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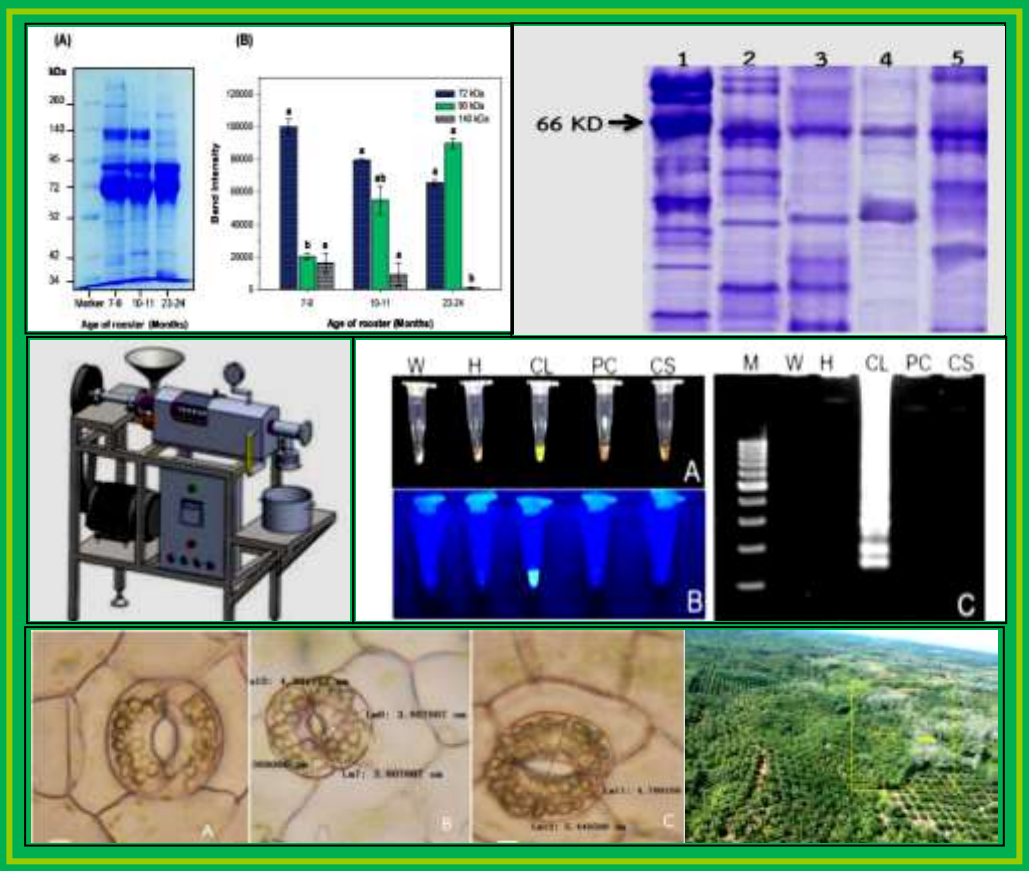

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The Application of tannin extract from plants to reduce the concentration of arsenic

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Aroonsrimorakot, S. and Whangchai, N. (2019). The application of tannin extract from plants to reduce the concentration of arsenic. International Journal of Agricultural Technology 15(2): 207-214.

Abstract This research prepared the tannins extract from agricultural wastes i.e. rice straw, tea leaves, longan leaves and banana leaves with fresh water for 2 months. The highest concentration of tannin was banana leaves (6,464.98 mg/L) followed by longan leaves (4,478.99 mg/L), rice straw (4,000.00 mg/L) and tea leaves (1,397.95 mg/L) respectively. Tannins extract were used for arsenic treatment in synthetic waste water. The result showed the efficiency of arsenic removal by 10, 20, 30, 40 and 50 mg/L of tannins concentration from banana leaves were 50.82%, 52.54%, 54.56%, 58.42% and 51.01% respectively, tea leaves were 69.74%, 73.42%, 60.29%, 63.60% and 70.54% respectively, longan leaves were 61.03%, 56.07%, 54.36%, 40.01% and 51.80% respectively and rice straw were 53.68%, 46.70%, 55.23%, 49.22% and 54.11% respectively which indicated that there were not significantly differed to arsenic reducing. In addition, increasing of pH in solution from 2.5 to 5.5, 6.5, 7.5 and 8.5 led to decrease of arsenic removal efficiency.

Keywords: Tannin extract, Agricultural wastes, Arsenic

Introduction

The expansion of community and development of technology has led to increase of the discharge of wastewater, which is leading to a serious environmental problem. Heavy metals in wastewater from plating, paint manufacturing and mining etc. cause the presence of high toxicity to human, animals, plants and environment, that can be accumulated in living organism and transfer in food chain (Mishra, 2014; Wan Ngah and Hanafiah, 2008). Arsenic is one of the most hazardous heavy metal. The toxicity of arsenic can cause acute and chronic health effects such as hyperkeratosis and pigmentation changes, circulatory disorders, diabetes and cancer in the liver, lungs, skin, bladder, kidneys (Bang *et al.*, 2005; Zhao *et al*., 2012).

To date, several techniques for heavy metal removal from wastewater have been established such as chemical precipitation, ion exchange, membrane

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filtration, electrochemical, and reverse osmosis (Arief *et al.,* 2008). Even though, these processes have high efficiency to reduce metal ion, they are usually difficult to apply in developing countries due to the limitations of high cost for setting and maintenance system. Biosorption is a good alternative for removal of heavy metals in wastewater because it is inexpensive and highly efficient (Diola and Orozco, 2014). In the recent time, researchers are interested in the application of biosorption process with tannin extract because it can be used as an effective agent in water treatment in developing countries (Mailoa *et al.,* 2013). Tannin is generally defined as polyphenol compounds that can be found in different parts of plants and agricultural waste such as seeds, roots and leaves (Sengil and Ozacar, 2009). The structure of tannin can be divided into two classes as: 1) Hydrolyzed tannins and 2) Condensed tannins (Figure 1) (Huang *et al.,* 2009). Recently, many reports presented the high efficiency of tannins to reduce the concentration of various heavy metals such as Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} (Sengil and Ozacar, 2009; Huang *et al.,* 2009; Heredia and Martin, 2009). Therefore, tannins extraction from 4 types of plants as banana leaves, longan leaves, tea leaves and rice straws were studied in this research. The details of each tannin extracted were presented to see its efficiency to reduce the concentration of arsenic (III) ion in synthetic wastewater.

In this study, we aimed to extract tannin from 4 types of plants as banana leaves, longan leaves, tea leaves and rice straws**.** To study the effect of tannin concentration on arsenic reduction. To study the effect of pH in solution on arsenic removal.

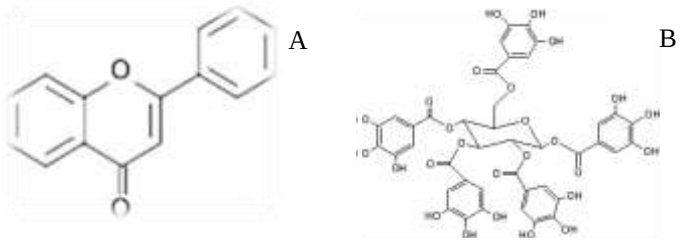

Figure 1. Structures of tannin: A= Hydrolyzed tannins and B = Condensed tannins (Ramkul, 2010)

Materials and methods

Tannin extraction

Maceration extraction is the method for extracting tannins from banana leaves, longan leaves, tea leaves and rice straws. 5.0 Kg of each material were

soaked in 100 L of fresh water for 2 months at room temperature and filtered under vacuum using a Buchner funnel. The polyphenols in tannins extract were reacted by Folin & Ciocalteaus reagent for 95 minutes to form a blue complex (Figure 2) that can be quantified by visible-light spectrophotometry (Blainski *et al.*, 2013).

Figure 2. Tannins analysis by Folin & Ciocalteaus reagent method

Preparation of synthetic wastewater

Stock solution of heavy metal ions was prepared by diluted arsenic standard solution (1000 mg/L) with deionized water (DI) to produce a 10 mg/L concentration for the experiment.

Effect of tannin concentration on arsenic removal

The experiment of arsenic removal was conducted by mixed 10 mg/L of arsenic solutions with 0, 10, 20, 30, 40 and 50 mg/L of tannins extract from banana leaves, longan leaves, tea leaves and rice straw respectively (Figure 3). The mixed solutions were filtered through a 0.45 µm nylon filter after 30 minutes at room temperature and the residual of arsenic concentration in the filtrate was estimated by Atomic Absorption Spectrometer (AAS) (APHA, AWWA and WEF, 1995).

Effect of pH on arsenic reduction

The effect of pH on arsenic removal with tannin extract was studied by varying the pH of arsenic solution from 2.5 to 5.5, 6.5, 7.5 and 8.5 respectively. 10 mg/L of tannin extract from each material were added into arsenic solution and mixed to make it homogeneous. The homogeneous solution was filtered by 0.45 µm nylon filter after 30 minutes and the residual of arsenic was analyzed by AAS.

Statistical analysis

The concentrations of As^{3+} in three replicate were presented as mean value ±SD. Analysis of Variance (ANOVA) was employed for the comparison among groups. Least Significant Difference (LSD) post hoc comparison was tested to show the differences between groups.

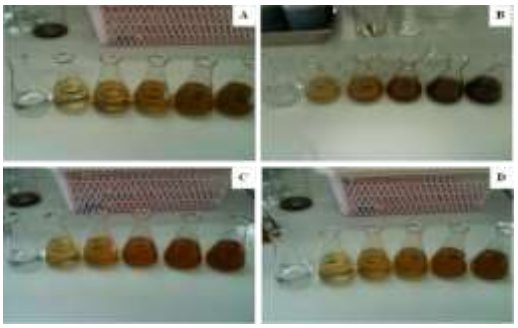

Figure 3. Arsenic treatments with tannins extract from A = banana leaves, B = tea leaves, C= longan leaves and D = rice straws

Results

The quantity of tannins extract

The amounts of extracted tannin from banana leaves, longan leaves, tea leaves and rice straws were extracted by fresh water for 2 months at room temperature. The result showed that banana leaves are having the highest tannin concentration followed by longan leaves, rice straw and tea leaves respectively (Table1).

Effect of tannins extracts concentration

The effect of tannins extract concentration on arsenic removal in synthetic wastewater was shown in Figure 4. The tannins extract from each material were mixed with arsenic solution and analyzed residual of arsenic concentration by AAS. The result showed the efficiency of tannins from banana leaves, tea leaves, longan leaves and rice straws, in reducing arsenic were 50.81%±1.31, 69.74%±5.56, 61.02%±26.83 and 53.68%±23.88 respectively at 10 mg/L of tannin concentration. The ANOVA test was presented in Table 2. It can be seen that there was no significant differences (p-value > 0.05) in removal of arsenic with different ratio of tannin concentration.

Moreover the test of LSD post hoc presented no significant differences on tannins efficiency from tea leaves compared with banana leaves and longan

leaves (p-value > 0.05). However, the efficiency of reducing arsenic by tannins from tea leaves was different with tannins from rice straws due to p-value was less than 0.05.

Table 1. The amounts of extracted tannin from agricultural wastes

Materials	Amounts of tannin (mg/L)
- Banana leaves	6,464.98 ±23.45
- Tea leaves	1,397.95 ±16.98
- Longan leaves	4,478.99 ±59.61
- Rice straws	4,000.00 ±42.77

Table 2. ANOVA test of tannins efficiency from 4 plants

Source	Sum of squares	df	Mean square	F	p-value
Materials	2476.63	3	825.544	2.041	0.042
Concentration	218.186	4	54.546	0.135	0.968

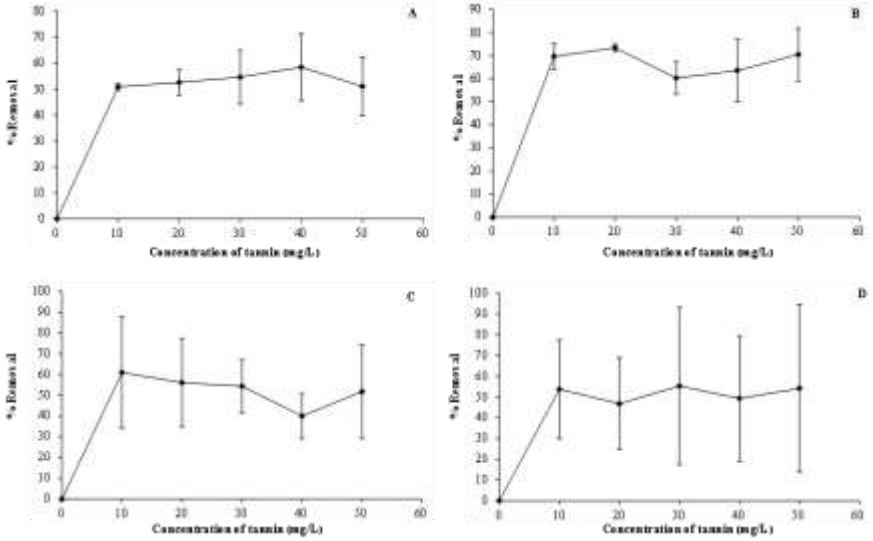

Figure 4. Effect of tannins concentration on arsenic removal:A = banana leaves, B= tea leaves, C= longan leaves and D =rice straws

The reaction mechanism is ion exchanged with hydroxyl functional group of polyphenols structures in tannins that can be seen as shown in equation 1 (R is the structure of polyphenol in tannin extract). That was similar to study of Nakano *et al.* (2001) who found the adsorption process between tannin and chromium ($Cr_2O_7^{2-}$) as ion exchanged with hydroxyl functional group (Nakano *et al.* 2001).

$$R\text{-}OH + H_2AsO_3^- \leftrightarrow R\text{-}H_2AsO_3 + OH^- \quad (1)$$

Effect of pH

The effect of pH in solution on arsenic removal was showed in Figure 5. That result showed the efficiency of arsenic reducing from each tannin extract. Banana leaves tannin able to reduce arsenic at pH 2.5 was 50.81%±1.31 which decreased to 37.10%±1.22, 40.40%±2.35, 38.17%±3.36 and 34.41%±2.42 with increasing pH to 5.5, 6.5, 7.5 and 8.5 respectively, similarly to tea leaves that can be reduced arsenic concentration was 69.74%±5.56, 34.30%±4.64, 37.7%±1.55, 39.89%±0.77 and 34.32%±3.27 at pH 2.5, 5.5, 6.5, 7.5and 8.5 respectively. While, longan leaves tannin at pH 2.5, 5.5, 6.5, 7.5 and 8.5 are able to reduce arsenic was 61.02%±26.83, 37.81%±0.40, 40.29%±3.85, 40.11%±3.60 and 39.29%±2.60 respectively and rice straws was 53.68%±23.88,37.04%±1.08, 39.54%±2.67, 44.11%±2.14 and 38.41%±1.74 respectively.

The statistical analysis is shown in Table 3. The result showed that there was no significant differences on the efficiency of tannin extract concentration on arsenic removal from the 4 types of plants. However, the effect of different pH values on arsenic removal was significant difference because p-value was less than 0.05. In addition, LSD post hoc showed tannin efficiency at pH 2.5 which indicates that there was significant difference with other pH in solution at 95% of confidence level.

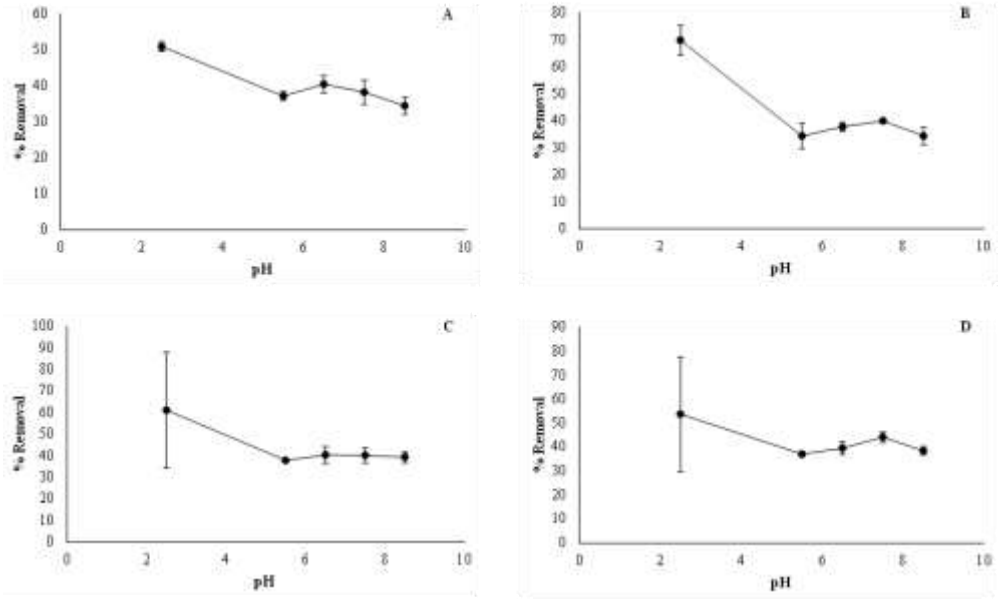

Figure 5. Effect of pH on arsenic removal by tannin extract from A = banana leaves, B= tea leaves, C= longan leaves and D =rice straws

Table 3. ANOVA test of arsenic removal at different pH values

Source	Sum of squares	df	Mean square	F	p-value
Materials	109.421	3	36.474	0.509	0.678
pH	4187.436	4	1046.859	14.607	0.000

Discussion

Extraction is the process to separate different substances in solubility of the two different liquids. The principle of extraction is the polar compounds dissolved in polar solvents and non-polar compounds dissolved in non-polar solvents (Mailoa *et al.,* 2013). The amounts of extracted tannin from agricultural wastes revealed that banana leaves were the highest tannin concentration followed by longan leaves, rice straw and tea leaves respectively. Each plants or different parts of plants had different amount of tannins such as Dwarf Cavendish Banana peels contain more tannins than Cultivated Banana peels and Pisang Maspeels while Black Tea leaves contain more tannins than Oolong Tea leaves and Green Tea leaves etc. (Khasnabis *et al.,* 2015; Surojanamethakul and Hiraga, 1994). In addition, the efficiency of tannins from banana leaves, tea leaves, longan leaves and rice straws was not significant differences in removal of arsenic. In accordance with Huang *et al*. (2009) found that a rise in the concentration of tannin from 1.0 g/L to 2.0, 3.0 and 4.0 g/L was no effect in reducing Hg2+. Moreover, the result of pH variation from each tannin extract, it can be seen that the efficiency of arsenic removal was decreased at basic (alkaline) condition consistent with Sornsathian *et al.* (2016) who found the efficiency of chromium removal by tannin are decreased with increasing pH value in wastewater.

Conclusion

Maceration extraction was used for extracting tannins from 4 types of plants as banana leaves, tea leaves, longan leaves and rice straws. Banana leaves were found with the highest tannin extract concentration followed by longan leaves, rice straws and tea leaves respectively. However, regarding the efficiency of tannin extracted from tea leaves, there was no significant difference with tannins extracted from banana leaves and longan leaves but different with rice straws. Moreover, raising tannin concentration from 10 mg/L to 20, 30, 40, and 50 mg/L does not lead to increase in percentage of arsenic removal because the ratio between arsenic concentration and tannin extracted were decreased. In addition, arsenic removal efficiency was decreased with increasing pH value in solution.

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Enhancing *Columnea latent viroid* detection using reverse transcription loop-mediated isothermal amplification (RT-LAMP)

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Bhuvitarkorn, S., Klinkong, S. and Reanwarakorn, K. (2019). Enhancing *Columnea latent viroid* detection using reverse transcription loop-mediated isothermal amplification (RT-LAMP). International Journal of Agricultural Technology 15(2): 215-228.

Abstract *Columnea latent viroid* (CLVd) is an important quarantine plant pathogen, it is of regulatory interest because of its high rate of seed transmission in tomato (*Solanum lycopersicum*). The detection of CLVd from leaves and seeds of solanaceous and cucurbitaceae species were investigated. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) method was developed for detection of CLVd within 1 h. The method is highly sensitive, being 2,000 times and 100 times more sensitive than conventional RT-PCR in detecting CLVd from leaves and seeds, respectively. The results showed that this detection method was species specific to CLVd with no cross reactivity to other viroids in the same *Pospiviroid* genus. It was successfully applied to detect CLVd from field samples. This appears to be the first application of RT-LAMP for detecting CLVd.

Keywords: Detection, RT-LAMP, viroids, leaves, seeds

Introduction

Viroids are the smallest plant pathogens and consist only of non-encapsidated, single-stranded circular RNA of 250-400 nucleotides (Bussie *et al*., 1999; Gozmanova, 2003; Tsagris *et al*., 2008). Viroids autonomously replicate in susceptible cells and are divided into two families, *Pospiviroidae* and *Avsunviroidae*. The *Columnea latent viroid* (CLVd) is a member of the genus *Pospiviroid* in the *Pospiviroidae* family, has a rod-like genome structure, an asymmetric circle replication mechanism in the nucleus and a wide host range. It was first reported from asymptomatic *Columnea erythrophae* in the

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USA, its genome was characterized and it was reported as transmissible to purple velvet plant (*Gynura aurantiaca*), cucumber (*Cucumis sativus*) tomato (*Solanum lycopersicum*) (Hammond *et al*., 1989), *Nemathanthus wettsteini* (Singh *et al*., 1992) and Gloxinia species (Nielsen and Nicolaisen, 2010). Severe visible symptoms in tomato are difficult to differentiate from those symptoms expressed by other pospiviroids and a universal primer set was not able to distinguish CLVd from other viroids (Bostan *et al*., 2004). Its host range in horticultural plants was further studied and numerous other species may also be hosts for CLVd because most of the currently identified host plants are symptomless (Matsushita and Tsuda, 2015). Seed transmission in tomato and *Petunia* × *hybrida* (Matsushita and Tsuda, 2016) was reported.

In Thailand, CLVd causes damage to tomato seed production and has been reported as having a low percentage of plant infection (Tangkanchanapas, 2005). It caused severe symptoms of necrosis on leaf veins, plant stunting and yield reduction on 'Rutgers' tomato when tested in greenhouse conditions (Tangkanchanapas, 2013). A CLVd isolate was inoculated onto 'Seedathip3' and 'Cherry' tomato plants and they showed severe symptoms of leaf epinasty, plant stunting and severe necrosis on stems and petioles (unpublished). The Department of Agriculture of Thailand has reported that CLVd has damaged a number of tomato cultivars and Bolomaka (*Solanum stramonifolium* Jacq.), and the risk has been included on the Thai plant pest list (Tangkanchanapas, 2013).

An essential component of viroid disease control is the development and use of sensitive diagnostic procedures that help to prevent crop infection and to eliminate infected materials. Many diagnostic techniques have been developed, such as nucleotide sequencing (Hammond *et al*., 1989; Owens *et al*., 2003), molecular hybridization (Stark-Lorenzen *et al*. 1997; Lin *et al*., 2011; Tiberini and Barba, 2012; Torchetti *et al*., 2012) and conventional RT-PCR using specific primers (Bostan *et al*., 2004; Rizza *et al*., 2009; Matsushita *et al*., 2010; Monger *et al*., 2010; Botermans *et al*., 2013; Olivier *et al*., 2014; Papayiannis, 2014). However, RNA extraction from plant tissue is required (Stark-Lorenzen *et al*., 1997) and gel electrophoresis and UV detection are time consuming. To decrease the time needed for viroid detection, reverse transcription loop-mediated isothermal amplification (RT-LAMP) was developed. The LAMP technique amplifies cDNA with high specificity, efficiency and rapidity under isothermal conditions. The main components of the reaction include a DNA polymerase, a set of four specific primers of six distinct sequences on the target DNA, which consist of inner primers containing sequences of the sense and antisense strands of the target DNA (Notomi *et al*., 2000). The efficiency of many RT-LAMP protocols for specific targets has been confirmed (Lan *et al*. 2009; Hassan *et al*., 2013; Mohammad and Dehabadi, 2013; Suzuki *et al*.,

2016). RT-LAMP was developed and reported for some viroids such as *Peach latent mosaic viroid* (PLMVd) (Boubourakas *et al*., 2009), *Potato spindle tuber viroid* (PSTVd) (Tsutsumi *et al*., 2010; Lenarcic *et al*., 2013), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Park *et al*., 2013) and *Coconut cadang-cadang viroid* (CCCVd) (Thanarajoo *et al*., 2014).

Objectives: This research aimed to develop a specific, highly sensitive and efficient RT-LAMP procedure for CLVd detection in both leaves and seeds of vegetable crops. It was also hoped that the technique developed in the current research would be useful for routine viroid detection in a range of other economic plant species.

Materials and methods

Viroid sources, plant materials and RNA preparation

The viroid isolates were obtained from a collection at the Department of Plant Pathology, Kasetsart University, Thailand; CLVd isolate NK-KUKPS1, accession no. KY235369; *Pepper chat fruit viroid* (PCFVd) isolate PCFVd-Thai, accession no. JF446893 (Reanwarakorn *et al*., 2011); and *Chrysanthemum stunt viroid* (CSVd) isolate RCS, accession no. MF803029; and were propagated using mechanical inoculation of 'Rutgers' tomato. The CLVd isolate NK-KUKPS1 was used as the primary viroid inoculum source. The cultivars used in the study were: tomato (*S. lycopersicum* cv. Seedathip 4), eggplant (*S. melongena* cv. Farmer Long), pepper (*Capsicum annuum* cv. PBC365), cucumber (*Cucumis sativus* cv. Bingo) and melon (*C. melo* L. cv. Pot Orange). The third leaf of the solanaceous plants and first true leaf of the cucurbitaceae species were inoculated mechanically using crude sap of CLVd-infected tomato leaves in 0.1M phosphate buffer pH 9.0 at 1 g: 5 mL, mixed with carborundum dust. All CLVd and mock inoculated (buffer) plants were maintained in an insect-proof nursery for 8 weeks after inoculation. Viroid infection was confirmed by conventional RT-PCR.

Total RNA was extracted from 100 mg of leaves or seeds from each sample following a modified CTAB method (Reanwarakorn *et al*., 2011) using the TLES extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0 M, 100 mM LiCl, 1% (v/v) SDS and 2% (w/v) Na2SO3) instead of the CTAB extraction buffer.

Conventional RT-PCR for CLVd detection

The conventional RT-PCR in this study used the CLVd specific CL-P2 primer set (cCL-P2 and hCL-P2), for which the target sequence was the whole

CLVd genome (370 nts). An internal control, amplification of the mitochondrial NADH dehydrogenase gene, based on previously reported primers (Lee and Chang, 2006) was used to monitor RNA extraction. The primers that were used are listed in Table 1. The PCR reaction mixture consisted of 1X of the Green PCR Master Mix (Thermo ScientificTM) as per manufacturer's recommendations, 0.1 µM forward and reverse primers, 2 µL of cDNA template, adjusted to a final volume of 20 µL with RNase-free water (Thermo ScientificTM). The PCR program included 5 min at 96 ℃, followed by 40 cycles of 40 s at 94 ℃, 40 s at 54 ℃ and 40 s at 72 ℃ and a final extension of 7 min at 72 ℃ for the CL-P2 primers. To monitor RNA extraction, plant *nad5* mRNA was determined using the Nad primers followed by the RT-PCR reaction using the CL-P2 primers. The PCR products were analyzed by 2% agarose gel electrophoresis with the RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology) and visualized under UV light.

Design of primer sets and optimization of the two-step RT-LAMP method

The RT-LAMP primers were designed using the CLVd isolate MC-M-11 accession no. AM698095 sequence using PrimerExplorer version 4 software from Eiken Chemical (https://primerexplorer.jp/elamp4.0.0) to provide four primer sets that covered the whole genome of CLVd after multiple sequence alignment of DNA using ClustalW alignment tools (http://www.ebi.ac.uk/Tools/msa/clustalo/) of the GenBank database (data not shown). The best sets of outer primers (F3 and B3), inner primers (FIP and BIP) and gene specific primers are shown in Table 1.

The cDNA was synthesized using the B3 primer and followed the same procedure as the RT reaction using the CL-P2 primers. The 25 µL LAMP reaction mixture contained 6 mM MgSO4, 1X Thermo polymerase buffer (Biolab), 1.4 mM dNTPs mixture, 1.6 µM of FIP and BIP, 0.2 µM F3 and B3, 1M Betaine, 8 units Bst DNA Polymerase Large Fragment (New England Biolabs), 2.0 µL of cDNA and 3.4 µL of RNase-free dH2O. The LAMP cycle was performed at 61 ℃, 63 ℃ and 65 ℃ for 60 min followed by heat inactivation at 80 ℃ for 10 min to terminate the reaction (Boubourakas *et al*., 2009). After the reaction, the products were analyzed by adding 1 µL of 1,000X SYBR green I to the reaction mix to observe the color change from orange to green, and under a blue light transilluminator (LED wavelength: 468 nm) to observe fluorescence for positive samples, and then confirmed by 1.2 % agarose gel electrophoresis using gel staining by the RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology) and visualization on a UV-transilluminator. Each sample was analyzed at least three times.

Table 1. The primer names, sequences and position of each primer set used in this study.

Target	Primer name	Primer	Sequence(5'- 3')	Genome positions[3/]
CLVd	SK-175[1/]	F3	CAGGGTTTTCACCCTTCCTT	175-194 (sense)
		B3	CGGTTCCAAGGGCTAAACAC	337-356 (antisense)
		FIP	CAGAACCTGCGCTGGTCAAGA-TTCTGGTTTCCTTCCTCTGC	237-257 (antisense), 197-216 (sense)
		BIP	CCGGTGGCATCACCGAGTTT-CCCGGAGACCAAGCTAGA	264-283 (antisense), 314-331 (sense)
CLVd	CL-P2[2/]	cCL-P2	CTGCAGCCATGCAAAGA	23-39 (antisense)
		hCL-P2	GGTCAGGTGTGAACCAC	40-56 (sense)

[1]/ RT-LAMP primers obtained from GenBank accession no. AM698095
[2]/ CL-P2 primers - target size at 370 bp used in this study
[3]/ The position of the RT-LAMP primers obtained from GenBank accession no. AM698095

Specificity and sensitivity of the RT-LAMP method to detect CLVd from leaves and seeds

The specificity and sensitivity of CLVd detection by the two-step RT-LAMP method was compared with conventional two-step RT-PCR. The specificity of both methods for the detection of CLVd, PCFVd and CSVd from infected tomato leaves was also compared. (PSTVd was not included in this study as it is a quarantine pest and has never reported in Thailand).

Two groups of sensitivity tests for CLVd detection were studied. With the first group, sensitivity was determined using a 10-fold serial dilution of the total RNAs from CLVd infected leaves with RNAs from healthy leaves of tomato, eggplant, pepper, cucumber and melon. The total RNA was measured and adjusted to 200 ng μL^{-1} using a nanodrop spectrophotometer, and the 10-fold serial dilutions were prepared at 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng μL^{-1}.

With the second group, total RNA from single seeds of viroid-infected and viroid-free seeds of tomato and eggplant was extracted and adjusted at 150 ng μL^{-1}. The serial dilution of total RNAs from viroid infected tomato and eggplant seeds and from viroid-free seeds was performed at: 1:100, 1:200, 1:400, 1:1,000, 1:2,000, 1:10,000, 1:20,000, 1:40,000 and 1:100,000 (volume: volume). The RT-LAMP reactions were conducted as previously described.

Sequencing

RT-LAMP products were cloned into the pGemT-easy vector following the manufacturer's protocol and sent to SolGent Co. Ltd. (Korea) for sequencing. The similarity of the sequences was determined by blast with the

GenBank database by using Blastn tools (http://www.ncbi.nlm.nih.gov/BLAST).

Application of the RT-LAMP method for a field survey for CLVd

To confirm the usefulness of the RT-LAMP method for CLVd detection, 15 field tomato plants from a previous viroid monitoring project in Thailand, that was conducted in 2015 to 2016, were used for assay with the RT-LAMP. These field samples included five PCFVd and 10 CLVd positive samples as determined using the conventional RT-PCR method. Total RNAs of leaves were used to detect the presence of CLVd using the conventional RT-PCR and RT-LAMP methods as previously described.

Results

Optimization of the two-step RT-LAMP procedure

Investigation of the optimal conditions for the RT-LAMP procedure was conducted using total RNA from CLVd infected leaves. The four primer sets were generated and tested at 61 ℃, 63 ℃ and 65 ℃ for 60 min. The results showed that primer set "SK-175" amplified the CLVd amplicons most efficiently at 65 ℃ (data not shown).

Specificity and sensitivity of the RT–LAMP method for detection of CLVd from leaves and seeds

The specificity of RT-LAMP using the SK-175 primer was tested to determine the cross reactivity between viroid species in the same Pospiviroid genus: CLVd, PCFVd and CSVd from infected tomato leaves. Total RNAs were used for the comparison. The results showed that CLVd was detected by RT-LAMP, but neither PCFVd nor CSVd was detected, indicating that the RT-LAMP primers are specific to CLVd (Figure 1).

Two groups were used to the sensitivity for CLVd detection by conventional RT-PCR and RT-LAMP. The detection of CLVd in the first group was determined using a 10-fold serial dilution of total RNA from infected and healthy leaves of tomato, eggplant, pepper, cucumber and melon. The results showed that conventional RT-PCR could detect CLVd from leaves in a range of 100 to 200 ng μL^{-1} from all samples and RT-LAMP could detect the viroid in the range of 0.001 to 200 ng μL^{-1}. The detection of the viroid using the cCL-P2 primers in the conventional RT-PCR, therefore, would require 700 ng (200 ng

μL^{-1}) of total RNA for the cDNA synthesis step while the outer primer (B3) of the SK-175 primer needs an initial RNA concentration of at least 0.35 ng (0.1 ng μL^{-1}). These investigations showed that the RT-LAMP was 2,000 times more sensitive than the conventional RT-PCR for CLVd detection from leaf samples (Table 2).

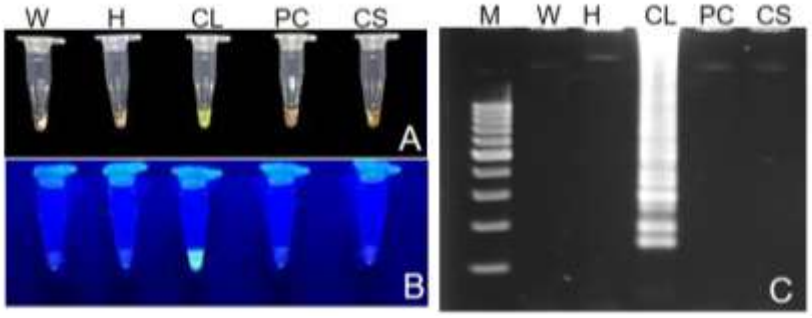

Figure 1. The specificity of RT-LAMP using the SK-175 primer for CLVd detection at 65˚C, (A) visual detection with SYBR Green I stain - the color changed from orange to light green color under normal light in the CLVd positive samples, (B) visual detection using SYBR Green I stain under the Blue Light Transilluminator showed fluorescence in only the CLVd positive sample, (C) agarose gel showing the DNA ladder of the CLVd amplicons. M = 100 bp molecular weight markers; W = dH_2O; H= healthy tomato; CL = CLVd infected tomato; PC = PCFVd infected tomato; CS = CSVd infected tomato

Table 2. Comparison of the sensitivity of conventional RT-PCR and RT-LAMP to detect CLVd from leaves.

Method	**Total RNA concentration (ng μL^{-1})**										
	Plant sp.	dH_2O	H[1/]	200	100	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Conventional RT-PCR	Tomato	-[2/]	-	+[3/]	+	-	-	-	-	-	NT[4/]
	Eggplant	-	-	+	+	-	-	-	-	-	NT
	Pepper	-	-	+	+	-	-	-	-	-	NT
	Cucumber	-	-	+	-	-	-	-	-	-	NT
	Melon	-	-	+	-	-	-	-	-	-	NT
RT-LAMP (SK-175)	Tomato	-	-	+	+	+	+	+	+	-	NT
	Eggplant	-	-	+	+	+	+	+	-	-	-
	Pepper	-	-	+	+	+	+	+	+	+	-
	Cucumber	-	-	+	+	+	+	+	+	-	NT
	Melon	-	-	+	+	+	+	+	-	-	NT

[1]/ H = Healthy plant RNA
[2]/ (-) = negative result
[3]/ (+) = positive result
[4]/ NT= no test

Sensitivity of detection of CLVd in the second group was determined using a serial dilution of total RNA from CLVd infected tomato and eggplant seeds in comparison with viroid-free seeds. The conventional RT-PCR could detect CLVd in seed at a minimum ratio of 1:100 from tomato and of 1:1,000 from eggplant, while RT-LAMP could detect CLVd from seed at ratios of 1:10,000 and 1:40,000 from infected tomato and eggplant seed, respectively. The technique was, therefore, 100 times more sensitive than conventional RT-PCR for CLVd detection from seed samples (Table 3).

Sequencing assay

To confirm the RT-LAMP products obtained when using the SK-175 primer, the sequences of the RT-LAMP products were blasted with the GenBank database using Blastn tools from the website (http://www.ncbi.nlm.nih.gov/BLAST). These results confirmed that the RT-LAMP primer was specific to CLVd due to the high similarity (96-98% identity; data not shown) of the product sequences to those of the CLVd isolate NK-KUKPS1.

Table 3. Comparison of the sensitivity of conventional RT-PCR and RT-LAMP to detect CLVd from seeds.

Method	**Plant sp.**	**Total RNA ratio (infected : healthy)**												
		dH$_2$O	H [1/]	1	1:100	1:200	1:400	1:10	1:2x10^3	1:1$0^4$	1:2x10^4	1:4x10^4	1:1$0^5$	1:2x10^5
Conventional RT-PCR	Tomato	-[2/]	-	+[3/]	+	-	-	NT[4/]	NT	NT	NT	NT	NT	NT
	Eggplant	-	-	+	+	+	+	+	-	-	-	NT	NT	NT
RT-LAMP (SK-175)	Tomato	-	-	+	+	+	+	+	+	+	-	-	NT	NT
	Eggplant	-	-	+	+	+	+	+	+	+	+	+	-	-

[1]/ H = Healthy plant RNA
[2]/ (-) = negative result
[3]/ (+) = positive result
[4]/ NT= no test

Application of the RT-LAMP method for field survey for CLVd

Conventional RT-PCR and RT-LAMP methods were able to detect CLVd in infected samples (Figure 2). However, the conventional RT-PCR method detected CLVd from most but not all of the CLVd infected samples; sample tube no. 12 (Figure 2A), while RT-LAMP detected all of the CLVd infected samples and a PCFVd infected sample, which may have been a mixed infection with CLVd; sample tube no. 3 (Figure 2B).

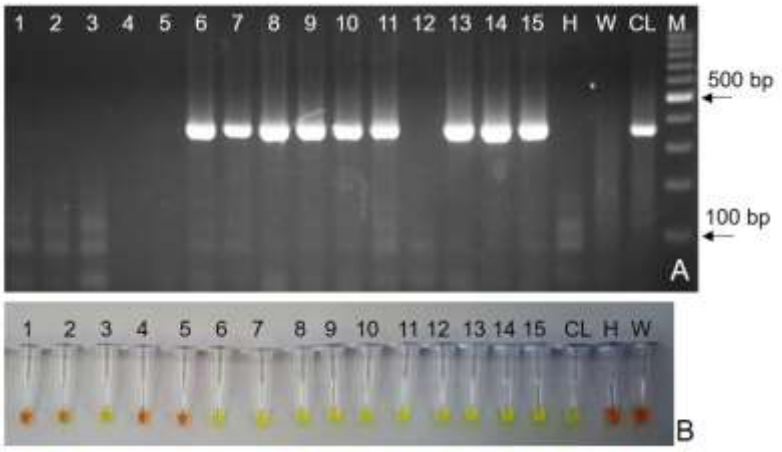

Figure 2. Application of conventional RT-PCR and RT- LAMP for detection of CLVd from field collected samples. (A) DNA products at 370 bp of CLVd using CL-P2 primers, (B) visual detection LAMP products with SYBR Green I stain; the color changed from orange to light green color under normal light in the CLVd positive samples. M=100 bp molecular weight markers; W= dH_2O; H= healthy tomato; CL= CLVd positive control; 1-5= PCFVd infected tomatoes; 6-15= CLVd infected tomatoes

Discussion

This study found that one set of primers could detect CLVd and it showed that the position of the viroid sequences was one critical factor for RT-LAMP primer development. The optimal conditions for viroid detection has previously been shown to depend on the viroid species and their genome sequence. Previous validations have included 62.5 ˚C for 32 min for *Peach Latent mosaic viroid* (PLMVd) (Boubourakas *et al*., 2009), 65˚C for 15-25 min for PSTVd detection (Lenarcic *et al*., 2013), 65˚C for 90 min for *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Park *et al*., 2013) and 60 ˚C for 60 min for *Coconut cadang-cadang viroid* (CCCVd) (Thanarajoo *et al*., 2014). Although four

primer sets for CLVd were designed by the program as specific to certain segments of the viroid genome, the software does not indicate the possibility of primer specificity and the efficiency of detection, so the evaluation of each primer set was compared under different conditions to select the best one.

This investigation proved that the CLVd accumulation in leaf and seed tissue varied with species; it accumulated to a higher level in solanaceous than in cucurbitaceae species and in eggplant seed more than in tomato seed. The results showed that the assay was highly sensitive for detection of CLVd from both leaves and seeds. This RT-LAMP assay provided results in 1 h instead of a few hours when compared with conventional RT-PCR. Similarly, the RT-LAMP for PLMVd was shown to be a sensitive detection method that has been applied to peach, plum, apricot, pear and quince samples with 100 times more sensitivity than the two-step RT-PCR method (Boubourakas *et al*., 2009). Likewise, because PSTVd detection using RT-PCR takes more time and is more complicated than RT-LAMP. This streamlined method was developed for specific PSTVd detection which could be completed in 1 h using the RNA from various parts of potato (leaves, tubers and true seeds) and tomato (leaves and seeds), and was shown to be 10 times more sensitive than the RT-PCR method (Tsutsumi *et al*., 2010). This method could be performed in the field using portable instruments, making it more cost effective (Lenarcic *et al*., 2013). The RT-LAMP method is very sensitive and contamination, due to carry-over of the LAMP product from one assay to another, is of concern because the product is increased three-fold every half cycle (Notomi *et al*., 2000) False positives can, therefore, be a problem (Zhao *et al*., 2018). In this study the false positives were minimized by separating the pipette set and working area from other work, sterilizing the pipette between samples and thoroughly cleaning the working area.

