Chamuang (*Garcinia cowa* Roxb.) Leaf Extract and Selected Non-thermal Processing Technologies for Inhibition of Melanosis and Quality Changes of Pacific White Shrimp (*Litopenaeus vannamei*) during Refrigerated Storage

Khursheed Ahmad Shiekh

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Inhibition of Pacific white shrimp (PWS) polyphenoloxidase (PPO) with Chamuang leaf extract (CLE) was studied. CLE was rich in polyphenolic glycosides, in which chrysoeriol 6-C-glucoside-8-C-arabinopyranoside and 2-feruloyl-sinapoylgentiobiose were dominant. It also contained organic acids including hydroxycitric acid and oxalosuccinic acid. CLE with copper chelation activity could inhibit PPO in a dose dependent manner. PWS treated with 1% CLE had the lower melanosis score than 1.25% sodium metabisulfite (SMS) treated sample and the control throughout the refrigerated storage of 12 days at 4 °C ($p<0.05$). Lower total volatile base (TVB) and thiobarbituric acid reactive substances (TBARS) along with lower counts of microbial (mesophile, psychrophile) and spoilage bacteria (*Pseudomonas*, Enterobacteriaceae and H_2S-producing bacteria) were obtained with 1% CLE treatment than the control and SMS treated sample during entire storage of 12 days at 4 °C.

When pulsed electric field (PEF) at varying specific energy densities (54–483 kJ/kg) and pulse numbers (200–600) was applied on PWS, PPO activity in cephalothorax was decreased as both parameters increased ($p<0.05$). Shrimp treated with PEF at highest level (PEF-T3) (483 kJ/kg, 600 pulses) had lower melanosis score than other samples, packaged in polystyrene trays and wrapped with shrink film, during 10 days of storage at 4 °C ($p<0.05$). Highest shear force values were noticed for the PEF-T3-treated sample at Day 10 ($p<0.05$). Microstructural gaping between shrimp muscle fibers was higher in PEF-T3. No protein degradation was observed for all samples. Lower total viable count (TVC) and psychrophilic bacterial count (PBC) in shrimp were obtained when PEF-T3 was implemented. After 10 days, higher sensory scores of PEF-T3-treated samples were attained, compared to others ($p<0.05$).

The combined effect of PEF treatment applied on PWS before soaking in CLE at different concentrations (0.5 and 1%) for 30 min was investigated. Sample pre-treated with PEF and soaked with 1% CLE (PEF-1 CLE) showed lower melanosis score than that with 1.25% SMS treatment, PEF treated sample or those soaked in CLE without prior PEF and the control during storage of 10 days ($p<0.05$). PEF-1 CLE sample showed lower TVB, PV and TBARS, but high sensory scores than others ($p<0.05$). Lower increases in mesophile, psychrophile and spoilage bacterial counts were obtained in PEF-1 CLE. Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside and veranisatin-C were found in PEF-1 CLE sample and were plausibly involved in keeping quality of shrimp.

The effects of PEF and 1% CLE treatment on PWS packed under various modified atmospheres (MAP) including absolute N_2, Ar or CO_2 were studied. Lower melanosis score was evidenced in PEF pre-treated shrimp, followed by soaking in 1% CLE and packing under CO_2-MAP (PEF-CLE-CO_2) than the control and other treated samples during storage at 4 °C ($p<0.05$). PEF-CLE-CO_2 showed lower pH, protein carbonyl content (PCC), TVB, peroxide value (PV) and TBARS, but exhibited higher likeness scores ($p<0.05$).. Spoilage and lactic acid bacterial (LAB) counts were less than 3 log CFU/g, which was lower than those of other samples at day 10 ($p<0.05$). Oxidation of fatty acids (EPA and DHA) was prevented in PEF-CLE-CO_2 than the control and other treated samples ($p<0.05$). Volatile compounds in PEF-CLE-CO_2 sample were negligible, compared to the control which was abundant in 3-methyl-1-butanol.

The impact of high voltage cold atmospheric plasma (HVCAP) using dielectric barrier discharge (DBD) was implemented on PWS pre-soaked in 0.5 or 1% CLE solutions. Lower melanosis scores were found in the samples treated with 1% CLE and HVCAP in the presence of Ar and air (80:20) than the control and other samples throughout the storage of 15 days at 4 °C ($p<0.05$). Chemical quality changes in HVCAP treated PWS along with 1% CLE packaged in Ar and air MAP (CP-Ar/Air-1 CLE sample) were not significantly found at day 15. Microbiological and spoilage bacterial counts were less or equal to 5 log CFU/g meat in CP-Ar/Air-1 CLE sample, which were lower than the control and other treated samples at day 15 ($p<0.05$). CP-Ar/Air-1 CLE sample also exhibited higher likeness scores for all the tested attributes.

Lipid and protein oxidation of HVCAP treated sample was prevented by 1% CLE, irrespective of gas composition.

The effects of PEF pre-treated PWS were soaked in CLE solutions (1 or 2%), followed by HVCAP (under Ar + Air at 80:20) for 10 min, were elucidated. Least melanosis scores were attained in the samples pre-treated with PEF-T2 (800 pulses) and CLE (2%) prior to HVCAP (PEFT2-CLE2-Ar/Air-CP), compared to the control, during 18 days at 4 °C ($p<0.05$). The lowest microbial load and spoilage bacteria count (≤ 5 log CFU/g meat) were attained in PEFT2-CLE2-Ar/Air-CP sample ($p<0.05$). PEFT2-CLE2-Ar/Air-CP sample showed higher quality and likeness scores were noted in PEFT2-CLE2-Ar/Air-CP sample for all the attributes. Oxidation of polyunsaturated fatty acids and proteins was alleviated by CLE treatment.

Effect of vacuum impregnation (VI) assisted penetration of CLE (1 or 2%) was studied at vacuum pressure of 5 kPa with various vacuum times (VT; 7.5-15 min) and restoration times (RT, 15-22.5 min) towards PEF treated PWS prior to MAP (Ar/Air; 80:20). Least melanosis scores were attained in samples pre-treated with PEF, VT-2 (15 min) and CLE (2%) (PEF-VI2-CLE2-MAP) than the control during 18 days, at 4 °C ($p<0.05$). Lower microbial load and spoilage bacteria counts were attained in PEF-VI2-CLE2-MAP sample ($p<0.05$). PEF-VI2-CLE2-MAP sample showed higher quality and likeness scores of all attributes when HVCAP was applied to treat PWS which were penetrated with CLE (1 or 2%) with the aid of VI (VT: 15, RT: 15 min) under Ar/Air (80:20) atmosphere for 10 min. Least melanosis scores and microbial load were attained in sample 2% CLE with the aid of PEF and VI and exposed to HVCAP (PEF-VI-CLE2-HVCAP) than the control during 18 days at 4 °C ($p<0.05$). This sample also showed lower lipid oxidation, pH, TVB and PCC than others ($p<0.05$). Higher likeness scores of all attributes were noted in aforementioned sample. Thus, shelf-life of PWS could be extended by the combination of non-thermal processes along with CLE up to 18 days.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is a vital crustacean with high market potential and is an excellent income of Thailand (Gulzar and Benjakul, 2020). Among all shrimp products, there is currently an increasing demand for whole shrimp in the markets. Despite their delicacy and popularity, this high value crustacean is highly perishable with short shelf-life (Sea-Leaw and Benjakul, 2019). Quality loss in shrimp is due to the onset of melanosis and microbial spoilage (Gokoglu and Yerlikaya, 2008). The blackening or melanosis in shrimps is caused by the formation of melanin (Benjakul *et al.*, 2005). This quality loss in shrimp is induced by a biochemical reaction, associated with phenol oxidation catalyzed by polyphenoloxidase (PPO), with subsequent formation of melanin (Haard and Simpson, 2000). Although the presence of melanin is not harmful, it considerably reduces the market value of the crustacean (Haard and Simpson, 2000). Sulfiting agents such as sodium metabisulphite (SMS) have been used to prevent browning reaction in foods, including crustaceans. Although sulfites including SMS have been generally recognized as safe (GRAS) for food applications, they can be allergenic for some sulfite sensitive people (Kronberg, 2008). To avoid aforementioned problem, PPO inhibitor, especially 4-hexylresorcinol, has been employed (Martínez-Alvarez *et al.*, 2008). Nevertheless, it is still costly when used as additive.

Over the past decades, several additives, both natural and synthetic, have been used to lower or prevent melanosis and to retard microbial growth in raw shrimp stored at refrigerated temperature (Nirmal and Benjakul, 2009; 2010). In addition to the low temperature storage, thermal processing of shrimps have been also done to ensure the better quality shrimp (Dabadé *et al.*, 2015). Plant extracts rich in polyphenols have been brought into application to inhibit melanosis and quality changes in order to prolong shelf-life of shrimps. However, efficacy in inhibiting PPO varied with type or structure of plant phenols. Sae-Leaw *et al.* (2017) documented that inhibition effect of epigallocatechingallate (EGCG) on PPO activity was higher than catechin, epicatechin,

epicatechin gallate and epigallocatechin. Both green tea and grape seed extracts rich in phenolics were used to treat shrimp for shelf-life extension, especially by lowering melanosis (Gokoglu and Yerlikaya, 2008; Nirmal and Benjakul, 2009a; Nirmal and Benjakul, 2009b). Additionally, plant extracts also exhibit antimicrobial activity towards spoilage microflora, thus maintaining the quality and sensory property of shrimp during the extended refrigerated storage. Moreover, cashew leaf extract (CE) has been proven to be the effective antimelanosis, antimicrobial and antioxidative agents for Pacific white shrimp during refrigerated storage (Sae-leaw and Benjakul, 2019).

Chamuang (*Garcinia cowa* Roxb.) is the indigenous plant growing in the southern part of Thailand and tropical countries (Lim, 2012). Its leaves have been used for cooking into several dishes (Wisootthipaet, 2015). The leaves are rich in organic compounds such as (-)-hydroxycitric acid and polyphenols (Jena *et al.*, 2002). Additionally, the extract from Chamuang leaves had antimicrobial properties and could retard microbial growth (Sakunpak and Panichayupakaranant, 2012). The Chamuang leaves rich in polyphenols and organic acids might be used as an alternative PPO inhibitor, in which melanosis in Pacific white shrimps can be prevented.

Non-thermal processes have gained importance in recent years due to the increasing demand for minimally processed seafoods with a high nutritional value and fresh-like characteristics (Olatunde and Benjakul, 2018). These technologies are also eco-friendly and cost effective in the food system (Kate *et al.*, 2016). Pulsed electric field (PEF) is one of the novel non-thermal technology, that can reduce microbial load of foods (Lado and Yousef, 2002). PEF is based on electroporation and cell disintegration by the application of repeated pulses when the food is placed between the two parallel electrodes (Gudmundsson and Hafsteinsson, 2001; Puertolas *et al.*, 2013). The main cause of cell destabilization is based on the imbalance in transmembrane potential due to electrochemical gradient maintained across the cell membrane. Moreover, the electroporation becomes more pronounced, if the field strength (E) exceeds the reversible threshold (Ec) with sufficient duration of exposure time that favors reversible electroporation (Čorović *et al.*, 2012). Excessive field strength and level of energy result in an irreversible electroporation (Davalos *et al.*, 2005; Al-Sakere *et al.*, 2007; Al-Sakere *et al.*, 2007). Microstructural and textural

changes have been reported as induced by electroporation associated with the leakage of cellular fluids in the tissue matrix (Gudmundsson and Hafsteinsson, 2001).

Nevertheless PEF treatment had no effect on protein structure in fish and chicken meat (Gudmundsson and Hafsteinsson, 2001). Without heat applied, foods retain high quality after being treated with PEF (Ho and Mittal, 2000). PEF can be combined with packaging, particularly modified atmosphere packaging (MAP) to assure the quality and extend shelf-life of shrimp during refrigerated storage. MAP refers to the replacement or alteration in the gaseous composition surrounding the food placed inside a sealed package (McMillin, 2008). MAP can collectively work with cold temperature conditions at low oxygen content. It could delay melanosis and prevent quality loss in Pacific white shrimp (Kalleda *et al.*, 2013; FAO, 2018). Packaging is another important hurdle, which can preserve food commodity and ensure the quality and safety for consumption (Sivertsvik *et al*., 2002). MAP has been considered as the potential means for shelf-life extension of deepwater pink shrimp when used in combination with several chemical additives (Gonclalves *et al.*, 2003). Natural extracts such as green tea and thymol essential oil with antimelanosis, antimicrobial and antioxidant agents jointly with MAP have been employed to prolong shelf-life of *Litopenaeus vannamei* and *Palaemon serratus* (Mastromatteo *et al.*, 2010; Nirmal and Benjakul, 2011). Pacific white shrimp packaged under high CO_2 (60% CO_2 + 5% O_2) inhibited melanosis and retarded quality changes during 12 days of chilled storage (Kimbuathong *et al.*, 2020). PEF can be used in combination with vacuum impregnation technology to facilitate the penetration of bioactive compounds.

