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CONTENT

Price risk perceptions and management strategies in Vietnamese pangasius production

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ABSTRACT

Vietnamese pangasius prices have become more volatile over the past decade exposing the business to risk and uncertainty. This study explored Vietnamese pangasius farmers' perceptions about the sources of price risk and the effectiveness of price risk management strategies through Likert scales. The relationships between farm and farmer socioeconomic characteristics and their perceptions were also examined by using Chi-square analysis. Data were obtained through a structured survey with 110 farmers in three provinces of An Giang, Can Tho, and Dong Thap. Results suggested that pangasius farmers mostly concerned the instability of input prices, the volume of input supply, the instability of output prices, and the legislation of sales contracts between farmers and processors. We further found that price risk management strategies were not perceived as less effective measures to mitigate the price risk. Gender, farmers' experience, farm size, crop period, farm types, and access to credit are positively related to the farmers' perceptions. Findings on the price risk perceptions and management strategies are useful to support public and private of price risk management decision towards increasing the sustainability of pangasius production.

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1. Introduction

Pangasius (*Pangasius hypophthalmus*) has become one of the most important export products of Vietnam. In 2015, exports of pangasius were valued more than 1.7 billion USD (FAO, 2017); the total of production to 1.1 million tons. Though it is one of the top export markets for Vietnamese pangasius, EU imports of Vietnamese pangasius decreased from 398,339 thousand USD in 2012 to 268,878 thousand USD in 2016 (Seafood trade intelligence portal, 2017). This decline could be due to a consequence of recent claims about the negative environmental impacts of pangasius production and food safety issues (Bush & Duijf, 2011; Little et al., 2012; Rico & Van den Brink, 2014). Furthermore, customers increasingly demand labelled pangasius, such as those of the Aquaculture Stewardship Council (ASC), GlobalG.A.P., Best Aquaculture Practices (BAP), and Naturland organic for farm operations, aim to guarantee consumers that the product is produced sustainably (Sahota et al., 2009; Bush & Duijf, 2011; Bush et al., 2013).

To mitigate environmental sustainability concerns and to keep up with the increasing demand for certified pangasius, technological innovations could be applied to reduce water pollution from pangasius farming. Initial investment costs for water purification technologies are relatively high (Pham et al., 2016). In addition, pangasius prices have become increasingly volatile due to the elastic nature of the supply for live pangasius (Pham et al., 2018). Price volatility implies price risk which in turn lead to reduced investment in sustainable production ways (Assefa et al., 2017).

Several studies have estimated risk perception and management in fish farming (Bergfjord, 2009; Dahl & Oglend, 2014; Asche et al., 2015). Literature showed that the risk from price volatility was perceived as the most important risk source in fish production. The main price risk management strategies considered in fish farming were sale contracts, insurance, diversification, future and options, and forward contracts. Although price risk was rated as the most important factor, many authors did not find a mismatch between farmers' perceptions of price risk and the price risk management strategies (Bergfjord, 2009; Le & Cheong, 2010). This raises the question that either fish farmers are not aware of the benefits of price risk management strategies (Le & Cheong, 2010) or the strategies considered are not adopted to mitigate the price risk. While these are no doubt important price risk, it is unknown about the sources of price risk that fish farmers consider to be important and how they manage the particular source of price risk.

The objective of this study was to provide insights into: (1) Vietnamese pangasius farmers' perceptions of the sources of price risk and the effectiveness of management strategies on particular source of price risk; and (2) the relationship between farms' and farmers' socioeconomic characteristics and farmers' perceptions of price risk in pangasius farming. Insight into the price risk and corresponding management strategies in pangasius farming is expected to provide useful information for policy makers and private sectors, which can be used to design policies and measures to help farmers improve their farm management.

2. Materials and Methods

2.1. Data collection

Data for this study were gathered in February 2018 through a questionnaire survey of 110 pangasius farmers. The surveyed pangasius farmers were randomly selected with the help of local aquaculture officers and were from three main production provinces in Vietnam, including An Giang, Can Tho, and Dong Thap. Of the 110 questionnaires, 8 were excluded from the analysis due to incomplete information. The questionnaire was pre-tested with 10 farmers to ensure that the questions were clear and understandable. In total, a sample of 92 farmers were available for statistical analyses. For the identification of sources of price risk and price risk management strategies in designing the questionnaire survey, we first created a long-list of price risk sources and price risk management strategies from the literature review. Second, 40 stakeholders (30 pangasius farmers, 6 local aquaculture extension officers, and 4 aquaculture researchers) were interviewed through either face-to-face and e-mail to select the existing sources of price risks and management strategies from the list and were asked for additional sources of price risk and management strategies in Vietnamese pangasius farming. The stakeholders were all knowledgeable on pangasius farming. The selected sources of price risk and management strategies to the particular sources of price risk are presented in Table 3 and Table 4, respectively. The questionnaire survey consisted of three parts, i.e. questions related to 1) farmers' perceptions of price risk sources, 2) farmers' perceptions of various strategies to manage price risk, and 3) socioeconomic characteristics of the farm and farmer. Most questions were close questions, mainly in the form of Likert-type scales ranging from 1 to 5 (Churchill & Iacobucci, 2006).

2.2. Data analysis

This study used the descriptive statistical methods, using the means and the standard deviations to measure the perceptions of Vietnamese pangasius farmers of price risk and the efficacy of price risk management strategies. Previous quantitative risk management studies which measured risk perceptions and risk management strategies, also rated the relative impact and effectiveness of different risk sources through Likert scales (Meuwissen et al., 2001; Hall et al., 2003; Assefa et al., 2017). Chi-square analysis was also conducted to explore the relationship between farmers' perceptions of price risk and farm and farmer socioeconomic characteristics.

3. Results and Discussion

3.1. Sample description

The descriptive statistics of the sample of farms and the socioeconomic characteristics of farmers are shown in Table 1. The average age of farmers was fairly young, forty-eight years old, with completed secondary school education and an average experience of 14 years. Farmers were mainly male

Table 1. Summary statistics of surveyed farmers' socioeconomic and farm characteristics (n = 92)

Characteristics	Mean	Percentage (%)	Standard deviation	Minimum	Maximum
Age (years)	48		12.56	23	74
Education (years)	8.81		3.12	3	16
Gender (1-males, 0-females)					
Male		91.7			
Female		8.3			
Experience (years)	13.38		5.08	2	25
Farm size (ha)	1.58		2.03	1	10
Number of months/crop	8.24		1.55	6	12
Access to credit (1-yes; 0 – No)	0.56		0.5	0	1
Technical support (1-yes, 0-No)					
Yes		20.8			
No		79.2			
Source of information support	0.56		0.5	0	1
Extension staffs		20.8			
Input suppliers		48.6			
Processors		1.4			
Aquacultural engineers		8.3			
Friends/relatives		47.2			
Farm types					
Independent		54.2			
Partially integrated		18.1			
Fully integrated		27.7			

(91%) and mostly owed small scale farms with average farm size of 1.6 ha. Seventy-nine percent of farmers took external technical consultancy, mainly from the input suppliers, friend, and extension staff. The majority of surveyed farmers were from independent and fully integrated farming: 54% independent farms, 28% fully integrated farms with processors, and 18% partially integrated farms with input suppliers. Partially integrated farms mean that an input supplier advances feed to an independent farmer for repayment with an interest at harvest or another agreed time when cash available. While fully integrated farms prefer to the vertical integration of pangasius farmers with processors.

3.2. Perceptions of price risk attitude and price volatility

To get insight into a farmer's perception of his or her price risk attitude, the five statements as shown in Table 2 were used. Huirne et al. (2000) and Valeeva et al. (2011) also used similar statements (Huirne et al., 2000; Valeeva et al., 2011). The statement of "Despite of pangasius market volatility, I keep producing live pangasius" was used to check the consistency of responses. This statement covers not only input and output price volatility, but also the volatility of live pangasius demand. Table 2 shows the percentage distribution of the farmers' answers in relation to each statement.

Table 2 shows that for most of statements, the majority of farmers indicated a 4 (agree) or 5 (totally agree). This implies that most farmers are willing to take risk when the input and output prices volatile. However, when the selling price of live pangasius increases, most of farmers hesitate to expand their pangasius farm size. The hesitation in expanding the pangasius production area could also be explained by the relatively high degree of volatility of pangasius price. Our study found that about 40% of farmers perceived that the average price fluctuated in the past 5 years ranging from 10% to 24%. According to the farmers, the price volatility in pangasius production was mainly caused by the variation in the supply and demand of the market (54%), and the disqualification of the pangasius products for export standards (46%).

Table 2. Farmers' perceptions of price risk attitude and the percentage distribution of farmers over categories (1= totally disagree, 5 = totally agree)

	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)
Despite of input price volatility, I keep producing live pangasius	8	8	9	25	51
Despite of decreased output price, I keep producing live pangasius with expectation of increased output price in the next crop	8	9	16	32	35
When the output price increases, I want to expand my pangasius farming	35	16	20	21	9
Despite of decreased output pangasius price, I will not produce other fish	16	8	10	27	39
Despite of pangasius market volatility, I keep producing live pangasius	9	9	10	24	49

3.3. Perceptions of sources of price risk

In total, 12 sources of input and output price risk were considered in this study. The second, third and the fourth columns of Table 3 show the frequency of appearance of input and output sources related to price risk, the average scores of farmers' perceived impacts of each source of price risk, and their ranks, respectively. The impacts of risks, which were estimated by the frequency of risk occurrence multiplies with severity, were ranked by their means in descending order to evaluate the perceived importance of sources of price risk. The results show that high and unstable input prices, instability of the volume of input supply, and low and inconsistent quality of input supply were the greatest concerns by pangasius farmers, with mean scores of impact at 4.13, 3.10, and 2.33, respectively. A study of Loc (2016) also found that unstable input price at farm stage was the key challenge for upgrading the Vietnamese pangasius value chain. When the pangasius selling price is high, many farmers enter the industry. This often leads to the over-demand of fingerlings supply in the period of increased selling price. Furthermore, the result by Le & Cheong (2010) and Loc (2016) suggested that uncontrolled input quality (fingerlings and feed) was highly associated with the price risk (Le & Cheong, 2010; Loc, 2016). This over-demand of pangasius fingerlings in a particular period of time together with the inconsistent input quality inevitably drive the unstable supply of the volume of inputs and unstable input prices. Poor on time delivery of inputs purchase, imbalance of bargaining power with input suppliers, and water in-pond pollution related to the input quality were less concerns by pangasius farmers with mean score of impact less than 3. This is likely explained by the fact that there is a competition between various input suppliers. As such, are generally accepted the offers by input suppliers.

With regard to the output selling related risks, low and unstable output prices, instability in demand volume, and weak legislation on sales contracts between farmers and processors gave the highest scores with mean scores of impact at 5.00, 3.79, and 3.43, respectively. Concern about the instability in output price and weak legislation on the sales contracts are likely to reflect that pangasius farmers were producing their fish without any guarantee of a sales price. Variability in prices were also found as the most important risk source in pangasius farming (Le & Cheong, 2010; Trifković, 2014). Imbalance bargaining power with the processors, lack of capacity of fulfill high quality specifications of customers, and poor on-time payment to the farmers are less concerns by pangasius farmers with mean score of impact less than 3. This likely reflects that farmers have relatively equivalent power in negotiating prices with processors which was similarly found by Pham et al. (2018). Also there does not often have delayed payment from processors. In addition, farmers perceived that they are capable of meeting the requirements of customers.

3.4. Perceptions of price risk management strategies

Farmers' perceptions of particular source of price risk management strategies are summarized in the fourth and fifth column of Table 4. In order to manage the input sourcing related risks, pangasius farmers mainly partially inte-

Table 3. The percentage of price risk appearance, mean (1 = very low impact, 5 = very high impact), and rank by mean for sources of price risk.

ID	Source of risk	Percentage (%)	Mean	Rank by mean
	Input sourcing related risks			
1.1	High and unstable input prices	93	4.13	1
1.2	Instability of the volume input supply	56	3.10	2
1.3	Low and inconsistent quality of input supply	60	2.33	3
1.4	Poor on time delivery of inputs purchase	17	2.25	4
1.5	Imbalance of bargaining power with input suppliers	29	2.06	5
1.6	Water in-pond pollution related to the input quality	51	0.83	6
	Output selling related risks			
2.1	Low and unstable output prices	85	5.00	1
2.2	Instability in demand volume	60	3.79	2
2.3	Weak legislation on sales contracts between farmers and processors	33	3.43	3
2.4	Imbalance bargaining power with the processors	39	2.26	4
2.5	Lack of capacity of fulfill high quality specifications of customers	36	1.68	5
2.6	Poor on-time payment to the farmers	63	1.27	6

grated with input suppliers (33%), cut production (32%), diversified suppliers (42%), bought inputs from suppliers with certification (32%), carefully checked the quality of inputs (56%); while adopting to prolong the sales (58%), follow market information (47%) and choose the prestige retailers (58%) were popularly used to manage the output selling related risks.

Table 4 clearly shows that all the strategies are perceived as the less effective way to manage the sources of price risk. The standard deviation of less than 1 indicates a high level of consensus among surveyed farmers. The current strategies that Vietnamese pangasius farmers used to manage price risk are relatively limited into two categories (survival and adaptive strategies) (Assefa et al., 2017). Survival and adaptive strategies aimed at minimizing production losses, improving efficiency, and flexible adaptation to market price movements (Assefa et al., 2017).

There is mismatch between farmers' perceptions of output selling related risks and the perceived importance of risk management strategies in dealing with output price volatility. For example, farmers mentioned that selling price volatility was their most concerns. Surprisingly, they did not perceive the contract farming by fully integrated farms with processors as an important risk management strategy. Although contract farming is often argued to minimize risk and uncertainty (Heyder et al., 2010; Trifković, 2014). The less effectiveness of contract farming (with average score of below or equal 2.00) confirms the findings of Le and Cheong (Le & Cheong, 2010), who indicated that the law governing sales contracts between pangasius farmers and processors is weak and unreliable. The less effective price risk management in pangasius production confirmed the fact that pangasius farmers were producing their fish without any guarantee of a sales price.

3.5. Variables that relate to the perceptions of price risk

Table 5 shows the results of the chi-square analysis carried out to explore the relationship between the perceptions of price risk and pangasius farm and farmer socioeconomic characteristics. With respect to the input sourcing related risks (risk ID from 1.1 to 1.6), the table shows that gender, farmers' experience, farm size, crop period, farm types, and access to credit have statistically significant and positive impacts to the farmers' perceptions at 1%, 5%, and 10% level. This explains that male farmers with more experience, accessibility to credit and their farms with larger scale, longer crop length perceived input sourcing related risks as important.

Similarly, in the case of the output selling related price (risk ID from 2.1 to 2.6), the table shows that gender, crop period, farm types, tech-

Table 4. The percentage of price risk management strategies application, mean (1 = very low effective, 5 = very high effective), and standard deviation

ID	Source of risk	Risk management strategies	Percentage (%)	Mean	Standard deviation
	Input sourcing related risks				
1.1	High and unstable input prices	Substitute expensive inputs	7	1.08	0.44
		Store input in the short time	8	1.11	0.68
		Partially integrate with input suppliers	33	2.00	1.54
		Fully integrate with processors	8	1.15	0.64
1.2	Instability of the volume input supply	Cut production	32	1.78	1.26
		Adjust farming practices	28	1.68	1.23
		Fully integrate with processors	7	1.18	0.68
1.3	Low and inconsistent quality of input supply	Diversify suppliers	42	2.17	1.48
		Buy inputs from suppliers with certification	32	2.04	1.57
		Carefully check the quality of inputs	56	1.56	1.53
		Fully integrate with processors	6	1.15	0.71
1.4	Poor on-time delivery of inputs purchased	Contract with suppliers	8	1.21	0.75
1.5	Imbalance of bargaining power with input suppliers	Compare prices between different input suppliers	17	1.45	1.09
1.6	Water in-pond pollution related to the input quality	Select the improved ingredients in input supply based on farmers experience	14	1.31	0.83
		Efficiently use the inputs	31	2.00	1.57
	Output selling related risks				
2.1	Low and unstable output prices	Prolong the sales	58	2.19	1.27
		Fully integrate with processors	13	1.38	1.04
		Diversify production	17	1.38	1.04
		Follow market information	47	2.15	1.36
2.2	Instability in demand volume	Cut production	29	1.56	0.95
		Follow market information	11	1.24	0.72
		Temporary stopped farming pangasius	24	1.35	0.77
		Diversify production	3	1.08	0.52
2.3	Lack of capacity to fill high quality specification of customers	Apply best farming practices	14	1.47	1.23
		Financial management for best farming practices	2	1.04	0.35
		Follow information about standards	6	1.14	0.61
		Fully integrate with processors	11	1.39	1.14
2.4	Imbalance bargaining power with the processors	Fully integrate with processors	6	1.19	0.83
2.5	Poor on-time payment to the farmers	Strong sales contracts	24	1.53	1.14
		Choose the prestige retailers	58	2.78	1.63
2.6	Weak legislation on sales contracts between farmers and processors	Strong sales contracts	14	1.35	0.94
		Choose the prestige retailers	28	1.82	1.44

nical support, and access to credit have statistically significant and positive impacts to the farmers' perceptions at 1%, 5%, and 10% level. This explains that male farmers with accessibility to credit and their farms with longer crop length, technical support perceived output selling related risks as important.

Results also reveal that there is a significant relationship between types of farm (i.e. independent farms, partially integrated with input suppliers, and fully integrated with output suppliers) and the perception of input sourcing related risks and output selling related risks. This is in line with the study of Ahsan (2011) and Trifković (2014) who stated that farmers tend to do contract farming as a market risk management strategy (Ahsan, 2011; Trifković, 2014).

4. Conclusions

The management of price volatility and uncertainty is considered to be an important step to increase sustainable investments in Vietnamese pangasius farming. The goal of this study was to obtain empirical insight into Vietnamese pangasius farmers' perception of price risk and the effectiveness of risk management strategies and variables related to price risk perceptions.

Results suggest that pangasius farmers mostly concerned the instability of input prices and volume of input supply, the instability of output prices and volume of market demand, and the legislation of sales contracts between farmers and processors. Nevertheless, price management strategies were perceived as less effective by pangasius farmers. The price risk management strategies are limited to few traditional instruments such as cut production, diversified suppliers, bought inputs from suppliers with certification, carefully checked the quality of inputs, prolong the sales, follow market information, and choose the prestige retailers. In terms of the relationship between perceptions of price risk and farm and farmer socioeconomic characteristics, gender, farmers' experience, farm size, crop period, farm types, and access to credit are positively related to the farmers' perceptions of input sourcing related price risk. While gender, crop period, farm types, and access to credit positively related to the farmers' perceptions of output selling price risk.

For price stabilizing policy interventions, pol-

Table 5. Results of chi-square analysis for price risk

Variables	Input sourcing related risks (ID1.1 to 1.6) and output selling related risks (ID 2.1 to 2.6)											
	1.1	1.2	1.3	1.4	1.5	1.6	2.1	2.2	2.3	2.4	2.5	2.6
Age (years)	0.78	0.88	0.27	0.32	0.82	0.82	0.38	0.44	0.97	0.41	0.38	0.38
Education (years)	0.60	0.51	0.61	0.25	0.24	0.10	0.28	0.22	0.88	0.56	0.27	0.90
Gender (1-males, 0-females)	0.15	0.37	0.20	0.09*	0.09*	0.14	0.08*	0.41	0.66	0.60	0.17	0.90
Experience (years)	0.05*	0.04	0.12	0.74	0.04**	0.19	0.59	0.70	0.40	0.41	0.16	0.39
Farm size (ha)	0.07*	0.17	0.47	0.25	0.43	0.42	0.55	0.63	0.65	0.53	0.46	0.64
Number of months/crop	0.02**	0.37	0.42	0.08*	0.18	0.49	0.07*	0.34	0.86	0.17	0.15	0.79
Technical support (1-yes, 0-No)	0.49	0.51	0.90	0.70	0.38	0.12	0.30	0.54	0.80	0.90	0.03**	0.54
Farm types	0.09*	0.19	0.01**	0.28	0.39	0.02**	0.00***	0.02**	0.06*	0.05*	0.01**	0.02**
Access to credit (1-yes; 0 – No)	0.65	0.33	0.00***	0.67	0.23	0.49	0.04**	0.03**	0.78	0.48	0.05**	0.40

Values with *, **, *** statistically significant at 10%, 5%, 1% are in bold.

icy makers could provide the timely dissemination of improved and accessible market price data and predictions. Such price data could be used to support pangasius farmers in production decision, as well as contract decisions. Given the importance of socio-economic characteristics of farmers and their farms on the input and output price risk perception, effort should be stepped up at introducing more effective price risk management strategies to male farmers with more experience, accessibility to credit and their farms with larger scale. These farmers concern more about the price risks and thus likely increase their willing to adopt the better risk management, such as strengthening the conditions of contract farming, improved information system, and price insurance.

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Efficacy of white rice-based diets in nursery pigs

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ABSTRACT

Rice is a staple cereal consumed by much of the world's population but has received relatively little attention as a potential feedstuff for the animal industries in many parts of the world. It may be because its price is relatively high and only a small amount of rice produced is traded (6.2%). India, Thailand and Vietnam play a major role in the world rice export market. Rice is characterized by its high starch content, low fat and dietary fiber content, and lower crude protein content in comparison to other cereals. Rice-based diets have a higher apparent digestibility of nutrients than corn-based diets. Complete replacement of corn with rice in weaned pig diets does not affect growth performance, but feed efficiency is improved when corn is replaced with brown rice. Heat processing of rice does not influence digestibility and growth performance of pigs. Due to rice's high digestibility and low fiber content, pigs fed rice-based diets have lower concentrations of volatile fatty acids and viscosity of intestinal digesta compared to other cereal-based diets. Moreover, rice has been shown to have potential to ameliorate diarrhea, colonization of pathogens, severity of enteric bacterial diseases, and pig removals. The mechanism for this protective function is not fully understood, but it may be, to a certain extent, related to lower fiber content and high digestibility of rice and a so-called "rice factor". In practice, when availability and cost of rice permits, pork producers can benefit from the use of rice-based diets for piglets.

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1. Introduction

Rice is a staple cereal consumed by much of the world's population, and a plethora of studies exist investigating the physical and chemical properties of cooked rice for man. Most of these studies relate to the starch properties of rice, presumably because starch constitutes more than 75% of rice's composition (Pluske et al., 2007; Stein et al., 2016), and hence forms the major carbohydrate consumed. The high starch content of cooked rice coupled with a very low non-starch polysaccharide (NSP) level makes cooked rice a ready source of absorbable glucose, and hence energy, for the human population. More recently, there is interest in the use of rice-based oral rehydration formulas for controlling enteric diseases in children (Iyngkaran & Yadav, 1998; Ramakrishna et al., 2000; Gregorio et al., 2016) and animals (Hampson et al., 2001).

In contrast, there is less information pertaining to the feeding of rice to animals, especially the pig, with respect to effects on production and intestinal "health", which incorporates enteric disease. This is predominately because other cereal sources, such as wheat, barley, corn, and sorghum, are used in pig production and can be fed to pigs cheaper than rice. Nevertheless, given the information available from the human literature with respect to the cooking and milling

properties of rice, potential exists for the use of processed (cooked) rice in certain diets for pigs, especially the young pig. This is particularly when the intestine is compromised by enteric pathogens such as *Escherichia coli*, the agent of post-weaning colibacillosis (PWC) or, as it is more commonly recognized, post-weaning diarrhea (PWD). Incorporation of processed rice into such diets has potential to add value to the world rice industry and reduce the pig industry's reliance on the use of growth promoting antibiotics. Furthermore, spin-offs into the biomedical field in the control of human enteric pathogens may be possible.

Antimicrobial agents are presently the main tool used for control of PWD, and are provided to pigs to treat overt disease, to provide prophylaxis in situations where disease is liable to occur, and to improve growth rates in the absence of disease. However, problems are arising over the use of antimicrobials in the pig industry. Their long-term use eventually selects for the survival of resistant bacterial species or strains, and genes encoding this resistance also can be transferred to other formerly susceptible bacteria. Currently, a variety of bacterial pathogens of pigs are showing resistance to a range of antimicrobial drugs. Not only is this reducing the number of antimicrobials available to control bacterial diseases in pigs, but this resistance also poses risks to human health. Risks include the transfer of multidrug resistant zoonotic pathogens (e.g., *Salmonella* spp. and *Campylobacter* spp.) from pigs to humans, the direct or indirect transfer of resistance genes from the porcine intestinal microflora to human bacterial strains, and the presence of antimicrobial drug residues in pig meat (Hampson et al., 2001). Public concern about these issues is leading to reduced availability or the complete banning of certain antimicrobial agents for use in pig production, as has occurred in certain parts of Europe. Although there are currently no total bans on the use of growth promoting antibiotics in the Vietnam pig industry, it is imperative to develop alternative means, such as the use of nutrition, both of controlling bacterial infections and promoting growth in pigs without recourse to the use of antimicrobials.

Swine rations usually contain a large amount of cereal grains such as corn, barley, wheat, oat, and rice. Among these ingredients, corn is the cereal grain preferred by most pork producers in Vietnam and many regions of the world. However, other cereal grains may be considered, at times, due to their lowered costs or their positive effects on growth performance and health of young pigs. Cereal grains have different carbohydrate composition which may affect the health of the digestive tract by providing different substrates for microbial activity (Jensen & Jorgensen, 1994; Bach Knudsen et al., 2012). Unfortunately, there are few reliable data to support intelligent selection of the most appropriate cereals for the health of young pigs. Oat, wheat, and barley are ingredients with high content of non-starch polysaccharides which can stimulate the growth of commensal gut flora (Bach Knudsen, 1991), leading to a healthy digestive tract. In other words, studies of McDonald et al. (1999 & 2001), Hopwood et al. (2004), and Mateos et al. (2006) indicate benefits of rice, which contains almost no fiber. Apparently, more information is needed on both the practical and physiological effects of various cereal grains in the diet of young pigs.

The aim of this paper is to review the effects of rice-based diets on growth performance, digestibility, gastrointestinal parameters, and health of weaned pigs in comparison to other cereal-based diets.

2. Global Rice Production and Trade

Rice is widely grown all over the world and a staple food for humans. Approximately 673.8 million metric tons (MMT) of rice are produced annually in the world, with overwhelming majority of this entering the human food markets. Only about 6.2% of rice produced is traded in the global markets. As with most crops, China has a major role in rice production and use, but a minor role in trade. In the 2016 marketing year China accounted for 31.0% of world production of 673.8 MMT (Table 1). The second largest rice producer is India, with a total production of 165.2 MMT in 2016. It may be surprising that India is now emerging as the world's largest rice exporter with an amount of 10.1 MMT. With a large population, strong economic growth and internal food price pressures, China could quickly disappear from the rice export market. In contrast, Thailand and Vietnam, though with smaller amounts of rice production as compared to China, play a major role in the world rice export market. The second largest rice exporter is Thailand at 9.9 MMT for 2016. Vietnam is the third rice exporter

at 6.1 MMT, 15.8% of the world total in 2016.

Most of the current price problems are related to exporters withdrawing supplies from the market and the general rise in all commodity prices. The longer-term structural question of who will produce rice for international markets will continue to influence market prices for years to come. It appears that India, Thailand and Vietnam are more committed to export markets.

3. Chemical Composition of Rice

Rice is characterized by its high starch content, low fat and dietary fiber content, and lower crude protein content in comparison to other cereals (Table 2). In term of crude protein, rice (8.1-8.6%) is comparable to corn (8.1%), even with a better balanced essential amino acid profile (Figure 1). Both brown rice and white rice has a higher concentration of essential amino acids, except for histidine and leucine, than corn. Piao et al. (2002) and Li et al. (2002), however, found that the balance between isoleucine and leucine is better in brown rice than that in corn. Barley (10.8%), oat (11.3%), and wheat (14.0%) have a greater content of crude protein than corn and rice.

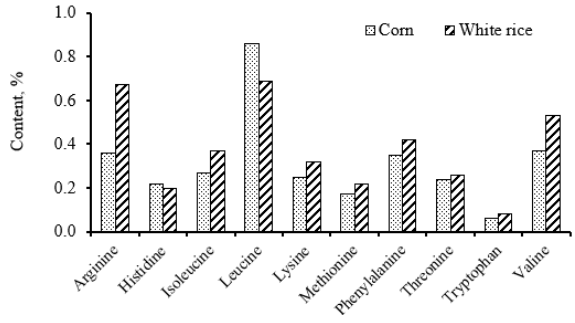

Figure 1. Essential amino acids in corn and white rice. Data from: Bach Knudsen (1997); Kim et al. (2007); Che et al. (2012).

Apart from high contents of crude protein, other cereals also contain a considerable amount of total dietary fiber (> 9.0%) which is much higher than that (1.2%) in rice (Table 2). In contrast, rice contains a significantly higher level of starch (75.3-87.4%) than other cereals. Regarding the energy content, rice has a higher level (3.54 Mcal/kg) of metabolizable energy (ME) than barley, oat, and wheat. In comparison to corn, although both rice and corn have the same gross energy content (Li et al., 2002; Vicente et al., 2008), the ME of rice is lightly greater than that of corn. The higher ME content of rice might be resulted from its higher digestibility. In addition, other cereals, particularly barley, oat and corn, have higher lipid content than white rice.

Because of its low fiber content and high starch content, rice might be a good alternative to other cereals in the pig's diet immediately after weaning. It may have a major impact on the digestibility of dietary nutrients and the microbial populations through providing fewer substrates for bacterial fermentation in the intestinal tract (Pluske et al., 2003; Montagne et al., 2004; Vicente et al., 2008). This in turn may prevent the proliferation of pathogenic bacteria. The interaction between the components of diet (e.g. fiber) and the development of intestinal bacteria and gut is complex. Thus, a rice-based diet does have an important role to play in intestinal disease and health of young pigs.

4. Effects on Growth Performance

Replacement of other cereals with rice in weaning diets for pigs has been conducted by several researchers, but most of the research has focused on comparing the effect of substituting rice for corn in the weaned pig's diet. In a series of experiments carried out at the same commercial farm testing whether corn, barley, rolled oat, or rice as the main energy source in the diet for weaned pigs affects growth performance, Che et al. (2012) reported that average daily gain (ADG) of pigs fed the rice diet was significantly higher than that of pigs fed barley or rolled oat diets, but not different from that of pigs fed the corn diet (Table 3). No difference in feed/gain (F/G) was seen among the treatment diets. Average daily feed intake (ADFI) of pigs fed corn, rolled oat, and rice diets were similar, but was significantly higher than that of pigs fed barley diet. In the second experiment, Che et al. (2012) investigated effects of complete replacement of corn with rice in diets and length of rice feeding on growth performance of weaned pigs (Table 3). The results showed that there were no significant differences in ADG, ADFI, and F/G. This suggests that rice can substitute for corn in the diet for weaned pigs, reared under commercial conditions, without affecting the growth performance of pigs.

However, with studies conducted at the univer-

Table 1. Top paddy rice producers-2016 and rice exporting countries worldwide in 2016[1]

Producers	Amount		Exporters	Amount	
	Million metric ton	%		Million metric ton	%
China	208.7	31.0	India	10.1	24.3
India	165.2	24.5	Thailand	9.9	23.9
Indonesia	72.7	10.8	Vietnam	6.1	14.7
Bangladesh	52.1	7.7	Pakistan	4.0	9.6
Vietnam	43.6	6.5	USA	3.3	8.0
Thailand	32.6	4.8	Myanmar	1.4	3.4
Myanmar	28.6	4.2	Cambodia	1.2	2.9
Philippines	18.5	2.7	Uruguay	0.9	2.2
Japan	10.7	1.6	Brazil	0.6	1.4
Brazil	10.6	1.6	Argentina	0.5	1.2
Pakistan	10.3	1.5	Others	3.5	8.4
USA	10.2	1.5			
Cambodia	10.0	1.5			
World total	673.8	100.0		41.5	100.0

[1]Data from FAO (2017).

Table 2. Chemical composition of cereal grains (as fed)

	Barley[1]	Oat[1]	Wheat[1]	Corn[3]	Rice[2]	Rice[3]
Protein, %	10.8	11.3	14.0	8.1	8.1	8.6
Ether extract, %	3.0	4.0	1.1	2.9	0.9	2.4
Starch, %	49.7	40.1	57.6	62.1	75.3-87.4	n.a.
Dietary fiber, %	18.8	22.8	9.8	9.5	1.2	n.a.
Ash, %	4.1	2.6	2.0	1.4	0.5	1.1
ME, Mcal/kg	2.91	2.60	3.30	3.39	3.54	n.a.

[1]Stein et al. (2016).
[2]Pluske et al. (2007); Stein et al. (2016).
[3]n.a.: not available; Li et al. (2002).

Table 3. Effects of cereals on growth performance of pigs from d 0 to 42 post-weaning[1]

Experiment 1	Dietary treatments			
	Corn	Barley	Rolled oat	Rice
ADG, g	331[a]	307[c]	323[bc]	337[a]
ADFI, g	495[a]	462[b]	489[a]	504[a]
F:G, g/g	1.49	1.49	1.52	1.49
Experiment 2	Dietary treatments[2]			
	Corn (6 wk)	Rice (1 wk)	Rice (2 wk)	Rice (4 wk)
ADG, g	307	315	318	307
ADFI, g	455	459	468	446
F:G, g/g	1.35	1.33	1.33	1.33

[1]12 pens of 21 pigs/treatment. Data from Che et al. (2012).
[2]Pigs were fed rice diets for 1, 2 or 4 weeks and then on a corn diet until the end of experiment.
[a-c]Means within a row with different superscripts differ ($P < 0.05$).

sity research farms, better performance of weaned pigs has been often reported. Mateos et al. (2006) showed that pigs fed the cooked-rice diet grew faster (12.3%) than those fed the cooked-corn diet. In another experiment using brown rice, Li et al. (2002) found that 50% or complete replace-

ment of corn with brown rice in nursery diets improved the feed efficiency. In comparison to wheat, pigs fed rice-based diets from 46-63 days of age, regardless of low or high dietary protein, ate more, gained faster, and had better feed efficiency than those fed the wheat-based diets (Bonet et al., 2003).

Rice has a high level of starch, thus gelatinization of the starch portion of the grains might improve nutrient utilization and thereby resulting in a better growth performance. Vicente et al. (2008) evaluated effects of cooked-flaked corn, raw-ground rice, cooked-ground rice, and cooked-flaked rice on performance of weaned pigs for 28 days post-weaning (Table 4). They showed that pigs fed rice consumed more feed (678 vs. 618 g/d), grew faster (466 vs. 407 g/d), and tended to have lower F/G than those fed corn. No differences in growth performance due to heat processing of rice were observed. This suggests that heat processing does not affect growth performance of pigs fed rice-based diets.

