benefits of fermentation in terms of feed preservation, feed sanitation as well as significant improvements in animal health and productivity.

2.3. Gasification at level 4

Biochar produced at Level 4 has a surface area that ranges from 250 to 650 square meters per gram. Such a large surface area provides microbes active at the first three levels of waste transformation with a lot of space for biofilm formation. When biochar is incorporated into fermented feed at Level 1, we see increases in poultry, animal and fish growth ranging from about 17% to 40%. When biochar finally makes its way into the soil, we see increases in plant growth ranging from 30 to 400% (Fig 4). Biochar remains in the soil for hundreds to thousands of years – an important dynamic in terms of carbon sequestration. Dr. Thomas R. Preston and co-workers have done more than twenty studies with biochar from my gasifiers, and he wrote that "Olivier gasifier stoves have been the driving force for nearly all the work we have done on biochar" (personal communication).

Fig 3. A Beautiful Blue Flame

Fig 4. Soil, Biochar and Charcoal (Southavong et al., 2012)

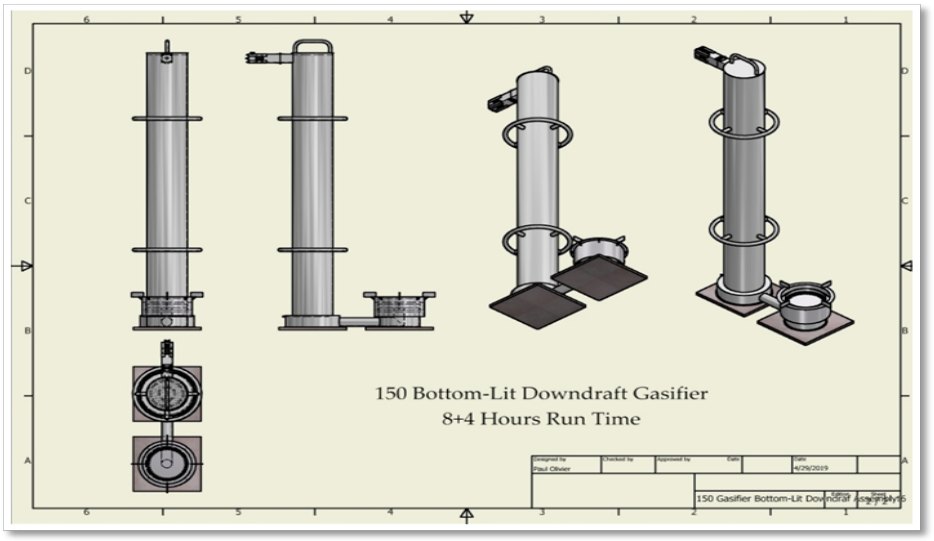

Fig 1. 150 Bottom-Lit Downdraft Gasifier

On the safety of my gasifiers in terms of emissions, see The Role of Small-Scale Crop Residue Gasifiers in Southeast Asia's Clean and Improved Cooking Sector (Devlin, 2018). Cost here is not at all an issue. A gasifier of a 7kW output sells for about $55 US, and a gasifier of an 80kW output sells for less than $500US. These gasifiers are made out of high-quality stainless steel (304 and 310). They produce a beautiful blue flame (Fig 3). We will soon start making bottom-lit downdraft gasifiers with a run time of 12 hours (Fig 5).

2.4. Type 2 and type 3 waste transformation

Type 2 waste is fresh manure. To facilitate the isolation and collection of fresh manure, animals are housed indoors on soft bedding. Bedding might vary in depth from 60cm to one meter. The bedding is composed of loose waste biomass in combination with about 5 to 10% biochar by volume. Each day bedding and animals are lightly sprayed with a probiotic liquid. The bedding remains at all times fly-free, pathogen-free and completely odorless. Antibiotics and other pharmaceuticals need never be used. This bedding concept is not experimental by Dr. Le Van Hien in Bao Loc (Lam Dong province, Vietnam) raised over 500 pigs on bedding over a period of several years. Small farmers remove fresh manure off bedding each day and place it in a biopod right next to the pen (Fig 6) where it is quickly devoured by larvae of the black soldier fly in several states of growth. Each day mature prepupal larvae neatly self-harvest into a bucket. Larvae do not have to be sieved out of their residue. When larvae eat fresh manure, not a drop of liquids is released. All within the biopod stays dry, aerobic and free of odor. The residue of the larvae is then fed to red worms or earthworms.