Vacuum impregnation (VI) is a promising non-thermal technique to improve food quality by enhancing the penetration of various bioactive compounds through porous tissues, under low pressure in a solid-liquid food system (Zhao *et al.*, 2019). VI involves the removal of gases or liquid inside pores of solid foods under vacuum condition and then replacing them with VI immersion solutions under restored atmospheric pressure (Yang *et al.*, 2017; Mao *et al.*, 2017). This technology has been proven as an effective means to replace the tradition immersion practices for food preservation. VI has been industrially coupled with antioxidant from natural extracts on fish and seafoods for prevention of chemical and microbial spoilage during storage (Zhao *et al*., 2019). Additionally, high intensity PEF pulses applied on sea bass skin

has been reported to facilitate the migration of VI solutions more effectively via pores generated by electroporation (Chotphruethipong *et al.,* 2019). Thus, PEF could aid in the passage of CLE in PWS during VI process, followed by restoration.

Cold atmospheric plasma (CAP) has been employed as non-thermal treatment technology among diverse foods to combat quality loss in food industry (Chizoba Ekezie *et al.*, 2017). Different types of reactive species produced in the cold plasma, such as free radicals including reactive oxygen species-ROS and reactive nitrogen species-RNS, ultraviolet (UV), negative and positive ions have been known to inactivate microorganisms (Liao *et al.*, 2017; Liao *et al.,* 2018). CAP has demonstrated bactericidal effects against spoilage microorganisms in food products, including fruits, vegetables, juices, cereals, meat and poultry, and spices (Mir *et al.*, 2016; Liao *et al.*, 2018). Recently, atmospheric cold plasma generated plasma activated water has been used in ice form for the preservation of shrimp (*Metapenaeus ensis*) (Liao *et al.*, 2018). Shelf-life of different perishable fruits including strawberries, grapes and Chinese bayberries was extended by decontamination using cold plasma technology (Guo *et al.*, 2017; Liao *et al.*, 2018; Sarangapani *et al.,* 2018). However, there are only limited studies concerning the application of cold plasma for the preservation of aquatic food (Chen *et al.*, 2019).

Thus, the quality and safety assurance of Pacific white shrimp in view of melanosis or blackening control, oxidative stability, antimicrobial efficacy and sensorial quality could be achieved by the application of natural extract from plant leaves coupled with non-thermal technologies and modified atmosphere packaging. Henceforth, aforementioned approaches could be the potential means to preserve quality and extend shelf-life of Pacific white shrimp at refrigerated storage condition.

1.2 Literature Review

1.2.1 Quality deterioration of crustaceans

Any change in the initial condition of seafood or crustacean that results in an unpalatable odor, taste, appearance and texture are referred to as spoilage. This change can be attributed to enzymatic, chemical or microbial activities in the marine foods (Ghaly *et al.*, 2010). Indigenous protease and lipase activities of spoilage microorganisms and oxidation of proteins and lipids could be responsible for spoilage

or deterioration during post-rigor mortis storage (Ghaly *et al.*, 2010; Grant *et al.*, 2003). Chemical, microbial and enzymatic spoilage in seafood can be controlled by non-thermal pre-treatment, natural preservatives and modified atmosphere packaging, that could aid in the retention of sensory and nutritional properties of the food product with extended shelf-life.

1.2.1.1 Pacific white shrimp (*Litopenaeus vannamei*)

Global shrimp production now stands at approximately 6 million metric ton (MMT) out of which Pacific white shrimp (*Litopenaeus vannamei)* accounts for approximately 76% or roughly 5 MMT of global shrimp production. Asia alone produces by 71% (Anderson *et al.*, 2016). Pacific white shrimp is widely consumed in Thailand and accounts for 90% of the global aquaculture production of Pacific white shrimp (Nirmal and Benjakul, 2009). Pacific white shrimp was introduced to Asia from America. This species has bloomed in Thailand in the last decade, resulting in an almost complete shift from the native giant tiger prawn (*Penaeus monodon*) to this introduced species (FAO, 2011). World production of crustaceans was relatively even in distribution among brackish water (2.4 million tonnes, or 47.7 percent), freshwater (1.9 million tonnes, or 38.2 percent) and marine water (0.7 million tonnes, or 14.1 percent). Pacific white shrimp formerly known as *Penaeus vannamei* accounts a total production percentage of 53% of seafood crustaceans as documented in Table 1 (FAO, 2018).

1.2.2 Polyphenoloxidase (PPO) in crustaceans

Polyphenoloxidase (PPO) or tyrosinase (EC 4.14.18.1) is a copper containing metalloprotein involved in the phenol oxidation to intermediate quinones, leading to the onset of melanosis formation in shrimp (Jang *et al.*, 2003). PPO enzyme belongs to oxidases and is widely distributed in the phylogenetic range of crustacean species (García-Molina *et al.*, 2007). PPO plays a vital role in physiological functions, particularly hardening of the chitin shell after molting (sclerotization) in shrimp and lobsters (Terwilliger and Ryan, 2006). Phenolic oxidation producing *o*-quinones in crustacean such as tyrosine shows cross-linkages with histidyl residues of chitin and cuticular proteins referred as sclerotization resulting in hardening of outer shell (Xu *et*

al., 1997). PPO shares an important part in the self-recognition and boosting immune system of crustaceans (Huang *et al.*, 2010).

Table 1 Major crustaceans produced in the world aquaculture

Species item	2010	2012	2014	2016	% of total (2016)
Pacific white shrimp (*Penaeus vannamei*)	2688	3238	3697	4156	53
Red swamp crawfish (*Procambarus clarkia*)	616	598	721	920	12
Chinese mitten crab (*Eriocheir sinensis*)	593	714	797	812	10
Giant tiger prawn (*Penaeus monodon*)	565	672	705	701	9
Oriental river prawn (*Macrobrachium nipponense*)	226	237	258	273	4
Giant river prawn, (*Macrobrachium rosenbergii*)	198	211	213	234	3
Other crustaceans	700	606	654	767	10
Crustaceans total	5586	6277	7047	7862	100

Source: Food and Agriculture Organization United Nations (2018)

The cell wall material such as lipopolysaccharides and β1, 3-glucans plays a vital role in the enhanced enzyme activity (Perazzolo and Barracco, 1997). Zotos and Taylor (1997) reported that PPO activation is mainly influenced by the three proteases namely protease I (possibly Zn-thiol protease), protease II (thiol protease) and protease III (Zn-serine protease). Undesirable discoloration mediated by PPO appearing on the outer surface of crustaceans is a tough challenge for food processing industries that results in quality deterioration and disliking by consumers (Sae-leaw and Benjakul, 2019).

1.2.2.1 Melanosis in crustaceans

Melanosis or blackening of the outer surface of the crustaceans especially shrimps is a major problem after capture that is triggered by activation of PPO to synthesize a high molecular weight pigment called melanin. Melanin appears underneath the shell or carapace of the cephalothorax and on the appendages of shrimp (Nirmal and Benjakul, 2011). Melanin is not toxic or hazardous to consumers, it degrades the sensory attributes of crustaceans and subsequently reduces the market value (Kim *et al.*, 2000; Gómez-Guillén *et al.*, 2005). According to the definition of Codex Alimentarius Commission melanosis can be defined as occurrence of dark spots at the mechanically bruised parts and joints of crustacean segments, triggered by a oxidation of phenols followed by synthesis of polymerized high molecular weight melanin (Rotllant *et al.*, 2002). The onset of melanosis in shrimp, crabs and lobsters occurs after their body surface contact with the molecular oxygen (Gokoglu and Yerlikaya, 2008).

Over the years, melanosis in the crustaceans is the major problem in seafood processing industries that induces deleterious changes in the sensory attributes with short shelf-life and reduces consumer acceptability (Kim *et al.*, 2000; Nirmal and Benjakul, 2009). Generally, cephalothorax, abdominal exoskeleton and zones of cuticular segments joined with the pleopods are the indicators of melanosis. Moreover, caudal zone, telson and uropods have been reported be affected by melanin (Ogawa *et al.*, 1984; Montero *et al.*, 2001; Nirmal and Benjakul, 2012). Melanosis in Pacific white shrimp (*Litopenaeus vannamei*) has been illustrated in Fig. 1. Melanosis occurrence varies with species, depending on the biological factors such as molting cycle (Cintra *et al.*, 1999). During harvesting to handling period, capture or mishandling of the caught crustacean triggers the defense mechanism during traumatic or bruised conditions results in enhanced melanin formation (McEvily, 1991; Bartolo and Birk, 1998; Gonçalves and de Oliveira, 2016). The amount of substrate and enzyme concentration varies with specie type and seasonal variation of the crustacean (Benjakul et al., 2005; Nirmal and Benjakul, 2012). Due to rapid spoilage during post-harvest storage and transportation, crustaceans must be cooled to the low temperature such as melting ice temperature (Pardio et al., 2011). Refrigeration or iced storage retards the rate of browning reaction (Martınez-Alvarez *et al.*, 2007).

Figure 1. Melanosis (black spots) appearance on the cuticle and telson of shrimp.
Source: Gonçalves and de Oliveira (2016)

The freezing or thawing processes can either inactivate or facilitate the function of enzymes in the hemolymph and digestive glands to act on substrate for prevention or increment in melanosis (Nirmal and Benjakul, 2010). Improper temperature maintenance and mis-management in cold chain corresponds to enzymatic and microbial spoilage. However, it has been reported that melanosis development occurs rapidly than microbial spoilage (Thepnuan *et al.*, 2008). Hence, it retards its appealing quality in the seafood market without direct hazard to human health.

1.2.2.2 Melanosis pathways

PPO exists as proPPO in crustaceans and triggers the formation of melanin (Garcia-Carreno *et al.*, 2008; Zamorano *et al.*, 2009). PPOs in crustaceans as exist zymogens (proPPO) and acts as a defensive system helps in immunity boosting and self-recognition processes in the crustacean (Kim *et al.*, 2000). Activation of ProPPO occurs due to serine proteinase cascade triggered by microbial cell components such as lipopolysaccharides, carbohydrates and other proteins (Wang *et al.*, 2006). Ramírez (2003) documented two types of PPO activity involving monophenol oxidase and *o*-diphenol oxidase. Monophenolase activity and diphenolase activity of tyrosinases are proceeded by the *o*-hydroxylation of mono-hydroxyphenols and the oxidation of *o*-dihydroxyphenols to *o*-quinones, respectively (Zamorano *et al.*, 2009). Schematic representation for the development of melanosis in crustaceans is shown in Fig. 2. Molecules associated to pathogens such as PGBP: peptidoglycan binding protein from gram positive bacteria, LGBP: lipopolysaccharide and beta-1,3-glucan binding protein from gram negative bacteria, and BGBP: beta-1,3-glucan binding protein

(fungi) trigger a serine proteinase cascade (1), which induces ProPPO to convert into activated PPO (2). In return, PPO (3) catalyzes the conversion of phenols into colorless quinones (4), that when oxidized become dark pigments called melanin. Melanin is the substance responsible for the darkening of crustaceans known as melanosis (5).