5. Effects on Nutrient Digestibility

Rice-based diets have a higher apparent total tract digestibility of nutrients than corn-based diets. Mateos et al. (2006) found that the digestibility of GE, OM, DM, and fat was higher for rice- than for corn-based diets (Table 5), which agrees with the results of Li et al. (2002), Piao et al. (2002) and Vicente et al. (2008). It was also shown that heat processing did not affect the digestibility of nutrients in the rice-based diets (Table 6). A similar result was obtained when corn was replaced with 50% or 100% of brown rice in the diets. The corn-based diet had a significant lower apparent digestibility of dietary components than the brown rice-based diet or the diet with 50% replacement of corn. The higher digestibility of a rice-based diet would be likely to explain the improved growth performance in weaned pigs fed rice diets compared to corn diets. It is pointed out that fewer substrates for bacterial fermentation might be resulted from a rice-based diet, but ileal digestibility of rice vs. other cereal diets needs to be determined.

6. Gastrointestinal Effects

With high digestibility of nutrients and low fiber content, rice-based diets may greatly influence activity of microbial fermentation and intestinal environment. Hopwood et al. (2004) reported that the barley-based diet or the diet with high inclusion level of barley fed to pigs caused a significant decrease in pH of distal colon and feces compared to pigs fed the rice-based diet (Table 7). However, no differences were observed in digesta pH in duodenum and ileum of pigs among the treatments. It is obvious that a diet containing high fiber ingredients, like barley, increases the pH in the large intestine via providing fermentable substrates to the microbial activity as compared to the rice-based diet. In another experiment, different types of fiber such as high-amylose corn starch, lupin isolate, or a combination of both included in a rice-based diet reduced the digesta pH in cecum, proximal colon, and distal colon (Table 8). Further, a rice-based diet resulted in a numerically higher pH in the large intestine as compared to a wheat-based commercial diet (Pluske et al., 2003). The inclusion of animal or plant protein in a rice-based diet also significantly influenced the digesta pH of the large intestine. The rice diet with animal protein had a higher cecum and colon pH than that with plant protein.

The increase in pH is likely to be because of the increased pool of volatile fatty acid (VFA) through the high activity of microbial fermentation in the large intestine. The rice-based diet had a lower total pool of VFA than that with increasing levels of barley. It was further indicated that rice-based diets with inclusion of various types of fiber sources produced different amounts of pooled VFA (Table 9). In order to prove that the fiber components added to rice diets increase the production of VFA, McDonald et al. (2001) added a viscous but unfermentable component, carboxymethylcellulose (CMC) to a rice-based diet. They found that no differences in concentration of VFA of digesta in the large intestine of pigs.

The high level of fiber in the cereals, e.g. barley, caused not only an elevated total pool of VFA and decreased pH but also an increased viscosity. The rice-based diets with different inclusion levels of barley resulted in an increase in viscosity in the small intestine of pigs (Figure 2). The viscosity in pigs fed the rice-based diet was lower than that in those fed the barley-based diet or the diet with the inclusion of 500 g/kg of barley. Hopwood et al. (2004) reported that the intake of non-starch polysaccharide was positively

Table 4. Effects of cereals and heating processing on performance of pigs from d 0 to 28 post-weaning[1]

Item	Corn		Rice	
	Cooked-flaked	Raw-ground	Cooked-ground	Cooked-flaked
ADG, g[2]	407	459	482	456
ADFI, g[2]	618	680	680	672
F:G, g/g	1.52	1.49	1.41	1.47

[1]8 pens of 5 pigs/treatment. Data from Vicente et al. (2008).
[2]Corn vs. mean of the 3 rice treatments ($P < 0.01$).

Table 5. Effects of cereals on total tract apparent digestibility of dietary components[1]

Item	Cereal source	
	Cooked rice	Cooked corn
DM, %	83.8[a]	80.6[b]
OM, %	86.2[a]	82.9[b]
GE, %	82.7[a]	79.0[b]
Ether extract, %	60.9[a]	58.8[b]
CP, %	72.9	72.9
Starch, %	99.2	99.4

[1]8 pens of 4 pigs/treatment; Average of d 6 & 16 post-weaning. Data from Mateos et al. (2006).
[a-b]Means within a row with different superscripts differ ($P < 0.05$).

Table 6. Effects of cereals and heat processing on apparent total tract digestibility of dietary components[1]

Item	Corn	Rice		
	Cooked-flaked	Raw-ground	Cooked-ground	Cooked-flaked
DM, %	86.7[a]	88.1[b]	88.8[b]	88.3[b]
OM, %	88.6[a]	90.6[b]	91.1[b]	90.8[b]
GE, %	86.5[a]	88.4[b]	89.3[b]	88.8[b]
CP, %	80.8	80.9	81.6	81.0

[1]8 pens of 5 pigs/treatment; Average of d 5, 14 & 28 post-weaning. Data from Vicente et al. (2008).
[a-b]Means within a row with different superscripts differ ($P < 0.05$).

Table 7. Digesta pH in various sections of the intestinal tract in pigs fed rice-based diet with different levels of barley[1]

Item[2]	Rice:barley (g/kg)			
	703:0	497:250	275:500	0:750
Duodenum	5.7	5.9	5.7	5.8
Ileum	6.7	6.3	6.6	6.1
Distal colon	6.8[a]	6.6[a]	6.1[b]	5.7[b]
Feces	6.9[a]	6.9[a]	6.5[b]	6.4[b]

[1]6 pigs/treatment; [2]10 d after weaning. Data from Hopwood et al. (2004).
[a-b]Means within a row with different superscripts differ ($P < 0.05$).

correlated with the viscosity of small intestinal content of pigs. The viscosity of digesta is also dependent on, in addition to fiber sources, types of fiber combined in the diet. Addition of high-amylose corn starch and lupin isolate combined to the rice-based diet greatly increased the vis-

Table 8. Digesta pH in different sections of the intestinal tract in pigs fed rice-based diets[1]

	Diet				
	R+AP	R+HACS	R+LI	R+HACS+LI	Com
Ileum	7.1	7.1	7.3	7.4	6.8
Cecum	6.2^{a}	5.3^{b}	5.5^{bc}	5.4^{b}	5.8^{ac}
Proximal colon	6.3^{a}	5.2^{b}	5.4^{b}	5.3^{b}	6.0^{a}
Distal colon	6.6^{a}	5.7^{b}	6.0^{b}	6.1^{b}	7.0^{bc}

[1]6 pigs/treatment. Data from Pluske et al. (2003).
R = rice, AP = animal protein; HACS = high-amylose corn starch, LI = lupin isolate, Com = commercial diet containing wheat.
[a-c]Means within a row with different superscripts differ ($P < 0.05$).

Table 9. Pools of VFA of digesta in the large intestine in pigs fed rice-based diets[1]

VFA pool (mmol per pig)	Diet				
	R+AP	R+HACS	R+LI	R+HACS+LI	Com
Cecum	8	18	15	12	11
Colon	19^{a}	45^{b}	45^{b}	27^{ac}	36^{bc}

[1]6 pigs/treatment. Data from Pluske et al. (2003).
R = rice, AP = animal protein; HACS = high-amylose corn starch, LI = lupin isolate, Com = commercial diet containing wheat.
[a-c]Means within a row with different superscripts differ ($P < 0.05$).

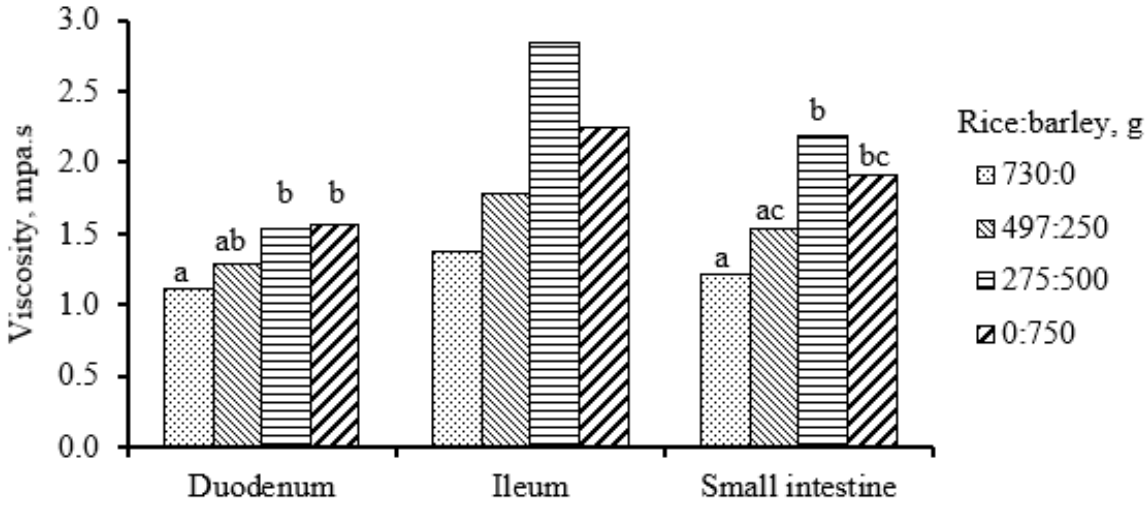

Figure 2. Viscosity of intestinal contents of pigs fed rice-based diets containing different levels of pearl barley. 6 pigs/treatments; 10 d after weaning. Bars with different superscripts differ ($P < 0.05$). Data from Hopwood et al. (2004).

cosity of ileal digesta if compared to diets with the inclusion of high-amylose corn starch or lupin isolate individually (Pluske et al., 2003).

7. Effects on Pig Health and Diarrhea

Rice, when compared to other cereals, has been shown to reduce the diarrhea, intestinal colonization of pathogens, and the severity of enteric bacterial diseases when pigs were challenged with enterotoxigenic *Escherichia coli* (ETEC) or *Brachyspira pilosicoli.* Hopwood et al. (2004) investigated the effect of rice-based diet with high inclusion level of barley with or without NSP enzyme supplementation. They showed that the fecal DM did not differ among dietary groups (Ta-

ble 10), but the post-infection fecal consistency score was different, with pigs receiving the rice-only diet having firmer and better-formed feces than pigs fed either of barley diets. Mateos et al. (2006) reported that pigs fed the cooked-rice diet had a lower diarrhea score than those fed the cooked-corn diet. This indicates that under normal or disease conditions rice-based diets fed to pigs reduce the moisture content of feces. When pigs challenged with ETEC, the ADG for the experimental period was negative for those pigs consuming diets with barley, and positive for those fed the rice-only diet (Table 11). The intestinal viscosity was also greater in infected pigs fed 500 g/kg of barley compared with those fed the rice-based diet. In another ETEC challenge study, Montagne et al. (2004) showed that ileal and cecal viscosity of pigs fed rice-based diets with animal or plant protein was lower than that of pigs fed wheat-based diet with plant protein.

In term of intestinal colonization of pathogens, culture of mucosal scrapings revealed greater proliferation of ETEC within the small and large intestines of pigs consuming diets containing barley than those eating the rice-only diet (Table 12). In addition, the ETEC were more dominant within the microbiota of pigs eating barley compared with that within pigs eating rice. At each of the intestinal sites swabbed there were more ETEC on the culture plates from pigs eating the barley diets compared with those not receiving barley. With *Brachyspira pilosicoli* inoculation (Figure 3), the period of fecal excretion ranged from 1 to 25 days. The pigs fed the rice diet excreted *Brachyspira pilosicoli* for a significantly shorter period than those fed the standard diet containing wheat and barley, regardless of diet forms. They also observed that a higher incidence of fecal excretion in all the groups fed the standard diet was accompanied by a significantly higher number of pigs showing clinical signs of disease compared to the pigs fed the rice diet. A similar protective effect of rice-based diets has been seen in pigs experimentally infected with the intestinal spirochete *Brachyspira hyodysenteriae*, the agent of swine dysentery (Pluske et al., 1996) and *Brachyspira pilosicoli*, the agent of porcine intestinal spirochetosis (Hampson et al., 2000).

The protective effect of such a diet against bacterial infection has been attributed in part to the high digestibility of its protein and carbohydrates (Siba et al., 1996; Pluske et al., 1998). In piglets,

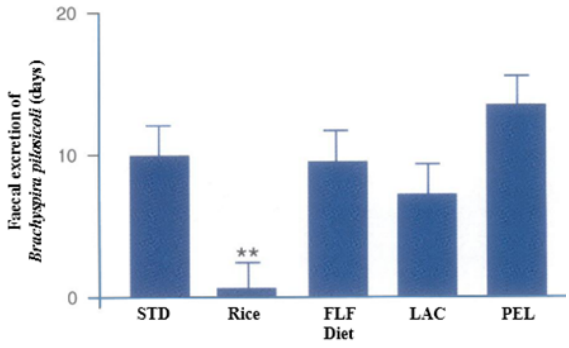

Figure 3. Fecal excretion of *Brachyspira pilosicoli* by pigs fed various diets and infected experimentally in 2 trials. STD=standard diet containing barley and wheat, FLF=fermented liquid feed, LAC=STD + lactic acid, PEL=pelleted STD; 6 pigs/treatment. Adapted from Lindecrona et al. (2004).

it is generally thought that diets containing less fiber and highly digestible ingredients, thereby limiting the quantity of fermentable substrates entering the large intestine, are associated with a decrease in the incidence of PWC (Montagne et al., 2003). Such diets may result in less accumulation of potential bacterial substrate in the upper small intestine, the primary site of proliferation of the pathogenic *E. coli* causing PWC (Francis, 2002).

One of the primary mechanisms by which toxin-producing bacteria, such as *E. coli* or *Salmonella*, initiate secretory diarrhea is the increase of water secretion by the small intestinal crypt cells, by a pathway involving cAMP (Keely et al., 2009). In young pigs, the large intestine is incompletely developed and may not be capable of absorbing enough fluid to prevent clinical diarrhea and dehydration. A component of boiled white rice recently identified and named the rice factor has been shown to block the secretory response of intestinal crypt cells to cAMP in guinea pigs (Macleod et al., 1995; Mathews et al., 1999). A potential effect of this rice factor has not been demonstrated in other animal species; however, boiled rice has been used for many years in the treatment of diarrhea in humans and is included in various oral rehydration products (Gregorio et al., 2016).

The reduction in diarrhea and intestinal colonization of enteric pathogens may help prevent infections and improve the pig health. In a series of experiment conducted by Che et al. (2012)

Table 10. Fecal dry matter and consistency score in weaned pigs infected with enterotoxigenic *Escherichia coli* and fed different diets

Item	Rice:barley, g/kg[1]		
	703:0	275:500	275:500 + Enzyme
Fecal DM (g/kg)			
Over 6d post-weaning	304	295	299
Post-infection	301	292	277
Fecal consistency score[2]			
Pre-infection	1.5	1.8	1.7
Post-infection	2.9[a]	3.6[b]	3.7[b]

[1]n = 11, 13, & 12 for 0, 500, & 500+NSP Enzyme groups, respectively. Data from Hopwood et al. (2004).
[2]Score 0-5.
[a-b]Means within a row with different superscripts differ ($P < 0.05$).

Table 11. Growth and digesta viscosity of weaner pigs killed 3-4 d after infection with enterotoxigenic *Escherichia coli*

Item	Rice:barley, g/kg[1]		
	703:0	275:500	275:500 + Enzyme
Gain, g/d	10.5	-7.8	-27.0
Viscosity, mpa.s			
Duodenum	1.8	2.1	2.6
Ileum	1.6[a]	2.3[b]	2.2[ab]
Small intestine	1.7	2.2	2.6

[1]n = 11, 13, & 12 for 0, 500, & 500+NSP Enzyme groups, respectively. Data from Hopwood et al. (2004).
[a-b]Means within a row with different superscripts differ ($P < 0.05$).

Table 12. Proportion of β-hemolytic enterotoxigenic *Escherichia coli* (ETEC) cultured from intestinal swabs in weaner pigs infected with ETEC and fed different diets

Item	Rice:barley, g/kg[1]		
	703:0	275:500	275:500 + Enzyme
Viable CFU/g ($\log_{10}$)[2]			
Mid-small intestine	1.0[a]	4.1[b]	3.5[b]
Proximal colon	2.3[a]	5.2[b]	6.0[b]
ETEC (%), intestinal swabs			
Duodenum	7.5	22.1	26.5
Ileum	11.0[a]	47.6[b]	21.4[ab]
Cecum	16.5[a]	53.2[b]	53.0[b]
Feces	27.9	44.5	38.8

[1]n=11, 13, & 12 for 0, 500, & 500+NSP Enzyme groups, respectively. Data from Hopwood et al. (2004).
[a-b]Means within a row with different superscripts differ ($P < 0.05$).

at the same commercial pig farm, feeding rice-based diets to weaned pigs significantly reduced the pig removal by half, even when pigs were fed rice diets for only one week immediately after weaning (Figure 4). Furthermore, Pluske et al. (2003) showed that the number of antibiotic treatments of pigs was also reduced in pigs fed the rice-only diet compared to a commercial diet and rice-based diets with the inclusion of various fiber sources. Obviously, feeding a rice-based diet improves pig health with evidence of reduced pig removal and number of antibiotic treatment.

8. Conclusions

Rice, widely grown over the world, is a highly digestible ingredient and has high potential to be a good feed ingredient for animals. Rice can sub-

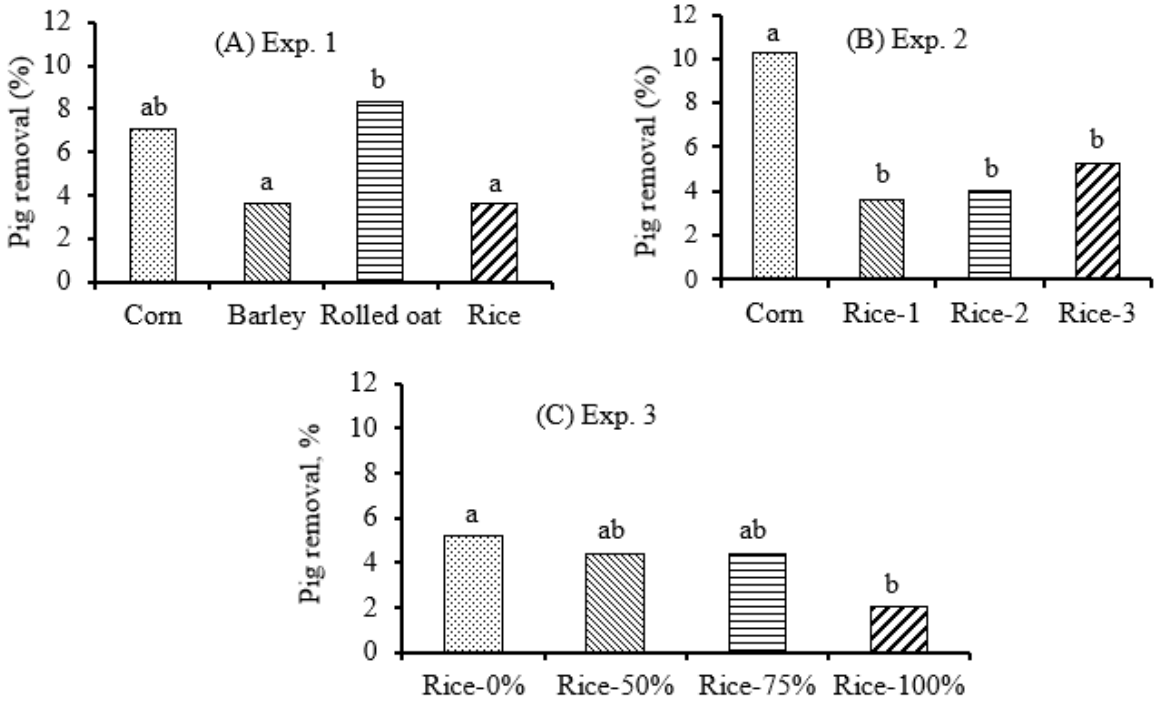

Figure 4. Effect of different cereal-based diets on pig removals 6 weeks post-weaning. (A) Pigs fed diets with different cereals as a main source of energy for 6 weeks post-weaning. (B) Pigs fed corn-based diets for 6 weeks or rice-based diets for 1 (Rice-1), 2 (Rice-2) or 4 (Rice-3) weeks post-weaning. (C) Effects of feeding rice with 0 (Rice-0%), 50 (Rice-50%), 75 (Rice-75%), and 100% (Rice-100%) replacement of corn in diets for 1 week on the overall pig removal over 6 weeks post-weaning. 252 pigs/treatment. $^{a\text{-}b}$Means with different superscript letters within each experiment differ ($P < 0.05$). Data from Che et al. (2012).

stitute for corn in diets for weaned pigs without affecting the pig's performance. The rice-based diet appears to be better in growth performance and feed efficiency than a barley- or wheat-based diet. Rice included in diets makes feces less moist and reduces the incidence of diarrhea. Inclusion of rice in diets causes less viscous digesta and appears to prevent the proliferation of pathogens. Feed ingredients in weaning diets that excessively increase the viscosity of the intestinal digesta may be detrimental to pig and production. Rice appears to improve pig health with evidence of reduced pig removal and antibiotic treatment.

9. Implications And Recommendations

Reduced viscosity, diarrhea, and proliferation of pathogens by rice would be likely due to its low fiber content, high digestibility, and rice factor. In practice, when availability and cost of rice permits, pork producers can benefit from inclusion of rice in diets for pigs immediately after weaning.

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Experimental application of surgical technique for perineal urethrostomy in male cats

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ABSTRACT

The objective of this study was to evaluate the efficiency of PU in male cats. The perineal urethrostomy (PU) was performed in 10 home-breed male cats, in which 9 cats were healthy and 1 cat had cystolith (2.5 ± 0.5 kg BW), from May 2017 to April 2018 at the Department of Clinical Veterinary Science of Nong Lam University, Ho Chi Minh City. The criteria for evaluating the experiment included the successful rate, wound healing time, post-operative pain assessment, time of return to normal urination and defecation and post-operative short-term complications. The results showed that 9 out of 10 cats completely recovered their urinating ability in approximately 14 days post-operation, in which 2 cats experienced wound infection and required a surgical intervention. One cat died due to unknown causes at the 10^{th}-day post operation. The average pain scores based on the Feline Glasgow Composite Measure Pain Scale (CMPS – Feline) in the first 5 days were relatively high (scored as 11) and gradually decreased; until 14 days postoperation, 100% of cats recovered had no sign of pain (scored as 1). It was recorded that all cats experienced pain and difficulty in urination and defecation for a few days after the surgery. After removing the urinary catheter, the cats needed 3 to 5 days to get used to the new urethral stoma and normally self-urinated ability. In general, the successful rate of this surgical method was 90% (9 out of 10 fully recovered cats) in which most of the cats were healthy and returned to normal urination after 14 days post-operation.

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1. Introduction

Nowadays, one of the most common concerned health problems of cats was the obstruction of urinary tract, especially in male cats. The Osborne's research indicated three percent (3%) of dogs seen at veterinary hospitals in the USA were affected by urolithiasis while these figures were over 7% of the feline case (Osborne et al., 1995). Therefore, it could be seen that the urinary obstruction rate was higher in cats than in dogs. Moreover, because of the narrow and long urethra of cats, male cats have a higher risk of urinary tract infection, urolithiasis or many life-threatening conditions related to the urinary system and the bigger chance of recurrence of those conditions.

The urethral anatomy of a male cat is divided into 3 main parts: the pre-prostatic (close to the urinary bladder), post-prostatic and penile urethra. These parts are orderly arranged based on the decreasing size of the lumen diameter: 2 mm, 1.3 mm and 0.7 mm, respectively (Cullen et al., 1983).

Because urinary tract diseases occur commonly in male cats, treatment methods require a plenty

of time, work, and money. The cured animals have to suffer physical and mental pain (for instance in the case that the animal has recurrent the urinary tract infection (UTI) and needs catheterization). That is why finding and applying another supportive solution for treating urinary diseases in male cats are extremely necessary.

Many methods are used to treat urinary diseases in cats including changing diets, increasing the water intake, medication and operation to remove the urinary stone. However, there are cases in which the urethra is completely blocked and catheterization cannot be applied due to the obstructive object that locates at the extremely narrow part. Most of the above treatments are unable to apply.

Regarding the previous researches, the perineal urethrostomy technique was firstly studied in 1963 by Carbone and modified in 1971 by Wilson & Harrison as a support treatment for urinary tract diseases in male cats. It was also a surgical treatment (Carbone, 1967; Blake, 1968) that effectively worked on severe traumatic penile cases. The technique was described as an artificial formation of a new bigger urine pathway after removing the penis and suturing the inner layer of the urethra with the perineal skin that allowed for obstructed materials to pass through.

Until now, there have not been any studies related to PU technique in Vietnam yet. This study was conducted to assess the successful rate and the recovery time of normal urination and defecation of cats after surgery. This would be offered offer more data about an alternative solution to assist the treatment of urinary tract diseases for eterinarians in Vietnam.

2. Materials and Methods

The study was conducted from May 2017 to April 2018. Nine healthy intact male cats and 1 intact cat with bladder stones (BW from 2.5 ± 0.5 kg) were undergone the perineal urethrostomy surgery at the Department of Clinical Veterinary Sciences of Nong Lam University, Ho Chi Minh City. All cats were fed the same diet for one week and dewormed before the surgery.

The evaluation criteria included the complications rate during and after the surgery (%), the time period to recover the ability to urinate and defecate, the time period of wound healing (days), the pain assessment based on Glasgow Feline Composite Measure Pain Scale (score), the success rate of surgery (%).

2.1. Surgical procedure

2.1.1. Before surgery

All of the cats before surgery must undergo the process of fasting of food for at least 8-12 hours and water for at least 3-4 hours, and were generally checked for their health conditions (temperature, respiratory rate and heart rate) in order to assure their appropriate condition for anaesthesia.

The cats were administered with atropine (0.2 mg/kg, IM) as premedication, Cefotaxime (25-50 mg/kg, IM) 30 minutes before surgery and 5 mg/kg IV of Zoletil during the induction of surgery. The cats' fur was shaved off from the navel to the whole groin and extended around 5-10 cm to the tail. Then, they were positioned supinely with the hip lifted a little bit by a pad under the back. Their hind limbs were fixed towards the front limbs in order to fully expose the surgical site. The surgical site was sterilized with Povidine 10% solution mixed with NaCl 0.9% solution, and was placed upon with a surgical clamp to ensure the complete sterilization.

2.1.2. During surgery

First, an ellipse incision was made around the scrotum. Then, the subcutaneous tissues were separated and the testes were removed in the manner similar to the procedure for common male cats' castration. The scrotum and testicles were also discarded. After that, the retractor muscle was cut in order to release the penis (Figure 1).

In the next step, an urinary catheter with Vaseline at the end was gently inserted into the penis to the bladder. The urine inside was drawn out slowly using a 5-cc syringe, which was kept intact to the end of the urinary catheter to prevent urine from dripping and contaminating the surgical site. Then, ischiocavenosus muscle, lying on the sides of the penis was identified by separating the perineal muscle area. In order to check whether the right muscle was found, the penis was moved a little bit (Figure 2). After being identified, the muscle was cut, so that the penis, which was hanging loosely from the perineal mus-

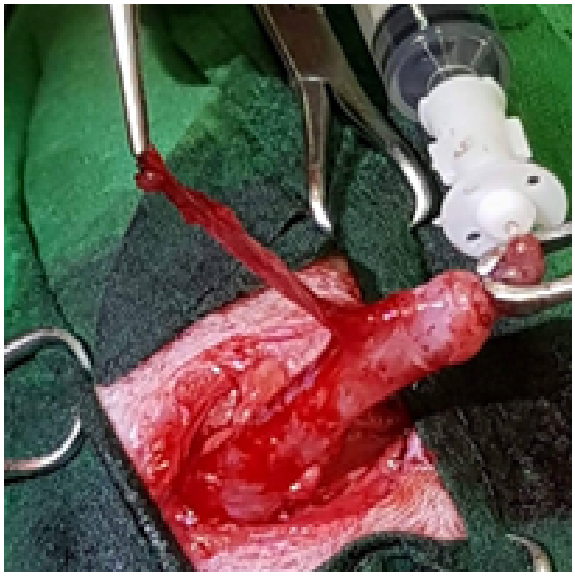

Figure 1. Penis was released by incising retractor muscle.

Figure 3. Scalpel was used to cut along the penal shaft based on the urinary catheter.

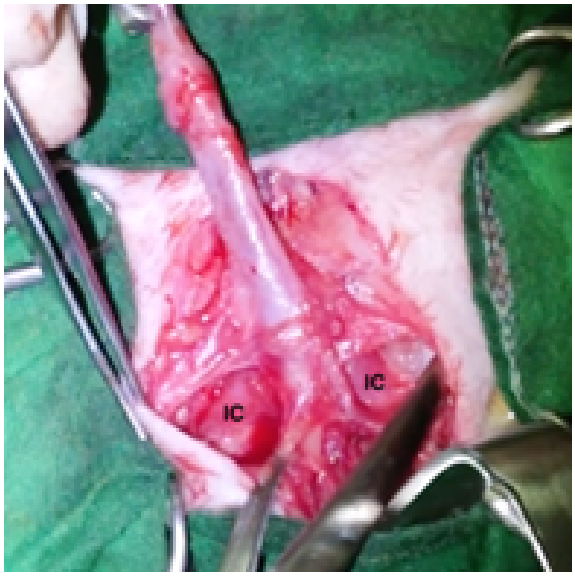

Figure 2. Identifying ischiocavenosus muscle.

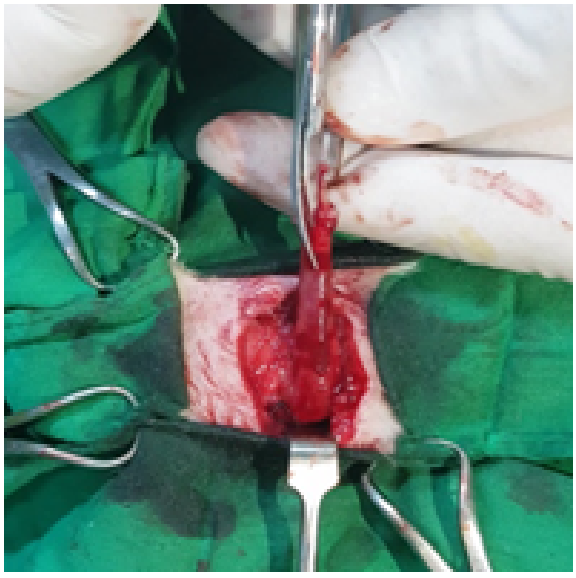

Figure 4. Urethra was cut by using scissor without the support of a urinary catheter.

cle, must be held straight by tissue forceps. Next, an incision was made with a scalpel on the penis ventrally based on the urinary catheter from the glans penis to a position around 3 mm from the bulbourethral gland (Figure 3). Before safely cutting off the penis, transfixation ligature was tied tightly.

After the above 8 steps of the surgery, the method of cutting penis was improved by using a pair of scissors to cut along the penal shaft instead of using scalpel as in the above mentioned method (Figure 4).

In the final steps, the urethral mucosa and the nearby most dorsal skin area were sutured together at 3 positions (10, 12 and 2 o'clock) cranially with a simple interrupted pattern (Figure 5). Here, it is obliged to make sure that the mucosa, muscle tissue and skin were all sutured simultaneously. The urinary catheter was with-

drawn and the surgical site was closed up routinely from the suture at 12 o'clock towards the abdomen. The urinary catheter was once more inserted into the urethra and fixed position with 4 suture lines lying symmetrically by the catheter (Figure 6).

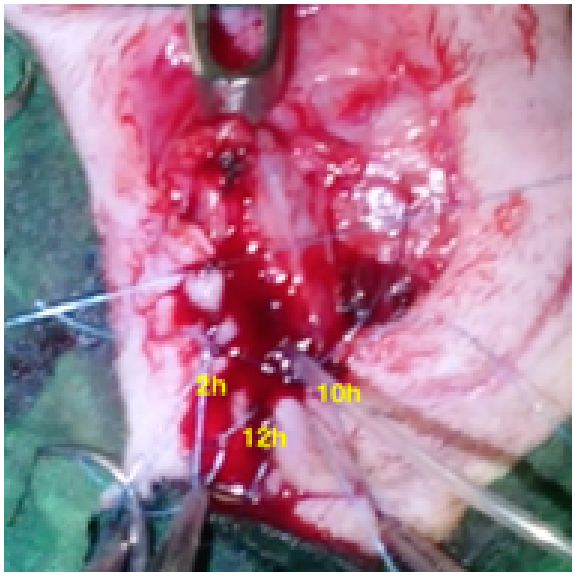

Figure 5. Suturing the mucosa and the nearby skin at 3 positions.

Figure 6. Suturing the mucosa to the nearby skin.

The wound was sterilized and bandaged carefully. The cats were obliged to wear Elizabeth collar post-operation throughout the healing process.

2.1.3. Post-operation care

After surgery, the cats were administered meloxicam (anti-inflammatory) for 5 consecutive days, cefotaxime for 5-7 consecutive days, and Lactate Ringer or glucose 5% intravenously is needed. The condition of the surgical sites (sutures, fluid, healing process...), and the general health condition (pain score, temperature...) were evaluated and documented. In case of a good healing process and as well as a regained ability to urinate on their own, the urinary catheter was withdrawn on day 5 and the sutures were removed on day 14. In case of complications due to the sutures being ruptured by the cats which caused necrosis at the surgical site, another surgery was required to place back the urinary catheter as well as the care routine. All 10 cats were kept strictly in cages to restrict their movements. For the cats which did not defecate in the first 3 days after surgery, Duphalac and laxative catheter were required.

As for the food, the cats were given a diet containing high levels of easily digested nutrients. For the cats which suffered from anorexia, intravenous fluid transmission and nutrition gel were obliged.

2.1.4. Pain assessment method

The cats' pain was daily rated post-operation according to Glasgow Feline Composite Measure Pain Scale: CMPS – Feline (Jacky, 2015). This method comprises of answering a series of 20 questions about the reaction and the behaviors of the cats in order to rate how much it was hurting them on the 20-score scale. In case they scored over 6, they would be classified as painful and analgesic measures must be taken.

3. Results And Discussion

3.1. Complications rate during and after surgery

Complication rate after surgery was displayed below in Figure 7, which consists of surgical site infection (36%), urethra blockage (21%), hematuria (7%), uncontrolled urination (7%), surgical site tearing (7%), and no complications (21%).