Fig 6. A Biopod

Fig 7. Mature prepupal larvae raised on pig manure

Fig 8. A portable, no-mix dry toilet

Larvae eat fresh manure, something that worms cannot do, and the worms eat the more fibrous biomass that the larvae cannot digest. Together they form an outstanding partnership in nutrient extraction, waste valorization and waste sanitization. Larvae and worms also effect the surface activation of biochar. Surface activation enhances the capacity of biochar to chemisorb nutrients, minerals and dissolved organic matter. The products of Type 2 waste transformation are larvae (feed) and worms (feed), and vermicompost (fertilizer). Vermicompost containing activated biochar is by far the finest fertilizer that exists. Chemical fertilizers along with conventional pesticides, herbicides and fungicides are poisons and should never be used in agriculture.

Animal urine gets rapidly immobilized in bedding, and spent bedding is a great fertilizer. The immobilization of urine can be understood as mesophilic composting at Level 3. In other words, animals are raised in large mesophilic compost bins. At least 12 square meters of bedding should be allotted to a cow, and at least two square meters of bedding should be allotted to a grow-out pig. A sow with piglets, however, should be granted at least 12 square meters of bedding where she and her piglets can build a nest.

Pigs run a play on bedding, they get plenty of exercise. There is very little anti-social behavior for the simple reason that pigs are happy and are not under stress. Tooth-clipping, tail-clipping

and castration are cruel and unnecessary. Pigs love to root through bedding, and as they do so, they quite often eat bedding. Fermented feed can be served to them on banana leaves in the form of individually formulated piggy packets. Troughs that might cut and bruise are not used. In this soft bedding concept, all of the behavioral, nutritional and comfort needs of an animal are met. The pen remains dry at all times. Biodigesters that waste water and transform nutrients at the wrong level are not needed. Type 3 waste transformation also includes the thermophilic composting of green waste that cannot be transformed into feed or fed to larvae. As much as 50% of a thermophilic composting mix might consist of biochar (Fischer and Glaser, 2012).

3. WHAT AQUAPONICS TEACHES US

We can learn a lot from aquaponics. If fish swim outdoors in streams or rivers, their waste cannot be accessed and directed to the growing of plants for human consumption. Aquaponics solves this problem. Fish are enclosed in tanks or ponds so that their fresh waste can be accessed and immediately directed to growing plants. For every kg of fish produced aquaponically, about 12kg of vegetables are produced. The waste of a fish over its lifetime can generate more value than the fish, for the simple reason that there is no such thing as a perfect feed conversion ratio.

Just as the raising of fish in tanks does not allow fish excreta to dissipate in stream or river, so too, the raising of animals indoors on soft bedding does not allow animal excreta to dissipate in yard or pasture. Urine is rapidly immobilized within bedding and can be used as fertilizer. At the same time, high-quality feed in the form of larvae and worms can be produced from fresh manure collected off bedding. Mature prepupal BSF larvae (Fig 7) have a protein content of about 42% protein and a fat content of about 34%. BSF lipids can contain up to 54% lauric acid, an acid active against lipid coated viruses (including HIV and measles), as well as Clostridium and many pathogenic protozoa. For every 5 or 6kg of pig manure, there are sufficient nutrients to cultivate about 1 kg of larvae and about a half kg of worms. With the millions of tons of fresh animal manure produced in Vietnam each year, Vietnam could easily grow enough larvae and worms so as to completely offset and replace the import of commercial feedstuffs such as corn and soybean. Here the creation of feed does not involve photosynthesis or the harvesting of industrial fish.