The four primers tested, which recognized the six distinct LAMP set sequences, ensure that there is a high specificity to the target amplification as described previously by Natomi et.al. (2000). This result confirmed that the primers used in this study were specific for CLVd detection from leaves and seeds. RT-LAMP has also been developed for detection of CCCVd from infected oil palm leaves; the sequencing assay proved that the primers are specific for CCCVd and did not detect other viroids such as CEVd and PSTVd (Thanarajoo *et al.*, 2014). Another RT-LAMP was developed for CChMVd, which causes serious diseases in chrysanthemum in Korea. It is a rapid detection method as its primers are specific to the CChMVd genome and have the ability to detect non-symptomatic and symptomatic CChMVd isolates (Park *et al.*, 2013). Other assays using RT-LAMP detection for viruses have been developed as effective genotype-specific techniques, which avoid lengthy

sequencing, for *Pepino mosaic virus* (PepMV) (Ling *et al.*, 2013), *Potato Virus Y* (PVY) (Mohammad and Dehabadi, 2013) and *Grapevine leafroll-associated virus* type 3 (GLRaV-3) (Walsh and Pietersen, 2013). A specific detection method for *Tomato necrotic stunt virus* (ToNStV) was also reported that had no cross-reactivity to other potyviruses or other common tomato viruses (Li and Ling, 2014).

Therefore, a CLVd detection procedure using RT-LAMP was developed. The results showed that the assay was highly sensitive for detection of CLVd from leaves and seeds. It was also more sensitive than conventional RT-PCR when detecting CLVd from leaf and seed samples from the various vegetable species in this study. This RT-LAMP assay gave results within an hour of sampling instead of a few hours when compared with conventional RT-PCR. This supported the objective of decreasing the detection time. The validation of this assay will lead to enhanced efficiency and sensitivity of CLVd detection from leaf and seed samples in both the laboratory and the field.

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Chemical properties, antioxidant activities and sensory evaluation of mango vinegar

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Abstract This study was carried out to examine the chemical properties, antioxidant activities and sensory scores of mango vinegar produced from five mango cultivars, namely 'Nam Dokmai', 'Kalon', 'Kaew', 'Chok Anan' and 'Maha Chanok'. Mango vinegars were produced via a two-stage (alcoholic and acetous) fermentation process. The initial soluble solid contents in the mango juice were adjusted to 22 °Brix use refractometer before the fermentation. Alcoholic fermentation was conducted using *Saccharomyces cerevisiae* as the inoculant while *Acetobacter pasteurianus* was used for acetous fermentation. As observed for all samples during the alcoholic fermentation the levels of soluble solids decreased continuously and the levels of alcohol were found to increase at the end of fermentation process. Notably, the wine produced from 'Nam Dokmai' cultivar exhibited the highest levels of alcohol (14.82%) and antioxidant activity (DPPH method) (80.21%). Similar results were observed for all samples during the acetous fermentation, in which the levels of alcohol dropped continuously and the levels of acetic acid were noted to elevate at the end of the fermentation process. The highest levels of acetic acid (6.96%) was detected in the vinegars produced from 'Kalon' cultivar while those produced form 'Maha Chanok' cultivar exhibited the highest levels of antioxidant activity (91%). Sensory evaluation based on the 9-point hedonic scales showed that the vinegars produced from 'Kaew' cultivar displayed the highest overall acceptability with an average score of 6.23, equivalent to the hedonic scale of 9, which indicated the slightly pleasant levels of the vinegar preference of the consumers.

Keywords: Antioxidant activity; Fermentation; Fruit vinegar; Mango; Sensory evaluation

Introduction

Vinegar is one of the most widely used seasoning in the world. In addition to being primarily used as food seasoning, vinegar plays an important role in the production of food products since it is applied in a wide variety of products, including sauces, ketchups and mayonnaise (Ho *et al.*, 2017). Moreover, vinegar has long been used in the treatment of many common

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ailments with claims of anti-infective, antitumor, and hyperglycemic properties (Johnston and Gaas, 2006).

The beneficial effects of vinegar might be due to bioactive substances such as amino acids, organic acids or phenolic compounds derived from its raw materials (Budak *et al.*, 2014; Ghosh *et al.*, 2016). Moreover, the bioactive compounds in vinegars can be produced and/or increased through the overall vinegar fermentation process (Solieri and Giudici, 2009), where phenolic compounds are transformed into new antioxidative molecules (Shahidi *et al.*, 2008).

Recently, the demand for fruit vinegars has increased due to their reputation as health food products, which help to promote different kinds of beneficial effects to consumers, such as having antidiabetic effects and lowering cholesterol levels in blood by inhibiting the oxidation of low density lipoproteins (LDLs), among other benefits (Chen *et al.*, 2017; Ho *et al.*, 2017). Owing to its excellent sensorial properties and nutritional compositions having different health-promoting properties, mainly from the antioxidant activities (Kim *et al.*, 2009), mango is an appealing ingredient for the production of vinegar. Mango (*Mangifera indica* L.) is rich of bioactive compound such as proteins (0.36 – 0.40) g 100 g^{-1} fresh weight (FW) of pulp, vitamin A (0.135 – 1.872) mg 100 g^{-1} FW pulp, vitamin C (7.8 – 172.0)mg 100 g^{-1} FW of pulp, carotenoids (0.78 – 29.34) µg g^{-1}FW of pulp; phenolic compounds, dietary fibre (DF), carbohydrates, minerals, and other anti-oxidants known to have medicinal, nutritional, and industrial benefits (Rymbai *et al.*, 2015).

For this purpose, this study was carried out to compare the chemical properties, antioxidant activities and sensory scores of the mango vinegars produced via a two-stage fermentation process from five cultivars, namely ‘Nam Dokmai’, ‘Kalon’, ‘Kaew’, ‘Chok Anan’ and ‘Maha Chanok’. In this context, chemical properties were assessed in terms of alcohol contents, glucose and fructose contents, and acetic acid contents. Antioxidant activities were determined by DPPH radical assays and total phenolic contents. Sensory evaluation was performed based on the 9-point hedonic scale.

Materials and methods

Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was purchased from Sigma–Aldrich (Steinheim, Germany). Folin-ciocalteau reagent was from Merck (Darmstadt, Germany) and sodium carbonate (anhydrous) from Univar (Downers Grove, IL, USA). All other chemicals and solvents were purchased

from local manufacturers. Deionized water was prepared by a Milli-Q Water Purification system (Millipore, MA, USA).

Material and fermentation

Mango pulp of five cultivars, namely 'Nam Dokmai', 'Kalon', 'Kaew', 'Chok Anan' and 'Maha Chanok', was used for the production of mango vinegars via a two-stage (alcoholic and acetous) fermentation process. Mango pulp of each cultivar was crushed and mixed with water at a ratio of 1:1 to prepare mango juice. After adjustment of the pH to 4.5 by acetic acid and sugar content up to 22 °Brix by sugar, the mango juice was pasteurized for 30 min at 60 ℃. Alcoholic fermentation was conducted for 8 days at room temperature under static conditions in plastic vessels containing 3 L of the mango juice inoculated with Lalvin ICV D-47 wine yeast, *Saccharomyces cerevisiae,* (Wine & Scientific Equipment Ltd., Part., Ratchaburi, Thailand) at a ratio of 0.75% (v/v). Preparation of yeast inoculum was carried out by mixing 5 g of yeast powder with 60 mL of warm water. At the end of the fermentation process, the obtained wine was separated from the sediment by allowing it to settle in glass bottles, followed by pasteurization for 30 min at 60 ℃ and clarification for 45 days at 10 ℃. Prior to acetous fermentation, the alcohol content of the obtained wine was adjusted to 7% by sterile distill water. Acetous fermentation was performed for 15 days under the aforementioned conditions in glass vessels containing 135 mL of the mango wine inoculated with *Acetobacter pasteurianus* TISTR 521 at a ratio of 10% (v/v). Sampling was performed at given timepoints to collect the two-stage fermented mango vinegars by allowing them to settle in microtube and storage at 4 ℃ in microtubes before the analyses.

Chemical analysis

Analysis of alcohol, acetic acid, glucose and fructose contents was performed on a Shimadzu HPLC-RID system (Shimadzu, Japan) consisting of Shimadzu LC-20AD pumps and RID-10A refractive index detector. The analytical column was Aminex HPX-87H column (300 mm × 7.8 mm i.d., 9 μm, Bio-Rad Laboratories, Inc., USA) coupled to a cationic exchange precolumn (Bio-Rad Laboratories, Inc., USA). H_2SO_4 (5 mM) was used as the mobile phase. The injection volume was 20 mL with a flow rate of 0.6 mL/min. The column temperature was set at 45 ℃.

Total phenolic contents

Total phenolic contents of the mango vinegars were determined using Folin-Ciocalteu reagent as described by (Singleton *et al.*, 1999). Briefly, 1 mL of each sample was diluted with 9.5 mL of distilled water and was then mixed with 0.5 mL of Folin-Ciocalteu reagent and 2 mL of 10% Na_2CO_3 solution. After 30-min incubation at room temperature, absorbance was measured at 765 nm using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). Results were expressed as mg gallic acid equivalents in 1 mL of sample (mg GAE/mL).

DPPH radical-scavenging activity

Antioxidant activities of the vinegars were evaluated by DPPH radical assay (Brand-Williams *et al.*, 1995), in which 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical was used as a stable radical. In brief, 1.5 mL of each sample was added to 1.5 mL of 0.1 mM DPPH radical solution prepared in ethanol, and the mixture was incubated for 20 min at room temperature in the dark. After incubation, absorbance was measured at 517 nm using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan), and the DPPH radical scavenging activities were expressed as the percentage of the DPPH radical elimination effect of vitamin C. Control solutions were prepared by dissolving 0.004 g of DPPH in 95% ethanol, followed by adjustment of the solutions to a final volume of 100 mL. DPPH radical scavenging capacity (RSC) was calculated using the equation $\%RSC = (1 - A_S/A_C) \times 100$, where A_C and A_S denote the absorbance of control and sample, respectively.

Sensory analysis

About 200 g of the mango vinegars were mixed with 150 g of honey and 150 g of water to make drinking vinegars and the obtained drinking vinegars were subjected to the sensory evaluation based on the 9-point hedonic scale by using 30 untrained panelists. The panelists were asked to rank the 9-point scale of affective tests of clearance, color, odor, taste and overall acceptance with the scale 9 representing like extremely, 5 representing neither like nor dislike and 1 representing dislike extremely.

Statistical analysis

A randomized block design, with three replicates and five samples per replicate, was used to compare the chemical properties, antioxidant activities

and consumers' preference of the mango vinegars produced from five mango cultivars. The results are expressed as the mean ± one standard deviation (SD) of three replicates and data were analyzed using one-way analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) to determine the significance between samples. In all cases, $p < 0.05$ was considered significant.

Results

Chemical properties of the mango juice, wines and vinegars

The mango wines produced from five mango cultivars via a 8-day alcoholic fermentation process using *Saccharomyces cerevisiae* as an inoculant were analyzed for their chemical compositions, and the results are presented in Fig. 1A. It was observed that at the end of the fermentation, high alcohol content was detected in all the mango wines, indicating that sugars in the mango juice were rapidly converted to alcohol. Acetic acid content of the mango wines was quite low but significantly increased at the end of the fermentation, ranging from 0.30% to 1.32% (Fig. 1D). The mango wine produced from 'Chok Anan' cultivar contained the highest level of acetic acid of 1.32 ± 0.47% while that from 'Maha Chanok' cultivar had the lowest acetic acid content of 0.30 ± 0.15%.

As given in Fig. 1B, glucose was rapidly utilized during the production of the mango wine as observed for all samples, with the most rapidly utilized glucose observed after 1 day of the fermentation in 'Kalon' cultivar. Notably, glucose was completely depleted in all the mango wine samples after 5 days of the fermentation. Fructose was likely to be utilized more slowly as compared to glucose (Fig. 1C). During an 15-day acetous fermentation process, the mango vinegars produced from the five mango wines using *A. pasteurianus* were analyzed for their chemical compositions, and the results are given in Fig. 2. As illustrated in Fig. 2A, all the mango vinegars showed a significant decrease in the alcohol content as it was converted to acetic acid by acetic acid bacteria, which was consistent with the increased acetic acid content, as depicted. On the other hand, at the end of a 15-day acetous fermentation process, acetic acid content was found to range from 4.88% to 6.96%, with the highest value of 6.96 ± 0.08% observed in the mango vinegar produced from 'Kalon' cultivar and the lowest 4.88 ± 0.00% in that produced from 'Nam Dokmai' cultivar (Fig. 2B).

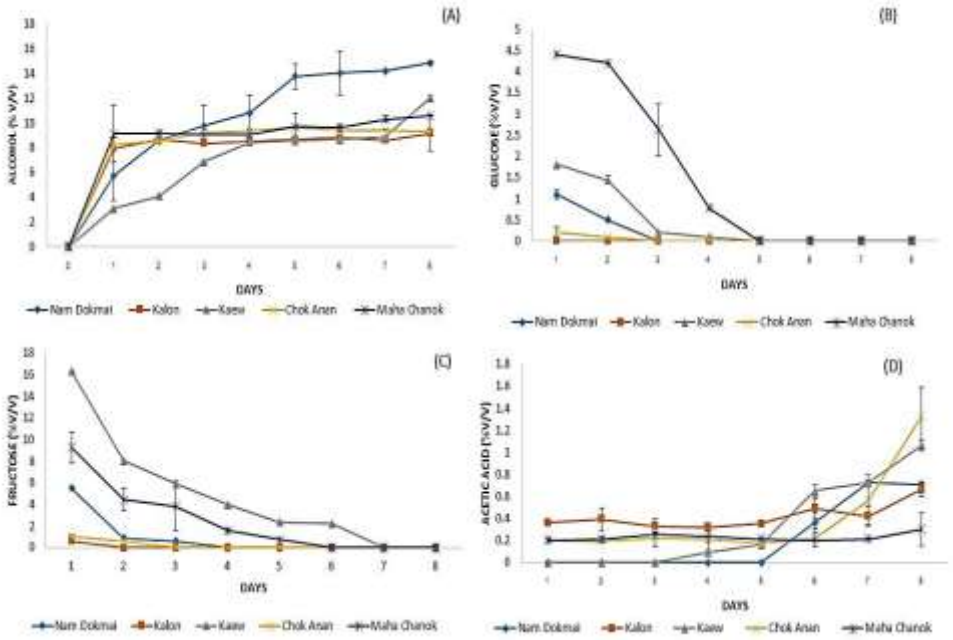

Figure 1. Physicochemical properties of mango wine during a 8-day fermentation process Alcohol (A) Glucose (B) Fructose (C) and Acetic acid (D)

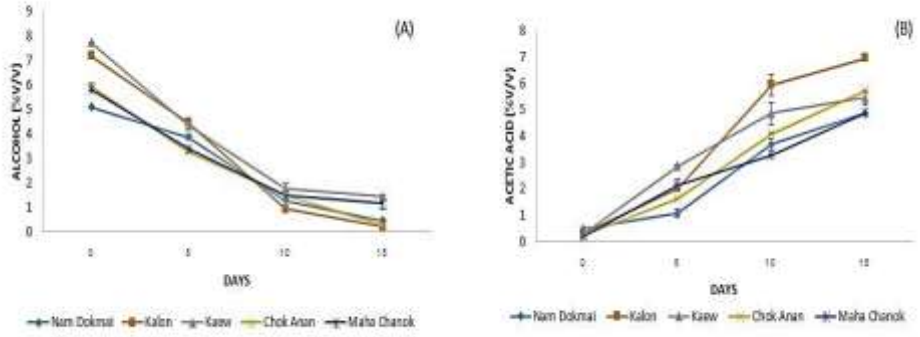

Figure 2. Physicochemical properties of mango vinegar during a 15-day fermentation process Alcohol (A) and Acetic acid (B)

Total phenolic contents and antioxidant activities

The levels of antioxidant activities of the mango vinegars are presented in Table 1. The results showed that the mango juice derived from 'Kalon' cultivar

exhibited the highest antioxidant activity of 69.13 ± 0.07%. Meanwhile, the wine produced from 'Nam Dokmai' cultivar had the highest antioxidant activity of 84.16 ± 6.72%. On the other hand, the vinegar produced from 'Maha Chanok' cultivar was observed to exhibit the highest antioxidant activity of 91.00 ± 2.37%.

The levels of total phenolic contents detected in the mango vinegars produced from different mango cultivars via a two-stage fermentation process are given in Table 2. It was noted that the mango juice derived from 'Kaew' cultivar contained the highest levels (117.20 ± 0.48 mg/L) of total phenolics. Similar results were observed for the mango wine produced from the same cultivar, in which the wine measured at the end of alcoholic fermentation exhibited the highest total phenolic content of 94.30 ± 0.19 mg/L. On the other hand, the vinegar produced from 'Chok Anan' cultivar was found to contain the highest total phenolic content of 117.81 ± 2.48 mg/L.

Table 1. Antioxidant activities of the five mango vinegars produced via a two-stage fermentation process

Cultivars	DPPH (% inhibition)		
	Juice	**Wine**	**Vinegar**
Nam Dokmai	33.83 ± 0.07^{d}	84.16 ± 6.72^{b}	88.30 ± 5.32^{a}
Kalon	69.13 ± 0.07^{a}	58.29 ± 0.43^{b}	68.51 ± 2.66^{b}
Kaew	66.87 ± 1.40^{b}	54.88 ± 3.09^{b}	65.73 ± 3.09^{c}
Chok Anan	43.54 ± 0.29^{c}	43.44 ± 0.00^{c}	86.37 ± 4.60^{a}
Maha Chanok	66.58 ± 0.07^{b}	80.21 ± 2.23^{a}	91.00 ± 2.37^{a}

Values with different letters in the same column are significantly different according to Duncan's multiple range test ($p < 0.05$).

Table 2. Total phenolic contents of the five mango vinegars produced via a two-stage fermentation process

Cultivars	Total phenolic content (mg/L)		
	Juice	**Wine**	**Vinegar**
Nam Dokmai	92.74 ± 0.29	84.77 ± 0.10^{b}	71.46 ± 0.19^{c}
Kalon	98.82 ± 0.10	72.14 ± 0.19^{e}	82.47 ± 0.29^{b}
Kaew	117.20 ± 0.48	94.30 ± 0.19^{a}	65.72 ± 2.58^{d}
Chok Anan	108.22 ± 28.48	78.92 ± 1.15^{d}	117.81 ± 2.48^{a}
Maha Chanok	77.27 ± 0.76	80.99 ± 0.10^{c}	83.08 ± 0.57^{b}

Sensory evaluation

The levels of consumers' acceptability based on the 9-point hedonic scale. The original 9-point scale, developed by the U.S. army for menu planning for their canteens, consisted of a series of nine verbal categories representing degrees of liking from 'dislike extremely' to 'like extremely'. For subsequent quantitative and statistical analysis, the verbal categories are generally converted to numerical values: 'like extremely' as '9', 'dislike extremely' as '1'. The levels of consumers' acceptability based on the 9-point hedonic scale of the drinking vinegars, a blend of the vinegars made from different mango cultivars and honey, are depicted in Fig. 3. The results showed that significant ($p < 0.05$) differences in color and clearance were observed among the drinking vinegars produced from different mango cultivars. The drinking vinegar produced from 'Kaew' cultivar displayed the highest level of consumers' preference, with the mean overall acceptability score of 6.23 ± 1.86.

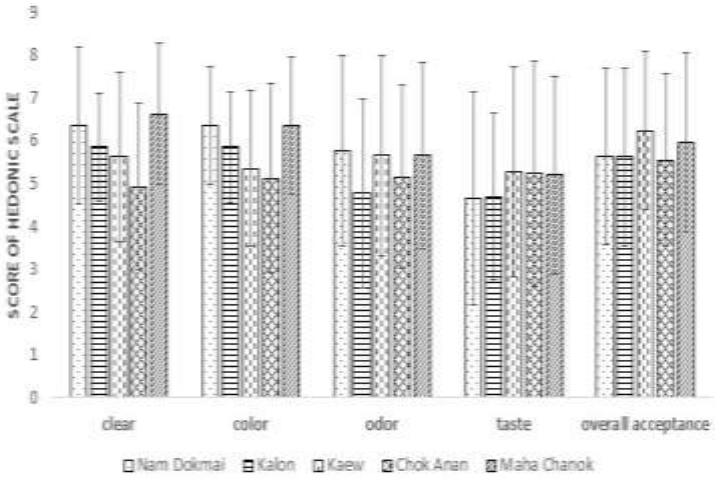

Figure 3. Sensory scores of the drinking vinegars blended from the five fermented mango vinegars

Discussion

Chemical properties of the mango juice, wines and vinegars

The mango wine produced from 'Nam Dokmai' cultivar contained the highest alcohol content of 14.82%, with the lowest alcohol content of 9.14%

observed for that produced from 'Kalon' cultivar, which was much greater than that (6.8%) detected in the mango wines produced in an earlier study (Reddy and Reddy, 2011). The difference in alcohol content in mango wines between Nam Dokmai' cultivars and 'Kalon' cultivars may be due to different of in its cultivars nutrients. Therefore, the efficiency of sugar consumption of yeast was differently. The mango wine produced from 'Chok Anan' cultivar contained the highest level of acetic acid of 1.32 ± 0.47% while that from 'Maha Chanok' cultivar had the lowest acetic acid content of 0.30 ± 0.15%, which was higher than that detected in lychee wine containing the acetic acid content of 0.04 g/100 mL (Chen and Liu, 2016). As given in Fig. 1B and 1C the most rapidly utilized fructose was observed in the mango wine produced from 'Kalon' cultivar which was completely depleted after 2 days of the fermentation. Meanwhile, fructose was completely depleted in all wine samples after 8 days of the fermentation. The rapid utilization of glucose and fructose and the consequent increase in the levels of alcohol confirmed that the yeast dominated the fermentation, which was supported by an earlier study (Taniasuri *et al*., 2016) which elucidated the rapid utilization of glucose and fructose in the production of wine durian, in which at the end of the fermentation fructose was completely depleted while glucose remained at 0.046 g/100 mL. In Fig. 2B., the alcohols were not completely depleted, in which at the end of acetous fermentation the vinegar produced from 'Kaew' cultivar contained the highest alcohol content of 1.44 ± 0.10% while that produced from 'Kalon' cultivar had the lowest alcohol content of 0.21 ± 0.19%, which was in disagreement with an earlier study (Li *et al*., 2014) which elucidated that the alcohol content in the *Hericium erinaceus* vinegar was 0% after 9 days of acetic fermentation. The highest acetic acid value of 6.96 ± 0.08% observed in the mango vinegar produced from 'Kalon' cultivar and the lowest 4.88 ± 0.00% in that produced from 'Nam Dokmai' cultivar (Fig. 2B), which was much lower than that obtained in a previous study (Li *et al*., 2014), in which an acetic acid content of 21.56 mg/mL was detected in the *H. erinaceus* vinegar after 9 days of acetous fermentation.

Total phenolic contents and antioxidant activities

The levels of antioxidant activities of the mango vinegars are presented in Table 1. the wine produced from 'Nam Dokmai' cultivar had the highest antioxidant activity of 84.16 ± 6.72%, which was greater than that produced from jack fruit (69.44 ± 0.34%) (Jagtap *et al.*, 2011). Interestingly, the antioxidant activity of the mango wine obtained from 'Kaew' cultivar was higher than that of white wine imported from Czech Republic since 2006 (90

mg/L) (Stratil *et al.*, 2008). On the other hand, the vinegar produced from 'Maha Chanok' cultivar was observed to exhibit the highest antioxidant activity of 91.00 ± 2.37%, which was much greater than that detected in the purple sweet potato makgeolli vinegar (67.63 ± 0.17%) (Chun *et al.*, 2014). Mango juice is naturally rich in polyphenols include ellagic acid, gallic acid, quercetin, catechin, epicatechin, chlorogenic acid, mangiferin and kaempferol which are powerfull antioxidants. (Santhiraseggaram *et al.*, 2015), and the polyphenol compound occur in wine and vinegar have antioxidant activity and free-radical scavenging capacity

On table 2 the vinegar produced from 'Chok Anan' cultivar was found to contain the highest total phenolic content of 117.81 ± 2.48 mg/L, which was much greater than that detected in the purple sweet potato makgeolli vinegar (24.73 ± 0.04 mg/L) (Chun *et al.*, 2014).

Sensory evaluation

In Fig. 3. The results showed that significant ($p < 0.05$) differences in color and clearance were observed among the drinking vinegars produced from different mango cultivars. The drinking vinegar produced from 'Kaew' cultivar displayed the highest level of consumers' preference, with the mean overall acceptability score of 6.23 ± 1.86, which was equivalent to the hedonic scale of 9. The 9-point hedonic scale has been the primary method of hedonic scaling in food science, which has been widely used for assessment of consumers' acceptability of foods and drinks (Cardello, 2017). For instance, (Varakumar *et al.*, 2012) employed the 9-point hedonic scale to evaluate the consumers' acceptability of mango wine produced using a novel yeast-mango-peel immobilized biocatalyst system, and the average values were recorded for the four evaluated attributes of which the aroma is the one with a slightly higher value, followed by the taste, appearance, and overall acceptance, with respective notes of 7.9, 7.7, 7.6, and 7.5.

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Effect of potassium chlorate combined with paclobutrazol, monopotassium phosphate and mepiquat chloride on fruit quality of Longan (*Dimocarpus longan*)

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Abstract The effect of potassium chlorate combined with paclobutrazol, monopotassium phosphate and mepiquat chloride on fruit quality of longan was investigated. Four treatments were potassium chlorate (control), potassium chlorate + paclobutrazol (PP), potassium chlorate + monopotassium phosphate (PM) and potassium chlorate + mepiquat chloride (PMC). The potassium chlorate was sprayed on 16 longan trees and the other chemicals were sprayed seven days after spraying with potassium chlorate. Results showed that there was no significant difference in fruit length, fruit diameter, fruit circumference, peel thickness, pulp thickness, total soluble solids, fresh weight of peel and pulp, and dry weight of peel, pulp and seed among treatments. Fruit set was increased by PP treatment as compared to the control. Fruit firmness resulting from PP, PM and PMC treatments were greater than fruit firmness resulting from the control. Results indicated that the sprayed chemicals did not reduce fruit quality of longan.

Keywords: longan, potassium chlorate, paclobutrazol, monopotassium phosphate, mepiquat chloride, fruit quality

Introduction

Longan (*Dimocarpus longan* Lour.) is an economic important fruit of Thailand. Longan can be sold as fresh fruit, dried fruit with peel, dried pulp, longan canned and frozen longan. The main export market is China where the highest price is during the Chinese New Year period. The income from longan in Thailand is more than 156 million dollars per year (Hanviriyapant, 2008). More than 80 percent of the growing area is in the north of Thailand. However, the highest amount of production in 2015 was at Chanthaburi province in east Thailand (Official of agricultural economics, 2016).

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Normally, the flowering of longan in the orchard requires a temperature of 15-20 ℃. Presently, farmers are able to induce longan flowering throughout the year by using chlorate. Farmers in Chanthaburi province also used this chemical to produce the longan off-season for selling during the Chinese New Year period (January-March). However, the decrease of flowering and fruit set in the rainy season as compared to the other seasons is still a problem of longan off-season production (Chanlueng, 2017).

Growth retardants are classified as plant growth regulators that have the capacity to reduce plant growth. They are organic compounds which were synthesized for agricultural utilization. Each of these compounds has different chemical properties. Such compounds effect many different physiological processes in plants, such as flowering, fruiting, drought-resistance, yield increase of vegetables, dark green leaves and thick leaves (Techapinyawat, 2001). Paclobutrazol and mepiquat chloride are also growth retardants that promote fruit set and fruit quality (Yeshitela *et al.*, 2004; Sridhar *et al*., 2009; Lim *et al*., 2004; Curry and Williams, 1986). Moreover, chemical fertilizers such as monopotassium phosphate can also promote fruit set and quality of fruit (Sarrwy *et al.*, 2010; Nerson *et al*., 1997; Chapagain and Wiesman, 2004). Therefore, the purpose of this experiment was to investigate fruit set and quality of longan during the rainy season as influenced by paclobutrazol, mepiquat chloride and monopotassium phosphate.

Materials and methods

The experiment was conducted from July 2017 to February 2018 in longan orchard at Soidao, Chanthaburi province, Thailand. The experimental design was a Completely Randomized Design (CRD). Four treatments were control (water), 2000 ppm of paclobutrazol (Srihara, 2014), 1% of monopotassium phosphate (Thongaon *et al.*, 2013) and 3000 ppm of mepiquat chloride (Nasee *et al*., 2017). Four longan trees were used for each treatment. Sixteen longan trees, approximately 10-12 years old and 6-8 m in canopy diameter were selected for the experiment. All longan trees were watered with potassium chlorate at 20 g/m^2 (Kativat, 2008) on 24 July 2017 during the rainy season in Thailand. Water, paclobutrazol, monopotassium phosphate and mepiquat chloride were spayed 15 days after spraying potassium chlorate.

Forty fruit bunches per longan tree were harvested on 3 February 2018 to measure fruit set, diameter, circumference and length of fruit. Peels were removed and data collected on peel thickness. Pulp firmness and total soluble solids were checked by using a fruit penetrometer and refractometer, respectively. Pulps were removed to measure pulp thickness. The length and

diameter of seed were also determined. Then fresh weight of peel, pulp and seed were determined. All parts of the fruit were dried in a hot air oven at 80 ℃ for 48 hours. Then all parts were weighted for data collection. All results were analyzed by analysis of variance and means separated by Duncan's multiple range test (DMRT).

Results

Results showed that there was a significant difference in percent fruit set and fruit number per bunch. The highest percent of fruit set resulted from the PP treatment, but did not differ significantly compared to fruit set resulting from the PMC treatment. However, percent fruit set in the control and PM treatment was significantly lower than fruit set resulting from the PP treatment (Fig. 1). Fruit number per bunch in the control was not significantly different as compare with PP and PMC treatments. The lowest number of fruits per bunch resulted from the PM treatment and was 8.26 fruits per bunch (Fig. 1).

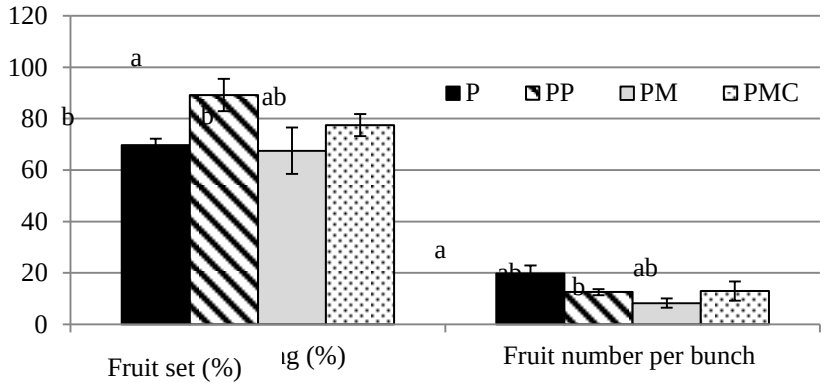

Figure 1. Fruit set and fruit number per bunch of longan at the end of the experiment. Bars with different letters in each plant part indicate significant differences in different treatments at the 0.05 probability level, according to DMRT.

There was no difference in fruit diameter, fruit height and fruit circumference among treatments. However, fruit diameter tended to be high in the PP treatment as compared to the control. Smaller fruit diameter resulted from the PMC treatment (Table 1). Fruit height tended to be high with the PP treatments compared to the control. The lowest fruit height resulted from the PM treatment (Table 1). The control treatment resulted in greater fruit circumference compared to the remaining treatments (Table 1).

Table 1. Fruit diameter, fruit height and fruit circumference of longan at the end of the experiment

Treatment	Fruit diameter (mm)	Fruit height (mm)	Fruit circumference (mm)
Potassium chlorate (P)	28.78 ±1.01	26.22 ±0.36	9.17 ±0.34
Potassium chlorate + Paclobutrazol (PP)	29.62 ±0.55	26.46 ±0.26	9.15 ±0.22
Potassium chlorate + Monopotassium phosphate (PM)	29.03 ±1.42	25.84 ±0.94	9.07 ±0.34
Potassium chlorate + Mepiquat chloride (PMC)	28.33 ±0.59	26.16 ±0.46	9.05 ±0.15
F-test	ns	ns	ns
CV (%)	3.31	2.17	3.05

ns = not significant at P≤0.05.

There was no significant difference in peel thickness and pulp thickness between the control and treatments. Peel thickness ranged from 0.57 to 0.83 mm and pulp thickness was 4.94 to 5.03 mm, respectively (Fig. 2). Pulp firmness was increased by the treatments as compared to the control. The highest pulp firmness resulted from the PM treatment. The control resulted in the least pulp firmness of 1.18 kg/cm^2 (Fig. 2).

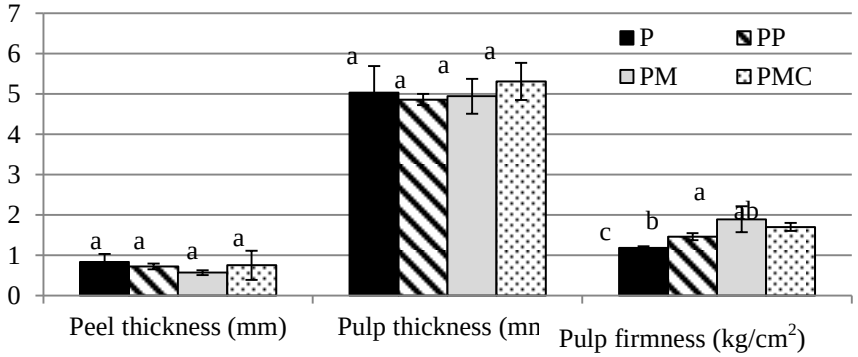

Figure 2. Peel thickness, pulp thickness and pulp firmness of longan at the end of the experiment. Bars with different letters in each plant part indicate significant differences in different treatments at the 0.05 probability level, according to DMRT

Total soluble solids in the control tended to be higher followed by PP, PM and PMC treatments which ranged from 18.51 to 18.87 ºBrix. However, there was no significant difference in total soluble solids among treatments

(Table 2). In case of fresh weight, there was no difference in fresh weight of peel and pulp between the control and treatments. Peel fresh weight ranged from 1.70 to 2.16 g while pulp fresh weight ranged from 7.75 to 7.92 g (Table 2). Fresh weight of seed was reduced by the PMC treatment as compared to the remaining treatments. Fresh weight of seed in the control did not differ as compared to the PP and PM treatments (Table 2).

Table 2. Total soluble solids, fresh weight of peel, pulp and seed of longan at the end of the experiment

Treatment	Total soluble solids (°Brix)	Fresh weight (g)		
		Peel	Pulp	Seed
Potassium chlorate (P)	18.93±0.49	2.16±0.40	7.75±1.15	1.64±0.06[a]
Potassium chlorate + Paclobutrazol (PP)	18.87±0.65	2.02±0.17	8.46±0.77	1.62±0.05[a]
Potassium chlorate + Monopotassium phosphate (PM)	18.64±0.66	1.89±0.20	8.08±1.28	1.63±0.04[a]
Potassium chlorate + Mepiquat chloride (PMC)	18.51±0.39	1.70±0.40	7.92±0.48	1.50±0.10[b]
F-test	ns	ns	ns	*
CV (%)	3.00	16.02	12.09	3.95

Means with different letters in each column are significantly different according to DMRT.
* = significant at P≤0.05. ns = not significant at P≤0.05.

Table 3. Dry weight of peel, pulp and seed of longan at the end of the experiment

Treatment	Dry weight (g)		
	Peel	Pulp	Seed
Potassium chlorate (P)	0.98±0.12	1.48±0.34	1.16±0.24
Potassium chlorate + Paclobutrazol (PP)	1.07±0.16	1.54±0.21	1.04±0.03
Potassium chlorate + Monopotassium phosphate (PM)	1.01±0.75	1.49±0.28	1.08±0.01
Potassium chlorate + Mepiquat chloride (PMC)	0.90±0.13	1.48±0.14	0.98±0.07
F-test	ns	ns	ns
CV (%)	12.32	17.15	11.87

ns = not significant at P≤0.05.

There was no significant difference in dry weight of peel, pulp and seed among treatments. However, peel dry weight resulting from the PP treatment tended to be higher from the PP treatment and lowest in PMC treatment (Table 3). Pulp dry weight was highest with the PP treatment as compared to the remaining treatments (Table 3). Results of seed dry weight were similar compared to the results of seed fresh weight. Namely, seed dry weight resulting from the PMC treatment was the lowest compared to the other treatments (Table 3).

Discussion

Yadava (2012) reported that paclobutrazol resulted in the highest percentage of fruit set in cape gooseberry. The highest fruit set maybe linked due to a balance between the C:N ratio and auxins. Results in this experiment showed that paclobutrazol increased percent fruit set in longan grown in the upland area in the east Thailand during the rainy season. These results were in agreement with Sripinta *et al.* (2009) who reported that fruit set of longan grown in upland area in north Thailand was increased by paclobutrazol.

In case of monopotassium phosphate, results showed that chemical fertilizer increased fruit firmness of longan. The result was in agreement with increased fruit firmness in mango (Taha *et al.,* 2014). This may be due to potassium in the fertilizer which is often described as the quality element for crop production (Usherwood, 1985). In addition, fruit firmness of longan was also increased by mepiquat chloride. This result is in accordance with that of Sherif and Asaad (2014) who reported that the firmness of pear was increased by mepiquat chloride.

Several researchers have shown that plant growth retardants improved some parameters of fruit quality and did not affect other parameters (Kim, *et al.,* 2008; Na Nakorn, *et al.,* 2017). In this experiment, there was no significant difference in fruit length, fruit diameter, fruit circumference, peel thickness, pulp thickness, total soluble solids, fresh weight of peel and pulp, and dry weight of peel, pulp and seed among treatments. Moreover, some experimental reports showed that plant growth retardants decreased fruit quality. Bhutia *et al.* (2017) who reported that high concentration of paclobutrazol reduced fruit set and yield. Results from this experiment indicated that 2000 ppm of paclobutrazol, 1% of monopotassium phosphate and 3000 ppm of mepiquat chloride did not ha a detrimental effect on fruit quality of longan. However, the effective method for increase fruit set and fruit quality of longan in rainy season should be further investigated.

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Effect of colchicine on survival rate and ploidy level of hydrid between *Dendrobium santana* x *D. friedericksianum* orchid

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Abstract The effect of colchicine on survival rate and ploidy level of hydrid between *Dendrobium santana* and *D. friedericksianum* orchid was studied. *In vitro* polyploid induction of this hydrid orchid was carried out by soaking nodal explant in different concentrations of colchicine for 24, 48 and 72 hours. The results showed that LD_{50} of colchicine for 24, 48 and 72 hours was 0. 052%, 0.041% and 0.011%, respectively. The treatment of colchicine at the above concentrations and durations increased DNA content from 33 to 50 % of the original DNA content as analyzed by flow cytometry (FCM) technique. This result was in accordance with physiological characteristics, which revealed the bigger size of guard cells but lower density and increase in number of chloroplasts in guard cell.

Keywords: *Dendrobium* hydrid, colchicine, survival rate

Introduction

Dendrobium is a popular orchid among all growers because it is easy to grow, bloom year round and various sizes of flowers and psuedobulbs. Apart from the benefits of using as an ornamental plant, some *Dendrobium* also be applied for medical purposes. *D. santana* is a hydrid between *D .moniliforme* (dwarf Nobile) and *D. friedericksianum* (Thai wild orchid), produces flowers many times in a year and form flower buds since small size at seedling stage. The mature orchids give numerous odor flowers of thick yellow petals. The hydrid of *D. santana* and *D. friedericksianum* should be heat tolerant orchid that is very easier to grow in tropical and beautifully marked in lip colours, which is suitable for growing as economic orchid in these areas. The hydrids can be used as a model for studying *in vitro* flowering that make value added produced as souvenirs.

So far, orchid is an economically important ornamental plant of Thailand. The export values of both flowers and seedlings of Thai orchids are over billion

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Baht per year. The orchid business has drastically increased in terms of quantity and value during the recent years. The competition among orchid production business is very intense, so a study on quality and quantity developments of orchids must be performed for commercial and conservative purposes. Moreover, plant breeding is very essential for creating bigger flowers by increasing chromosome using colchicine that can change plant characteristics to desirable traits.

The induction of polyploid or increase in chromosome set are plant breeding techniques for creating more biodiversity by increasing cell size affecting plants in having bigger size of vegetative parts and reproductive organs. These characteristics are different from polyploid plants that are developed from natural pollination, which can produce just a few polyploid plants.

The induction of polyploid plant can be performed by applying some chemicals inhibit the formation of spindle fibers. By this mechanism sister chromatid can not be pulled to the opposite pole of the cell during cell division at anaphase stage. Those chemicals are colchicine, orizaline, trifluralin, amiprophosemethyl and nitrous oxide. Among those chemicals colchicine is commonly used for ploidy induction purpose. Colchicine, a poisonous alkaloid extracted from the seeds and corms of *Colchicum autumnale*, is used in various times and concentrations. It is highly soluble in water, a desirable target in application is microtubule because it is matched with tubulin protein that is a part of microtubule, resulting in miss producing long fibers called spindle fibers during cell division. At anaphase, chromatic fiber cannot be attached at cell polar, so the chromosomes are increased from 2x to 4x, or polyploid (Starr and Taggart, 1995). Several attempts have been made to induce polyploidy orchids with colchicine. The first success of induced polyploid in orchid (*Laelia tranaei* var *alba*) was done by MacLeod (1947). The resulting plants were chimeras composed of predominantly diploid tissue and had low commercial value. The application of colchicine for polyploid induction in orchid development is the important method for plant development in order to increase sizes of flower, pseudobulb or other desired characteristics. The commonly used concentrations are 0.01–1.0%. The concentration of colchicine for polyploidy induction is species-specific (Silva *et al*., 2000; Kim *et al*., 2003; Vichiato *et al*., 2007; Atichart and Bunnag, 2007).

Therefore, the objective of this study was to evaluate the effect of colchicine at different concentrations and durations on changes of chromosome of hydrid between *D. santana* and *D. friedericksianum* orchid and to investigate the changes in some physiological characters in order to fulfil market requirements. In addition, this study can be used as guideline for the

development of other Thai orchids in the future.

Materials and methods

Plant material

Six-month-old seedlings of hydrid between *D. santana* and *D. friedericksianum* induced from culturing seed on VW medium were used in this study. Subculture in same medium was rountinely carried out at two-month-intervals. The culture was maintained at 25±2℃ under 2,000 lux/m^2/sec of fluorescent lamp at 14 hours phototperiod.

Effect of cochicine on survival rate of hydrid between D. santana and D. friedericksianum and physiological characteristics

Survival rate

Nodal segment from *in vitro* seedling of hydrid between *D. santana* and *D. friedericksianum* was excised at size of 1.0 cm consisting of a stem with one node and soaked in 0.01%, 0.05% and 0.10 % colchicine, then placing on shaker (100 rpm) at 26±2^{o}C in the dark for 24, 48, and 72 hours. After that the explants were transferred to sterile paper for blotting dry and then transferred to culture on VW solidified medium without plant growth regulators. The survival rate and 50% lethal dose (LD_{50}) were recorded after one month of culture and statistically compared among those concetrations of colchicine and periods of soaking. In addition, physiological characters in terms of guard cell size, chloroplast number and stomatal density were evaluated and statistically compared among those treatment using 3-months-regenerated-shoots after colchicine treatment.