Our results was consistent with complications reported in researches of Hauptman (1984), Smith (2002), & Bass (2005).

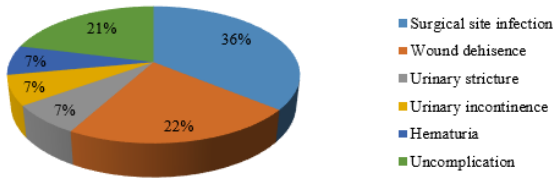

Figure 7. Complications rate after surgery.

The most commonly seen post-surgery complication was surgical site infection. There were in total 5 cases with the presence of pus which usually appeared on day 5 after withdrawing the urinary catheter. This, in turn, caused the urine to drop directly onto the surgical site, weakening the adhesion between the urinary mucosa and the skin. The amount of pus depended on the position of the surgical site combined with other complications such as wound dehiscence or urinary incontinence. This agreed with the findings of other studies (Baines et al., 2001). The first case, due to the lack of experience, the surgical site was located near the anus, causing the consecutive discharge of inflammatory fluid for 8 days after surgery. There were 3 out of 10 cases in which the Elizabeth collar was slipped, allowing the cats to lick and bite at the surgical site, causing the rupture of the suture, bleeding and prolonging the healing process. There was a case in which the cat lost its ability to urinate on its own due to being in pain, making the urine to drop on the surgical site and extend the healing process (8 days post-surgery).

In cases of surgical site infection, the wound was cleaned regularly with saline solution and Povidine iodine, and bandaged to prevent the contact with the cage floor. Plus, antibiotics were also used for 5-7 days to minimize the infection. The cage floor bars were disinfected with disinfectant-VirKon. In cases of surgical site tearing, the following surgery was performed to remove necrotic tissues, suture back, and replace the urinary catheter.

In case no. 8, there was a stricture of the newly formed urinary opening, due to the cat biting the suture and tearing the surgical site, which widened the necrotic area. Therefore, it was difficult to distinguish the position of the previous surgical site, as well as to suture the wound, causing a stricture. During the healing process, the urinary opening was narrowed down, and covered by a layer of scab, making the cat difficult to urinate without the aid of urinary catheter. After cutting the sutures and clearing off the scab, the cat's urinary ability returned to normal.

In case no. 10, during post-operation care, hematuria was spotted on the first 2 days but was gone later on. Hence, in spite of operating on a cat with bladder stones, there were no huge changes in the post-surgery parameters.

3.2. Evaluation of the time period to recover the ability to urinate and defecate

The average time for cats to recover the ability to urinate and defecate was 10.6 and 5.2 days, respectively (Table 1).

Throughout the first few days after surgery, all 10 cats showed symptoms of being in pain, and difficulty in urinating and defecating by pushing because they were used to their new urinary flow yet. After removing the fixed urinary catheter, the cats needed aid in catheterization for 3-5 days on average, whereas some of them recovered faster and were able to urinate right after catheter removal (2/10). However, in the first case, due to the surgical site being very close to the anus, the wound was strained during the cat's excretion process, hurting it to the point it refused to defecate or urinate. Therefore, the healing process in this case was extended to 19 days. In case no. 8, as a following surgery was needed to create a new urinary opening, the time period to recover the urination ability was reset and it took longer than the other 9 cases.

In order to encourage the cats to use their newly formed urinary stoma as well as to recover their defecating ability, they were provided with plenty of water and were limited to catheterize by urinary catheter and defecating catheter.

3.3. The time period of wound healing and the pain assessment

On average, the cats' wounds took up around 9 to 21 days to heal, based on the 9 surviving cats after surgery as the cat no. 3 died with no apparent reason. There were 7 out of 10 cats with the wound completely healed on day 17. In cases of cats with infected wound which required a further

Table 1. The time period to recover the ability to urinate and defecate of cats after surgery

Cats	1	2	3	4	5	6	7	8	9	10	Average
Return to normal defecation	14	10	0	4	3	8	2	5	2	4	5,2
Return to normal urination	19	5	0	9	10	8	8	31	6	10	10,6

surgery, the healing time would be prolonged (31 days) (Figure 8).

Wounds that healed completely had a bigger diameter of the new urinary opening than their glans penis. This would play a huge role in benefiting the cats' urination due to bigger urine flow than normal cats (Figure 9).

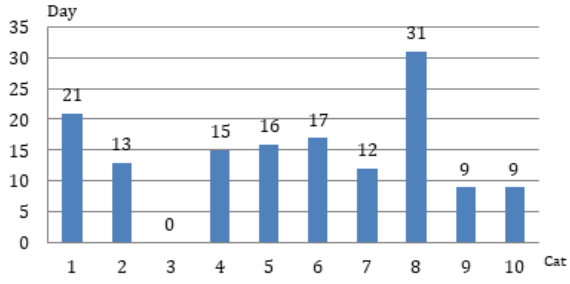

Figure 8. Wound healing time after surgery.

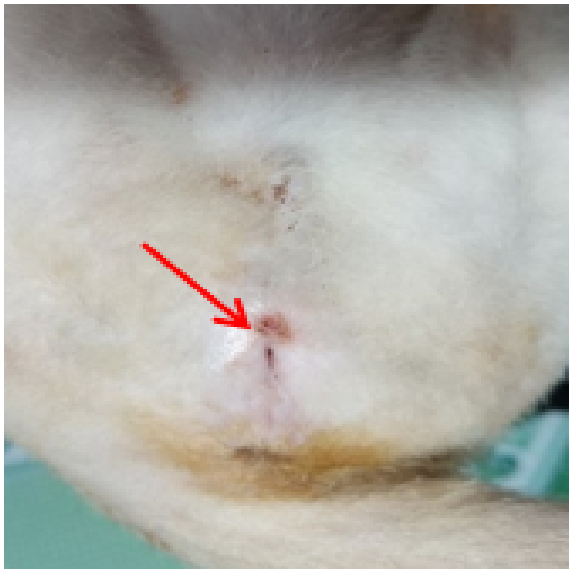

Figure 9. The new urinary opening after 21 days.

3.4. The pain rating evaluation after surgery

Based on Glasgow pain scale, the pain rate of cats after surgery would fall around 9 to 13, in which, 40% of them scoring 9, 20%, scoring 11 and 13, 10% scoring 10 and 12. On the subsequent days, the pain rate decreased gradually until reaching 1 on day 14. However, in cases with a further surgeries (cat no. 6 and 8) or with suture rupture sooner than planned (5 days), the pain rate was reset on the next surgery.

In general, after surgery, the cats suffered from a great deal of pain. Accordingly, they were provided with analgesic measures such as anti-inflammatory medicine, Meloxicam in order to calm them down, minimizing the strain of wounds.

3.5. Improvement of surgical techniques

The surgical technique was introduced by Wilson & Harrison (1971), we have had 2 improvement in the modified surgical technique, which helped the healing time and the operation time shorter.

Improvement of urethra opening location expression: In the first case, the penis was only 1mm away from the bulbourethral gland, the cat suffered from great difficulty in defecating in the early stage of the healing process, with feces kept coming out uncontrollably. This, in turn, prolonged the healing time compared to the cases in which the penis was cut 3 mm from the bulbourethral gland.

Improvement of surgical technique: Cutting along the penal shaft without urinary catheter inside by a pair of scissors was proved to be more effective than using a scalpel which was based on the urinary catheter, as it helped saving surgery time, anesthesia as well as easing the surgeons because of a much cleaner cut.

4. Conclusion

The results from this study indicated the PU technique has highly successful rate with 9 out of 10 cats recovered completely after 9 to 21 days. The male cats returned to normal urinary and defecate frequency ability within 10.6 days and 5.2 days, respectively. The complications could be

controlled by giving a good post-operative care, pain relief or surgical intervention in some severe cases. The research has provided new information of a surgical method and gave more choices for Vietnamese veterinary practitioners in relieving severe urethral obstruction cases in male cats.

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Effects of lysine, methionine, threonine and tryptophan on growth performance and serum antibody titers to Gumboro disease of color-feathered chickens

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ABSTRACT

The experiment was conducted to determine the effects of four amino acids (lysine, methionine, threonine and tryptophan) supplementation on growth performances and serum antibody titers to Gumboro disease (IBD, infectious bursal disease) in broiler chickens. Chicks were randomly assigned to 5 dietary groups (5 chicks/group as 12 replicates of 5 chicks) in a complete randomised experimental design. Group I served as control group, was fed a diet without any supplementation. Groups II and III were supplemented 10% of four amino acids (lysine, methionine, threonine and tryptophan) of the recommended requirements for 45 and 42 days, respectively. Meanwhile, Groups IV and V were supplemented 20% of four amino acids (lysine, methionine, threonine and tryptophan) of the recommended requirements for 45 and 42 days, respectively. The supplementation was started at 3 days of age in chicks. The chickens were vaccinated against Gumboro disease at day 12 and day 19. The antibody titer of the chickens in each group was assayed using IBD anti-body ELISA. Besides, the live body weight, average feed intake and feed conversion (FCR) were determined at 0, 21, 42 and 84 days of age. The results showed no significant different between groups in performance parameters such as body weight and FCR. However, the present work showed highest dose of lysine, methionine, threonine and tryptophan supplementation for 42 days may be improved feed intake and immune response of chicken against Gumboro disease vaccination.

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1. Introduction

In order to be effective in chicken production, preventive medicine, genetic selection and improved nutrition and management should be concerned carefully. Nutritional supplements (carbohydrate, protein and fats) should be provided to ensure the growth, repair of damaged tissues as well as daily maintenance. However, deficiency or excess of dietary protein or amino acids alters immune responses (Payne et al., 1990). Infectious bursal disease (or Gumboro disease) is one of the economically most important diseases that affects commercially produced chickens worldwide (Eterradossi & Saif, 2008). Chickens infected with IBDV between 3 and 6 weeks of age mostly show clinical signs and mortality accompanied with bursal atrophy. According to Muller et al. (2003), strain and the amount of the virus, age and the breed of chickens, the route of inoculation, the presence or absence of neutralizing antibodies, intercurrent primary and secondary pathogens

and environmental and management factors affect the level of serious clinical signs in chicken. In chicken, infected with IBDV can cause immunosuppression, which makes the birds vulnerable to a variety of secondary infections chickens also develop a poor immune response to vaccination against other pathogens (Mazariegos et al., 1990). Therefore, strict hygiene management and vaccination programmes have been used to prevent IBD. For optimal growth and immune response, lysine and methionine are required for protein synthesis by mammals and avian species (Rubin et al., 2007). Tryptophan can be considered as a third limiting amino acid for poultry, followed by methionine and lysine (Peganova et al., 2003). According to Kidd & Hackenhaar (2006), tryptophan deficiency not only affects carcass quality but it also impairs the synthesis of important neurotransmitters such as serotonin and melatonin. Besides, threonine is a major component of intestinal mucin and plasma gamma-globulin in animals (Kim et al., 1999). Thus, the aims of this study was to evaluate the effects lysine, methionine, threonine and tryptophan requirements on growth performances and serum antibody titer to Gumboro disease in broiler chickens.

2. Materials and Methods

A total of 300 day-old chicks (DOC) of color feather breed named "Huynh De" were obtained from Binh Minh breeder farm to use in all experiments. The chicks were weighed on arrival, and randomly allocated into 5 equal groups with 12 replicates each (5 chicks per replicate). Feed and water were provided ad libitum. The basal diet (Table 1) was formulated to contain all essential amino acids at recommended levels (NRC, 1984), modified by Duong Duy Dong (unpublished materials). In addition, chemical composition of the basal diet used in chicken feeding was shown in Table 2. Group I served as control group, was fed a basal diet without any supplementation. Groups II and III were supplemented 10% of four amino acids (lysine, methionine, threonine and tryptophan) of the recommended requirements for 45 and 42 days, respectively. Meanwhile, Groups IV and V were supplemented 20% of four amino acids of the recommended requirements for 45 and 42 days, respectively. The supplementation was started at 3 days of age in chicks. The chicks were vaccinated against Newcastle disease on 4, 20 and 42 days of age; and Gumboro diseases on 12 and 19 days of age. Response variables measured during the experiment included body weight, body weight gain; feed intake, feed conversion ratio on day 0, 21 42 and 84 days of age. On 11, 18, 27, 34, 41, 49, 56, 63, 70, 77 and 84 days of age, five birds from each group were chosen at random and blood samples were collected from the brachial vein. Serum was separated by centrifugation (3000 g, 15 min) and antibody titre against IBD were performed using commercially available ELISA kits (IDEXX, Labs Inc., Westbrook, Maine, USA) according to manufacturer's instructions. The data obtained were analyzed by Tukey's test and one-way analysis of variance (ANOVA) using Minitab 16.0.A P value < 0.05 was considered statiscally significant.

3. Results and Discussion

3.1. Growth performance

As shown in Table 3, the body weight and average daily gain of Group III was higher than those at 84 days of age. However, no significant difference about the body weight and average weight gain was found among treatment Groups. The body weights of this study reached the standards of Binh Minh company in which chickens at 100 days of age were gained from 1.7 to 1.9 kg (Vu, 2015). The highest food consumption in Group I (control group) and the lowest of this found in Group IV with the increment level of 20% for 45 days of 4-amino acid mixture supplementation were also found. Compared with Control Group, Groups I and II, it was observed that dietary treatment Groups IV and V had significant effects on the feed intake ($P < 0.001$) and feed conversion ratio ($P < 0.05$). Bouyeh (2012) also confirmed that the increment levels of 10%, 20% and 30% lysine and methionine would increase body weight, cardiac and liver weight as well as decrease feed intake of chicken. On the contrary, the increment level of 40% of these two amino acids would decrease body weight of chicken. Rogers & Pesti (1990) also reported that tryptophan-deficient diets caused a reduction in weight gain; but, the excess tryptophan in the diet caused a numerical decrease in bird weight gain (Koelkebeck et al., 1991). Therefore, the standard and the amount of amino acid consumed by the birds have a great influence on weight gain and feed intake (Teeter et al., 1993).

Table 1. Ingredient composition of the experiment diets (basal diet)

Ingredients (%)	0 - 21 days of age	22 – 42 days of age	43 – 84 days of age
Corn	63.219	63.513	62.930
Rice bran I	5.791	2.387	28.796
Soybean meal 46	23.175	26.129	2.500
Fish oil	2.460	2.500	0.353
L-Lysin-HCl 98%	0.212	0.135	0.790
DL-Methionin	0.210	0.249	0.249
L-Threonin 98.5	0.100	0.210	0.117
L-Tryptophan 98	0.029	0,097	0.035
$NaHCO_3$	0.817	0.820	0.805
Choline chloride 60	0.120	0.120	0.120
Antioxidants	0.020	0.020	0.020
Limestone powder	1.186	1.170	1.157
Premix BA112	0.250	0.250	0.250
Poison absorption	0.100	0.100	0.100
Herb extracts	0.015	0.015	0.015
Precursor creatine	0.060	0.060	0.060
DCP 18	2.086	2.077	2.065
Organic acids	0.100	0.100	0.100
Probiotics	0.050	0.050	0.050

Table 2. Chemical composition of the basal diet used in chicken feeding

Composition	Unit	0-21 days of age	22 – 42 days of age	43 – 84 days of age
Dry matter	%	87.699	87.628	87.581
Metabolisable energy	Kcal/kg	2900.000	2900.000	2900.000
Crude protein	%	19.000	18.000	17.000
Crude lipid	%	5.614	5.651	5.811
Linoleic acid	%	1.367	1.259	1.179
Gross fiber	%	2.911	2.975	3.086
Total mineral	%	5.805	5.899	6.052
Calcium	%	0.950	0.950	0.950
Total phosphorus	%	0.703	0.719	0.743
Available phosphorus	%	0.400	0.400	0.400
Sodium	%	0.226	0.230	0.230
Chlorides	%	0.140	0.134	0.128
dEB	meq	240.000	240.000	240.000
Total lysine	%	1.174	1.090	0.996
Total methionine	%	0.538	0.489	0.477
Total Met. + Cys.	%	0.857	0.797	0.773
Total threonine	%	0.813	0.758	0.724
Total tryptophan	%	0.235	0.189	0.205
Digestible lysine	%	1.080	1.000	0.910
Digestible methionine	%	0.512	0.464	0.452
Digestible Met.+ Cys.	%	0.778	0.720	0.697
Digestible threonine	%	0.686	0.635	0.604
Digestible tryptophan	%	0.210	0.166	0.182
Digestible leucine	%	1.480	1.416	1.340
Digestible isoleucine	%	0.707	0.665	0.619
Digestible valine	%	0.777	0.738	0.695

Table 3. Body weight, average daily gain, feed intake and feed conversion ratio

Group	0 day of age	21 days of age	42 days of age	84 days of age
		Body weight (g)		
I	35.25 ± 0.96	221.33 ± 15.10	602.50^{ab} ± 35.20	1489.17 ± 96.90
II	35.75 ± 1.21	233.67 ± 12.35	617.50^{ab} ± 35.50	1502.50 ± 99.30
III	35.00 ± 1.04	218.17 ± 19.99	621.67^{a} ± 49.70	1520.83 ± 122.10
IV	35.66 ± 1.23	224.00 ± 15.63	575.00^{b} ± 34.25	1510.00 ± 112.20
V	35.83 ± 0.83	228.00 ± 16.88	570.83^{b} ± 56.00	1516.67 ± 91.10
P	0.263	0.172	0.011	0.952
Group	0 - 21 days of age	22 – 42 days of age	43 – 84 days of age	0 – 84 days of age
		Average daily gain (g)		
I	8.45 ± 0.68	18.15^{ab} ± 1.46	21.11 ± 2.27	17.10 ± 1.14
II	8.99 ± 0.53	18.27^{ab} ± 1.60	21.07 ± 2.01	17.25 ± 1.16
III	8.33 ± 0.88	19.21^{a} ± 2.35	21.40 ± 3.02	17.47 ± 1.43
IV	8.56 ± 0.68	16.71^{b} ± 1.19	22.26 ± 2.57	17.34 ± 1.31
V	8.73 ± 0.76	16.32^{b} ± 2.07	22.52 ± 2.13	17.42 ± 1.07
P	0.195	0.001	0.459	0.953
		Feed intake (g/day)		
I	19.03^{b} ± 2.42	41.72^{a} ± 2.64	69.20 ± 0.37	49.43^{a} ± 0.98
II	21.67^{a} ± 1.72	39.92^{b} ± 3.06	67.84 ± 2.55	48.99^{b} ± 1.88
III	18.01^{b} ± 1.52	39.07^{ab} ± 3.37	68.87 ± 1.71	48.34^{cb} ± 1.64
IV	18.97^{b} ± 2.43	34.64^{bc} ± 3.63	66.62 ± 1.54	46.38^{cb} ± 1.34
V	19.08^{b} ± 1.93	35.60^{c} ± 4.38	68.07 ± 5.23	46.68^{c} ± 1.49
P	0.001	0.000	0.199	0.000
		FCR (kg feed/kg weight gain)		
I	2.25 ± 0.27	2.30 ± 0.17	3.31 ± 0.36	2.90 ± 0.21
II	2.41 ± 0.21	2.19 ± 0.19	3.24 ± 0.29	2.85 ± 0.18
III	2.18 ± 0.28	2.07 ± 0.36	3.28 ± 0.52	2.78 ± 0.26
IV	2.23 ± 0.34	2.08 ± 0.29	3.02 ± 0.33	2.69 ± 0.19
V	2.19 ± 0.18	2.20 ± 0.29	3.04 ± 0.29	2.67 ± 0.17
P	0.216	0.207	0.217	0.049

[a-c]Mean values for control and amino-acid-supplement groups within a column not sharing a common superscript letter were significantly different at $P < 0.05$.

3.2. Serum antiboy titres against Gumboro disease in broiler chickens

According to IDEXX laboratories (2010), the antibody titres against Gumboro disease of around 1000-4000 would be sufficient to protect chickens from this disease. As shown in Table 4, the antibody titre against Gumboro disease after the first vaccination was lower than the minimum protective of 1000 in treatment Groups, except for the high antibody titre against Gumboro disease was also found in 18-day-old chicken of Group V (1099 ± 1144). The lower antibody titres following the primary vaccination could be due to the young age of chicken when the immune function of young animal is not well developed (Rubin et al., 2007). The higher antibody titre in Group IV was significantly different than those of the remaining Groups at 63 days of age. In addition, the antibody titre in Group IV was still higher than those of the other Groups at the end of this experiment; although, no significant difference was found. This observation was consent with the study conducted by Lidiya et al. (2015), the higher dose (140% of the recommended dose) of lysine and methionine improved immune response of chicken against infectious bursal disease vaccination. Furthermore, the increasing total methionine levels from 0.35 to 1.2% in the diet for chickens will enhance the aspects of the immune responses including T-cell proliferation in response to mitogen stimulation, plasma levels of immunoglobulin G; leucocyte migration and antibody titre (Swain & Johri, 2000). On the con-

Table 4. Serum antiboy titres against Gumboro disease in broiler chickens

Days of age	Item	Group I	Group II	Group III	Group IV	Group V	P
11	(X ± SD)	1277 ± 869	939 ± 310	563 ± 470	713 ± 365	854 ± 523	0.122
	CV (%)	68.02	33.05	83.38	51.19	61.28	
	Min	332	632	91	215	144	
	Max	2688	1543	1505	1334	1750	
18	(X ± SD)	400^{ab} ± 398	231.8^{b} ± 198.6	212.9^{b} ± 229.1	228.6^{b} ± 267.1	1099^{a} ± 1114	0.013
	CV (%)	99.56	85.71	107.61	116.84	101.35	
	Min	0	0.0	0.0	0.0	260	
	Max	1095	486.0	559.0	828.0	3200	
27	(X ± SD)	1900 ± 969	1764 ± 747	1726 ± 1171	2222 ± 1486	2181 ± 1985	0.915
	CV (%)	51.00	42.35	67.83	66.87	91.00	
	Min	574	626	130	639	0	
	Max	3134	2940	3339	4869	4946	
34	(X ± SD)	4019 ± 1354	3165 ± 1121	2889 ± 357	3177 ± 1621	4190 ± 1289	0.155
	CV (%)	33.69	35.40	12.36	51.02	30.77	
	Min	2466	1416	2489	1341	2003	
	Max	6323	5106	3569	5290	5919	
41	(X ± SD)	5163^{a} ± 2770	3559^{ab} ± 1463	2250^{b} ± 1529	3718^{ab} ± 919	2362^{b} ± 1271	0.110
	CV (%)	53.65	41.12	67.93	24.71	53.79	
	Min	1730	2030	144	2161	358	
	Max	9752	5459	4901	4994	4248	
49	(X ± SD)	4470 ± 2283	4874 ± 1733	3184 ± 1440	3694 ± 830	4611 ± 1201	0.198
	CV (%)	51.06	35.56	45.23	22.46	26.05	
	Min	2191	1760	307	2161	2891	
	Max	9003	6952	4625	4917	6565	
56	(X ± SD)	5146 ± 1187	3951 ± 1970	4240 ± 1721	3992 ± 1182	4567 ± 1698	0.576
	CV (%)	23.06	49.85	40.59	29.62	37.19	
	Min	2883	1083	1604	2298	2815	
	Max	6119	6494	6439	5941	7780	
63	(X ± SD)	4328^{ab} ± 1679	3008^{ab} ± 1565	2819^{b} ± 2021	5542^{a} ± 2281	3501^{ab} ± 1526	0.033
	CV (%)	38.80	52.02	71.71	41.17	43.58	
	Min	1899	435	59	3131	1279	
	Max	7347	5534	5413	8607	6073	
70	(X ± SD)	3494 ± 1770	3598 ± 1152	3134 ± 1639	5298 ± 2741	3547 ± 1599	0.178
	CV (%)	50.65	32.02	52.31	51.74	45.09	
	Min	1706	2124	1418	3278	231	
	Max	6775	5229	6736	11526	5472	
77	(X ± SD)	3030 ± 1742	3111 ± 1445	2753 ± 2473	4982 ± 2719	3719 ± 1008	0.192
	CV (%)	57.50	46.43	89.86	54.59	27.11	
	Min	0	971	324	1655	1529	
	Max	5336	4715	7515	10121	4702	
84	(X ± SD)	2983 ± 1482	2950 ± 1487	3256 ± 2399	3864 ± 2469	3191 ± 1416	0.878
	CV (%)	49.69	50.42	73.69	63.89	44.38	
	Min	1464	693	379	1548	242	
	Max	6404	5652	7270	8802	4869	

[a-c]Mean values for control and amino-acid-supplement groups within a row not sharing a common superscript letter were significantly different at $P < 0.05$.

trary, the high supplemental levels of methionine or cysteine (1.45% in the diet) were detrimental to the growth and immune responses of chickens (Tsiagbe et al., 1987), probably due to the excess production of highly toxic substances (e.g. homocysteine and sulphuric acid) (Wu & Meininger, 2002). Besides, the antibody responses and cell-mediated immunity in chickens were reduced by the lack of dietary lysine (Chen et al., 2003).

In conclusion, the present study showed that the supplementation of 20% of lysine, methionine, threonine and tryptophan for 42 days seemed better in improving feed consumption, feed conversion ratio and antibody titre against Gumboro disease. Furthermore, evaluation of the effect of these amino acids under various management systems and in different breeds is recommended.

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Evaluation of different diets to replace *Artemia* nauplii for larval rearing of giant freshwater prawn (*Macrobrachium rosenbergii*)

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ABSTRACT

A study was conducted on *Macrobrachium rosenbergii* larvae to evaluate the efficiency of different diets to replace *Artemia* nauplii in the feeding scheme. The study included two experiments performed at pilot scale in 12–L tanks using a recirculating system. Larval stocking density was 100 larvae/L. After 7 days of feeding by *Artemia* nauplii, different diets, included wet and dry diets and decapsulated *Artemia* cysts, were tested to replace *Artemia* nauplii. An extra treatment using only decapsulated *Artemia* cysts throughout the complete larval rearing was also included. The results showed that feeding larvae exclusively decapsulated cysts for the complete rearing cycle was not appropriate. When gradually replacing up to 50% of the *Artemia* nauplii ration with wet or dry diets, good results in terms of growth, survival and quality of the larvae were obtained, similar to the control treatment receiving only *Artemia* nauplii. However, abruptly replacing 50% of the *Artemia* nauplii ration with artificial diets negatively affected larval development. Weaning could start from larval stage V, with about 25% of the *Artemia* nauplii replaced with artificial diet. Subsequently, the weaning ration could be increased up to 50% from stage IX to postlarva stage. Artificial diets should be provided in different particle size ranges based on the larval stage, gradually increasing from 250 to 1000 μm from stage V to postlarva stage. The results obtained in the present study may aid future research and serve as a baseline for further optimization of feeding strategies in prawn larviculture.

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1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* is a commercially important species in freshwater aquaculture in Vietnam and other Southeast Asian countries. Freshwater prawn farming has been pinpointed as one of the major target species of the aquaculture sector. The Ministry of Fisheries of Vietnam has put forth that the annual production of *M. rosenbergii* must reach 50,000 tons utilizing 50,000 ha by the year 2025. The seed production demand of freshwater prawn will be of sufficient quality and quantity from 2 to 3 billion per year in 2025 to serve farming (GOV, 2018). Freshwater prawn culture has great potential for rural aquaculture, generating considerable employment and income, thereby bringing prosperity to rural poor. Giant freshwater prawn farming is environmentally sustainable, since it is practiced at lower grow–out density (New, 1995). A majority of seed used in grow out farming of *M. rosenbergii* comes from hatcheries (Murthy et al., 2004; Phuong et al., 2006). Existing hatcheries in the country are however not producing up to their installed capacity due various constraints.

Artemia nauplii are the preferred live food source used in the larviculture of many crustaceans of commercial value. Lavens et al. (2000) demonstrated that *Artemia* nauplii suffice to pro-

duce *M. rosenbergii* postlarvae. However, others showed that *Artemia* nauplii do not completely fulfil the nutritional requirements of larvae during the last larval stages and therefore recommend the use of supplemental diets (Valenti & Daniels, 2000). As a feed source, decapsulated *Artemia* cysts have a higher energy and nutritional value than live *Artemia* nauplii (Bengtson et al., 1991). Leger et al. (1987) showed that decapsulated *Artemia* embryos have 30–50% more energy than newly–hatched nauplii (instar I). Sorgeloos et al. (1977) suggested the use of decapsulated cysts as a direct source for fish and crustacean larvae. Subsequent studies demonstrated that decapsulated cysts are a good feed similar to freshly hatched *Artemia* nauplii for the larvae of marine shrimps and freshwater prawn, such as *Penaeus monodon* (Mock et al., 1980), and *Macrobrachium rosenbergii* (Bruggeman et al., 1980).

Although live food such as *Artemia* nauplii has proven successful for raising the larvae of many species, inherent problems remain such as the potential introduction of pathogens into the culture system or the high costs of labour and equipment required for preparation. In addition, the nutritional quality and physical properties of *Artemia* nauplii are depending on the source and time of harvest of cysts (Sorgeloos et al., 1983). Imported *Artemia* cysts are predominantly used, which are expensive and uncertain in availability. Dependence entirely on *Artemia* as feed not only makes hatchery operations expensive, but also unsustainable (Murthy et al., 2008). The dependence on *Artemia* is also a major constraint in the expansion of *Macrobrachium rosenbergii* hatcheries (New, 1990). Hence, there is a need to look for acceptable alternative diets to replace *Artemia* and reduce the cost of prawn larval rearing. Several alternative foods, both live and inert, are being investigated as either supplement or replacement for *Artemia* nauplii in crustacean hatcheries. Wan (1999) developed several semi–purified spray–dried diets and evaluated their performance with larval striped bass, *Morone saxatilis* and freshwater prawn *Macrobrachium rosenbergii*. Larvae of both species consumed the diets, but growth and survival were significantly less than that of *Artemia*–fed larvae. However, Kovalenko et al. (2002) reported that larval growth of freshwater prawn fed a microbound diet was 90% of that achieved for larvae fed newly–hatched nauplii of *Artemia*. Survival of the larvae fed the microbound diet was not significantly different from that of *Artemia*–fed larvae. Several studies also investigated supplementation of Artemia with prepared feed in prawn larval rearing (Sick & Beaty 1975; Corbin et al., 1983). However, no standard substitute for *Artemia* has been developed for freshwater prawn hatcheries. Barros & Valenti (2003a) developed an ingestion rate model of *Artemia* nauplii for *M. rosenbergii* larvae based on the individual ingestion rate and prey density. However, this equation indicated that *Artemia* is not an adequate prey for later larval stages and that there is a necessity for a supplementary diet from stage IX onwards. Several studies indeed confirm this finding, however controversy still exist concerning the best timing to introduce formulated feeds in the feeding schedule. Daniels et al. (1992) recommend diet supplementation from stages V–VI. Barros & Valenti (2003b) reported supplementation should start from stage VII onwards. The development of the larval digestive tract and the increase of enzyme activity from stage VI onwards (Kumlu & Jones, 1995) may explain the acceptance of inert diets, since digestion processes become thoroughly functional. In order to further optimize the feeding schedule for *M. rosenbergii* larval rearing, a series of experiments were performed in the present study to evaluate the use of formulated larval diets to supplement or partially replace *Artemia* nauplii.

2. Materials and Methods

2.1. Experimental animals

Two experiments were conducted at the experimental hatchery of the Faculty of Fisheries, Nong Lam University, Vietnam. *M. rosenbergii* breeders bearing yellow eggs were obtained from culture ponds in Ben Tre province, Southern Vietnam and acclimated to the hatchery conditions for egg incubation. The water quality parameters of the broodstock tanks, photoperiod, and feeding were adjusted in accordance with the recommendations for prawn rearing (New, 2003). In both experiments, the larvae were obtained from several oviparous female breeders to ensure that enough the quality larvae was supplied for the pilot scale experiments. Twenty four hours after hatching, larvae were collected and stocked into the experimental tanks.

2.2. Experimental design

Experiment 1 consisted of seven treatments, which originated from the combination of different diets (*Artemia* nauplii, decapsulated *Artemia* cysts, two commercial dry diets and a wet egg custard diet (Table 1). Experiment 1 was performed in pilot–scale 12–L cylindro–conical rearing tanks with three replicates per treatment. Three separate recirculation systems were installed, with one replicate of each treatment assigned to each system. Each recirculation system consisted of 120–L cylindro–conical reservoir tank connected to a 160–L submerged biological filter and a 60–L overhead tank. Water was continuously pumped from reservoir tank to the overhead tank and then forced back through the bottom of the rearing tanks by gravity at 0.3 L/min. An outlet screen (150 μm) at the surface of the rearing tank led the water back to the biological filter tank and at the same time retained the larvae and *Artemia* within the rearing tank. The filter screen was cleaned daily to avoid water overflow. Water with a salinity of 12 g/L was obtained through mixing deionised water (tap water source) and natural seawater. Aeration in the rearing tanks and filter tanks maintained the oxygen level above 5 mg/L. Ammonia, nitrite and nitrate were always below 0.1, 0.03 and 50 mg/L respectively, while pH varied from 7.8 to 8.2. The waste and uneaten food in rearing tanks were removed every morning before feeding by siphoning. The same amount of prepared water (mixed water) was added into the system to keep the water volume constant. Light was supplied for 12h per day at 800–1000 lx at the water surface. Larvae were stocked at an initial density of 50 larvae/L. Experiment 2 consisted of four treatments. In three treatments 25–50% of the *Artemia* nauplii ration was replaced with different artificial diets based on the larval stage of the animals. A control treatment was fed 100% *Artemia* nauplii (Table 2). Experiment 2 was performed in pilot–scale 12–L cylindro–conical rearing tanks with three replicates per treatment at initial larval density of 50 larvae/L using the same recirculation system and rearing condition as described in experiment 1.

2.3. Diet preparation and feeding

M. rosenbergii larvae in the two experiments were fed different diets including *Artemia franciscana* nauplii (Great Salt Lake strain, Crystal Brand, Ocean Star International, Inc. USA); a wet egg custard–like diet following the formulation of Hien et al. (2002); and two kinds of commercial shrimp larval diets (1) Brine Shrimp Flakes (Ocean Star International, Inc. USA) and (2) Gromate (Fantai company, Taiwan). The formulation of the wet diet and the proximate composition of the three different substitution diets are presented in Table 3.