The idea of enclosing poultry and animals indoors on bedding so that their fresh waste can be properly accessed and transformed is not so readily understood, especially by cowboys. Some people cling to romantic notions of pigs, chickens and cows roaming freely outdoors on a pleasant, sunny day in springtime. But if we let livestock roam outdoors, only pastureland gets fertilized, and this fertilization can be highly inefficient due to losses of P in runoff as well as losses of N in forms of ammonia, nitrous oxide, nitrogen oxide and nitrate. Cows produce globally about 205 trillion liters of methane each year – one of the leading killers of our planet. "Researchers say cows are producing twice as much methane gas as scientists previously believed Ross (2013), also see on the publication of Wolf *et al*. (2017). However, if cows are kept indoors, their diets can be carefully formulated with biochar and other nutrients so as to reduce the production of enteric methane, sometimes by as much as 42%, and any methane that they produce can be captured in biochar filters inoculated with methane-eating bacteria.

4. COMMON MISTAKES IN WASTE TRANSFORMATION

Type 1 waste should not be fed to larvae and worms at Level 2, unless it has spoiled and can no longer be preserved as feed. BSF larvae have a protein conversion ratio of 2 to 1, and when food waste is fed to larvae instead of being used directly as feed, there is a substantial loss of nutrients. Why make a feed out of a feed, especially when there is no shortage of Type 2 waste? Type 1 waste should not be composted at Level 3. Composting food waste creates an inefficiency of over 80% in the utilization of nutrients compared to transforming food waste directly into feed. Likewise, one should not make feed out of food: for example, feeding soybean or corn to insects, pigs, chickens, cows or fish. "Feeding grain to farm animals

is a particularly egregious practice, argued Stevenson. It is inherently wasteful of calories: for every 100 calories of human edible cereals fed to farm animals, just 17-30 calories enter the human food chain as milk or meat" (van der Zee, 2017). Finally, there is the total nonsense of making fuel out of food: for example, making ethanol out of corn, or making biodiesel out of palm oil, soybean oil or larval fats (Malins, 2017). More than one third of the entire corn crop in the United States is devoted to ethanol production. Using palm oil used as fuel is also a disaster for the environment: "For every megajoule of energy used, the study finds that palm oil emits 231g of CO_2 equivalent and soybean oil 150g/CO_2e, far higher than the UN climate science panel's estimates for any fossil fuel" (Neslen, 2016).

5. THE LOCALIZATION OF FEED, FERTILIZER AND FOOD PRODUCTION

At the same time that farmers provide comfort and dignity to poultry and animals by raising them indoors on soft bedding, farmers can make use of larvae, worms, vermichar and spent bedding to help fulfill their need for feed and fertilizer. The buying and selling of these intermediate products make no sense, since, on small farms where multiple plant and multiple animal systems are properly integrated, farmers need both feed and fertilizer. Why would small farmers sell intermediate products, when they could use them on their farms to create additional revenue? The buying and selling of feed and fertilizer, as takes place within industrialized agriculture, is aberrant and does nothing more than enrich big corporations, traders and middlemen. These neo-colonial parasites create two unnecessary monetary streams grounded in the lack of integration of plant and animal systems. This drives up the cost of food, and many people go hungry and malnourished, even in the developed world. Industrialized agriculture along with industrialized animal husbandry is the most destructive force on our planet.

Since fresh biodegradable waste cannot be transported economically long distances, the food derived from it must be locally raised or grown. Food is not a just another commodity to be traded in the global marketplace. When all of the environmental benefits of waste transformation are correctly factored into the production of food, food is a lot more than the nutrition it provides. If waste transformed locally is the best thing we've got in producing food, so too, food produced locally is the best thing we've got in cleaning up certain types of waste.

All nations must learn to deindustrialize the production of food, to localize the production of food and to decommodify the sale of food (Helena, 2018). How else can they achieve food security in the context of political instability, war or climate change? The market value of food should never override broader issues relating to food safety, food security, food justice, food sovereignty, income inequality, the health of the environment and the biodiversity of our planet. Since no country should depend on another country to transform its waste, no country should depend on another country to produce its food. When consumers buy food produced locally, they know well the small farmers who produce it, and they know that it is safe. Organic certification is not needed.