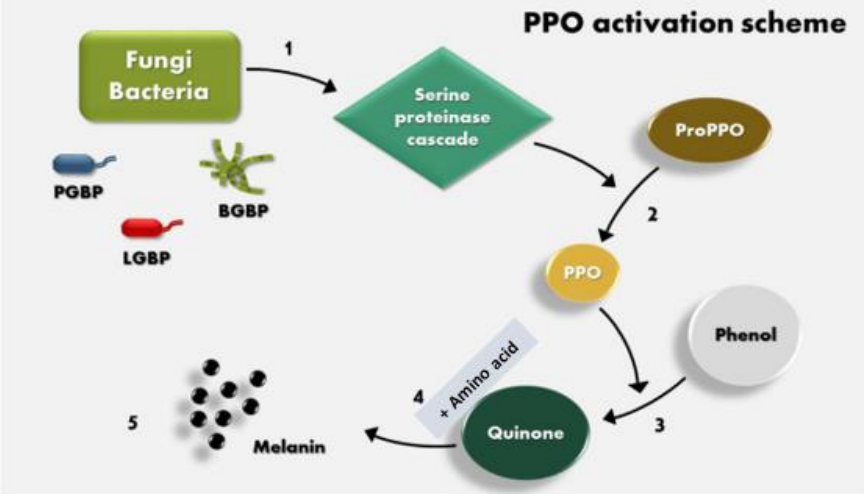

Figure 2. ProPPO activation scheme in crustaceans.
Source: Amparyup *et al.* (2013)

Reaction initiated by PPO in the presence of oxygen changes monophenols (colorless) to diphenols and are further converted to highly colored quinones, which react with amino acids to form complex brown polymers (Haard and Simpson, 2000). Apart from PPO in crustaceans, hemocyanin (Hc) a copper-binding protein is also found in the hemolymph of mollusks and arthropods responsible for oxygen transport (Kim *et al.*, 2000; Fan, 2009). It accounts for 90-95% of the total plasma protein in crustaceans. PPOs were regarded earlier as the only inducers of diphenols oxidation to form *o*-quinones in the hemolymph, however the different mechanism induced by hemocyanin was HcPO system (García-Molina *et al.*, 2007). Indigenous or microbial Proteolytic enzymes during post-mortem storage can activate pro-PPO by the protease hydrolysis providing substrates for active PPO (Montero *et al.*, 2001; Zamorano *et al.*, 2009). The optimum temperature and pH of PPO activity have been reported between 20-25 °C and pH 6-8, respectively. The stability of the PPOs also depended on the substrate type, ionic strength, buffer system and the incubation time (Kim *et al.*, 2000). The oxidative reactions induced by PPO and HcPO cause the rapid development of melanosis during post-harvest storage. Therefore antimelanosis agents have been used to stop these reactions to avoid the losses of used

quality (Encarnacion *et al.*, 2011; Encarnacion *et al.*, 2011). Nakagawa and Nagayama (1981) found that L-tyrosine and catechol were main substrates for PPO activity in various body parts of different crustaceans such as taisho-ebi shrimp (*Penaeus orientalis*), deep sea crab "nihon-ibaragani" (*Neolithodes nipponensis*), crayfish (*Procambarus clarkii*), tiger shrimp (*Penaeus japonicus*), snow crab (*Chionoecetes opilio*), swimming crab (*Portunus trituberculatus*) and deep sea crab "ibaraganimodoki" (*Lithodes aequispina*). Hemolymph and gill evidenced to develop melanosis in crayfish while as, hemolymph only tiger shrimp showed PPO activity towards L-tyrosine (Nakagawa and Nagayama, 1981).

1.2.2.3 Inhibition of melanosis

Prevention of enzymatic browning can be accomplished through various techniques and mechanisms that have been developed over the years to prevent PPO action in crustaceans (Kim *et al.*, 2000). These techniques aim to eliminate one or more of the essential components (oxygen, enzyme, copper, or substrate) from the reaction (Gokoglu and Yerlikaya, 2008). Chemical and natural compounds have also been used to prevent PPO action to control melanosis (Mendes, 2006; Sae-leaw and Benjakul, 2019).

Retardation of melanosis in shrimp have been done by alteration in pH or temperature as well as use of appropriate antimelanosis agents to inhibit PPO for quality maintenance during low temperature storage (Nirmal and Benjakul, 2012). PPO inhibitors according to their primary mode of action, have been classified into different categories such as ascorbic acid and ascorbyl derivatives termed as reducing agents, acidulants including phosphoric acid and citric acid or chelating agents such as ethylenediaminetetraacetic acid (EDTA), occupying the free active sites of copper ion to avoid melanosis (Gómez-Guillén *et al.*, 2005). Sulphites, especially sodium metabisulphite (SMS) at 1.25% level, are the additives most widely and effectively used to prevent melanosis in shrimp and other crustaceans (López-Caballero *et al.*, 2006; Nirmal and Benjakul, 2009; Bono *et al.*, 2012). Sulfur dioxide and its salts have been used since ancient Greece for preservation (Rotllant *et al.*, 2002). The inhibition of melanosis in shrimp dates back to the Greece in 1950s (Fieger, 1951) and remain until today as worldwide practice. In the case of catechol as a substrate, nucleophilic attack

by sulfite ion in position 4 of the *o*-quinone to give 4-sulfocatechol after subsequent addition of a hydrogen ion possibly aids in the prevention of browning reaction. Therefore, the quinone are reduced by the addition of proton (Kim *et al.*, 2000). Nevertheless, sulphites have potentially pathological effects (López-Caballero *et al.*, 2006; Nirmal and Benjakul, 2009; Bono *et al.*, 2012). In clinical practice, metabisulphite is believed to trigger asthmatic attacks, causing serious allergic reactions (Collins-Williams, 1983). Fishermen or expert skill full workers at fishing sites or crustaceans farms are exposed to higher doses of sulphites on daily basis of their business activities of crustacean handling and distribution. After crustaceans are delivered to industries, these are washed before storage to eliminate residual SO_2. Even though SO_2 levels may be reduced with storage time, however the initial high concentration still favor trimethylamine oxide (TMAO) degradation into dimethyl amine (DMA) and formaldehyde (FA), which are fetal health and prohibited in foods (Cintra *et al.*, 1999).

Traditionally, the preservation of fresh aquatic food products in general practices involve the usage of flaked ice or slurry ice (Aubourg *et al.*, 2007). Frozen storage has been practiced to delay crustacean spoilage, but it adversely affects the physical and chemical changes thereby alters sensory characteristics during extended storage (Pardio *et al.*, 2011). However, the technique was not sufficient during iced storage between 2 and 4 days, thawed frozen Rose Shrimp (*Aristeus antennatus*) had black spots (Rotllant *et al.*, 2002). Thermal inactivation of PPO is another means to control melanosis in shrimp. Pre-cooking inactivates PPO associated with quality loss, thereby providing more stable products (Manheem *et al.*, 2012; Sae-leaw *et al.*, 2018). At boiling temperature, 2 min were enough to deactivate PPO in deepwater pink shrimp (*Parapenaeus longirostris*) (Martínez-Alvarez *et al.*, 2009). High pressure treatment may also be recommended for prevention of melanosis, inhibiting microbial growth, thus extending shelf-life and improving product quality (Encarnacion *et al.*, 2010). Previous studies has shown that prawns, *Penaeus japonicus*, treated with high pressure technology, successfully prevented the appearance of black spots (Montero *et al.*, 2001).

After the capture, crustaceans on board may be processed immediately immersion methods in chemical additive solutions followed by dusting or spraying

(Martínez-Alvarez *et al.*, 2005) or using blend of antimelanosis agents such as chelating agents, sulphite derivatives and organic acids (Gómez-Guillén *et al.*, 2005). Furthermore, crustaceans may be partially bleaching to maintain an acceptable appearance during distribution process to the seafood markets (Rotllant *et al.*, 2002). These chemicals prevent quinones polymerization, and tend to be colorless compounds. However, these chemical or bleaching agents are not stable for longer period of time and need to be reapplied for a continuous effect.

Safe additive has been introduced to control or prevent melanosis. 4-hexylresorcinol (4-HR) has been viewed as a safe alternative to metabisulphite and other sulphite treatments (Lopez-Caballero *et al*., 2006; Montero *et al.*, 2004; Montero *et al.*, 2006). The use of 4-HR is permitted in United States, Australia, Canada and some Latin American countries (Martínez-Alvarez *et al.*, 2007). The potential use of 4-HR has been investigated to be safe after capture and in combination other methods of crustacean processing for quality control (Montero *et al.*, 2004; McEvily *et al.*, 1991; Thepnuan *et al.*, 2008). 4-HR has several advantages over sulphites due to specific mode of inhibitory action even at lower doses and is stable for longer time (Kim *et al.*, 2000). To show an effective synergistic antimelanosis effect, 4-HR is used in combination with ascorbic acid causing the loss in enzymatic activity (Kim *et al.*, 2000). Although 4-HR has no evidence of any health hazard, but its applications in shrimp may impact taste during sensory evaluation (Kim *et al.*, 2000). Therefore, awareness and strict regulations of chemical additives have promoted a matter of concerning for their replacement by natural alternatives to be the antimelanosis (Gokoglu and Yerlikaya, 2008).

1.2.3 The use of plant extracts or phenolic compounds as additives in seafood

1.2.3.1 Phenolic compounds

Plant phenolics have been paid increasing attention as potential natural additives, since they have both antioxidant and antimicrobial activities (Banerjee, 2006). Phenolic compounds are naturally occurring as the secondary metabolites that are derivatives of the pentose phosphate, shikimic acid, and phenylpropanoid pathways in plants (Ryan and Robards, 1998). Mostly phenolic compounds are present in plant based products such as vegetables, fruits, leaves, herbs and spices containing essential

oils that exhibit antioxidant activity (Sellappan *et al.*, 2002). Natural phenolics of plant origin with antioxidant activity are predominantly found in culinary herbs, spices, vegetables, fruits and oilseed products (Shahidi and Zhong, 2010). These compounds are of considerable physiological and morphological importance in plants (Balasundram *et al.*, 2006). Phenolics of plant origin mainly constitute tocopherols, flavonoids, cinnamic acid derivatives, and coumarins. Naturally occurring plant derived phenolics are abundantly found in grape, green tea and leaves and contribute to antioxidant effects and PPO inhibition (Kim *et al.*, 2000). The extract from edible enokitake mushroom (*Flammulina velutipes*) was reported to be a promising source of natural antioxidants and antimelanosis agents (FAO, 2011). The radical scavenging activity and suppression of lipid oxidation remarkably achieved by using mushroom extract containing 2-thiol-L-histidine-betaine (ergothioneine, ERT) at a level of 3.03 ± 0.07 mg mL^{-1} (Encarnacion *et al.*, 2010). Phenolic compounds such as catechins, epicatechins, and epicatechin-3-ogallate with dimeric, trimeric, and tetrameric procyanidins were investigated in grape seeds (Gokoglu and Yerlikaya, 2008). Natural food flavorings and antioxidant agents used in foods, were obtained from Rosemary, a herb from the *Lamiaceae* family (Seabra *et al.*, 2011). Rosmanol, epirosmanol, rosemary quinone, rosemary diphenol, carnosol, and carnosic acid are primarily responsible for the antioxidant activity of rosemary (Hadolin *et al.*, 2004). Lead (*Leucaena leucocephala*) is another source of carotenoids, xanthophylls and mineral employed in shrimp melanosis control and shelf-life extension (Nirmal and Benjakul, 2011). Besides containing phenolic antioxidative compounds, this plant consists of a non-protein amino acid called mimosine (b-(3-hydroxy-4-pyridon-1-yl)-L-alanine) (Lalitha *et al.*, 2006), employed as natural antimelanosis agent in Pacific white shrimp (Nirmal and Benjakul, 2011).

Chemically, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl groups, and range from simple phenolic molecules to highly polymerized compounds (Dykes and Rooney, 2006). Additionally, phenolic compounds are found to be conjugated with saccharides (mostly mono and disaccharides), linked via acid-labile hemiacetal bond joining many phenolic compounds (Ryan and Robards, 1998; Balasundram *et al.*, 2006).

The various classes of phenolic acids include hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids (C_6-C_1) include protocatecguic, vanillic, gallic, *p*-hydroxybenzoic and syringic acids. Hydroxycinnamic acids comprise of C_6-C_3 structure which is constituted of coumaric, caffeic, ferulic, and sinapic acids (Dykes and Rooney, 2007). Flavonoids are a large class of compounds, ubiquitous in plants with low molecular weight compounds with a fifteen carbon atoms arrangement (C_6-C_3-C_6) configuration (Rice-Evans *et al.*, 1997). Based on the several phenolic hydroxyl functional groups attached to ring structures are named as B and C, such that aromatic ring A emerge from malonate pathway, while as ring B is formed from phenylalanine through shikimate pathway (Balasundram *et al*., 2006). Tannins are comprise of high molecular weight compounds may be further divided into hydrolyzable tannin with gallic acid esters and condensed tannins belonging to polymers of polyhydroxy flavanol monomers (Shahidi and Naczk, 2004).