Artemia naupllii were hatched according to standard techniques following Van Stappen (1996). *Artemia* nauplii were collected as instar I stage and kept in a refrigerator at 4–6^{0}C with gentle aeration in order to maintain instar I stage nauplii for feeding throughout the day. Decapsulated *Artemia* cysts used in the experiment 1 were prepared following Tunsutapanich (1979). The ingredients of the wet diet were weighed and blended. The resulting mixture was placed in a pan and cooked in a water bath to pudding consistency. After cooling, it was cut into small pieces, individually wrapped with polyethylene film and kept in a freezer for use the next 1–2 weeks. Before being fed to the larvae, the pieces were made into smaller particles, which were then sieved with different mesh screens to obtain three size classes of 250–500, 500–750 and 750–1000 μm for feeding based on the larval stages IV–VI, VII–IX and X–XII respectively. The Brine Shrimp Flake diet was also sieved into different size classes using mesh screens to obtain the desired sizes for feeding. The Gromate feed had a particle size from 150–500 μm and could directly be fed to the larvae. All supplemental or substitution diets were fed to the larvae from day 8 after hatching onwards (about larval stages V–VI). The artificial diets were fed several times daily following the feeding schemes in Tables 1 and 2. The different substitution and supplementation treatments were based on a standard *Artemia* ration of 6, 8 and 10 *Artemia* nauplii/mL/day for the periods from day 1–7; day 8–15 and day 16–PL stage respectively. The amount of formulated feeds given was based on visual observation of the larval tanks upon feeding. Special care was taken not to overfeed, as this may cause degradation of the water quality.

2.4. Evaluation parameters

At day 10 and 15, a larval stage index (LSI) was determined following Maddox and Manzi (1976)

Table 1. Different diets and feeding schedules used in experiment 1

Treatment[1]	Feeding scheme										
	Day 1–7		Day 8–PL								
	7h	17h	7h	9h	10h	11h	12h	13h	14h	15h	17h
100N	50N	50N	50N								50N
50N+50C	50N	50N	50C								50N
100C	50N	50N	50C								50C
75N+F	50N	50N	25N		F		F		F		50N
75N+W	50N	50N	25N		W		W		W		50N
50N+F	50N	50N	F	F		F		F		F	50N
50N+W	50N	50N	W	W		W		W		W	50N

[1]N: *Artemia* nauplii; C: Decapsulated *Artemia* cysts F: Brine Shrimp Flakes; W: Wet diet. Values represent the percentage of the standard daily *Artemia* nauplii/cysts ration, which constitutes 6, 8 and 10 *Artemia* nauplii/cysts/mL for day 1–7; day 8–15 and day 16–PL stage respectively.

Table 2. Different artificial diets and feeding schedules used to supplement or substitute *Artemia* nauplii in experiment 2

Treatment[1]	Larval rearing day	Feeding scheme				
		7h00	10h00	12h00	14h00	17h00
Control treatment (1) 100N	1–PL	50N				50N
Replaced *Artemia* treatments was applied the same feeding regime in below						
(2) N+W; (3) N+F; (4) N+G	1–7	50N				50N
	8–15	25N	*x*	*x*	*x*	50N
	16–PL	*x*	*x*	*x*	*x*	50N

[1]N: *Artemia* nauplii; W: Wet diet; F: Brine Shrimp Flake; G: Gromate; "*x*": time points when artificial diet was fed. Values represent the percentage of the standard daily *Artemia* nauplii ration, which constitutes 6, 8 and 10 *Artemia* nauplii/mL for day 1–7; day 8–15 and day 16–PL stage respectively.

to assess larval development. (LSI was determined during larval stage from 1-11 when has not any PL occurred). For this at least 30 larvae were sampled from each treatment and the average larval stage determined. The larval stage was recorded based on the description by Uno and Kwon (1969). The duration of the rearing cycle (days) was determined for each rearing tank. For this the duration from larval stocking up to the time 90% of the larvae in the rearing tank had metamorphosed into postlarvae was recorded. At the same time the final larval survival rate in each treatment was recorded. Larvae were also subjected to a total ammonia nitrogen (TAN) toxicity test following the procedure described by Armstrong et al. (1978) in order to assess larval quality.

Where:

$[NH3] = [TAN] / (1 + 10^{[pK–pH]})$

pK = 9.31 at temperature of 28^0C and salinity of 12 g/L.

pH = mean of values measured at the beginning and the end of test.

The test was performed on postlarvae in a series of 1–L glass cones at 28 ± 1^0C. Groups of 30 animals from each treatment were exposed during 24h to 4 increasing concentrations of total ammonia and a control (no ammonia added). As the toxicity of TAN is a function of temperature and pH, the pH of the test solution was adjusted at 7.8–8.0. Based on the mortality rates, the mean lethal concentrations for 50% of the population (24h–LC_{50}) were estimated.

2.5. Statistical analyses

Larval stage index; duration of rearing cycle; survival and ammonia toxicity data were analyzed by analysis of variance (one–way ANOVA) and, if significant differences were found ($P < 0.05$), the least significant differences (Weller–Duncan) test was applied for post hoc comparison. All percentage data were normalized by square root–arcsine, but only non–transformed means are presented.

Table 3. Formulation of the wet diet and proximate composition of the three formulated diets

Formulation of wet diet (%)		Proximate composition of formulated diets (% dry weight)			
			Wet diet	Flakes*	Gromate*
Milk powder	53.8	Protein	48.6±1.2	53	57
Chicken egg yolk	41.7	Lipid	25.5±0.7	9	8
Squid oil	3.0	Ash	5.8±0.1	4	13
Lecithin	1.5	Mineral	6.5±0.1	2	2
Vitamin C	200 mg/kg	Fiber	0.3±0.0	2	4
		Moisture	57.7±2.5	9	9

*Composition based on the product label.

3. Results

3.1. Experiment 1

Larval development rate in terms of larval stage index in experiment 1 showed significant differences between treatments. At day 10, three different groups had formed based on larval stage index ($P < 0.05$). The lowest performance was observed in the treatments 50N+50C and 100C. In contrast to the fastest growth was found for treatments 100N, 75N+F and 75N+W. Treatments 50N+F and 50N+W showed intermediate development rates. At day 15 of the experiment, the larval development rate in treatment 100C was significantly lower compared to all others treatments ($P < 0.05$). The treatment 50N+50C had a significantly higher LSI than the treatment 100C but lower than treatment 75N+W (Figure 1). Larval survival rate at the end of rearing cycle also showed significant differences. Three different groups could be distinguished. The lowest survival (30%) was observed in the treatments 100C and 50N+F. The highest survival (43–45%) was observed in the treatments 100N, 75N+F and 75N+W. Intermediate values around 35% were found in the treatments 50N+50C and 50N+W (Figure 2). Considering the duration of the rearing cycle, an opposite trend as for survival was noted. Larvae in the treatments 75N+F and 75N+W needed around 24–25 days of rearing to reach the postlarval stage, which was significantly shorter than for treatments 50N+50C and 100C, in which the duration of the rearing cycle was extended up to 28–29 days (Figure 2). The results of the ammonia stress test showed differences in postlarval tolerance (LC_{50}) ($P < 0.05$). The group containing treatments 100C and 75N+F presented the lowest values (136–138 mg/L TAN), intermediate tolerance levels were found in treatments 50N+50C and 50N+W (165–168 mg/L TAN), while the highest tolerance was found in treatments 75N+F and 75N+W (185–189 mg/L TAN) (Figure 3). In general, the treatments 100N, 75N+W and 75N+F showed the best overall results in term of larval development, survival and larval quality. While the treatments 100C and 50N+F showed the lowest results.

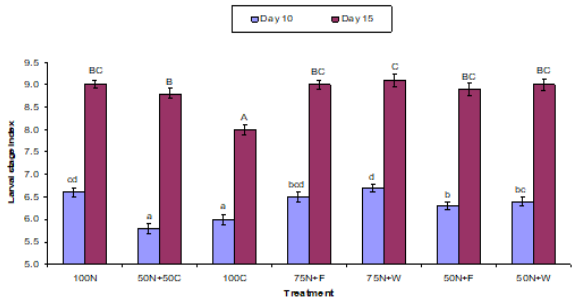

Figure 1. Larval stage index at day 10 and 15 of*M. rosenbergii* larvae reared according to different feeding schedules in experiment 1. Different letters between treatments denote significant differences ($P < 0.05$). For description of treatments refer to Table 1.

3.2. Experiment 2

At day 10 of the rearing period, the larvae in the different treatments showed the same development rate ($P > 0.05$). However, larval development rate in treatments 100N and N+W became significantly higher compared to treatment N+G ($P < 0.05$) by day 15 of the rearing cycle (Figure 4). Survival rate results at the end of the experiment revealed a significantly higher survival in treatments 100N and N+W (53–54%) compared to treatment N+G, which had a survival of only 40% ($P < 0.05$). Evaluation of the

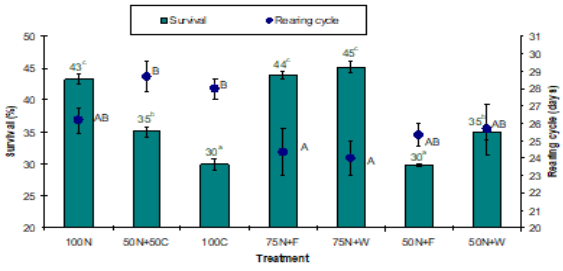

Figure 2. Survival and duration of the rearing cycle of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 1. Different letters between treatments denote significant differences ($P < 0.05$). For treatment descriptions refer to Table 1.

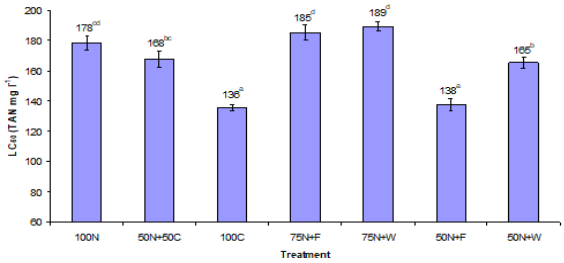

Figure 3. Ammonia tolerance (expressed as 24 hour LC_{50}–TAN) of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 1. Different letters between treatments denote significant differences ($P < 0.05$). For treatment descriptions refer to Table 1.

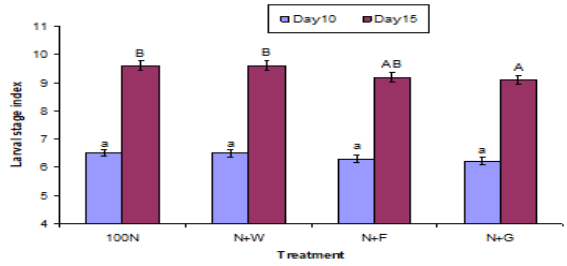

Figure 4. Larval stage index at day 10 and 15 of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 2. Different letters between treatments denote significant differences ($P < 0.05$). For treatment descriptions refer to Table 2 and 3.

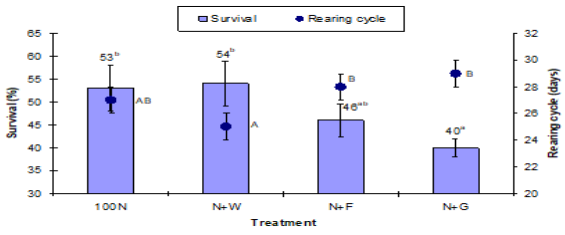

Figure 5. Survival and rearing cycle of *M. rosenbergii* larvae reared according to different feeding schedules in the experiment 2. Different letters between treatments denote significant differences ($P < 0.05$). For treatment descriptions refer to Table 2 and 3.

duration of rearing cycle showed that larvae in the treatment N+W completed the rearing cycle in 25 days, which was significantly shorter than in the treatments N+F and N+G which needed 28 and 29 days respectively (Figure 5). Postlarval tolerance to total ammonia was significantly higher in treatments 100N and N+W (190 and 214 mg/L TAN respectively), compared to treatment N+G for which the LC_{50} was only 145 mg/L TAN ($P < 0.05$) (Figure 6). In general, the treatments 100N and N+W showed better results in terms of larval development, survival, rearing and larval quality compared to treatment N+G.

4. Discussion

In experiment 1, the results of larval development, survival, duration of the rearing cycle and larval quality distributed the treatments into three distinct groups. The best group included the treatments fed exclusively *Artemia* nauplii and the treatments in which around 25% of the *Artemia* ration was replaced with artificial wet or dry diets. Consequently, the replacement of a part of the live food in the feeding schedule did not affect performance of the larvae. However, treatments in which 50% of the live feed was replaced from day 8 onwards reduced survival rate and larval quality. Especially, the use of an exclusive diet of decapsulated *Artemia* cysts seemed not appropriate for *M. rosenbergii* larval development. Although *Artemia* cysts are reported to contain higher energy and nutrient levels than *Artemia* nauplii (Sorgeloos et al., 1977; Leger et al., 1987; Bengtson et al., 1991), it was observed that they rapidly sink to the bottom upon feeding, thus reducing their availability for the larvae to feed upon in the water column (Lavens & Sorgeloos, 1996). This while the behavior of prawn larvae is rather to swim in the upper part of the water column or at the water surface. Increasing the aeration in the rearing containers may keep these particles better in suspension, however the increased turbulence may make it

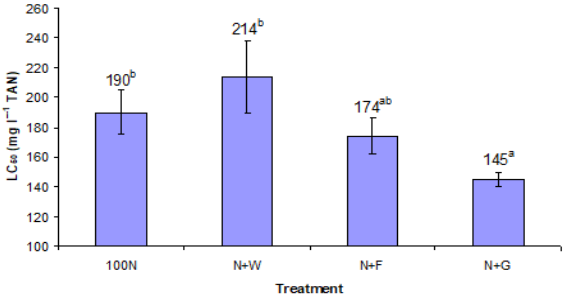

Figure 6. Ammonia tolerance (expressed as 24hour LC_{50}–TAN) of *M. rosenbergii* larvae reared according to different feeding schedules in experiment 2. Different letters between treatments denote significant differences ($P < 0.05$). For treatment descriptions refer to Table 2 and 3.

more difficult for the larvae to capture and ingest the prey. Decapods larvae do not specifically orientate towards a food source, they depend on chance encounter to capture food (Kurmaly et al., 1989). In addition, *Artemia* cysts have a round shape, which may be difficult for the larvae to capture and hold on to during eating. In contrast, the mobility of *Artemia* nauplii allows its permanence in the water column, thus, increasing the chances of encounter (Barros & Valenti, 2003a). Using exclusively decapsulated cysts, which have a narrow size range (210–260 μm, Tackaert et al., 1987) may also not be appropriate for all larval stages during development. Barros & Valenti (2003a) suggested that live food supplementation should start from stage VII onwards, using food particles increasing from 250 to 1190 μm. Therefore, the dimensions of decapsulated cysts may be appropriate for stage VII and VIII *M. rosenbergii* larvae only.

Replacing *Artemia* nauplii by artificial diets at a constant ratio of 50% from larval stage V–VI onwards (in experiment 1) negatively affected survival rate, but did not affect larval growth. This may be explaining by the drastic and sudden reduction of live feed in these treatments. In these treatments live feed was supplied only one time per day in the evening, and consequently the live feed density during the day time was low. Especially in the early period of weaning, the larvae may not have been adapted yet to non–living feed, probably resulting in low survival due to increased cannibalism. Indeed, when the larvae were more gradually weaned from *Artemia* onto formulated feeds (experiment 2), better results were obtained. Therefore, it is recommended to replace only 25% of the *Artemia* ration at the start of the weaning period to allow the larvae to adapt to the new diet. Subsequently, the weaning ration may be increased up to 50%, spread over several feedings per day. The replacement diets need to be offered with increasing particle sizes in function of the larval stage. In this respect, it was found that the Gromate feed, which had a rather narrow particle size range of 150–500 µm showed lower results compared to the wet and flake diets. Although the Gromate feed contained a higher protein level than the other diets, the narrow particle size range may have been a disadvantage for later *M. rosenbergii* larval stages. In contrast, the wet and flake diet could easily be sieved into the desired particle sizes using sieves with different mesh sizes.

In the present study, artificial diets were supplied from day 8 (stage V–VI) onwards. It was noticed that the larvae readily accepted the inert feeds. In this respect, the wet diet seemed to be more attractive to the larvae than the dry diets. Barros & Valenti (2003a) stated that the larvae only accepted inert feed from stage VII onwards and suggested that the live feed could totally be replaced with wet or dry diets from stages VII and IX onwards respectively. However, it is necessary to evaluate final survival rates and productivity when applying total substitution of *Artemia* for commercial larviculture. Murthy et al., (2008) suggested that using wet diets which contain shrimp and clam meat fed to larvae in combination with *Artemia* nauplii showed larval survival rates of 40% in 150–l rearing tanks. Islam et al. (2000) reported that freshwater prawn larvae reared in a recirculation system with 140–l rearing tanks fed *Artemia* nauplii supplemented with egg custard obtained a survival of 30%, which was higher than larvae fed exclusive *Artemia* (only 12%). However, Kamarudin et al. (2002) studied the use of artificial diets containing various ratios of cod liver and corn oil to replace 25-100% of the standard *Artemia* nauplii ration from stage III to XI. The results showed that there were no significant differences in survival between the substitution treatments and the control treatment fed solely *Artemia* nauplii. In the current study, a gradual replacement of up to 50% of the *Artemia* nauplii ration with wet and dry diets showed similar compared to a 100% *Artemia* control in terms of larval development, survival and larval qual-

ity. However, performance was impaired when the *Artemia* diet was abruptly replaced at a constant rate of 50% from day 8 onwards. In practice production efficiency depends on the production cost, which is based on the feed source and cost, labour cost, etc., cost–effectiveness may therefore vary from one region to another. Therefore, the feeding strategy in *M. rosenbergii* larviculture cannot be standardized. The results obtained in the present work may however serve as a guideline for practical considerations of feeding strategies.

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Evaluating the growth capacity and heavy metal absorption of sweet sorghum and grain sorghum at the seedling stage

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ABSTRACT

In recent years, the use of plants for clean-up and recovery (phytoremediation) has been studied and used in many countries of the world. In this study, E-Tian sweet sorghum (ET) and BT x 623 (BT) sorghum were treated with heavy metal cadmium at 5 concentrations (0, 5, 10, 25, 50 mg/kg). The growth of plant; absorption, accumulation of cadmium (Cd) heavy metals in plant parts at the seedling stage have been identified and assessed. The results showed that Cd affected the height and number of leaves of the plant. Especially, Cd accumulation in the plant decreased in sequence: root, stem, leaf. When comparing the heavy metals accumulation in the two cultivars, the results showed that the BT cultivar had higher Cd uptake and accumulation potential than ET. Therefore, BT can be used for phytoremediation of heavy metals in soil but not for providing food and feed.

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1. Introduction

Nowadays, heavy metal contamination in soil has become a great concern for global and every country due to human's mining and using fertilizer and pesticide application, and fuel production... (Garbisu & Alkorta, 2003). Excessive heavy metals, for example, lead (Pb), chromium (Cr), zinc (Zn), cadmium (Cd), copper (Cu), and nickel (Ni), in agricultural areas seriously threaten food safety and public health. Elimination or remediation of heavy metal contamination in soil is urgently in request to prevent human and animals from toxicity. The treatment for these heavy metals meets many difficulties and costs much. In current years, phytoremediation is a cost-effective and eco-friendly technique to clean up heavy metal pollution in soil (Li et al., 2004).

Sorghum (*Sorghum bicolor* L.) consists of natural variant cultivars of sorghum with abundant sucrose storage in culm and great biomass, and is thereby considered an ideal feedstock for biofuel production (Murray et al., 2008). Sweet sorghum will be a competitive candidate species for soil remediation due to its great biomass and strong resistance to adverse environmental conditions. To preliminarily evaluate its potential for phytoremediation, a number of morphological and physiological characteristics of sorghum were investigated under heavy metal stresses (Cd, Pb, Zn, Cu) in previous studies (Zhuang et al., 2009; Liu et al., 2011; Soudek et al., 2013). The results indicate that sweet sorghum can grow well, absorb heavy metals, and clean up contaminated soil (Zhuang et al., 2009).

Grain sorghum is an important cereal in temperate semi-arid countries with high yield and rapid growth (Rawy et al., 2013). However, no research has been done on the absorption and accumulation of heavy metals in this important food crop.

In this paper's scope, we study the accumulation of Cd heavy metals in plant parts, then, compare the capacity of Cd heavy metal accumulation of two sorghum kinds. The research results contribute to providing the scientific basis for the application of sorghum for the purpose of restoring agricultural land contaminated with heavy metals.

2. Material and Methods

2.1. Plant material and experimental design

The elite line of sweet sorghum E-Tian (ET) and grain sorghum BT x 623 (BT) were used for experiments. The ET originated from China, which was introduced in 1970s possesses rich sugar storage in stem. The BT x 623 (BT) originated from America with high yield and good quality (Zheng et al., 2011).

The soil was fertilized with base fertilizers (urea, diammonium phosphate and potassium sulfate), contained 2.0 g nitrogen, 0.26 g phosphorus (P_2O_5) and 0.35 g potassium (K_2O) for high-yield land application. Soil is crushed, put into the pots (2 kg/pot, pot sizes are 30 cm in diameter, 25 cm in height). Soil was amended with $CdCl_2$ at final concentrations of 0, 5, 10, 25, 50 mg/kg. The group not treated with $CdCl_2$ was the control group.

Seeds were soaked in warm water at 28^0C, then placed on a moist filter paper tray in a warm place for germination. After 3 days, the seedlings were transferred to the potted soil, 2 seedlings/pot. The pots were placed in the greenhouse of the Institute of Environmental Resources, Southwestern University, China at a temperature of 28-32^0C during lighting time of 14-16 h; and at 22-26^0C in the dark for 8-10 h. Water content was adjusted daily. The water-holding capacity of the soil was never exceeded, therefore no leaching occurred. The same care conditions and procedures were used for all experimental and control plants. Each experiment formula and control formula consisted of 12 plants with 3 replications. On day 35 after planting, roots, stems, and leaves were collected and analyzed in the laboratory.

2.2. Analysis of Cd heavy metal content

The plant samples were dried in a ventilated oven at 105^0C for 30 mins and 70^0C for 48 h and subsequently grinded into powders. Approximately 0.1 g of the grinded sample was soaked in a mixture of HNO_3 and $HClO_4$ (3:1; v/v) according Sun et al. (2008). Cd concentration was determined using a flame atomic absorption spectrometry HITACHI Z5000 (Tokyo, Japan).

2.3. Data analysis

The data were calculated using Statistix (version 9.0). Significant differences were determined by least significant differences (LSD) at 5% level of probability.

3. Results

3.1. Effect of Cd on plant height

Although there was no significant difference between the results, low Cd concentrations had a slight effect on the growth of the plant height in the sweet sorghum ET (average height at 5 mg Cd/kg was 66.67 ± 3.05 cm, Table 1). While high Cd levels inhibited the growth of plant height (at 25 and 50 mg/kg Cd, the mean plant heights were 57 ± 7.21 cm and 48 ± 3.06 cm, respectively).

The plant height of BT was inhibited by Cd very clearly, the higher the Cd level was, the lower the plant height was (from 64.00 ± 10.54 cm in the control, down to 46.5 ± 3,04 cm in the experimental formula Cd 5 mg/kg and reached the minimum value 40.33 ± 9.87 cm at Cd 50 mg/kg). The results showed that Cd significantly affected the growth of both sorghum experimental varieties.

Comparison between the two varieties, it can be seen that ET can be better tolerated than BT when it was treated with low concentrations (5 mg/kg Cd), expressed by higher of plant height than the control group.

3.2. Effect of Cd on number of leaves

At the seedling stage, the response of sorghum to Cd was similar in both experimental varieties. The number of leaves of both varieties decreased in comparison with the control group. However,

for ET varieties, as well as the plant height index, the number of leaves increased at Cd 5 mg/kg, and decreased gradually as the Cd concentration increased (Table 1). The results show that Cd affects the number of leaf, which can affect other growth characteristics such as photosynthesis, sugar content and starch content of plants.

In previous studies also showed that the higher the Cd level was, the lower the gowth rate. Liu et al. (2011) reported that low Cd concentrations could promote plant growth and height of sweet sorghum and experimental sudan grass variety, plant height reached high values at Cd 25 mg/kg while at high concentrations (50 and 100 mg/kg), Cd inhibits the growth of plant height. In other studies in rice, high concentrations of Cd (50 and 100 mg/kg) strongly inhibited the height of the plant (Herath et al., 2014). A study by Liu et al. (2014) showed that, after 3 to 7 days of treatment with Cd (25, 50 and 100 mg/kg), the growth of young cotton plants were significantly inhibited, reflecting a sharp drop in height, biomass and leaf area.

3.3. Ability to absorb heavy metals in plants

The experimental results showed that, at the seedling stage, both ET and BT showed strong Cd absorption from contaminated soil (Figure 1a and b). In the control treatments of both cultivars, there was not Cd. However, in the experimental formulation, the Cd concentrations in the leaves, stems and roots of both varieties also increased when Cd levels were treated accordingly. There was a significant difference in Cd concentration accumulated in the plants between the Cd treatments. In both varieties, roots absorbed and accumulated the highest Cd, followed by stems and leaves. This suggests that the plant has the mechanism to prevent the transfer of Cd from the roots to the shoots.

In ET, the lowest Cd concentration accumulated was recorded in leaf of plant. Cd concentrations increased from 0.005 μg/g DW to 0.453 μg/g DW when the Cd level increased from 5 to 50 mg/kg (Figure 1a). Cd concentration in roots is 6 times as high as Cd accumulated in stems. The highest Cd concentration in roots was recorded in the experimental Cd concentration 50 mg/kg (4.51 μg/g DW).

Comparison between the two types, the results showed that BT varieties can absorb and accumulate Cd content is nearly 5 times as high as than ET can (Figure 1b). Cadmium content in leaves of BT was so high, increasing 2.559 μg/g DW to 6.788 μg/g DW when Cd treatment increased from 5 to 50 mg/kg. Cd content accumulation in the stem was also much higher than ET, reaching the highest value of 10.332 μg/g DW at Cd 50 mg/kg, 3 times as high as ET's. Cadmium was absorbed and accumulated at the highest level in roots, the highest accumulated Cd value was 14.719 μg/g DW.

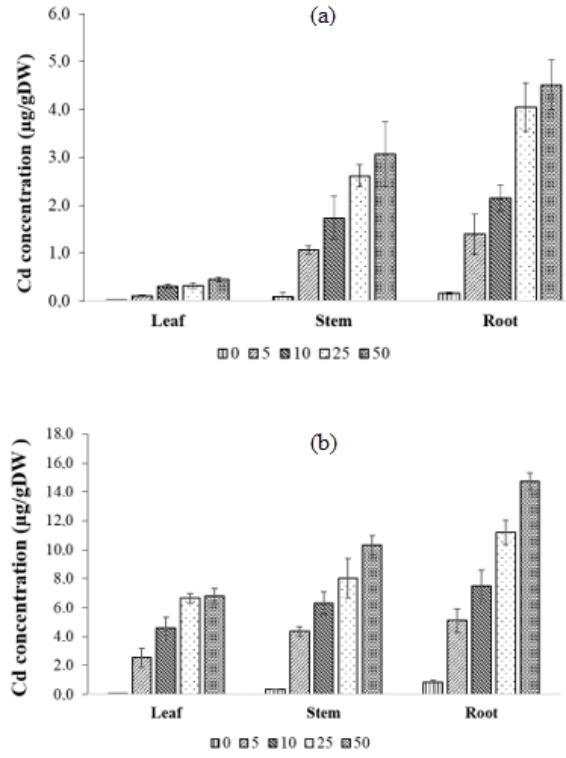

Figure 1. Cd concentration accumulated in parts of ETian sweet sorghum (1a) and BT grain sorghum (1b) (*DW: dry weight*).

4. Discussion

The distribution of Cd in different parts of vegetation plays an important role in minimizing the harms of heavy metals to plant. At the seedling stage of the study, both sorghum varieties showed their Cd absorption ability was high. The results of this study are consistent with previous studies on different vegetation species (Barros et al., 2009; Zhuang et al., 2009; Angelova et al., 2011). Tu et al. (2013) investigated the concentration

Table 1. Effect of Cd on plant height and leaf number

Cd treatment		Plant height (cm)	Number of leaf
ET	0	63,67 ± 9,07^{a}	7,00 ± 0,00^{ab}
	5	66,67 ± 3,05^{a}	7,30 ± 0,57^{a}
	10	57,33 ± 8,08^{ab}	6,00 ± 0,00^{c}
	25	57,00 ± 7,21^{ab}	6,30 ± 0,57^{bc}
	50	48,00 ± 3,06^{b}	6,67 ± 0,57^{abc}
BT	0	64,00 ± 10,54^{a}	6,67 ± 0,57^{a}
	5	46,50 ± 3,04^{b}	6,00 ± 0,00^{ab}
	10	48,67 ± 5,51^{ab}	5,67 ± 0,57^{b}
	25	44,33 ± 11,01^{b}	5,33 ± 0,57^{c}
	50	40,33 ± 9,87^{b}	4,67 ± 0,57^{bc}

$^{a\text{-}c}$The different letters in the same column of a variety show a significant difference at $P < 0.05$.

of Cd in leaves, roots and stems of two sweet sorghum varieties which increased with prolonged treatment time with Cd. Both varieties exhibited high Cd accumulation in roots, followed by stems and leaves. However, the results of studies by Izadiyar and Yargholi (2010) on Cd absorption and accumulation in sorghum are the highest in roots and lowest in stems. The results show that the response to heavy metals is different depending on each type of sorghum.

Cadmium is absorbed and accumulated firstly in the roots, then transported to the stems and leaves. The BT sorghum has a lower average plant height than the ET sweet sorghum, BT also has strongly ability of growth, thus, the Cd can be transported from the roots to the stem and to leaves with more strength. It is possible that the defense mechanism of ET against Cd is better than that of BT. As a result, the Cd content accumulated in parts of the plant is lower. Therefore, further research is needed on the molecular and biochemical mechanisms and physiology of these two sorghum varieties. In a study by Pinto et al. (2006), increasing Cd pollution caused the rise of phytochelat content in plants.

Phytochelatins are an important layer of proteins, which are produced by the plants to increase their response to heavy metal ions such as Hg and Cd, in order to reduce the damage of these metals in the plant (Pinto et al., 2006). The results of Soudek et al. (2013) showed that the roots have a mechanism to prevent Cd transport to shoots. Cadmium accumulation in plants is limited by several factors, such as: 1) biological activity of roots; 2) the speed of transportation to the roots through apoplastic and symplastic pathways; 3) Cd fixation in roots such as Cd-phytochelatin complex formation and accumulation in vacuoles; 4) Transport speed in xylem and Cd distribution to plant parts (Rahat et al., 2012).

5. Conclusions

The experiment helped to determine, compare the absorption and accumulation of Cd heavy metals in parts of sweet sorghum and grain sorghum. It can be concluded that, Cd affects the growth of both sorghum varieties, through reduction of plant height and number of leaves. Each plant has different levels of Cd accumulation in order: root, stem, leaf. Comparison between the two sorghum varieties, BT has higher ability of Cd absorbtion and accumulation than ET. Therefore, BT can be used for phytoremediation of heavy metal in soil but could not use for providing food and feed. If combined with the purpose of treating heavy metal pollution and biofuel production, ET sweet sorghum could be used.

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The use of water spinach (*Ipomoea aquatica*) in domestic wastewater treatment

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ABSTRACT

The main objective of this study was to examine the efficacy and capacity of using hydroponic systems in municipal pollutant removal at household scale. Three pilot scaled hydroponic systems [dimension for each system: 4.5 m (L) x Φ 114 mm] were installed to investigate the optimal age of vegetable, planting density and retention time for household wastewater treatment, respectively. Water spinach (*Ipomoea aquatica*) planted in 27 plastic cups throughout 4.5-m-length and 114-mm-diameter uPVC pipes filled with wastewater was employed as the treating agent of pollutants. The averaged influent contained proximately 32.5 mg/L suspended solids (SS), 76.0 mg/L biological oxygen demand (BOD_5), 220.5 mg/L chemical oxygen demand (COD), 26 mg/L NH_4^+, 5.0 mg/L NO_3^-, and 8.5 mg/L PO_4^{3-} at pH 7.3. Results showed that a designed system consisting of 10 plants of 15-day-old water spinach pre-planted in baked clay in each cup was capable of treating 30 L of domestic wastewater meeting the current municipal wastewater discharge standards in Vietnam (column A standards of QCVN 14:2008/BTNMT) after 4 days of wastewater retention time. If operated under conditions of the above parameters, the pilot-plant hydroponic system can achieve the removal of 65% SS, 82% BOD_5, 74% COD, 90% NH_4^+, 30% NO_3^- and 86% PO_4^{3-}. The result of this study has provided an applicable domestic wastewater treatment system eco-friendly and suitable for small and medium household areas.

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1. Introduction

The proportion of domestic wastewater treated is at low levels, and raw wastewater is usually discharged directly to environment in urban areas of Vietnam (MONRE, 2016). Currently, 37 collective wastewater treatment plants have been in operation in urban centers of grade III or higher cities (MONRE, 2016). Wastewater drainage systems, however, have not been completed, causing difficulties in collecting and leading wastewater to treatment plants (MONRE, 2016). Hence, a domestic wastewater treatment plant at household scale is necessary to reduce pollutant loads to environment.

Domestic wastewater can be treated in different ways: mechanically, chemically or biologically (Luong, 2011; Hoang & Tran, 2014). Among biological treatments, the hydroponic system is a potential way for wastewater treatment at household scale because it is easy to establish and requires small space and harvested vegetable can be used as food (VEA, 2010). Hydroponic crops can be almost any type of plants such as vegetables, fruits, flowers, garden trees, herbs, ivy, and perennial that crops are harvested after a short planting period (Lem et al., 1990). It is easy to control various environment parameters as nutrients, pH, temperature, oxygen, etc. (Lem et al., 1990). Wastewater would be used instead of chemical fertilizers for growing vegetables. However, hydroponics has disadvantages such as higher initial costs than planting in soil and diseases could spread to the other plants root easily and are dif-

ficult to control in the case of planting with recirculation systems (Lem et al., 1990).

Ipomoea aquatica, or water spinach, is a herbaceous perennial trailing vine (Patnaik, 1976). It has hollow stems that grow floating or prostrate (Patnaik, 1976). The roots from the nodes penetrate the soil or mud, and the leaves are simple and alternate (Patnaik, 1976). This plant species grows well as a crop in regions where the mean temperature is above 25^0C (Patnaik, 1976). Hence, hydroponics in Vietnam is a conducive environment for water spinach to flourish.