Analysis of DNA cotent by flow cytometry technique

The leaves from 10 explants of putative polyploid hydrid and 10 control explants were used for DNA content analysis. The leaf laminar was separated from midrib, sliced into 1-2 cm in size and placed in 6-cm diameter Petri-plate. Polyvinylpirrolidone (PVP) solution at volume of 700 µl was added to the plate and the leaves were chopped with sharp razor blade for 100 times. The mixture of chopping leaf laminar and PVP solution was filtered through 20 µm nylon mesh placed on funnel to centrifuge tubes. The filtrate was added with 20 µl propidium iodide (PI) solution, mixed by shaking the tubes gently for 2-3 times, then incubatedon ice 10 minutes. The sampling solutions were feeded into

Beckman Coulter Flow Cytometry. The DNA content was counted, recorded and compared between putative and control shoots in comparison with DNA of Japanese rice of Nipponbare [(*Oryza sativa*) cv. 'Nipponbare']. DNA content from both sources of shoots was calculated using the following equation:

$$\text{Sample (2C DNA)} = \frac{\text{G1 peak channel of sample}}{\text{G1 peak channel of } \textit{Oryza sativa}} \times 108 \text{ pg}$$

Statistical Analysis

The experiments were set up in completely rancomized design (CRD) with three treatments consisting of three replications per treatment. The test of significant differences among treatments were detected using Duncan's multiple range test (DMRT) at 5% confidence level.

Results

Effect of cochicine on survival rate and ploidy level

In vitro nodal explants of hydrid between *D. santana* and *D. friedericksianum* could survive at 100% in control treatment (without soaking in colchicine solution). Soaking the nodal explants in various concentrations of colchine at different period of times caused the decreament of survival rate. The explants treated with 0.01%, 0.05% and 0.10% colchicine for 24 hours had the survival rate of 60.00%, 60.00%, and 13.33%, respectively. The explants treated with the same concentration of colchicine for 48 hours had the survival rate of 76.67% 16.67% and 6.67 %, respectively. After the explants were treated with colchicine for 72 hours, the explants had lower survival rate of 13.33%, 6.67% and 16.67%, respectively (Table 1).

The analysis of LD_{50} of colchicine treatment at different concentrations and durations showed that the values of LD_{50} of colchicine treatment for 24, 48 and 72 hours were 0.052%, 0.041% and 0.011%, respectively (Figure 1). Multiple shoots developed from the colchicine-treated nodal explants of hydrid between *D. santana* and *D. friedericksianum* were illustrated in Figure 2.

Table 1. Effect of different concentrations and durations of colchicine treatment on survival rate of nodal explants and shoot formation after cultured on VW for 30 days

Colchicine (%)	Duration (hrs)	Explants	Survival rate (%)	Shoot formation (%)
0	-	30	100.00[a]	93.33
0.01	24	30	60.00[c]	33.33
	48	30	76.67[b]	39.29
	72	30	13.33[d]	50.00
0.05	24	30	60.00[c]	39.52
	48	30	16.67[d]	66.67
	72	30	6.67[d]	66.67
0.1	24	30	13.33[d]	83.33
	48	30	6.67[d]	66.67
	72	30	16.67[d]	100.00
F-test			*	ns
C.V(%) .			18.04	59.25

*=significantly different at $P \leq 0.05$
Ns = non significant difference
Mean values followed by the same letter(s) within a column are not significantly different. ($P \leq 0.05$)

Physiological characteristics

Colchicine at different concentrations and durations caused the alteration of some physiological charcteristics, especially guard cell size, density and number of chloroplasts in guard cell. For guard cell size the results showed that shoots obtained from colchicine-treated nodal explants at higher concentrations and longer durations had bigger size of guard cells. Whereas the density of guard cell decreased. The key parameter indicating increase in ploidy level is number of chloroplasts in guard cell. The result showed that the explants treated with 0.1% colchicine for 72 hours had the highest number of chloroplasts at 68 chloroplasts following by 0.01% colchicine for 72 hours had the number of chloroplast at 48.33 chloroplasts which was significant ($p \leq 0.05$) higher than that of control (without colchicine treatment, 32.56 chloroplasts) (Table 2, Figure 3).

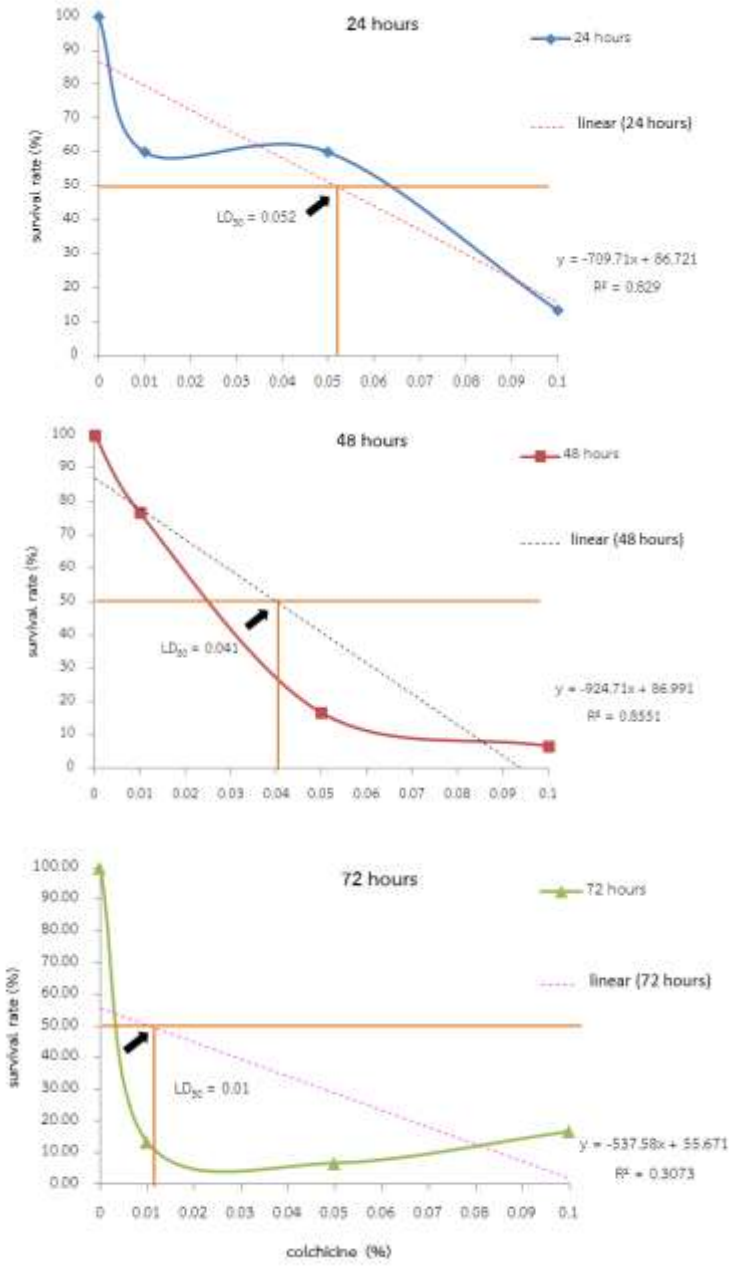

Figure 1. Survival rate at 50 % (LD_{50}) of hydrid between *D. santana* and *D. friedericksianum* after soaking nodal explants in different concentrations and durations of colchicine subsequent to culture on solidified VW medium for 30 days

Figure 2. Multiple shoots developed from nodal explants of hydrid between *D. santana* and *D. friedericksianum* treated with 0.1% colchicine for 48 hours subsequent to culture on solidified VW medium for 60 days (bar = 1 cm)

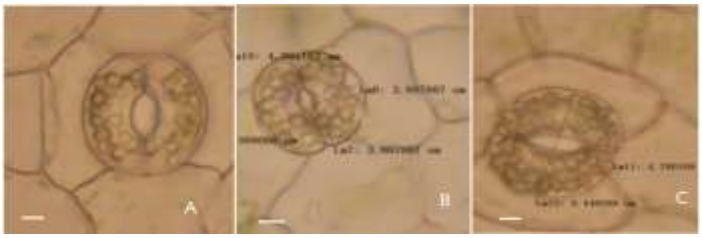

Figure 3. Chloroplasts in guard cell of hydrid between *D. santana* and *D. friedericksianum after* 8 months culture on VW medium (bar = 1 μm); (A) Control; (B) Hydrid obtained after treatment with 0.05% colchicine for 72 h.; (C) Hydrid obtained after treatment with 0.1% colchicine for 72 h.

Table 2. Effect of different concentrations and durations of colchicine treatment on some physiological characteristics of hydrid between *D. santana* and *D. friedericksianum*

colchicine		**Guard cell size (μm)**		**Stomatal density (mm^2)**	**Chloroplast number**
Concentration (%)	**Duration** (**hr**)	**lenght**	**width**		
0	24	3.81^{c}	3.75^{de}	19.67^{a}	32.56^{c}
0.01	24	3.58^{d}	4.14^{c}	14.33^{c}	30.33^{cd}
	48	3.67^{d}	4.69^{b}	17.25^{c}	21.67^{e}
	72	4.94^{a}	4.73^{b}	9.00^{f}	48.33^{b}
0.05	24	3.66^{d}	3.28^{f}	20.67^{a}	21.00^{e}
	48	4.30^{b}	3.86^{de}	13.00^{c}	33.00^{c}
	72	3.94^{c}	3.93^{d}	8.67^{e}	35.67^{c}
0.10	24	4.30^{b}	3.38^{f}	14.33^{c}	26.00^{de}
	48	3.61^{d}	3.72^{e}	21.00^{a}	24.67^{e}
	72	5.04^{a}	5.22^{a}	11.00^{d}	68.68^{a}
F-test		*	*	*	*
C.V.(%)		3.41	2.76	7.65	8.92

*=significant different at $P \leq 0.05$

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.05$).

Flow cytometry analysis revealed that DNA content of hydrid between *D. santana* and *D. friedericksianum* were mixoploid. DNA content from leaves of shoots derived from non-treated nodal explants was diploid at quantity of 3.07±0.11 picogram. The base pair size was at $1.48x10^9$ bp. While the DNA content of the hydrid treated with colchicine at every concentration and duration was between 2.98±0.04 to 3.64±0.09 picogram. The base pair size was between $1.44x10^9$ bp to $1.76x10^9$ bp. (Table 3, Figure 4).

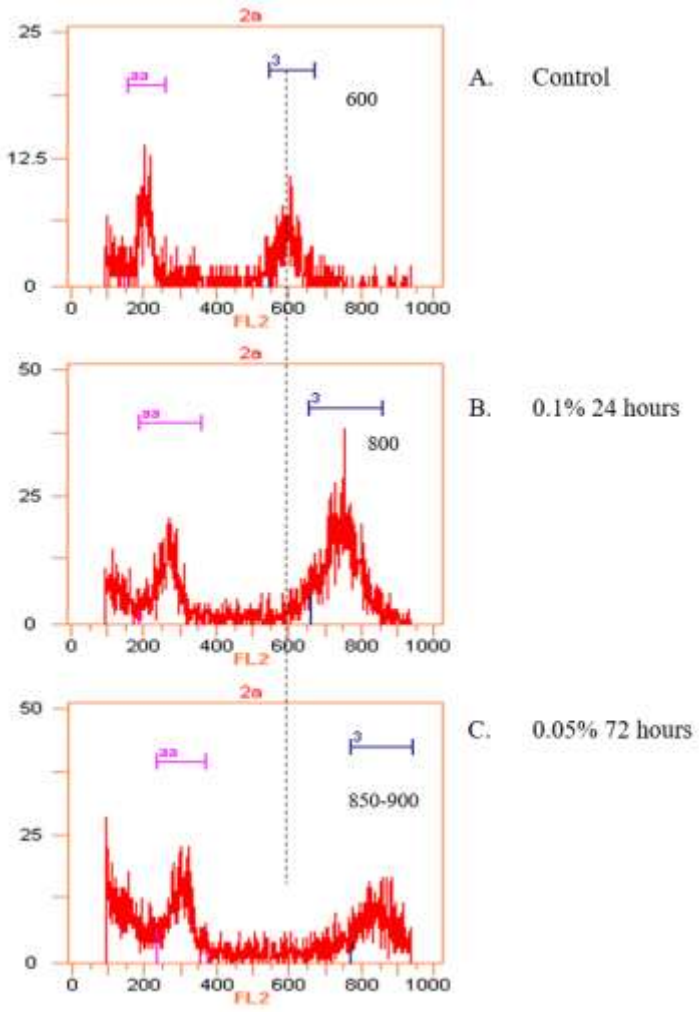

Figure 4. Histograms showing DNA content of hydrid between *D. santana* and *D. friedericksianum* treated with different concentrations and durations of colchicine in comparison with DNA content of Nipponbare rice

Table 3. Mean values of DNA content of hydrid between *D. santana* and *D. friedericksianum* treated with different concentrations and durations of colchicine

Colchicine		Treatment number	DNA content ±SE (pg $2C^{-1}$)	Mbp*
Concentration (%)	Duration (h)			
0	24	3	3.07±0.11	1.48
0.01	24	3	3.18±0.03	1.53
	48	3	3.64±0.09	1.76
	72	3	3.19±0.12	1.54
0.05	24	3	3.61±0.27	1.74
	48	3	3.09±0.07	1.49
	72	3	3.01±0.07	1.45
0.10	24	3	3.31±0.19	1.60
	48	3	2.98±0.04	1.44
	72	3	3.16±0.21	1.52

*1 picogram DNA =965 Mbp

Discussion

The nodal explant of hydrid orchid between *D. santana* and *D. friedericksianum* soaked in colchicine with different concentrations and durations for 30 days showed different response in survival rate. High concentration of colchicine with longer duration of application yielded low survival rate of cultured explant subsequent to shoot development (multiple shoot formation). The result was in accordant with *D. scabrilingue* Lindl. (Sarathum *et al*., 2008) and *D. formosum* (Petchang, 2010). It is assumed that high concentration of colchicine would be toxic to plant cell, caused imbalance of cell and affected to internal process of cell causing plants died eventually. So far, increase in ploidy of *Dendrobium* from protocorm and protocorm-like bodies (PLBs) had been reported. For example, Sanguthai *et al.* (1973) could induce numerous hexaploid and mixoploid *Dendrobium* by soaking PLBs in 0.1% colchicine solution. While, *D. scabrilligue* gave a good results in survival rate of protocorm at 36.8% from soaking PLBs in 0.075% colchicine for 14 days. After treating with colchicine ploidy level or DNA content was investigated with flow cytometry technique. The results showed that the polyploid could be induced at 43.1% (Sarathum *et al.,* 2010). Watrous and Wimber (1988) could induce tetraploid of *Paphiopedilum* for more than 50% when soaking protocorm in 0.05% colchicine for 3-10 days. For other orchids Silva *et al*. (2000) reported that soaking of PLBs of *Cattleya* in 0.05-0.1% colchicine for 4 days was the optimum treatment for the increment of ploidy level, but longer duration at 10-14 days was required for *Phalaenopsis*

(Griesbach, 1981). However, polyploid induction from nodal explant of *Dendrobium* had never been reported. In this present study, soaking of nodal segment of hydrid between *D. santana* and *D. friedericksianum* in 0.05-0.1% colchicine for 24-72 hours could promote the increment of DNA content at 33-50% of the original content as revealed by flow cytometry techniques. This could be assumed that hydrid plantlets obtained from treating nodal explants with colchicine are mixoploid, and some plants would be triploid because the DNA content was increased more than 50% from the control plants.

After comparing stomatal size and density including chloroplast number of hydrid between *D. santana* and *D. friedericksianum* orchid treated with different concentrations of colchicine and durations, the results showed that the explants treated with higher concentration and longer duration of colchicine had higher guard cell size and chloroplast numbers. This result was in accordant with guard cell size of *Doritis* treated with colchicine. Octaploid plants of *Doritis* had the longest guard cell followed by tetraploid plants. The diploid had the shortest guard cell (Jiwanit, 2009). The results were in accordant with *D. secuntum* (Atichart and Bunnag, 2007), *Cattleya intermedia* (Silva *et al*., 2000), and *Phalaenopsis* (Chen *et al*., 2009). However, in *Cymbidium*, the results showed that guard cells of diploid, triploid, and tetraploid did not have different sizes. (Kim *et al.*, 2003) The comparison of stomatal density of hydrid between *D. santana* and *D. friedericksianum* orchid in this study showed that the treatment with higher concentration of colchicine with longer duration affecting the decrement in stomatal density. Similar result was also reported in *Doritis*. Stomatal density was decreased when ploidy level was increased. (Jiwanit, 2009). However, in Charng Daeng orchid, stomatal density was increased with the higher concentration of colchicine. (Kerdsuwan and Te-chato, 2012). However, the key parameter that indicates the duplication of chromosome is number of chloroplast in guard cell. Generally, increase in number of chromosome have a close relation with the increase in number of chloroplasts in guard cell. The result obtained in this present study showed that chloroplast number of mixoploid plants was increased slightly higer than 2 times when compare with diploid plants. DNA analyse also showed the increament of DNA content or chromosome but not two times like the number of choloroplasts. Next investigation will be carried out by root tip chromosome counting to make sure about the duplication of chromosome.

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Spatial variability in soil water under adjacent mature oil palm and rubber plantations: application of a new dielectric method in evaluating soil water

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Abstract Many people have commonly addressed that oil palm plantations release much more water from the ground by evapotranspiration compared to other crops. The current research evaluated the spatial variability in soil water content under adjacent oil palm and rubber plantations established in 2005 and 1995, respectively. We collected ten pairs of soil electrical impedance data (Z, in kΩ) from the oil palm and rubber sites using a newly-developed electrical impedance meter, then converted to soil water content (θ, in $g.g^{-1}$) using the equation of $\theta = 0.45.Z^{-0.2}$. The impedance measurements took place at 0-10 and 10-20 soil depths to allow the comparisons of actual soil wetness between the two crops at the rooting zones. At the same time, we also collected disturbed soil samples from the measurement points for the laboratory determination of soil water using the standard gravimetric method. Results showed that soils under oil palm plantation were consistently more wet than under rubber in all pairs of measurements. At the 0-10 cm depth, the average soil water content at the time of measurements was 0.04 $g.g^{-1}$ higher for oil palm than for rubber. The field water content ranged from 0.310 to 0.384 and 0.268 to 0.318 $g.g^{-1}$ for oil palm and rubber, respectively. The standard deviations of samples were about 0.02 $g.g^{-1}$ for both crops indicating the statistical confidence that the oil palm site contained more water than the rubber site. Similar trends were found at the 10-20 cm soil depth suggesting the consistent benefit of the oil palm plantation in preserving soil water in the 0-20 cm rooting zone. Results in soil water variability gained from the dielectric method were similar to those obtained using the standard gravimetric method.

Keywords: Electrical impedance, oil palm, rubber, soil water

Introduction

Increasing demand in the European Union biofuels market is stimulating a vast expansion of oil palm (*Elaeis guineensis* Jacq.) cultivations in Indonesia,

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particularly in Sumatera and Kalimantan Islands. The expansion may lead to unsuccess efforts of government in safeguarding the water resources on which the local livelihoods depend on (Larsen *et al*., 2014). In oil palm plantations, water supply is the main limiting factor that controls the crop growth and yields. Therefore, this phenomenon has been widely adopted to frame the opinion that the plantations have caused a significant shortage of water resources in many countries. In many cases in Indonesia, the substantial expansion in oil palm plantations often takes place by conversing the water-converting forest and arable land as reported by Anggraini and Grundmann (2013).

Limited studies are focusing on the relations between oil palm production and the soil water use, therefore, it is difficult to conclude oil palm plantations as a factor responsible for the shortage of water resources. A study by Carr (2011), however, reveals the rates of 4-5 mm d^{-1} in the evapotranspiration from the mature oil palm trees in the monsoon months. The study also shows the 10 per cent loss of fresh fruit bunch for every 100 mm increase in soil water deficit. Kospa *et al.* (2017) report that total water use in all stages of the production of crude palm oil (CPO) and kernel was 1.36 m^3 ton^{-1} of fresh fruit bunch produced, while the wastewater in the production area was approximately 306.81 m^3 ton^{-1}. A question raised from these findings is whether any possible shortage of water resources can be related directly to the use of water for oil palm growth and yield or indirectly to the loss of water by land conversions.

In Sumatera, Indonesia, many studies have been conducted regarding the effects of tropical rainforest conversion into annual crop plantations especially oil palm and rubber plantations. Microbiological biomass, including the fungi and bacteria species, is the first soil characteristics to change when the rainforest is converted to farms (Krashevska *et al*., 2016). Direct exposure of the soil surface to rainfall water as a consequence of leaving the soil surface uncovered at the crop plantations during the rainy seasons may cause the physical degradation of soils such as the soil aggregate disintegration and the presence of pounding water following the rainy season (Hermawan and Bomke, 1997). The effects of converting the protected land into more exposed arable land to chemical properties of soils particularly the nitrogen cycle have been reported elsewhere by Allen *et al*. (2015). The above findings suggest that the accelerated expansion of oil palm and rubber plantations in Sumatera and other parts of Indonesia may lead to land degradation and low crop production.

The areas of plantations in Bengkulu Province is about 557,000 ha, including those owned by private and state companies as well as by the local people in a smallholders scale. The oil palm and rubber plantations occupy about 75 per cent of the planting areas, i.e. 290,000 and 125,000 ha, respectively (BPS, 2016). Therefore, comparing the soil water use of oil palm

cultivation to the use by other crops is urgent, as the water dependence of these two major crop yields also applies to all crop production systems. Intensive production in the oil palm and rubber commodities of Bengkulu Province may affect the hydrological characteristics of arable land and the sustainability of crop yields.

Generally, oil palm has been attributed qualitatively as a high water-consuming crop that is responsible for the degradation of water resources. Studies on quantifying the loss of soil water from oil palm and rubber plantations are very limited in Indonesia. However, the common assumption suggests that oil palm rather than rubber plantations are responsible for decreasing water resources. On the other hand, a study by Nodichao *et al.* (2011) indicated that oil palm plants with a high total root length might uptake soil water more efficiently, as well as cause a slower drying out of soils surrounding the roots compared to those with less rooting systems.

This research aimed to compare the spatial variability in soil water under adjacent oil palm versus rubber plantations. The comparisons between both crop plantations involved soil water data determined by using a dielectric method in the field and a standard gravimetric method in the laboratory. Theoretically, both crops should have contrasting patterns of soil water uptake due to differences in the root characteristics, such as shallow-fine root systems for the oil palm versus deep-coarse conditions for the rubber.

Materials and methods

The study took place in the adjacent mature oil palm and rubber plantations in Bengkulu Province, during a dry period in August 2017. The dual study of oil palm and rubber plantations were established about 11 and 20 years ago, respectively, by the local people, occupying about 20 ha of undulating areas (slopes of less than 15 per cent) on Inceptisols at the altitude of about 100 m above sea level (Figure 1). Both areas of these smallholder plantations were located at the latitude of -3.697699 S and 102.316371 E, about 10 km to the north of the provincial capital, Bengkulu City.

The physical and chemical characteristics of study soils at three positions of slopes are available in Table 1. Study soils were characterized by high sand fractions, medium levels of total nitrogen, low phosphorus and potassium, high organic carbon, and deficient aluminium. High contents of sand in the study soils indicated that both crops might not be severed to water logging during the rainy seasons due to good drainage conditions in the root zones. However, the land in the plantation areas might suffer from nutrient deficiences such as phosphorous and potassium, as well as lead to yield dissatisfaction for farmers.

Figure 1. Sites of soil water measurements (inside the box) at adjacent oil palm and rubber plantations

Table 1. Physical and chemical characteristics of study soils at three slope positions

Variables	Slope positions		
	Upper	Middle	Lower
pH (H2O)	4.60	4.90	4.85
C-org (%)	3.35	3.73	3.33
N-tot (%)	0.28	0.33	0.30
P-Bray (ppm)	14.11	10.32	10.44
K (me/100g)	7.76	2.98	6.53
Al (me/100g)	2.39	2.64	1.98
Sand (%)	49.62	47.23	48.17
Silt (%)	30.43	27.95	27.01
Clay (%)	19.95	24.82	24.82

Data collection

Data were obtained using a purposive sampling technique, as the sample crops were chosen according to the closest distance between a pair of oil palm and rubber. Ten pairs of measurement and sampling points of study soils were assigned for the bordering oil palm and rubber plantations, respectively, during a discharged period of soil water or about ten days after the last rain event. Fundamental physical and chemical characteristics of study soils were provided from a previous study by Hermawan *et al.* (2017a). The pair of sample crops were at a distance of about 20 m to each other, assuming that they grew in a similar soil type. Therefore, any differences in soil water content were attributed to the crop factor. Both measurement and soil sampling were conducted at about 1 m from each tree.

For each crop, gravimetric soil water content data were collected from the 0-10 and 10-20 cm depths by measuring the soil electrical impedance. The measurement was conducted according to a newly developed dielectric method by using the impedance meter. The working mechanism of the technique in determining soil water content in the field has been reported by authors elsewhere (Hermawan *et al*., 2017b). A pair of sensors connected to the impedance meter was inserted into the soil up to a depth of 10 cm, and then the device was switched on to allow the electric current to travel at a frequency of 300 kHz. The soil electrical impedance value appeared within five seconds on the LCD screen of the device (as illustrated in Figure 2) and recorded. At the same time, a disturbed soil sample was taken from the same depth using an earth borer, put into a plastic bag and tightened with rubber for the laboratory measurement of water content. Both impedance measurement and soil sampling at the 0-10 cm depth were repeated for the 10-20 cm layer.

Figure 2. A newly developed impedance meter for measuring soil water content using the dielectric method in the field

Gravimetric soil water content (θ in $g.g^{-1}$) was calculated from the measured soil electrical impedance (Z in kΩ) using the following nonlinear equation.

$$\theta = a.Z^b \quad (1)$$

Where a was intercept and b slope in the θ-Z line graphic. The intercept value was 0.45 and slope was -0.2 as found from a previous study on the same soil (Hermawan *et al*., 2017a). Therefore, Equation 1 was written as

$$\theta = 0.45.Z^{-0.2} \quad (2)$$

The proposed model has been tested for hundreds of pairs of soil water and impedance data and indicated the closeness of their relations (R^2>0.80).

The laboratory measurement of soil water content was applied to the soil samples taken at the same depths of the impedance readings. Soil water content was determined using a standard gravimetric method (Gardner, 1986), while its calculation was based on the ratio between the weight of water existing at soil samples and that of soils subjected to the oven drying treatment for at least 24 hours. The mass of water in the soil was determined from the weight differences between the field moist and oven-dried soil samples. Soil water content determined using this method has been usually used as the corrector for any alternative techniques in measuring soil water. The corrections were required since the alternative methods in determining soil water content, including the procedure proposed in the current study, predict the amount of water in soils by measuring other variables, while the laboratory gravimetric scheme directly measures the soil water content.

Data analyses

Soil water data obtained from the new device were analyzed descriptively by comparing the patterns of their spatial variability under mature oil palm and rubber plantations. The comparisons were made by plotting pairs of soil water data for both crops on the line graphs. This analysis technique allowed us to calculate the gaps in soil water content between pairs of oil palm and rubber trees chosen as study samples.

Differences in predicted soil water content under oil palm and rubber plantations were then compared to those for the laboratory measured soil water. This data analysis technique was used to evaluate the accuracy of the new device in predicting soil water content in the field. The accuracy of the proposed method was evaluated according to the closeness of the spatial variability patterns in soil water between the two methods.

Results

Spatial variability in soil water

Spatial variability in soil electrical impedance, as a dependent variable to calculate the in-situ soil water content in the field under mature oil palm and rubber canopies, was presented in Figure 3. The electrical impedance values

under oil palm were consistently lower over ten measurement points compared to those under rubber plantations. The differences in the impedance for each pair of oil palm and rubber readings ranged from 3.0 to 9.0 kΩ for the uppermost 0-10 cm soil depths and 0.5 to 9.0 kΩ at the lower layers. The results suggested that the electric current injected from the impedance meter travelled with less significant resistance under oil palm than under rubber trees. The trend in the electrical impedance variability existed up to the depth of 20 cm from the soil surface. The spatial variability in soil electrical impedance values might indicate the distribution of soil components at the measuring zones such as variations in soil wetness, the presence of plant roots, (Wang *et al*., 2019) as well as lime, bentonite, and polymer (Raheem *et al*., 2017).

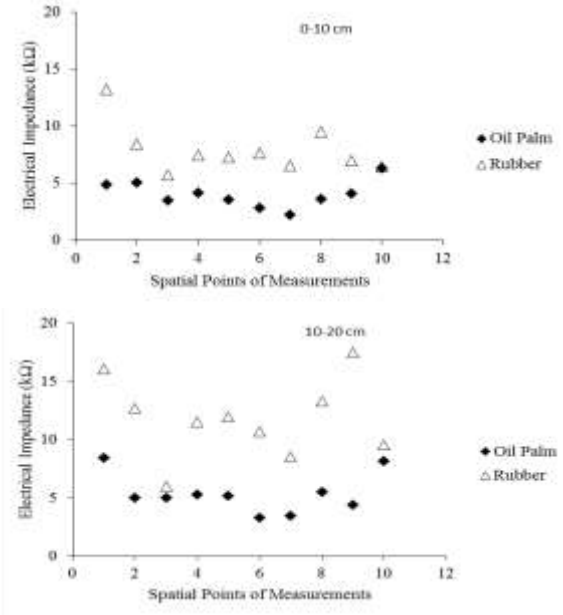

Figure 3. Spatial electrical impedance of soils measured under oil palm and rubber plantations

In the current study, the soil electrical impedance was measured using a newly developed impedance meter operated at a low (3.0 kHz) frequency of sinusoidal voltage generator. Since soil dielectric variables such as impedance and capacitance were frequency dependent (Kelleners and Verma, 2010), the electrical impedance data presented in Figure 3 might indicate the real values of the dielectric properties, despite different frequency bases of the measurement devices. In relations to soil water content, the electrical impedance had negative exponential correlations with the proportion of water occupying the soil pores. Increasing electrical impedance in the porous media, including soils,

would be followed by decreasing content of soil water and increasing occupation of highly-resistant air to the electricity transmission. The exponential relations between soil electrical impedance and soil wetness were similar to those found by Wang *et al.* (2019), indicating that this dielectric parameter can serve as an indicator of the substrate's water content.

The purposes of measuring the electrical impedance of study soils was to determine the in-situ soil wetness variations under mature oil palm and rubber plantations. When the dielectric data in Figure 3 were converted to soil water content using Equation 2, the spatially predicted water was higher for oil palm compared to rubber soils (Figure 4). Results showed that soils under oil palm plantation were consistently wetter than soils under rubber in all points of measurements. At the 0-10 cm depth, the average soil water content at the time of measurements was 0.04 $g.g^{-1}$ higher for oil palm than for rubber, ranging from 0.310 to 0.384 and 0.268 to 0.318 $g.g^{-1}$, respectively. The standard deviations of samples were about 0.02 $g.g^{-1}$ for both crops, indicating the statistical confidence that the oil palm site contained more water than the rubber site. The superiority of soil water under mature oil palm plantation might offer crucial information regarding the common assumption among many environmental activists worldwide that the plantation has been responsible for a shortage of water resources, as well as for water pollution due to high fertiliser applications (Comte *et al.*, 2015).

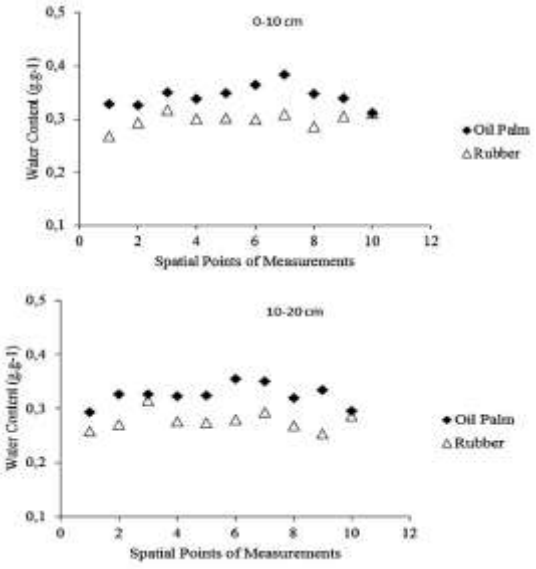

Figure 4. Spatial variability in water content at 0-10 and 10-20 cm soil depths under two crops as calculated from the electrical impedance values

Gravimetrically-measured soil water distribution

The second objective of the current study was to evaluate the comparative differences between dielectric-based predicted and gravimetrically-measured soil water content under two different plantations. As shown in Table 2, the averages in laboratory-measured soil water content were about 0.05 and 0.04 $g.g^{-1}$ higher for mature oil palm trees at the 0-10 and 10-20 cm depths, respectively. Standard deviation for each site was lower at the 10-20 cm depth suggesting that differences in measured water content were statistically more significant at lower rather than upper soil layers.

Table 2. Spatial variability in soil water ($g.g^{-1}$) under oil palm and rubber plantations as determined by the gravimetric method

Points of measurements	0-10 cm depth		10-20 cm depth	
	Oil palm	Rubber	Oil palm	Rubber
1	0,373	0,322	0,282	0,307
2	0,270	0,287	0,275	0,300
3	0,419	0,291	0,327	0,290
4	0,375	0,328	0,358	0,299
5	0,342	0,289	0,320	0,309
6	0,421	0,365	0,416	0,342
7	0,420	0,315	0,398	0,330
8	0,394	0,307	0,363	0,312
9	0,364	0,322	0,310	0,282
10	0,286	0,287	0,298	0,287
Mean	0,336	0,311	0,335	0,306
Stdev.s	0,054	0,025	0,048	0,019

Trends of gravimetrically-measured data in Table 2 were relatively equal to those predicted from Equation 2 (Figure 4), suggesting the closeness between predicted and measured soil water content in the study area. These features indicate that dielectric variables such as the electrical impedance can be used in predicting the field variations in soil water content, in comparison to the conventional technique. Prediction models that determine soil water content in the field are useful tools to help us better understand soil-water interactions, as they can obtain large numbers of soil water data in a short period. The models can be applied to a wide range of soil and water management techniques, such as irrigation, by improving water uptake by the plant (Karandish and Šimůnek, 2016).

Discussion

Wetter soils under the oil palm plantation were consistently found at all measurement points, suggesting slower drying processes of rooting-zone soils by this crop during the drying period. Well-distributed fine roots of mature oil

palm, in comparison to the coarse roots of rubber, might have better access to a large volume of soil to absorb water, and therefore could slow down the drying process of soil in the rooting zone (Nodichao *et al*., 2011). The presence of massive roots under oil palm might be responsible for the more homogenous soil water profile up to a 20 cm depth, as shown in Table 2. On the other hand, the fast-drying soils under the rubber plantation found in this study could be attributed to the fact that rubberwood absorbed more water vapour than the oil palm trunk (Zaihan *et al*., 2011), and might enhance water loss by evaporation from the soil surface. Canopy openness of plantation trees could also be the reason for soil wetness differences under oil palm and rubber sites. Meijide *et al.* (2018) reported that some microclimate variables were better under oil palm than under rubber canopies, air humidity was 1.1% higher and vapour pressure deficit was 33 Pa lower under oil palm, and likely responsible for less evaporation and soil water loss for oil palm. Our field observation during measurements also found that oil palm canopy covered more land surfaces than rubber trees.

Results shown in Figure 4 suggested that the mature oil palm plantation used in this study conserved more water in the rooting zone of 0-20 cm soil depth compared to the monoculture rubber plantation. The possibility that water penetration might be limited by evapotranspiration at the vegetated soil, as suggested by Wang *et al.* (2008), did not happen for vegetated study soils with well-distributed fine root systems such as oil palm. Lateral-distributed root system development had a greater ability to maintain soil water availability and crop production (Suralta *et al.,* 2015). Also, the application of several water conservation practices under oil palm trees could result in more soil water availability during water deficit periods. Applying the terrace bund in the mature oil palm plantation has increased soil water content and fresh fruit bunch production by 13.4 per cent compared to the control site (Murtilaksono *et al.,* 2011). Mulching application using materials from oil palm residues are often suggested by many researchers to improve water conservation and land productivity in the plantations. Moradi *et al.* (2015), for example, recommended the use of empty fruit bunches for mulching due to an increase in distributed water throughout the soil profile, particularly on hill plantations. Management practices for soil water conservation are the main key to the oil palm development since annual soil water deficit is the most significant limiting factor in the land suitability classification for oil palm production.

Conclusion

Spatial variability of soil water within each plantation as predicted from the measured electrical impedance values was lower than the variability

between the two farms. Soils under oil palm plantation were consistently wetter than under rubber in all pairs of measurements. The average soil water content at the 0-10 cm layer was 0.04 $g.g^{-1}$ higher for oil palm than for rubber, ranging from 0.310 to 0.384 and 0.268 to 0.318 $g.g^{-1}$, respectively. The standard deviations of samples were about 0.02 $g.g^{-1}$ for both crops, indicating the statistical confidence that the oil palm site contained more water than the rubber site. Similar trends were found at 10-20 cm soil depth, suggesting the consistent benefit of the oil palm plantation in preserving soil water in the 0-20 cm rooting zone. Predicted water variability gained from the dielectric method were similar to those measured in the laboratory using the standard gravimetric method, suggesting that the laboratory determination of soil wetness variability can be shifted to the field measurement.

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Creation of a knowledge management process in broiler production of Thailand

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Abstract The Delphi technique is employed in this study to find an appropriate process of knowledge management in broiler production of Thailand. It is a specific agricultural knowledge management, which is different from other types of knowledge that are mostly accumulated knowledge gained from practice and problem solving. Different factors based on current situation are used to select an appropriate process with tacit knowledge management. It can aid problem solving in practice as the explicit knowledge can be transferred and connected to a method of cause analysis and thus address challenges related to broiler production. This helps develop the production system and knowledge transfer to benefit a new generation of animal husbandry practitioners. Results of the study show that the creation of the knowledge management process in broiler production involved seven steps: knowledge indication, knowledge creation and acquisition, knowledge codification and refinement, systematic knowledge management, knowledge access knowledge sharing, and learning process. A very high level ofconsistency in the value of opinions of specialists on knowledge management was found. Specific knowledge must be kept to construct innovative ideas for broiler production and development. This is to ensure safe food production and food security support, which results in sustainable production of safe meat.

Keywords: knowledge management, broiler production, sustainability

Introduction

Broiler domestication to produce chicken meat, which is a good protein food source, is important in human nutrition.Thailand is in a position to become the world kitchen in terms of geography, access to raw materials, and satisfying the needs of the country. Broiler production thus plays an important role in income generation and food security of the country (Parsertsak, 2012) and the world community in response to the high demand for food by the rapidly

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increasing world population (Injana *et al.*, 2015). Consequently, broiler production must be enlarged to develop the production system of agro-industry (Office of Agricultural Resources, Chulalongkorn University, 2017). This necessitates farmers or farm owners adapting themselves to becomeentrepreneurs. Also, they must add more animal husbandry technical workers and improve all aspects of animal husbandry.. This will result in an effective operation of the animal husbandry farm that has the appropriate size. It is because broilers are economic animals having a rapid growth rate; feed conversion ratio can be observed after only 38-42 days of rearing. In other words, a newborn chick weighing 35-42 g will be mature and ready to be dissected when it reaches 2,800-3,200 g (Cobb-vantress, 2017a). Also, breeders can produce 166-182 chicks (Cobb-vantress, 2017b). There is thus a need for a body of knowledge and skills in broiler management to have good production control. This knowledge can be sorted into two: tacit knowledge and explicit knowledge at the ratio of 80:20 (Suanpleng, 2009; Sakroongposakul and Yuttananwiboonchai, 2006). Tacit knowledge is difficult to explain and transfer to other people (Namprasertchai, 2015); it is lost when people who possess it pass away. This loss affects the production system and have an impact on the sustainability of the business. Therefore, entrepreneurs give importance to process seeking and creation of a tool that can collect tacit knowledge and transform it into explicit knowledge that benefits the production process.

As knowledge management is a tool that can transform tacit knowledge into explicit knowledge, it is interesting to investigate what knowledge management process is needed for broiler production. This is a specific agricultural knowledge that can bring about production innovation and efficiency in the broiler industry.

Conceptual Framework of Study

Figure 1. Conceptual framework of study

Materials and Methods

Step 1. Review of related literature and Delphi technique to formulate conceptual framework about knowledge management.

Step 2. Specialist selection: one specialist obtained from purposive sampling; the obtained specialist proposes three otherspecialists and the three specialists propose another three specialists each (snowball sampling). The number of population having least classification error was 17 persons (Srinantawong, 2005; Khongkhanoi, 2010).

Step 3. Investigation step based on Delphi technique. Open-ended questionnaire was used for data collection, administered to 20 specialists. It involved the knowledge management process and various components of knowledge management. Obtained data were analyzed and synthesized and then used to constructa rating scale questionnaire to assess the appropriateness of knowledge management activities in each process. The rating scale questionnaire was then checked by five specialists. Other specialists considered consistency together with reason.

Step 4. Making conclusions based on consistency value. For items having low consistency (IR value range was 2.00-2.99), the specialist tries to get reasons by using an open-ended questionnaire; a discussion is then conducted.

The answers were used for the construction of a rating scale questionnaire, whichwas administered with the experts based on level of appropriateness of activities in each process (from most to least or from 5 to 1). Obtained scores of appropriateness were analyzed to find the median, mode, and interquartile range to interpret a level of consistency. Then, it was given to specialists for confirmation and evaluation of consistency of answers of other specialists. The following were the criteria of consistency:

Interquartile range		**Answers of specialists**
3.00 and above	=	Have no consistency
2.00 – 2.99	=	Low consistency
1.01 – 1.99	=	Moderate consistency
0.51 – 1.00	=	High consistency
0.50 and below	=	Very high consistency

The accepted criterion at the interquartile range was between 1.01 and 1.99. This implies that answers of the specialists had moderate consistency.

Step 5. Final conclusions are made.

Results

Results of the study showed that more than half of the 20 specialists (75%) were female and that they belonged to age ranges 20-40 and 41 years old

and above (10 persons each). More than one-half (60%) were bachelor's degree holders and the rest had above bachelor's degree.

It was found that the 20 specialists had 12 issues in knowledge management: identification of knowledge needed for practice; acquisition of needed knowledge; improvement of knowledge to make it appropriate for practice; application of knowledge to be beneficial to practice; knowledge exchange; knowledge extraction for dissemination used in the organization; knowledge arrangement to be systematic; knowledge organizing; learning; and determination of the person responsible for the task. For data synthesis, seven processes of knowledge management were obtained: 1) knowledge indication, 2) knowledge creation and acquistion, 3) knowledge organizing, 4) knowledge management to be systematic, 5) knowledge access, 6) knowledge exchange, and 7) learning. All of these confirmed the findings of Gretsch *et al.* (2012).