1.2.3.2 Extraction of phenolic compounds

Diversity of polyphenols from simple to complex structures are distributed randomly in plants at the tissue, cellular and subcellular levels, and may be conjugated with carbohydrates and proteins (Luthria *et al.*, 2006). Phenolic compounds have been extracted from plant source, especially leaves, seeds, rind of fruits or other parts from the root or shoot system is influenced by their chemical nature, mode of extraction, sample particle size, extraction time and storage conditions (Shahidi and Naczk, 2004). Therefore, phenolic extracts of plant origin are always mixture of different classes of phenolics that are soluble in the solvent system used. Type of solvent whether non-polar or polar, degree of polymerization of phenolics and interaction of phenolics with other chemical constituents governs the solubility of phenolic compounds. Therefore, diverse extraction modes of phenolic substances have been employed in plant materials (Luthria *et al.*, 2006). Solvent extraction using distilled water, methanol, ethanol, acetone, ethyl acetate and their combinations are frequently used for the extraction of phenolics (Olatunde *et al.*, 2018). Ultrasonication, maceration, stirring and microwave assisted extraction have been employed to facilitate the extraction of plant based polyphenols (Sutivisedsak *et al.*, 2010; Nirmal and Benjakul, 2011; Chotphruethipong *et al.*, 2019).

Row and Jin (2006) postulated the recovery of catechin compounds from Korean tea by solvent extraction with an optimum extraction at 80°C for 40 min using pure water as extraction solvent. The extract was partitioned with water/chloroform (1:1 v/v) to remove caffeine impurity from the extract that aids in the purification of the catechin compounds. Torre *et al.* (2008) extracted ferulic acid from corn cobs by alkaline hydrolysis. Rusak *et al.* (2008) extracted phenolics from bagged and loose leaves of white and green tea at 80 °C using distilled water and aqueous ethanol (10, 40 and 70 %). Addition of lemon juice (5 ml) could enhance the extraction of phenolic from white tea and aqueous ethanol (40 %) was most effective in the extraction of catechins. Extraction of vanillin and ferulic acid form flax shives, wheat bran and corn bran by non-pressurized alkaline hydrolysis (0.5 M NaOH) and pressurized solvents (0.5 M NaOH, water, ethanol and ammonia) extraction methods (Buranov and Mazza, 2009). Corn bran (5 g) extracted using pressurized 0.5 M NaOH extraction condition yielded higher vanillin (165 mg/100 g) as depicted by its higher content of ferulic acid (2510 mg/100 g).

1.2.3.3 Identification and characterization of phenolic compounds

Several analytical techniques have been employed for the separation and characterization of phenolic compounds such as high performance liquid chromatography (HPLC). Liquid chromatography (LC) in connection with electrochemical detection (EC), fluorescence (F), and mass spectrometry (MS) have been commonly used for identification. Phenolic compounds have absorbance maxima at numerous wavelengths. Most of the benzoic acid derivatives, gallic acid, salicylic and syringic acid show the maximum absorbance around 254, 275, 310 and 280 nm, respectively. Charrouf *et al*. (2007) studied the separation and characterization of phenolic compounds in argan fruit pulp using liquid chromatography (LC) equipped with negative electrospray ionization (ESI) mass spectroscopy (MS/MS). A C-18 (50 x 2.1 mm, i.d 3.5 μm) was used for the separation and gradient elution was performed with water/0.05% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 600 μL/min. Sixteen compounds were identified, mainly flavonoid aglycons and their glycosides. Chlorogenic, caffeic and *p*-coumaric acid have the maximum absorbance close to 325 nm, and ferulic acid near to 310 nm (Herrera *et al.*, 2005). Weisz *et al*.

(2009) extracted eleven phenolic compounds from defatted sunflower (*Helianthus annuus* L.) kernels and shells and characterized by HPLC with diode array and electrospray ionization (ESI) mass spectrometric detection in negative mode. The column used was 150 mm x 3.0 mm inner diameter, 4 μm particle size, C18 Hydro-Synergi. Ma *et al*. (2009) detected seven phenolic compounds of two families including cinnamic acids and benzoic acid from citrus peel extract using C-18 reverse phase column and elution was performed with the mobile phase consisting of 4 % (v/v) acetic acid in water: 100 % methanol (80:20, v/v) at solvent flow rate of 1 mL/min. The mobile phase consisted of 2 % (v/v) acetic acid in water (eluent A) and 0.5 % acetic acid in water and acetonitrile (50:50, v/v, eluent B) with a flow rate of 0.4 mL/min. UV-visible absorption spectra were recorded from 200 – 600 nm during HPLC analysis. UV-Visable spectra were recorded in the range of 200-600 nm. Profiling and identification of cashew leaf extract was performed by LC-DAD-ESI-MS/MS based on m/z ratio of phenolic compounds (Sae-leaw and Benjakul, 2019). Samples were eluted with gradient system consisting of solvent A (2 % acetic acid, v/v) and solvent B (acetonitrile: methanol, 10:15, v/v), with a flow rate of 1 mL/min from C-18 column (250 x 4.6 mm, 5 μm particle size). Epifisetinidol-4alpha-ol and hinokiflavone were identified as the most dominant polyphenols in cashew leaf extract. Five phenolic compounds, namely gallic acid, procyanidin B2, (-)-galllocatechin, (-)-epicatechin, and (-)-epicatechin-3-gallate were identified from 50 % ethanol extract of litchi (*Litchi sinensis* Sonn.) seeds (Prasad *et al*., 2009).

1.2.3.4 Antioxidant activity of phenolic compounds

Phenolic compounds are known to be potential antioxidants and their activity depends on the structure and the number of the hydroxyl groups and substitution nature of the aromatic rings. The chemical activity of phenols depends on their ability of reducing properties as hydrogen or electron donating properties to claim as antioxidants (Rice-Evans *et al.*, 1997). Radical-scavenging activity and total antioxidant activity of grape (*Vitis vinifera*) seed extracts in the ratio of acetone: water: acetic acid (90:9.5:0.5) and methanol: water: acetic acid (90:9.5:0.5) were quantified at concentrations of 25 and 50 ppm. Acetone: water: acetic acid (90:9.5:0.5) extract showed the higher radical scavenging activity than methanol: water: acetic acid

(90:9.5:0.5) extract (Jayaprakasha *et al.*, 2003). Negi *et al.* (2005) reported that extract of seabuckthorn (*Hippophae rhamnoides* L.) seeds was extracted by using ethyl acetate, acetone, chloroform and methanol using Soxhlet extractor for 8 h. Shahidi *et al*. (2007) reported that extracts of hazelnut byproducts such as skin, green leafy cover, hard shell and tree leaf) possess the stronger antioxidant activities than hazelnut kernel. Among all the tested samples, extracts of hazelnut skin, in general, showed superior antioxidative efficacy and higher phenolic content to other extracts. Prasad *et al.* (2009) documented that 50% ethanol extract of *Litchi sinensis* Sonn. seeds showed total antioxidant capacity, scavenging the 1,1-diphenyl-2-picryl hydrazyl radical and prevented lipid oxidation by inducing strong antioxidant potential (25-100 μg/mL) equivalent to butylated hydroxyl toluene. Green tea extract had the higher reducing and DPPH radical scavenging activities, compared with mulberry tea extract ($P < 0.005$). (Nirmal and Benjakul, 2011a).

1.2.3.5 Metal-chelating activity of phenolic compounds

Complex structure of phenolic compounds with numerous hydroxyl group in ortho position had the tendency of chelating metal ions to prevent oxidation (Maqsood and Benjakul, 2009). Maqsood and Benjakul (2009) reported that catechin showed the highest metal chelating activity, followed by caffeic acid, tannic acid and ferulic acid. Green tea extract had the higher copper chelating activity, compared with mulberry tea extract ($P < 0.005$) (Nirmal and Benjakul, 2011a). Moreover, tannin fractions of almonds, hazelnuts and walnuts possess higher chelation capabilities (Karamac, 2009). Similarly, copper chelating activity of ferulic acid, catechin and mimosine was reported by Nirmal and Benjakul (2011b; 2011c). The stability of metal-phenolic compound complex is higher in six-membered than five-membered ring complexes (Wettasinghe and Shahidi, 2002). Borage and evening primrose crude extracts and their fractions I aqueous medium exhibited strong metal chelating activities. (Wettasinghe and Shahidi, 2002). PPO is a metalloprotein, containing copper metal ion responsible for enzyme activity. Therefore, the copper chelating capacity of phenolic compounds is one of the important inhibitory mechanisms involved in the inhibition of PPO. However, the efficiency in metal chelation varied with the type of

phenolic compounds. Recently, Cashew leaf extract (0.5%) was documented to exhibit copper chelation activity (Sae-leaw and Benjakul, 2019).

1.2.3.6 Mechanism of antimicrobial effect of plant polyphenols

Phenolic compounds in plant extracts can disrupt the cell membrane integrity by interacting with membrane proteins of bacteria or fungi. By increasing the permeability of the cell membrane, these compounds cause the leaching of potassium ions and other cytoplasmic components thus ultimately causing cell death (Bajpai *et al.*, 2008; Simoes *et al.*, 2009). The cell membrane of bacteria contains phospholipids, which can be partitioned by the insertion of hydrophobic compounds of polyphenols plant extract, thereby rendering the cell membrane permeable and disrupting the cell structure (Jose *et al.*, 2014; Sikkema, *et al.*, 1994). Cell death is also caused by extensive disruption of bacterial cell wall (Ajaiyeoba *et al.,* 2003). However, the sensitivity of Gram-negative bacteria and Gram-positive bacteria to polyphenols differs. The former have thinner cell wall containing single cell membrane made of lipopolysaccharide and membrane proteins, attached to a thick peptidoglycan layer compared to the later having double layer of lipopolysaccharide and membrane proteins in which peptidoglycan layer is embedded between the inner and outer cell membranes, resulting in extra barrier properties for penetration of plant polyphenols. Thus, Gram-negative bacteria are more susceptible to the antimicrobial compounds due to less barrier properties (Abdollahzadeh *et al.,* 2014). The potential mechanism of antimicrobial action of plant polyphenols for the inhibition of Gram-positive and Gram-negative bacteria has been proposed (Olatunde and Benjakul, 2018) as given in Fig 3.

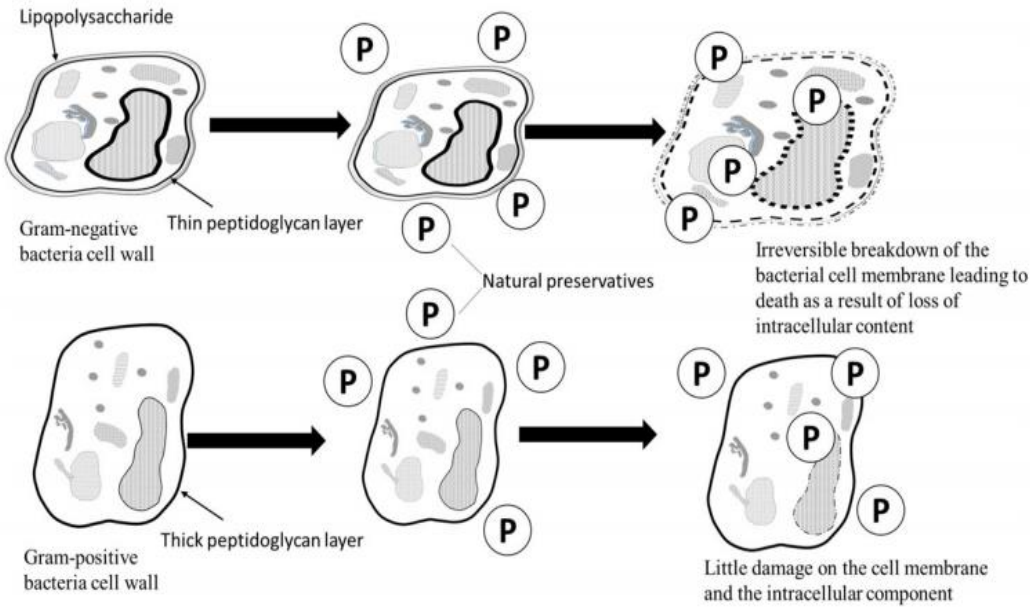

Figure 3. Response of Gram-positive and Gram-negative bacteria to preservatives.
Source: Olatunde and Benjakul (2018)