Previous studies have demonstrated that planting *Ipomoea aquatica* in fishponds can efficiently remove nutrients and improve water quality (Li & Li, 2009; Dai et al., 2012). Accordingly, the current study expected that water spinach could use the nutrients in domestic wastewater for growing and reducing water pollutant loads. Pilot hydroponic systems with water spinach were established to examine the removal percentages of municipal pollutants in wastewater from an apartment. Moreover, the optimal age of water spinach, planting density and retention time were also determined for household guidelines.

2. Materials and Methods

2.1. Domestic wastewater characteristics

Domestic wastewater was collected from collecting tank of Sunview Apartment, Cay Keo Street, Thu Duc District, HCMC, Vietnam in the morning from January to June 2017 according to TCVN 6663-1:2011 and ISO 5667-1:2006. The wastewater parameters included: water temperature 29^0C, pH 7.3, SS 32.5 ± 1.5 mg/L, BOD_5 76.0 ± 8.0 mg/L, COD 220.5 ± 25.5 mg/L, NH_4^+−N 26.0 ± 4.0 mg/L, NO_3^-−N 5.0 ± 1.0 mg/L, and PO_4^{3-} 8.5 ± 1.5 mg/L and did not vary much throughout the experiments. Wastewater was pre-filtered through a kitchen sieve to remove large particles, contained in 30-L plastic buckets and transferred to Environmental Technology Laboratory of Faculty of Environment and Natural Resources, Nong Lam University. The wastewater was then analyzed and employed for the experiments immediately.

2.2. Conditions of water spinach

Prior to the experimetns, water spinach was grown hydroponically in baked clay at Institute of Biotechnology and Environment (IBE), Nong Lam University. Water spinach seeds were provided by Phu Nong Seeds Company.

2.3. Experiments

2.3.1. Hydroponic systems

Three pilot scaled experiments consisting hydroponic systems [dimension for each system: 4.5 m (L) x Φ 114 mm] were installed with water spinach to investigate the optimal age of vegetable, planting density and hydraulic retention time (HRT) for household wastewater treatment, respectively (Figure 1). Water spinach (*Ipomoea aquatica*) planted in 27 plastic cups throughout 4.5-m-length and 114-mm-diameter uPVC pipes filled with wastewater was employed as the treating agent of pollutants. A similar designed pipe without water spinach was used to make the control.

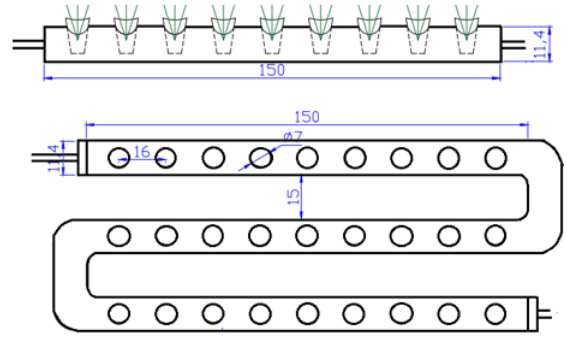

Figure 1. Hydroponic pilot (sizes in cm).

The pre-experiments were executed to choose ranges of vegetables' optimal age (10, 15 and 20 days old), optimal planting density (5, 10 and 15 plants per cup) and optimal retention time (2, 4 and 6 days).

2.3.2. Determination of the optimal age of vegetables

After 10, 15, and 20 days pre-planted in baked clay at IBE, water spinach was transferred to three hydroponic systems, respectively in 27 plastic cups. Each cup contained 10 plants. The control system was made without vegetables. Thirty liters of domestic wastewater were added to each hydroponic systems with HRT = 4 days. Treated wastewater was collected after HRT to analyze

SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentrations remaining.

2.3.3. Determination of the optimal planting density

Fifteen-day-old water spinach was planted in 27 plastic cups with 3 different densities of 5, 10 and 15 plants per cup throughout the pipes, respectively. The control system was made without vegetables. Thirty liters of domestic wastewater was added to each hydroponic systems with HRT = 4 days. Treated wastewater was collected after HRT to determine SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentration residues.

2.3.4. Investigate the optimal retention time

Thirty liters of domestic wastewater was added to each hydroponic systems. Fifteen-day-old water spinach was removed from baked clay and put in 27 lastic cups with the density of 10 plants/cup. There were 3 hydroponic systems with 3 different HRTs of 2, 4, and 6 days, respectively. A control system was made without vegetables. Treated wastewater was collected after HRT to analyze SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentrations remaining.

2.4. Water analysis

The concentrations of SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} and pH of the wastewater out of the hydroponic systems were checked after hydraulic retention time. The water sample was collected stochastically from three locations of each hydroponic system from 8 AM to 9 AM with 100 mL per model.

Chemical oxygen demand was analyzed according to SMEWW 5220 D (2012). BOD_5 was analyzed according to TCVN 6001-1:2008 and ISO 5815-1:2003. NH_4^- (LoD = 0.2 mg/L, LoQ = 0.5 mg/L), NO_3^- (LoD = 4 mg/L, LoQ = 10 mg/L) and PO_4^{3-} (LoD = 0.04 mg/L, LoQ = 0.1 mg/L) concentrations were determined by Sera Test Kits (Germany). In addition, the samples have concentrations of NO_3^- less than 20 mg/L were determined by Tropic Marin Test Kits (Germany) with LoD = 0.5 mg/L and LoQ = 1.5 mg/L. pH was measured by LAQUAtwin portable pH meter (HORIBA Scientific, Japan). Temperature was measured by mercury thermometer. Each measurement was made 3 times.

3. Results

3.1. Optimal age of water spinach

After 4 days, SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentrations of wastewater in the hydroponic systems containing 10, 15, and 20-day-old water spinach were 13.0 ± 1.5, 15.0 ± 2.0, 61.0 ± 5.0, 4.0 ± 1.0, 3.0 ± 0.5 and 2.0 ± 0.5 mg/L; 11.8 ± 1.3, 13.5 ± 2.5, 57.5 ± 5.5, 2.5 ± 0.5, 3.5 ± 0.5 and 1.2 ± 0.2 mg/L; and 16.0 ± 1.0, 15.5 ± 2.0, 67.5 ± 6.5, 3.5 ± 0.5, 4.0 ± 1.0 and 2.5 ± 0.5 mg/L, respectively (Figure 2). The pH values ranged from 7.9 to 8.1 in the three systems. As a result, the efficiency of the system with 15-day-old water spinach was greater than that of the other systems. Therefore, 15-day-old water spinach was employed for the next experiments.

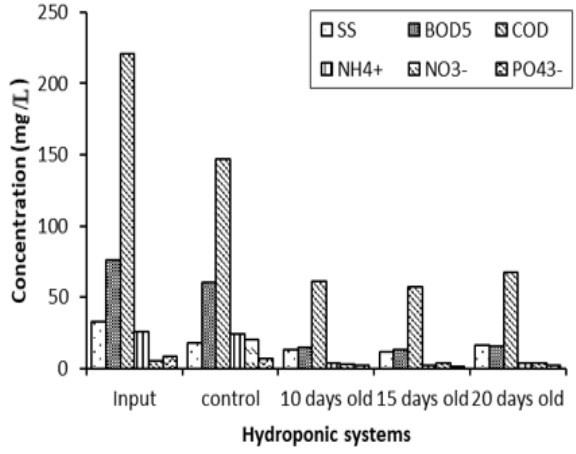

Figure 2. Treated wastewater parameters in hydroponics with different initial ages of water spinach.

3.2. Optimal planting density

After 4 days, treated SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} values of hydroponic systems with 5 plants/cup, 10 plants/cup, and 15 plants/cup were 15.0 ± 1.5, 16.0 ± 2.0, 68.0 ± 7.0, 3.0 ± 0.5, 4.0 ± 0.5 and 1.5 ± 0.5 mg/L; 11.0 ± 1.0, 14.0 ± 2.0, 55.0 ± 5.0, 2.5 ± 0.5, 3.0 ± 1.0 and 1.2 ± 0.2 mg/L; 10.0 ± 1.0, 14.0 ± 2.0, 57.5 ± 5.5, 2.5 ± 0.5, 3.5 ± 1.0 and 1.4 ± 0.2 mg/L, respectively (Figure 3). The pH values ranged from 7.5 to 8.0. Consequently, the optimal density was 10 plants each cup and used in the last experiment.

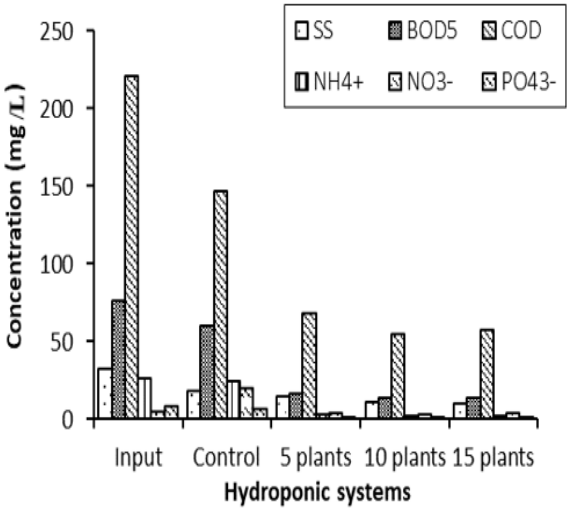

Figure 3. Treated wastewater parameters in hydroponics with different planting densities.

3.3. Optimal retention time

After HRT = 2 days, SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentrations of wastewater in the experimental hydroponic system were 19.5 ± 1.5, 53.0 ± 6.0, 97.0 ± 15.0, 3.0 ± 0.5, 4.0 ± 1.0 & 2.0 ± 0.5 mg/L, respectively (Figure 4a) and pH was 7.5 ± 0.1 while those of the control system were 24.0 ± 1.0, 68.0 ± 8.0, 160.0 ± 20.0, 24.0 ± 4.0, 5.0 ± 1.0 and 7.5 ± 0.5 mg/L, respectively (Figure 4b) and pH was 7.1 ± 0.2. After HRT = 4 days, SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentrations of wastewater in the experimental hydroponic system were 11.5 ± 1.5, 13.5 ± 5.5, 57.0 ± 8.0, 2.5 ± 0.5, 3.5 ± 0.5 and 1.2 ± 0.3 mg/L respectively (Figure 4a) and pH was 7.8 ± 0.1 while those of the control system were 18.0 ± 1.5, 60.0 ± 6.0, 146.5 ± 18.0, 24.0 ± 4.0, 20.0 ± 2.0 and 7.0 ± 0.5 mg/L respectively (Figure 4b) and pH was 6.8 ± 0.1. These parameters met the current municipal wastewater discharge standards in Vietnam (column A standards of QCVN 14:2008/BTNMT).

After HRT = 6 days, SS, BOD_5, COD, NH_4^-, NO_3^-, and PO_4^{3-} concentrations of wastewater in the experimental hydroponic system were 3.5 ± 0.5, 6.0 ± 1.0, 36.0 ± 7.0, 2.5 ± 0.5, 3.0 ± 0.5 and 1.2 ± 0.5 mg/L respectively (Figure 4a) and pH was 8.1 ± 0.1 while those of the control system were 7.0 ± 1.0, 52.0 ± 6.0, 112.0 ± 15.0, 22.0 ± 4.0, 25.0 ± 3.0 and 7.0 ± 1.0 mg/L respectively (Figure 4b) and pH was 6.5 ± 0.1.

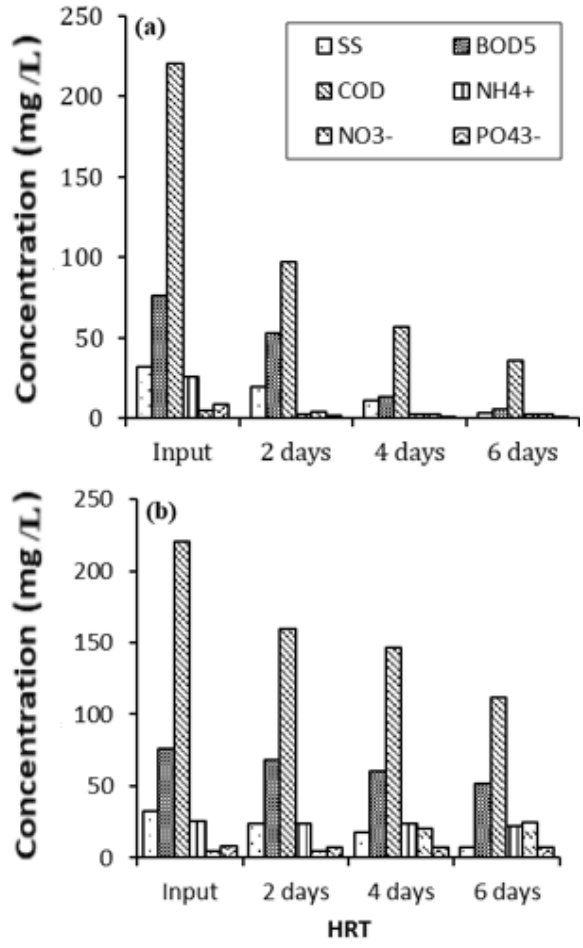

Figure 4. Treated wastewater parameters in (a) hydroponics with different HRTs and (b) the control system.

4. Discussion

4.1. Hydroponics with water spinach

In general, a hydroponic system consisting of 10 plants of 15-day-old water spinach pre-planted in baked clay in each cup could process 30 L of domestic wastewater to meet the current municipal wastewater discharge standards in Vietnam (column A standards of QCVN 14:2008/BTNMT) at a HRT of 4 days.

4.1.1. pH

pH of the wastewater out of the hydroponic systems increased slightly from 7.3 to over 7.5 in all experiments. That was because the water spinach in the hydroponic systems absorbed

CO2 for photosynthesis, so the pH of water was increased. CO2 in the water reacts with water to produce H+ and bicarbonate to decrease pH of water according to the mechanism: : $CO_2 + H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^-$ (Kanabkaew & Puetpaiboon, 2004). Because CO_2 for photosynthesis of aquatic plants is absorbed faster than the amount of CO_2 generated from the respiratory process of the quatic plants, plants must take CO_2 from the metabolism of HCO_3^- ($2HCO_3^- \rightarrow CO_2 + CO_3^{2-} + H_2O$) (Kanabkaew & Puetpaiboon, 2004). Therefore, the pH of water increases.

4.1.2. SS removal

The SS concentration decreased from 32.5 ± 1.5 mg/L to 11.8 ± 1.3 mg/L (Figure 4a), which means 65% of SS was removed from the wastewater. The removal of SS may be due to sedimentation or/and breakdown of microorganisms and plants.

4.1.3. COD and BOD_5 removal

Previous research has show that COD and BOD5 can be assimilated by plants (Vymazal & Kropfelova, 2009). The microbes around the roots can also contribute to the purification. The flourishing roots can provide a comfortable environment for microbes. Thus, the organic matter can be removed effectively. The concentrations of COD and BOD_5 decreased from 220.5 ± 25.5 mg/L to 57.5 ± 5.5 mg/L and from 76.0 ± 8.0 mg/L to 13.5 ± 2.5 mg/L, respectively (Figure 4a). 74% of the COD and 82% of the BOD_5 were removed from the wastewater. The efficiency of removal at different HRTs was quite difference. The efficiency of short HRT (2 days) was lower than that of middle HRT (4 days) (Figure 4). This could be because the plants needed a period of time to adapt to the new environment. When the roots grew flourishing, the plants could purify the water by assimilation of organic matters and nutrients.

4.1.4. Nitrogen removal

The concentrations of $NH_4^+$and NO_3^- in wastewater decreased from 26.0 ± 4.0 mg/L to 2.5 ± 0.5 mg/L and from 5.0 ± 1.0 mg/L to 3.5 ± 0.5 mg/L, respectively (Figure 4a). 90% of the NH_4^+−N and 30% of the NO_3^-−N were removed from the wastewater. The nitrogen in wastewater existed in the form of organic nitrogen, NH_4^+−N and NO_3^-−N. In the current study, the removal of odd nitrogen in wastewater relied on the assimilation of these compounds by water spinach in hydroponic systems. Firstly, NH_4^+ was converted to NO_3^- and a portion of NO_3^- would then be denitrificated to N2 by microorganisms. Another NO_3^- portion was absorbed by water spinach via roots for growing. However, which process contributed more to the NO_3^- removal was not clarified. In other words, NO_3^- could be assimilated by plants or sent back to the atmosphere by the effect of denitrifying microorganisms (Xu et al., 1999).

4.1.5. Phosphorus removal

Phosphorus is the essential nutrient for plant growth. It can be assimilated by plants and be converted into various kinds of organic matter of plants (Gu et al., 2008). Water spinach, therefore, could assimilate PO_4^{3-} in wastewater and make a reduction from 8.5 ± 1.5 mg/L to 1.2 ± 0.2 mg/L. Eighty six percent of PO_4^{3-} were removed from the wastewater.

4.2. Control system

On one hand, after HRT we observed moss stricking on the inner surface of pipes in the control system. On the other hand, SS created a visible layer of sediment on the inner surface. Moreover, activities of microorganisms could also break organic matters down in wastewater. Consequently, SS, BOD_5 and COD decreased (Figure 4b). Level of pH declined from 7.3 to 6.5. That was probably because NH_4^+ was nitrificated to NO_3^- as evidenced by decreasing NH_4^+ and increasing NO_3^- concentrations at the end of the experiment.

4.3. Suggested household hydroponic system

A family with 4 people release approximately 400 L of wastewater a day (MONRE, 2016). A tank of 1600 L is needed to store wastewater in 4 days. According to the design in this study, 240 m of Φ14-mm uPVC pipe are enough to treat the total amount of wastewater in 4 days. Pipes can be arranged as in Figure 1 or in tower shapes to save space. Total pipe investment costs VND 18,163,200.

5. Conclusions

The averaged influent contained proximately 220.5 mg/L chemical oxygen demand (COD), 76.0 mg/L biological oxygen demand (BOD5), 32.5 mg/L suspended solids (SS), 26 mg/L NH4+, 5.0 mg/L NO_3^-, and 8.5 PO_4^{3-} at pH 7.3. The designed system consisting of 10 plants of 15-day-old water spinach pre-planted in baked clay in each cup was capable of treating 30 L of domestic wastewater meeting the current municipal wastewater discharge standards in Vietnam (column A standards of QCVN 14:2008/BTNMT) after 4 days of wastewater retention time. If operated under conditions of the above parameters, the pilot-plant hydroponic system can achieve the removal of 74% COD, 82% BOD_5, 64% SS, 90% NH_4^+, 30% NO_3^- and 86% PO_4^{3-}. The result of this study has provided an applicable domestic wastewater treatment system eco-friendly and suitable for small and medium household areas.

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Isolation and optimization of the growth conditions of thermophilic microorganism from hot springs

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ABSTRACT

The aim of this study was to isolate and optimize the growth conditions of thermophilic microorganism from hot springs. The isolation was conducted by using the mineral salt basal medium supplemented with 0.6% yeast extract at 50^0C. Totally, 33 isolates of thermophilic microorganism were isolated from hot springs at Truong Xuan (Khanh Hoa province) and Binh Chau (Ba Ria - Vung Tau province). The effects of temperature (45 - 80^0C), pH (pH 6 - 9) and carbon sources (malate, pyruvate, acetate, glucose, fructose, or carbon dioxide) on the growth of isolates were examined. In addition, the isolate morphology was also investigated by Gram and spore staining. The isolated thermophilic microorganism showed the diversity in colony morphology and color appearance. Most of them were rod shaped, spore-forming and most grew well at 50^0C and pH 7. The highest growth of all isolates was observed under malate, glucose, or fructose, as an organic carbon source and unable to use carbon dioxide. Six out of 33 thermophilic microorganism isolates (namely BM7, BS5, NS1, NS3, NS4, and NW6) grew rapidly under high temperatures from 50 - 55^0C and their morphology characteristics showed high similarity to *Bacillus* sp. The study evidenced the polymorphic diversity of thermophiles in the geothermal hot spring ecosystems.

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1. Introduction

Hot springs, the emerged water bodies produced by geo-thermally heated groundwater, are scattered all over the globe, in every continent and even under the sea. In Vietnam, there are more than 287 hot springs and water containing dissolved minerals distributed in different regions of the country (Cao et al., 1998). There are many previous studies that focus on investigation about geological features of geothermal areas (Rastogi et al., 2010; Tran et al., 2012; Tulasi et al., 2013). Besides, the geothermal ecosystems such as hot springs and volcanic eruption areas are the habitat of thermophilic microorganisms. Based on the range of optimal growth temperature, thermophiles are classified into the following groups: moderate thermophiles (40-60^0C), extreme thermophiles (60-85^0C) and hyperthermophiles (>85^0C) (Tulasi et al., 2013). These thermophilic-derivative products could be applied in biotechnology as industrially valuable compounds. Extremophiles have provided an interesting and challenging platform for researchers since they were explorered. Besides growth under the extreme conditions, extremophiles could pro-

duce thermophilic enzymes, biogradable plastic, biofuel, etc. (Tulasi et al., 2013). Thermophilic microorganisms capable of biosynthesis of heat-resistant enzymes are widely used in the industry where production conditions require high temperatures (Gaughran et al., 1947). During the past few years, the interest in diversity, ecology, and physiology and biochemistry of thermophiles has increased rapidly in Vietnam. The thermophilic bacterium species Geobacillus caldoxylosilyticus was isolated from sedimental sludge of My Lam hot spring in Tuyen Quang province, Vietnam (Tran et al., 2012). Furthermore, this strain became promising candidate in industry due to its capability of producing thermostable enzymes such as cellulase and amylase (Tran et al., 2012).

The southern of Vietnam is very rich in hot springs. One is Truong Xuan hot spring (M' Dung village, Ninh Hoa, Khanh Hoa), and another is Binh Chau hot spring (Binh Chau commune, Xuyen Moc district, Ba Ria – Vung Tau) that is very famous in Vietnam. The diversity of microbial communities in these hot springs has not yet been fully studied. This study aimed to isolate, optimize, and evaluate the carbon utilization of thermophilic microorganism isolated from these locations. Results from this study were a preliminary step to apply thermophilic microorganism and their products in biotechnology.

2. Materials and Methods

Soil, muddy, and water samples were collected at Truong Xuan hot spring ($12^0$31'20"N, $108^0$59'00"E, Ninh Hoa, Khanh Hoa), and Binh Chau hot spring ($10^0$36'21"N, $107^0$33'29"E, Xuyen Moc, Vung Tau). Hot water in Truong Xuan hot spring was bubbled from the vein in the rock with temperature ranging from 37^0C to 67^0C. The pH was recorded in the range of 7.7-8.0 indicating alkaline environment. Binh Chau hot spring is the largest hot spring (more than 1 km^2) in Vietnam. Water temperature in the veins ranged from 43^0C to 65^0C with many bubbles, and smell hydrogen sulfide (H_2S). The pH was recorded in the range of 7.8-9.2 indicating alkaline environment. The temperature of the sampling site is unstable, normally, the temperature at the sampling sites was lower than that at the veins.

Samples were randomly collected from different sites of off flow and stored in 500 mL sterile containers (Hildur et al., 2011). They were immediately brought into the laboratory and analyzed within 24 hours. In total, 24 samples were collected from Truong Xuan (14 samples) and Binh Chau (10 samples) hot springs. The samples (soil, muddy, and water) were collected separately in the vacuum flask, transported to laboratory and analyzed within 24 hours.

The mineral salt basic (MSB) medium used for microorganism growth and trace element solution with the components are shown in Table 1 and Table 2 (Goto et al., 1977). The isolation medium was MSB supplemented with 0.6% yeast (w/v) so-called as MSBY medium, pH 7.

Table 1. The components of basic cultivation medium

Deionized Water	1.0 L
$(NH_4)_2SO_4$	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	2.0 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.0011 g
$CaCl_2$	0.03 g
Trace elements solution	0.5 mL
Final pH	7.0

Table 2. The components of trace elements solution

Deionized Water	1.0 L
MoO_3	0.004 g
$ZnSO_4.7H_2O$	0.028 g
$CuSO_4.5H_2O$	0.002 g
H_3BO_3	0.004 g
$MnSO_4.5H_2O$	0.004 g
$CoCl_2.6H_2O$	0.004 g

2.1. Isolation of thermophilic microorganisms from hot springs

The thermophilic microorganisms were isolated based on the possible growth at 50^0C. Fifty Celsius degree was chosen to make the initial isolation temperature to isolate those microorganisms that were capable of growing by 50^0C or more.

The procedure of enrichment was as follows: 1 gram of soil, sludge or 1 mL of water was diluted in 5 mL of MSB supplemented with 6 g/L of yeast extract and incubated at 50^0C for 48

hours. The growth of microorganisms was observed and recorded via estimation of the environmental opacity in test tubes. A five tenfold serial dilution was performed, and then spread on MSBA plates (MSB medium supplemented with agar 3% (w/w) and incubated at 50^0C for 72 hours. Single colonies growing on plates were transferred into freshly prepared MSBA slants and kept at -20^0C for further study. The isolates were investigated by observation of colony morphology, Gram stain, and sporulation (Goto et al., 1977).

2.2. Optimization of the growth condition of the isolated thermophilic microorganisms

In order to determine the optimal temperature for the growth of isolated thermophilic microorganisms, each isolate was inoculated in 5 mL of MSBY medium (pH 7) in a test tube in range of temperature from 45^0C to 80^0C, shaken at 180 rpm for 12 hours. Then, the optimum pH value was examined between 6 and 9 at the optimal temperature. The pH value was adjusted by using 1M NaOH solution. The microorganism growth was determined at 3-hour intervals by measuring the optical density (OD) of the cultures at 540 nm and streaked onto freshly prepared MSBA plate (Goto et al., 1977). The high thermo-tolerance isolates were selected for further experiments.

2.3. Investigation of the potential use of different carbon sources of the isolated thermophilic microorganism

The carbon sources were used in this study including organic substrates [acetate (C_2), pyruvate (C_3), malate (C_4), glucose (C_6), or fructose (C_6)] and inorganic substrate (CO_2). The concentration of carbon in the organic compounds was equivalent to 15 mM. In order to evaluate the use of CO_2, the isolates were cultured in MSB medium with the addition of H_2: O_2: CO_2 (80%: 10%: 10%) (Goto et al., 1977).

The cultures were incubated in a reciprocating shaker at the optimal temperature and pH. The initial OD value at 540 nm was 0.04-0.06. The microorganism growth in various carbon sources was recorded within 72 hours. The mean value OD_{540} of triplicates for each experiment was analyzed by using Microsoft Excel 2013 software.

3. Results and Discussion

3.1. Isolation of the thermophilic microorganisms from hot springs

Thirty three isolates that could grow at 50^0C were isolated from 24 soil, muddy, and water samples from two hot springs in Khanh Hoa (16 isolates) and Ba Ria – Vung Tau (17 isolates) provinces. Of 33 isolates, 11 isolates were obtained from soil (33.3%), 8 isolates from muddy (24.3%) and 14 isolates from water (42.4%) samples (Table 3).

The colonies were appeared in various of color (beige, white, yellow, or pink) including 7 isolates were beige-colored, 11 were white, 14 were yellow, and 1 was pink on MSBA medium (Figure 1).

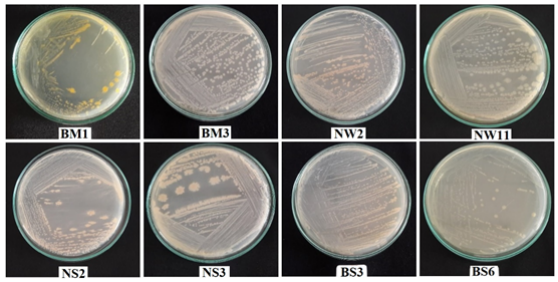

Figure 1. Diversity of colonial morphology of isolates microorganism from hot springs on MSBA.

The Gram-positive isolates were 18 isolates /33 (54.5%) of the collection. Of the 33 isolates, 31 (94%) were rod-shaped, with the size of cells in range of 0.16 – 0.8 ± m, 20 isolates (60.6%) were able to form oval endospores and had the size of the spores in range of 0.10 – 0.41 ± m (Figure 2). This result showed the diversity of the thermophilic microorganism communities in geothermal area.

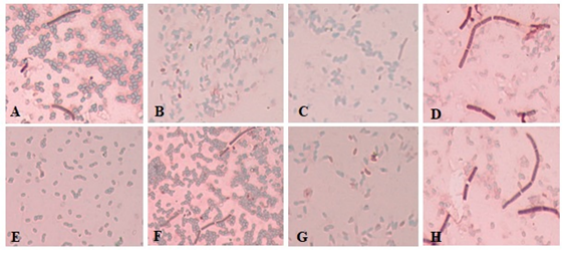

Figure 2. Spore shapes of the isolates under microscope observation (magnificent 1000X).
A: BS2; B: NW7; C: NW6; D: BM5; E: BS5;
F: BM5; G: BS4; H: BM8.

Table 3. Thermophilic microorganism isolated from hot springs

Hot spring	Sample	Isolate	Notation
Binh Chau (B) (Ba Ria – Vung Tau)	Water (W)	3	BW1,2,3
	Soil (S)	6	BS 1,2,3,4,5,6
	Mud (M)	8	BM 1,2,3,4,5,6,7,8
Truong Xuan (N) (Nha Trang, Khanh Hoa)	Water (W)	11	NW1,2,3,4,5,6,7,8,9,10,11
	Soil (S)	5	NS1,2,3,4,5
	Mud (M)	0	
Total		33	

3.2. Optimization growth conditions of thermophilic isolates

In order to optimize the temperature and pH for microorganism growth, the isolates were cultivated at temperature range from 45^0C to 80^0C and pH range from 6 to 9. The result was shown in details in Table 4. The aim of this study was to isolate the microbes that were capable of growing from 50^0C, therefore the intended study temperature range was 45, 50, 55, 60, 65, 70, 75, 80^0C. However, at the temperature higher than 55^0C the growth of microorganisms isolated was very weak. So, we focused on testing from 50 to 55^0C. At pH 9, the growth of microorganisms could not be observed, then the data were not shown in Table 4.

After 12 hours of incubation, the OD_{540} values of six isolates including BM7 (0.73 ± 0.06, at 50^0C), BS5 (0.67±0.02, at 52^0C), NS1 (0.71 ± 0.03, at 55oC), NS3 (1.04 ± 0.05, at 50^0C), NS4 (0.93 ± 0.04, at 50^0C), and NW6 (0.82 ± 0.09, at 55^0C) were higher than the others. Of these, isolates, BM7, NS3, NS4 grew optimal at 50^0C with OD540 from 0.73 to 1.04, while growth of isolate BS5 was optimal at 52^0C with OD540 at 0.67 ± 0.02. Isolates NS1 and NW6 were optimal at 55^0C with high OD_{540} at 0.71 ± 0.03 and 0.82 ± 0.09, respectively. The pH investigation also showed that isolate BS5 grew optimal at pH 6, isolates BM7, NS1, NS3, NS4 grew optimal at pH 7 while NW6 was optimized at pH 8. Moreover, the highest OD_{540} (1.18 ± 0.08) was recorded in isolate NS3 at pH 7.

3.3. Evaluation of the use of different carbon sources

Of the 33 isolates, 9 (27.3%) grew on acetate, 15 (45.5%) grew on pyruvate and 24 (72.7%) were able to use malate. All isolates were seen growth well in MSB medium with the supplement of glucose, or fructose. Under CO_2 condition, microorganism were grown in MSB medium (pH 7) with a final gas phase consisting of H_2: O_2: CO_2 (80%: 10%: 10%) at 50^0C. However, none of isolate was able to grow under autotroph condition. After 7 days of continuous observation, the turbidity environmental change was not found in the test tubes. This result suggested that there is no isolate could be fixed CO_2 or grown autotrophically. Experimental results showed that most isolates developing favorably in the presence of malate, glucose, or fructose. Malate acts as an intermediary helps the microbes produce energy as well as metabolite to produce amino acids via the tricarboxylic acid cycle, whereas glucose or fructose is easily metabolized via glycolysis pathway (Kim et al., 2008). Hence, the isolates could favorably utilize this organic substrate.

It is now very well-known that extreme thermophiles are mostly distributed among the genera of *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Thermotoga*, *Aquifex* (Tulasi et al., 2013). In which, Bacillus is a large and diverse genus that is widely distributed in soil and thermal water areas (Claus & Berkeley, 1986). During the past few decades, a great diversity of microorganisms has been discovered that exist in hot environments. In a previous report of Nguyen et al. (2015), 64 aerobic isolates of thermophilic microorganism were identified from muddy and hot water of Binh Chau hot spring. The percentage of microorganism with cellulase, amylase and protease activities is 19%, 67% and 24% of total 64 microorganism isolates, respectively.