Table 1. Knowledge identification process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Having a meeting to make clear conclusion about practice form	5.00	4.00	4.00	5.00	1	High
2	Explanation of tasks of each section	5.00	4.00	3.00	5.00	2	Low
3	Identifying sections that must work together	4.00	4.00	4.00	5.00	1	High
4	Formulating mutual mission	5.00	4.00	3.00	5.00	2	Low
5	Identifying clearly the needs of the organization	5.00	4.00	4.00	5.00	1	High
6	Having annual project planning every year	4.00	4.00	4.00	5.00	1	High
7	Preparingcontinual operational report	4.00	4.00	3.25	5.00	1.75	Moderate
8	Having conclusion about problems in practice after a task is finished	5.00	5.00	4.00	5.00	1	High
9	Having conclusion aboutfactors that ensure successful task operation	4.00	4.00	4.00	5.00	1	High

Confirming the opinions of the specialist group, there was a high level of consistency in terms of 1) having a meeting to make clear conclusion about practice form─e.g.,daily chicken health checking, checking amount of feed and water; 2) having needs told among sections that work together─e.g.,making a record of dead chickens; 3) telling the organization about needs such as percentage of damage from broiler rearing, feed conversion ratio, and growth rate per head per day; 4) having annual project planning every year to come up with a calendar of chicken rearing for each production cycle and a calendar of pen break; 5) making conclusion regarding problems in practice after finishing tasks such as percentage of loss in each batch; and 6) making conclusions about

factors contributing to a successful task operation such as reporting of findings on each batch.

Table 2**.** Knowledge creation and acquisition process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Knowledge acquisition from specialists for use in practice	4.00	4.00	4.00	4.75	0.75	High
2	Coordination among various sections of the organization for mutual knowledge acquisition	4.00	4.00	4.00	5.00	1	High
3	Coordination from external organizations for new knowledge acquisition	4.00	4.00	4.00	4.00	0	Very high
4	Continual knowledge acquisition from staff in the organization	4.00	4.00	4.00	5.00	1	High
5	Staff acquiring knowledge through various media such as internet	5.00	4.50	4.00	5.00	1	High
6	Knowledge acquisition for practice	4.00	4.00	4.00	5.00	1	High
7	Acquiring existing knowledge in the organization for improvement of practice	3.00	4.00	3.00	5.00	2	Low
8	Seeking for specialists to institute practices in the organization	4.00	4.00	4.00	5.00	1	High

This confirmed the high level of consistency found by the specialist group: coordination from external organizations for new knowledge acquisition such as in-house seminars on biological safety system and farm sanitation; knowledge acquisition from specialists regarding practice; coordination among various sections in the organization for mutual knowledge acquisition; continual knowledge acquisition among organization staff such as fixing date and time for weekly meetings; and staff acquiring knowledge from various media such as internet, publications, etc.; and seeking for innovation in practice.

Table 3**.** Knowledge codification and refinement process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Continual review of practice steps	4.00	4.00	3.25	5.00	1.75	Moderate
2	Definition of terms and disseminating such throughout the organization	3.00	4.00	3.00	4.75	1.75	Moderate
3	Keeping knowledge in the same form	4.00	4.00	3.00	4.75	1.75	Moderate
4	Creation of a form of body of knowledge for the organization	5.00	4.00	3.00	5.00	2.00	Low

The specialist group found a moderate level as follows: continual review of practice steps; definition of terms and disseminating such throughout the organization; and keeping knowledge in the same form (e.g., making conclusions about problems in chicken rearing in each production cycle; preparation of reports on efficiency in daily chicken rearing; preparation of a farm news board; and establishment of similar groups for spreading farm news.

Table 4**.** Knowledge creation and acquisition process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Clear formulation of knowledge keeping structure in the organization	4.00	4.00	3.00	5.00	1	High
2	Clear policy formulation on knowledge keeping for use in practice and learning	5.00	4.50	4.00	5.00	1	High
3	Preparation of database on skills of staff in each aspect which is beneficial to systematic practice	4.00	4.00	3.25	4.75	1.50	Moderate
4	Preparation of knowledge acquisition system which is beneficial to work performance of staff	5.00	4.00	3.50	5.00	1.75	Moderate
5	Clear form of data collection used for practice and task transfer	4.00	4.00	3.00	4.00	1	High

Table 5. Knowledge access process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Continually inform the organization staff about knowledge access	4.00	4.00	3.00	4.00	1	High
2	Continually send data/information to organization staff	4.00	4.00	3.00	4.00	1	High
3	Construct website of the organization	5.00	5.00	4.00	5.00	1	High
4	Clearly prepare the organization unit for knowledge dissemination	5.00	4.50	3.25	5.00	1	High
5	Continually update data	5.00	4.50	4.00	5.00	1	High
6	Encourage participation in gathering and dissemination of data/information among organization staff	5.00	4.50	3.25	5.00	1.75	Moderate
7	Continually develop communication channels in the organization	5.00	4.50	3.25	5.00	1.75	Moderate
8	Continually call for meetings between administrators and organization staff or workers	3.00	4.00	3.00	4.00	1	High

A moderate level of consistency was found in terms of the following: clear formulation of knowledge keeping structure in the organization; clear policy formulation on knowledge keeping for use in practice and learning; and clear form of data collection for practice and task transfer. Examples were preparation of a manual on broiler production; formulation of a teaching method for new animal husbandry technical workers and chicken rearing staff; and clear designation of important tasks in data collection and management for broiler production.

The consistency level of opinions of the specialist group was found to be high based on the following: continually informing organization staff about knowledge access; continually sending data/information to organization staff; constructing the organization website; establishing the knowledge dissemination unit of the organization; continually updating data; and continuous conduct of meetings between administrators and organization staff.

Table 6. Knowledge sharing process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Engaging in activities to encourage organization staff to share knowledge	5.00	4.50	4.00	5.00	1	High
2	Preparing information to enable organization staff to learn and transfer knowledge to others in the organization	5.00	4.50	4.00	5.00	1	High
3	Creating networks for learning and transferring knowledge to others in the organization	5.00	5.00	3.25	5.00	1.75	Moderate
4	Creating networks for learning and transferring knowledge to others outside the organization	4.00	4.00	3.25	4.00	0.75	High
5	Presenting and sharing suggestions about practice among sections in the organization	4.00	4.00	4.00	4.00	0	Very high
6	Having meetings for learning and sharing of body of knowledge arising from practice	5.00	4.00	3.00	5.00	2	Low
7	Activities for knowledge sharing among various sections in the organization	4.00	4.00	4.00	5.00	1	High
8	Knowledge sharing from section heads and co-workers	5.00	4.00	4.00	5.00	1	High
9	Having a team for knowledge transfer and problem solving	4.00	4.00หป	3.25	5.00	1.75	Moderate

The following were found at a high consistency level: activities promoting organization staff to share knowledge; preparing information

enabling the organization staff learn and transfer knowledge to others in the organization; creating networks for learning and transferring knowledge to others outside the organization; activities for knowledge sharing of various sections in the organization; and knowledge sharing from section heads and co-workers. Examples are the conduct of meetings between teams of each section; making conclusions about the needs of concerned units in each section; preparing documents on practice objectives of each section; clear definition of tasks of each position and section; and clear preparation of success indicators for each duty.

Table 7. Knowledge learning process

No	Step in knowledge management	Mode	Mediun	Q1	Q3	IR	Level of consistency
1	Adoption of prepared knowledge in practice	5.00	4.50	4.00	5.00	1	High
2	Creation of innovation in practice	4.00	4.00	4.00	5.00	1	High
3	Practice development of sections	5.00	4.50	4.00	5.00	1	High
4	Having practice assessment of all sections	4.00	4.00	4.00	4.00	0	High
5	Having internal monitoring in the organization	4.00	4.00	4.00	5.00	1	High
6	Development of co-workers between sections	4.00	4.00	4.00	5.00	1	High
7	Integration of practice in the organization	4.00	4.00	4.00	5.00	1	High
8	Investigation and analysis between sections	4.00	4.00	3.00	4.75	1	High

Table 8. Level of consistency in the process of knowledge management in broiler production

No	Step in knowledge management	Mode	Mediun	IR	Level of consistency
1	Knowledge identification process	5	4	0	Very high
2	Knowledge creation and acquisition process	4	4	0.5	Very high
3	Knowledge codification and refinement process	4	4	0.5	Very high
4	Knowledge organization process	4	4	0	Very high
5	Knowledge access process	5	4.5	0.5	Very high
6	Knowledge sharing process	5	4	0.25	Very high
7	Knowledge learning process	4	4	0	Very high

The consistency level of opinions of the specialist group was found at a high level in terms of the following: adoption of prepared knowledge in practice; creation of innovation in practice; practice development of sections; having internal monitoring in the organization; development of co-workers between sections; integration of practice in the organization; investigation and analysis between sections; practice planning in the organization; andpractice from preparation.

The consistency level of the entire process of knowledge management in broiler production can be deduced from data obtained from the specialists (Table 8).

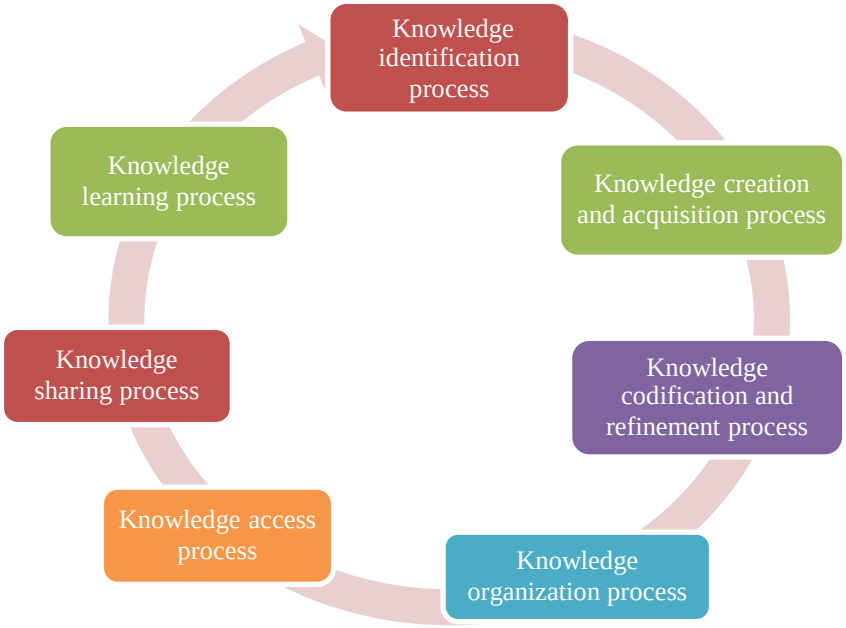

Figure 2. Process of knowledge management in broiler production

Discussion

The process of knowledge management facilitation consisted of seven steps: knowledge creation and acquisition, knowledge codification and refinement, knowledge organization, knowledge access, knowledge sharing, and knowledge learning. This may be classified based on the form of management in each process called the D.I.K. Model (Siemieniuch *et al*. (1999); Tian *et al*. (2009); Huei Tse Hou (2012); Rennolls *et al.* (2008). The model has three major parts:

Part 1. Data management process comprising knowledge identification, knowledge creation and acquisition, knowledge codification and refinement, and knowledge organization.

Part 2. Information management process comprising knowledge sharing.

Part 3. Knowledge management process comprising knowledge access and knowledge learning.

Therefore, knowledge management in broiler production is in the hands of individuals (tacit knowledge) and they use various information to turn this into explicit knowledge, which can be transferred to others in the organization. In other words, it can be used to create innovation in practice and thereby increase production efficiency. Interested farmers can benefit by following this model of knowledge management in broiler production.

1. Knowledge identification in broiler production. The Information Technology and Communication Center (2017) states that knowledge identification entails consideration of the target vision and mission and that, in order to achieve these, it is crucial that we know what existing knowledge we have, in what form, and with whom it resides. This conformed to the opinions of the specialist group, which were found at a high level of consistency in terms of having meetings for clear conclusion of practice form; identifying the needs of concerned sections; clearly showing organization needs; continual annual operational planning; making conclusion regarding problems encountered after finishing the tasks; and making conclusion about factors effecting a successful practice. For example, there was identification of broiler production elements: i.e., rearing form, number of chickens per area, live weight of broilers needed by the slaughter house, weight and size at each age, vaccination program, period of feed changing, time span of lighting in each period, setting a production goal for each batch, etc.

2. Knowledge creation and acquisition in broiler production. Choochan (2017) stated that this process involvescreation and acquisition of knowledge from various sources dispersing inside and outside the organization. This aims to arrange the content of knowledge to meet the needs of the staff in an organization. This conformed to opinions of the specialist team, which were found at a high level of consistency on the basis of the following: coordination from external sources to acquire new knowledge and find existing knowledge in the organization. The following were activities having a high level of consistency: acquiring knowledge from specialists for use in practice; coordination among various sections of the organization for knowledge acquisition; continual knowledge acquisition for co-workers in the organization; staff of the organization acquiring knowledge from various

sources; and seeking for innovation for use in practice. These entail continuous training on knowledge about broiler production management; presentation of various innovations; and support on data source creation.

3. Knowledge codification and refinement in broiler production. Pasukyud (2007) identifies this process as improvement of documentary form to meet standards, language use, and content of knowledge. This conformed to opinions of the specialist group which were found at a moderate level of consistency on the basis of the following: continual review of practice steps; defining terms related to practice and disseminating these throughout the organization; and arranging the same knowledge for future use. For example, there were conclusions made regarding problems encountered in each batch. Moreover, there was preparation of daily/weekly broiler rearing forms and news dissemination boards; a dissemination group was likewise set up for news/information sharing.

4. Knowledge organization in broiler production. A study by the faculty of Pharmaceutical Science, Ubon Ratchathani University (2010) revealed that knowledge organization involves laying out a knowledge structure for systematic knowledge keeping in the future. This conformed to opinions of the specialist group which were found at a high level of consistency on the basis of the following: formulation of a clear structure of knowledge keeping for use in practice. Examples included the preparation of a manual on broiler production for new animal husbandry technical workers and designation of data collection tasks for broiler production.

5. Knowledge access in broiler production. The Office of Public Sector Development and the Thailand Productivity Institute (Office of the National Research Council of Thailand, 2003) revealed that knowledge access is to make knowledge users gain easy access to information technology system, web board, and public relations board. This conformed to opinions of the specialist group, which were found at a high level of consistency based on the following: continually giving knowledge access to staff in the organization; creation of organization website; establishment of an agency for knowledge dissemination; continual update of data; and regular meetings between administrators and workers.

6. Knowledge sharing about broiler production. Noiwat (2011) claimed that the knowledge sharing process involves management of a cross-functional team, group activities on quality and innovation, learning community, escort system, task shifting, knowledge sharing venue, etc. This conformed to opinions of the specialist group, which were found at a very high level of consistency in terms of sharing suggestions about practice between sections in the organization. The following were found at a high level: activities

encouraging organization staff to share knowledge; preparing information to enable the organization staff learn and transfer knowledge to others in the organization; and knowledge sharing between heads and co-workers. Examples were holding meetings between teams of each section; assessing the needs of each section; preparing a document on the objectives of each section; clearly defining the tasks of each position and section; and clearly identifying success indicators.

7. Knowledge learning about broiler production. It is the adoption of knowledge for problem solving, improvement, and development of work performance. Problems encountered must be discussed for improvement of procedures and assessment of practice must be done. This conformed to opinions of the specialist group, which were found at a high level on the basis of the following: application of knowledge to practice; creation of innovation for practice; development of work performance; internal assessment; practice development between sections; practice integration in the organization; investigation and analysis of practice between sections; creation of practice plan in the organization; and creation of common practice.

Conclusion

This study aimed to seek guidelines for future practice in order to reduce conflicts among specialists with respect to broiler domestication. This study showed that the knowledge management process in broiler domestication comprised seven steps: 1) knowledge identification, 2) knowledge creation and acquisition, 3) knowledge organizing, 4) knowledge management to be systematic, 5) knowledge access, 6) knowledge exchange, and 7) knowledge learning. All of these processes had a high level of consistency. This could help animal husbandry workers develop efficiency in broiler production. This would result in decreased production cost and creation of innovative ways to further improve the broiler industry. Besides, this body of knowledge could be transferred from senior animal husbandry workers to beginners. In fact, the learning process or the development of knowledge management on broiler domestication could happen in every step, depending on the experience, problem solving ability, and knowledge management skills of each animal husbandry worker**.**

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Age-related difference changes semen quality and seminal plasma protein patterns of Thai native rooster

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Abstract The association between age and semen quality of Thai native roosters (Leung hang khao) in relation to patterns of seminal plasma proteins were determined. Eighteen of the native roosters were grouped according to the ages 7-8, 10-11 and 23-24 months. Semen was collected by abdominal massage and evaluated for different physical parameters. Results showed that semen volume, color, pH and sperm concentration were not significantly different among the different age groups. In contrast, sperm viability was found to be significantly different. Sperm viability was significantly higher in the 7-8 ($93.34^{a} \pm 2.17$) than the 23-24 months age category ($71.38^{b} \pm 7.44$). Seminal plasma proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Based on our findings, proteins with molecular weight fractions of 72, 90, 140, 220, and 260 kDa showed patterns of expression in 7-8, 10-11, and 23-24 month old roosters, respectively. Moreover, proteins with molecular weight fractions of 72, 90, and 140 kDa were shown sharply patterns of expression among age of roosters. These results indicate that sperm vitality (percentage of live sperm or dead sperm) and proteins with molecular weight fractions of 90, and 140 kDa are associated with age. Proteins with molecular weight fractions of 90 and 140, kDa may serve as markers to determine semen quality in Thai native roosters.

Keywords: seminal plasma proteins, semen quality, Thai native rooster

Introduction

In poultries and mammals, semen is a complex fluid containing spermatozoa and seminal plasma (Marzoni *et al.,* 2013). The precise observation of semen quality is required for successful artificial insemination

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(Almahdi *et al.,* 2014). Semen quality may be affected by various environmental factors such as feed, temperature, age, and strain thereby influencing fertilization rate (Karaca *et al.,* 2002; Shanmugam *et al.,* 2014; Zhang *et al.,* 1999). Aging playing an essential role in male fertility in all species of animals appears to have a significant impact on reproductive behavior (Avital-Cohen *et al.,* 2013). Fertility of domestic roosters is highest at 32 weeks of age and declines at about 45 weeks of age. Previous studies showed that older gobblers exhibit poor semen quality characterized by low sperm volume, number, motility, viability, and membrane integrity (Iaffaldano *et al*., 2008). Broiler breeders have been reported to produce the maximum number of sperm at 36 weeks of age and progressively declined until 55 weeks of age (Sarabia Fragoso *et al.,* 2013). Another findings in Iranian indigenous broiler breeders showed that spermatozoa concentration between 26 and 34 weeks of age maintain at the basal level. However, significant reductions were found in these broiler breeders at 45 weeks of age (Tabatabaei *et al.,* 2010). Semen volume, sperm concentration, and total sperm per ejaculation of Indian red jungle fowl were significantly higher at 43-54 months of age followed by the age groups of 31-42, 19-30, and 6-18 months (Rakha *et al.,* 2017).

The properties of proteins, nutrients, and buffer systems in seminal plasma connect with several sperm parameters, such as motility, capacitation, sperm transport, survival and longevity, protection against damages, and the formation of the sperm reservoir inside the female reproductive tract, all of which determine the semen quality (Novak *et al.,* 2010; Pilch and Mann, 2006; Troedsson *et al.,* 2005), (Al-Aghbari *et al.,* 1992; Brandon *et al.,* 1999; Marzoni *et al.,* 2013; Slowinska *et al.,* 2008). Several studies research seminal plasma proteins with fertility in various species (Borziak *et al.,* 2016; Marzoni *et al.,* 2013). The protein profiles of bovine seminal plasma have been investigated by using two-dimensional polyacrylamide gel electrophoresis, and two proteins (26 and 55 kDa) are indicators for bulls with high fertility (Killian *et al.,* 1993). Examining canine seminal plasma proteins by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two proteins (67 and 58.6 kDa) were correlated with sperm motility and other semen properties (de Souza *et al.,* 2007). In addition, studies involving seminal plasma of south Indian jersey and hybrid bulls showed that protein group 1 (205 and 97.4 kDa) and protein group 2 (22.0 and 14.3 kDa) were positively and negatively correlated with sperm concentration, respectively (Sundaram *et al.,* 2016). In this context rare data is available on semen quality and seminal plasma protein expression pattern of Thai native rooster at different ages. Therefore, the present study was performed to investigate the properties of semen and seminal plasma proteins in Thai native roosters, Leung hang khao.

Materials and methods

Animals and Semen collection

Eighteen Thai native roosters, Leung hangs khao, were divided into three groups at three different age, 7-8, 10-11, and 23-24 months of age. They were housed in individual cages in an open-sided house under natural photoperiod and climatic conditions. Each rooster was daily fed with 130 g of commercial feed, and water was provided *ad libitum*. Prior to the experiment, the roosters were accustomed to the environment. The semen was collected by using abdominal massage method.

Semen evaluations

Semen samples were collected in 1.5 mL microtubes and evaluated for total volume, color, pH value, concentration, and percentage of live and dead sperm. Semen color was evaluated based on a visual scoring scale of 1 - 5 (1 = watery semen, 2 = watery semen with white streaks, 3 = medium white semen, 4 = thick white semen, and 5 = very viscous chalky white semen sample) (McDaniel and Craig, 1959). The pH value was investigated by using a p-Hydrion test paper (pH value ranging from 0.0 to 13.0) and a color chart meter. The concentrated sperm was diluted 1:1000 in 4% Sodium chloride solution and was loaded into hematocytometer. The count was done at 400x magnification under bright-field microscopy. The percentage of live and dead sperm was determined by using eosin-nigrosin staining (Campbell *et al.*, 1953) Figure 1. A minimum of 200 live or dead sperms were counted on each slide (Shanmugam *et al.*, 2014).

Seminal plasma preparation

The seminal plasma from the native rooster in each group was harvested and centrifuged at 8,000 rpm for 10 min at 4 ℃. The supernatant was transferred into 2 mL microtubes and was centrifuged at 12,000 rpm for 20 min at 4 ℃. Total protein was determined by Bradford method (Bradford, 1976). The seminal plasma was stored at -80 ℃ until electrophoresis was performed.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

The seminal plasma proteins from the native roosters in each group were subjected to SDS-PAGE. The total proteins were denatured by using the sample

buffers (10% SDS; 0.5 M Tris, pH 6.8; 20% v/v glycerol; 0.2% w/v bromophenol blue). After boiling at 100 ℃ for 5 min, proteins (20 µg) from each sample were analyzed on a stacking gel containing 4.5% and 10% polyacrylamide gel. To stain proteins, the PAGE gels were placed in a coomassie brilliant blue R250 dye solution for a few hours and were de-stained until the protein molecules was separated and clearly visualized. Each sample was normalized by divided the density of the band by Image J Software (Version 1.6; NIH).

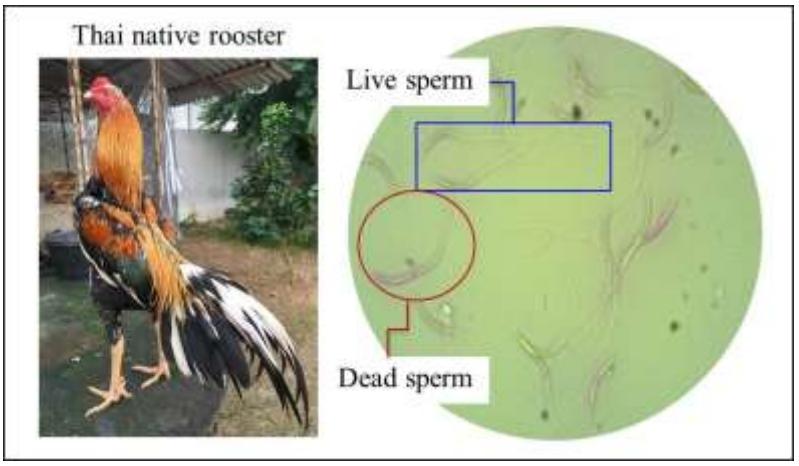

Figure 1. Using eosin-nigrosin staining to determine sperm viability. The live sperms were colorless and the dead were pink

Statistical analysis

Data were analyzed by using the Statistical Package for the Social Sciences (SPSS, version 16). One way analysis of variance (ANOVA) was used to compare semen parameters between the various groups of roosters. Duncan's multiple comparison test was performed to evaluate differences in the semen quality parameters among the different age groups. Results were reported as Mean ± SEM. P values less than 0.05 were considered statistically significant.

Results

Age affected sperm live percentage in Thai native roosters, Leung hang khao

Semen samples from three different ages, 7-8, 10-11, and 23-24 months of age. of Thai native roosters were collected and evaluated for semen quality

parameters. The semen quality parameters of roosters in the each group were shown in Table 1. The parameters of volume, color, and pH value and sperm concentration of the semen derived from younger or older roosters did not show significant differences. However, the age had significant effects on live and dead sperm ($P < 0.05$).

Table 1. Mean ± SE of semen volume, color, pH and sperm concentration of Thai native rooster, Leung hangs khao at different ages

Semen parameters	Age of rooster (months)		
	7-8	10-11	23-24
Volume (mL)	0.255 ± 0.09	0.208 ± 0.06	0.167 ± 0.04
Color	3.80 ± 0.18	3.80 ± 0.18	3.80 ± 0.18
PH value	7.00 ± 0.00	7.0 ± 0.00	7.5 ± 0.27
Concentration ($x10^9$/mL)	2.28 ± 0.47	2.17 ± 0.63	2.14 ± 0.63

Mean within a row with different superscripts differ significantly ($p < 0.05$)

The percentage of live sperm in the group of 7-8 months of age (93.34^a ± 2.17) was significantly higher than that in the group of 23-24 months of age (71.38^b ± 7.44) ($P < 0.05$). However, the results in the group of 10-11 months of age (88.95 ± 2.11) showed no significant differences in the groups of 7-8 months and 23-24 months of age (Fig 2.). The percentage of dead sperm in the group of 7-8 months of age (6.66^a ± 2.17) was significantly lower than 23-24 months of age (28.62^b ± 7.44).

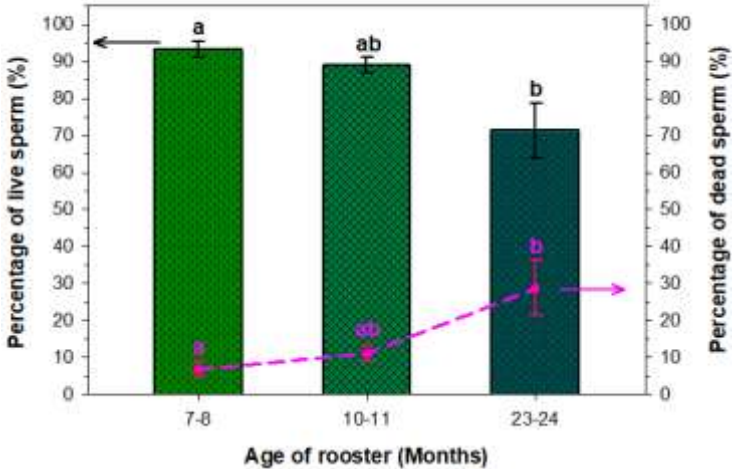

Figure 2. The effect of age on the percentage of live and dead sperm of Thai native rooster (Leung hangs khao)

Younger rooster expressed high level of high molecular weight proteins

The analysis of seminal plasma protein derived from native roosters was performed by SDS-PAGE. A total of 12 protein bands with the molecular weight ranging from 34 to 260 kDa were identified in Thai native rooster seminal plasma. According to the results, the expressions of proteins with molecular weights of 72, 90, and 140 kDa were more predominant than other proteins. The protein bands with molecular weight of 72 kDa was high at 7-8 months of age and slightly decreased at 10-11 months of age but non-significant different among the different age groups. The expression of 140 kDa proteins at 7-8 and 10-11 months of age were higher than that at 23-24 months of age. However, the expression of 90 kDa proteins at 7-8 months of age was significantly lower than that at 23-24 months of age (Fig. 3). Therefore, sperm live and dead was correlated to two bands (90 and 140 kDa) and the protein bands with molecular weight of 72 kDa were correlated with semen volume and sperm concentration.

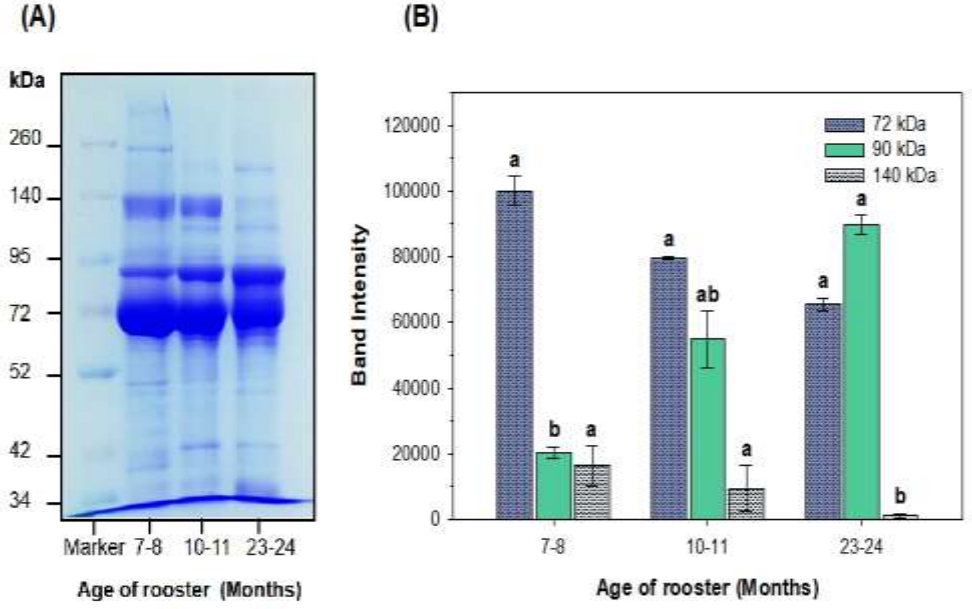

Figure 3. (A) Separation of seminal plasma proteins derived from Thai native roosters by using polyacrylamide electrophoresis gel (SDS-PAGE). Lane 1; protein marker, lane 2; the group at 7-8 months of age, lane 3; the group at 10-11 months of age, and lane 4; the group at 23-24 months of age.(B) Intensity of protein bands (72, 90, and 140 kDa) by using ImageJ software analysis

Discussion

The relation between age and semen quality in avian species has been extensively discussed (Shanmugam *et al.,* 2012). Generally, the low sperm viability and motility will occur between 31 and 52 weeks of age (Douard *et al.,* 2003), and age shows negative correlation with sperm concentration, motility, and viability (Tabatabaei *et al.,* 2010). In the present study, age did not significantly effect on volume, color, sperm concentration, and pH value of semen (Table 1). The previous study showed that sperm concentration of naked neck at 24 weeks of age (5.18 $\pm$ 0.55) was higher than that at 48 weeks of age (3.81 $\pm$ 0.48) but non significantly different between these ages (Shanmugam *et al.,* 2012). In the present study, the highest concentration of rooster sperm was found in 7-8 months of age (2.28 $\pm$ 0.47) but no significantly different with 10-11 months of age (2.17 $\pm$ 0.63) and 23-24 months of age (2.14 $\pm$ 0.63). Another study found that semen concentration in white leghorn cocks was higher at 2 years of age (2.92 $\pm$ 0.85[a]) than that at 1 year of age (2.85 $\pm$ 0.72[a]) although the results showed no significant differences (Elagib *et al.,* 2012). Moreover, we also found that the percentage of live sperm decreased as age increased. This was consistent with the results reported by Tabatabaei and his colleagues (2010). Older age of indigenous roosters also results in low sperm viability rates (that is, 90.64 $\pm$ 1.47, 82.30 $\pm$ 1.62, and 74.11 $\pm$ 1.35% for roosters at 26, 34, and 45 weeks of age, respectively) (Tabatabaei *et al.,* 2010).

Moreover, previous studies have reported that seminal plasma plays a role in the regulation of semen quality such as sperm morphology, motility, sperm concentration, acrosome reaction, and fertility (Mann, 1981). This is because it contains many proteins that originate from the testis, the rudimentary epididymis, and the ductus deferens which provide an optimal environment for cell survival while others serve in the defense against oxidative or microbiological damage and assist in sperm interactions with different microenvironments in the female genital tract (Atikuzzaman *et al.,* 2017; Marzoni *et al.,* 2013). Therefore, we assessed the relation between age and semen quality of roosters at different ages, and found that their seminal plasma protein pattern changed with age. The apparent molecular masses were 72, 90, and 140 kDa. Protein bands at 72 and 140 kDa were abundant in the group at 7-8 months of age and slightly decreased with age increased. However, protein bands at 90 kDa were low at 7-8 months of age and slightly increased with age increased (Fig. 3). Therefore, seminal plasma proteins with molecular weights of 90 and 140 kDa might be associated with live sperm or viability. It could be concluded that the semen quality of Thai native roosters (Leung hangs khao) is affected by age of the bird. The roosters in different age groups contain specific

seminal plasma proteins that can be used as valuable markers of semen quality. This might serve as a better alternative for other qualitative methods.

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Virulence and genetics at the molecular level of an indigenous strain of *Beauveria bassiana* affected by artificial and mass production media usage for sustainable insect control

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Abstract Molecular biology of indigenous strain of entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillenim (Ascomycota: Hypocreales) (strain Bbs01) based on DNA fingerprinting and enzymatic studies and based on respective RAPD-PCR and acrylamide gel electrophoresis were conducted. The results showed that neither artificial media (PDA, SDA, SDAY, MEA, NA and WA) nor mass production material (cooked rice, paddy, millet and dog feed) effect the fungal genetic stability and protease enzyme activity. Correlation analysis indicated that pathogenicity of Bbs01 to cabbage aphids, *Lipaphis erysimi* (Hemiptera: Aphididae) and flea beetle, *Phyllotreta sinuata* (Coleoptera: Chrysomelidae) were increased with protease activity. Accordingly, the most suitable respective artificial and culture material for *B. bassiana* Bbs01were SDAY and dog food.

Keywords: *Beauveria bassiana*, genetic stability, mass production media, sustainable insect control, virulence

Introduction

Beauveria bassiana (Balsamo) Vuillenim (Acomycota: Hypocreales), a major widespread entomopathogenic fungus has been applied as microbial insecticides for several insect pest species including Order Hemiptera Lepidoptera, Diptera, Hymenoptera and Coleoptera (Tanada and Kaya, 1993). An example is European corn borers (*Oatrinia nubilalis*) (Lewis *et al.*, 1996) in which some strains were utilized for testing microbial insecticides; e.g., Bb147 screening from *O. nubilalis*, commercialized under various trademarks; e.g., Bio-Power, Beauverin, Boverol and Boverosil as well as Naturalis-O and Naturalis-T and Back-off recommended for control of Asiatic corn borer, coffee borer, white

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grubs, bollworm, cutworm, brown planthopper (Copping, 2009). Nevertheless, the microbial insecticide market share is limited and not widespread due to higher cost and shorter self-life compared with chemical insecticide resulting, it's hard to be afforded by low-income farmers. Therefore, in developing countries, the major limitations have been expanded by the benefits of indigenous strains applied with local material cultures (Lopez-Llorca *et al.,* 1999; Posada-Flores, 2008; Sahayaraj and Namadivayam, 2008).

Recently, many efforts have been made with the aim for the effectiveness of native entomopathogenic *B. bassiana* isolate Bbs01 and enrichment culture techniques by using local mass production materials for insect pest control in Thailand as well (Saengyot and Napompeth, 2007). However, several factors; namely, pathogenicity and environmental tolerances, (e.g., light, temperature and moisture) according to the genotypes (Butt *et al.,* 2001) have affected the performance of *B. bassiana* at field condition. Additionally, some reports indicated that an artificial cultural condition influence virulence of *B. bassiana* and may affect its genetic variation (Rodríguez-Gómez *et al.,* 2009). Thence, the types of cultural materials would not only affect the amount of the enzyme production and the molecular genetic, but would also be concerned the crucial factors in the economic investment for the production of a further commercial formula.

The research aimed to point out the suitability of artificial media and culture materials for mass production of Bbs01 involving evaluation on the effects of the different media on pathogenicity and some molecular characteristics including genetic stability and enzymatic activity for resulting in sustainable local resources to maximize the benefits and worthy investment. Hence, this article would lead to crucial conclusion of a suitable artificial medium and materials of mass production of a quantitative and phenotypic molecular quality, which would directly be involved in the effectiveness of the *B. bassiana* isolate Bbs01.

Materials and methods

Pathogenicity of Beauveria isolate 01 (Bbs01) to the target insect pests effected by different artificial media and cultural materials

Cabbage aphids, *Lipaphis erysimi* (Hemiptera: Aphididae), flea beetle, *Phyllotreta sinuata* (Coleoptera: Chrysomelidae) and cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) were collected from the field and mass cultured according to the appropriate methods of each pest species (information not shown). The spore suspension of the Bbs01 was obtained from stock culture

culturing on different artificial media comprising of potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), Sabouraud dextrose agar supplemented with yeast extract (SDAY), malt extract agar (MEA), nutrient agar (NA) and water agar (WA), as well as four types of sterilized mass production media including, cooked rice, paddy, millet and dog feed. Then, the fungus was subjected to pathogenicity test on those three insect pests according to Lacey (1997) and Tanada and Kaya (1993). The experiments were divided into two series: study of the spore suspension obtained from respective six artificial media and the four type of mass production materials as described above compared to an untreated control (0.02% of distilled water mixed with Tween 80). Death and healthy population data were collected and corrected Percent Cumulative Mortality (PCM) were calculated using Abbott's formula (Abbott, 1925) as follows:

$$\text{Corrected (\%)} = 1 - \frac{\text{n in T after treatment}}{\text{n in Co after treatment}} \times 100$$

Where: n = Insect population, T = treated, Co = control

The experimental design was a completely randomized design (CRD) with five replications. Corrected mortality of the target insects of both studies were subject to statistical analysis using Duncan's Multiple Range Test (DMRT).

Genetic study of B. bassiana isolate Bbs01 grow on different artificial media and local cultural materials

The genetic pattern of the *B. bassiana* isolate Bbs01 was obtained by deoxyribonucleic acid (DNA) fingerprinting with a random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). Initially, the Bbs01 was grown in a broth media including PDB, SDB, SDBY, MEB, NB and WB in accordance with Alves *et al.* (2002). Growing materials cooked rice, paddy, millet and dog feed, was prepared by grinding in distilled water at the ratio of 1:1. Then, 100 milliliters of each solution was poured separately into 125 ml flasks and sterilized. Spores from 30-day-old cultures on respective media were collected for the genomic DNA extraction following the modified procedure of Maurer *et al.* (1997). Next, fungal proteins were removed by a lysis buffer containing proteinase K at 65°C for one hour. Then, the genomic DNA was extracted using chloroform: isoamyl alcohol or isopentyl alcohol with a ratio of 24:1 and three replications, centrifuged at 12,000 rpm for 15 minutes, and washed with 70% of ethanol, and the water was removed with a vacuum dryer. The fungal DNA was dissolved in 300-500 µl of a Tris EDTA buffer, and the content of the RAPD markers were increased by PCR with primers; namely, OPA-3 and OPB-10 (Operon Technologies, Alabama, USA). The investigation of the genetic pattern of the

fungus was done in accordance with Castrillo *et al.* (2003) with a PCR. After the initial denaturation at 35 cycles of 95 ℃ for four minutes, denaturation at 94°C for 40 seconds followed by annealing at 37°C for 40 seconds, and an extension at 72°C for 40 seconds with final extensions at 72°C for four minutes were conducted, respectively. The PCR products was checked through with 1.5% of agarose gel electrophoresis and stained gel with ethidium bromide (EtBr). The DNA bands were detected under ultraviolet radiation and compared with the DNA marker bands of 500 and 1,000 bp for estimating the number of DNA molecules.

Enzymatic study of B. bassiana isolate Bbs01 grow on different artificial media and local cultural materials

Protease production of the Bbs01 was studied according to method by Kucera (1971). Bbs01 grown on respective liquid media was incubated at a 150 rpm shaker at 35°C for three days before percolation and centrifugation (10,000 rpm for 15 minutes). The protease molecule was determined by 12.5% of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) that contained 0.3% of the gel without induction at room temperature for an hour in 2.5% of Triton X-100 solution for washing SDS and enzyme denaturation. The gel was incubated in 0.01 M of Tris-HCl buffer pH 8 and 10 mmol of CaCl2 pH 8.0 for five-eight hours. Then, the gel was stained with 0.1% of Coomassie brilliant blue R-250 (CBB G-250) for three hours, and the reaction was stopped by the addition of methanol and acetic acid for one hour. Finally, the protein molecular weight was estimated by comparing the gel bands with a standard marker.

To determine protease activity, a virulent factor of the *B. bassiana* isolate Bbs01. The fungus was cultured on respective artificial media and local cultural materials. Briefly, adjusted $1x10^6$ spores/ml spore suspension (dissolved in distilled water) of Bbs01 obtained from 15 days old was prepared. Then one milliliter of spore suspension was added to 250 ml Erlenmeyer flask each contained 100 ml of respective artificial medium, PDB, SDB, SDBY, MEB, NB, and WB and the flask were on a shaker at 28 ±1°C at 180 rpm for 24 hours. The pH of each artificial medium was adjusted to 8.0 and incubated at 28°C with 180 rpm for 56 hours. The hypha was corrected with filtering cloth, washed with sterilized water two times and separated by centrifugation at 4,000 rpm for five minutes. The supernatant obtained from each flask was evaluated enzymatic activity according the method of Kunitz (1947). The substrate of the protease mechanisms was prepared by mixing 2 g of casein (Sigma) in 0.01 M of Tris-HCl pH 8.0 and 10 mmol of CaCl2 pH 8.0 was adjusted to 100 ml by

the distilled water. Then, 400 µl of the substrate solution was added to the supernatant that dissolved in 200 µl of the mixture of 0.01 mol of Tris-HCl pH 8.0 and 10 mmol of 10 mM $CaCl_2$, and incubated at 35°C for 10 minutes. Then, 1.2 mol of trichloroacetic acid (TCA) was added, centrifuged at 4,000 rpm for 5 minutes, which measured the absorbance of the fungal samples by a spectrophotometer at 280 nm. The protease activity was calculated by the rate of producing tyrosine per minute. Enzymatic activity by mean of unit per minus were recorded from 5 replications in CRD before statistically analyzed using Duncan's Multiple Range Test (DMRT) for means comparing.