1.2.3.7 Antimelanosis activity of phenolic compounds

Several phenolic compounds and plant extracts have been shown as natural antimelanosis agents since they act as PPO inhibitors. Some phenolic compounds inhibit PPO activity by interacting with active site of the enzyme (Janovitz-klapp *et al*., 1990). Benzene ring containing carboxylic acids of cinnamic acid and other compounds such as ferulic, p-coumaric, and sinapic acids are known to be the best competitive inhibitors of PPO (Kim *et al*., 2000). Hydrogen bonding or hydrophobic interaction is possible cause of interaction with the PPO to arrest its activity in foods (Prigent, 2005). Chen *et al*. (1991) found that kojic acid showed a mixed-type inhibition for white, lobster polyphenoloxidase grass prawn, and shrimp. Among the various phenolic acids tested, kojic acid showed the highest inhibitory effect on browning in apple slices (Son *et al*., 2001). Cuminaldehyde (p-isopropyl benzaldehyde) was identified as potent mushroom tyrosinase inhibitor (ID50 =7.7 μg/mL) in cumin (Kubo and Kinst-Hori, 1998). Kubo *et al*. (2003) reported that 1.55 mM dodecyl gallate exhibited the inhibitory activity towards mushroom tyrosinase, in which 50 % activity loss was obtained. Prasad *et al*. (2009) identified gallic acid, procyanidin B2, (-)-gallocatechin, (-)-epicatechin, and (-)-epicatechin-3-gallate in the extract of litchi. Seeds. This litchi seed extract showed inhibitory activity of tyrosinase in a

concentration dependent manner (25-100 μg/mL). Jang *et al*. (2003) reported that shrimp (*Trachypenaeus curvirostris*) treated with 70% acetone extract of enokitake mushroom (*Flammulina velutipes*) (2.5 g wet enokitake/mL, test sample) had the delayed darkening at 24 °C for 20 h as compared to control. Gokoglu and Yerlikaya (2008) found that shrimp (*Parapenaeus longirostris*) treated with ethanolic extract of grape seed (*Vitis vinifera sp.*) at 1.5 % had the retarded melanosis for 3 days at 4 °C. They possess inhibitory activity towards mushroom tyrosinase (Likhitwitayawuid, 2008). Different types of phenolic compounds such as ferulic acid, catechin, mimosine green tea and lead seed extracts have been reported in the past to retard the melanosis and overall quality of Pacific white shrimp during low temperature storage (Nirmal and Benjakul, 2009a,b; Nirmal and Benjakul, 2011a,b). The various plant extracts and phenolic compounds employed to prevent melanosis in crustaceans are illustrated in Table 2.

1.2.3.7 Chemical constituents in Chamuang (*Garcinia cowa* Roxb.)

Chamuang (*Garcinia cowa* Roxb.) tree belongs to family *Guttiferae* widely distributed in Thailand. *Garcinia cowa* is an abundant source of bioactive phytochemicals. Among plant parts, the fruit, twig and stem are the best sources of secondary metabolites, providing flavonoids, phloroglucinols and xanthones, respectively. Seventyeight compounds have been identified from the plant and several of them have pharmacological activities (Ritthiwigrom *et al.*, 2013). It has been used since decades in the folk medicine for various purposes. The bark has been used as an antipyretic and antimicrobial agent.

Table 2 Antimelanosis agents for quality preservation in crustaceans

Agents	Concentration	Crustacean	Comprehensive results	References
Catechin	0.05-0.1%	Pacific white shrimp (*L. vannamei*)	Catechin showed almost 90% melanosis inhibition	Nirmal and Benjakul (2009)
Ferulic acid	2-3%	Pacific white shrimp (*L. vannamei*)	Lower melanin formation was observed up to 8 days, when ferulic acid was used in the reported levels	Nirmal and Benjakul (2010)
Green tea (GTE) and mulberry tea extract (MTE)	1.0%	Pacific white shrimp (*L. vannamei*)	GTE showed higher PPO inhibitory activity and antioxidative activity, compared with mulberry tea extract	Nirmal and Benjakul (2011d)
*Green tea extract	1.0%	Pacific white shrimp (*L. vannamei*)	Shrimp treated with GTE prior to MAP had the lowest melanosis scores compared to the untreated control shrimp	Nirmal and Benjakul (2011c)

Table 2 (continued)

Agents	Concentration	Crustacean	Comprehensive results	References
ME	0.00205 ppm	Kuruma shrimp (*P. japonicus*)	Shrimps treated with ME did not present melanosis for 4 days of ice storage	Encarnacion *et al.* (2010)
ME	0.5%	Kuruma shrimp (*P. japonicus*)	The effect of ME was equivalent to that of 500 ppm 4-HR and greater than that of 500 ppm of AA)	Encarnacion *et al.* (2011); Encarnacion *et al.* (2011)
Grape (*Vitis vinifera*) seed extract	15 %	Deepwater pink shrimp (*Parapenaeus longirostris*)	The lowest scores for melanosis were obtained in treatment with grape seed extract of	Gokoglu and Yerlikaya (2008)
Rosemary (*Rosmarinus officinalis*)	0.5%	Pacific white shrimp (*L. vannamei*)	Addition of dehydrated rosemary can improve the quality of peeled shrimp stored at freezing temperature	Seabra *et al.* (2011)

Table 2 (continued)

Agents	Concentration	Crustacean	Comprehensive results	References
Lead (*Leucaena leucocephala*) extract	0.5%	Pacific white shrimp (*L. vannamei*)	Lead seed extract retarded melanosis up to 12 days of low temperature storage	Nirmal and Benjakul (2011a)
Mimosine	3.7 mM	Pacific white shrimp (*L. vannamei*)	PPO inactivated in a dose dependent manner; mixed type reversible inhibition on PPO followed by copper reduction and chelating capacity	Nirmal and Benjakul (2011b)
Pomegranate peel extract	0, 7.5 and 15%	Pacific white shrimp (*L. vannamei*)	PPE at 0, 7.5 and 15 g/L achieved lower scores on melanosis inhibition than that of SMS at 12.5 g/L	Fang *et al.* (2013)

Table 2 (continued)

Agents	Concentration	Crustacean	Comprehensive results	References
Zn^{2+}, Mg^{2+}, EDTA and DETC	20 mM	Crab (*Charybdis japonica*)	Zn^{2+}, Mg^{2+}, EDTA and DETC jointly inactivated phenoloxidase from hemolymph	Liu *et al.* (2006)
Cu^{2+}, Zn^{2+}, EDTA and DETC	20 mM	Crab (*Charybdis japonica*)	Hemocyanin (Hc)-derived phenoloxidase (HdPO) - PO activity of HdPO was also strongly inhibited by Cu^{2+}, Zn^{2+}, EDTA and DETC	Fan *et al.* (2009)
Ergothioneine (ESH)-rich mushroom extract (*Flammulina velutipes*) solution	1.0%	Red queen crab (*Chionoecetes japonicus*)	Significant inhibition of haemolymph by masking gene expression in haemocytes for controlled melanosis in the crabs during ice storage	Encarnacion *et al.* (2011)

Table 2 (continued)

Agents	Concentration	Crustacean	Comprehensive results	References
Cashew leaf extract	0.5%	Pacific white shrimp (*L. vannamei*)	Cashew leaf extract showed higher PPO inhibitory activity and copper chelation activity	Sae-leaw and Benjakul (2019)
**Epigallocatechingallate (EGCG)	0.25 %	Pacific white shrimp (*L. vannamei*)	EGCG solution with the aid of ultrasound had lower melanosis	Sae-leaw *et al.* (2018)

Footnote: GTE=Green tea extract; MAP = Modified atmosphere packaging; 4-HR = 4-Hexylresorcinol; AA=Ascorbic acid; ME= Mushroom extract; PPO = Polyphenol oxidase; SMS = Sodium metabisulfite; PPE = Pomegranate peel extract

*Used in combination with MAP

**Used with the aid of ultrasound

The latex has been used as antifever agent (Na-Pattalung *et al.,* 1994).Some pharmacological properties of the crude extracts of leaves, e.g., antitumor-promoting activity (Murakami *et al.,* 1995) and inflammation induction (Ilham *et al.,* 1995) have been reported. Previous investigations of the latex and stem bark of *Garcinia cowa* Roxb revealed the presence of prenylated xanthones, namely cowaxanthone, cowanin, cowanol, 1,3,6-trihydroxy-7-methoxy-2,5-bis (3-methyl2-butenyl)xanthone, b-mangostin, 7 methyl-garcinone E and norcowanin (Mahabusarakam *et al.,* 2005). Some of these compounds showed antimalarial activity and antimicrobial activity (Likhitwitayawuid *et al.,* 1998). Polyprenylated acylphloroglucinol derivatives unsubstituted at C-2 and C-6, garcicowin A, together with three other new (garcicowins B-D) and nine known analogues, were isolated and characterized from the twigs of *Garcinia cowa* and their structures were elucidated. The compounds isolated from twigs of *Garcinia cowa* were evaluated for their cytotoxicity against two cancer cell lines (HT-29 and HCT116) and against normal colon cells (CCD-18Co), and the results demonstrated their selective toxicity toward the cancer cells (Xu-Gang *et al.,* 2010).

Garcinia cowa leaf extract exhibited antibacterial activity against gastrointestinal pathogenic bacteria (Sakunpak and Panichayupakaranant, 2012). Purification of the ethyl acetate extract of *cowa* leaves using an antimicrobial assay-guided isolation indicated that a new polyprenylated benzophenone, chamuangone, that exhibited satisfactory antibacterial activity against *Streptococcus pyogenes* with minimum inhibitory concentration (MIC) (7.8 μg/ml),-*Streptococcus viridans* and *H. pylori* (MICs 15.6 μg/ml), and *Staphylococcus aureus*, *Bacillus subtilis* and *Entero-coccus* sp. (MICs 31.2 μg/ml). Antibacterial agent of Chamuang leaves against gastrointestinal pathogenic bacteria was identified to be polyisoprenylated benzophenone, chamuangone (Sakunpak and Panichayupakaranant, 2012).

Two new xanthones, 1,5,6-trihydroxy-3-methoxy-4- (3-hydroxy-3-methylbutyl) xanthone and 1,5-dihydroxy-3-methoxy-6 ', 6'-dimethyl-2-Pyrano (2 ', 3': 6, 7)-4-(3-Methylbut-2-enyl) xanthone, were isolated together with previously known xanthones such as 1, 3, 5-Trihydroxy-6 '; 6'-dimethyl-2-pyrano (2', 3 ': 6, 7) xanthone; Dulxanthone A; 1, 5, 6-Trihydroxy-3, 7-dimethoxyxanthone; 1,7-dihydroxyxanthone; 1,3,5-trihydroxy-6-methoxyxanthone and 1,3,6,-7-tetrahydroxyxanthone, from the

stems of *Garcinia cowa* (Shen and Yang, 2006). Acetone extract from the inflorescences of *Garcinia cowa* led was purified and a new benzophenone derivative, cowanone, together with seven known xanthones, α-mangostin, β-mangostin, cowanin, fuscaxanthone A, 9-hydroxy-calabaxanthone, garcinianone A and cowanol were found. Those compounds showed antibacterial activities against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) (Trisuwan and Ritthiwigrom, 2012).

Previous studies have reported the biological activities of the crude extracts/purified compounds from various parts of *Garcinia cowa*. Dried fruit rinds of *Garcinia cowa* were extracted with hexane and chloroform and the extracts were used to evaluate their antioxidant and anti-mutagenic activities (Negi *et al.,* 2010). Using β-carotene-linoleate-model system, at 200 ppm concentration, hexane, chloroform extracts and butylated hydroxyanisole (BHA) showed 91.7, 93.7, and 98.0% antioxidant activity, respectively, whereas, at 50 ppm, DPPH radical scavenging activity was 83.3, 86.3, and 88.5%, respectively.

At concentrations of 5000 μg/plate or 2500 μg/plate, hexane and chloroform extracts exhibited strong anti-mutagenicity against the mutagenicity of sodium azide in both the tester strains of *Salmonella typhimurium* (TA-100 and TA-1535). Thus the extracts from the fruit rinds of *Garcinia cowa* possess antioxidant and anti-mutagenic properties. Higher radical scavenging activity of the crude latex from *Garcinia cowa* extracted with ethanol was due to high content of antioxidants (Mahabusarakam *et al.,* 2005).