6. TRANSFORMING HUMAN WASTE

When human waste is correctly transformed and safely returned to agriculture, sustainability kicks in as never before. "For 4,000 years, human excrements and urine were considered extremely valuable trade products in China, Korea and Japan" (De Decker, 2010). There is no way to define sustainability in agriculture without the transformation of human waste at high levels, otherwise agriculture will always be dependent on chemical fertilizers and other external inputs. To achieve sustainability in an economical, safe and efficient manner, all toilets should be no-mix dry toilets, and septic tanks and sewage treatment plants that waste human waste and precious water resources should be abolished. The high-level transformation of human waste is not optional. It is absolutely necessary if ever humans are to live in harmony with the natural world. No-mix, portable, dry toilets can be fabricated in 304 stainless steel for

less than $25US a piece (A No-Mix, Dry Toilets). The open-front toilet shown in Fig 8 works in conjunction with a simple and inexpensive hand-held urinal. There are many simple ways to reduce odor down to zero when urine and feces are temporarily stored indoors.

7. TRANSFORMING BONE WASTE

In Vietnam, cow and pig bones are boiled anywhere from three to six hours to make stock for noodle soups such as Pho, Bun Bo, Hu Tieu... Such intensive boiling demands a great deal of energy - energy usually derived from the toxic combustion of coal, charcoal or wood. However, if gasifiers are used to boil bone, combustion is clean, and energy cost becomes an energy profit due to the value of the biochar produced. In Vietnam waste bone usually gets discarded along road and streets, or it ends up in landfill (Tuoitrenews, 2018). The discarding of spent bone is incredibly wasteful, since about 80% of the phosphorous within an animal is found in its bone.

So instead of dumping spent bone along streets or in landfills, I suggest that bone be crushed into a fine powder and pelleted along with agricultural biomass. These pellets are then gasified at temperatures as high as 85°C. Such a high temperature is easily reached in my gasifiers when pellets are used as fuel. This high temperature also destroys prions that might contaminate bone. As fresh bone is being boiled in a pot above the gasifier, spent bone is charred at the same time. This results in the simultaneous production of broth, bone char and biochar. About 80% of bone char consists of highly valuable tricalcium phosphate (www.en.wikipedia.org). When gasifier heat is used to make bone char, heat is not wasted as in dirty biochar or bone char kilns. In contrast to rock phosphates which might contain uranium, cadmium, lead, copper, arsenic and other heavy metals, bone char is generally free of such contaminants. Bone char can be added to a feed fermentation mix, or it can be crushed and incorporated into the soil. It is referred to as a "soft" phosphate in that it is not as soluble and leachable as commercial phosphate. The high-level transformation of bone is not optional. Apatite reserves are rapidly dwindling. In as little as 20 years, many of these reserves will no longer be economically exploitable. Some predict that massive world-wide starvation will follow in the decades to come.

8. CONCLUSION

In conclusion, small-scale multifunctional farms can be 10 to 30 times more productive and more lucrative per hectare than large-scale mono-functional farms, and when small farmers are incorporated into social enterprises that educate, train, equip and sell on their behalf, small farmers in developing countries, with no more than an hectare of farmland, could each make $50,000 to $100,000US per year (A Wheel of Life). If small farmers provide land, they should receive 40% of the end value of their food to consumers. If they provide the labor needed to produce food, they should receive another 40% of the end value. The social enterprise then carries out the home and restaurant delivery of food, and in so doing, it should make 20% of the end value to consumers. Markets and supermarkets are not needed in procuring food. Since it has no employees, a social enterprise does not need to be unionized. Even though it is not a charity, it stands as a staunch defender of economic and environmental justice.