Protease activity assay in different local cultural materials, cooked rice, paddy, millet and dog feed

The paddy and millet were prepared by washing with water and soaked in distilled water for 24 hours. Then, the materials were 50% steamed cooked for 15 minutes and dried on a bamboo tray for 30 minutes. The adult dog food brand of 'Plemo' with a liver flavour was soaked in water for 1.5 minutes and dried. Then, each material was ground with a grinder adding some water while grinding before 100 g of each was tranfered into 250 ml flasks. The fungal spores were scratched into the distilled water and adjusted to $1x10^6$ spores/ml. The suspensions were incubated on a shaker at 150 rpm at 35℃ for 72 hours, and the fungal hyphae were filtered through a filter cloth. Then, collected hyphae were rinsed with distilled water two times and centrifuged at 10,000 rpm for 15 minutes. This was followed by the determination of the Bbs01 protease activity by acrylamide gel electrophoresis. Enzymatic activity by mean of unit per minus were recorded from 5 replications in CRD before statistically analyzed using Duncan's Multiple Range Test (DMRT) for means comparing.

Correlation analysis of pathogenicity and protease activity for B. bassiana isolate Bb01 associated with different artificial media and local cultural materials

The study was carried out through the series of 1) the relationship of different artificial mediums and 2) growing materials on protease reaction. The correlation of the protease activity and pathogenicity of the Bb01 culture on PDA, SDA, SDAY, MEA, NA and WA as well as of the difference type of mass production materials: cooked rice, paddy, millet and dog feed were analyzed for the restriction on *Lipaphis erysimi* (Kal.), *Phyllotreta sinuata*, and *Spodoptera litura* using linear regression according to Le Clerg *et al.* (1966) Snedecor and Cochran (1967) Wadley (1967).

Results

Effect of artificial media and local cultural materials on DNA fingerprinting of the B. bassiana isolate Bbs01

The DNA fingerprints of the *B. bassiana* isolate Bbs01 used RAPD-PCR, OPA-3, OPB-10 for growing in PDA, SDA, PDA, MEA, and NA in which there were differences in the transmitted pattern. In this case, five DNA bands of 500-1,000 bp were shown (Figure 1) while the molecular genetics of the Bbs01 growing in different materials (cooked rice, paddy, millet and dog food) showed the same genetic pattern (Figure 2).

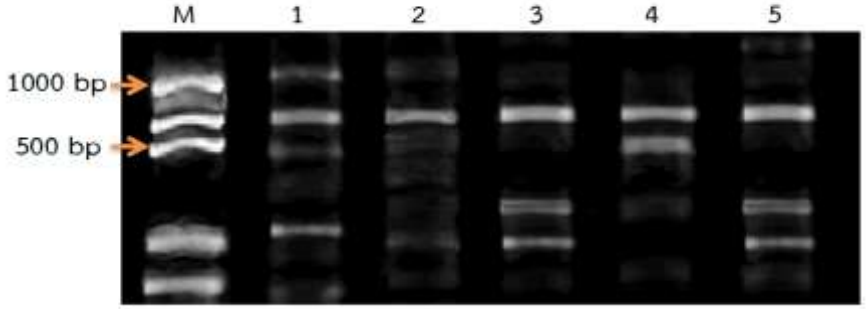


Figure 1. RAPD-PCR product using primer OPA-3 of fungi *B. bassiana* (M) and the gene of the *B. bassiana* Group 1 that was the same gene from fungi growing in PDA (1), SDA (2), PDA (3), MEA (4), and NA (5), respectively

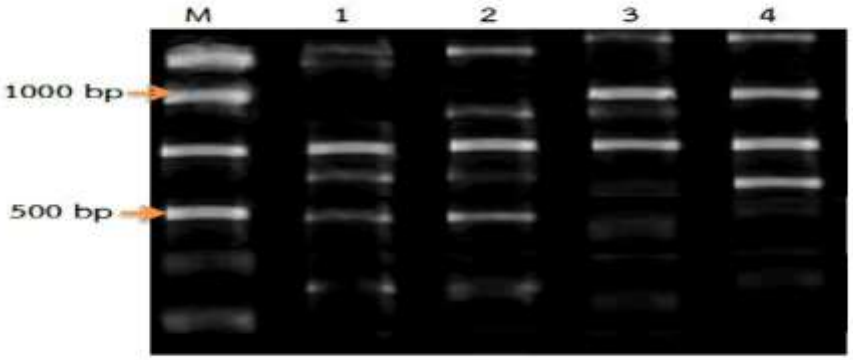


Figure 2. RAPD-PCR product using primer OPA-3 of fungi *B. bassiana* (M) and the gene of the *B. bassiana* group 1 that had the same genetic pattern growing in cooked rice (1), paddy (2), millet (3), and dog feed (4), respectively.
***** M meant a marker with a molecular size of 500-1,000 bp

Confirmation of the protease enzyme in culture media

The protease activity was indicated as the virulence of the Bbs01. After growing the Bbs01 under different artificial media; namely, PDA, SDA, SDAY, MEA and NA, the protease content was detected using 12.5% of SDS-PAGE. The Bbs01 recorded the same band of the protease molecular weight of 66 kDa fragment determined by SD-PAGE as shown in Figure 3 and 4.

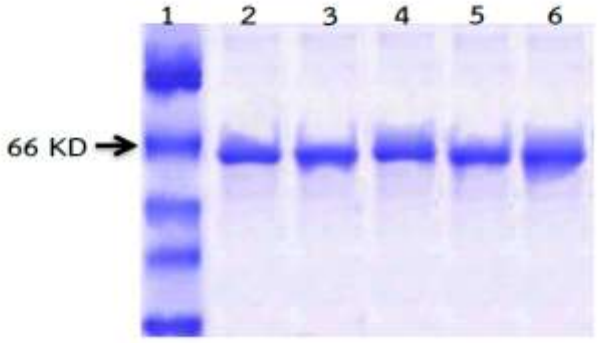


Figure 3. Protease production of the *B. bassiana* isolate Bbs01 using 12.5% of SDS-PAGE and stained with Coomassie brilliant blue R-250 for three hours. The 1st band was a protein marker while the other bands were protein extracted from various fungal inoculated artificial media including PDA, SDA, SDAY, MEA, and NA, respectively

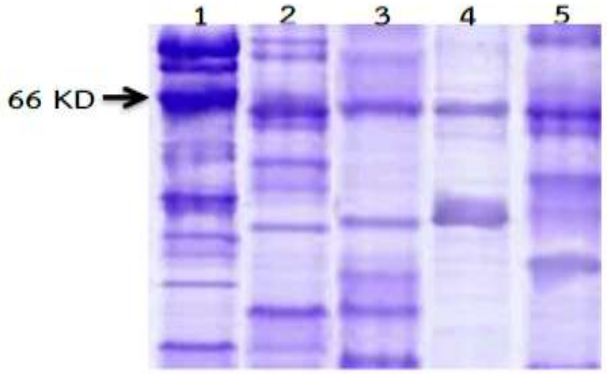


Figure 4. Protease production of the *B. bassiana* isolate Bbs01 using 12.5% of SDS-PAGE and stained with Coomassie brilliant blue R-250 for three hours. The. 1st band was a protein marker while the other bands were protein extracted from various fungal inoculated material including cooked rice, paddy, millet, and dog feed, respectively

Protease activity of the Bbs01 grown on difference artificial media and local cultural materials

The proteolytic enzyme produced by Bbs01 was detected by using a casein substrate. In laboratory scale, both the artificial media and growing materials had a high significance on the protease content (p=0.01). This enzyme remained within the range of 0.85±0.16 - 1.99±0.09 U/ml. The most production of protease was found in SDAY similar to those of MEA and SDA whereas PDA had the lowest protease activity (Table 1). For the growing materials, the highest protease reaction was found in the dog food (1.86+0.08 U/ml) similar to millet (1.79±1.16 U/ml). However, the activity of the protease ranged from 1.17 ± 0.10 U/ml (growing in cooked rice) to 1.86±0.08 U/ml (growing in dog feed) (Table 2).

Table 1. Protease activity produced from the B. bassiana isolate Bbs01 growing in different artificial media

Media	**Protease Activity (unit/ml ± SD)**
Potato Dextrose Agar (PDA)	0.85 ± 0.16c
Sabouraud Dextrose Agar (SDA)	1.43 ± 0.08a
SDA with Yeast Extract (SDAY)	1.99 ± 0.09b
Malt Extract Agar (MEA)	1.91 ± 0.06a
Nutrient Agar (NA)	1.32 ± 0.08b
Water Agar (WA)	inability to cultivate the sufficient amounts for testing

***** The mean values in each column followed by the same letter are not significantly different by DMRT at $p \leq .01$

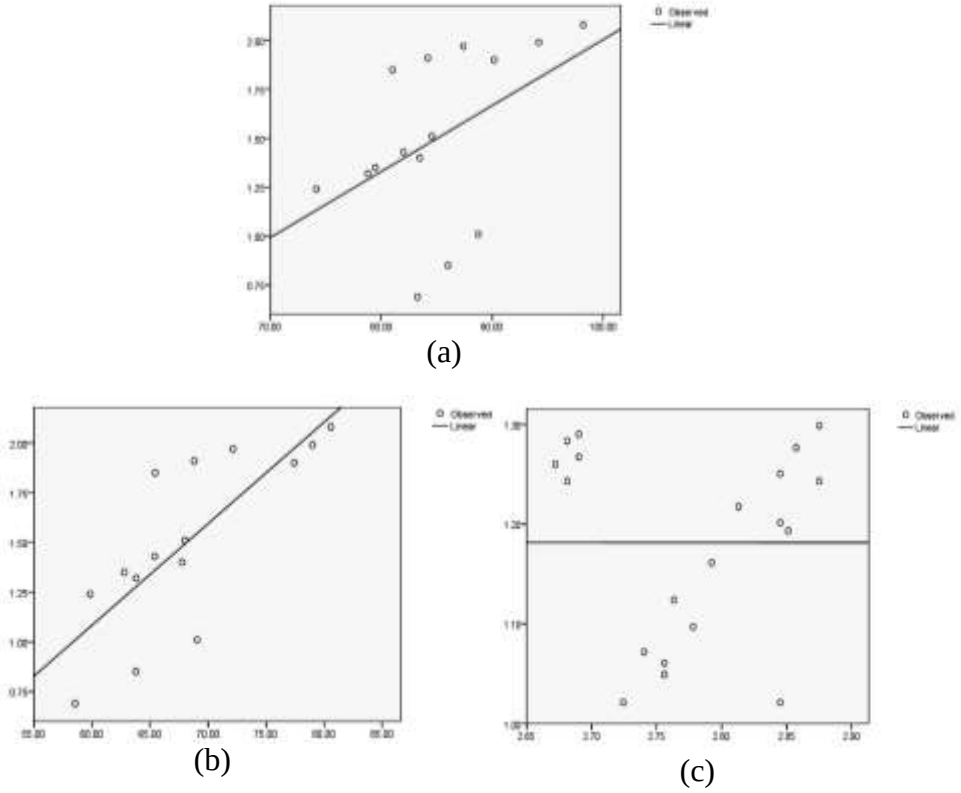
Table 2. Protease activity produced from the B. bassiana isolate Bbs01 growing in growing in different cultural material

Growing Media	Protease Activity (unit/ml ± SD)
cooked rice	1.40 ± 0.25b
paddy	1.17 ± 0.10c
millet	1.79 ± 0.16a
dog feed	1.86 ± 0.08a

***** The mean values in each column followed by the same letter are not significantly different by DMRT at $p \leq .01$

Correlation of pathogenicity and protease activity of the Bb01 grown on difference artificial media and local cultural materials

This research established the greater association of the increasing protease activity and of the virulence of the Bb01 to *L. erysimi* and *P. sinuata*, respectively. The regression coefficients for *L. erysimi* and *P. sinuata* were 0.420 and 0.438, while the lowest regression was found on *S. litura* (0.01) (Figure 5). The correlation analysis indicated that local cultural materials, cooked rice, paddy, millet, and dog food was positively associated with both protease content or pathogenicity of the Bb01 Infested *L. erysimi*, and *P. sinuata* with respective coefficients of 0.447 and 0.772. However, *S. litura* differed from the other two target insects (0.027) (Figure 6).



(a)

(b)

(c)

Figure 5. Linear regression showed the relationship between the protease production by the *B. bassiana* isolate Bbs01 growing in different artificial culture media and the Percent Cumulative Mortality (PCM) of (a) aphid (*Lipaphis erysimi*), (b) flea beetle (*Phyllotreta sinuata*), and (c) cutworm(*Spodoptera litura*)

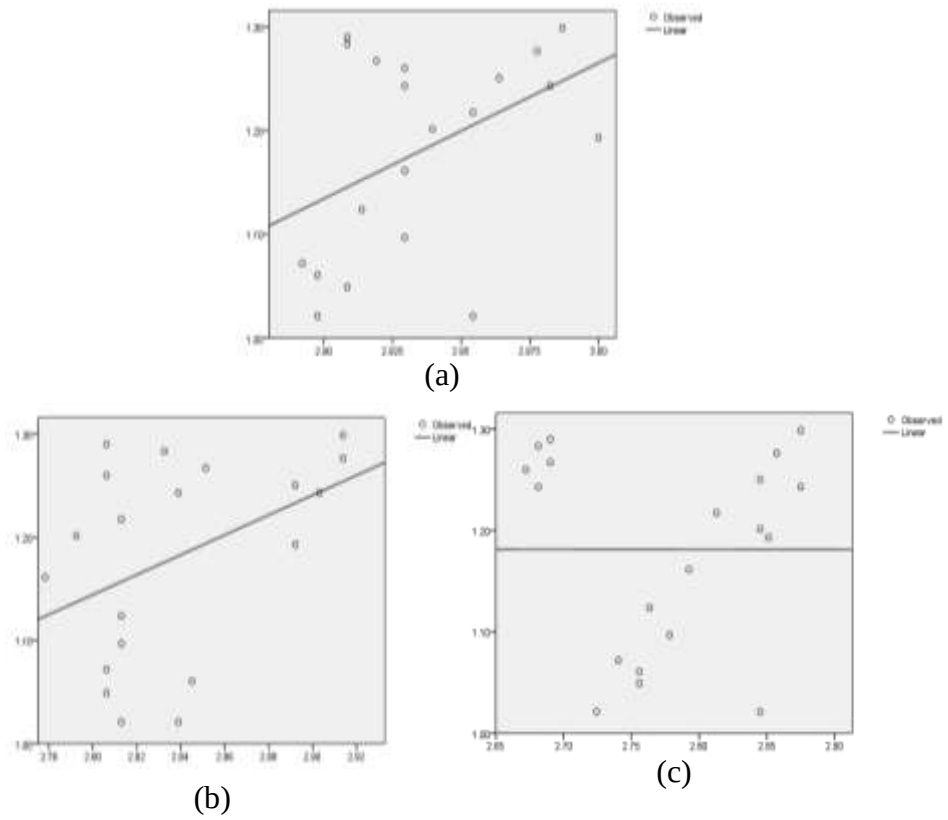


Figure 6. Linear regression showed the relationship between the protease production by the *B. bassiana* isolate Bbs01 growing in different cultural material and the Percent Cumulative Mortality (PCM) of (a) aphid (*Lipaphis erysimi*) (b) flea beetle (*Phyllotreta sinuata*) (c) cutworm (*Spodoptera litura*)

Discussion

Although some reports showed that fungal pathogenicity could be controlled by molecular genetics (Zhang *et al.*, 2009), for this study, the researchers investigated the DNA fingerprinting with RAPD-PCR of the Bbs01 that did not have any significant effect from the difference artificial media and local cultural materials. There was evidence that showed various protease activities from *B. bassiana* were caused by many factors; for instance, strain, geographic origin, and culture medium (Kucera, 1971). Therefore, this research focused on some other substantial factors, which were found to have been caused by the medium and growing materials as well. Accordingly, the artificial medium and growing material may involve the protease activity of the

B. bassiana isolate Bbs01, and fungal pathogenesis. Protease was an enzyme affecting virulence (Inglis *et al.*, 2001). From an experiment conducted in 2012, it was found that the Bbs01 could produce a proteolytic enzyme in all artificial media (PDA, SDA, SDAY, NA, and MEA) and growing materials (cooked rice, paddy, un-millet rice, and dog food) by using 12% of SDS-PAGE and a 66-KD protein marker (Figures 1 and 2). Additionally, the present observation confirmed that the protease activities had a significant difference depending on the artificial medium and growing materials. In this study, the protease activities of SDAY (1.99 ± 0.99 U) were similar to that of MEA (1.91 ± 0.06 U) while that grown in dog food (1.86 ± 0.08 U) did not differ from that of millet (1.79 ± 0.16. U). However, some studies confirmed that the culture medium type was the crucial cause of the virulence of *B. bassiana* (Fan *et al.*, 2007). Thus, there is a positive relationship of the protease activity with the pathogenesis of the *B. bassiana* isolate Bbs01. The results showed the same trend that was the artificial culture media and growing materials were positively related to the virulence to aphid, flea beetle, and cutworm. This meant that the *B. bassiana* isolate Bbs01 could be virulent when protease was being produced.

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The relationship of hematological values with Newcastle disease antibody in Thai indigenous chicken: strain Leung Hang Khao

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Abstract The relationship between hematological values and Newcastle disease immunity of Thai indigenous chicken was studied in strain Leung Hang Khao which attractive to consumers in Thailand. The blood and serum samples were collected from 153 chickens of this strain (mixed sex: male = 70, female = 83) at 4 and 7 months of age raising in free-range condition. Hematological value of the strain Leung Hang Khao at 4 month of age showed the mean corpuscular volume (MCV) in male (126.80±0.51 fl.) was statistically significant (p<0.05) higher than in female (123.30±0.46 fl.). While, the total red blood cell (TRBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell count (TWBC), heterophil, lymphocyte, monocyte and H:L ratio were no statistically significant (p>0.05) between sexes at 7 month of age. The average titers were no statistically significant (p>0.05) between sex at 4 month (male: 806.40±97.70 and female: 756.70±87.65) and 7 month (male: 5,372±799.08 and female: 6,561±709.28) of age, respectively. In addition, the hematological values of this strain at 4 month of age was relative to Newcastle disease immunity. The titer was statistically significant (p<0.05) correlation with the hematological value. The titers with the low, medium and high levels of immunity (63.15±23.47, 421.77±28.04 and 1525±131.92, respectively) were negative relationship with lymphocyte (71.00±2.46, 69.79±0.81 and 67.72±1.29 percentages of white blood cell (WBC), respectively) but statistically significant positive (p<0.05) relationship with heterophil (25.17±2.34, 27.76±0.86 and 29.41±1.05 percentages of WBC, respectively). The relationship between the amounts of lymphocyte and heterophil, and Newcastle disease immunity were indicated with the coefficients of phenotypic correlation of -0.17 and 0.18, respectively. From these results showed that, the relationship between hematological values and Newcastle disease immunity could be used as a primary health assessment Newcastle disease index for Thai indigenous chicken strain Leung Hang Khao.

Keywords: Thai indigenous chicken, immunology, hematological values, Newcastle disease

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Introduction

Thai indigenous chicken is very popular to feed in Thailand due to low cost of egg production and housing enables low-income farmers to provide a source of meat protein with low investment. These chicken are rared not for desmetic consumption but also for expert in Asean (Singapore, Brunei, Malaysia, Phillippines, Vietnam, Laoss, Cambodia and Myanmar) (Jaturasitha *et al.,* 2016). In general, the chickens are reared by free ranging under backyard system or on the farm as live birds and slaughtered a few at a time as needed. The Thai indigenous chicken (*Gallus domesticus*) originates from the Red jungle fowl (*Gallus gallus*) of Southern Asia (Parkhurst and Mountney, 1988; Wight and Siller, 1997). One of the most popular Thai indigenous chicken is Leung Hang Khao because it is a good fighting cock strain. This strain has been selected as a pure breed line by Department of Livestock Development. The special properties of this chicken are easy to parenting, big shape, good growth and high disease resistance (Duangjinda, 2015). Therefore, it is suitable for feeding in a tropical weather condition like Thailand. In addition, its meat has unique taste and texture with low cholesterol, free of antibiotic and hormones chicken production. This leads to become very popular among Thai consumer and a higher price than commercial broilers. However, one of the important problems in the indigenous chicken is outbreak of virus infectious disease due to the lack of control and poor disease prevention (Duangjinda *et al.,* 2009).

The seriously disease in Thai indigenous chicken is Newcastle disease, which is found worldwide and including Thailand. Newcastle disease virus (NDV) contains of one serotype and is also known as avian paramyxovirus serotype 1 (PMV-1). It is the most common RNA virus causes of respiratory problems in chicken. This disease is found to be compatible with the chicken of any age, but in the chicks will have the illness and high mortality rate may be up to 100 % (Horning *et al.,* 2003; Alders and Peter, 2001). Breeding of the chicken with a good immune level and high disease resistance is the alternative way to solve this problem in farming system. The vaccination of NDV vaccines in the chicken is one of the best way to protect Thai indigenous chicken from Newcastle disease. The NDV vaccines stimulate an immune response that minimizes or completely prevents the occurrence mortality of the disease (Miller *et al.,* 2009). This method is not only helpful in diagnosis of specific poultry diseases but also provide basic knowledge for study the immunology in chicken. The study on hematology of Thai indigenous chicken could be help for protection and reduction of this disease. The hematological data is one of the significant basic data that indicates animal health called “Health Indices” and

some blood biochemical and electrolyte contents are also indicate the deviation from normal organ function.

Since, there is a little information about the basic biological data of Thai indigenous chicken, the aims of this study was to examine the average standards of hematological values and the relationship between hematology and Newcastle disease immune system in Leung Hang Khao strain.

Materials and methods

Chicken, vaccine and nutrition

One hundred fifty-three Thai indigenous chickens (*Gallus domesticus*) with 1 day to 7 month of age were provided from Kabinburi Animal Husbandry and Research Center Thailand. These Thai indigenous chickens were housed using semi intensive rearing system in the farm of Suranaree University of Technology, Nakhon Ratchasima, Thailand. For the first 3 weeks they were fed by starter diet containing 21% crude protein after that the diet was contained 17% crude protein. The chickens were vaccinated with the live vaccine against Newcastle disease (ND: Lasota strain) and Infectious bronchitis (IB: H 120 strain) at 1, 2, 8 and 12 weeks. After that they were vaccinated every 3 months according to the recommendation from the local veterinarians.

Sample collection

The serum samples were collected for examination the presence of antibodies against Newcastle disease virus. Firstly, the 3 mL of blood samples were collected from the jugular vein using sterile disposable needles and syringes into the microtubes from the chickens at 4 to 7 months. After a few minutes the blood samples were formed a clot. Next, the serum was separated from the clot by centrifugation at a speed of 3,000 rpm for 15 minutes at 25◦C. Finally, the supernatant (serum) was transferred into the new microtube and stored at -20 ◦C to determine the Newcastle disease antibodies by ELISA kit for assessing antibody titer against NDV. (Synbiotics Corporation, San Diego, Canada).

Hemotological technique

The differential WBC counts were analyzed by spreading blood on monolayer blood films, fixed and stained with Giemsa-Wright's stain. For total red blood cell (TRBC) and total white blood cell counts (TWBC) were

determined using haemacytometer (Campbell, 1995; Pierson, 2000). The packed cell volume (PCV) was measured using microhematocrit capillary tubes and centrifugated at a speed of 2,500 rpm for 5 minutes. In addition, the hemoglobin concentration (Hb), erythrocyte indices of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were examined using HYCEL® automatic blood counting machine (Pierson, 2000).

Statistical analysis

All hematological data were expressed as mean ± standard error. The comparison of hematological data both sex and age was statistically analyzed by student's *t*-test using SPSS Statistics software (Version 20) (SPSS, Inc., Chicago, III, USA). Pearson's correlation coefficients (r) was analysed to determine the relationship between titer antibody and hematological parameters of the chicken at the age of 4 month. The Differences among the mean values were tested by Duncan's multiple range test.

Results

Titer and Hematological parameters

The average hematological values in Thai indigenous chickens strain Leung Hang Khao age of 4 month by sex were shown in Table 1. The results indicated the MCV in male (126.80±0.51 fl.) was statistically significant ($p<0.05$) higher than female (123.30±0.46 fl.). While, TRBC, HGB, HCT, MCH, MCHC, TWBC, heterophil, lymphocyte, monocyte and H: L ratio were no statistically significant ($p>0.05$) between sexes. The average titers of this strain were no statistically significant (male: 806.40±97.70 and female: 756.70±87.65).

At 7 month of age, the TRBC, HGB, HCT, MCV, MCH, MCHC, TWBC, heterophil, lymphocyte, monocyte and average titers (male: 5,372±799.08 and female: 6,561±709.28) were no statistically significant ($p>0.05$) between sexes (Table 2).

The relationship of Hematology with Newcastle disease immunity in Thai indigenous chickens strain Leung Hang Khao

The results showed that the titer was statistically significant ($p<0.05$) relative with hematological values. At 4 month of age the titer of this strain

Table 1. The hematological values and titers of Thai indigenous chicken strain Leung Hang Khao at 4 month of age and other strains

Parameters	Sex		Total (n=153)	Range	Simaraks *et al.* (2004)
	Male (n=70)	Female (n=83)			
Total RBC ($\times 10^6$ µl)	4.20±0.84	4.09±0.76	4.15±0.80	1.5-5	2-3
Hemoglobin (g/dl)	10.98±0.21	11.34±0.19	11.16±0.20	7.6-14	8-10
Hematocrit (%)	30.46±0.61	31.83±0.55	31.15±0.58	21-40	28-37
MCV (fl)	126.80±0.51**	123.30±0.46	125.05±0.49	116-147	126-163
MCH (pg)	45.78±0.42	44.21±0.37	45.00±0.40	39-60	35-45
MCHC (g/dl)	35.70±0.42	35.83±0.38	35.77±0.40	25-36	24-31
Total WBC ($\times 10^4$ µl)	13.07±0.60	14.19±0.53	13.63±0.57	6-25	1.6-2.5
Lymphocyte (%)	69.32±0.70	68.82±0.64	69.07±0.67	38-83	54-73
Heterophil (%)	28.17±0.67	28.31±0.61	28.24±0.64	14-44	16-31
Monocyte (%)	2.47±0.17	2.54±0.16	2.51±0.17	1-6	1-7
H: L ratio	0.42±0.01	0.24±0.01	0.42±0.01	0.17-0.82	0.23-0.57
Titer	806.40±97.70	756.70±87.65	781.55±88.19	62.1-1,694	-

Table 2. Comparison of hematological values between Thai indigenous chicken strain Leung Hang Khao at 7 month of age and other species

Parameters	Thai indigenous chickens strain Leung Hang Khao		Thai fighting strain[/2]		Vietnam fighting strain[/2]		Thai indigenous chickens in northeastern Thailand[/3]			Jain (1993)
	Male (n=56)	Female (n=67)	Male (n=15)	Female (n=15)	Male (n=15)	Female (n=15)	Male (n=20)	Female (n=20)	Range	Range
Total RBC ($\times 10^6$ µl)	2.84±0.10	2.72±0.09	2.99±0.16	3.16±0.12	2.79±0.14*	2.04±0.10*	2.32±0.31	2.19±0.26	2-3	2.5-3.5
Hemoglobin (g/dl)	13.24±0.53	12.73±0.47	12.90±0.40*	11.40±0.20*	12.20±0.30*	8.90±0.40*	9.27±1.42*	8.52±0.85	8-10	7.0-13.0
Hematocrit (%)	36.38±1.29	34.07±1.14	40.10±1.10*	35.70±0.70*	38.90±1.00*	29.70±0.90*	33.55±4.72*	30.80±3.96	28-37	22.0-35.0
MCV (fl)	126.40±18.08	144.10±16.04	137.60±6.40*	115.00±4.50*	144.30±8.40	152.50±12.10	147.87±18.43	141.39±19.17	126-163	90.0-140.0
MCH (pg)	45.70±1.16	44.61±1.03	44.30±2.30*	36.70±1.51*	45.30±2.80	46.10±4.20	40.26±5.16	39.11±4.42	35-45	33.0-47.0
MCHC (g/dl)	36.25±0.81	35.83±0.38	32.20±0.70*	31.90±0.60*	31.50±0.90	30.00±0.70	27.93±4.56	27.79±1.77	24-31	26.0-35.0
Total WBC ($\times 10^4$ µl)	18.08±1.46	16.23±1.29	11.81±1.04*	21.04±1.69*	18.42±1.86*	17.32±1.37*	2.05±0.39	2.05±0.53	1.6-2.5	1.2-3.0
Lymphocyte (%)	-	-	49.10±3.40	49.30±2.50	57.30±0.50	57.30±3.20	60.30±5.33	67.05±1.49*	54-73	45.0-70.0
Heterophil (%)	-	-	43.30±3.60	38.80±3.30	33.00±3.00	37.30±3.30	25.40±5.12	22.00±8.78	16-31	15.0-40.0
Monocyte (%)	-	-	1.80±0.20*	2.50±0.20*	6.30±1.30*	3.30±0.60*	4.35±3.15	4.05±3.39	1-7	5.0 - 10.0
H:L ratio	-	-	-	-	-	-	0.43±0.12	0.36±0.21	0.23-0.57	-
Titer	5,372±799.08	6,561±709.28	-	-	-	-	-	-	-	-

[1]/* Mean ± SE with superscript, within row between sex in each parameter differ significantly ($p< 0.05$); [2]/ Reference values of Salakij (2004); [3]/ Reference values of Simaraks *et al.* (2004)

could be divided into 3 levels including low (63.15±23.47), medium (421.77±28.04) and high (1,525±131.92), respectively. The lymphocyte was negative statistically significant (p<0.05) relationship with Newcastle disease immunity (71.00±2.46 69.79±0.81 and 67.72±1.00 percentages of WBC, respectively), but positive statistically significant (p<0.05) relationship was found in heterophil (25.17±2.34, 27.76±0.86 and 29.41±1.05 percentages of WBC, respectively) (Table 3). The relationship between the amounts of lymphocyte and heterophil, and Newcastle disease immunity were indicated with the correlation coefficient of -0.17 and 0.18, respectively (Table 4).

Table 3. The relationship of immunity group to titer, lymphocyte and heterophil of Thai indigenous chicken strain Leung Hang Khao at 4 month of age

Group of Titer (N=153)	Titer		Lymphocyte		Heterophil	
	Male	Female	Male	Female	Male	Female
Low (N=8) 0-100	64.19±20.48	62.10±26.45	73.00±2.16	69.00±2.79	25.00±2.04	25.33±2.63
	Average = 63.15±23.47		Average = 71.00±2.46		Average = 25.17±2.34	
Medium (N=91) 101-800	438.18±28.50	405.35±27.57	69.93±0.82	69.64±0.79	27.46±0.87	28.06±0.84
	Average = 421.77±28.04		Average = 69.79±0.81		Average = 27.76±0.86	
High (N=54) >800	1694±146.76	1356±117.08	67.76±1.43	67.67±1.14	29.91±1.16	28.91±0.93
	Average = 1525±131.92		Average = 67.72±1.29		Average = 29.41±1.05	

Discussion

Thai indigenous chicken is in the group of *Gallus domesticus* which containing of various chicken species and each species have a different characteristics. The 8,600 species of poultry group have a different anatomy and physiological characteristics and hematological value. Therefore, these characteristics are widely studied at the present in order to animal health check and farming development for using as food. However, it is still a lack of information about the effects of season, age, gender and reproduction which are initially studied and these data will support for further development of this knowledge (Bounous and Stedman, 2000). The biological information of indigenous chicken is studied to farming development for using as food. Recently, Thai indigenous chickens are increased a role as live stock which are developed for Thai farmer. The results of this study are very useful for feeding Thai indigenous chicken both at the present and in the future.

Table 4. Coefficients of phenotypic correlation between titer and haematological parameters of Thai indigenous chickens at 4 month of age

	Titer	Total RBC ($\times 10^6$ µl)	Total WBC ($\times 10^4$ µl)	Hemoglobin (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Lym (%)	Het (%)	Mono (%)	H:L
Titer	1.00											
Total RBC	0.04	1.00										
Total WBC	0.12	0.02	1.00									
Hemoglobin	0.09	0.08	0.20*	1.00								
Hematocrit	0.05	0.03	0.18*	0.91**	1.00							
MCV	0.09	-0.01	-0.08	-0.44**	-0.41**	1.00						
MCH	0.13	0.01	-0.01	-0.16*	-0.50**	0.47**	1.00					
MCHC	0.09	0.11	0.03	-0.03	-0.25**	-0.03	0.47**	1.00				
Lym	-0.17*	-0.01	0.01	-0.21**	-0.19*	0.11	0.03	-0.01	1.00			
Het	0.18*	0.04	0.06	0.18*	0.14	-0.11	0.02	0.01	-0.86**	1.00		
Mono	0.00	-0.13	-0.09	0.05	0.04	0.08	0.07	-0.19*	0.10	-0.37**	1.00	
H:L	0.19*	0.04	0.03	0.20*	0.20*	-0.12	0.01	0.08	-0.95**	0.97**	-0.28**	1.00

[1]/** Mean ± SE with superscript, in each parameter differ very significantly ($p \leq 0.01$);[2]/* Mean ± SE with superscript, in each parameter differ significantly ($p \leq 0.05$)
RBC = Total red blood cell; WBC = Total white blood cell; HGB = Hemoglobin; HCT = Hematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular hemoglobin; MCHC = Mean corpuscular hemoglobin concentration; Lym = Lymphocyte; Het = Heterophil; Mono = Monocyte; H:L = Heterophil to lymphocyte ratio

Lumsden (2000) reported in the study on biological standard value for using as a reference value of each animal species which could be indicated abnormal in the animals by comparison of the minimum and maximum values of normal animals (normal values). Therefore, normal values of normal animals was important and must be accurate and reliable affecting by various parameters including methods of analysis, sample collection and maintenance,

and statistical analysis. In addition, the normal values were affected by animal species, age, gender, feed stock and farm system. The findings were basic information of Thai indigenous chicken under free-range and semi free-range raising systems.

The relationship between hematological values and Newcastle disease immunity in Thai indigenous chickens strain Leung Hang Khao was determined to find the average hematological value and titer for use as a primary health assessment Newcastle disease index of Thai indigenous chicken. The hematological values of Thai indigenous chicken strain Leung Hang Khao at 4 month of age showed the highly statistically significant ($p<0.01$) of MCV between male and female chickens. The amount of MCV was higher in male more than in female. The high MCV values were found in Thai indigenous chicken which could be used as a tool for chicken health check. If the MCV value was lower than normal value (small red blood cells) may be caused by anemia from lack of iron of disorder of blood cells. In contrast, if the MCV values was higher than normal value (big red blood cells) may be due to the abnormal of liver. In this study, at 4 month of age male indicated higher MCV than female but MCV values were in the standard range which is similar to the report of Jain (1993) (126-163 and 90-140, respectively).

The comparison of hematological values between Thai indigenous chicken strain Leung Hang Khao and other chicken strains was performed. The different of haematological values in each chicken strains was found and the average values were shown in Table 2. The titer and haematological values were no statistically significant ($p>0.05$).

The amount of lymphocyte and heterophil were relative to Newcastle disease immunity with the coefficients of phenotypic correlation of -0.17 and 0.18, respectively. In Table 4, chicken with high titer was negative relative to lymphocytes but positive relative to heterophil. These hematological values could be used as additional information for evaluation the state of Newcastle disease immunity. The function of heterophil in chicken are a part of immune that helps fight infection, defend the body against other foreign materials and uses to diagnose body infection. Using hematological value to determine the state of Newcastle disease immunity in the vaccinated Thai indigenous chicken must be very difficult. The amount of lymphocytes and heterophil were not identified that directly produced by immune system due to the very low coefficient correlation of immunity and amounts of lymphocytes and heterophil. In addition, the separation of blood cell type in chicken was no tools to get the accuracy and use a long time to count. Therefore, the evaluation of immunity level in chicken using ELISA remains a cost effective method to save time, precision and specification at the present.

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Potentially harmful elements in lebanese fattoush salad

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Abstract The estimation of potentially harmful element availability in Fattoush ingredients cultivated in Lebanon is essential for evaluating impending risks for human and ecosystem health. In this study and for the first time, the selected plant species were the ingredients of the traditional Lebanese salad, Fattoush, composed of lettuce, cucumber, tomato, onion, purslane, radish, lemon and sumac in order to fulfill all the requirements for the assessment of the contamination levels in vegetables from soils with potentially harmful elements. The major physicochemical properties of topsoil including pH, organic matter content and texture showed that studied soils were almost neutral pH of 7.0, very poor in organic matter (organic matter percent <0.05 %) and sandy respectively(based on soil texture triangle). Concentration ranges of As, Cu, Fe, Zn, Mn, Ni, V, Cd, Co, Cr, Pb, P, Sn and Al in soils and edible parts of plants collected from urban allotments in the South, Damour, Ghazir and Akkar areas were determined and assessed by Inductively Coupled Plasma Optical Emission Spectroscopy following microwave assisted digestion by HNO_3/H_2O_2. Also, weak acetic acid digestion and aqua regia digestion were similarly measured following microwave assisted digestion. Transfer factors from sandy soils to vegetables because of their health risk were calculated accordingly. Results showed that concentrations of most of studied elements in soil and plant samples were recorded above the permissible limits set by World Health Organization especially in purslane, lemon and sumac. For this reason, amounts of accumulated Cu, Fe, Zn, Mn, Ni and V in the studied plant – soil systems (mg/kg) were separately shown. Furthermore, the sumac, purslane, lemon and lettuce plants were found to be "heavy metal hyperaccumulating plants". Finally, soil to plant transfer is the major path way of human exposure to PHEs contamination and safety measurements should be strictly applied.

Keywords: pseudototal, mobilizable, assisted digestion, aqua regia, transfer factor

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Introduction

The concerning in soil-plant relationships about potentially harmful elements loading from soils to plants has increased over the past few decades as awareness of metal contamination in human food sources has increased (Sarma, 2011). Vegetables are common diet taken by populations throughout the world especially in the Middle-East where salads are of high interest, being rich sources of essential nutrients, antioxidants and metabolites. It is known that both essential and toxic elements are absorbed by vegetables from the soil (Hajeb *et al.*, 2014). The ingestion of heavy metal contaminated vegetables may expose humans to many health risks; therefore the heavy metal contamination of food is one of the most important aspects of food quality (Khan *et al.*, 2008). Bioavailability of heavy metals in soils is critically dependent on the chemical speciation of the metals and plants respond only to the fraction that is "phytoavailable". The readily soluble fraction of heavy metals in soil is generally considered to be phytoavailable, but there is growing awareness that the various methods for assessment of "soluble" and "phytoavailable" fraction need reevaluation. It is generally known that there are variations in the rates of soil to plants transfer between different plant species but also between the same plant species from different areas (McLaughlin *et al.*, 2011). Lebanese diet is characterized, as in most of the Mediterranean regions, by a dominating contribution of fruits and vegetables (42 %), cereals (34 %: bread 14 %; pastries 5 %) and legumes (7 %) in the daily food ration. This fact makes us sure that salads are highly present at the Lebanese table such as the famous 'Fattoush' made up of many vegetables cultivated in Lebanese soils which may expose the consumers to many threats (Tueni *et al.*, 2012).

Fattoush is a salad made from toasted or fried pieces of Arabic flat bread mixed with vegetables and herbs according to season, region and taste. The vegetables are cut into relatively large pieces compared to Tabbouleh which requires ingredients to be finely chopped. Sumac is the herb usually used to give Fattoush its sour taste (Ariel, 2012). The Fattoush salad mixture is essentially made from a garniture of several vegetables, more precisely, a net hundred gram of "Fattoush" plate (DW basis) is made of the following quantities of vegetables: 20 g of cucumber, 5 g of onion, 20g of tomato, 10g of radish, 25 g of lettuce, 15g of purslane, 2.5g of sumac and 2.5 g of lemon.

Soil in Lebanon is young and is characterized by fragility, poor consistency and shallowness especially on slopping terrains (Fadel *et al.*, 2017). Average elemental concentrations in Damour urban allotments were published low (Fadel *et al.*, 2017). The heavy metal concentrations in the bed sediments of the Lower Litani River Basin in the South were published remarkably high

(Nehme *et al.*, 2014) while in Oustouan River in Akkar region publications referred an enrichment of dissolved salts throughout the course of the river from the source to the mouth (Bouaoun and Nabbout, 2016).

The selected plant species were the ingredients of the Lebanese Fattoush salad (lettuce, cucumber, tomato, onion, purslane, radish, lemon and sumac) collected from allotments in South Lebanon (Bourj Al Moulouk and Deirmimas), Damour, Ghazir and Akkar areas in order to fulfill all the requirements for the assessment of the pseudototal and mobilizable concentrations of the same elements in the rhizosphere soil of the edible parts of collected plants and the assessment of the contamination levels in soils and vegetables with potentially harmful elements (zinc, chromium, arsenic, manganese, cadmium, barium, aluminum, iron, cobalt, nickel, copper and lead).

Materials and methods

Field sampling and chemical analysis

Soil and plant samples were collected in the spring of 2017. The samples were collected from four communal allotments whereof vegetables were grown organically: site A (Borj Al Mlouk town, 85 km south of Beirut), site B (Damour town, 24 km south of central Beirut), site C (Ghazir town, 27 kilometers north of Beirut) and site D (Akkar, 110 km north of Beirut) as shown in Figure 1.

In the laboratory of soil sciences of the Faculty of Agriculture in the Lebanese University, plants were thoroughly washed three times with deionized water and air dried at room temperature. The edible parts of the plants were separated before drying, chopped manually with a cutter into very thin pieces and kept for further analysis. All plant and soil samples were ground in an agate mill (less than 2 μm diameter) in the Faculty of Geology and Geoenvironment, Athens-Greece and concentrations of heavy metals (zinc, chromium, arsenic, manganese, cadmium, barium, aluminum, iron, cobalt, nickel, copper and lead) were measured by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) in the University of Portsmouth, UK following microwave digestion by HNO_3/ H_2O_2, 6:1 v/v. All samples were oven dried at 40^{o}C for 3 days in a thermostatically controlled oven and kept for further analysis. Pseudototal heavy metal concentrations in soil (less than 2 μm diameter) were measured by ICP-OES following digestion by a mixture of HNO_3, H_2O_2 and HCl (US-EPA, 2002). Acetic acid (0.43 M) extractable concentrations of heavy metals were also measured by the same analytical technique after mixing 1g of the soil samples (less than 2 μm diameter) with 40 ml acetic acid and shaking

for 16 h at room temperature in an overhead shaker. All utensils which were used during laboratory work were thoroughly cleaned between the samples in order to avoid cross contamination. Analytical quality control procedures included the performance of duplicate analysis, the inclusion of blank solutions and certified reference materials of soils (NIST SRM 2709 and NIST SRM2711a for the total analysis and BCR-483 and BCR-484 for the acetic acid extraction) at random positions within the analytical batches. The obtained results from analytical control were found within acceptable limits for all geochemical results.