1.2.3.8 Impact of plant extracts on sensory acceptance of seafood

Changes in the appearance, taste, and color of seafood treated with natural additives, particularly plant extract are a major drawback for their application/utilization. For instance, chlorophyll, a natural pigment in plant leaves, is co-exacted with polyphenols, regardless of the extracting media. These contributes to the characteristic dark greenish color of the extract, which induces unacceptable color changes in treated seafood. Olatunde *et al.* (2018) reported a change in color and appearance for Asian sea bass slices treated with ethanolic coconut husk extract, particularly at high concentration, which was attributed to the color of the extract.

Nevertheless, the extraction of polyphenols with water has been effective in mitigating the amount of co-extracted chlorophyll. This is associated with

the hydrophobic nature of chlorophyll (Olatunde *et al.,* 2020a). Polyphenols from cashew leaves extracted using water with the aid of ultrasonication had the low chlorophyll content in the extracted. Cashew leaf extract exhibited pale yellow color and had no negative impact on the appearance of Pacific white shrimp (Sea-leaw and Benjakul, 2019). Apart from the contribution to undesirable color of the extract, chlorophyll can act as pro-oxidant (Namal Senanayake, 2013), thus limiting the efficacy of its applications in foods. Ethanolic guava leaf extract obtained by the sedimentation process in aqueous medium had light pale yellow color and had no effect on the appearance or other sensory attributes of Pacific white shrimp (Olatunde *et al.,* 2020). Therefore, minimizing of chlorophyll or other natural pigment in leaf extracts has become an essential process for maximizing the antioxidant properties as well as improving the color of the resulting extract for wider uses without any constraints. Additionally, liposomes has been used to mask undesirable characteristic of plant extracts. Olatunde *et al.* (2020b) used liposome loaded with coconut husk extract and found that it could extend the shelf-life of sea bass slices without any negative impact on sensory property or acceptance.

1.2.4 Non-thermal processing and preservation technologies

1.2.4.1 Pulsed electric field

Non-thermal processes such as pulsed electric field (PEF) is a novel technology aimed to extended shelf-life with excellent nutritional quality, and acceptable sensory attributes (Kumar *et al.,* 2016). PEF is a minimal processing antimicrobial technology to substitute traditional thermal processing of foods to deliver safe and quality sensory food products, without compromising on the nutritional quality PEF has been referred as eco-friendly technology that does not create any hazard during food processing (Mohamed and Eissa, 2012; Kumar *et al.,* 2016).

1.2.4.2 Principle

PEF treatment involves the exposure of short duration of pulses (µs to ms) of moderate electric voltage (10 to 60 kV/cm) to food placed between a pair of parallel electrodes (Kumar *et al.,* 2016). High electric field voltage (5-50 kV/cm) has been applied for the preservation, enzyme and microbial inactivation (Mohamed and Eissa, 2012). The exposure of biological cell (plant, animal and microbial) to the high-

voltage pulses induces pores that could be temporary or permanent on the cell membrane. The phenomenon named electroporation causes the permeabilization of cell membrane (Gudmundsson and Hafsteinsson, 2001). The increase in permeability in cell membranes initiates a loss of barrier function, intracellular content leakage, and loss of vitality. Electroporation becomes well pronounced which confirmed electric field thresholds of 480 V/cm, 1,050 V/cm and 240 V/cm, 430 V/cm for reversible (E_{rev}) and irreversible (E_{irrev}) electroporation of the skin and muscle cell model system, respectively (Čorović *et al.,* 2012). Excessive field strength and level of energy resulted in an irreversible electroporation (Al-Sakere *et al*., 2007). The treatment is applied in a chamber with a top and bottom electrode containing product is exposed to the high-voltage pulses in liquid at high voltage and high energy pulses with a duration of less than a second (Luengo *et al.,* 2015). Intensity of the PEF favors electroporation depends on the range of voltage and specific energy input and field strength, but also by product composition and temperature (Puertolas *et al.,* 2013). PEF can be applied at slightly above, below, or at ambient temperature in the form of bipolar, an exponentially decaying, oscillatory pulse, and square wave (Butz and Tauscher, 2002).

1.2.4.2 Antimicrobial efficacy and application

Pulse electric field used for fractions of seconds could breakdown and induce structural changes of the cell membrane, so called electroporation as illustrated in Fig 4. High intensity pulses results in irreversible breakdown of the cell membrane, which leads to microorganism inactivation or death, can be possible with the utilization of PEF, particularly at higher treatment intensity (Toepfla *et al.,* 2007: Faridnia *et al.,* 2015). PEF technology has been successfully applied for the pasteurization of foods such as fish soups, tomato juice, and liquid eggs (Dunn, 2001). PEF processing offers good-quality, fresh, or raw-like liquid foods with excellent shelf-life, nutritional value, and flavor. Because PEF application on food involves no heat, the treated foods with PEF maintain their appearance, taste, and fresh aroma with that of the fresh products and no loss in nutritional content (Nagarajarao, 2016).

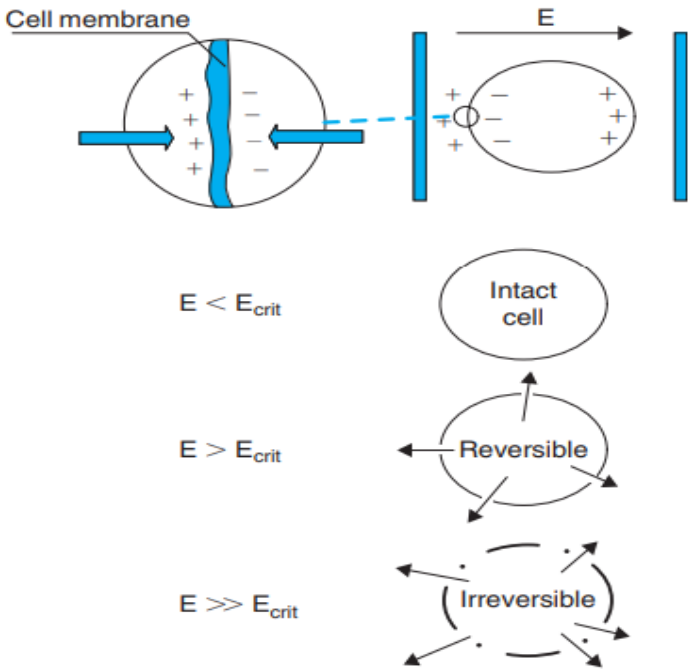

Figure 4. Schematic representation of PEF electroporation.
Source: Toepfl *et al.* (2006).

The growth and physiological states influence PEF efficacy for the target killing of microorganisms includes shape, size and type of microorganism and their sensitivity towards PEF treatment. In general, Gram-negative vegetative cells are less resistant to PEF due to thinner cell wall and single layer pf peptidoglycan in comparison with Gram-positive bacteria (Saldana *et al.,* 2010). PEF sensitivity is higher in yeasts than bacteria (Mohamed and Eissa, 2012). The induction of electric fields into cell membranes of large cells is greater than small cells when exposed to PEF treatment (Zhang *et al.,* 1994). PEF has been employed to treat the foods, especially raw materials for quality improvement and shelf-life extension. Gudmundsson and Hafsteinsson (2001) reported that an electric field as high as 18.6 kV/cm for 7 pulses at room temperature did not affect the primary structure of the treated salmon fish protein as compared with the control. Moreover, the research studies pay attention to bacteria cell inactivation as affected by PEF, although only a few reports are accessible on the inactivation of spores, describing a limited effect of PEF in foods. Pagan *et al.* (1998) found that *Bacillus cereus* spores were not affected with PEF treatment of 60 kV/cm for 75 pulses at room temperature. However, Marquez *et al.* (1997) reported 5 log and

3.42 log reductions of *Bacillus cereus* and *Bacillus subtilis* spores, respectively, with PEF treatment of 50 kV/cm for 50 pulses at 25 °C in salt solution. PEF treatment (2.0 kV/cm and 90 pulses/min) prevented water loss in fish samples (frozen cod loins, Iceland cyprine, cod fresh fillets, common whelk, frozen haddock loins, and pollock fillets), compared to the samples only injected with brine (Klonowski *et al.,* 2006).

1.2.5 Modified atmosphere packaging (MAP)

MAP of food has gained considerable popularity as a potential method for packaging (Fernández *et al.*, 2009; Soldatou *et al.*, 2009). MAP has long become an increasingly popular preservation technique. Shelf-life extension of whole Norway lobster was achieved when packed in the optimal modified atmosphere (Gornik *et al.*, 2013). The shelf-life of numerous fishery products can be extended by MAP via lowering the growth of various microorganisms (Nirmal and Benjakul, 2011b). Its effect on microbial growth is attributed to the presence of carbon dioxide (CO_2), which extends the lag phase and reduces bacterial growth rate during the logarithmic phase of facultative and anaerobic microorganisms. Additionally, O_2-free MAP effectively delayed enzymatic spoilage, i.e. melanosis in crustaceans (Bono *et al.*, 2012). MAP was used to prevent melanosis in Pacific white shrimp, *L. vannamei* (Nirmal and Benjakul, 2011). MAP has been employed in combination with plant polyphenols or extracts to inhibit melanosis in crustaceans during prolonged storage. Shrimp treated with GTE prior to storage under MAP had the lower melanosis score than those stored under MAP (without GTE treatment) or the control, irrespective of ascorbic acid incorporated. The control or those stored under MAP had shelf-life of 6 days, while that treated with GTE or GTE combined with ascorbic acid prior to storage under MAP was acceptable up to 10 days. Nirmal and Benjakul (2011b) studied the impact of green tea extract (GTE) without and with ascorbic acid in combination with MAP (50% CO_2; 5% O_2; 45% N_2) on melanosis prevention of Pacific white shrimp during refrigerated storage of 10 days.

Combined effects of freezing and MAP with gas mixtures (100% N_2 or 50% N_2–50% CO_2) of deep-water rose shrimp (*Parapenaeus longirostris*) and giant red shrimp (*Aristaeomorpha foliacea*) were reported during 12-months of frozen storage (Bono *et al.*, 2012). TBA in giant red shrimp and deep-water rose shrimp ranged between 0.15 to 0.30 mg MDA/kg and 0.15 to 0.23 mg MDA/kg, respectively over 12

months of frozen storage packaged in 100% N_2 modified gaseous composition with decreased melanosis (Bono *et al.*, 2012). Pacific white shrimp pretreated with cold ozonated water (1 ppm, 10 min, 15 °C) and chlorinated water (5 ppm, 10 min, 15 °C) packaged in MAP (100% CO_2) under refrigerated storage was studied for 12 days (Gonçalves *et al.*, 2019). It was further assessed that mesophilic and psychrophilic counts in ozone treated sample and stored under MAP (100% CO_2) were reported to be 2.55×10^3 and 4.85×10^4 log CFU g^{-1}, respectively. According to Wang *et al.* (2016) regardless of packaging conditions, mesophilic and psychrotrophic bacterial counts were notably inhibited ($p<0.05$) by increased CO_2 levels after 2 days of storage and the highest inhibitory effects were observed in Pacific white shrimp packaged with 100 mL CO_2 per 100 mL atmosphere, which may be attributed to the inhibitory effect created by the presence of CO_2 on microbial growth. MAP with gaseous composition of 60% CO_2, 10% O_2 and 30% N_2 was used for the retention of physicochemical attributes and shelf-life extension of crab during storage at -3 ± 1 °C (Sun *et al.*, 2017). It was revealed that superchilling with MAP (10% O_2/60% CO_2/30% N_2) prevented microbiological growth, reduces lipid and protein oxidation, and maintains tight morphology of crab. Furthermore the carbonyl content of the proteins increased from 1.21 nmol/mg of protein (0 day) to 2.03, 1.87, 1.66 nmol carbonyl/mg protein on the 6th day for control, superchilling treatment and combined treatment of MAP and superchilling, respectively (Sun *et al.*, 2017).