Cellular structure and enzyme activities are deeply affected by temperature of habitat. For any microbe to grow at high temperature, their proteins must be able to resist heat. Hence, thermophiles have accumulated various thermostable enzymes that are high potential application in biotechnology. The thermophilic strains of *Bacil-*

Table 4. OD_{540} values of 33 isolates at different temperatures and pH

No.	Isolate	Temperature			pH		
		50^0C	52^0C	55^0C	6.0	7.0	8.0
1	BM1	0.40 ± 0.03	0.30 ± 0.02	0.23 ± 0.03	0.10 ± 0.02	0.41 ± 0.04	0.09 ± 0.02
2	BM2	0.59 ± 0.04	0.33 ± 0.02	0.15 ± 0.01	0.16 ± 0.02	0.57 ± 0.06	0.08 ± 0.01
3	BM3	0.44 ± 0.01	0.34 ± 0.03	0.29 ± 0.01	0.25 ± 0.01	0.44 ± 0.02	0.19 ± 0.03
4	BM4	0.36 ± 0.03	0.45 ± 0.02	0.23 ± 0.02	0.02 ± 0.01	0.37 ± 0.03	0.06 ± 0.01
5	BM5	0.24 ± 0.02	0.18 ± 0.04	0.14 ± 0.05	0.26 ± 0.04	0.27 ± 0.02	0.24 ± 0.05
6	BM6	0.40 ± 0.03	0.22 ± 0.01	0.22 ± 0.07	0.15 ± 0.03	0.44 ± 0.04	0.13 ± 0.03
7	BM7	0.73 ± 0.06	0.50 ± 0.02	0.47 ± 0.03	0.54 ± 0.05	0.72 ± 0.04	0.57 ± 0.04
8	BM8	0.45 ± 0.02	0.42 ± 0.03	0.22 ± 0.04	0.02 ± 0.01	0.47 ± 0.03	0.12 ± 0.02
9	BS1	0.41 ± 0.05	0.26 ± 0.02	0.25 ± 0.03	0.14 ± 0.03	0.42 ± 0.02	0.16 ± 0.02
10	BS2	0.43 ± 0.02	0.10 ± 0.03	0.05 ± 0.02	0.23 ± 0.02	0.45 ± 0.03	0.27 ± 0.02
11	BS3	0.53 ± 0.03	0.42 ± 0.07	0.34 ± 0.06	0.15 ± 0.03	0.50 ± 0.03	0.13 ± 0.03
12	BS4	0.35 ± 0.03	0.27 ± 0.03	0.18 ± 0.03	0.17 ± 0.01	0.37 ± 0.01	0.10 ± 0.01
13	BS5	0.46 ± 0.07	0.67 ± 0.02	0.30 ± 0.02	0.71 ± 0.05	0.65 ± 0.06	0.52 ± 0.04
14	BS6	0.35 ± 0.03	0.43 ± 0.05	0.23 ± 0.03	0.14 ± 0.03	0.39 ± 0.02	0.31 ± 0.03
15	BW1	0.40 ± 0.01	0.34 ± 0.02	0.24 ± 0.04	0.50 ± 0.03	0.43 ± 0.03	0.30 ± 0.03
16	BW2	0.42 ± 0.03	0.38 ± 0.03	0.22 ± 0.06	0.18 ± 0.06	0.44 ± 0.02	0.08 ± 0.01
17	BW3	0.60 ± 0.03	0.22 ± 0.01	0.29 ± 0.06	0.11 ± 0.02	0.58 ± 0.05	0.17 ± 0.04
18	NS1	0.45 ± 0.02	0.59 ± 0.01	0.71 ± 0.03	0.32 ± 0.05	0.76 ± 0.03	0.40 ± 0.04
19	NS2	0.62 ± 0.03	0.41 ± 0.03	0.23 ± 0.02	0.34 ± 0.04	0.61 ± 0.05	0.08 ± 0.01
20	NS3	1.04 ± 0.05	0.52 ± 0.05	0.56 ± 0.03	0.83 ± 0.07	1.18 ± 0.08	0.73 ± 0.09
21	NS4	0.93 ± 0.04	0.46 ± 0.01	0.30 ± 0.04	0.92 ± 0.04	0.94 ± 0.10	0.57 ± 0.05
22	NS5	0.43 ± 0.05	0.34 ± 0.06	0.30 ± 0.03	0.50 ± 0.03	0.43 ± 0.01	0.13 ± 0.02
23	NW1	0.51 ± 0.01	0.20 ± 0.02	0.36 ± 0.03	0.14 ± 0.03	0.54 ± 0.03	0.11 ± 0.02
24	NW2	0.26 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.24 ± 0.01	0.05 ± 0.01
25	NW3	0.30 ± 0.04	0.38 ± 0.02	0.23 ± 0.03	0.23 ± 0.03	0.31 ± 0.03	0.14 ± 0.03
26	NW4	0.55 ± 0.02	0.03 ± 0.01	0.34 ± 0.05	0.19 ± 0.04	0.53 ± 0.06	0.12 ± 0.04
27	NW5	0.42 ± 0.02	0.24 ± 0.02	0.22 ± 0.01	0.15 ± 0.02	0.41 ± 0.05	0.07 ± 0.02
28	NW6	0.68 ± 0.03	0.46 ± 0.06	0.82 ± 0.09	0.48 ± 0.03	0.65 ± 0.06	0.98 ± 0.12
29	NW7	0.48 ± 0.02	0.30 ± 0.04	0.06 ± 0.02	0.16 ± 0.01	0.51 ± 0.04	0.09 ± 0.02
30	NW8	0.42 ± 0.05	0.12 ± 0.03	0.31 ± 0.05	0.26 ± 0.02	0.40 ± 0.01	0.14 ± 0.03
31	NW9	0.61 ± 0.02	0.04 ± 0.01	0.51 ± 0.02	0.12 ± 0.01	0.59 ± 0.03	0.06 ± 0.01
32	NW10	0.60 ± 0.04	0.16 ± 0.03	0.40 ± 0.03	0.13 ± 0.03	0.64 ± 0.04	0.05 ± 0.02
33	NW11	0.53 ± 0.05	0.48 ± 0.04	0.35 ± 0.02	0.19 ± 0.01	0.52 ± 0.05	0.22 ± 0.01

Table 5. Characteristics of six selected thermophilic microorganism isolates

Characteristic	BM7	BS5	NS1	NS3	NS4	NW6
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Color	Cream	Yellow	Orange	Cream	White	White
Gram/Spore	+/+	+/+	+/+	+/+	+/+	+/+
Optimum growth temperature (^{0}C)	50	52	55	50	50	55
Optimum growth pH	7	6	7	7	7	8
CO_2	–	–	–	–	–	–
Acetate	–	–	–	–	–	–
Pyruvate	–	–	–	–	–	–
Malate	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+

+: positive; –: negative

lus that synthesized cellulase, amylase and protease have a great significance for many fields of industry (Rastogi et al., 2010).

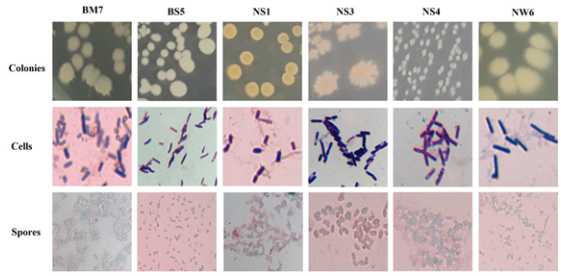

Figure 3. Image profiles of six selected thermophilic microorganism isolates.

Table 5 and Figure 3 described the profile of six selected thermophilic microorganism isolates that were selected from thermophilic microorganism collection in this study in details. In order to explore the potential application of six selected thermophilic microorganism isolates, the identification of microorganism to species as well as enzyme activity screening is required. Recently, the most effective approach to microorganism taxonomy may be analysis of 16S rDNA molecules by oligonucleotide sequencing. Detailed information of the molecular identification for six selected microorganism isolates will be announced very soon elsewhere.

4. Conclusions

From the sources of samples collected form the geothermal areas, we have successfully constructed the collection of thermophilic microorganism including of 33 isolates that are evaluated in terms of morphology, microscopy, and growth test on different substrates. Six selected isolates were Gram-positive, rod-shaped, and spore-forming. These characteristics of six selected isolates with the optimum growth temperature from 50-55^{0}C were found highly similar to *Bacillus* species. The achievement in collection of thermophiles is the preliminary step in effort to be able to apply the thermophilic microbes into the biotechnology sector.

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The science foundation for the establishment of cooperation mechanism on trans-boundary water management

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ABSTRACT

The Mekong Delta (MD) is of socio-economic importance for both Vietnam and Cambodia. The trans-boundary dimension and respective management issues are highly relevant for the economic development and security of each country. Because the economic development of each country is different in the boundary region of MD, there are many kinds of natural hazard occurred in MD (including flood, drought, acid sulphate soils, and saltwater intrusion), the impact of upstream development, a myriad of flood controlling infrastructure, and the implications of climate change. To resolve these above problems and maintain the boundary security condition, the two countries are implementing many cooperation and coordination mechanisms (CMs) at central and provincial levels. In this article, the current CMs between the two countries are presented to clarify the implementing status, effective and coherent level of the trans-boundary water management (TWM). From that, some recommendations will be proposed to set up a new CM closed with TWM for the boundary provinces in MD.

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1. Introduction

The Mekong Delta extends from central Cambodia to Vietnam, where the Mekong River empties into the sea. As the "rice bowl" of both countries, the delta is essential for their food security and income. However, the upstream hydropower development and delta's flood controlling infrastructures affect fisheries, the floodplains, sediment movement, and water quality. Impacts of climate change, including severe floods and droughts and saltwater intrusion, also threaten the delta's natural resources (MRC, 2017).

Due to the need for economic development, security as well as the mitigation of adverse impacts at the border areas of Vietnam and Cambodia, the Governments of the two countries as well as the border provinces' administrations have carried out many institutional activities to promote a cooperation and dialogue. The most raised problems at present are the issues related directly or indirectly to water in Mekong River and they still not have a solution or still not have solved to cause many misunderstanding and complex for each other. To better manage the shared resources in the Mekong Delta, Cambodia and Vietnam work together to develop joint planning and harmonised mechanisms for the management of water resources through a bilateral project supported by the Mekong River Commission (MRC). After consultations with government officials and local stakeholders and field surveys, the two countries have identified six main issues to address in cooperation. Details of the six main

issues are recorded in the joint technical paper, Trans-boundary Water Management Issues in the Mekong Delta of Cambodia and Vietnam, published in September 2017 (MRC, 2017).

The six joint issues on TWM between Vietnam and Cambodia are:

- Flood and drought strategic planning.
- Infrastructure development and investment.
- Coordination and Communication Mechanisms.
- Human and institutional capacity building at the managerial and technical level.
- Environmental, social and economic impacts of development and climate change.
- The implementation of the Navigation Agreement between Cambodia and Vietnam.

Therefore, for the solution all above issues, it should review the institute content of current cooperation and dialogue mechanism between two countries. Based on the analysis, to propose the CM on the TWM, which will aims to improve and enforce the quality of existing coordination and cooperation mechanisms and to focus on the boundary provincial level in MD's boundary region.

2. Advantages and Disadvantages on The Implementation Existing CMs Among Vietnam and Cambodia

At present, the trans-boundary cooperation and dialogue mechanisms between Vietnam and Cambodia are established at two levels, which are the central and boundary provincial levels. They are:

- The CMs at the national level: The central coordination and cooperation mechanisms are based on many agreements, treaties or memorandum of understanding. These documents are included as (Bui, 2015):

◇ The Treaty of Peace, Friendship and Cooperation between the Socialist Republic of Vietnam and the People's Republic of Cambodia signed on February 18, 1979. The main content of the treaty laid the foundation for peace and cooperation between the two neighboring countries.

◇ The Agreement on the Historical Waters of the Socialist Republic of Vietnam and the People's Republic of Cambodia signed on July 7, 1982, delineates the boundaries of coastal and marine waters belonging to the two countries.

◇ The Agreement on the principle of resolving the Vietnam-Cambodia border issue (signed on July 20, 1983).

◇ The 1995 Agreement on Mekong River low basin's cooperation in sustainable development between the Governments of the Kingdom of Cambodia, the Lao People's Democratic Republic, the Socialist Republic of Vietnam and the Kingdom of Thailand.

◇ The Agreement on Water transport on the Mekong River and Transit at the common boundary region between Vietnam and Cambodia, signed on 17 December 2009.

◇ The Memorandum of Understanding (MoU) between the Governments of the Kingdom of Cambodia, the Lao People's Democratic Republic and the Socialist Republic of Vietnam for Road Transport signed on 17 January 2013. The Memorandum of Understanding favorable transportation of goods and people between the participations; cooperate in socio-economic development and bring stability for each country.

- The CMs at the bordering provincial levels: The boundary local CMs are based on many agreements, memorandum of understanding. These documents are included as (Bui, 2015):

◇ The Agreement on activities at border water region between provinces of Kien Giang Provincial People's Committee and the Kampot State Administration under the UNEP / GEF project "Prevention of environmental degradation trends in the East Sea and Gulf of Thailand" (Kien Giang PPC, 2003).

◇ The Fisheries Cooperation Plan between Kampot Fisheries Department and Department of Agriculture and Rural Development of Kien Giang Province, signed on 29/05/2014 (Kien Giang DARD, 2014).

◇ The agreement on trans-boundary cooperation between An Giang province (Vietnam) and Kandal province (Cambodia) on 6 February 2007. The main content of the search and rescue agreement; exchange of forecast information on storms, floods and warnings; health care; Cooperation in agriculture, fisheries and veterinary (An Giang PPC, 2007).

◇ The Cooperation Agreement between the Long An Provincial People's Court and the Court of First Instance of Svay Rieng Province - Kingdom of Cambodia dated 31 May 2013. The cooperation agreement between the two provinces includes the following contents: exchange of experi-

ence of the two parties; information on crime situation, especially drug trafficking, transnational human trafficking and border crossing between Long An and Svay Rieng (Long An PPC, 2013).

◇ The Cooperation signatures between Dong Thap (Vietnam) and Prieveng (Cambodia). For example, the 2016 cooperation agreement between the two provinces was signed on 10/5/2016. Minutes of cooperation in 2016 include 05 sectors with important contents such as: strengthening the exchange of information between the two provinces to ensure political security and social order in the border area; To create favorable conditions and coordination for the protection of landmarks and positions of demarcated landmarks, stabilizing the entire borderline;...

2.1. The advantages

Based on the Memorandums of Understanding (MoU), Agreements or Bilateral Cooperation Plans between two boundary provinces sharing border lines, the sectors of cooperation, dialogue, etc., are diverse and specific from national security and activities on the exchange and sharing convenient information, knowledge and data for their line agencies to set up specific projects and activities in the common border areas. The agreements between two countries have oriented/founded indeed the cooperation and dialogue on the trans-boundary water management for the central organizations (ministries, institutes) and local administration (provinces, departments).

The most highlight cooperation institute is the Mekong Agreement 1995 (1995 agreement) signed by four Low Mekong Basin's (LMB) countries. Vietnam and Cambodia both are very active members and have expressed their concenrs of the water and related development in the Mekong Delta in line with the 1995 agreement. The MRC is the international regional organization established by the 1995 agreement. The 1995 agreement is a dynamic "framework agreement" that enables and requires the MRC to adopt and refine rules and procedures to carry out its work in close cooperation and coordination with relevant agencies and member countries. It identifies key activities and mechanisms that support the sustainable and equitable use, utilization and protection of the Mekong water and water related resources. Under the water utilization procedure (WUP), the MRC and the Member Countries agreed to develop sets of rules for water utilization for the LMB (MRC, 2017).

The Articles 1 and 3 of the 1995 agreement (MRC, 1995) have affirmed the importance of the fields of development the riparian countries benefit from the MRB resources priovided that they have to pursuit the protection of environment as stipulated in Article 3. Besides the Article 1 and 3, the MRC countries can apply the article 7 (prevention and cessation of harmful effects) and 8 (state responsibility for damages) of the Agreement to be able to solve the issues of transboundary Impact caused by the development and management of water resources, especially in the situatuion of emerging development. The example about the effect of MRC's supporting to the bilateral cooperation in LMB is as the below program.

A further joint project, between Cambodia and Vietnam, proposes to promote Community-based trans-boundary fisheries management in the border areas of the MD, through the fishermen and local governments in the bordering provinces of Prey Veng in Cambodia and Dong Thap in Vietnam contributing to better management and sustainable utilization of fisheries resources through cross-border cooperation. The implementation of joint Neural Information Processing System (NIPS) projects is to occur between 2016-2020 and pending an appropriate funding source. The issue of future development upstream and especially the impact of cascading hydropower projects remains a significant concern to the management of the MD. Climate change and saltwater intrusion also pose significant challenges.

2.2. The disadvantages

However, the content of all remain bilateral agreements, MoU or joint plans is general and only orientation for the cooperation and dialogues on the economic, social and security sectors, some elements (sectors) have concerned or been affected by trans-boundary waters (transboundary rivers, main streams) with some central organizations only as the Ministry of Agriculture and Rural development (MARD), the Ministry of Natural Resource and Environment (MONRE). The TWM is indirectly identified or be as independent sector. The exchange or sharing hydro-meteorology, communication information and knowledge related to TWM are not

touch specially.

In Vietnam, the extensive irrigation systems in the Mekong Delta are used to manage floods for economic benefits and minimize flood impacts, and during the dry season, limit shortages of water for irrigation and prevention (or reclamation) of acid sulphate soils (which is naturally occurring) (Bui, 2017). Therefore, the improvement of existing canal systems is a necessity. The shortage of water also causes salinity intrusion in the main rivers affecting agriculture and living conditions (mostly in Vietnam) will be exacerabated by the impacts of climate change.

The flood control dyke systems built in Vietnam contribute to extensive flooding in the Mekong Delta region of Cambodia and vice versa. Vulnerable provinces in Cambodia include Takeo, Prey Veng and Svay Rieng with flood damage occurring in six out of ten districts in the Takeo province and in Vietnam vulnerable locations along the boundary canals include: Vinh Te canal, Tra Su canal, Bay Xa canal, and Bao Ke canal. The future construction of flood management infrastructure such as road heightening and embankment or water diversion in Cambodia and Vietnam may further increase the water level and prolong the flood period in the flood prone areas in the Mekong Delta. The adverse impacts of flood control infrastructure are discussed and agreed by two sides in many bilateral meetings. But the joint study/research of two countries is still delayed due to the lack of the effective mechanism on the information and data sharing and exchange.

Additionally, there are many raised adverse impacts related to the management of TWM in the boundary region as the trans-boundary water use conflicts, the connection of irrigation canals, the erosion bank at natural boundary line – canals or rivers. And the most of them has been discussed and solved through the establishment of high level, committees and councils, and multilateral agreements where more than 2 countries cover parts of a river basin. For the Mekong River, this includes the establishment of the MRC Council, the MRC Joint Committee and various agreements including the Procedures for Data and Information Exchange and Sharing (PDIES). These are necessary platforms for raising trans-boundary water sector related issues and setting out regional and national strategies to address them. With membership at the Minister and/or senior executive level, these types of arrangements are generally held no more than 2-3 times per year due to the many other commitments of its members and resources required to bring Ministers and government executive staff together. The less focus has been on establishing the processes to support implementation of trans-boundary strategies, including monthly, weekly and even day-to-day information sharing and discussions that is often required.

3. The Proposal CM on TWM between Vietnam and Cambodia for The Mekong Delta

3.1. The general characteristics of CM on TWM

ACM is considered to be an arrangement for sharing and discussing water resources information between two or more countries to help address trans-boundary issues. It includes, but is not restricted to, high level decision-making committees, technical work groups, emergency response teams, day-to-day storage (dam) operation groups, email groups, forums, workshops and websites. They can be temporary or permanent, focus on addressing one or many issues, and include other characteristics.

Based on the current trans-boundary coordination and cooperation mechanism between Vietnam and Cambodia in MD, the common foundation for the CM on TWM are (Bui, 2017):

• Principle to Develop the CM is some current institutes such as: The Mekong Agreement 1995 and its 5 procedures approved; The existing bilateral cooperation Cambodia – Vietnam is at two levels – central and boundary provincial; The cooperation level is implemented at the basin level and also provincial level.

• Objectives: To promote cooperation between the border provinces of Vietnam and Cambodia in some fields such as: The share and exchange the data and information on water resource and relative issues are required from the boundary provinces of two countries and upper Mekong River Basin; The technical support and capacity building to help the boundary provinces and other stakeholders to address all relative issues on TWM; and The advisory and support decision-making of the boundary provincial administrations.

• Scope of CM: The CM is implemented in

boundary region of MD between two countries; It is established for the boundary provinces of two countries; The CM should focus on the priority main fields (planning, development, assessment and navigation).

• Forum of cooperation: The CM on TWM should include the bilateral and multi-lateral activities of the National Mekong Committees (NMCs), in which, there are Cambodia Mekong Committee and Vietnam Mekong Committee. It means that, the content of CM on TWM takes the Mekong Agreement 1995 with 5 Procedures and Techinal Guilines (Procedure of Water Use Management, Procedure of Data Information Exchange and Sharing, Proceduce for Notification, Prior Communication and Agreement, Procedure of Mitigation Flood Management, Procedure of Water Quality) as its conferences.

The boundary provincial administrations implement some contents of CM on TWM, which should include some main items such as:

• Management: existing border province mechanism Joint meeting (Decision making, Advisory and Resolve conflict). Oversees water resources planning and management processes including river basin planning, flood management planning etc. Generally mid-to-high level groups within government/organization.

• Technique: Provides technical advice to support water resources planning and on-going management, including hydrological, meteorological, engineering, economic and aquatic ecology. Exchanging relevant information and documentation (ex: Flow, Water Quality,); Exchanging visits of experts and delegations; Joint Study/ research (ex: Fish migration, Bio-diversity, Env. flow...); Joint organizing seminars, workshops and trainings; Capacity building - apply MRC's Decision Support Framework tools for Impact assessment; Joint raising awareness on Flood forecasting.

• Operational: Supports on-going, including day-to-day, water resources management processes including operation of water infrastructure in-line with agree water releases rules and monitoring of water resources. Joint managing the shared water resources and other related natural resources.

• Frequency: This is how often the CM is to meet (if a group) and/or information to be shared (if an online tool). For example, a trans-boundary water resources committee may only need to meet twice a year whereas provincial water departments may need to share information weekly or even daily.

• Membership: Positions and representatives of a CM. For example, a trans-boundary technical working group could comprise of senior hydrologists and hydrogeologists, whereas and a weekly river basin update report could be circulated to a restricted group of government and non-government stakeholders, or even made public via publishing online.

3.2. Identification of CM on TWM

A framework is developed to help ensure a systematic and iterative approach to identifying and ensuring close alignment of the CMs to address the joint trans-boundary issues. The example is the first step, which is to identify information and people needs, including skill sets, to support resolution of the trans-boundary issues. Reviews, surveys, meetings and workshops are undertaken by the National Mekong Committees to identify what information is needed, available and accessible, and how best to use it at the trans-boundary level. The assessments of the effectiveness of existing CMs are also undertaken (Figure 1).

3.3. The proposal CM on TWM

The mechanism should address all the requirements related to the six joint trans-boundary issues on water management in boundary region of Mekong Delta, which are identified such as table 1 (Bui, 2017).

The flow chart of cooperation mechanism on the trans-boundary water management applied for the boundary provinces in Mekong Delta (Figure 3).

The main detail coordination and cooperation mechanism implemented by the boundary provinces of both countries for the Mekong delta are proposed as followings:

• Objectives: To promote cooperation between the boarder provinces of Vietnam and Cambodia in the field such as (1) Exchange information on water resource; (2) Technical Support and capacity building; (3) Advisory and support decision-making.

• Areas of Cooperation: Mekong Delta, the boundary provinces are Vietnam's Long An, Dong Thap, An Giang and Kien Giang; Cambodia's Takeo, Prey Veng, Sey Rieng and Kandal.

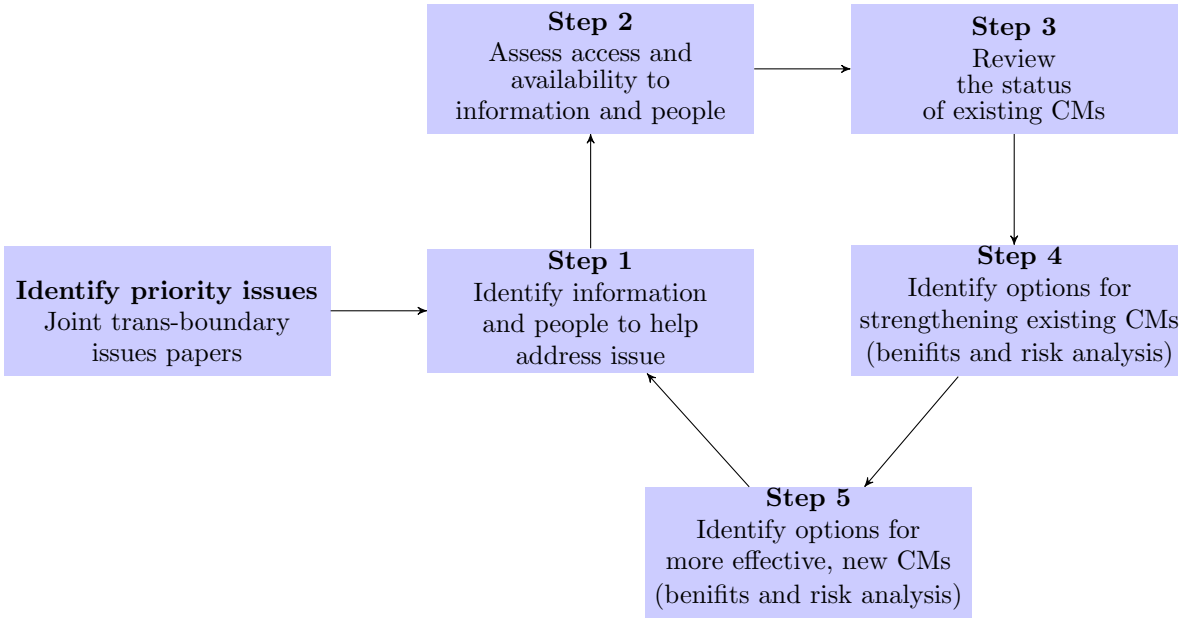

Figure 1. Framework for identifying trans-boundary coordination mechanisms (Bui, 2017).

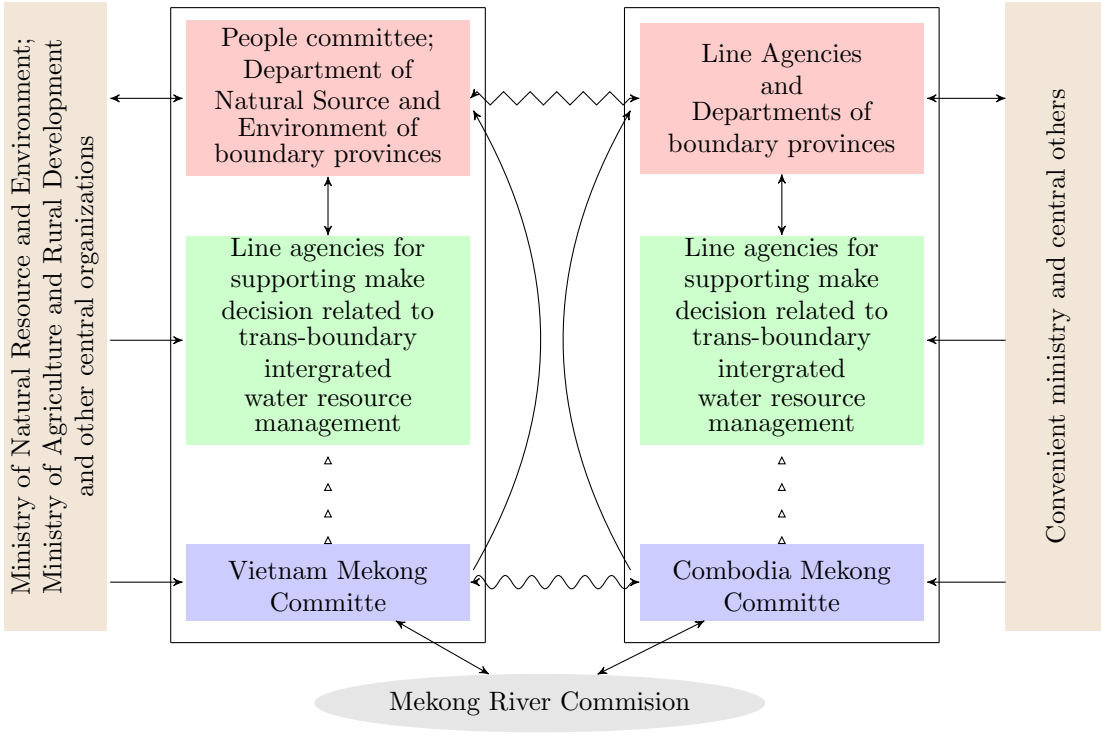

Figure 2. Framework for identifying trans-boundary coordination mechanisms (Bui, 2017).

• Prioritized areas of cooperation: (1) Flood and drought strategic planning; (2) Infrastructure development and investment; (3) Environmental, social and economic impacts of development and climate change; and (4) The implementation of the Navigation Agreement between Cambodia and Vietnam.

• Form of Cooperation: Exchanging relevant

Table 1. The content of proposal cooperation mechanism on trans-boundary water management between Vietnam and Cambodia for Mekong Delta

Elements	Definitions
The addressed requirements related to TWM	Make the understanding of hydrological regime. Make the joint coordination and other convenient activities in the Mekong Delta. Make the understanding of environmental, social and economic variances in boundary region of two countries of Mekong Delta. Make the agreement or acceptable conditions on the identifying and implementing changes to the flow, water level and hydrological regime necessary. Make the advantage for the navigation in local river network. Make the best and advantage condition of canal and river networks in the border areas. Implement the measures to address the shortage of water during the dry season for irrigation, domestic water supply and prevention of salinity intrusion. Implement the bilateral cooperation. Implement the consideration trans-boundary implications and impacts of the infrastructure development and investment related to the water management sector in boundary region of Mekong Delta. Implement the support and build the decisions capacity. Implement the exchange and sharing data, information and knowledge related to the trans-boundary water management. Implement the communicating about the flood and drought strategic plan.
The data, information and knowledge	The convenient data includes: Hydrology of mainstream; Hydrology of important trans-boundary tributaries; Meteorology data; Geology data/information; Topography map; Geophysic data/information; Water quality data/information; and Groundwater data/information;.... The convenient information and knowledge include: Infrastructure information; Planning information; Water using damage information; Development planning information; and Social/economic data/information. The convenient information and knowledge include: Infrastructure information; Social/economic data/information; and Hazard impacts' information.
The special implement groups	The general management council/group: The group is an active group. The technical group: The group is a supporting group, which is set up when has the issue. The operation group: The group is a supporting group, which is set up when has the issue or being an active group. The group should implement for the multiple purposes or many different issues.

information and documentation on 4 Prioritized areas of cooperation through various means (including, but not restricted to, emails and official channel); Exchanging visits of experts and delegations; Jointly organizing seminars, training workshops and meetings attended by scientists, experts, policy makers, regulators, law enforcers and others concerned; (Capacity building - apply MRC's Decision Support Framework tools for Impact assessment); And developing detailed joint action plans to implement the agreed priority actions.

• Implementation Mechanism: At the national level, it is MRC under the Mekong Agreement 1995. At the provincial level, it is the existing bordering provincial mechanism; And Vietnam Mekong Committee will encourage and facilitate their respective water use organizations, law enforcement agencies, and research institutions, to establish and develop direct contacts and focal points with each other in the field of 4 Prioritized areas of cooperation other related agreements to implement the following tasks: i) develop the annual action plan; ii) monitor and evaluate the implementation of the agreed action plan.

• The boundary provinces' tasks are i) the ap-

point a coordinator for the purpose of implementing coordination and cooperation mechanism; ii) the implementation of the coordination and cooperation mechanism will be reviewed; iii) the venues and times of coordinators' meetings shall be agreed upon in writing between the coordinators; and iv) each party shall bear its own costs and expenses arising from the implementation of this coordination and cooperation mechanism.

4. Conclusions and Recommendations

The establishment of CMs on TWM between Vietnam and Cambodia should be via legislation, international treaties, formal (e.g. Memorandum of understanding) and informal (e.g. handshake) agreements and other arrangements, which are already established and available.

Integrated water resource management program of MRC supports a multi-CMs approach to achieve desired trans-boundary outcomes. All levels of government, and non-government and private sector should be considered in establishing CMs, and approval and on-going resourcing requirements. The proposal CMs between Vietnam and Cambodia should be aligned to support the resolution of priority trans-boundary issues on TWM in MD. This includes consideration of what specific information; people and skill-sets are needed and how often required. Multiple CMs could be required to address one or a number of issues on TWM.

These cooperation mechanisms are based on the current state of the database and new interesting findings may be found as the database continues to expand, as the number and type of actors increases. There is also potential to develop search functions and other features to provide a more 'user-friendly' interface. By doing so, the hope is that through the database contribute to improved coordination, cooperation and learning between two countries working with TWM and provide insights into new opportunities to address knowledge gaps that can lead to more informed decision-making on trans-boundary waters.

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Changes of total polyphenolics and vitamin C in acerola during storage and spray drying process

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ABSTRACT

Acerola fruit is known to have a high vitamin C concentration. Polyphenolics are also natural oxidants occurring in plants. Understanding changes of these components during storage conditions and processing steps become important. Results of this research showed that there was significant difference in vitamin C and total polyphenolic concentration and three popular varieties of acerola fruits from Tien Giang province. Concentrations of both vitamin C and polyphenolics reduced rapidly during storage at room temperature. After three days, vitamin C reduced about 40% whereas total polyphenolics reduced about 70%. The losses at refrigerated temperature after 3 days were less than 15% and less than 30%, for vitamin C and total polyphenolics, respectively. Frozen storage of the fruit maintained quite well vitamin C and polyphenolics. Acerola pomace juice was concentrated before spray drying and, at the same vacuum pressure, temperatures influenced significantly the retention of vitamin C and total polyphenolics. Optimization of spray drying conditioners including inlet hot air temperatures and added ratio of maltodextrin (drying carrier) was also carried out to obtain high recovery of dry matter, total polyphenolics and vitamin C.

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1. Introduction

Acerola is known as an excellent source of vitamin C (Mezadri et al., 2008). Estimatedly, a cup (180 ml) of acerola compressed juice, containing potentially 35 mg/mL ascorbic acid, is equivalent to the amount of vitamin C of 14 L orange compressed juice (Johnson, 2003). According to Decarvalho & Manica (1994), the concentration of vitamin C in acerola fruit was about 5 – 20 times higher compared to guava, about 10 – 15 times compared to mango. Especially, vitamin C occurs mainly in the pulp of acerola while it occurs at higher concentration in the peel of guava. Concentration of vitamin C in compressed juice of acerola juice is higher than that in compressed juice of oranges, lemons, grapes,... Therefore, acerola fruit could be used as a commercial source of vitamin C for daily diet or a supplement to other foods. As well, acerola juice can be added to other fruit juices to increase the vitamin C content.

Polyphenols are of secondary metabolites widely found in the plant kingdom. These compounds have received great attention nowadays mainly due to their antioxidant potential and the relation between their consumption and the prevention of some diseases associated with oxidative

stress, including cancer, and others such as cardiovascular diseases and osteoporosis. Polyphenols found in acerola (*Malpighia emarginata DC.*) include anthocyanins, quercitrin, hyperside, flavonols, astilbin and proanthocyanidin (Hanamura et al., 2005). Rufino et al. (2010) reported 1063 mg gallic acid equivalents/100 g pulp of Brazil acerola. Because of the large amount of vitamin C and polyphenols, acerola has a high antioxidant capacity (Mezadri et al., 2008).

Tien Giang and Ben Tre are two primary plantation areas of acerola in Vietnam and the three varieties are sweet (*Malpighia punicifolia L.*), traditional sour (*Malpighia glabra L.*) and imported sour variety (which is locally called new sour variety) which is also called Brazil (*Malpighia emarginata D.C.*) variety. In different parts of the world, acerola can be processed into powder, juice, applied as vitamin C pills or applied in facial cosmetics... In Vietnam, most of acerola is stored at room temperature for selling as fresh fruit. This storage condition could not be good to preserve natural antioxidants like vitamin C and polyphenols. Processing of the fruit into different products may help increase the value of acerola and the products can be stored for longer time for consumption. It has been known for long time that acerola is a good source of vitamin C, as discussed. Recently, acerola can be also a good source of polyphenols. These components are antioxidants and good for health. However, they are sensitive to processing as well as storage conditions. Therefore, suitable storage and processing conditions should be considered to preserve as much as possible the bioactive components.