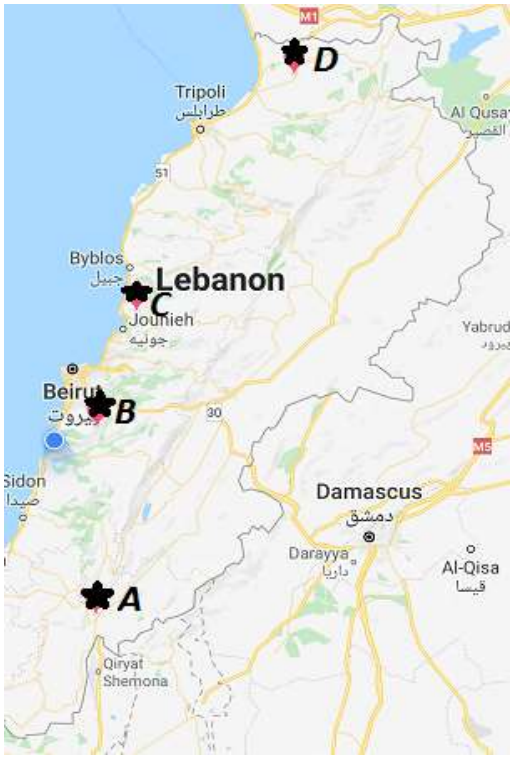

Figure legend:
Navigation *Scale* 1:2000000
: Capital of Lebanon
: Sampled allotment
: International boundary

Figure 1. The locations of sampled allotments (with named Site A, B, C and D with its related coordinates degrees and decimal minutes) on the Lebanese Map (Scale 1:200 000) with site A (Borj Al Mlouk town: 33.324432, 35.563296), site B (Damour town: 33.731625, 35.453765), site C (Ghazir town: 34.011270, 35.668439) and site D (Akkar: 34.544370, 36.081058)

Measurement of physicochemical parameters of soil

The determination of major soil physicochemical properties has been assessed including pH, electrical conductivity, organic matter content and soil texture (sand, silt, clay). The pH was measured in a solid -to-liquid ratio of 1:2.5 by a calibrated pH meter (ISO, 1994). Organic matter content of the soil samples was estimated by the loss-on-ignition (LOI) method by heating 1 g of each sample to 450 ^{0}C for 4 hours in a furnace oven(US-EPA, 2002). Since the method determines the organic matter content in the soil, a conversion factor of 1.724 has been used to convert organic matter to organic carbon based on the assumption that organic matter contains 58% organic C (i.e., g organic matter/1 .724 = g organic C). The grain size distribution in the sand, silt and clay fractions or the soil texture technique (triangle) was determined using the Bouyoucos Hydrometer Method (Bouyoucos, 1962).

Statistical treatment

Statistical treatment of vegetable ingredients of Fattoush salad and soil data from South region of Lebanon, Damour, Ghazir and Akkar areas was carried out using Microsoft Excel (Means ±standard deviation) and Minitab 18 (Coefficient of variation) statistical software. Descriptive statistics of each of the studied potentially harmful element and of their extractability percentages were estimated and were presented in terms of the ISO 9001: 2015 in accordance with the requirements of the quality assurance system. Water Certified Reference Material or Water (CRM), Plant CRM (Recovery percentage range ~100%), Aqua Regia Soil Digestion (Recovery percentage range ~100%) were assessed with a significance level <0.05. The transfer Factor (TF) was calculated. The Coefficient of variation (CV) was also calculated. Analytical quality control procedures included the performance of duplicate analysis, the inclusion of blank solutions and certified reference materials of soils and plants samples at random positions within the analytical batches.

Results

The famous “Fattoush salad” is made up of several mixed ingredients and the amount of potentially harmful elements have been assessed in this study showing measurable concentration of the potentially harmful elements (As, Cu, Fe, Zn, Mn, Ni, V, Cd, Co, Cr, Pb, P, Sn, Al) after plants acid digestion and determined by microwave assisted digestion. A net hundred gram

of “Fattoush” plate (DW basis) is normally made of the following quantities of vegetables: 20 g of cucumber, 5 g of onion, 20g of tomato, 10g of radish, 25 g of lettuce, 15g of purslane, 2.5g of sumac and 2.5 g of lemon. The “Table 1” showed the amount of potentially harmful elements in each used vegetable in 100 g of Fattoush mixture. The maximum value of potentially harmful element (Zn, Cu, Ni, Cr, Co, Mn, Fe, Ba, Zn, Pb, Cd, As, Al and V) concentration (mg/100 g DW) in every Fattoush salad ingredient (sumac, lemon, cucumber, tomato, lettuce, onion and radish) was shown in the figure below (Figure 2).

Table 1. The amount of PHEs of each vegetable used in a 100g dry weight “Fattoush” plate

PHEs	**Cucumber (mg/20g)**	**Onion (mg/5 g)**	**Tomato (mg/20 g)**	**Radish (mg/10 g)**	**Lettuce (mg/25 g)**	**Purslane (mg/15 g)**	**Sumac (mg/2.5 g)**	**Lemon (mg/2.5 g)**	**Total mg/100 g**
Zn	0.54	0.12	0.92	0.44	0.725	27.82	0.73	0.71	31.465
Cu	0.10	0.01	0.24	0.31	0.225	3.34	0.18	0.08	4.485
Ni	0.38	0.06	0.06	0.16	0.095	2.00	0.07	0.01	2.835
Cr	#*	0.09	#*	0.12	#*	2.20	0.03	0.00	2.44
Co	0.22	0.47	0.1	0.25	0.53	0.16	0.00	0.00	1.73
Pb	0.01	0.004	0.008	0.006	0.025	0.51	0.21	0.12	0.893
Mn	0.39	0.0815	1.28	0.16	1.82	26.05	0.81	0.17	30.7615
Fe	9.7	2.80	11.52	9.34	16.2	1139.9	14.32	1.15	1204.93
As	-	-	-	-	-	#*	#*	#*	#*
V	-	-	-	-	-	1.99	0.06	#*	2.05
Cd	-	-	-	-	-	0.04	#*	#*	0.04
Sn	-	-	-	-	-	#*	#*	#*	#*
Al	-	-	-	-	-	725.83	38.00	35.18	799.01
				Total					2080.64

#* Concentration below the detection limit (0.1 mg/kg DW)

The selected data for purslane, lemon and sumac was presented in a separated table because of its extremely high rate of potentially harmful elements found in Fattoush salad (Table 2).

No Arsenic nor Tin traces detected in the tissues of three studied plants

which is quite satisfactory since. As is very toxic for humans when consumed. The highest concentration of cupper was registered in purslane (222.75 mg/kg) compared to a much lower value in lemon (34.41 mg/kg).

Iron concentration was excessively high in purslane 75986.55 mg/kg, high in sumac 5729.18 mg/kg and acceptable in lemon crop 460.29mg/kg.

The highest concentration of Zn was found in purslane (1854.59mg/kg) and the lowest one in lemon (284.42mg/kg) that strongly exceeded the acceptable limits.

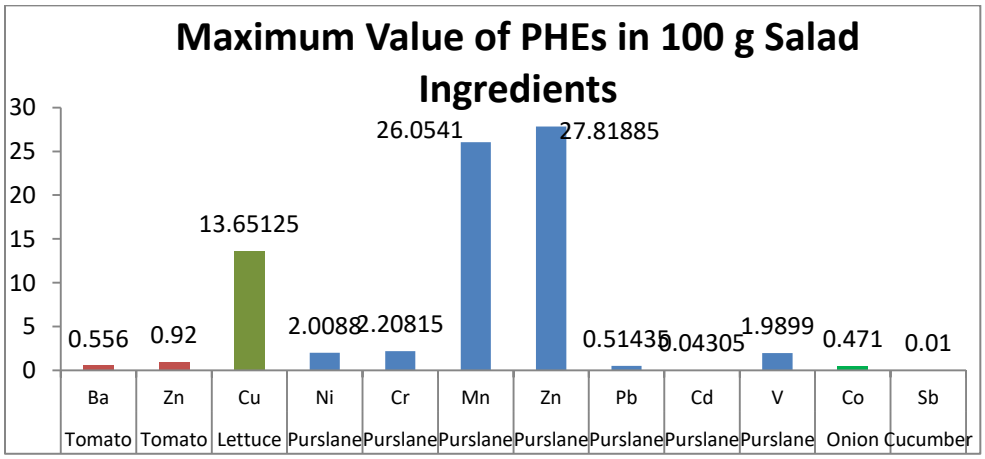

Figure 2. Potentially harmful elements (Zn,Cu, Ni, Cr, Co, Mn, Fe, Ba, Zn, Pb, Cd, As, Al and V) concentration (mg/100 g DW) in Fattoush Salad ingredients (Sumac, Lemon, Cucumber, tomato, lettuce, onion and radish)

Manganese recorded extremely high levels in purslane (1736.94 mg/kg) even though high levels were registered in sumac (324.38 mg/kg) and lemon (69.53mg/kg) crops and strongly exceeded the acceptable limits.

When it comes to Ni the levels in the three crops exceeded the acceptable limits especially in purslane and sumac crops where amounts of 133.92 mg/kg and 29.63 mg/kg were recorded respectively. Chromium registered levels that strongly exceeded the limits in the three studied crops with purslane accumulating the highest amount (147.21 mg/kg).

Total absence of V was recorded in lemon crop, whereas in purslane an amount of 132.66 mg/kg was recorded followed by a level of 25.66 mg/kg in sumac plant that strongly exceeded the acceptable limits. Usually the range of V in vegetables is 6.7–78.9 mg/kg which makes the content in purslane higher than normal.

Cadmium as well as cobalt uptake were high only in purslane crop and recorded higher values (2.87mg/kg Cd and 10.7 mg/kg Co) than the acceptable

limits (0.24 mg/kg Cd and 0.5 mg/kg Co).

When it comes to lead, sumac showed the highest uptake with an amount of 85.8 mg/kg that strongly exceeded the acceptable limits (2.11 mg/kg).

Table 2. Distribution of potentially harmful elements concentrations (means ± standard deviation in mg/kg DW) in purslane, sumac and lemon plant sample samples.

PHE	Purslane	Sumac	Lemon
As (mg/kg)	#*	#*	#*
Cu (mg/kg)	222.75	74.23	34.41
	±4.67	±0.35	±0.08
Fe (mg/kg)	75986.55	5729.18	460.29
	±152.2	±2.1	±0.26
Zn (mg/kg)	1854.59	295.24	284.42
	±29.70	±11.13	±1.32
Mn (mg/kg)	1736.94	324.38	69.53
	±18.47	±2.2	±2.84
Ni (mg/kg)	133.92	29.63	3.85
	±6.8	±0.92	±0.02
V (mg/kg)	132.66	25.66	#*
	±0.3	±0.43	
Cd (mg/kg)	2.87	#*	#*
	±0.11		
Co (mg/kg)	10.73	0.02	0.10
	±0.06	±0.00	±0.00
Cr (mg/kg)	147.21	14.14	1.52
	±12.1	±3.1	±0.08
Pb (mg/kg)	34.29	85.80	50.37
	±1.26	±5.2	±0.21
Sn (mg/kg)	#*	#*	#*
Al (mg/kg)	48383.98	15203.71	14075.82
	±56.7	±42.1	±100.2

#* Concentration below the detection limit (0.1 mg/kg DW)

Al high content is clearly shown in this study.

One approach to assess the mobility of potentially harmful elements by plants is to calculate the transfer factor (TF), as defined in the following equation (Chojnacka *et al.*, 2005):

$$TF = \frac{C\ plant}{C\ total - soil}$$

where C_{plant} is the concentration of an element in the plant material (dry weight basis) and C_{total} is the total concentration of the same element in the soil (dry weight basis) where the plant was grown. The higher the value of the TF, the more mobile/available the element is. The TF values of the elements for the plants studied are presented in Table 3.

If the ratios >1, the plants have accumulated elements, the ratios around 1 indicate that the plants are not influenced by the elements, and ratios < 1 show that plants exclude the elements from the uptake (Olowoyo *et al*., 2010).

Table 3. The Transfer Factor (TF) values of potentially harmful elements in purslane, sumac and lemon plant samples

PHE	Samples		
	Purslane	**Sumac**	**Lemon**
As	-	-	-
Cu	1.05	0.95	0.97
Fe	0.85	0.93	0.84
Zn	1.06	0.94	0.97
Mn	0.96	0.99	1.00
Ni	1.26	1.30	0.84
V	0.83	0.95	-
Cd	0.75	-	-
Co	0.97	2.15	0.1
Cr	0.99	0.96	0.86
Pb	0.16	2.63	1.08
Sn	-	-	-
Al	0.95	1.01	0.99

TF value of Cu in plants was the highest in purslane (1.05) with sumac and lemon showing approximately the same value for TF (0.95 and 0.97). As for Fe a TF less than 1 was recorded for the three crops with sumac recording the highest TF value (0.93). When it comes to Zn, purslane accumulated the highest amount with a TF of 1.06 followed by lemon (0.97) and sumac (0.94). Mn uptake was the highest in lemon with a TF value of 1.

The TF values of Ni were high in both sumac (1.3) and purslane (1.2). V also accumulated in purslane and sumac crops while Cd accumulated only in

purslane. The lowest accumulation for Co was in lemon with a TF value of 0.1 compared to a much higher accumulation in sumac with a TF value of 2.1. The TF values of Cr were below 1 for the three crops while Pb was transferred to the crops in higher quantities since the TF obtained for sumac was 2.6 and for lemon 1.08.

The coefficient of variation (CV) which is the ratio of the standard deviation to the mean was calculated (Figure 3). Purslane CV values ranged from 0.1% for Al to 8.2% for Cr. When it comes to sumac samples, the highest CV was registered for Pb with a 6.0% value and lowest one was for Fe with a 0.03% value. Moreover, Cr registered the highest CV for lemon crop with a percentage of 5.2 while P had the lowest CV with a value of 0.1%.

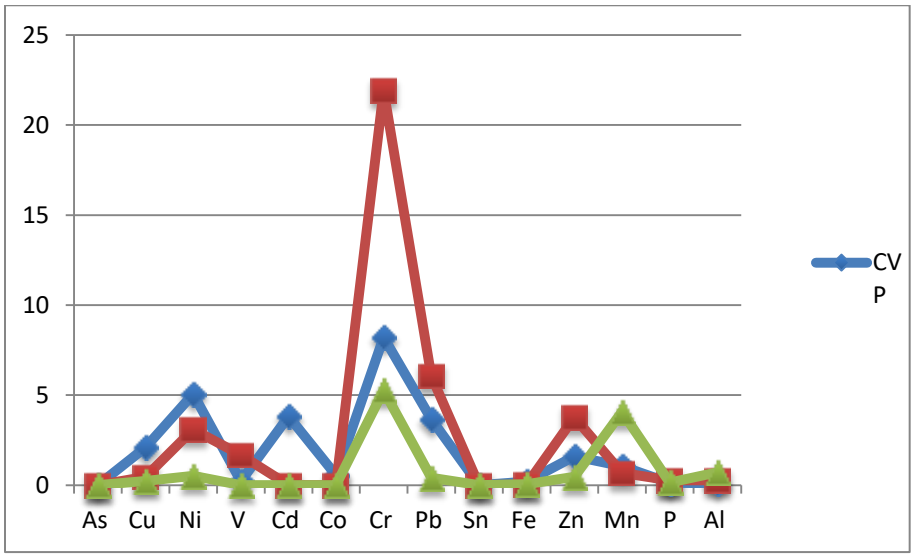

Figure 3. Variation Coefficient for PHEs in Plant Samples

Discussion

"Fattoush salad" case is alarming and leads us to posing many questions about the nature and the quality of many other consumed foods since the daily requirements of the trace elements range between 50 micrograms and 18 milligrams (Bini and Besh, 2014). The studied salad contains approximately an amount of 2080.64 mg/100g of harmful elements equivalent to the amount 4.08g which seems not acceptable and way higher than the required or acceptable daily needs. These obtained values are shocking and clearly indicate that the soils mainly in the South region are really poor in organic matter and extremely rich to a toxic extent in harmful elements that are causing

contamination to the cultivated vegetables. The highest concentration of cupper in purslane compared to that found in lemon could be linked to the studied soils containing high levels of Cu that strongly exceeded the acceptable limits (4 mg/Kg). The recommended values of iron concentration by WHO/FAO are 400-500 mg/kg. In general, it is a common belief that most of the vegetables are enriched with Fe and the same was recorded here (Demirbas, 2010). Adsorption rate of Zn differed between the crops and similar results were obtained by (Zhang *et al*., 2010). Cadmium as well as cobalt uptake were high only in purslane crop and recorded higher values (2.87mg/kg Cd and 10.7 mg/kg Co) than the acceptable limits (0.24 mg/kg Cd and 0.5 mg/kg Co). Such values strongly exceeded the acceptable limits. Some published studies linked the accumulation of Cd with that of Zn (Cherif *et al.*, 2011). Al high content may be due to the application of high levels of fertilizers (Yanling, 1989).

Since the essential trace elements daily requirements range between 50 micrograms to 18 milligrams/ day depending on the element (Bini and Besh, 2014), those amounts are exceeded only in the three studied crops exposing though human beings to many threats. Such results demonstrated that metals pollution is dominating in the studied sites showing also high amounts accumulated in plants (but below the detection limits) grown in agricultural soil in South region of Lebanon (Bourj Al Moulouk and Deirmimas), Damour, Ghazir and Akkar. The presented data showed clearly the highest accumulation of heavy metals in the edible tissues of purslane sumac and lemon. The three crops accumulated high amounts of PHEs and since they are ingredients of the famous Fattoush they may constitute a potential threat to the consumers.

Sumac had a high TF value of Ni (1.3), of Co (2.15) and for Pb (2.6) when compared to other studied plants which can make us consider it as a "hyperaccumulator plant". A "hyperaccumulator plant" is considered the plant that can take up toxic metal ions at very high levels and should not be cultivated in the South region (Bourj Al Moulouk and Deirmimas). Purslane also accumulated high amounts of harmful elements and may present a threat for human when ingested. Lemon also showed to be an accumulator but less than the other two studied crops.

Similar results were obtained by Antoniadis *et al.* (2015) showing that the metal soil-to-plant transfer coefficient was lower in the contaminated soil than that of uncontaminated soil, thus, indicating slower metal uptake with increased metal concentrations in lettuce, purslane and geranium grown in a Cd and Zn contaminated soil. In all these studied plants, Zn was absorbed in favour of Cd, irrespective of the fact that Cd was more mobile than Zn (judged by metal TC values).

According to results of the present study our crops were planted in harmful elements enriched soils. Harmful elements accumulation varies and depends on many factors such as, soil properties (e.g., soil pH, organic matter, clay content and metal concentration), plant factors (e.g., plant type and panting mode) and other environmental conditions (e.g., atmosphere and industrial pollution) (Xu *et al.*, 2016). Therefore, caution must be taken when making specific agricultural planting plans especially when it comes to crops destined for human or animal consumption.

Conclusion

Potentially harmful element contamination in soil is receiving increasing attention all over the world. The cultivation of healthy edible plants starts by adequate protection, restoration remediation of soil ecosystems contaminated by potentially harmful elements. Concentration of different elements in edible plants depends upon the relative level of exposure of plants to the contaminated soil as well as the deposition of toxic elements in the polluted air by sedimentation. The concentration of potentially harmful elements in studied soils and plants exceeded the acceptable limits. The contamination levels in edible parts of plants were also assessed with the calculation of the transfer factor value (TF) of zinc, chromium, arsenic, manganese, cadmium, barium, aluminum, iron, cobalt, nickel, copper and lead. TF value showed that there are external sources of harmful elements threatening the cultivated vegetables. Consequently, sumac, lemon and purslane are considered "potentially harmful elements accumulating plants" and are considered toxic for consumption, so growing such plants in contaminated soils should be restricted. These values of high TF could be due to long-term use of fertilizers, pesticides application and bad watering practices in agricultural lands. Obtained results indicate clearly that high accumulators are unsuitable to be grown in such contaminated soils.

Therefore, it is recommended that the study of potentially harmful elements in environmental components in the selected studied areas should be often repeated and sources of contaminants should be studied. The remediation of the contamination of soil and plants is necessary not only to preserve soil and plants but also to safeguard ecosystem and protect the humans and animals from the dangers of consumption. In addition, it is mandatory to grow the plants in healthy soils and irrigate with clean water because eating such plants in salads will lead the society to severe health problems.

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Effect of glycerol on improving quality of ready to eat Nham jerky, an innovation of Thai fermented meat product

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Abstract Different concentrations of glycerol were investigated for improving qualities of ready to eat pork Nham jerky, an innovation of Thai fermented meat product. Three concentrations at 0 (control), 5 and 10% of glycerol were applied in pork Nham. Microbiologica and physicochemical qualities were recorded during fermentation period and after drying. The results showed that addition of 10% glycerol displayed higher pH particularly on the third day of fermentation ($p<0.05$), causing lower total acidity and purge loss in pork Nham during fermentation process. However, lactic acid bacteria population were not different in all treatments. Greater red color (a*) and lower lightness (L*) was observed in Nham with 5% and 10% glycerol adding ($p<0.05$). After 3 days of fermentation process, pork Nham was cooked and dehydrated until the internal temperature reached 72˚C and water activity (a_w) < 0.75. This product, ready to eat Nham jerky is a novel meat product as normally Nham consume as raw product. Percentage of drying yield, a_w, shear force, color and sensory evaluation were compared among treatments. The results demonstrated that drying yield percentage of jerky Nham containing 5% and 10% glycerol was significantly higher than that of the control ($p<0.05$), while a_w and shear force obtained the lower value than control ($p<0.05$). Sensory evaluation indicated that the highest score in overall acceptance was observed in Nham jerky containing 10% glycerol. In conclusion, adding glycerol in Nham jerky improved qualities and sensory acceptance.

Keywords glycerol, Nham, Thai fermented meat product

Introduction

As our research focus on develop innovation product, therefore, the favourite product in Thailand was concerned. Nham is the most favourite traditional fermented pork product in Thailand (Luxananil *et al*., 2009; Kingcha *et al*., 2012). The ingredients of Nham composed of minced pork (60% w/w), cooked pork ride (35% w/w), fresh garlic (4.3%), cooked rice (4.3%), salt (1.9%

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w/w), sugar (0.3% w/w), sodium tripolyphosphate (0.2%), erythobate (0.25% w/w), potassium nitrite (0.01%) and monosodium glutamate (0.2% w/w). After all ingredients were mixed well, Nham was packed in an air-tight casing which reacted in oxygen-limited but still maintains the water activity throughout the process. The fermentation process occur a room temperature (30-37°C) for 3-5 days which is carried out by lactic acid bacteria (Visessanguan *et al*., 2005; Chokesajjawatee *et al*., 2009; Pringsulaka *et al*., 2011; Swetwiwathana and Visessanguan, 2015). As Nham is consumed as raw product without heating (Swetwiwathana and Visessanguan, 2015). The incidence of pathogenic bacteria is currently concerned for consumer safety (Chokesajjawatee *et al*., 2009). Even though the pH of Nham is lower than 4.6 (According to standard TIS 1219-2547, 2004 issue by the Thai industrial standard institute, Ministry of industry) which this pH is useful to inhibit the growth of pathogenic bacteria. However, there have been reported on pathogens during the fermentation of pork products as well (Zhao *et al*., 2016). Pathogenic bacteria can be observed during early phase of Nham fermentation (Chokesajjawatee *et al*., 2009). Therefore, to overcome this problem, raw Nham was cooked and dried for ready-to-eat Nham jerky. Subsequently, Nham jerky is an innovation Thai fermented meat product.

Nowadays, the market for meat snack has rapidly grown and those meat snack, meat jerky is very popular because it has high protein content and can be purchased easily in retail shops worldwide and has shelf stability. (Church *et al*., 2013; Kim and Kim, 2017). Jerky is one of the typical intermediate-moisture food which is favourite for ready to eat meat product in the world. There are different type of jerky which made from slice hole muscle meat and formed from ground meat, follow by marinated or mixed with spice, and then dried (Choi *et al*., 2008; Sorapukdee *et al*., 2016). This product was dried by reduction in water a_w to 0.70-0.75 to preserve the content.This properties resulted in prolong shelf-life of jerky without refrigerate during storage (Yang *et al*., 2009; Jang *et al*., 2015; Zhao *et al*., 2016). However, disadvantages of semi-dried meat product were undesirable of color, a tough texture and reduction chewibility of product (Yang *et al*., 2009). Generally, consumers prefer products with a softer texture (Chen *et al*., 2000). To overcome this problem, humectant is used in semi-dried meat product particularly 5-10% concentration of glycerol (Chen *et al*., 2000; Jang *et al*., 2015; Sorapukdee *et al*., 2016). The properties of humectant improve water holding capacity, texture and appearance of dry meat products. It has been reported that adding 5 to 10% glycerol was effective in reduction a_w and improved texture (Kim *et al*., 2010; Sorapukdee *et al*., 2016). Another research reported that adding glycerol 3, 6

and 9% in chinese-style pork jerky improved texture as shear force value decreased (Chen *et al*., 2000).

Therefore, the objectives of this research was to investigate the effect of glycerol on improving qualities and sensory of ready to eat semi-dried Nham (Nham jerky), an innovation of Thai fermented meat product.

Materials and Methods

Nham Jerky preparation samples

The ingredient of Nham composed of ground pork from ham portion, cooked pork ride, cooked rice, fresh garlic, salt, sugar, sodium tripolyphosphate, sodium erythobate, potassium nitrite and monosodium glutamate. Salt and Sodium tripolyphosphate were added into ground pork and mixed well, then followed by other ingredients. The mixture divided into 3 groups: 1) control (without glycerol), 2) adding 5% and 3) adding 10% glycerol. The mixture was formed into strip ($3 \times 10 \times 0.5$ cm^2) and subsequently packed in vacuum pack and kept at 30 ^oC for 3 days. After 3 days, Nham was dried in an oven at temperature 85 ^oC for 2 hours (h) until the internal temperature reached to 72 ^oC and continued at 60 ^oC for 1 hr to reduce a_w

Determination of Nham quality during fermentation process

Microbiological determination

Lactic acid bacteria, *Staphylococcus aureus* and yeast/mold in pork Nham were counted. Twenty- five grams of each treatment was homogenized and diluted to 10-fold serial dilution with 225 ml of 0.1% (w/v) peptone solution. Appropriate dilution was used for bacterial enumeration. 100 µl of dilution was spreaded on to media. Lactic acid bacteria was analyzed by MRS agar (de Man-Rogosa-Sharp; Merck, Germany) containing 0.5% calcium carbonate (AOAC, 2006). *S. aureus* was carried out by Baird-parker agar (Merck, Germany) adding egg yolk tellurite emulsion and the plate was incubated at 37 ^oC for 24-48 hr. The black colonies were selected for continue biochemical test as described by BAM (2001). For yeast/mold investigation, method of AOAC (2005) was followed using Malt agar (Merck, Germany) acidified with lactic acid and subsequently the plates were incubated at 26 ^oC for 3-5 days. Yield of bacteria expressed as log CFU/g.In addition, total plate count using plate count agar (Merck, Germany), *s. aureus* and yeat/mold were detected after drying.

Determination of pH and total acidity

Two grams of sample was blended and homogenized (Ultra tarrax model IKA T 25 digital, Germany) with 20 ml of distilled water. The mrat suspension was directly applied for pH measurement using a pH meter (Mettler Toledo, Switzerland) (AOAC, 1984). Total acidity of pork Nham determination was described by Friedrich (2001). Two gram of pork Nham was homogenized with 20 ml distilled water. The homogenate was centrifuged at 4000 g (4 ^{o}C). The supernatant was filter through filter paper (Whatman No. 1) and the filtrate was titrated subsequently with standardized 0.1 N NaOH with phenolphthalein as indicator.

Determination of purge loss

Purge loss was performed as modified by Nakao *et al.*, (1998). Twenty-five gram of pork Nham before fermentation at day 0 (A) was weight. Then, Nham was packed in vacuum pack during fermentation process for 1, 2 and 3 days. During fermentation period, pork Nham was removed from package, the exudate was dried with paper towels and consequently weight again (B). The purge loss was calculated by weight difference and expressed as a percentage of the initial weight as following

$$\text{Purge loss (\%)} = \frac{(A-B)}{A} \times 100$$

Determination of Nham jerky quality after dried

Percentage of drying yield

Drying yield was evaluated by calculating the weight differences from jerky Nham before and after drying as follows:

$$\text{Drying yield (\%)} = \frac{\text{Weight of dried sample (g)}}{\text{Weight of raw sample before dry (g)}} \times 100$$

Measurement of a_w

Water activity was recorded in triplicate on 2 gram of sample using a water activity meter (Novasina® Labmaster aw, Axair Ltd, Switzerland).

Shear force measurement

Sample size of Nham jerky 1×3×0.5 cm was applied to texture analyzer with Warner-Bratzler shear blade (Instron, 1011, USA). The shear force was express in Newton (N).

Color measurement

Three random area of surface sample (size of sample around 3x8x0.5 cm) were measured by the lightness (L*), redness (a*), and yellowness (b*) system mode of CIE, using a Hunterlab Mini Scan Ez (Germany).

Sensory evaluation

Ready to eat Nham jerky samples were served to 30 undergraduate and graduate students of Department of Animal Production Technology and Fisheries, KMITL as panelists. The hedonic test was used to evaluate the color, appearance, texture, flavor, sourness and overall acceptability. A score ranged from 1 to 7 indicating as the following: 1= undesirable, 2= moderately undesirable, 3= slightly undesirable, 4= indifferent, 5= slightly desirable, 6= moderately desirable and 7= extremely desirable.

Statistic analysis

All data were analyzed using the analysis of variance (ANOVA). Comparison of treatment means was based on a Duncan's Multiple Range Test. The statistical analyzed were carried out by using the Statistical Package for Social Science (SPSS for windows version 11.5, SPSS Inc., USA).

Results

Effect of glycerol on microbial counts in pork Nham during fermentation process

Lactic acid bacteria, total plate count, *S. aureus* and yeast/mold during fermentation process (0 and 3 days) in control, 5% and 10% glycerol treatments were detected. There were no significant differences in number of lactic acid bacteria among 3 treatments ($p>0.05$) as shown in Table 1 This indicated that glycerol at 5% and 10% had no effect on lactic acid bacteria growth. Although, the initial lactic acid bacteria count that analyzed on the first day of fermentation in control, 5% and 10% glycerol trails were 4.89, 4.70 and 4.42 log cfu/g respectively ($p>0.05$). Lactic acid bacteria population in all treatments after 3 days of fermentation increased with significant difference ($p<0.05$) at 7.63, 7.48 and 7.39, respectively. The initial number of *S. aureus* in pork Nham was 3.04, 2.74 and 1.85 log cfu/g, while initial number of yeast/mold was 3.97, 3.95 and 4.09 log cfu/g in control, 5% and 10% glycerol treated, respectively. However, both *S. aureus* and yeast/mold decreased less than 1 log cfu/g which were undetectable after 3 days of fermentation and oven-dried.

Table 1. Effect of glycerol on microbial in pork Nham during fermentation process and after dried

Microorganisms	Fermentation period (day)	Microbial counts (log cfu/g)		
		Control	5% Glycerol	10% Glycerol
Lactic acid bacteria	0	4.89 ± 0.99 ^{a,B}	4.70 ± 0.71 ^{a,B}	4.42 ± 0.21 ^{a,B}
	3	7.63 ± 0.32 ^{a,A}	7.48 ± 0.11 ^{a,A}	7.39 ± 0.06 ^{a,A}
	After dried	<1	<1	<1
Total plate count	0	5.10 ± 0.18 ^{a,A}	4.71 ± 0.39 ^{a,A}	4.57 ± 0.63 ^{a,A}
	3	4.26 ± 0.20 ^{a,A}	4.92 ± 0.21 ^{a,A}	5.24 ± 0.69 ^{a,A}
	After dried	<1	<1	<1
S. aureus	0	3.04 ± 0.54 ^{a}	2.74 ± 0.25 ^{a}	1.85 ± 0.72 ^{a}
	3	<1	<1	<1
	After dried	<1	<1	<1
Yeast/mold	0	3.97 ± 0.93 ^{a}	3.95 ± 0.75 ^{a}	4.09 ± 0.21 ^{a}
	3	<1	<1	<1
	After dried	<1	<1	<1

^{ab} different letters in the same row with different letters are significant different among glycerol treatment (p<0.05)

^{AB} different letters in the same column with different letters are significant different among fermentation period (p<0.05)

The Effect of glycerol on physicochemical properties of pork Nham during fermentation process

Effect of glycerol on pH and total acidity

The studies of pH value in pork Nham with 3 various concentrations of glycerol at 0, 5 and 10% on 1 and 3 days of fermentation process were carried out. The pH value of the product increased with significant difference (p<0.05) along on more glycerol adding. At the beginning, pH values of pork Nham without glycerol (control), 5 and 10% glycerol were not different (5.98, 6.11, and 6.0, respectively). After fermentation process for 3 days, the highest pH values were observed in pork Nham containing 10% glycerol (p<0.05). The pH of the control group was the lowest (<4.6), whereas pH of pork Nham with 0 and 5% glycerol were not different (4.51 and 4.65, respectively). The total acidity was not significant difference (p>0.05) in all treatments during pork Nham fermentation. After fermentation for 3 days, the lowest pH value was corresponding to the highest total acidity in control group. As pork Nham adding glycerol 5 and 10% revealed higher pH than control, resulting in lower total acidity (Table 2).

Table 2. Effect of glycerol on pH value and total acidity during fermentation process of pork Nham

Parameters	Fermentation period (days)	Control	5% Glycerol	10% Glycerol
pH	0	5.98 ± 0.08 ^{a,A}	6.11 ± 0.25 ^{a,A}	6.00 ± 0.10 ^{a,A}
	1	4.70 ± 0.26 ^{c,B}	5.13 ± 0.34 ^{ab,B}	5.62 ± 0.18 ^{a,A}
	2	4.60 ± 0.05 ^{b,B}	4.68 ± 0.08 ^{b,C}	4.96 ± 0.12 ^{a,B}
	3	4.51 ± 0.08 ^{b,B}	4.65 ± 0.09 ^{b,C}	4.89 ± 0.07 ^{a,B}
Total acidity	0	0.82 ± 0.30 ^{a,B}	0.83 ± 0.23 ^{a,B}	0.85 ± 0.25 ^{a,B}
	1	1.28 ± 0.18 ^{a,A}	1.19 ± 0.16 ^{a,AB}	1.08 ± 0.22 ^{a,AB}
	2	1.51 ± 0.27 ^{a,A}	1.28 ± 0.23 ^{a,A}	1.17 ± 0.26 ^{a,AB}
	3	1.69 ± 0.18 ^{a,A}	1.49 ± 0.17 ^{a,A}	1.38 ± 0.27 ^{a,A}

^{ab} different letters in the same row with different letters are significant different (p<0.05)
^{ABC} different letters in the same column with different letters are significant different (p<0.05)

Effect of glycerol on purge loss

The results revealed that the higher concentration of glycerol reduced purge loss during fermentation as shown in Table 3. Pork Nham with 10% glycerol released the lowest purge loss when compared to control and 5% glycerol treatments with significant difference (p<0.05). The purge loss percentage on the third day of fermentation was 9.46, 5.07 and 2.55 in control, 5 and 10% glycerol treated, respectively. In addition, the longer fermentation period, the higher of purge loss percentage were found.

Table 3. Effect of glycerol on purge loss during fermentation process of pork Nham

Fermentation peroid (day)	% Purge loss		
	control	5% Glycerol	10% Glycerol
1	1.54^{a,C} ± 0.12	1.25^{ab,B} ± 0.25	1.07^{b,B} ± 0.16
2	4.64^{a,B} ± 0.49	3.77^{a,B} ± 0.52	2.13^{b,A} ± 0.62
3	9.46^{a,A} ± 0.39	5.07^{b,A} ± 0.20	2.55^{c,A} ± 0.23

^{abc} different letters in the same row are significant different among treatments (p<0.05)
^{ABC} different letters in the same column with different letters are significant different among fermentation peroids (p<0.05)

Effect of glycerol on color

There were not differenes in lighteness (L*), redness (a*) and yellowness (b*) on Nham with glycerol on the first day of fermentation. However, at 2 and 3 days, L* value of glycerol group was lower than control which was not

different in yellowness. On the contrary, a* values were higher in glycerol adding than control with significant difference ($p<0.05$) as shown in Table 4. However, fermentation time increasing, L* and a* values also increased (Table 4).

Table 4. Effect of glycerol on color of pork Nham during fermentation process

Parameters	Fermentation peroid (day)	Control	5% Glycerol	10% Glycerol
Lightness (L*)	0	$23.74 \pm 0.60^{a,x}$	$24.06 \pm 1.19^{a,D}$	$25.15 \pm 0.84^{a,A}$
	1	$33.40 \pm 0.39^{a,C}$	$30.36 \pm 1.15^{b,C}$	$31.05 \pm 1.06^{a,C}$
	2	$41.04 \pm 0.23^{a,B}$	$37.05 \pm 1.23^{b,B}$	$34.19 \pm 0.98^{c,B}$
	3	$44.03 \pm 0.53^{a,A}$	$41.19 \pm 0.78^{ab,A}$	$38.59 \pm 0.92^{c,A}$
Redness (a*)	0	$12.91 \pm 0.79^{b,B}$	$15.07 \pm 0.21^{a,C}$	$15.84 \pm 0.13^{b,B}$
	1	$14.23 \pm 1.03^{a,AB}$	$15.30 \pm 0.33^{a,C}$	$16.06 \pm 0.17^{a,B}$
	2	$15.12 \pm 0.58^{b,A}$	$16.20 \pm 0.08^{ab,B}$	$16.91 \pm 0.06^{a,A}$
	3	$16.04 \pm 0.45^{c,A}$	$17.00 \pm 0.42^{ab,A}$	$17.56 \pm 0.12^{a,A}$
Yellowness (b*)	0	$17.29 \pm 0.69^{a,A}$	$15.81 \pm 0.24^{a,A}$	$16.50 \pm 0.18^{a,A}$
	1	$14.24 \pm 0.42^{a,A}$	$13.67 \pm 0.01^{a,A}$	$14.66 \pm 0.29^{a,A}$
	2	$13.48 \pm 0.28^{a,A}$	$12.16 \pm 0.06^{a,A}$	$13.66 \pm 0.85^{a,A}$
	3	$12.26 \pm 0.59^{a,A}$	$11.98 \pm 0.24^{a,A}$	$13.52 \pm 0.33^{a,A}$

^{abc} different letters in the same row are significant different among treatments ($p<0.05$)
^{ABC} different letters in the same column are significant different among fermentation peroid ($p<0.05$)

Effect of glycerol on physicochemical characteristic of ready-to-eat Nham jerky after drying

The percentage of drying yield for all treatments was 35-40% and higher drying yield was observed in Nham jerky adding 5 and 10% glycerol compared to control ($p<0.05$). The a_w of ready-to-eat Nham jerky after oven-dried in all treatments was lower than 0.85 with the figures of 0.68, 0.70 and 0.73 in control, 5% and 10% glycerol groups, respectively. The a_w in 5 and 10% glycerol was lower than control with significant difference ($p<0.05$). The a_w of 10% glycerol adding was the lowest with significant difference ($p<0.05$) comparing to 5% glycerol and control (Table 5).The shear force value of Nham jerky decreased significantly ($p<0.05$) with increasing levels of glycerol. Shear force value was 45.53, 42.53 and 35.87 N in control, glycerol 5% and 10 %, adding respectively (Table 5). The glycerol in the product demonstrated the higher L* value with significant difference compared to control ($p<0.05$). In contrast to the a* value that was lower with significant difference to control ($p<0.05$). Sensory evaluation was performed on the effect of color, appearance,

texture, flavor, sourness and overall acceptance. As Nham is fermented meat product, the important parameter to detect was sourness.The color, appearance, texture, flavor and overall acceptance in Nham jerky with 10% glycerol exhibited the highest acceptance to other groups (p<0.05) with liking score of 5 (slightly desirable) to 6 (moderately desirable).

In addition, the analysis of total count, *S. aureus*, yeast and mold in the product were under detectable level (<1 log cfu/g) as data has not shown.

Table 5. Effect of glycerol on physicochemical characteristics and sensory attributes of Nham jerky from pork

Parameters	Control	5% Glycerol	10% Glycerol
Drying yield (%)	35.14 ± 0.38^{c}	37.70 ± 0.73^{b}	40.77 ± 0.10^{a}
water activity	0.749 ±0.05^{a}	0.684 ±0.07^{b}	0.602 ±0.04^{b}
Shear force (N)	45.53 ± 0.25^{a}	42.53 ± 0.17^{b}	35.87 ± 0.25^{c}
Color			
- Lightness (L*)	21.89 ± 0.40^{b}	23.87 ± 0.23^{ab}	24.89 ± 0.33^{a}
- Redness (a*)	5.38 ± 0.0^{a}	4.09 ± 0.03^{b}	3.72 ± 0.03^{c}
- Yellowness (b*)	4.45 ± 0.10^{c}	5.31 ± 0.05^{b}	6.19 ± 0.11^{a}
Sensory attributes			
- Color	4.63^{a}	4.79^{a}	4.85^{a}
- Appearance	4.67^{b}	4.49^{b}	5.10^{a}
- Texture	4.46^{b}	4.48^{b}	5.46^{a}
- Flavor	4.82^{b}	4.88^{b}	5.19^{a}
- Sourness	5.06^{a}	4.81^{ab}	4.65^{b}
- Overall acceptability	4.53^{b}	4.82^{a}	5.06^{a}

^{abc} different letters superscripts in the same row indicate significances differences among treatments
(p<0.05)

Discussion

Microbiological and physicochemical characteristic of pork Nham with glycerol 5 and 10% compared to pork Nham without glycerol (control) were studied during fermentation process. It was found that lactic acid bacteria in pork Nham during fermentation for 3 days were not different in all treatments. Even though number of lactic acid bacteria were not different, pH value of pork

Nham adding glycerol was higher than the control, resulting in lower total acidity and lower purge loss percentage. Lactic acid bacteria increased during longer fermentation time. After fermentation period 3 days, *S. aureus* and yeast/mold did not observed. Lactic acid bacteria are usually present in raw meat in small amount (10^2-10^3 cfu/g), but propogate rapidly in the fermented meat products under vauum-packed with NaCl and nitrite (Pringsulaka *et al*., 2011; Rantsiou and Cocolin, 2008). Lactic acid bacteria are the major producers of organic acid which respond to decrease in pH and increase in acidity during fermentation (Valyasevi and Rolle, 2002; Zhao *et al*., 2016; Thongruck *et al*., 2017). The pH value of Nham was gradually decreased during fermentation because organic acid was produced from carbohydrate by lactic acid bacteria, contributing of the inhibition of undesirable microorganisms (Visessanguan *et al*., 2004a; Komprda *et al*., 2004; Bozkurt and Bayram, 2006; Zhao *et al*., 2016). The pH of uncooked Nham should lower than 4.6 after fermentation according to standard TIS 1219-2547 issued by Thai Industrial Standard Institute, Ministry of Industry. This pH value is useful to inhibit the growth of pathogenic bacteria (Chokesajjawatee *et al*., 2009). Previous mentioned pH in Nham adding glycerol showed higer value. However, there was no report on effect of glycerol on pH in fermented meat product before. This could be explained that after adding glycerol in Nham formulation, they attract water to itself, resulting in lower concentration of acidity and high pH. Even though, pork Nham adding glycerol displayed higher pH, indicating delay fermentation period but this product was oven-dried (internal temperature reached to 72 ˚C) after fermentation to overcome the incidence of undesirable microorganisms. Therefore, this product named ready to eat Nham jerky, an innovation Thai fermented meat product.