Use of noble gases such as argon (Ar), helium (He), neon (Ne), xenon (Xe), and radon (Ra) has been into the preservation of foods (Donglin *et al.*, 2001). Argon is reported to be biochemically active, probably due to its enhanced solubility in water compared with nitrogen and possible interference with enzymatic oxygen receptor sites (Spencer, 1995). Tyrosinase activity with argon treatment was reduced by up to 14.2% more than nitrogen treatment when treated directly, and by up to 22.6% in the mixture of the enzyme and substrate Therefore, argon could block some chemically-active sites on the enzyme more effectively than nitrogen. Furthermore, argon displaces oxygen more effectively than nitrogen. This is possibly based on its similar atomic size to molecular oxygen and its improved water solubility (0.034 vs. 0.016 $g \cdot L^{-1}$) and higher density (1.650 vs. 1.153 kg/m3) compared with nitrogen (Spencer, 1995, 2005). Carbon dioxide (CO_2) gas has been used to extend the shelf life

of shrimp with minimum quality changes observed during cold storage. samples packaged with higher CO_2 levels (i.e. 100% CO_2, 67% CO_2 and 50% CO_2) had no statistically significant changes ($P > 0.05$) in pH values after 4 and 6 days of storage, which may be attributed to the dissolution of CO_2 in the shrimp samples, acidifying it via the formation of carbonic acid (Banks *et al.*, 1980). Regardless of packaging conditions, mesophilic and psychrotrophic bacterial counts were notably inhibited by increased CO_2 levels after 2 days of storage and the highest inhibitory effects on them were observed in Pacific white shrimp packaged with initial gas mixture of 100 mL CO_2 per 100 mL atmosphere, which may be attributed to the solubilization of CO_2 in water present in samples tends to create weakly acidic conditions the inhibitory effect created by the presence of CO_2, such that the bacteriostatic effect was exerted on aerobic flora growth (Laursen *et al.*, 2006). MAP with noble gases or their mixtures can be a potential means to tackle the crustacean melanosis and quality loss especially in shrimp stored under low temperature (Okapa *et al.*, 2016).

1.2.6 Vacuum impregnation

1.2.6.1 Principle

The hydrodynamic mechanism of vacuum impregnation (VI) involves the exchange of internal gas and/or occluded liquid in the open pores with an external liquid phase promoted by pressure changes. This mechanism enables the incorporation of liquid into the pores during VI. The porosity, mechanical properties, size, and shape of the food matrix used affects the incorporation of the liquid during VI in porous foods (Ostos *et al.*, 2012). Vacuum impregnation is a tool used in the development of vegetable or fruit products that enables a modification of the original composition of the food without destroying the cellular structure (Fito *et al.*, 2001; Guillemin et al., 2008; Hironaka *et al.*, 2014).

1.2.6.2 Application of vacuum impregnation in foods

VI is a process of penetrating bioactive compounds containing impregnating solutions, thus improving the physico-chemical properties and sensory attributes of products (Mei *et al.*, 2019). Various compounds can be impregnated via VI, such as cryoprotectants, antioxidants, bio-preservatives, enzymes, and probiotics (Martínez-Monzo *et al.*, 2000). In the fishery industry, this technology has been used

to develop fish and seafood with high structural and sensorial properties, as well as prolonged shelf life (Zhao *et al.*, 2019a). Fish gelatin along with grape seed extract (GSE) with the aid of vacuum impregnation retained freshness of chilled tilapia fillets including protein oxidation (Ca^{2+}-ATPase activity, disulphide bonds, sulfhydryl groups and carbonyl groups) and protein degradation of during 12 days storage (Zhao *et al.*, 2019b).

Industrial utilization of VI has been practiced in the food industries. This technique can incorporate the immersion solutions into the food through the subsequent pore spaces and aid in the improved keeping quality of foods (Fito *et al.*, 2001). It is a helpful method to develop food in term of firmness, high nutritional value and prolonging shelf-life (Guilleminn *et al.*, 2008). VI technology was applied along with the antimicrobial casing incorporated with chitosan could help in the foodborne pathogens inhibition (Kaowkum *et al.*, 2012). Additionally, nisin incorporated casing based on collagen was penetrated in ready-to-eat sausage with the aid of VI process to inhibit foodborne pathogens and food spoilage bacteria during refrigeration storage (4 °C) (Batpho *et al.*, 2017).

1.2.7. Cold plasma

1.2.7.1 Principle

Cold atmospheric plasma (CAP) is a novel non-thermal processing technology with great antimicrobial potential. Electricity and carrier gases such as oxygen, nitrogen, air, and argon are the key elements for this technology. The term “plasma” refers to a partially or wholly ionized gas. Although the gas contains high temperature electrons, the neutrals, ions, and radicals remain close to room temperature and as such they are considered cold plasmas with limited macro heating of material, to which they interface with (Misra *et al.*, 2015; Mishra *et al.*, 2016; Min *et al.*, 2017). Nonthermal or cold plasmas are considered to be in a state of nonthermal equilibrium (Schluter *et al.*, 2013; Surowsky *et al.*, 2015). Because of the charged particles, free radicals, photons, chemical reactive species, and ultraviolet radiations generated, CAP can be used for sanitizing foods and containers (Muranyi *et al.*, 2007).

CAP may be generated by a diversity of electrical discharges such as dielectric barrier discharge (DBD), DC glow discharge, radio frequency (RF)

discharge, atmospheric pressure plasma jet (APPJ), microwave and pulsed power discharge. Industrially, CAP has not been implemented in food industry for direct food contact but is employed for packaging and label modification. Plasma jet is typically operated with noble gases, which increases the treatment cost (Misra *et al.,* 2011).

A dielectric barrier discharge (DBD) between the two cylindrical parallel electrodes is referred to as a barrier discharge or a silent discharge, where at least one of the electrodes is covered with a dielectric material (Kogelschatz *et al.,* 2002). For most operating conditions, a DBD consists of a large number of discharge filaments, which have nanosecond duration and are randomly distributed over the dielectric surface. These filaments, also known as micro-discharges, are the active regions of a DBD, in which active chemical species and UV radiation can be produced (Pal *et al.,* 2008). DBD that plays a very important role in gas ionization is mainly composed of insulated glass, quartz, ceramics, and also thin enamel or polymer coating on the electrodes, acts as a current limiter and prevents the formation of a spark or an arc discharge with the electrical energy coupled into a DBD-plasma mainly transferred to energetic electrons, while the neutral gas remains closest to ambient temperatures (Pal *et al.,* 2008). DBD are generated when high voltage is applied across the electrodes. Ideally, ambient air can be used in the cold plasma technology. Application of DBD was achieved to generate atmospheric cold plasma inside sealed packages filled with air through the application of sufficiently high voltages (Misra *et al.,* 2014a; Misra *et al.,* 2014b; Misra *et al.,* 2013; Pankaj *et al.,* 2013). Another advantage, in comparison with other cold plasma's generators, is that the treatment takes place inside sealed packages, which eliminates the risk of post-process contamination and facilitates rapid treatment times as the resultant reactive species are contained within the package and continue to act after treatment (Misra *et al.,* 2014a; Misra *et al.,* 2014b). These discharges generate energetic electrons that dissociate oxygen molecules by direct impact. This single O atom combines with oxygen molecules (O_2) to form ozone gas (Misra *et al.,* 2014a; Misra *et al.,* 2014b).Mixtures of ions, electrons, and free radical species in gaseous plasmas are responsible for microorganism destruction (Chirokov *et al.,* 2005; Perni *et al.,* 2008). The ionization of gas because of the contact among the ions or electrons generated inside the sealed bag and emits fluorescent light during cold

plasma processing. The respective gas leads to the creation of new active species when passed through plasma (Critzer *et al.*, 2007) as illustrated in Fig. 5.

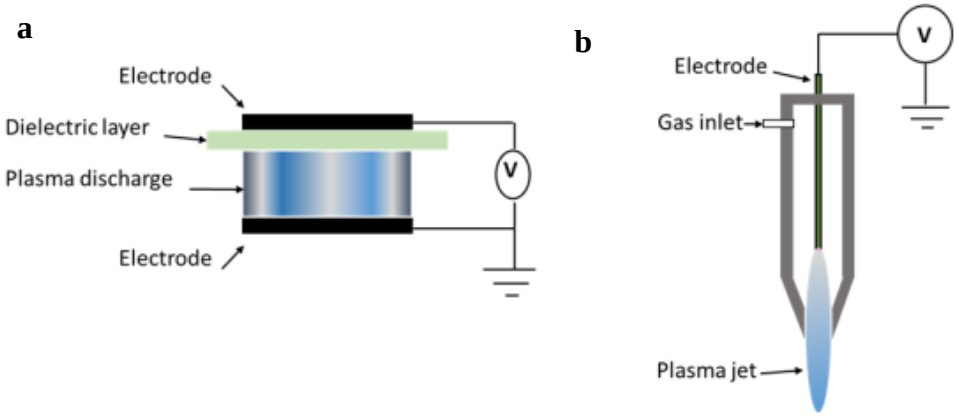

Figure 5. Schematic diagram of (a) dielectric barrier discharge; (b) plasma jet system. **Source:** Pankaj *et al.* (2018)

Numerous types of plasma sources have been introduced such as CAP jet devices application in foods (Critzer *et al.*, 2007; Deng, Cheng *et al.*, 2008). Besides the working gases, the type and source of plasma are also considered for particular food application. In general, the frequency range (kHz) generates atmospheric pressure plasma by dielectric barrier discharge (DBD) or CD. The microwave region is generated by plasma torches, although radiofrequency (RF) could be generated by an atmospheric pressure plasma jet or by inductive coupled plasma (ICP) (Frohling *et al.*, 2012).

DBD has been successfully applied on several foods to retain quality, especially in dry products such as wheat flour, legumes (peas) and dried laver (Misra *et al.*, 2015; Bubler *et al.*, 2015; Kim *et al.*, 2015). Fresh fruits and vegetables such as tomatoes, spinach, strawberries, grapes and orange juice and fresh-cut melon (Pankaj *et al.*, 2013; Misra, Keener *et al.*, 2014; Misra *et al.*, 2014a; Klockow and Keener, 2009; Misra *et al.*, 2014b; Moon *et al.*, 2016; Tappi *et al.*, 2015; Almeida *et al.*, 2015) were also subjected to DBD. The activity of microorganism is the main factor limiting the shelf-life of fresh fish (Olafsdottir *et al.*, 1997). Different plasma sources for decontamination of foodstuff such as meat, etc. have been reported (Kim *et al.*, 2013; Kim *et al.*, 2011; Noriega *et al.*, 2011; Rod *et al.*, 2012). However, these products are susceptible to detrimental effects of oxidation due to the formation of hydroxyl acids,

keto acids, short-chain acids and aldehydes (Misra *et al.,* 2011). Kim et al. (2011) did not find any significant changes due to plasma jet treatment (pH, TBARS, microscopic observation) except for color, in which L* values of the meat surface were increased. Rod *et al.* (2012) demonstrated that TBARS values of plasma treated samples increased with power and storage time but the plasma did not induce measurable color differences. However, Kim *et al.* (2013) found lower TBARS values in plasma treated bacon than the control upon treatment but increased subsequently as a function of storage time. Also, significant reductions in the sensory quality parameters were observed in plasma treated samples. Jayasena *et al.* (2015) observed minor deterioration of fresh pork and beef quality, but high exposure time (10 min) caused lipid oxidation. Furthermore L* value was not affected by DBD, whereas a* values were lowered significantly after 5 and 7.5 min of DBD exposure. Wang, Zhuang, and Zhang (2016) demonstrated that DBD was effective in inhibiting spoilage bacteria for inoculated chicken carcasses. The effect of DBD generated plasma on microbial reduction and quality parameters of fresh mackerel (*Scomber scombrus*) fillets were studied using different combinations of voltages (70 kV and 80 kV) and treatment time (1, 3 and 5 min) (Albertos *et al.,* 2017). Recently, high voltage cold atmospheric plasma (HVCAP) was implemented on sea bass slices, when treated with HVCAP using argon and oxygen (90:10) as gas composition for various times (2.5-10 min). Total viable count was reduced by 1.0 log CFU/g in slices treated with HVCAP for 5–10 min. Thus, HVCAP treatment for 5 min could extend shelf-life of refrigerated slices (Olatunde and Benjakul, 2019).