Since abundant food can be produced wherever there is waste biomass or co-cropped biomass from farmland, pastureland is no longer needed and can be returned to the wild. Wild animals should not be confined in circuses, zoos or parks. By doing away with pastureland, as well as agricultural land devoted to growing grain for poultry and livestock, we could return to the wild up to a third of the land surface of planet Earth. This might be a good place to start as advocated by Wilson (2015). Once humans stop taking from the pastureland, pastureland will rebound in an explosion of biodiversity. Wolves, tigers, lions and cougars, along with many other wild carnivores, deserve a home free of humans and their domesticated animals. As domesticated animals housed indoors would no longer compete for grass with wild ungulates, populations of ungulates would increase, and wild carnivores would have sufficient prey.

Similarly, humans should not take fish from the sea. If humans want to eat fish, fish can be grown aquaponically in fresh or salt water tanks or ponds. Once humans stop taking from the oceans, they, too, will beautifully rebound in an explosion of biodiversity.

When people let waste go to waste or fail to transform it at the right level, people often go hungry. How often do we see pictures of starving or malnourished children, and at the same, they are everywhere surrounded by waste? We do not have a food crisis, but a catastrophic failure to grasp the basics of waste transformation. The natural world is prepared to give us all the food that we need. We have only to give back to her the transformed biomass that she needs. Marvelous things happen when humans do not waste and when they live in harmony with the natural world.

(More detail, please see : https://www.dropbox.com/s/wbarsaz89mvtglw/Summary.pdf?dl=0

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CREATING ANIMAL HUSBANDRY PROFESSIONALS FOR THAILAND, THROUGH "WORK-BASED EDUCATION"

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ABSTRACT

This paper reviews "Work-based Education (WBE)" as an inspirational approach for Thai corporate organizations to secure animal husbandry professionals for their future needs. At present, Thai graduates are at a high risk of being unemployed upon graduation, as a result of choosing just to study programs that are popular amongst the student populace. Regrettably these programs are not in line with the current industry's trends and needs. Inevitably, "Work-based Education" a new concept and methodology in teaching and learning, can help the students find their true vocation, from tandem instruction both in the classroom and the workplace. The Faculty of Agro-Industry, Panyapiwat Institute of Management, Thailand, is providing a Farm Technology Management program, using a WBE approach, to create animal husbandry graduates for the CPF Company (Thailand's biggest Agri-Industrial conglomerate). Normally, the majority of students who graduate will only be good in theory, WBE as a consequence is becoming more important, to help cultivate work experiences and apply knowledge from 'on the job' training/work to the classroom by learning from real and experienced professionals. Therefore, every confidence may be placed on students/employees who have passed the WBE course, as they will be ready to work immediately upon graduation.

Keywords: *Work-based Education, Agro-Industry, Farm Technology Management.*

1. INTRODUCTION

The Corporate University (CU) is any educational entity that is a strategic tool designed to assist its parent company/organization in achieving its goals by conducting activities that foster individual and organizational learning and knowledge. At the same time seeking to strengthen their relationships with the higher education system. The Corporate University has become very popular among world-leading companies, for example and to name just a few: Walt Disney, Boeing and Motorola in the USA, Infosys in India, Huawei in China, Singapore Airlines in Singapore and Etisalat in the United Arab Emirates have well-established corporate universities/learning centers (Antonelli *et al*., 2013; Baporikar, 2015).

Panyapiwat Institute of Management (PIM) and the Faculty of Business Administration was established on the 9th of March 2007, as the first Corporate University in Thailand. It is operated under the umbrella of the CPALL Company (Thailand's leading retail business conglomerate) to supply a workforce for their 7-Eleven retail stores. Thereafter PIM expanded its academic capabilities to include Liberal Arts, Engineering and Technology, Communication Arts, Management Sciences, International College, Innovative Agricultural Management, Food Business Management, Education, and the Faculty of Agro-Industry (AGI), and Logistics Technology Management. All PIM's Academic Faculties and Colleges are accredited by Thailand's Ministry of Education. "Creating Professionals through Work-Based Education (WBE)" is PIM's unique vision and they network with several hundred private enterprises to provide scholarships and professional support, in particular the provisions of experienced and qualified instructors, and internship and work placement sites.