Spray drying of acerola juice into powder has a high potential since the powder can be applied in many forms of products; such as pills, cosmetic supplements or instant beverage. Temperature to do spray drying is a critical parameter, and its effects on the retention of the phytochemicals need to be investigated. It seems not possible to spray-dry the juice without adding carriers (maltodextrin, corn syrup, anhydrous starch, gum arabic, whey protein concentrate, whey protein isolate ...). Juice dry matter contains a substantial amount of sugars and the spray-dried products become very sticky, so they easily stick to the wall of the drying chamber and are difficult to be collected. The sugar perhaps also prevents the evaporation of moisture if no carrier is added. The use of carrier may also protect the sensitive components. Therefore, addition of carrier is necessary and more experiments should be done to find out suitable added concentration to give an efficient process.

The objectives of this research are to determine the concentrations of polyphenols and vitamin C in three acerola varieties grown in Vietnam and to investigate the changes of the components during storage, evaporation to concentrate the juice, and spray drying the juice into powder. Experiments were carried out to find suitable conditions to perform those processes with less loss of the antioxidants.

2. Materials and Methods

2.1. Materials and chemicals

Fresh acerola fruits were picked directly in gardens in Go Cong town, Go Cong district, Tien Giang province and were used for analysis or experiments within five hours after picking. The fruits selected were of similar ripeness (just ripened), characterized by a complete maturity, the peel of fruit near the stem was smooth and well out, light green to orange yellow with pink spots, and were hard with no damage due to insect or transportation. Maltodextrin was of Japanese product, in form of white powder with a moisture content of 6-7% and DE value of 20.

For chemicals used for analysis, metaphosphoric acid, acetic acid of $\geq$ 99.98%, thiourea ($CH_4N_2SO_4$), sulfuric acid H_2SO_4 of $\geq$ 99.98%, bromine, ethanol of $\geq$ 99.5%, acid clohydric (HCl), and sodium carbonate were of Chinese products. Other chemicals were 2,4-dinitrophenylhydrazine of $\geq$ 99.5% (Germany), standard ascorbic acid for food of $\geq$ 99.98% (India), Folin-Ciocalteu reagent of $\geq$ 99.8% (Merck, Germany), and standard gallic acid of $\geq$ 99.9% (Merck, Germany).

2.2. Experiments

2.2.1. Determination of concentrations of total polyphenolic compounds and vitamin C in three acerola varieties grown in Go Cong district, Tien Giang province

Fruits of three varieties; namely sweet variety (*Malpighia punicifolia L.*), sour variety (*Malpighia glabra L.*), and Brazil variety (*Malpighia emarginata D.C.*) were the subjects

of the analysis. Each variety was picked from three gardens and the whole experiment was carried out in triplicate. All measurements were performed in, at least, duplicate.

2.2.2. Changes of total polyphenolics and vitamin C during storage at various conditions

The experiment was designed to evaluate the effects of storage conditions on the evolution of content of total polyphenolic compounds and vitamin C in acerola fruits. The variety for this experiment was the sweet acerola (Malpighia punicifolia L.). The fresh fruits were put in Styrofoam trays and covered with a PE foil and stored under three different conditions, namely room temperature, 4 ± 2^0C, and freezing at -18 ± 2^0C. Representative samples were taken for analysis of total polyphenolic compounds and vitamin C after 1, 2, 3, 4 and 30 days of storage. The experiment was carried out in triplicate.

2.2.3. Effects of evaporation temperatures on the retention of polyphenolic compounds and vitamin C in acerola pomace juice

Concentration of diluted juice using evaporation before spray drying to obtain powder is more economical in term of energy than direct spray drying of the diluted juice into powder. This experiment was designed to evaluate the effects of evaporation temperatures, performed at the same vacuum pressure, on the retention of polyphenolic compounds and vitamin C.

Frozen sweet variety acerola was thawed and the seeds were removed using a stainless steel knife. The pulp (including the peel) was blended using a multifunction blender (Cornell Inc., USA) and filtered against several layers of a cheese cloth. The pomace juice was fast blanched for 1 minute at 80^0C and standardized at 7% dissolved solids. Each 200 g of the juice was subjected to evaporation to 15% dissolved solids at three different temperatures, namely 65, 75 and 85^0, using a rotary evaporator set at a vacuum pressure of 0.86 ± 0.02 kg/cm^2. The loss of polyphenolic compounds and vitamin C was determined. The experiment was carried out in triplicate.

2.2.4. Optimization of spray drying of the concentrated acerola pomace juice into powder in consideration of hot air temperatures and added ratio of maltodextrin

After screening the effects of hot air temperatures and the added ratio of maltodextrin using one factor experiments, an optimization experiment was carried out to evaluate simultaneously the effects of hot air temperatures and added ratio of maltodextrin on the recovery of dry matter, polyphenolic compounds and vitamin C.

Surface methodology using Central Composite design was applied. Two factors; x_1, hot air temperatures, and x_2, added ratio of maltodextrin (maltodextrin solids/ juice solids) were included with three levels as described in Table 2. The pomace juice was blanched and concentrated to 15% dissolved solids using the rotary evaporator set at 65^0C and 0.86 ± 0,02 kg/cm^2, as described previously, before added with maltodextrin and inspired into the spray dryer. The spray dryer used was a Labplant SD – Basic (Labplant Inc., UK). The operation conditions of the spray dryer were 0.15 ± 0,02 MPa for the compressed air to spray the juice and the input pump was set at 20 mL/min. The fixed settings and experimental parameters were taken in a way that the obtained powders had moisture content of 5.5% and below (3.5-5.5%), to meet the requirement of a stable powder during storage.

The full quadratic equation (Eq. 1) was fit to the obtained data to model the process

$$\begin{aligned} Y_i = a_{io} + a_{i1}x_1 + a_{i2}x_2 \\ + b_{il}x_1x_2 + c_{il}x_1^2 + c_{i2}x_2^2 \end{aligned} \quad (1)$$

Where a_{io}, a_{i1}, a_{i2}, b_{il}, c_{il}, and c_{i2} were regression coefficients and $i = l$–3, representing three responses, namely recovery of dry matter, recovery of total polyphenolic compounds, and recovery of vitamin C.

Recovery yield of dry matter was determined as the percentage of the obtained dry matter in the powder compared to the input dry matters (of the pomace juice and of the added maltodextrin, if used). Similarly, the recovery yield of polyphenolic compounds and vitamin C was the percentage of the components remaining in the obtained powder compared to their amount in the inspired (pumped into the spray dryer) juice.

2.3. Analyses

2.3.1. Chemical analysis

Moisture content or dry matter content of samples was determined using the method of drying to constant weight with drying temperature of 105^0C.

The content of dissolved solids in the juice was determined using a 0 – 32^0 Brix Atago refractometer.

Concentration of total polyphenolic compounds was determined using spectrometry method (UV-VIS 2502 spectrometer, LaboMed Inc, USA) at 700 nm after reaction with Folin-Ciocalteu reagent (Lima et al., 2005). Gallic acid was used as the standard to build the calibration curve. Content of total polyphenolic compounds was expressed as µg gallic acid equivalents (GAE) per gram of sample (pulp in case of analysis of the fruit).

Concentration of vitamin C was determined using spectrometry method (UV-VIS 2502 spectrometer, LaboMed Inc, USA) after reaction with 2-4 DNPH and the absorbance was recorded at 521 nm (Rufino et al., 2010). Ascorbic acid was used to build the calibration curve and the concentration of vitamin C was expressed as μg/g sample (pulp in case of analysis of the fruit).

2.3.2. Data analysis

Average calculation and plotting was performed with Microsoft Excel 2007. JMP software 9.2 (SAS Institute Inc, NC 27513, USA) was used for designing the two-factor experiment and for statistical analysis. The difference was considered significant at the $P < 0{,}05$.

3. Results and Discussion

3.1. Concentration of total polyphenolic compounds and vitamin C in acerola fruits of three varieties grown in Go Cong district, Tien Giang province

Concentrations of total polyphenolic compounds and vitamin C in acerola of the three varieties are shown in Table 1. There was variation in concentrations of total polyphenolic compounds and of vitamin C in the same varieties of different gardens; however, the difference was insignificant. Composition of acerola fruit is known to be influenced by environmental conditions and culturing practices (Mezadri et al., 2005). The fruits selected for the experiments were based on the same ripeness, but this could not be judged exactly by the appearance. Therefore, the variation in polyphenolics and vitamin C due to the difference in ripeness could not be ruled out (Vendramini & Trugo, 2000; Mezadri et al., 2005).

The concentrations of the components of the three varieties were significantly different (Table 1). The Brazil variety was characterized with the highest concentration of total polyphenolic compounds, followed by the sour variety and then the sweet variety. The same trend was observed with the concentration of vitamin C. Rufino et al. (2010) analyzed acerola (*M. emarginata D.C.*) grown in Brazil and reported vitamin C concentration of 1357 mg/ 100 g, which is quite in range with our results.

The results (Table 1) showed that, acerola was not only rich in vitamin C but also in polyphenolic compounds and that this fruit can be a good source for this antioxidant.

3.1.1. Changes of total polyphenol content and vitamin C content during storages at various conditions

The reduction of concentrations of polyphenolic coumpounds and of vitamin C in fruits of sweet variety (Malpighia punicifolia L.) during storage at three different conditions is shown in Figure 1. Concentrations of polyphenolic compounds and vitamin C were reduced during storage and storage conditions strongly influenced the rate of the reduction (Figure 1).

After 30 days of storage at – 18^0C, polyphenolic compounds were reduced of 16.15% while the vitamin C concentration was reduced of 6.29%. Both these changes were statistically significant. The reduction of the components during chilling storage and room temperature storage was much faster. Especially, after three days of storage at room temperature, the vitamin C concentration was reduced of 81.87% and polyphenolic compounds were reduced of 37.51%. It was observed that the fruits became too ripen (rotten) and mold started to grow at 4 days of storage at this condition.

For storage at 4 ± 2^0C, the reduction of both components was observed after each day of storage. After one month, the vitamin C concentra-

Table 1. Concentrations of total polyphenolic compounds and vitamin C in acerola fruits of three varieties grown in Tien Giang province[1]

Variety	Sweet variety (*M. punicifolia L.*)			Sour variety (*M. glabra L.*)			Brazil variety (*M. emarginata D.C.*)		
	G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9
Polyphenol (mg GAE/100g)	1153.2 ± 64.7	1195.7 ± 18.9	1295.7 ± 46.0	1441.3 ± 21.3	1336.2 ± 28.6	1226.2 ± 26.6	1563.8 ± 30.9	1429.8 ± 33.2	1534.0 ± 27.2
Average	1214.9 ± 73.2[b]			1324.59 ± 107.6[b]			1509.93 ± 70.4[b]		
Vitamin C (mg/100g)	725.4 ± 7.1	743.1 ± 15.6	762.7 ± 16.2	1226.7 ± 41.9	1093.3 ± 66.8	970.7 ± 32.8	1365.3 ± 35.3	1279.1 ± 18.7	1405.3 ± 17.5
Average	743.7 ± 18.7[c]			1096.9 ± 128.0[b]			1349.9 ± 64.5[a]		

[1]Data are expressed as means ± S.D. G1-9 represents gardens 1 to 9. Three samples of different days were taken for each garden. On the same row, values do not share a common superscript differ significantly.

tion was reduced of 77.26% while polyphenolics were reduced at a less extent of 26,09% (Figure 1). It can be concluded that during chilling storage, the loss of polyphenolics was slower than that of vitamin C. At a long time of storage under this condition, the color of acerola fruits already changed due to water loss.

The results of this experiment pointed out that storage conditions are critical for preservation of the antioxidants in acerola. In reality, e.g., in Vietnam, acerola fruits are displayed at room conditions during selling and this practice should be discouraged.

3.2. Effects of concentration temperatures on the retention of polyphenolic compounds and vitamin C in acerola pomace juice

The fresh juice of sweet variety for this experiment had 7% dissolved solids, and concentrations of vitamin C and total polyphenolic compounds were 1225.78 mg/100 g, 1302.13 mg/100 g, respectively. The fresh pomace juice was blanched at 80 oC for 1 min to inhibit the browning, and then concentrated to 15% of dissolved solids. The effects of evaporation temperatures on the retention/loss of polyphenols and vitamin C are illustrated in Figure 2.

It was observed that, blanching caused loss of polyphenols and vitamin C. Subsequent evaporation caused further loss of the components (Figure 2). At the same vacuum pressure, namely 0.86 ± 0.02 kg/cm2, evaporation at 65^0C retained 70.63% polyphenols and 56.5% vitamin C, compared to amounts occured in the fresh pomace juice. While evaporation at 75^0C and 85^0C retained 60.59% and 51.07% polyphenols, respectively, and 49.55% and 43.73% vitamin C, respectively, although the evaporation time was 5 and 10 min less than that at 65^0C.

It can be concluded that evaporation temperature is an important factor influencing the loss of antioxidants in the acerola pomace juice. It was interesting to note that, the loss of vitamin C was more pronounced than that of polyphenols.

3.3. Optimization of spray drying of the concentrated acerola pomace juice into powder in consideration of hot air temperatures and added ratio of maltodextrin

Two-factor experiment to evaluate the effect of hot air temperatures and added ratio of maltodextrin was carried out, as described previously. The results obtained with all the ten runs of the experiment are shown in Table 2.

Analysis using JMP software showed that, the models in Eq. 1 explained very well the obtained data shown in Table 2, as illustrated that all three responses had $P < 0.01$ and R^2 values of 0.98 and above.

"Parameter estimation" analysis to show the significance of regression coefficients is shown in Figure 3. Coefficients having *P values* < 0.05 were considered as significant and included in the established equations for Y_1, Y_2 and Y_3 (Table 3).

In the zone of experiment, x1 or hot air temperatures (^{0}C), had significant effects, both as linear term or quadratic term, to all the three responses (Figure 3 & Table 3). In the experiment zone, x2 or added ratio of maltodextrin had significant effect as linear term to only recovery yield of dry matter (Figure 3 & Table 3). There was an in-

Table 2. Effects of hot air temperatures and added ratio of maltodextrin on recovery of dry matter, polyphenolic compounds, and vitamin C

Run	Code	x_1 (^{o}C)	x_2 (w/w)	Dry matter recovery yield (%)	Polyphenols recovery yield (%)	Vitamin C recovery yield (%)
1	- -	130	1.5	84.34	49.54	43.52
2	a0	130	2	84.02	50.97	44.13
3	+ +	130	2.5	83.34	46.36	44.46
4	0a	140	1.5	84.62	54.35	44.84
5	00	140	2	84.55	55.56	46.17
6	00	140	2	84.38	54.48	46.25
7	0A	140	2.5	84.23	55.25	42.11
8	+ -	150	1.5	82.35	30.66	31.30
9	A0	150	2	82.65	40.64	31.40
10	+ +	150	2.5	82.02	44.38	31.22

Table 3. Established regression equations and their peak parameters for the three experimented responses[1]

Response: Recovery of	Established regression equations	Response maximum value	At values of	
			x_1 (^{0}C)	x_2 (time)
Dry matter (%)	$Y_1 = 84.55–0.78x_1–0.29x_2–1.29x_1^2$	84.81	136.4	1.57
Polyphenols (%)	$Y_2 = 55.83–5.2x_1 + 4.22x_1x_2–10.84x_1^2$	56.60	138.2	2.16
Vitamin C (%)	$Y_3 = 45.46–6.37x_1–6.96x_1^2$	46.93	135.4	1.96

[1] x_1 is hot air temperatures ^{0}C, x_2 is added ratio of maltodextrin (maltodextrin solids/juice solids).

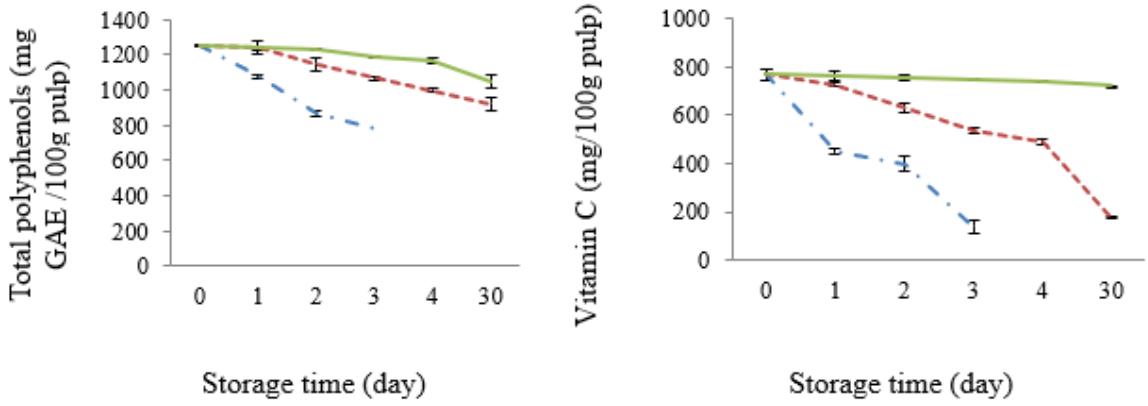

Figure 1. Changes of concentrations of total polyphenolic compounds (above) and of vitamin C (below) during storage of sweet variety acerola fruits at room temperature (–.–), 4 ± 2^{0}C (– –), and -18 ± 2^{0}C (——).

teraction of x_1 and x_2 on the recovery yield of polyphenols. All the three models were quadratic, meaning that the response surfaces were curve ones and maximal values could be inferred.

The spray drying conditions to obtain separately maximum values of the three responses are

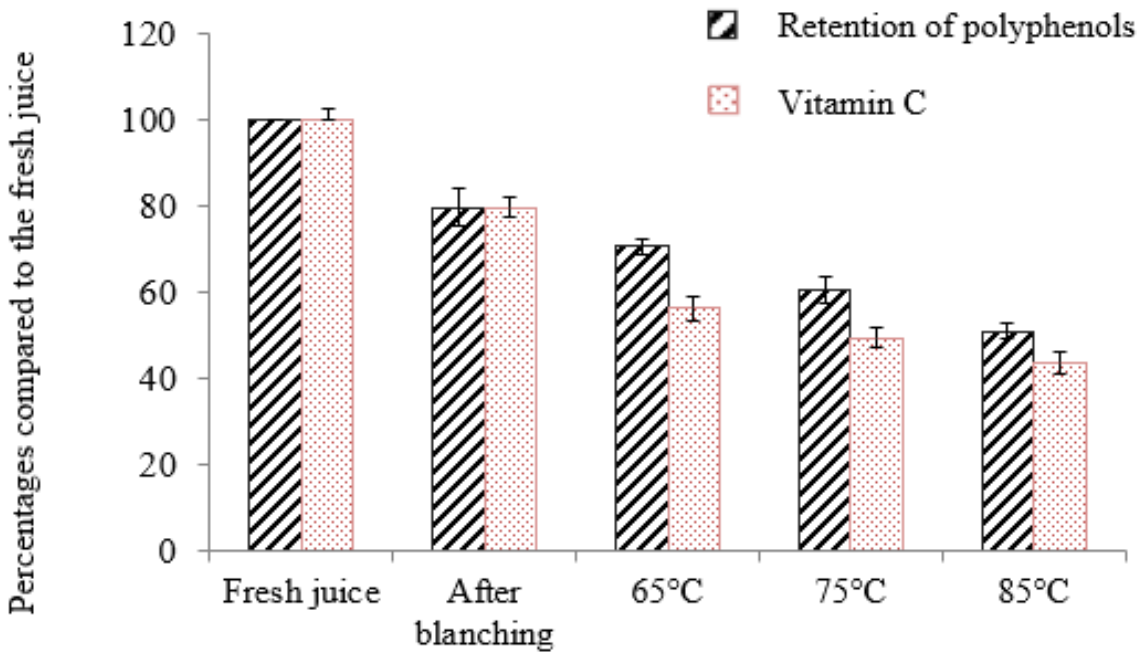

Figure 2. Retention of polyphenols and vitamin C (expressed as percentage compared to the components occurring in the starting material – the fresh pomace juice) under the effects of evaporation temperatures at 0,86 ± 0,02 kg/cm^2.

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	84.545714	0.10015	844.19	<.0001*
X1(130,150)	-0.78	0.068416	-11.40	0.0003*
X2(1.5,2.5)	-0.286667	0.068416	-4.19	0.0138*
X1*X2	0.1675	0.083792	2.00	0.1162
X1*X1	-1.291429	0.109709	-11.77	0.0003*
X2*X2	-0.201429	0.109709	-1.84	0.1402

(a)

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	55.834286	1.017419	54.88	<.0001*
X1(130,150)	-5.198333	0.69503	-7.48	0.0017*
X2(1.5,2.5)	1.9066667	0.69503	2.74	0.0517
X1*X2	4.225	0.851234	4.96	0.0077*
X1*X1	-10.84357	1.114527	-9.73	0.0006*
X2*X2	-1.848571	1.114527	-1.66	0.1725

(b)

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	45.468571	0.800655	56.79	<.0001*
X1(130,150)	-6.365	0.546952	-11.64	0.0003*
X2(1.5,2.5)	-0.311667	0.546952	-0.57	0.5993
X1*X2	-0.255	0.669876	-0.38	0.7228
X1*X1	-6.962143	0.877074	-7.94	0.0014*
X2*X2	-1.252143	0.877074	-1.43	0.2266

(c)

Figure 3. Retention of polyphenols and vitamin C (expressed as percentage compared to the components occurring in the starting material – the fresh pomace juice) under the effects of evaporation temperatures at 0,86 ± 0,02 kg/cm^2.

shown in Table 3. The conditions were quite similar on hot air temperatures but quite different on added ratio of maltodextrin (Table 3). Therefore, setting a drying condition where all the three responses got the maximum values would be impossible. Compromised conditions, as suggested by JMP software, to obtain simultaneously as highest as possible recovery yields of dry matter, polyphenols, and vitamin C were 137.1 – 138.9 oC for hot air temperatures and 2.02 – 2.19 for added ratio of maltodextrin solids compared to juice solids.

4. Conclusions

Experiment results showed that concentrations of polyphenols and vitamin C were different in the three acerola varieties, and the Brazil variety had highest concentrations of both phytochemicals, 1509 mg/100 g pulp for polyphenolics and 1350 mg/100 g pulp for vitamin C. Sour acerola variety was richer in concentrations of the components than sweet acerola variety. Storage conditions influenced the reduction rate of the components. After one month of storage of sweet variety at -18 ± 2^oC, polyphenols were reduced by 16.2% and vitamin C reduced 6.3%. These values were actually much smaller compared to the loss of the components during storage at chilling and room temperatures. At room temperature, sweet acerola variety could only be stored for less than 4 days and at three days about 81.9% of polyphenols and 37.5% of vitamin C were lost. At the same vacuum pressure to concentrate juice of 7% to 15% dissolved solids, 0.86 ± 0.02 kg/cm^2, lower evaporation temperatures (65^0C was better than high temperatures (e.g., 75 and 85^0C in term of retention of polyphenolic compounds and vitamin C, even though the former condition had longer processing time. Hot air temperatures and added ratio of maltodextrin, the carrier, influenced the drying processing efficiency. In the experiment zone (temperatures ranged from 130 – 150^0Cand added ratio of maltodextrin ranged from 1.5 to 2.5 times) to spray dry 15% dissolved solids juice, temperatures influenced more pronouncedly to the recovery yields of dry matter, polyphenols, and vitamin C in the obtained powder. The optimal conditions to obtain simultaneously as highest as possible the values for the three recovery yields were 137 – 139^0C for temperatures and 2 – 2.2 for added ratio of maltodextrin.

Results of the research confirmed that acerola is rich in both vitamin C, as known for a long time, and polyphenolic compounds. Processing conditions are critical to the loss of these bioactive components. Further research is needed to evaluate the changes of the components during storage of the powder.

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Optimization of maltodextrin and carrageenan gum concentration added in spray drying process of *Pouzolzia zeylanica* extract by response surface methodology

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ABSTRACT

Pouzolzia zeylanica is a kind of medicinal plant which is generally cultivated in Mekong Delta region. It owns many bioactive compounds that are known to possess antioxidant, antimicrobial and anticarcinogenic properties. This study aimed to optimize additional carrier concentration for spray drying of *Pouzolzia zeylanica* extract. Response Surface Methodology (RSM) with central composite design (CCD) was applied for optimization and investigation of the influence of maltodextrin (5÷15%, w/v) and carrageenan gum (0.06÷1.0%, w/v) concentration on the physicochemical characteristics of spray dried powder (bioactive compounds, moisture content as well as particle size distribution). The results showed that the optimum concentrations of maltodextrin and carrageenan gum were 8.8% w/v and 0.082% w/v, respectively. At these optimal conditions, the anthocyanin, flavonoid, polyphenol, tannin, moisture content and particle size of obtained spray dried powder were 5.77 mg cyanidin-3-glycoside equivalents (CE)/100 g; 29.49 mg quercetin equivalents (QE)/g; 28.35mg gallic acid equivalents (GAE)/g; 27.44 mg tannic acid equivalents (TAE)/g, 6.55% and 6.09 μm, respectively.

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1. Introduction

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products and in the last decade much attention has been shifted to search for phytochemicals of native and naturalized plants for pharmaceutical and nutritional purposes (Oktay et al., 2003; Wangensteen et al., 2004). *Pouzolzia zeylanica* was reported that it had no oral acute toxicity at the oral dose of 10 g extract powder/kg (Tran et al., 2010) and can be used to treat cough, pulmonary tuberculosis, sore throat, enteristis and dysentery (Vo, 2012). Many researches showed that this plant contains flavonoids, tannin, carotenoids, quercetin, vitexin, isovitexin, phylanthin, metylsterate, β-sitosterol, oleanolic acid, epicatechin, scopolin, apigenin, alkaloids, steroids, glycosides and saponins (Ghani, 2003; Le, 2007; Fu et al., 2012; Saha & Paul, 2012). Therefore, it will be an important material source for processing functional products as beverage, instant tea, etc.

Spray drying is one of the most commonly used techniques in transforming a large amount of liquid foods into powder form, due to commercially costs and final product quality and stability (Favano et al., 2010). Food powders have many benefits and economic potential over their liquid forms such as volume reduction and packaging easier handling and transportation, stable structure and much longer shelf life (Sarabandi et al., 2014). The physicochemical properties of spray-dried powders depend on the process variables

such as the characteristic of liquid feed including viscosity, flow rate and the drying air in term of pressure and temperature as well as the type of atomizer (Tee et al., 2012). In order to achieve a successful drying process, high molecular weight of drying agent such as maltodextrin, gum needed to be used for reducing stickiness and wall deposition in the dryer chamber. Moreover, the drying carrier agent may improve powder recovery and production yield (Goula & Adamopoulos, 2005; Langrish et al., 2007; Martineli et al., 2007).

The objective of this study was to evaluate the impact of maltodextrin and carrageenan gum concentration added to spray drying process of *Pouzolzia zeylanica* extract on the anthocyanin, flavonoid, polyphenol, tannin, moisture content and particle size distribution of dried powder product. The other variables of spray drying process were maintained constant.

2. Materials and Methods

2.1. Sample preparation

Pouzolzia zeylanica plants were collected in March 2015 from An Giang University. They were harvested after one and a half month cultivation, with 20-30 cm in height. The plants were then washed with tap-water, air-dried until the final moisture content about 12%, cut into small pieces with the length of about 2-3 cm, were extracted with water using airtight extractor. The stirring rate, temperature, time and ratio of solvant and raw material of extraction process were maintained in 90 rpm, 81^0C, 30 min and 27:1 v/w, respectively. The hot extract was filtered through cotton cloth and their quantity was determined. The extract was next blended with maltodextrin and carrageenan gum at different concentration following experimental design before spray drying process. The inlet hot air temperature and feed flow speed of spray drying process were 180^0C and 18 rpm, respectively. Drying process was carried out in a laboratory scale spray dryer (SD-05, LabPlant^{TM}, United Kingdom), with co-current flow regime, the flow rate of drying air was fixed at 60 m^3h^{-1} and the atomizing air was 1.1 bar.

2.2. Powder product analysis

Physical characteristics: residual moisture content and total content solids of the product were measured using the infrared humidity analyzer (model AND MS-50, Japan). The particle size of the different samples were obtained in the particle analyzer (model ZEOL-5500, Japan).

Bioactive compounds: the anthocyanin content was determined by using the pH differential method (Ahmed et al., 2005; Santos et al., 2013). The results were expressed as mg cyanidin-3-glycoside equivalents (CE) per gram product. The aluminum chloride colorimetric method was used for flavonoids determination and the amount of flavonoid was calculated as quercetin equivalent (QE) per gram of product (Eswari et al., 2013; Mandal et al., 2013). The polyphenol content was determined by Folin-Ciocalteu reagent method and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of product (Hossain et al., 2013). Tannin content was determined by Folin-Denis method and the results were showed as milligrams of tannic acid equivalents (TAE) per gram of product (Laitonjam et al., 2013).

2.3. Experimental design and data analysis

In order to evaluate the effect of maltodextrin (5 to 15%, w/v) and carrageenan gum (0.06 to 0.10%, w/v) concentration on moisture content, particle size distribution and bioactive compounds (anthocyanin, flavonoid, polyphenol and tannin content), a full factorial design (3^2) was applied with five replicates in the center point of the experiment design to fit the surface plot for the responses and to estimate the pure error of the multiple regression models, totaling 13 sample preparations (Table 1). The experimental design and statistical analysis were performed using Statgraphics Plus version 15.0 (SYSTAT Software Inc., Richmond, CA, USA) (Myers et al., 2009). A quadratic equation (second degree polynomial equation) was used to fit the results:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad (i < j)$$

Where Y is the predicted response parameter, β_0 is a constant, β_i, β_{ii} and β_{ij} are the regression coefficients. X_i and X_j are the levels of the independent variables (maltodextrin and carrageenan gum concentration). Experimental data

were then fitted to the selected regression model to get a clear understanding of the correlation between each factor and different responses. This was obtained by estimating the numerical values of the model term (regression coefficients), whose significance was statistically judged in accordance with t-statistic at confidence interval of 95%. Non-significant ($P > 0.05$) term was deleted from the initial equation and data were refitted to the selected model. The quality of the mathematical models fitted by RSM was evaluated by ANOVA, based on the F-test, the probability value (P_{value}) of lack-of-fit and on the percentage of total explained variance (R^2) and also on the adjusted determination coefficient (R^2_{adj}), which provide a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their linear and quadratic interactions (Table 2). A simultaneous optimization using the desirability function was performed in order to maximize the anthocyanin, flavonoid, polyphenol, tannin content and to minimize moisture content and particle size distribution.

3. Results and Discussion

3.1. Effect of matodextin and carragennan concentration on bioactive compounds

In spray drying processing, bioactive compounds can be often destroyed by thermal air. Thus, the supply of maltodextrin and carrageenan gum into *Pouzolzia zeylanica* extract to reduce bioactive compounds damage and improve physicochemical characteristics of obtained powder by gel particle formation mechanisms of polysaccharide to protect bioactive compounds during spray drying process (Burey et al., 2008) (Figure 1).

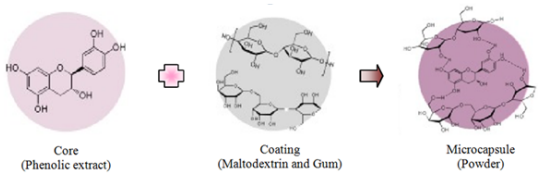

Figure 1. Schematic of gel particle formation mechanisms.

The result showed that anthocyanin, flavonoid, polyphenol and tannin content changed from 2.42 to 5.85 mg CE/100g; 20.63 to 29.30 mg QE/g; 27.39 to 28.35 mg GAE/g and 25.83 to 27.43 mg TAE/g powder product (Table 1), respectively. The bioactive compounds were presented in final products which depended on the supplemental carrier percent of maltodextrin and carrageenan.

The concentration of carrageenan gum and maltodextrin had a positive quadratic effect ($P < 0.01$) on anthocyanin content. The anthocyanin content increased with increasing carrageenan gum concentration in approximately 0.075 to 0.095% (w/v) and achieved optimal values at 0.083%. Besides, anthocyanin content was also achieved high values in maltodextrin concentration approximately 7 to 10% (w/v) and reached an optimum of 8.88% (Figure 2a). It could be noticed in Figure 2b that levels of carrageenan gum had slight quadratic influence ($P < 0.05$) to flavonoid content in product. The flavonoid content achieved high values with carrageenan gum concentration of range from 0.065 to 0.10% (w/v) and reached optimum values in the carrageenan gum concentration of 0.082%. Whereas the concentration of maltodextrin had a clear quadratic impact ($P < 0.01$) on flavonoid content in product. The flavonoid content achieved high values in maltodextrin concentration from 5 to 9% (w/v) and the optimum values obtained at maltodextrin concentration of 7.38%. The carrageenan gum concentration had slight quadratic impact ($P < 0.05$) on the polyphenol content in the product. However, the maltodextrin levels had significant quadratic effect on polyphenol content ($P < 0.01$). The high polyphenol content was obtained when using the carrageenan gum concentration from 0.07 to 0.10% (w/v) and optimal values was achieved at 0.086%. In addition, the polyphenol content increased with maltodextrin concentration increases in the range from 5 to 11% (w/v) and the optimum value was found at 6.83% of maltodextrin concentration (Figure 2c). The response surface and contour plot in Figure 2d showed that carrageenan gum concentration had significant quadratic influence ($P < 0.01$) on tannin content in the product, whereas the maltodextrin levels had slight quadratic effect on tannin content.