Percentage of purge loss of pork Nham was higher during fermentation period. In addition, purge loss percentage was lower in Nham adding glycerol. Smilar result had been reported by Visessanguan *et al*. (2004b). Increasing in weight loss and released water were found as the fermentation proceeded. This could be the accumulation of lactic acid resulting in lower pH, an increasing in releasing water caused by denaturation of Nham protein. As the Nham adding glycerol displayed higher pH, leading to lower releasing water. Adding glycerol in Nham showed lower L* and higher a* value. Visessanguan *et al.* (2005) explained that characteristic color of meat is a function of meat pigments and light-scattering properties. Nitric oxide myoglobin (NOMb) was spontaneously formed in Nham during the mixing process and accounted for approximately 90% of total heme pigment. However, L* and a*value in Nham increased with longer fermentation period. Visessanguan *et al.* (2005) also explained that

acidification of meat proteins affectes color by increasing the light-scattering properties, thus meat becomes opaque and paler.

Physicochemical, microbiological and sensory were performed after pork Nham was dried. The percentage of drying yield was higher after adding glycerol in Nham jerky. This results agreed with Seol *et al.* (2003), Han *et al*. (2011) and Sorapukdee *et al.* (2016) who reported that humectant including glycerol, sorbitol, konjac, egg albumin, rice syrup enchanced in higher drying yield in meat jerky comparing to jerky meat without humectant. However, Jang *et al*. (2015) found that drying yield was not different after added 0, 2.5 and 5% sorbitol, glycerol and xylitol in semi-dried jerky. In this studied, a_w of Nham jerky decreased with high level of glycerol. Similarly, several previous studied such as Chen *et al.* (2000); Kim *et al.* (2010), Jang *et al.* (2015) and Sorapukdee *et al.* (2016) reported that adding humectant, glycerol, sorbitol ect., could reduce water activity in jerky meat. Kim *et al.* (2010) explained that glycerol is an effective additive for water activity control. The water activity of Nham with glycerol 5 and 10% were 0.684 and 0.602, respectively. The use of glycerol in Nham jerky corresponded to safety standard of recommendation of semi-dried meat product from American Food Safety and Inspection Service, FSIS, Ministry of Agriculture, USA (FSIS<2012) that water activity of jerky product should be at ≤ 0.70 in order to inhibit yeast and mold growth. The shear force of Nham jerky decreased with higher level of glycerol, indicated tenderness improvement of product. This finding supported by Chen *et al.* (2000); Sorapukdee *et al*. (2016); Kim *et al*. (2010) and Jang *et al.* (2015) who demonstrated that adding humectant such as glycerol, sorbitol and xylitol ect. improved tenderness of jerky meat as their experiment had showed lower shear force value in jerky meat. Chen *et al.* (2000), Mori *et al*. (2016) and Kim *et al*. (2010) also explained that tenderness is the main factor which contributed to overall eating quality of meat as consumer preferred products with a softer texture. In addition, Chen *et al*. (2000) and Sorapukdee *et al.* (2016) reported that humectants were a substance that attracted water to it self, they could retain water in food stuff, reduced water activity and improving food softness. Flavor scores were increased by the addition of glycerol. There has been a similar finding by Chen *et al.* (2000) and Kim *et al.* (2010), indicated that the glycerol addition increased acceptance score. In conclusion, adding glycerol in Nham jerky improved qualities including yield, a_w, texture and leading sensory acceptance.

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Effect of biogas effluent from pig manure and Longan (*Dimocarpus longan*) residues on growth of Marigold (*Tagetes erecta*)

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Prathumyot, W., Makboon, R., Yota, M., Chakhatrakan, S., Matta, F. B. and Chitaree, L. (2019). Effect of biogas effluent from pig manure and longan (*Dimocarpus longan*) residues on growth of marigold (*Tagetes erecta*). International Journal of Agricultural Technology 15(2): 347-358.

Abstract The efficiency of biogas effluent fermented with pig manure and longan residues on growth of marigold was investigated. Treatments were control (no fertilizer), and four concentrations (10%, 20%, 30% and 40%) of biogas effluent and chemical fertilizer. Plants were watered with 200 ml of each biogas effluent concentrationt every 4 days. Chemical fertilizers were applied as 15-15-15 and 8-24-24. Results showed that the growth of marigold in the control was lowest as compared to the other treatments. There was no significant difference in the plant height, stem diameter, bush diameter, fresh weight of blooming flowers and initiation of first flower between biogas effluents and chemical fertilizer. Fresh weight of whole plant and number of blooming flowers resulting from the chemical fertilizer treatment was highest compared to the control and effluent treatments. Dry weight of whole plant resulting from the chemical fertilizer treatment was not significantly different compared to 10%, 20% and 30% biogas effluent treatments. Total chlorophyll and chlorophyll per unit leaf area in the chemical fertilizer did not differ as compared to 30% and 40% biogas effluent treatments.

Keywords: biogas effluent, longan, marigold, growth

Introduction

Thailand is well-known as an agricultural country where fertilizer is necessary for farmers to increase their agricultural productivity. Thailand imports chemical fertilizers from abroad every year. In 2014, Thailand imported 5,415,020 tons of chemical fertilizers from abroad valuedat 2,030 million dollars (Department of Agriculture, 2015). Therefore, searching for chemical fertilizer replacement is important to reduce the expense of purchasing and importing chemical fertilizer.

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At present, the animal husbandry in Thailand has grown to meet the demand for meat. However, the expansion of animal husbandry has caused environmental pollution problems in farms and nearby communities, such as bad smell, flies, wastewater and diseases resulting from animal waste and other wastes from the unsuitable management of farm systems. Therefore, biogas production from animal waste was promoted for solving the pollution problems and the biogas is also a renewable energy source in farms. After biogas production is completed, the fermented waste or effluent can be used as organic fertilizer to improve the soil and increase crop yields (Furukawa and Hasegawa, 2006; Ausungnoen *et al.,* 2014; Stinner, Moller and Leithold, 2008; Hossain *et al.,* 2014). Therefore, using effluent from biogas fermentation as bio-fertilizer is markable strategy for reducing the expense and trade deficit from the import of chemical fertilizer.

Prathumyot *et al.* (2017) reported that the biogas effluent fermented with pig dung, peels and seeds of longan (*Dimocarpus longan*) contains nitrogen, phosphorus and potassium that are important nutrients for plant growth. However, the utilization of effluent after biogas production as bio-fertilizer has not been studied. Thus, in this experiment the effects of biogas effluent fermented with pig manure, peels and seeds of longan on the growth of marigold (*Tagetes erecta*), an attractive yellow flower that can tolerate and adapt to various environmental conditions, were investigated for potential use as bio-fertilizer.

Materials and methods

The experimental design was a Completely Randomized Design (CRD) with 4 replications. Six treatments were control (no fertilizer), four concentrations of biogas effluent (10%, 20%, 30% and 40%) and chemical fertilizer (15-15-15 and 8-24-24). Marigolds were planted in cell plug trays with peat moss. The plants were transferred to pots containing 2.4 kg of soil mixed with husk and burned husk at a ratio of 2:1:1, 10 days after planting (DAP). The chemical properties of soil and biogas effluent are shown in Table 1. Marigolds were watered with a total of 200 ml/pot of 10%, 20%, 30% and 40% biogas effluent solution every 4 days. The chemical fertilizer treatment consisted of 5 g of 15-15-15 formula fertilizer dissolved in 400 ml water to water pots 16, 23 and 30 days after planting (DAP) and 5 g of 8-24-24 formula fertilizer dissolved in 400 ml of water to pots 37, 44, 51, 58 and 65 DAP by following the cultivation method of the Department of Agricultural Extension (2002). Four hundred ml of water were added daily to all pots. The experiment was conducted for 69 days at the Agricultural Technology Faculty at Rambhai Barni Rajabhat University.

Table1. Chemical properties of soil and biogas effluent at the start of the experiment

Chemical properties	Soil	Biogas effluent
pH	5.44	5.57
Nitrogen concentration (ppm)	1033	4900
Phosphorus concentration (ppm)	161.68	1300
Potassium concentration (ppm)	754.48	3600

Soil pH was recorded at the beginning and the end of the experiment using a IQ-150 pH meter (IQ Scientific Instruments Inc., Carlsbad, CA). Data of plant height, stem diameter and bush diameter were collected weekly. After flowering, flower number, fresh weight and dry weight of blooming flowers was measured daily. The data of flower diameter was collected from 1st to 10th blooming flower. Total chlorophyll, chlorophyll a and chlorophyll b concentrations were measured by the method of Mackinney (1941). At the end of experiment, plants were sampled and washed in water. The fresh plants were weighted for collecting the data of fresh weight. Then plants were dried in an oven at 80℃ for 48 hours and measured dry weight of roots, branches and leaves. Results were analyzed by analysis of variance and means separated by Duncan's multiple range test (DMRT).

Results

Soil pH

Soil pH at the start of experiment (15 DAP) was not statistically different among the treatments and ranged from 5.40 to 5.44. However, the results showed a statistical difference in soil pH at 63 DAP. The soil pH was decreased by the chemical fertilizer and 40% effluent treatments compared to the remaining treatments (Table 2).

Growth parameters

There was a significant difference in plant height after 28 DAP until the end of the experiment. The chemical fertilizer and all effluent treatments increased in plant height compared to the control at 69 DAP. The control treatment resulted in the lowest plant height of 36.66 cm. There was no significant difference in plant height between all biogas effluent and chemical fertilizer treatments at 69 DAP and ranged from 68.16 cm to 77.66 cm. However, the plant height resulting from the 40% effluent treatment tended to be lower than the other effluent treatments (Fig. 1).

Table 2. The effect of treatments on soil pH, plant height, stem diameter and bush diameter at the end of experiment

Treatment	Soil pH		Stem diameter (mm)	Bush diameter (cm)
	15 DAP	63 DAP		
Control (0%)	5.44±0.15[a]	5.44±0.25[a]	16.00±1.00[c]	4.03±0.21[b]
Effluent 10%	5.44±0.03[a]	5.45±0.05[a]	38.50±1.80[b]	11.37±1.12[a]
Effluent 20%	5.36±0.26[a]	5.37±0.09[a]	46.33±5.50[a]	12.02±1.36[a]
Effluent 30%	5.42±0.04[a]	5.31±0.14[a]	44.00±4.58[ab]	11.99±1.35[a]
Effluent 40%	5.40±0.02[a]	4.60±0.33[b]	39.00±3.46[b]	10.86±0.86[a]
Chemical fertilizer	5.41±0.08[a]	3.72±.08[c]	42.66±1.52[ab]	12.36±1.24[a]
F-test	ns	*	**	**
CV (%)	1.92	8.38	9.04	10.58

Means with different letters in each column are significantly different according to DMRT.
** = significant at P≤0.01. * = significant at P≤0.05. ns = not significant at P≤0.05.

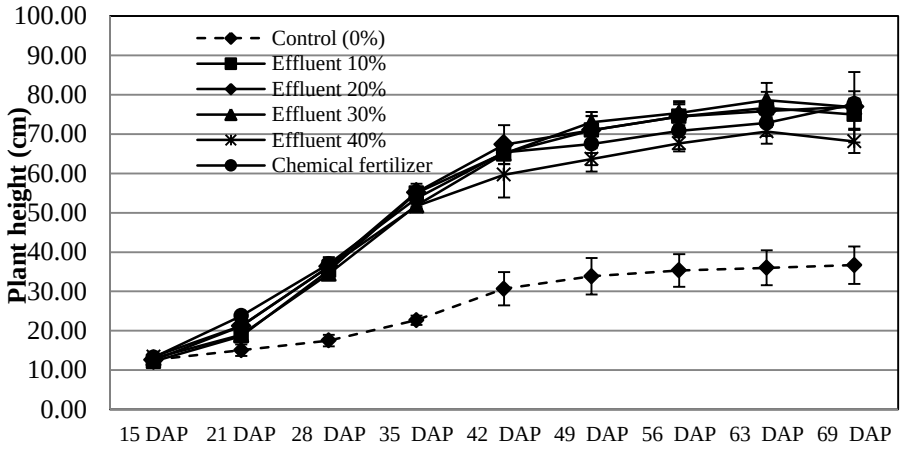

Figure 1. The effect of treatments on plant height during the experimental period

The greatest stem diameter resulted from the 20 % effluent treatment (46.33 mm) which did not significantly differ from the chemical fertilizer and 30% effluent treatments. The control treatment resulted in the lowest stem diameter of 16 cm (Table 2). Bush diameter was significantly increased by the chemical fertilizer and all effluent treatments compared to the control which resulted in the lowest bush diameter of 4.03 cm. There was no significant difference in bush diameter among all biogas effluent and chemical fertilizer treatments and ranged from 11.37 cm to 12.36 cm (Table 2).

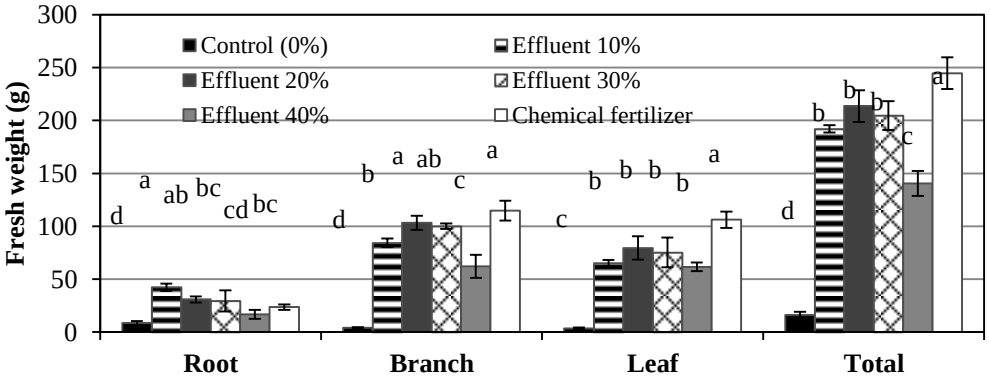

Figure 2. The effect of treatments on Fresh weight of root, branch, leaf and total of marigold plants at the end of the experiment (69 DAP). Bars with different letters in each plant part indicate significant differences in different treatments at the 0.01 probability level, according to DMRT.

Root fresh weight was significantly increased by the chemical fertilizers and all effluent treatments compared to the control. Root fresh weight resulting from the 10% effluent treatment was significantly higher than that resulting from the chemical fertilizer treatment. Effluent treatment of 20%, 30% and 40% increased root fresh weight to similar levels compared to the chemical fertilizer treatment (Fig. 2). In the case of branch fresh weight, the control treatment resulted in the lowest branch fresh weight of 3.96 g. 20% and 30% effluent treatments significantly increased branch fresh weight equivalent to the chemical fertilizer treatment and ranged from 100.12 to 114.90 g. Branch fresh weight resulting from 40% effluent treatment was lower compared to the remaining effluent treatments (Fig. 2).

Leaf fresh weight was significantly increased by the chemical fertilizer treatment compared to the other treatments. The control treatment resulted in the lowest dry weight of leaf and followed by the effluent treatments (Fig. 2). Total plant fresh weight was significantly increased by the chemical fertilizers and all effluent treatments compared to the control. Total fresh weight was greater in the chemical fertilizer treatment. Effluent treatment of 40% resulted in the lowest total fresh weight compared to the other effluent treatments. The control treatment resulted in the lowest total fresh weight of 16.16 g (Fig. 2).

Root dry weight was increased by the 10% and 30% effluent treatments compared to the chemical fertilizer treatment. Root dry weight of the control was similar compared to the chemical fertilizer and 40% effluent treatments (Fig. 3).

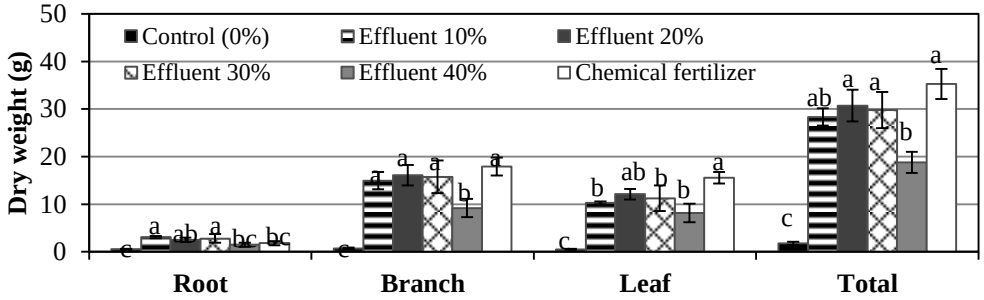

Figure 3. The effect of treatments on dry weight of root, branch, leaf and total of marigold plants at the end of the experiment (69 DAP). Bars with different letters in each plant part indicate significant differences in treatments at the 0.01 probability level, according to DMRT.

Branch dry weight was significantly increased by the chemical fertilizer treatment and the 10%, 20% and 30% effluent treatments. Effluent treatment of 40% resulted in the lowest branch dry weight compared to the other effluent treatments. The control treatment resulted in the lowest branch dry weight of 0.70 g (Fig. 3). The 20% effluent treatment increased leaf dry weight the same as the chemical fertilizer treatment. There was no difference in leaf dry weight among effluent treatments. Leaf dry weight was reduced by the control and was 0.51 g (Fig. 3). Total dry weight was increased by all effluent and chemical fertilizer treatments compared to the control. Total dry weight in the chemical fertilizer treatment did not differ as compared to 10%, 20% and 30% effluent treatments. Total dry weight in 40% effluent treatment and the control was significantly lower as compared to the chemical fertilizer treatment. The lowest total dry weight was 1.76 g in the control (Fig. 3).

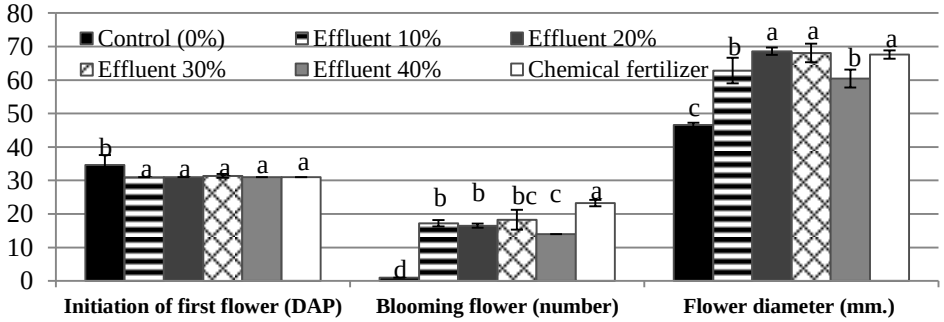

Figure 4. The effect of treatments on initiation of first flower, blooming flower number and flower diameter of marigold at the end of experiment. Bars with different letters in each plant part indicate significant difference in treatments at the 0.05 probability level, according to DMRT.

There was a significant difference in the initiation of first flower, blooming flower, and flower diameter. The chemical fertilizer and all effluent treatments significantly reduced the initiation of first flower compared to the control. The initiation of first flower in the chemical fertilizer and all effluent treatments was 31 DAP and 34.67 DAP in the control (Fig. 4). Blooming flower number was increased by the chemical fertilizer as compared to the remaining treatments. Number of blooming flowers resulting from the 40% effluent treatment tended to be lower compared to the other effluent treatments. The control treatment resulted in the lowest number of blooming flowers (Fig. 4). In the case of flower diameter, the control treatment resulted in the lowest flower diameter followed by 40% effluent and 10% effluent. Flower diameter was similar in plants treated with 20% and 30% effluent and the chemical fertilizer (Fig. 4).

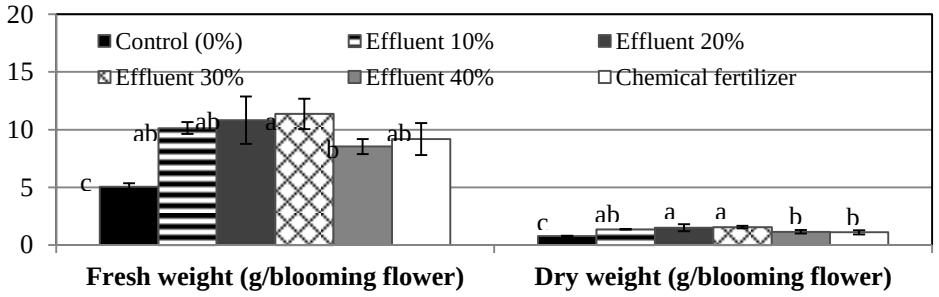

Figure 5. The effect of treatments on fresh weight and dry weight of blooming flower at the end of experiment. Bars with different letters in each plant part indicate significant difference in treatments at the 0.01 probability level, according to DMRT.

Fresh and dry weight of blooming flowers was increased by all treatments compared to the control. In case of fresh weight, the chemical fertilizer treatment did not differ significantly when compared with the effluent treatments. The lowest fresh weight was in the control at 5.06 g (Fig. 5). Dry weight of blooming flower in 20% and 30% effluent treatments was significantly higher than that in the chemical fertilizer treatment. The 10% and 40% effluent treatments resulted in dry weight similar to the chemical fertilizer treatment. The control resulted in the least dry weight of 0.77g (Fig. 5).

Plant physiological parameters

There was a significant difference in the concentrations of total chlorophyll, chlorophyll a and chlorophyll b per unit leaf area among

treatments. Total chlorophyll concentration resulting from the chemical fertilizer treatment was similar to the 30% and 40% effluent treatments. The 20% and 10% effluent and control treatments showed the lowest total chlorophyll concentration as compared to the chemical fertilizer treatment (Fig. 6). Similarly, the difference in chlorophyll a concentration per unit leaf area did not differ among the chemical fertilizer, 30% and 40% effluent treatments. Chlorophyll a concentration resulting from the 20% and 10% effluent treatments and the control was less than chlorophyll a resulting from the chemical fertilizer treatment (Fig. 6). Chlorophyll b concentration in the chemical fertilizer treatment did not differ significantly when compared to the effluent treatments. The lowest concentration of chlorophyll b was in the control at 4.92 µg/cm^2 (Fig. 6).

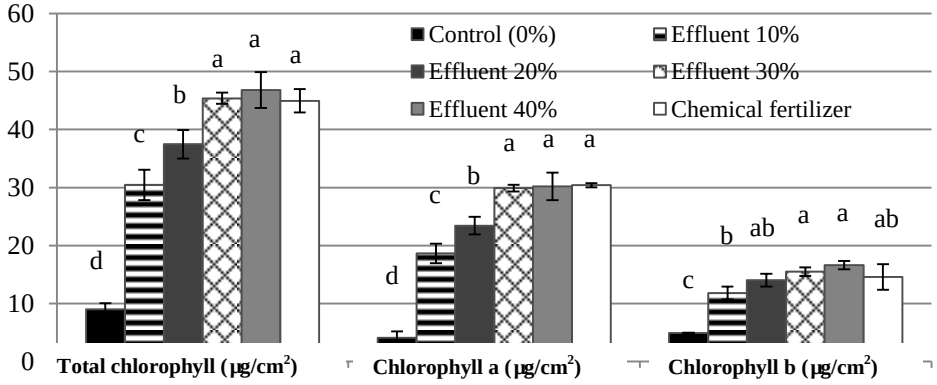

Figure 6. The effect of treatments on the concentrations of total chlorophyll, chlorophyll a and chlorophyll b per unit leaf area. Bars with different letters in each plant part indicate significant difference in treatments at the 0.01 probability level, according to DMRT.

Discussion

Nitrogenous fertilizers decrease soil pH (Liu *et al*., 2010; Hatiet *et al*., 2008; Darusman *et al*., 1991). This is mainly due to the fact that most fertilizers supply N as NH_4^+ first, which upon oxidation releases H^+ ions (Magdof *et al*., 1997). The soil chemical properties of this experiment showed that soil pH was reduced by the chemical fertilizer treatment compared to the remaining treatments. It may be due to the chemical fertilizers used in this experiment, 15-15-15 and 8-24-24, which are nitrogenous fertilizers. These results were in agreement with the findings of Chit-aree *et al.* (2017), who also reported the effect of chemical fertilizer on soil acidity.

Moreover, the results in this experiment also showed that soil pH in 40% effluent treatment decreased as compared to the other effluent treatments. This may be also due to the 40% effluent treatment consisted of the highest amount of nitrogen which affected soil acidity. However, this result was different as compared to the report of Chit-aree *et al.* (2017) which found that biogas effluent did not affect soil pH. This difference may due to high nitrogen concentration in biogas effluent of this experiment (4900 ppm) while it was 1000 ppm in the experiment of Chit-aree *et al*. (2017).

In this experiment, it was found that dry weight of roots in the control was the same as the dry weight of roots in the chemical fertilizer and 40% effluent treatments. These results agree with the research of Chit-aree *et al.* (2017) who found that the dry weight of marigold roots was decreased by soil acidity which was 3.72 and 4.60 in the chemical fertilizer and 40% effluent treatments, respectively. The dry weight of branch in 10%, 20% and 30% effluent treatments did not decrease as compared to the chemical fertilizer treatment. On the other hand, growth of leaf was reduced by all effluent treatments when compared to the chemical fertilizer treatment. The reduction of growth by the biogas effluent treatment was most severe in the leaf as compared to root and branch. Finally, total fresh and dry weights as influenced by all effluent treatments tended to be lower than total fresh and dry weight resulting from the chemical fertilizer treatment.

Plant nutrients are necessary for plant growth and survival. Nitrogen, phosphorus and potassium are essential nutrients for plant growth (Taiz and Zeiger, 2006). In this experiment, total amount of nitrogen, phosphorus and potassium which plants received from the chemical fertilizer treatments was 4.25, 8.25 and 8.25 g, respectively, while plants treated with 30% biogas effluent received 3.82 g of nitrogen, 1.01 g of phosphorus and 2.81 g of potassium. The total amount of nitrogen, phosphorus and potassium applied to plants gradually decreased with the concentration of biogas effluent. The treatment containing the lowest nutrient concentration was the control treatment. Thus, the lower nitrogen, phosphorus and potassium concentrations in 10%, 20% and 30% effluent treatments as compared to the chemical fertilizer treatment may be the cause of growth reduction resulting from these effluent treatments.

On the other hand, total amount of nitrogen, phosphorus and potassium which plants received from 40% biogas effluent treatment was 5.10, 1.35 and 3.74g, respectively. It showed that total amount of nitrogen, phosphorus and potassium was higher than that of 10%, 20% and 30% effluent treatments. However, total fresh and dry weights in 40% effluent treatment were lower as compared to 10%, 20% and 30% effluent treatments. A report by Chaikachang

et al. (2017) showed that the high concentration of effluent was also high in electric conductivity of effluent. When this high EC effluent was used to water the soil, soil EC also increased and resulted in low plant growth. The EC of 10%, 20%, 30% and 40% effluent in this experiment was 3.49, 4.40, 2.50 and 7.11 ds/m, respectively. The EC of 40% effluent was highest as compared to the other concentration. This may be the reason for growth reduction by the 40% biogas effluent treatment.

In this experiment, it was found that low plant nutrients in biogas effluent treatments effected to the number of blooming flowers while it did not affect initiation of first flower, fresh and dry weights of blooming flowers. These results were to agree with the findings of Chit-aree *et al*. (2017), who also reported the effect of low nutrient concentration in biogas effluent on blooming flower number.

Nitrogen is an important component of chlorophyll which is the major pigment for photosynthesis (Taiz and Zeiger, 2006). Results showed that total chlorophyll and concentrations were decreased by the control, 10% and 20% effluent as compared to the remaining treatments. This may be due to the low nitrogen concentration resulting from the control, 10% and 20% effluent treatments as explained above. This low chlorophyll concentration resulting from the control, 10% and 20% effluent treatments may also influenced total fresh and dry weights of marigold.

The growth parameters of marigold in this experiment were enhanced to a greater degree compared to the results of Chit-aree *et al.* (2017). For example, plant height, stem diameter, bush diameter, total chlorophyll, chlorophyll a and b concentrations and branch dry weight in 30% effluent treatment of this experiment was equivalent to those resulting from the chemical fertilizer treatment while they were lower those of the chemical fertilizer treatment in the report of Chit-aree *et al*. (2017). It may because of the biogas effluent in this experiment contained higher concentration of nitrogen as compared to the report of Chit-aree *et al*. (2017), 24% biogas effluent had only 1.70 g of nitrogen as bio-fertilizer. Moreover, the number of blooming flowers was still low in this and Chit-aree *et al*. (2017) experiments. Such results may be due to the low concentration of phosphorus and potassium in the effluent of both experiments. Therefore, identifying materials that can produce biogas and that contain high concentration of phosphorus and potassium should be further investigated.

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Design and experimental verification of a small sized machine for making Vietnamese fresh *Bun* rice noodle

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Son, T. D. and Naoki, U. (2019). Design and experimental verification of a small sized machine for making Vietnamese fresh Bun rice noodle. International Journal of Agricultural Technology 15(2): 359-374.

Abstract Fresh *Bun* is the quintessence of Vietnamese cuisine. Nowadays in Vietnamese, fresh *Bun* has been produced by specialized production lines in factories. In other Asian countries, *Bun* is mostly imported from Vietnam and Thailand in a dried form. Customers prefer fresh *Bun* over dried one due to its different taste and combination with other cooking ingredients. In order to produce fresh *Bun* directly in restaurants and hotels, the *Bun* making machine must be small enough and have suitable power consumption to be installed and operated in these environments, while having the same quality as *Bun* produced by the industry. The proposed design therefore has the following technical specifications: Length: 1000 mm, Width: 500 mm, Height: 1350 mm, main materials: Stainless steel 316 (SUS 316), *Bun*'s diameter: 1.5 mm; 2.0 mm; 2.5 mm**,** Elasticity: 5000 - 5500 Pa**,** Color: White, Capacity: 20 Kg/hour. The machine has been built and experimentally verified, and the fresh *Bun* produced by the machine has the same quality with the industrially produced *Bun*.

Keywords: *Bun*, swelling factor, screw extruder

Introduction

Fresh *Bun* is a type of Vietnamese rice noodle used to make soup with sprouts, dill, split water spinach, cilantro, onion and broth and either beef or pork. It is convenient, easy to cook, delicious and nutritionally rich product and is now gaining great appraisal outside Vietnam. Fresh *Bun* is different from other Vietnamese rice noodle due to its production technology and the rice type used to make it. Currently, fresh *Bun* is produced in industrial environment with available high-power machines. Small sized machines that can be placed in restaurants to produce fresh rice *Bun* noodle directly to serve customers is still a challenge. The developed small sized machines were found suitable to address the needs for restaurants and hotels in the world.

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Vietnamese fresh rice noodle has many types as *Pho, Bun, Banh hoi, Hu tieu,* which are categorized according to making processes. The process of making Bun is illustrated in Figure 1.

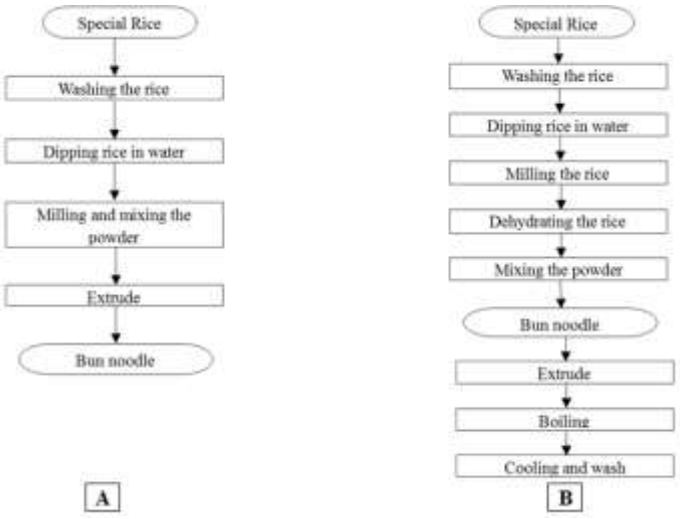

Figure 1. Making Processes of Fresh Bun Rice Noodle A-in proposed machine, B-in conventional industry

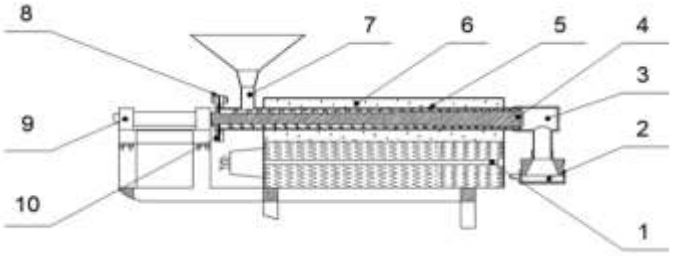

Figure 2. Equipment used in industrial production 1-Electrical resistor, 2-Extrusion mold, 3-Cylinder's cover, 4-Rice powder,5-Screw extruder, 6-Water, 7-Liquid powder feeder, 8-Seal housing, 9-Bearing, 10-Seal

Figure 2 showed the structure of the conventional industrial machine. When heating, the temperature of the screw extruder is the same along its longitudinal direction, it is usually from 75^0 C to 80^0 C. The *Bun*'s surface in contact with the inner surface of the cylinder can reach up to 95^0 C. The 75^0 C – 95^0 C range can complete only about 50% of *Bun* cooking, so the extruded *Bun* must go through further boiling. If heating is over 100^0 C, the powder is

instantly solidified and burnt, and it gets stuck in the cylinder and making the process of detaching from the screw extruder difficult.

The proposed machine is not a miniaturized version of its industrial counterpart but an entirely new design with an optimized heat transfer process suitable for swelling the rice (Bhesh *et al.*, 2013), and also is capable of changing key parameters like heat and pressure while maintaining product's quality. With this design, fresh Bun produced with the proposed machine does not need further boiling as in industrial production process.

Rice grain is the base ingredient for making *Bun*, which has a long round appearance and is highly expansive after cooking. The type of used rice is not sticky but dried rice.

The South Vietnamese market has many brands of rice, such as *504* and *Long Dinh*, while northern markets usually provides *DT10, CR203, 13/2, Moc Tuyen, VN10* and *Khang Dan* brands. In the United States there are many types of long grain rice imported from Mexico, Thailand and India that can be used to make *Bun* like *Basmati* (India), *Sarita* (Mexico). In Japan there is *Yume Toiro*, and so on.

The starch content in rice is around 60%, protein is from 7,57% to 10,84% and amylose is higher than 25% (Hoang and Ngo, 2006), (Juliano, 2004).

Materials and methods

Parameters requirement

Fresh *Bun* produced with the proposed machine should satisfy the technical and microbiological requirements is given in Table 1.

Table 1. Specifications for a small sized machine and produced *Bun*

Specifications for small sized machine		Required Specifications for Bun	
Length of a Machine (mm)	1000	***Bun*'s diameter (mm)**	1.5; 2.0; 2.5
Width of machine (mm)	500	**Elasticity (Pa)**	5000 – 5500
Height of machine (mm)	1350	**Color**	white
Production capacity (kg/hour)	20	**Microbiological indicators**	46/2007/QĐ-BYT

Conceptual design

Principles

In industrial production line, in addition to extrusion process performed by the screw extruder, there was pressing process performed by a piston-cylinder structure as illustrated in Figure 3.

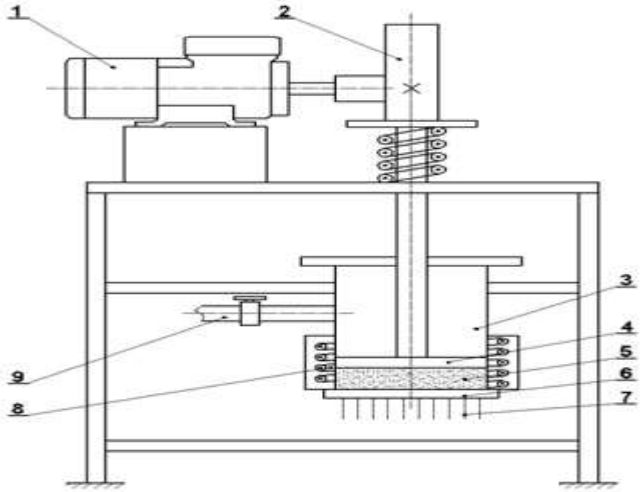

Figure 3. Fresh *Bun* pressing process by a piston – cylinder 1-Electric Motor, 2-Cam mechanism, 3-Cylinder, 4- Piston, 5-Rice Powder, 6-Mold, 7-Fresh Bun, 8-Electrical resistor, 9-Rice powder feeder

This process was simple in principle but difficult to design the mechanism and optimize the heat transfer process. Furthermore, it is complicated and time-consuming to clean the machine after shifts, and therefore unsuitable for restaurants and hotels environment.

In order to satisfy the specifications in Table 1, in the proposed design illustrated in Figure 4, the milled rice powder is poured into the feeder (5), and the extrusion screw (7) is heated by water boiled by the electrical resistor (3). When the temperature of water reaches 90^0 C, a valve on the feeder is opened and liquid rice powder flows into the cylinder. The motor (1) is turned on to actuate the screw driven by belt mechanism (2). Initially, the frequency converter is set to 20 Hz and the screw rotates slowly to convert the liquid rice powder to a solid form. After a few minutes, the frequency converter is set to 30-32 Hz. The cooked powder is forced through the hole in the mold (13). Fresh *Bun* falls into the sink and is ready for use. If fresh *Bun* elasticity was not

satisfactory, the cover (12) can be tightened or loosened manually to increase or decrease the extrusion pressure.

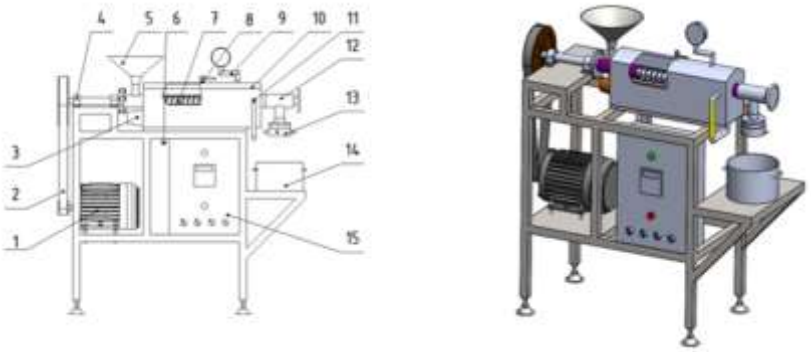

Figure 4. Schematic diagram of the proposed machine 1-Electric motor, 2-Belt drive, 3-Heating resistor , 4-Bearing housing, 5-Rice powder feeder, 6-Water outlet, 7-Screw shaft, 8-Water inlet, 9-Temperature gauge, 10-Water tank, 11-Water level indicator, 12-Cylinder's cover, 13-Mold, 14-Production tank , 15-Electric box.

Specific design

Cylinder Design

The cylinder encloses the extrusion screw, so that the rice powder can be compressed and moved from the feeder to the mold. In addition, the cylinder's wall acts as a medium for heat transfer from the boiled water to the extrusion screw. The structure and size of the cylinder corresponds to the change in the structures of the rice powder under the effect of heat illustrated in Figure 5.

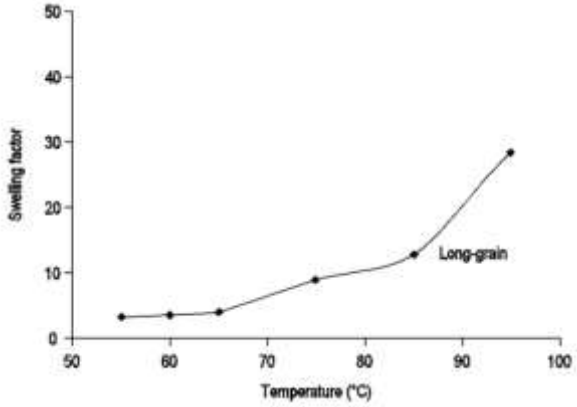

Figure 5. Swelling factor of rice type long grain (Bhesh *et al.*, 2013)

The long - grain curve in Figure 5 described the change in expansion of the powder by temperature. By considering that the temperature of steam is unchanged, the thickness of the cylinder's wall must change in order to provide different temperature range that corresponds with the temperature range shown in Figure 5.

The cylinder made of SUS 316 has a shape in Figure 6; inside radius r_1, outside radius r_2, mean radius r, thermal conductivity λ constant, temperature at walls t_{w1} and t_{w2}. Differential equation for heat conduction is given by (Mohr and Mallouk, 1959):

$$\frac{\partial t}{\partial \tau} = a \times \left(\frac{\partial^2 t}{\partial r^2} + \frac{1}{r} \times \frac{\partial t}{\partial r} + \frac{1}{r^2} \times \frac{\partial^2 t}{\partial \varphi^2} + \frac{\partial^2 t}{\partial z^2}\right) \quad (1)$$

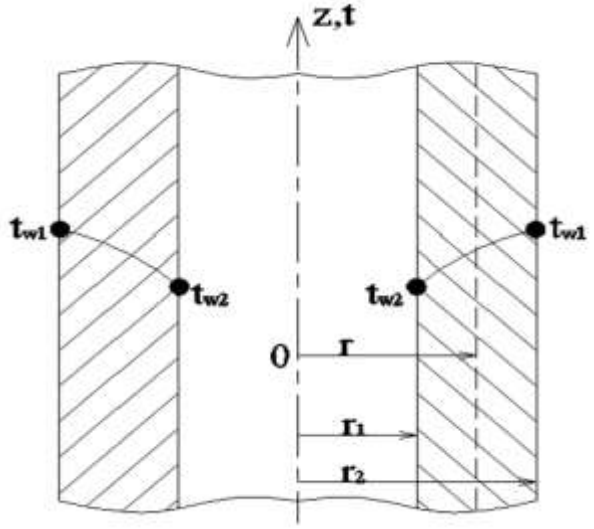

Figure 6. Structure of the cylinder used in calculation

The heat in the steamer should follow the long-grain line in Figure 5 to gradually swell and achieve good texture and elasticity after cooking. From this long – grain line, it can be seen that the expansion of flour during the ripening process did not change much between 55^0 C and 65^0 C, and began to vary gradually in the range 65-85^0 C, then changed rapidly in the range 85 -100^0 C. Therefore, during the ripening process, the amount of heat that the powder receives to increase its temperature should be appropriated to maintain the quality of *Bun*.

The slurry is boiled and cooked by steam during the process through the screw extruder. At the end of the process, *Bun* was ready to use and re-boiling or cooking are not required.