The WBE system allows students the opportunity to practice in a real working environment for more than 50% of their 4 year of BSc program. While half of the students are taking their theoretical courses at the PIM campus (Chaeng-Watana Rd, Nonthaburi-Bangkok Metropolitan), the other half are working at various work placement sites (switching back and forth every 3 months between their theory

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and practical courses) over the 4 year program. Graduates are expected to gain adequate theoretical knowledge and substantial hands-on work experiences, with graduates ready to work effectively and immediately upon graduation.

CPF (Thailand) PCL (Thailand's largest Agri-Industrial conglomerate) signed a MOU with PIM in 2014 requesting PIM's assistance to establish the Faculty of Agro-Industry (AGI). Agro-Industry began accepting the first intake of Thai undergraduates for the "Bachelor of Science Program in Farm Technology Management" (FTM) in June 2015. A year later, in June 2016, AGI began accepting undergraduate Thai students for a Bachelor of Science Program in Food Processing Technology Management or PTM. CPF's worldwide expansion and investment initiative in 18 countries, its vision to be the "Kitchen of the World" and as a prerequisite to international acceptance, required that the teaching medium be changed from Thai to English with effect from 2018.

2. FARM TECHNOLOGY MANAGEMENT (FTM) PROGRAM

Farm Technology Management is one of AGI-WBE programs specifically focused on producing animal husbandry professionals (Pig and Poultry) to staff the CPF Company. The entire FTM curriculum was designed by CPF professional farm executives according to PIM's motto, "Creating professionals by professionals", its innovative, forward thinking and highly relevant. Farm Technology Management provides students with a 'well balanced' learning experience of 43% theoretical study in the classroom and a 57% practical hand-on experience, by studying theory for 3 months and then rotating for 3 months with a practical experience and then returning to the theory class again, alternating like this over the full 4 year program. There is no long holiday as with regular Universities and colleges, however students have a short break of about 2 weeks during their rotation from theory to practical and visa-versa. Students may take a rest or return to their homes. Fourth year students will spend their final 6 months, before graduating, in one of many CPF farms, the last 3 month of which, students will be given 'problem issues' from the farm to create and inspire initiatives with their mentors and then spend those 3 months researching and applying this initiative as an "innovation". Thereafter students submit the complete and final report to AGI, after their advisor has finished checking the score. Once the AGI Board qualifies and quantifies the graduate, the Graduates will receive their degree. "Bachelor of Science" in Farm Technology Management.

3. RECRUITING THE RIGHT STUDENT FOR FUTURE FRAM PROFESSIONAL

Grade 12 students who wish to apply for an FTM Scholarship have to pass the SME (Science Math and English) test designed by AGI and thereafter prospective students have to attend a Freshmen Admission Camp (FAC) for 3 days 2 nights in a CPF pig and/or chicken farm to ensure that they are comfortable and really like animal farm life. This screening process will prevent and/or reduce the number of student drop-outs during the 4 years of their study. In fact, a few students have resigned from the beginning of the application process.

4. WORK PLACEMENT SITES FOR FARM TECHNOLOGY MANAGEMENT PROGRAM

For the FTM program, students are required to be trained practically every 3 months, between their classroom learning, in the following establishments: 7-Eleven retail store, broiler farm, breeder chicken farm, breeder pig farm, fattening pig farm, layer chicken farm during the first 1-3 years period. Finally 4th year students must select just one business unit that they wish to work for after graduation. The CPF HR department will coordinate and help to match this with the workforce needs for each business unit. Finally the students must spend the last 6 months practicing in the selected business unit, to qualify the graduates to work effectively.

5. CREATING ANIMAL HUSBANDRY PROFESSIONALS BY PROFESSIONALS

According to PIM's Guidelines of creating professionals by professionals, CPF has appointed special instructors from the operation of their various chicken and pig farms and/or from the operation of their food processing plants, to complement classroom instruction as

professional teachers in their respective fields for AGI. At any one time there may be 1-5 special teachers from CPF who teach an FTM course, these professional will ensure that FTM graduates can work professionally in the Farms.