A high tannin content was obtained when using carrageenan gum concentration from 0.07 to 0.095% (w/v) and the highest value was achieved at 0.084% of carrageenan gum. Moreover, the high tannin content was obtained when using the maltodextrin percent ranging from 5 to 15%

Table 1. Code and real values of maltodextrin and carragennan concentration and results from physicochemical properties analysis of spray dried powder

Number run	Factors		Responses variables					
	Maltodextrin (%, w/v)	Carragennan (%, w/v)	Anthocyanin (mg/100g)	Flavonoid (mg/g)	Polyphenol (mg/g)	Tannin (mg/g)	Moisture (%)	Particle size (μm)
1	10 (0)	0.08 (0)	5.59	29.05	28.13	27.43	6.45	6.04
2	15 (+1)	0.1 (+1)	3.29	23.38	27.39	25.93	7.31	6.51
3	15 (+1)	0.08 (0)	3.38	22.25	27.75	27.16	6.89	6.44
4	10 (0)	0.08 (0)	5.78	28.19	28.25	27.39	6.49	6.15
5	10 (0)	0.08 (0)	5.75	29.30	28.32	27.43	6.52	6.18
6	5 (-1)	0.06 (-1)	3.91	28.29	27.75	25.83	7.75	6.14
7	10 (0)	0.08 (0)	5.85	28.86	28.33	27.37	6.47	6.13
8	10 (0)	0.06 (-1)	4.25	27.57	27.92	26.63	7.21	6.23
9	5 (-1)	0.1 (+1)	4.15	28.20	28.26	27.20	7.61	6.19
10	10 (0)	0.1 (+1)	5.07	28.29	28.08	26.69	6.95	6.24
11	5 (-1)	0.08 (0)	4.94	29.18	28.32	27.31	7.25	6.09
12	10 (0)	0.08 (0)	5.66	29.14	28.35	27.24	6.52	6.09
13	15 (+1)	0.06 (-1)	2.42	20.63	27.43	26.71	7.68	6.54

Table 2. Mathematical equations that describe the response variables (anthocyanin, flavonoid, polyphenol, tannin, moisture content and particle size) in response to maltodextrin and carrageenan gum concentrations

Response variables	Regression equation[1]	R^2	R^2 (adjusted for d.f.)	P_{value} (lack-of-fit)
Anthocyanin (mg CE/100g)	$Y = -13.001 + 359.437X_1 + 0.862X_2 - 2244.4X_1^2 + 1.575X_1X_2 - 0.056X_2^2$	0.987	0.978	0.077
Flavonoid (mg QE/g)	$Y = 14.077 + 273.443X_1 + 1.189X_2 - 1976.72X_1^2 + 1.189X_1X_2 - 0.12X_2^2$	0.989	0.982	0.603
Polyphenol (mg GAE/g)	$Y = 21.473 + 139.345X_1 + 0.264X_1 - 752.155X_1^2 - 1.375X_1X_2 - 0.011X_2^2$	0.974	0.956	0.917
Tannin (mg TAE/g)	$Y = 9.434 + 370.73X_1 + 0.606X_2 - 1930.6X_1^2 - 5.775X_1X_2 - 0.007X_2^2$	0.997	0.995	0.066
Moisture content (%)	$Y = 17.857 - 222.667X_1 - 0.414X_2 + 1387.5X_1^2 - 0.575X_1X_2 + 0.022X_2^2$	0.994	0.989	0.081
Particle size (μm)	$Y = 7.869 - 39.655X_1 - 0.067X_2 + 247.845X_1^2 + 0.005X_2^2$	0.954	0.939	0.785

[1] X_1 = Carrageenan gum concentration (%, w/v); X_2 = Maltodextrin concentration (%, w/v).

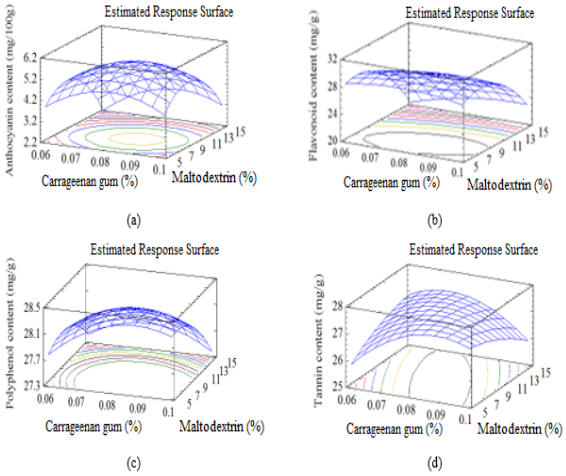

Figure 2. Response surface and contour plots for anthocyanin (a), flavonoid (b), polyphenol (c) and tannin (d) content in different maltodextrin and carrageenan gum concentrations.

(w/v) and an optimum value was found at maltodextrin concentration was 8.19%.

The content of compounds (anthocyanin, flavonoid, polyphenol and tannin) increased with increasing the maltodextrin concentration from 5 to 9% and this compound content decreased when using maltodextrin from 9 to 15%.

The maltodextrin and carrageenan gum concentrations were significant quadratic impact on bioactive compounds in product. The bioactive compounds achieved the high values when the concentrations of maltodextrin and carrageenan gum were added to the extract in the range from 6.8 to 8.8% and 0.082 to 0.086%, respectively. Bhusari & Kumar (2014) also showed the polyphenol content was increased when increasing the concentration of added carrier agent. Maasniza et al. (2013) reported that the best quality of Garcinia powder with additional maltodextrin concentration was 5%. The beetroot-orange juice powder was also obtained with the best functional properties and the conservation of betalain was high when using 5% of maltodextrin (Ochoa-Martinez et al., 2015). The best quality of Ber powder was obtained with encapsulating material, with 8% maltodextrin (Singh et al., 2014), whereas the use of maltodextrin/pectin with 10:1 ratio (11% w/v) led to encapsulate 3% w/v polyphenol-rich extract forming a stable powder made up of well-formed and micronized particles suitable for storage and handling (Sansone et al., 2011). The pink guava powder produced with 15% of maltodextrin was found to be more convenient than other concentrations. The obtained powder had a low moisture content and was more stable with the highest bulk density (Shishir et al., 2015). The obtained pequi pulp powder with high nutritional quality (vitamin C, carotenoid) found at additional maltodextin concentration was 18% (Santana et al., 2016).

3.2. Effect of maltodextrin and carragennan concentration on moisture content and particle size of powder product

The moisture content had an influence on the quality of the powder (Goula et al., 2004). The results in Figure 3a showed that the additional carriers concentration also had significant quadratic impact on the moisture content of spray dried powder product ($P < 0.01$). The moisture content was decreased in increasing maltodextrin and carrageenan gum concentration. The low moisture content was obtained when using maltodextrin and carrageenan gum at concentration varied from 9 to 12% and 0.075 to 0.09%, respectively. The lowest moisture content was achieved at maltodextrin of 10.59% and carrageenan gum of 0.082%. The study result was also similar to the result reported of Fernandes et al. (2012), Wang & Zhou (2013), & Sabhadinde (2014). The concentration of maltodextrin used for development of the *Pouzolzia zeylanica* powder varied between 5 to 15% (w/v). The maltodextrin concentration using in this study was less than 10 to 30% that were used by Abadio et al. (2004), Tonon et al. (2008), & Kha et al. (2010). Moisture content of sample decreased with increasing maltodextrin concentration from 5 to 9%. Abadio et al. (2004) also found a decrease in moisture content in final pineapple juice powder with an increase of the maltodextrin concentration from 10 to 15% (w/v). A higher concentration of maltodextrin used could increase the concentration of feed solids and could reduce the content of total moisture for evaporation (Grabowski et al., 2006). Carrageenan gum concentration had no effect on particle size with $P < 0.05$. The mean particle size was increased with increasing the maltodextrin concentration (Figure 3b). The result from Sharifi et al. (2015) revealed that concentration of maltodextrin increased from 7.5 to 15%, SEM micrographs of the powder indicated the increasing trend in particle size as a result of in-

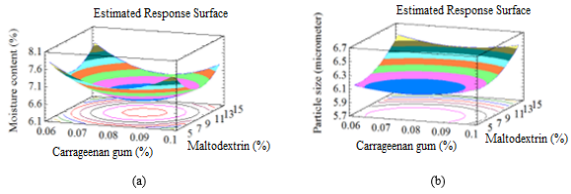

Figure 3. Response surface and contour plots for moisture content (a) and particle size (b) in different maltodextrin and carrageenan gum concentrations.

crease of concentration of maltodextrin as drying aid. However, Fernandes et al. (2012) reported that no correlation was found between particle size distribution and different carbohydrate concentration.

A statistical analysis was performed on the experimental results to obtain the regression models. ANOVA was used to evaluate the significance of each variable on the model. The quadratic model for all the response in terms of coded factors are shown in Table 2. The goodness-of-fit of the regression model showed that the experiment and predicted data were fitted and the coefficient of determination $R^2 > 0.8$ (Guan et al., 2008). In addition, the probability value of lack-of-fit was non-significant ($P > 0.05$) (Zabeti et al., 2009). The results of ANOVA analysis showed that the linear, quadratic and interaction factors of maltodextrin and carrageenan gum concentration had effects on anthocyanin, flavonoid, polyphenol, tannin and moisture content of obtained powder product with the reliability of 95%. However, the carrageenan levels were not effective on particle size, so regression equation of particle size did not have interaction factor of carrageenan gum and maltodextrin concentration. The coefficient of determination of the predicted models in the response was $R^2 > 0.954$, $R^2_{adj} > 0.939$ and lack of fit had $P > 0.05$. These values would give a relatively good fit to the mathematic model. Moreover, the correlation between experimental and predictable data of goal functions such as anthocyanin, flavonoid, polyphenol, tannin, moisture content and particle size are also shown in Figure 4.

3.3. Multiple response optimization

The simultaneous optimization of multiple responses might be a main concern for industrial applications (Tsai et al., 2010). The energy cost of the process significantly diminished when ex-

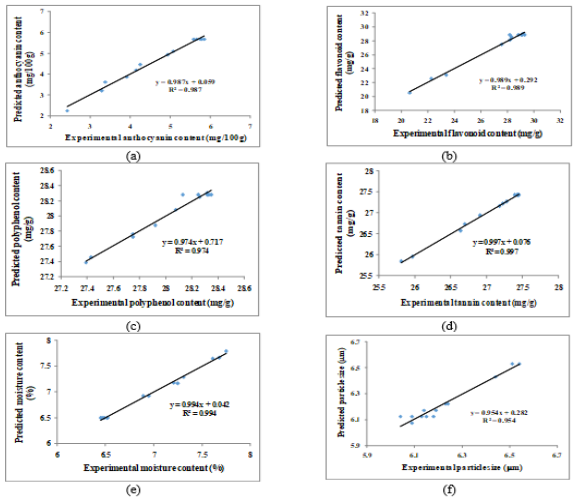

Figure 4. Correlation between the experimentally and the estimated values for anthocyanin (a), flavonoid (b), polyphenol (c), tannin (d), moisture content (e) and particle size (f) using the models described in equation 2, 3, 4, 5, 6, 7, respectively (as shown in Table 2).

traction parameters were optimized (Spigno et al., 2007). The response variables including anthocyanin, flavonoid, polyphenol, tannin, moisture content and particle size were optimized separately; therefore, they allowed the targeting of a certain class of compounds only by varying the spray drying process parameters. Yet, the desirability function in the RSM was utilized to reveal the combination of the parameters (maltodextrin and carrageenan gum concentration) which are capable of simultaneously maximizing or minimizing the responses. The overplay plot shows the outlines superposition of all the studied responses and the simultaneous optimum for all responses is shown by the black spot (Figure 5) showing the best experimental parameters that maximize bioactive compounds content and minimize powder product characteristics. The black spot showed the optimum for all the responses.

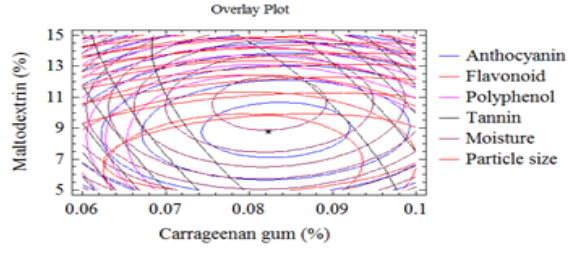

Figure 5. Superposition plots.

4. Conclusion

The effects of the concentration of maltodextrin and carrageenan gum on the powder quality of the spray dried *Pouzolziazeylanica* extract had successfully been investigated by factorial experimental design. The result of simultaneous optimum for all responses showed that the optimum supplemental carrier concentrations to produce spray dried powder with the highest content of bioactive compounds, the lowest moisture content and the smallest particle size were obtained when the blending of maltodextrin and carrageenan gum concentration was 8.8% and 0.082%, respectively.

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Effect of thawing methods on antioxidant capacity of frozen strawberry (*Fragaria x ananassa*) and mulberry (*Morusnigra*)

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ABSTRACT

The aim of this study was to evaluate the antioxidant capacity of thawed frozen strawberries (*Fragariaananasa*) and mulberries (*Morusnigra*). Both types of fruit were frozen in a freezer with a cooling rate of 1^0C/min and stored at -18^0C in 1 week before thawed at ambient temperature, cold temperature (4^0C) and in a microwave oven. ANOVA, LSD test and Principle Component Analysis (PCA) were applied to compare the effect of thawing methods. Results showed that different thawing methods significantly affected ($P < 0.05$) drip loss, vitamin C content, total phenolic content (TPC) and antioxidant capacity of strawberries and mulberries. The strongly negative correlation was found between the drip loss and the remaining vitamin C as well as between the thawing time and the antioxidant capacity of thawed fruit. Among three thawing methods, microwave was considered as the most effective method to retain antioxidant capacity, vitamin C and total phenolic content for both strawberry and mulberry.

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1. Introduction

Recently, the consumption of fruits increased because of the expanding customer's knowledge about their benefits (Mohammad et al., 2004). Strawberries and mulberriesare not only favoured by the taste but also possess high content of ascorbic acid and bioactive phenolic compounds (Hartmann et al., 2008; Oszmiański et al., 2009; Bobinaite et al., 2012). Many studies have proven that phytochemical compounds of these berries exhibit antioxidant capacity, thus they have a role in prevention of many diseases such as carcinogenic, cardiovascular and otherchronic diseases (Hollman, 2001; Anttonen & Karjalainen, 2005; Hartmann et al., 2008). Due tothe health promoting properties, these fruits are considered as natural functional products (Bobinaite et al., 2012).

In Vietnam, strawberries and mulberries are mostly grown at high land. Strawberries are harvested between December and April meanwhile harvesting time of mulberries is from February to March. Apart from being consumed fresh, they are also used in processed forms such as fruit juice, jam, concentrate or alcohol fermented juice. However, these berries are very perishable soft fruits and are harvested in season (Wills & Kim, 1995). Consequently, freezing is one of the most common ways to prolong shelf life of these fresh fruits and supply the year-round raw materials for food industry (Mohammad et al., 2004; Bobinaite et al., 2012).

However, frozen fruits after thawing often face many quality problems due to the loss of firmness, drip loss and nutrition loss (Jeremiah, 1996; Hui, 2006). Freezing process often results in osmotic

shrinkage and cell wall injuries which can lead to exposure of cellular compartments to oxidizing agents as oxygen, enzyme, light and heat (Hartmann et al., 2008; Oszmiański et al., 2009; Kruger et al., 2011). Industrial quick freezing can help to minimize damaged raw materials due to the formation of small ice crystals and less migration of water (Rhaman, 2007; Oszmiański et al., 2009). However, quick freezing does not always bring advantages, especially if thawing is not controlled carefully (Erickson & Hung, 1997). In quick freezing, the water in food is not totally crystallized and easy to achieve the glass state which is convenient for food preservation at low temperature. But this means when thawing, a re-crystallization can happen and leads to the devitrification which can damage cell membranes (Lozano et al., 2000; Rhaman, 2007).

Besides freezing, thawing method is also very important to the quality of frozen fruit (Hui, 2006; Rhaman, 2007; Krüger et al., 2011). Especially in developing countries, at the small scale, most fruits are preserved in household freezers which only attain the minimum temperature of -18^{0}C. In this case, thawing methods can contribute more to the physicochemical properties of final products. Normally, at low temperature, most chemical reactions are slowed down and this helps to limit the bio-chemical degradation of food, but during thawing some oxidative enzymes are re-activated at high concentration and promote these undesirable reactions (Jeremiah, 1996; Rhaman, 2007). Some studies showed that phenolic content and vitamin C are very unstable, thus they may be reduced in quantity under thawing conditions which leads to reduce antioxidant capacity and biological value (Garrote & Bertone, 1989; Oszmiański et al., 2009; Syamaladevi et al., 2011). To link the effect of freezing or thawing on the nutritional values of food products, principal component analysis is a simple and useful tool which was successfully applied in many previous studies (Alvarez & Canet, 2000; Soazo et al. 2013).

There were some studies about the effects of freezing and thawing on the quality of strawberries (Lindley, 1998; Holzwarth et al., 2012) but not much with mulberries. Moreover, understanding about the correlation between physicochemical properties of berry fruits after freezing-thawing is limited. Therefore, the aim of this study was to investigate the effects of different thawing methods (thawing at ambient temperature, at low temperature and in a microwave) on the physicochemical properties of frozen Vietnamese strawberries and mulberries. Moreover, statistical tool was also applied to build the correlation between some parameters such as drip loss, thawing time with vitamin C content, total phenolic content and antioxidant capacity of these products.

2. Materials and Methods

2.1. Chemicals

Most chemicals used in this study included: 2,2'-aziro-bis-(3–ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), FolinCiocalteu's phenol reagent, methanol (99.8%), oxalic acid, acetic acid, xylem, formaldehyde acid, potassium persulphate ascorbic acid and gallic acid were purchased from Sigma-Aldrich. The distilled water was used in all experiments.

2.2. Freezing and thawing samples

Strawberry (*Fragariaananasa*) and mulberry (*Morusnigra*) fruits grown at Lam Dong highland (Lam Dong province, Vietnam) were harvested at commercial ripeness before being frozen at -18^{0}C in a freezer (Sanaky, Japan) with a cooling rate of 1^{0}C/min. After 1 week storage, fruits were thawed at cold temperature (4 - 7^{0}C) in a fridge, at ambient temperature and in a microwave oven (NN-SM330 MYUE, Panasonic, Japan) with the power of 184W to reach to +4^{0}C at the core of fruits. The temperature was recorded by a digital thermometer (Barnant, USA). The thawing was stopped when the temperature of fruit reached the ambient temperature and thawing time was recorded for all samples.

2.3. Drip loss and moisture determination

Drip losses were determined by weighing immediately the exudates after thawing process completely using an analytical balance (TE214S, Sartorius, Germany). Moisture contents of fruits were determined by drying sample in a dryer (Memmert, Germany) at 105^{0}C until the constant weight. The experiments were carried out in triplicate.

2.4. Vitamin C content

Vitamin C content of thawed fruits was determined by using 2,6-dichlorophenol indophenols solution(ISO, 1984). Firstly, thawed samples were homogenized at speed of 8000 RPM during 20 minutes in a homogenizer (Ultra-Turrax T25, IKA, Germany) with oxalic acid (5%, w/v for strawberry and 2%, w/v for mulberry) and centrifuged (EBA 20, Hettich, Germany) at 6000 RPM for 15 minutes. Next, the 5 ml of supernatant was diluted with 5 ml of buffer pH 4 (potassium hydrogen phthalate solution), then added an excessive amount of 2,6-dichlorophenol indophenols and kept for 10 min. Finally, the solution was added with 10 ml of xylen and then centrifuged again. The absorbance final supernatant was measured by using a UV-VIS spectrophotometer (Genesys 20, Thermo Scientific, USA) at wavelength 500 mn to determine the remaining reagent. The amount of used reagent was the difference of the amount of reagent added and the amount of remaining reagent.

2.5. Samples preparation for measurement of total phenolic content and antioxidant capacity

Similar to previous studies (Hartmann et al., 2008; Holzwarth et al., 2012), 1.5 gram of thawed fruits (including exudates) were extracted with 10 ml of 80% methanol and 0.5 ml of HCl 0.1 N for 5 minutes using a homogenizer (Ultra-Turrax T25, IKA, Germany) at 4^0C in an ice bath. Next, the homogenate extract was centrifuged for 15 minutes at room temperature. Finally, the supernatant was transferred to a new tube and used for determining the antioxidant capacity and total polyphenols content.

2.6. Total phenolic content

Total polyphenol content was determined by using Folin-Ciocalteu reagent (FCR) (Singleton et al., 1999). A small amount of the extract (50 μl) was mixed with 250 μl of the FCR and 450 μl of distilled water and stand for 3 min. Next, 2 ml of a sodium carbonate 7.5% was added and mixed. The mixture was kept in dark for 2 hours at ambient temperature and then the absorbance was read at 765 nm using UV-VIS spectrophotometer (Genesys 20, ThermoScientific, USA). Total phenolic content was calculated from standard curves of gallic acidand was expressed in mg of gallic acid equivalent per 100 g of fresh weight (mg GAE/100 g F.W).

2.7. Antioxidant capacity

Antioxidant capacity of thawed berries was done according to the method of Thaipong et al. (2006) with some modifications. Antioxidant capacity was evaluated by using Trolox Equivalent Antioxidant Capacity (TEAC) assay, based on ABTS radical scavenging capacity of the antioxidant molecules in berries extracts. When being reduced by the antioxidant molecules of sample, the dark green color of $ABTS^{\bullet+}$ solution was decolorized. Briefly, $ABTS^{\bullet+}$ was prepared by mixing 7.4 mM ABTS with 2.6 mM potassium persulfate (ratio 1:1, v/v) in the dark at ambient temperature for 12 hours. Before measuring, $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance between 0.700 and 0.900. This solution (2850 μl) was mixed with 150 μl of extract sample and kept in dark for 2 hours. The absorbance was read at 734 nm using spectrophotometer (Genesys 20, ThermoScientific, USA). The Trolox was used as a reference and the antioxidant capacity of berries extract was expressed in mmol/L trolox equivalent per 100 g of fresh weight (mMTE/100 g F.W).

2.8. Statistical analysis

All experiments were done in triplicates. Data were reported as the mean ± confidence interval. Significant differences (α=0.05) among treatments were determined using one-way ANOVA and LSD test. The correlation between thawing methods and antioxidant capacity of samples was evaluated by PCA. SPSS software (version 16, IBM, USA) was used to run all statistical analysis.

3. Results and Discussion

3.1. Thermal behavior of strawberry and mulberry during thawing processes

The temperature changes of berries during different thawing processes are presented in Figure 1. From the thermal curves, it was showed that thawing conditions had considerable effects on the thermal behavior of frozen fruit. With slow thawing as in fridge, the thermal behavior of

these fruits had three stages including two sensible heating and one latent heating. The later stage did not appear when thawing at ambient temperature. Besides, the thawing time was also very different. At ambient temperature, the thawing time of strawberry and mulberry were 136 minutes and 113 minutes, respectively. These values reduced to 35 minutes (with strawberry) and 17 minutes (with mulberry) when thawing in fridge. When using microwave, the thawing time decreased dramatically to 2 minutes and 0.5 minutes for strawberry and mulberry, respectively. The difference of thawing time between two types of berries mainly depends on the size of fruits. With smaller size, mulberry required less energy than strawberry when thawing in same condition. Consequently, the thawing time of mulberry was shorter than that of strawberry.

According to the Figure 1, it was clearly showed the effects of freezing on the thermal behavior of both types of berry during thawing. With slow cooling rate (approximately 1°C/min), water in fruits was maximum crystallized before reaching the glass state at -18°C. This helped prevent the re-crystallization of water in fruits which could appear when thawing at slow rate. Thus, the temperature change of frozen fruits during cool thawing was stable and there were no signs of exothermic on thawing curves at this condition. This means that the tissue in fruits can be better protected from the devitrification.

Effect of thawing methods on drip loss of frozen fruit is presented Table 1 comparing the drip loss of strawberry and mulberry when thawing in a microwave oven, at ambient and cold temperature. It can be clearly seen that berries thawed in a microwave oven had the least drip loss. In comparison with strawberry, mulberry had a great amount of drip loss after thawing due to the difference in water content of fruits. Water content of mulberry was higher than that of strawberry (94.85 ± 2.60% comparing to 89.11 ± 2.53%), thus mulberry may contain more free water. The high amount of bound water in strawberry can lead to difficulties in freezing this fruit, thus drip loss was less after thawing. For both of berries, thawing in cold temperature (the longest thawing time) had the highest drip loss. This result was probably the consequences of dehydration when thawing. The slow freezing velocity led to the formation of large ice crystals in the extracellular space of fruits which causes tearing of cell walls, dislocation of cell contentsafter thawing (Lindley, 1998; Delgado & Rubiolo, 2005; Holzwarth, 2012). This makes cell contents lose their water-holding capacity, thus they cannot hold their existing water inside the cells and leakage normally occurs (Müftügil & Yigit, 1986). Consequently, when frozen fruits were thawed at slow velocity, water was lost gradually. However, if the thawing rate is higher, cells recover structure and perhaps absorb a part of water due to the reverse osmosis. Briefly, the longer thawing process results in the more drip loss.

Table 1. Drip loss (%) of Strawberry and mulberry at different thawing methods[1]

Thawing methods	Strawberry	Mulberry
Microwave	1.25 ± 0.11^{a}	3.94 ± 0.49^{c}
Ambient temperature	1.82 ± 0.30^{a}	15.95 ± 0.46^{d}
Cold temperature	2.55 ± 0.05^{b}	17.75 ± 1.82^{d}

[1]Results are expressed as mean of three replicate measurements ± standard deviation. Values followed with different superscript (vertical) were significant different ($P < 0.05$).

3.2. Effect of thawing method on remaining vitamin C, total phenolic content and antioxidant capacity

The effect of thawing methods on vitamin C and phenolic contents of both of berries are presented in Table 2. ANOVA and LSD tests showed that thawing methods affected significantly these compounds. Both types of fruit thawed in microwave remained the highest vitamin content (24.43 mg/100 g F.W for strawberry and 16.60 mg/100 g F.W for mulberry) whereas thawing in fridge resulted in the highest loss. This result agreed with that of Holzwarth et al. (2012) who reported that thawing of block frozen strawberry at 4^0C for 24 hours caused the highest loss of vitamin C (34.8%) while thawing in microwave oven attributed only 4% of vitamin C loss. Similarly, thawing methods affected the same tendency loss on total polyphenol content of mulberry as vitamin C losses, whereas regarding strawberry, thawing at ambient temperature caused the highest loss as coup and with thawing at cold temperature (Table 2).

After thawing, the content of phenolic compounds remaining in strawberry ranged from 227 to 331 mg GAE/100 g F.W. Li et al. (2003) reported that total polyphenol contents in 6 strawberry varieties grown in China stored at differ-

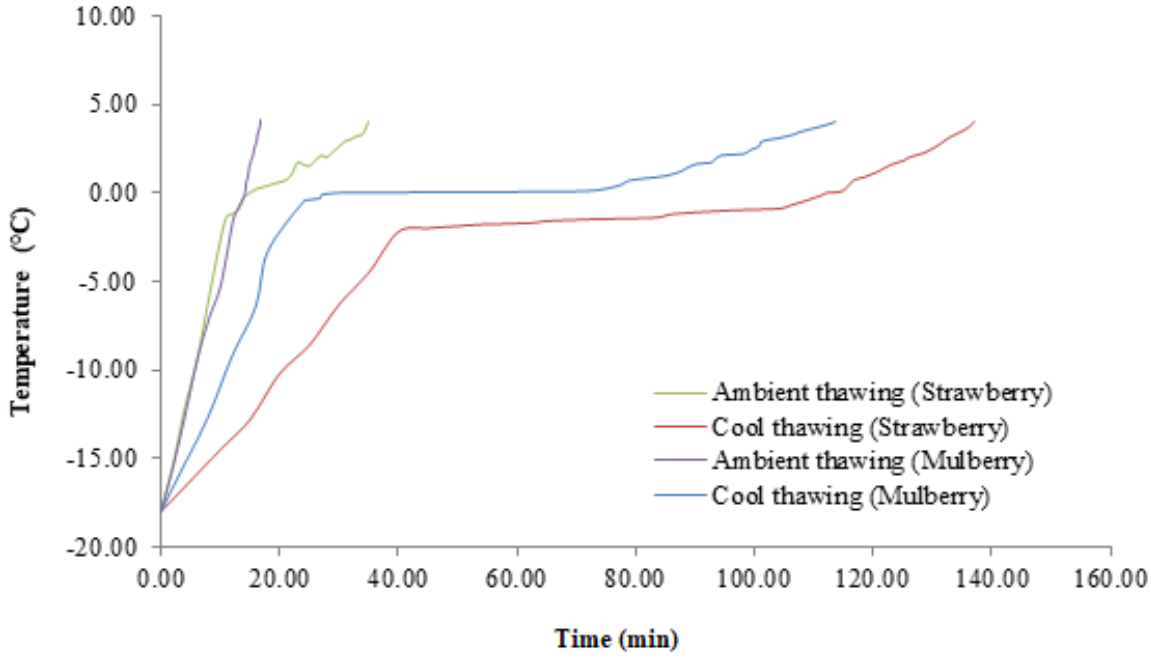

Figure 1. Thawing curves of strawberry and mulberry at different conditions (ambient thawing and cool thawing).

ent temperature and duration time were between 197-377 mg GAE/g F.W. However, the value in current studywas less than 2-5 times in comparison to that of 5 varieties grown at Korea, which ranged from 959 to 2500 mg GAE/100 g F.W (Bae & Suh, 2007). Variation in content of total polyphenol was that Bae et al. (2007) analysed in fresh strawberry while our study evaluated total phenolic content of thawed berries which were surely lost during thawing. Moreover, not only cultivars but also maturity, size and analyzing procedure could be factors contributing to the difference of these compounds(Olsson et al., 2004; Scalo et al., 2005; Bobinaitė et al., 2012).

There was a significant difference among the thawing methods for the antioxidant capacity of strawberry and mulberry (Table 3). In comparison with mulberry, strawberry had higher vitamin C and polyphenol contents, thus the antioxidant capacity of this fruit was also much higher. The lowest antioxidant capacity was found in berries thawed at cold temperature (0.88 mMTE/100 g F.W for strawberry and 1.59 mMTE/100 g F.W for mulberry). Microwave oven was also proved to be the best method to thaw frozen strawberry but not to mulberry. Surprisingly, the antioxidant capacity of mulberry thawed in microwave oven (2.90 mMTE/100 gF.W) was lower than that thawed at ambient temperature (3.22 mMTE/100 g F.W), although thawing time in the oven was only 30 seconds. Presumably, mulberry is very soft texture and small size, with the diameter about 0.8 cm. Thus, the degradation of antioxidant capacity was affected by heating of microwave oven.

3.3. Relation between physical and chemical properties of thawed strawberry and mulberry

The Principle Component Analysis (Figure 2) showed the correlation between drip loss, thawing time with vitamin C content, total phenolic content and antioxidant capacity of thawed fruit. Firstly, if the amount of drip loss was higher, the less vitamin C remained in thawed berries. This can be explained based on the high solubility of this vitamin in water. Therefore, microwave thawed fruits which have low drip loss often have a high retention fo vitamin C content. Secondly, thawing time seems to have considerable negative effect on total phenolic content and antioxidant capacity of thawed fruit. This result

Table 2. Vitamin C and total phenolic contents in thawed strawberry and mulberry

Fruit	Thawing methods	Vitamin C (mg/100gF.W)	TPC (mgGAE/100gF.W)
Strawberry	Microwave	24.43 ± 0.46^{a}	331.02 ± 1.11^{c}
	Ambient temperature	22.26 ± 0.38^{b}	227.66 ± 3.71^{d}
	Cold temperature	21.00 ± 0.48^{b}	277.56 ± 20.90^{e}
Mulberry	Microwave	16.60 ± 0.24^{f}	443.37 ± 37.47^{h}
	Ambient temperature	14.79 ± 0.88^{f}	377.16 ± 22.03^{i}
	Cold temperature	9.25 ± 1.05^{g}	278.49 ± 13.92^{j}

[a-j]Results are expressed as mean of three replicate measurements ± standard deviation. Values followed with different superscript (vertical) were significant different ($P < 0.05$).

Table 3. Antioxidant capacity of thawed strawberry and mulberry by TEAC assay (mMTE/100 gF.W)

Thawing methods	Strawberry	Mulberry
Microwave	1.31 ± 0.01^{a}	2.90 ± 0.10^{d}
Ambient temperature	0.98 ± 0.01^{b}	3.22 ± 0.03^{e}
Cold temperature	0.88 ± 0.01^{c}	1.59 ± 0.16^{f}

[a-f]Results are expressed as mean of three replicate measurements ± standard deviation. Values followed with different superscript (vertical) were significant different ($P < 0.05$).

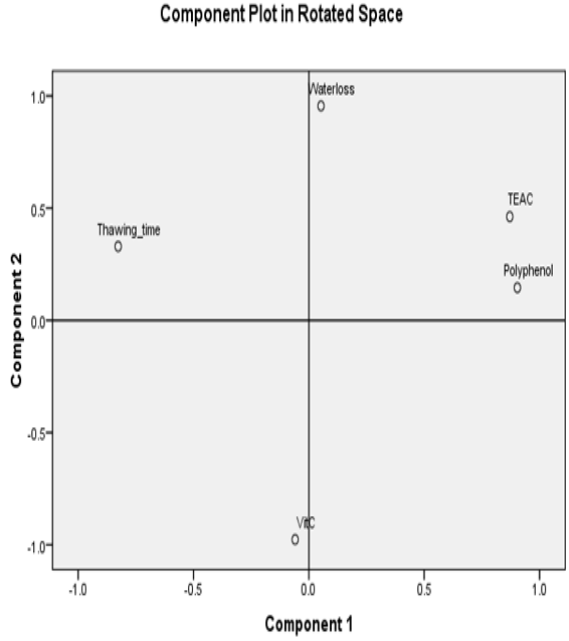

Figure 2. Principle Component Analysis of physical and chemical properties of frozen fruits after thawing.

is consistent with Oszmiański et al.(2009) who claimed that thawing frozen strawberry in microwave oven (the shortest thawing time) retaining more phenolic content than thawing at 20^0C for 20 hours. It is assumed that the degradation of anthocyanin by the activities of polyphenol oxidase (PPO) and peroxidase (POD) which is still active at low temperature during long thawing process (Cano et al., 1995; Chisari et al., 2007; Oszmiański et al., 2009). On the other hands, the exposure to oxygen during thawing can attribute to oxidation of phytochemical compounds. Consequently, faster thawing method in microwave oven is better to achieve high content of phenolic compounds in frozen berries (Oszmiański et al., 2009).

4. Conclusions

The study revealed that the microwave oven was the best method for thawing frozen strawberry and mulberry. Interestingly, berry fruits thawed at cold temperature (4^0C) increased drip loss and decreased vitamin C, polyphenol contents, thus reducing antioxidant capacity of berries. It was assumed that prolonged thawing process increased the exposure time of vitamin C and polyphenol compounds to oxygen and enzyme degradation. Furthermore, PCA showed that the retention of vitamin C was correlated to drip loss, whereas phenolic content and antioxidant capacity in thawed berries depended on thawing time. Thus, thawing regime is the important factor to assure quality of frozen strawberry and mulberry after thawing.

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