By assuming that the heat transfer through a cylinder radially in a steady state, the unidirectional condition is given by:

$$\frac{1}{r} \times \frac{dt}{dr} + \frac{d^2t}{dr^2} = 0 \quad (2)$$

Conditions $t_{r=r1} = t_{w2}$ and $t_{r=r2} = t_{w1}$ lead to:

$$t = t_{w1} - \frac{t_{w1} - t_{w2}}{ln\frac{r_2}{r_1}} \; ln\frac{r}{r_1} \quad (3)$$

Table 2 showed the parametric values for the cylinder design, where the thickness of cylinder's wall is $\delta = r_2 - r_1$.

Table 2. Outside radius design of the cylinder following Figure 5

t_{w1} (^{o}C)	100	100	100	100	100	100	100
t_{w2} (^{o}C)	55	60	65	70	75	80	85
t (^{o}C)	60	65	70	75	80	85	90
r_1 (mm)	21	21	21	21	21	21	21
r_2 (mm)	26	25,39	24,79	24,2	23,63	23,07	22,53
r (mm)	25,39	24,79	24,21	23,63	23,07	22,53	22,01
δ (mm)	4,39	3,79	3,21	2,63	2,07	1,53	1,01

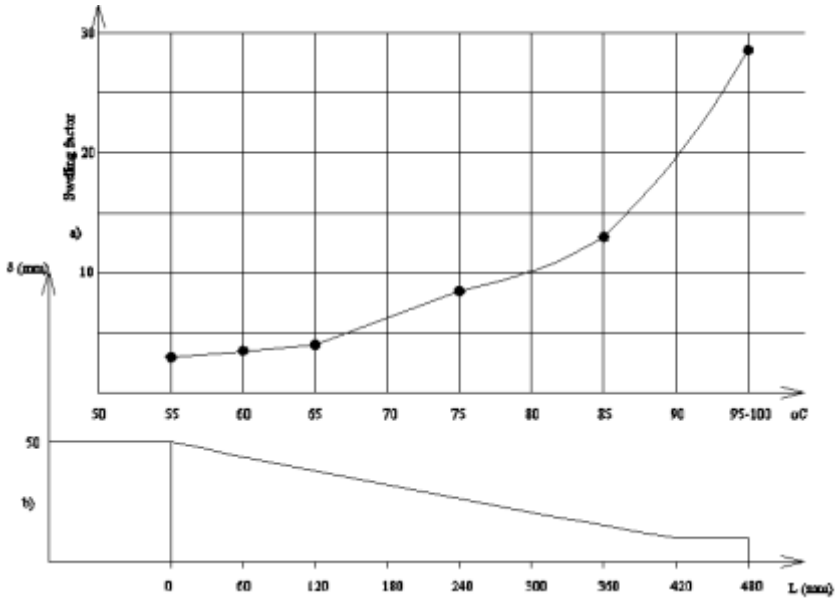

Figure 7. Cylinder's thickness satisfying Figure 5

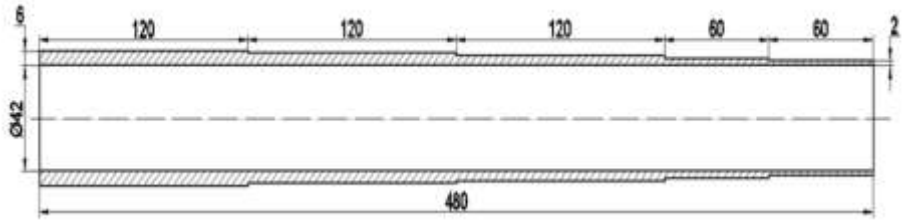

Figure 8. Cylinder structure corresponding to Figure 7

The cylinder shown in Figure 8, its diameter varied along its length to simplify the manufacturing process, although there were only 5 temperature regions in the real cylinder as opposed to 6 regions given in Figure 7. The resulting effect was acceptable. In addition to changing the outside dimension of cylinder, it was able to change the temperature that acts on the cylinder along its longitudinal direction.

Design of screw extruder

Extrusion is carried out by a single-screw extruder illustrated in Figure 9, which has the following parameter values:

Compression ratio of the screw: 2.58

Barrel diameter: D = 41.5 mm

Input core diameter: D_a = 21 mm

Output core diameter: D_b = 35 mm

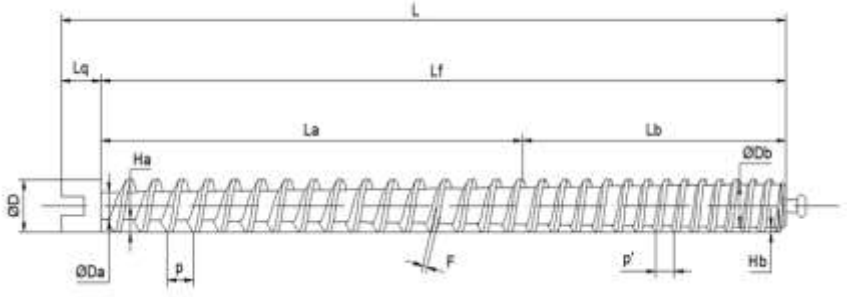

Figure 9. Screw Shaft

Design of enclosed steam chamber

The input parameters used to calculate heat and heat loss of the machine given in Table 3. Table 4 showed the results for heat and heat loss calculation.

Table 3. Parameters values used for design of steam chamber

Number	Parameter for calculation	Symbol	Value	Unit
1	Dimension of steam chamber	L x W x H	420 x 140 x 150	mm
2	The height of water in the chamber	H_{nc}	100	mm
3	Temperature of environment	t_{mt}	25	^oC
4	Elevated temperature of water	$t_s = t_{w1}$	110	^oC
5	Saturated pressure of water at 110 ^oC	p_{bh}	1.4326	bar
6	Specific heat of water	C_{pnc}	4.18	kJ/kg.K
8	Density of water	ρ_{nc}	1000	kg/m^3
9	Specific heat of steel SUV 304	c_{ptv}	0.46	kJ/kg.K
10	Specific volume of steel SUV 304	ρ_{tv}	7850	kg/m^3
11	Specific heat of steel used to make the chamber	C_{pb}	0.5	kJ/kg.K
12	Specific volume of steel used to make the chamber	ρ_b	7980	kg/m^3
13	The coefficient of convection of air	α_2	10	$W/m^2 K$
14	Thermal conductivity coefficient of SUV 304	$\lambda_1 = \lambda_3$	16.3	W/m. K
15	Thermal conductivity coefficient of insulator	λ_2	0.0425	W/m. K
16	Time to boil water to 110^oC	τ_1	8	minute
17	Specific heat of flour mixture	c_{pbot}	2.76	kJ/kg.K
18	Density of powder mixture	ρ_{bot}	1020	kg/m^3
19	Machine productivity	G_{bot}	20	kg/h
20	Temperature on the outside surfaces of the chamber	t_{w4}	40	^oC
21	Shell thickness of chamber	δ_1	3	mm
22	Thickness of stainless steel layer wrapped outside the chamber	δ_3	1	mm

Design of noodles mold

The quality of the *Bun* depends on the size, roughness and delivery ducts of the mold, which can be achieved by machining the mold with either High-Speed Machining or Electrical Discharge Machining (López De Lacalle *et al.*, 2002). The design of the mold must ensure that the *Bun* has good appearance and is smooth on the surface, and the fiber flows out of the mold quickly and easily. The design of the mold for 4 mm fresh *Bun* is given in Figure 10, which has hole-shape conical cylinder and the *Bun* is easy to pass through the holes.

Table 4. Design of noodles mold

Number	Values need to calulate	Symbol	Equation	Value	Unit
1	The mass of water in the chamber	G_{nc}	$V_{nc}.\rho_{nc}$	5.88	kg
2	Heat to boil the water	Q_{nc}	$G_{nc}.c_{pnc}.\Delta t$	2104.16	kJ
3	The amount of heat to heat the screw	Q_{tv}	$G_{tv}.c_{ptv}.\Delta t$	79.33	kJ
4	The amount of heat to heat the cylinder	Q_v	$G_v.c_{pv}.\Delta t$	49.72	kJ
5	The amount of heat to heat the chamber	Q_b	$G_b.c_{pb}.\Delta t$	282.2	kJ
6	Total heat to boil the water	Q_1	$Q_{nc} + Q_{tv} + Q_v + Q_b$	2515.41	kJ
7	Power consumption of resistors	$P_{đt}$	$\frac{Q_1}{\tau_1}$	5.24	kW
8	Thickness of wool insulator layer	δ_2	$q = \frac{t_{w1-}\ t_{w4}}{\frac{\delta_1}{\lambda_1} + \frac{\delta_2}{\lambda_2} + \frac{\delta_3}{\lambda_3}}$	22	mm
9	Heat loss in τ_2 = 1 hour	Q_{mt}	$q.F.\tau_2$	259.2	kJ
10	Total heat needed in τ_2 = 1 hour	Q_2	$G_{bot}.c_{pbot}.\Delta t$	4692	kJ
11	Total heat consumed in τ_2 = 1 hour	Q_t	$Q_{mt} + Q_2$	4951.2	kJ
12	Working time of resistors in τ_2 = 1 hour	τ_3	$\frac{Q_t}{P_{đt}}$	15.75	minute

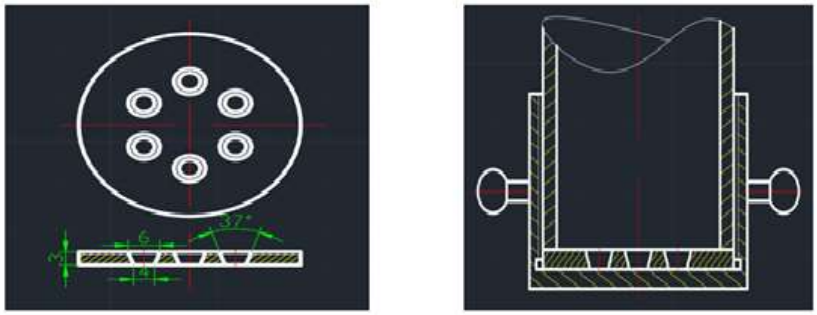

Figure 10. *Bun* mold C- The arrange the hole on the mold, D- Extruder assembly

Results

Special rice collected from markets is usually the 504 type of Vietnam and the long grain rice Sarita of Mexican and American Markets. Rice is dipped

in the water for 6 hours and it is milled (Figure 11) by the wet method with 1:1 ratio (rice/water). The moisture content of the slurry is maintained within the range of 50–55%. After the proposed machine was fabricated (Figure 12), fresh *Bun* was produced (Figure 13) in order to evaluate the reliability of the machine and the quality of fresh *Bun*.

Quality analysis of fresh Bun

There are many factors that determine whether the fresh Bun satisfies the requirements in Table 1 or not. Ultimately, the fresh Bun must also satisfy the customers in term of taste and food safety. The following three factors that affected to the quality of Bun are considered in this study.

Material viscosity, too high viscosity of material causes damage to the machine and making it difficult to press. In contrast, if the viscosity is too low, the molecules are less flexible, so they are difficult to align with each other to make the bond tight, making Bun cracked.

The temperature of the gelatinization of the powder in the screw: High gelatinization temperature of the powder in the screw makes the water molecules more dynamic, and it easily diffuses into the powder mix to shorten the gelatinization time. However, if the gelatinization temperature is too high, it hydrolyzes the starch and affects the toughness of the vermicelli later. In contrast, low temperature slows down the gelatinization time. If the temperature is too low, it cannot gelatinize the powder. Rotational speed of the screw extruder: Together with the gelatinization temperature, it determines whether the slurry is completely gelatinized and cooked or not.

Figure 11. Rice milling machine

Figure 12. Proposed fresh *Bun* making machine prototype

Figure 13 E-Small sized machine in the process of making *Bun*, F-Produced fresh *Bun*

Sensory evaluation

Sensory evaluation is an important criterion for the final acceptance of food quality. After introducing new processing equipment, it is essential to check the consumer acceptability of the processed products, in terms of "color", "taste", "texture" "overall acceptability", and so on. For fresh Bun, four samples were produced using the same type of rice (504 brand), using the same milling equipment and with the same moistures for the powder. The machine

temperature setting is different for each sample. Sample 1: T = 90^0 C, sample 2: T = 95^0 C, sample 3: T = 100^0 C, sample 4: T = 105^0 C. The evaluation team of four people judges the sample 2 to be the best, with similar qualities to sample 5 obtained from the market. The sample 2 is then used to analyze the quality of the products.

Microbiology of Bun

TỔNG CỤC TIÊU CHUẨN ĐO LƯỜNG CHẤT LƯỢNG
TRUNG TÂM KỸ THUẬT TIÊU CHUẨN ĐO LƯỜNG CHẤT LƯỢNG 3
QUALITY ASSURANCE & TESTING CENTER 3
QUATEST 3

KT3 – 03377AVS8

PHIẾU KẾT QUẢ THỬ NGHIỆM
TEST REPORT

24/09/2018
Page 01/01

1. Tên mẫu / *Name of sample*: BÚN
2. Mô tả mẫu / *Sample description*: Mẫu thử nghiệm do khách hàng lấy mẫu, tên mẫu và thông tin về mẫu do khách hàng cung cấp./*Testing sample was sampled by customer, sample name and sample information were supplied by customer.* Mẫu đựng trong hộp nhựa./ *As received sample contained in plastic box.*
3. Số lượng mẫu / *Quantity*: 01 (500 g)
4. Ngày nhận mẫu / *Date of receiving*: 15/09/2018
5. Thời gian thử nghiệm / *Testing duration*: 17/09/2018 –24/09/2018
6. Nơi gửi mẫu / *Customer*: **HO CHI MINH UNIVERSITY OF TECHNOLOGY**
268 Ly Thuong Kiet, Ward 14, District 10, Ho Chi Minh City
7. Kết quả thử nghiệm / *Test results*:

Tên chỉ tiêu / *Characteristic*		Phương pháp thử / *Test method*	Kết quả thử nghiệm / *Test result*
7.1. Tổng số vi sinh vật hiếu khí / *Total aerobic plate count*	CFU/g	ISO 4833 - 1 : 2013	$4,5 \times 10^2$
7.2. Coliforms,	CFU/g	ISO 4832 : 2006	< 10 (*)
7.3. *Escherichia coli*,	CFU/g	ISO 16649 – 2 : 2001	< 10 (*)
7.4. *Staphylococcus aureus*,	CFU/g	AOAC 2016 (975.55)	< 10 (*)
7.5. Tổng số nấm men, / *Total yeast,*	CFU/g	ISO 21527 – 1 : 2008	< 10 (*)
7.6. Tổng số nấm mốc, / *Total mould*	CFU/g	ISO 21527 – 1 : 2008	< 10 (*)

Ghi chú/*Notice*: (*) Theo phương pháp thử, kết quả được biểu thị nhỏ hơn 10 CFU/g khi không có khuẩn lạc mọc trên đĩa. / *According to the test method, the result is expressed as less than 10 CFU/g when the dish contains no colony.*

PHỤ TRÁCH PTN VI SINH - GMO
HEAD OF MICROBIOLOGY - GMO TESTING LAB.

Trần Thị Ánh Nguyệt

TL. GIÁM ĐỐC/ *PP.DIRECTOR*
TRƯỞNG PHÒNG THỬ NGHIỆM/
HEAD OF TESTING LAB

KỸ THUẬT TIÊU CHUẨN ĐO LƯỜNG CHẤT LƯỢNG 3

Ngô Quốc Việt

Head Office: 49 Pasteur, Q1, Hồ Chí Minh City, VIỆT NAM Tel: (84-28) 3829 4274 Fax: (84-28) 3829 3012 Website: www.quatest3.com.vn
Testing: 1 Road 1, Biên Hòa 1 Industrial Zone, Đồng Nai Tel: (84-251) 383 6212 Fax: (84-251) 383 6298 E-mail: tn-cskh@quatest3.com.vn

Figure 14. Test on microbiology content of *Bun*

Bun sample tested satisfied all requirement for microbiological content according to the standard set by Vietnamese Ministry of Health (Fig.14).

Tensile stress of Bun

The experimental setup for testing samples 2 and 5 is shown in Figure 15. The results were similar to each other so this paper only showed the testing result for sample 2 in Figure 16 and Table 4. Bun produced using the proposed machine has similar physical properties to Bun currently sold in the market, the machine enables to provide the satisfactory results.

Figure 15. Experimental set-up

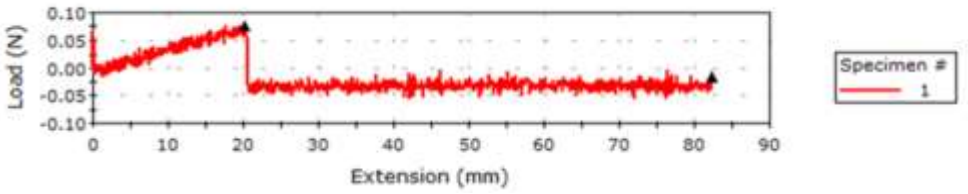

Figure 16. Tensile stress and extension of Bun samples 2 and 5

Table 5. Testing results for sample 2

	Specimen label	Load at Break (Standard) (N)	Maximum Load (N)	Tensile stress at break (Standard) (MPa)	Tensile extension at maximum load (mm)	Extension at break (Standard) (mm)	Tensile extension at break (Standard) (mm)	Tensile stress at maximum load (MPa)
Mean Value	Bun	-0.02	0.07757	-0.00533	20.1014	82.30218	82.32018	0.02469

Discussion

The developed small sized machines were found suitable to address the needs for restaurants and hotels in the world. The originality of study is that cylinder structure is calculated following the swelling of the long-grain rice (Bhesh *et al.*, 2013).This reaserch is not found in any reference cited and this study is different with industrial cylinder structure. Due to the thermal analysis and calculations performed in this article, quality of Bun is maintained with short heat exposure duration, and Bun produced does not need a following boiling operation. Other calculations performed in "Design of enclosed steam chamber" section are similar to other references (Yunus, 2003). The Bun noodle is produced with the proposed small sized machine satisfies sufficient textural and physical properties and safe microbiological content. The proposed small sized structure and the power source requirement ensure that most home, hotels and restaurants can afford to install and operate it. In addition, customers can enjoy seeing the process while eating.

Acknowledgement

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Number of topic: 09/2018/TN

The authors would like to thank the students Nam Phuong Thanh Le and Minh Cong Tong for technical support during the experimental verification**.**

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Probiotication of black grass jelly [*Mesona chinensis* (Benth.)] by encapsulated *Lactobacillus plantarum* Mar8 for a ready to drink (RTD) beverages

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Wulandari, N. F., Suharna, N., Yulinery, T. and Nurhidayat N. (2019). Probiotication of black jelly [*Mesona chinenesis* (Benth.)] by encapsulated *Lactobacillus plantarum* Mar8 for a ready to drink (RTD) beverages. International Journal of Agricultural Technology 15(2): 375-386.

Abstract *Lactobacillus plantarum* is an importantly probiotic bacteria for intestinal microbiota. The use of encapsulated the probiotic *Lactobacillus plantarum* Mar 8 by agar, carrageenan, Arabic gum, konjac (*Amorphophallus konjac* K.Koch), and black jelly [*Mesona chinensis* (Benth.)] for its application for RTD packaged beverages product was investigated. The probiotic bacteria was prepared by cultivation, biomass collection by suspension, and encapsulation. The result showed that 2% of carrageenan was the best encapsulant for the probiotic based on its suitable elasticity of the black jelly for RTD use. The probiotic was maintained at 8-9 log cfu/ml. This result would be potential for the application of encapsulated probiotic for RTD jelly black beverages.

Keywords: beverages, microcapsule, probiotic, storage, viability

Introduction

The black jelly grass is known as a healthy food or drinks (Handayani *et al.*, 2017). This jelly has a beneficial effect on health because that this jelly contains a high concentration soluble fiber and low-calorie composition. High soluble fiber has been proven to lower lipid profiles (Ramos *et al.*, 2011; Wahyono *et al.*, 2015; Handayani *et al.*, 2017). The Indonesian Nutrition Directorate Health Department confirms that 100 grams of grass jelly gel contains 6.23 grams of crude fiber. Hence, daily consumption of black grass jelly along with fruits and vegetables will fulfill personal needs in daily fiber (30 g). The black grass jelly also showed antioxidant activity due to its phenol content. This component has been reported to prevent DNA damage in human lymphocytes when it is exposed to free radicals such as hydrogen peroxide and UV radiation (Tasia and Widyaningsih, 2014; Handayani *et al.*, 2017).

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Recently, black grass jelly (*Mesona palustris* Bl) was reported to have anti-dyslipidemia in high cholesterol diet-fed rats (Handayani *et al*., 2017).

Although black grass jelly is good as a healthy food or drink, adding more beneficial effect on health such as by a probiotication is one of best choice supplements. Probiotics are live microorganisms, which when ingested in sufficient amount, could exert a health benefit on the host (FAO/WHO, 2001). Its benefits of health is as a result of the effect of their properties such as antimicrobial (bacteriosin) activity (Seddik *et al*., 2017; and adherent ability to specific mannose (*L. plantarum* in particular) (Adlerberth *et al*., 1996; Pretzer *et al*., 2005) to compete against harmful bacterial guts. Lactobacillus is grouped in to Lactic acid bacteria (LAB) which constitute a diverse group of Gram-positive, catalase-negative bacteria producing lactic acid as the main end-product of carbohydrate fermentation (Felis and Dellaglio, 2007). Lactobacillus has more than 220 valid species (http://www.bacterio.net/lactobacillus.html) definitely make this genus the main and diverse LAB group. Isolation of Lactobacilli has been made from different ecological niches, and diverse studies show diversities at their genetic and physiological levels (Seddik *et al.*, 2017; Liu *et al.*, 2018). *L. plantarum* is one of the potential probiotics which have a similar beneficial effect on health. *L. plantarum* fulfils the qualified presumption of safety (QPS) status from the European Food Safety Authorities (EFSA) and has been confirmed as the generally recognized as safe (GRAS) status from the US Food and Drug Administration (US FDA). This species also has been related with history of food use (Mogensen *et al.*, 2002; Seddik *et al.* 2017). With regard to a well-characterized bacterium with documented safety, *L. plantarum* is offering various applications along with which its use as probiotic. This highly versatile microorganism is found in wide range environments. This versatility is reflected in its genome plasticity. At present, numerous evidences are constituted on the role of *L. plantarum* in medical cases as diarrhea prevention, cholesterol lowering, and reduction in the IBS symptoms. More benefits value of *L. plantarum* comes from strains with capability to produce plantaricins, which are bacteriocins with specific structural and functional diversities. These bacteriocins present a wealth panel of applications in medical, veterinary, and food domains (Seddik *et al.* 2017).

Our potential *L. plantarum* Mar8 was originated from a tropical fruit and this strain showed to produce plantaricin A (Yulinery and Nurhidayat, 2015). This strain also had showed a stable mannose specific adhesin (MSA) (Nurhidayat, 2012). Based on these properties, this strain was ideal for use as a probiotic to deliver for gut health.

We continue to a new our assessment on another potential probiotic, *L. plantarum* Mar8 to apply as a probiotic supplement for black grass jelly as

RTD beverage. To meet with the condition that will affect the viability and stability of the probiotic, an encapsulation should be made to prolong span life of probiotic during processing or storage of the drink product. An encapsulation of probiotic is aimed at more resistance of the probiotic to such bad environment (very low pH and saline condition) and come to the gut environment at its adequate density for gut health.

We studied potential encapsulant which was suitable one for its application for RTD beverages, such as carrageenan, gum arabic, konjac and agar. The objectives of the research was to get the best microencapsulant for *L. plantarum* Mar 8 which was suitable for the black grass jelly RTD beverages.

Materials and methods

Cultivation of Probiotic Bacteria

One ose of isolate of *Lactobacillus plantarum* Mar8 from culture was inoculated on 200 ml of sterile *Lactobacillus* MRS Broth (Himedia RefM369-500g) as mother stock. After incubated for 24 hours, 200 µL were added to the 200 ml MRS Broth.

Biomass collection by suspension

The pellet of *L. plantarum* Mar8 were centrifugated at 10,000 rpm (round per minutes) for 15 minutes. The pellet were collected.

Encapsulation by Cincau and Stabilizer with Various Concentrations

Four grams of cincau are mixed in 1 L boiling water and stirred carefully. Encapsulants (konjac, carrageenan, gum arabic, commercial order) with different concentrations (0.5; 1; 1.5; 2) were added to the encapsulation process with *L. plantarum* Mar8 in ambient (37^oC) and cold temperature (4^oC).

Measurement of Syneresis Rate, Breaking Strength, Flow rate average, texture, pH

L. plantarum Mar8 which was encapsulated with grass jelly and varied encapsulant was then calculated the syneresis rate, breaking strength, flow rate average, texture and pH. Syneresis was measured by calculated the loss of weight during storage compared to initial weight (Latimer, 2012). The breaking

was measured by using Lloyd instrument, pH by pH meter, flow rate by time counting on syneresis, and texture by direct observation.

Viability test after Encapsulation

The encapsulation results were stored in pyrex, glass cups or microwavable plastic bowls so that they remain sterile. After the encapsulation were complete, the cincau/jelly mix were store ambient (37^oC) and cold temperature (4^oC). The Total Plate Count were conducted in serial dilution 10^{-4}, 10^{-5} and 10^{-6}. Colony counting were done after 36-48 hours of incubation period at room temperature and cold temperature.

Results

Rate of Syneresis

Figure 1 showed various rate of syneresis of encapsulants. Under ambient temperature the rate of syneresis was much lower compared to ambient temperature. Carrageenan and konjac showed lowest rate of syneresis compared to control, agar, Arabic gum and konjac.

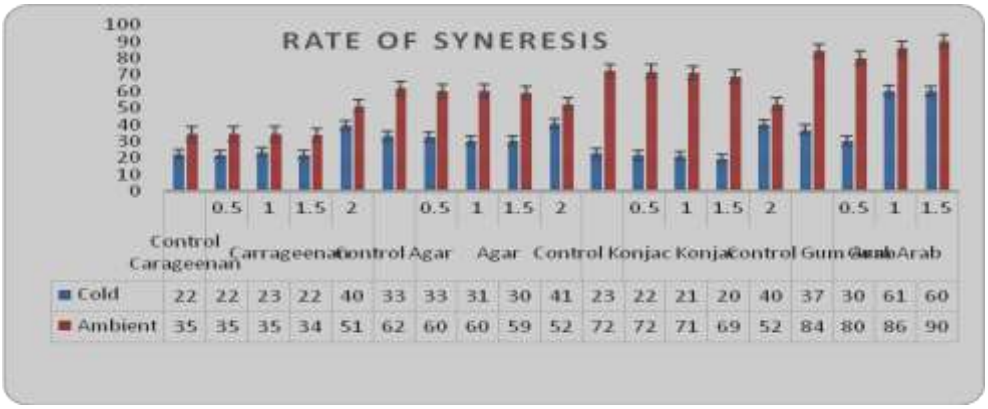

Figure 1. Rate of Syneresis of Various Encapsulants

Flow Rate of Syneresis

Figure 2 showed the average flow rate from concentration 0.5, 1.0, 1,5 and 2% of each encapsulants. Konjac has zero rate of syneresis but at 2% at 0.67, followed by carrageenan by increase from 0.2 to 0.67, agar by decrease from 1.1 to 0.56 and Arabic gum by increase from 1 to 7.

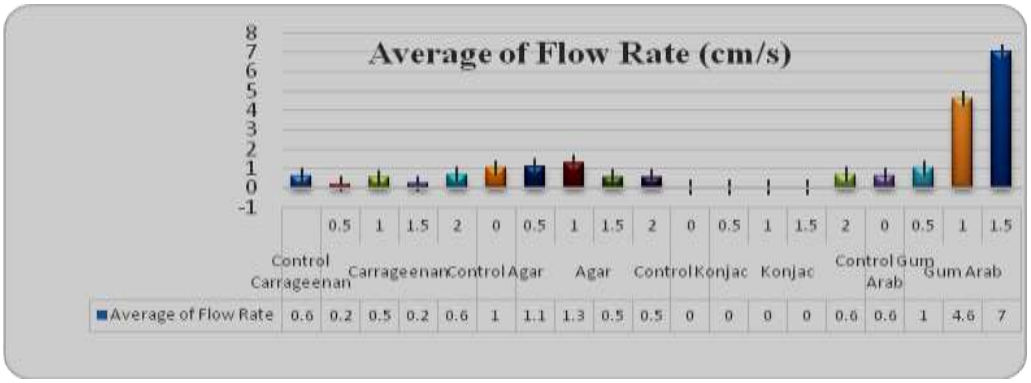

Figure 2. Average of Flow Rate of Various Encapsulants

Breaking Strength

Figure 3 showed that konjac was the highest value of breaking strength, followed by carrageenan, Arabic gum and agar. Konjac and carragenaan were almost the same breaking strength. But, Arabic gum showed decrease of its value at higher concentration. Arabic gum and agar have similar value of its breaking strength.

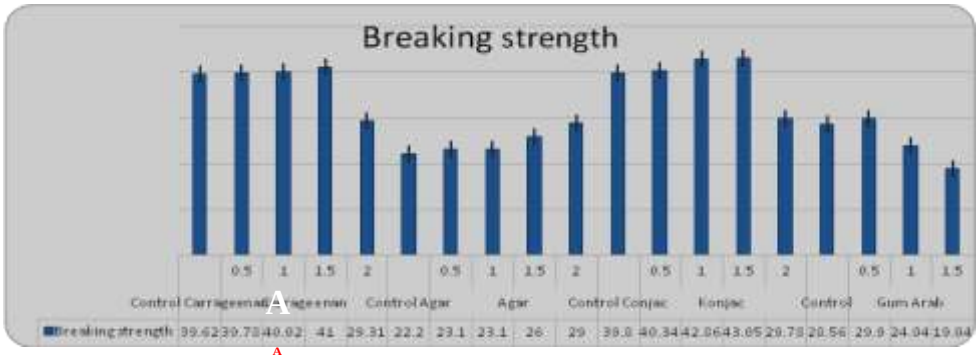

Figure 3. Breaking Strength of Various Encapsulants

Table 1 showed the profile of jelling agent of various encapsulants. All encapsulant have no differences of their pH value. But changes were occurred at ambient temperature to all jelling agent at every concentration, all pH value was decreased to 4. The textures were vary among the encapsulant. In general, carrageenan was the best jelling agent, followed by agar, konjac and Arabic gum. Similar jelling agent texture was showed by 0.5-1% concentration of each encapsulants except for konjac. At 1.5-2% concentration, only carrageenan had

stable jelling, konjac showed its texture to become stickier, while Arabic gum become easy to break down.

Table 1. Texture and pH value of encapsulants as jelling agent

Jelling agent (Encapsulant)		pH			Textures	
Treatment	**%**	**Before**	**Cold**	**Ambient**	**Cold**	**Ambient**
Control of carrageenan	0	7	7	4	-	-
carrageenan	0,5	7	7	4	j (+)	j (+)
	1	7	7	4	j (++)	j (++)
	1,5	7	7	4	j (+++)	j (+++)
	2	7	7	4	j (++++)	j (++++)
Control of Agar	0	7	7	4	-	-
Agar	0,5	7	7	4	j (+)	j (+)
	1	7	7	4	j (+)	j (+)
	1,5	7	7	4	J, ss (++)	J, ss (++)
	2	7	7	4	J, ss (++)	J, ss (++)
Control of konjac	0	7	7	4	-	-
konjac	0,5	7	7	4	s, d (+)	s, y (+)
	1	7	7	4	s, d (++)	s, y (++)
	1,5	7	7	4	s, d (+++)	s, y (+++)
	2	7	7	4	s, d (++++)	s, y (++++)
Control of Arabic Gum	0	7	7	4	-	-
Arabic Gum	0,5	7	7	4	j (+)	j (+)
	1	7	7	4	j (++)	j (++)
	1,5	7	7	4	b (+)	b (+)
	2	7	7	4	b (+++)	b (+++)

Note: j=jelly; s=solid; ss= somewhat solid; b= soft and easy breakdown; y=sticky; - = not measured; +=good; ++=better; +++= very good; ++++=excellent

Table 2 showed that all encapsulant were very good to keep the probiotic viable and stable. This result showed that viability was high (9 log cfu/ml). The best encapsulant was the mixture of cincau (black grass jelly) and agar (0.97 x 9 log cfu/ml) and followed respectively by cincau and konjac (0.84 x 9 log cfu/ml), cincau and carrageenan (0.70 x 9 log cfu/ml) and cincau and Arabic gum (0.67 x 9 log cfu/ml).

Table 2. The Viability of *Lactobacillus plantarum* Mar8 in Black Jelly Grass (Cincau) after Two Weeks of Storage at 4 ^{o}C

Cincau and Encapsulants	The Number of *Lactobacillus plantarum* Mar8 (CFU/ml)
Cincau and Konjac	0.84 x 9 log
Cincau and Carrageenan	0.70 x 9 log
Cincau and Gum Arab	0.67 x 9 log
Cincau and Agar	0.97 x 9 log

Discussion

The viability of microencapsulated probiotic is essential for probiotication as this state is the key point for the effectiveness of probiotication for an RTD beverage. The result showed a good indication that all encapsulant was suitable for the probiotic viability. At high density, 9 log cfu/ml, probiotication use was met with the effectiveness prerequisite for delivery to the gut. Probiotic has been known as all organic and inorganic food complexes, in contrast to harmful antibiotics, for the purpose of upgrading such food complexes as supplements. The term probiotic is technically defined as live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition (FAO/WHO, 2001; Mustafa, 2016; Seddik *et al.*, 2017). The definition of probiotics term has been in Guidelines for the evaluation of probiotics in food drafted by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Working Group as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). The guidelines give general *in vitro* tests to evaluate probiotic prospective, include resistance to acidity, bile acid resistance, adherence to mucus and/or human epithelial cells and cell lines, anti-microbial activity against potentially pathogenic bacteria, ability to decrease pathogen adhesion to surfaces, bile salt hydrolase (BSH) activity, and resistance to spermicides (appropriate to probiotics for vaginal use). In generally published reports, probiotics are commonly characterized by assessing acid resistance, bile salt tolerance, antimicrobial activity, antibiotic resistance, adhesion to intestinal epithelial cells, resistance to simulated gastric-intestinal juice and lysozyme. The population density of intestinal microorganisms elevate along the gastrointestinal (GI) tract: there are about 10^3 microorganisms/ml of luminal content in the duodenum, 10^8 microbes/g of ileum content, and 10^{12} microorganisms/g of colon content (O'Hara and Shanahan, 2006; Booijink *et al.*, 2007). Lactobacilli contribute the foremost probiotics in the human intestine, significantly playing a role by competing against pathogenic bacteria via adherence to and replication in the intestinal tract. Several characters were regarded as probiotic characters, such as resistant to acid, bile salt, simulated gastric and/or intestinal transit, and antibiotics, anti-microbial activity, ability of adhesion to intestinal epithelial cells, and inhibition of pathogen adhesion (FAO/WHO, 2002). Lots of data based on the probiotic criteria are fulfilled by *L. plantarum*. The high tolerance to acid and bile salt enables *L. plantarum* to transit through stomach and the upper gastrointestinal tract, while adherent ability to intestinal epithelial cells increase colonization in the lower gastrointestinal tract, and improve in competitive inhibition of pathogens (Liu

et al., 2018). Therefore, it is important to make sure that the viable probiotic must be alive and present in high number. In general, more than 10^9 cells is ingested daily dose (Vasudha and Mishra, 2013). Blurueanua *et al.* (2012) proposed that the minimal number of live probiotic minimally ranged from 10^5 to 10^6 CFU/ml or gram of the product as a functional food. While, other references showed adequately stimulation on the human health by consumption of 10^7-10^{10} live cells in a day (Kailasapathy and Chin, 2000; Arup and Shantanu, 2013). Certainly, every probiotic product should specify the minimum daily amount required for particular health benefit(s) (Vasudha and Mishra, 2013, FAO/WHO, 2002). More over, a study on the safety of two *L. plantarum* strains in Wistar rat model by a short-term oral toxicity assay was already reported. Rats have treated a normal dosage (about 9×10^9 CFU/kg/ day) or a high dosage (about 4.5×10^{10} CFU/kg/day) for 28 days. No adverse effect was observed after those periods, based on general behavior, growth, feed and water consumption, hematology, clinical chemistry indices, organ weights, or histopathologic analysis in the rats that have treated both dosages (Tsai *et al.*, 2014). Previously, investigation by Daniel *et al.* (2006) on mouse colitis model confirmed the safe trait of *L. plantarum* Lp-115 and its inability to translocate to extra intestinal organs. More recent, a study report of AB-LIFE® formulation containing three *L. plantarum* strains (CECT 7527, 7528, and 7529) stated its safety based on a 90-day repeated-dose study in rats. There was no adverse effect showed even after a high dose intake (1.85×10^{11} CFU/kg/day) (Mukerji *et al.*, 2016). These reports showed the safety of Lactobacillus consumption as probiotic even at very high dose (Seddik *et al.*, 2017). Numerous reports have exposed the effects of gut microbiota on cardiometabolic regulation and microcirculatory disturbances, and associating these with metabolic disorders such as hypercholesterolemia, obesity, diabetes, stroke and heart failure. (Turnbaugh *et al.*, 2006; Wang *et al.*, 2011; Mulders *et al.*, 2018). The use of probiotics ultimately alters gut environment and gut microbiota profiles (Liu *et al.*, 2018).

Syneresis is an important point to regard with before the encapsulant was applied that it might act as a jelling agent as well. The jelly could form different properties after applying the encapsulant, such as syneresis. Syneresis is related to water flow out from the substrate which caused by the presence of the pores. Syneresis is affected by several factors such as pH, temperature, set pressure, and properties of chemical composition of its solvent (Aurand and Woods, 1973), As the encapsulated probiotic which was trapped in the jelly which absorbed water, the syneresis will affect the presence of the encapsulated probiotic in the jelly; the probiotic will also come out from the jelly. The higher syneresis occurs at the jelly, the higher losses of the viable probiotic. The use

konjac or carrageenan as the encapsulant for probiotic was also good as the gelling agent as well. Konjac showed as the best gelling agent as it can keep water inside which means the probiotic might remain inside as well. While, the use of dextrin as an encapsulant for the *L. plantarum* Mar8 strain was already reported (Nurhidayat, 2012). However, this encapsulated probiotic was not suitable since this encapsulant will be damaged and discolored the drinks that are not desired besides the loss of its function as an encapsulant.

The physical effect of its encapsulant on the jelly grass will need consideration so as to make encapsulation and probiotication are ideal or meet with the prerequisite as probiotic for its application for RTD beverages. Therefore, the addition of the encapsulant must support the character of the jelly drink. The strength of the jelly is important to keep the jelly as in its unbreakable form. The breakdown of the jelly will also reduce the quality of jelly including the viability of the probiotics as they can come out causing decreasing of their viability. Carrageenan or konjac showed its good function to maintain the jelly in the good form as a result from its elasticity. Agar was not suitable for keeping the gel from breakdown. But the use of Arabic gum showed lower value in breaking strength of the gel compared to carrageenan or konjac. Another consideration of suitable jelling agent is the pH and texture of the jelling agent. The good jelling agent will show by the texture of a typical jelly agent. The pH is also essential for keeping the jelly drink well for RTD beverages. Carrageenan was the best jelling agent. The higher the concentration, the higher the value of the jelly agent was reached. Binding water capability of carrageenan in large amounts causes the space between particles to become tighter so that more water is bound and trapped to make the solution hard. The addition of carrageenan concentrations in rosella-sour sop jelly drink caused the level of caffeine to decrease due to the formation of a strong double helix structure so that it can capture and bind water so that the water molecules in the gel are not easily released which will reduce the occurrence of syneresis (Agustin and Putri, 2014).

The same value of pH was observed at all treatment indicated that cold condition was the best condition for keeping the jelly drink from pH changes. The changes of pH to a low value will reduce the quality of drinks such as by chemical reaction of an ionic condition that makes the jelly will be easy to break down or liquefaction. Black grass jelly contains organic acids that will contribute to lower pH (Yulianto and Widyaningsih, 2013). It might be correlated with elevation of syneresis by warm temperature. Agar was also a good was also a good jelling agent and become more solid at higher concentration. Arabic gum was also good jelling agent but at higher concentration, it was not suitable as a jelling agent as their textures became soft

and easy breakdown. Although konjac could make jelling well, but it was sticky that it made changed the jelly form besides undesirable for the jelly drink as RTD beverages.

Black grass jelly has 122 calories and 6 grams protein for each 100 grams. Therefore, its beneficial will follow if this jelly is consumed daily engaged. The healthy effect of the jelly is from its high fiber and low-calorie composition (Wahyono *et al.*, 2015). Not only does it contain fiber, the black grass jelly also contains active polyphenol. This component has been reported to prevent DNA damage in human lymphocytes when it is exposed to free radicals such as hydrogen peroxide and UV radiation. Black grass jelly extract has high antioxidant activity due to its phenol content (Tasia and Widyaningsih, 2014; Handayani *et al*., 2017). Black grass jelly can elevate antioxidant level because of their phenolic compounds (Chusak *et al.*, 2014; Lai *et al.*, 2001). Other studies have shown that black grass jelly prevents AGE (Advanced Glycation End Products) formation and protein oxidation, processes associated in diabetes (Adisakwattana *et al*., 2014). Decrease of lipid peroxidation with malondialdehyde (MDA) concentration was also reported (Lai *et al*., 2001).

Conclusion

Probiotication together with its microencapsulation by carrageenan showed a potential application as a health supplement for black grass jelly drink as RTD beverage. This application highly improved the status of healthy black grass jelly drink as RTD beverage after probiotication by encapsulated *L. plantarum* Mar8. Carrageenan was the suitable and ideal for microencapsulation of the probiotic, *L. plantarum.* The amount of live bacterium (9 log cfu/ml) was reached that it was ideal density to ingest so as to take the effect on gut health. The carrageenan has a function to stabilize the jelly and also hold the viability and stability of the probiotic and prevent it from coming out from the jelly.

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- Commonly used abbreviations are as follows: (i) volume: ml; (ii) length: km; (iii) concentration: M; (iv) weight: kg; (v) temperature:℃; (vi) others: Fig., Figs. Months are not abbreviated.
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- Words of non-English origin, like in vivo, et al., ibid. and scientific names should be italicized.
- Brackets are used in the following order: {level 3 [level 2 (level 1)]}.
- Citation of nomenclatural authorities for taxa is optional except for taxonomic papers. For abbreviation of authors' names, use Kirk and Ansell, Authors of scientific names, 1992.
- Multiple references cited in text should be in chronological order, while multiple references from the same year are cited in alphabetical order. Where there are three or more authors, cite the first name only in text, adding *et al.* for all citations (Armstrong, 2000; Bell and Bell, 2000; Armstrong *et al*., 2002a,b, 2003).
- References in reference list should be in ascending order of authors' names first, followed by year. Journal names and book titles should be spelt in full form.
- Figures must be mounted on cardboard and fit a maximum of 20 cm height by 13.5 wide including space for the legend after reduction. Figures should be numbered consecutively with Arabic numbers, and in the order that they are referenced in the text. Add a cover sheet to protect each plate. Write author's name and figure number on the cover sheet. The original artwork will not be returned to the authors.
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