Agro-Industry signed an MOU with Iowa State University in 2014 and invited Dr. David G. Acker to guide and teach the CPF's professionals to be able to teach effectively according to the philosophy of AGI "We do not teach agro-industry but we do teach how to learn Agro-industry". Agro-Industry has named this teaching technique as the ATP (Acker Teaching Platform) to honor Dr. David G. Acker. It combines various techniques such as Lectures, Demonstrations, Workshops, Debates, Videos, Interactive Learning Competitions, Flipped-Classrooms, Group Discussions, Student Presentations etc. ATP enables change in teaching and learning all the time, making it not boring, fun and a challenge (Acker, 1999). It also provides opportunities for students to learn and research on their own and create leaders that can start teaching friends. It has been found that CPF professional teachers are able to incorporate a variety of teaching styles and enable students to learn better at a satisfactory level.

6. CONCLUSION

Agro-Industry's "Work-based Education (WBE)" is an inspirational approach for corporate organizations to secure and ensure animal husbandry professionals for their future needs. This new concept and methodology in teaching and learning can help students find their true vocation, from tandem instruction both in the classroom and the workplace. Regular students who graduate will normally only be good in theory, whereas WBE, as a consequence, is becoming more important, to help cultivate work experiences and apply knowledge from 'on the job' training/work to the classroom by learning from real and experienced professionals. Therefore, every confidence may be placed on students/employees who have passed the WBE course, as they will be ready to work immediately upon graduation.

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THE SIZE OF HOUSEHOLDER PIG POPULATIONS IN VIETNAM

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Vietnam is a tropical country, where about 70-75% of 93 million of the population earn their living from agriculture. Livestock make a major, although largely underestimated contribution, to rural development in Vietnam. They produce meat, enhance crop production and provide additional economic goods and services as well as cash income. In 2018, the Vietnamese livestock was well developed: 28.2 million heads of pigs, in which, 3.97 million sows and 75 thousand boars, producing 3.82 million tons of live weight; 409.0 million chicken, producing 1097.0 thousand tons of chicken meat and 8.87 billion eggs; 5.65 million cattle, with the live weight of 334.5 thousand tons and 936.1 thousand tons of fresh milk; and 2.42 million buffalos, producing 92.1 thousand tons of live weight, formed an important part of the Vietnamese economic sector. Pigs play an integral part in the peasant economies in Vietnam.

Pig meat consumption is therefore very prevalent in Vietnam, accounting for about 70-75% of meat consumed. However, raising pigs in Vietnam is still mainly in small scales in householders.

Country, Bio-Regions and Provinces	Number of householders	1-9 pigs	10-29 pigs	30-99 pigs	100-299 pigs	≥300 pigs
Whole country	2 962 573	2 463 561	399 112	84 437	10 418	5 045
1. Red River Delta	599 572	430 641	127 514	36 176	4 066	1 175
Ha Noi	129 841	80 348	39 573	8 544	963	413
Vinh Phuc	51 368	37 414	9 590	3 882	382	100
Bac Ninh	28 715	20 944	6 802	826	108	35
Quang Ninh	40 643	34 826	4 730	902	153	32
Hai Duong	35 387	21 139	9 546	4 182	425	95
Hai Phong	34 851	27 939	4 909	1 807	123	73
Hung Yen	23 387	11 238	8 310	2 958	652	229
Thai Binh	108 623	82 925	19 447	5 775	417	59
Ha Nam	29 001	15 547	9 393	3 462	521	78
Nam Dinh	80 323	67 775	10 243	2 097	181	27
Ninh Binh	37 433	30 546	4 971	1 741	141	34
2. North-East	1 086 600	968 007	103 370	13 459	1 435	329
Ha Giang	115 631	108 718	6 531	372	8	2
Cao Bang	71 797	66 302	5 405	83	7	
Bac Can	44 311	42 006	2 178	122	4	1
Tuyen Quang	65 148	57 147	7 006	876	107	12
Lao Cai	69 993	62 253	7 170	436	122	12
Yen Bai	71 341	64 771	5 978	551	35	6
Thai Nguyen	90 075	69 230	17 277	3 168	320	80

Country, Bio-Regions and Provinces	Number of householders	1-9 pigs	10-29 pigs	30-99 pigs	100-299 pigs	≥300 pigs
Lang Son	63 465	59 632	3 557	266	7	3
Bac Giang	104 356	84 291	15 969	3 510	476	110
Phu Tho	91 661	78 220	10 480	2 672	227	62
Dien Bien	53 516	47 238	6 070	187	20	1
Lai Chau	51 952	50 512	1 348	75	12	5
Son La	119 893	111 246	8 097	490	47	13
Hoa Binh	73 461	66 441	6 304	651	43	22
3. Central and Northern of central	768 216	676 930	76 445	12 775	1 248	818
Thanh Hoa	127 622	108 517	16 162	2 404	356	183
Nghe An	176 729	163 250	11 760	1 549	131	39
Ha Tinh	38 174	30 570	6 505	902	70	127
Quang Binh	35 402	31 804	2 945	569	64	20
Quang Tri	47 574	41 896	4 581	1 010	64	23
Thua Thien - Hue	29 841	27 202	2 248	348	33	10
Da Nang City	11 239	9 501	1 573	157	3	5
Quang Nam	87 798	83 250	3 597	861	44	46
Quang Ngai	76 155	70 391	4 680	978	64	42
Binh Dinh	87 066	68 349	15 223	3 126	290	78
Phu Yen	15 081	13 324	1 526	187	11	33
Khanh Hoa	11 664	9 721	1 522	243	44	134
Ninh Thuan	11 440	10 756	567	73	9	35
Binh Thuan	12 431	8 399	3 556	368	65	43
4. West upland	186 056	160 004	21 261	3 835	494	462
Kon Tum	21 071	18 406	2 391	258	12	4
Gia Lai	73 670	67 707	5 103	751	75	34
Dak Lak	59 486	48 409	9 569	1 175	133	200
Dac Nong	11 009	9 185	1 342	376	58	48
Lam Dong	20 820	16 297	2 856	1 275	216	176
5. Southern East	53 287	26 320	18 049	5 381	1 582	1 955
Binh Phuoc	8 836	7 221	1 078	354	51	132
Tay Ninh	8 368	6 287	1 799	181	27	74
Binh Duong	3 525	1 288	1 383	290	151	413
Dong Nai	21 283	8 260	7 153	3 641	989	1 240
Ba Ria-Vung Tau	2 531	880	1 421	63	118	49
Ho Chi Minh City	8 744	2 384	5 215	852	246	47

Country, Bio-Regions and Provinces	Number of householders	1-9 pigs	10-29 pigs	30-99 pigs	100-299 pigs	≥300 pigs
6. Cuu Long River Delta	268 842	201 659	52 473	12 811	1 593	306
Long An	11 125	6 085	4 024	899	87	30
Tien Giang	36 084	23 532	9 807	2 385	294	66
Ben Tre	24 263	12 744	7 878	2 923	621	97
Tra Vinh	23 662	17 081	5 069	1 376	117	19
Vinh Long	14 017	10 278	2 890	730	101	18
Dong Thap	18 339	12 917	3 526	1 783	105	8
An Giang	7 879	5 353	2 099	397	28	2
Kien Giang	21 573	17 346	3 823	331	51	22
Can Tho	9 069	5 656	2 451	895	63	4
Hau Giang	14 764	10 005	3 998	644	96	21
Soc Trang	32 001	26 042	5 484	429	30	16
Bac Lieu	22 868	22 269	598	-	-	1
Ca Mau	33 198	32 351	826	19	-	2

Source: Ministry of Agriculture and Rural Development in April, 2019.