1.2.7.3 Antimicrobial efficiency and application

CAP is a chemical-free, contactless, and waterless method that can prevent and reduce microbial growth (Niemira, 2012). Depending on the plasma sources, the gases used, and processing parameters, the concentrations and the reactive species vary. Between plasma devices or the same device used, inactivation kinetics differs (Frohling *et al.,* 2012). It has been applied successfully to different food products due to its antimicrobial effectiveness (Fernandez-Gutierrez *et al.,* 2010; Grzegorzewski *et al.,* 2010). The strong oxidizing agents, such as ozone and atomic oxygen, generated in plasma (Kelly-Wintenberg *et al.,* 1999), as well as UV radiations, photons, chemical reactive species, and charged particles (Frohling *et al.,* 2012) affect the integrity of

microbial cellular membranes and spawn the antimicrobial effect (Brandenburg *et al.,* 2007; Perni *et al.,* 2008). Because of their ability to induce oxidation of microbial components and microbial cell membrane destruction, atomic oxygen species are the most effective (Han *et al.,* 2016). Oxidation of nucleic acids and amino acids of microorganisms can lead to death or damage of their cells (Smeu and Nicolau, 2014). Oxygen-reactive species affect lipid membranes, and this can be due to their ability to attach to the bacterial cell surface, which makes them more prone to attack by such strong oxidizing agents (Critzer *et al.,* 2007). Agricultural products (mango, lettuce, almond, apple, and melon), foods (cheese and cooked meat), and egg surface have been decontaminated by CAP in the food industry (Deng, Shi, Chen, and Kong, 2007). Lee *et al.* (2011) showed that CAP treatment for 2 min with helium gas (5 L/min) mixed with oxygen (100 mL/min) at 60 Hz and 30 kV/cm retarded the growth of *L. monocytogenes* on smoked salmon by 1 log CFU/g. Chen *et al.* (2011) reported that the population of *Photobacterium phosphoreum*, a bacterium associated with seafood spoilage in cold smoked salmon, was significantly reduced (< 3 log CFU/g) by CP (air and air + 7% CO_2 mixture) treatments operated at an applied voltage: 13 kV at 15 kHz frequency for in 60 to 120 s. This inactivation effect was comparable between different gas compositions used (air and air + 7% CO_2). However, CAP treatments did not significantly inactivate *L. monocytogenes* or *Lactobacillus sakei* in cold smoked salmon, irrespective of the gas used. Park and Ha (2015) reported a significant decrease in the counts of *Cladosporium cladosporioides* and *Penicillium citrinum* on dried filefish fillets with increasing cold oxygen plasma (air subjected to high-energy deep-ultraviolet (UV) light with an effective radiation spectrum between 180 and 270 nm) with treatment times of 3 to 20 min.

1.2.8 Changes in quality of seafood during storage

Any chemical or microbial, enzymatic and activities changes in the seafood in the initial quality of seafood that results in an unpalatable odor, taste, appearance, and texture during storage is referred to as spoilage (Ghaly *et al.,* 2010). Adebowale *et al.* (2008) reported that rigor mortis, which is a biochemical change in fish muscle that occurs immediately after death, led to the loss of muscle flexibility. Chemical, microbial, and enzymatic spoilage of seafoods including crustaceans could

be retarded by the combined huddle of non-thermal technologies, addition of natural or chemical preservatives and packaging for retaining sensory quality during low temperature storage (Olatunde and Benjakul, 2018).

Storage of fish and shellfish under refrigerated, frozen or molten ice storage conditions (chilling or super chilling) has been the routine practice of postharvest preservation from the capture site to whole sale seafood markets (Lakshmanan *et al.*, 2002; Nirmal and Benjakul, 2009). However, freezing technology has been widely used to store crustaceans for export (Lopez-caballero *et al*., 2007). Although freezing is an effective method of preserving foods, some deteriorations in frozen food quality occur during storage. Quality loss of foods many include several factors, including storage temperature, rate of freezing and thawing, freeze thaw abuse during storage and temperature fluctuations (Srinivasan *et al.*, 1997). The impact of iced and frozen storage on shrimp quality was reported to be temperature dependent (Erickson *et al*., 2007).

1.2.8.1 Lipid oxidation

Lipid oxidation produces rancid odors and off-flavors, decreases shelf life and can alter texture and appearance of muscle foods (Maqsood, 2010). Lipid oxidation is one of the main factors limiting the quality and acceptability of muscle foods (Morrissey *et al.*, 1998). This process leads to discoloration, drip losses, off-odor and off-flavor development, texture defects and the production of potentially toxic compounds (Morrissey *et al.*, 1998; Richards *et al.*, 2002). Lipid oxidation is a rather a complex process, in which unsaturated fatty acids react with molecular oxygen via a free radical chain mechanism, forming fatty acyl hydroperoxides, generally called peroxides or primary oxidation products (Gray, 1978). The primary auto-oxidation is followed by a series of secondary reactions, which lead to the degradation of the lipid and the development of oxidative rancidity. Lipids are also highly susceptible to oxidation in the presence of catalytic systems such as light, heat, enzymes, metals, metalloproteins and microorganisms (Shahidi and Zhong, 2010). The catalytic degradation of lipids leads to complex processes of autoxidation, photo-oxidation, thermal or enzymatic oxidation. Most of the reaction intermediates constitute of the free radicals and/or other reactive species (Halliwell, 2015)

Autoxidation is the most common process among all and is defined as the spontaneous reaction of lipids with atmospheric oxygen through a chain reaction of free radicals. The process can be accelerated at higher temperatures and light. Photooxidation involves excitation of a photosensitizer and energy transfer to lipid molecules or oxygen (Baptista *et al.,* 2017). Oxidation can also be catalyzed by certain enzymes such as lipoxygenases such as in gills of marine and fresh-water fishes (German *et al.,* 1992). Unsaturated fatty acids are the major reactants affected by such reactions, whether they are present as free fatty acids, simple alkyl esters, acylglycerols or phospholipids. The PUFA in membrane phospholipids and cholesterol, especially low density lipoprotein (LDL)-cholesterol, are the major target substrates of oxidation *in vivo*, causing irreversible cellular and tissue damage (Lu and Gursky, 2013).

The initiation of lipid oxidation requires the presence of initiators or catalysts (Schaich, 2013). The initiation process is quite complex, however, it is believed to involve loss of a hydrogen atom from the lipid molecule. The abstraction of hydrogen atom takes place most readily at the carbon next to the double bond fatty acids, due to lower C-H bond energy. The dissociation energy of hydrogen in fatty acid played a vital role for the onset of initiation reaction of lipid oxidation (Shahidi and Zhong, 2010). During propagation, lipid hydroperoxides are produced as primary products of oxidation. They are unstable and break down to a wide range of secondary oxidation products, including aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds leading to the development of off-odours. Therefore, rancidity is generally related to the secondary oxidation products. Meanwhile, alkoxyl (RO), peroxyl (ROO), hydroxyl (OH) and new lipid radicals (R) are generated from decomposition of hydroperoxides, and further participate in the chain reaction of free radicals. The cleavage of O–O bond in hydroperoxide forms alkoxyl and hydroxyl radicals is more likely favored due to requirement of less bond dissociation energy (44 kcal/mol) than that of the O–H bond which requires a higher activation energy (90 kcal/mol) (Hiatt *et al.,* 1968). The lipid changes in mud shrimp (*Solenocera melantho*) were affected by both freezing duration and temperatures. Mud shrimp stored at −20°C exhibited more lipid oxidative damage due to higher FFA (3.28 to 6.27 g/100 g), PV (3.02 to 9.35 meq/kg), TBARS (1.5-2 mgMDA/kg sample), and higher FCs compared to those stored at −30 and −40 °C during 12 weeks of frozen

storage (Zonglin *et al.,* 2018). Lower lipid oxidation was recorded for PWS pre-treated with guava leaf extract prepared by sedimentation process in aqueous medium, especially at 1%. The main aim of sedimentation process, a green method, showed promising efficacy for producing extract of guava leaves free from green color with improved antioxidant properties for effectively reducing lipid oxidation in PWS during refrigerated storage of 12 days (Olatunde *et al.,* 2020b).

1.2.8.2 Protein oxidation

Protein oxidation occurs as covalent modification of proteins either by reactive oxygen species (ROS) or via the interaction with secondary lipid oxidation products (Shacter, 2000). Protein oxidation begins with the abstraction of hydrogen atoms from a protein molecule through ROS to form a protein carbon-centered radical. This protein radical, in the presence of oxygen, is converted into an alkylperoxyl radical and ultimately to an alkylperoxide by obtaining a hydrogen atom from another susceptible molecule (Fig. 6). Oxidation of proteins is believed to proceed via a free radical chain reaction similar to that of lipid oxidation, although more complex pathways and a wider range of oxidation products may be involved in lipid oxidation (Davies and Dean, 2003). Subsequently, alkoxyl radical and its hydroxyl derivative are produced when they react with ROS or reduced forms of transition metals. Finally, carbonyl groups and acyl radicals are formed when these alkoxyl residues undergo *b*-scission or fragmentation reaction via cleavage of peptide bonds (Lund *et al.,* 2011).

Moreover, protein oxidation also occurs when lipid oxidation products (such as hydroperoxides, aldehydes, reducing sugars and ketone) interact with nitrogen or sulfur centers of reactive amino acid residues of protein (Viljanen, 2005) (Fig. 7). Protein fragmentation can occur as a result of a free radical attack of glutamyl, aspartyl and prolyl side chains (Hawkins and Davies, 2001). Oxidative modifications of proteins and amino acids can lead to the formation of cross-linked protein derivatives, alteration of side chains amino acids and oxidative cleavage of peptide chains. As a result, protein functionalities (e.g. solubility, water holding capacity, viscosity, and emulsification) and muscle quality can be changed (Xiong, 2000).

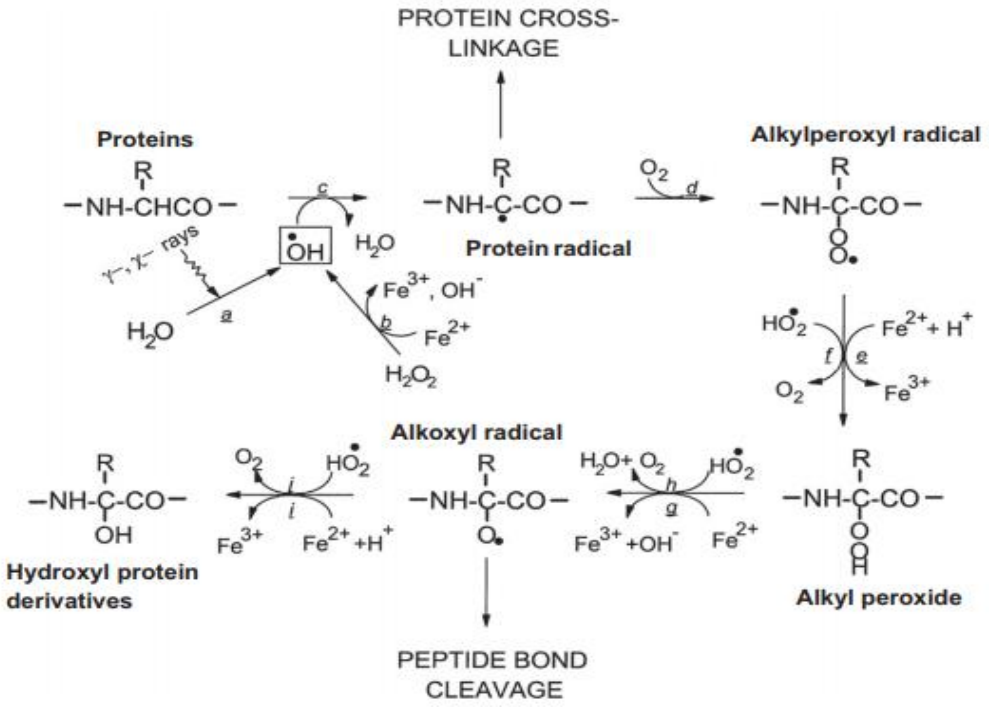

Figure 6. Radical mediated protein oxidation.

Source: Berlett and Stadtman (1997)

The consequences of protein oxidation in muscle foods have often been associated with changes in functional properties of proteins or may cause alterations in water holding capacity and increased drip loss (Xiong, 2000). The consequences of protein oxidation in muscle foods primarily depends on processing and storage conditions (Eymard *et al.*, 2009). Furthermore, oxidative modifications of proteins can lead to degradation and loss of essential amino acids with decreased digestibility, thereby affecting the nutritional quality of muscle foods (Morzel *et al.*, 2006; Sante-Lhoutellier *et al.*, 2008). Oxidation of numerous amino acids leads to the formation of carbonyls groups and other derivatives (Stadtman and Berlett, 1997).

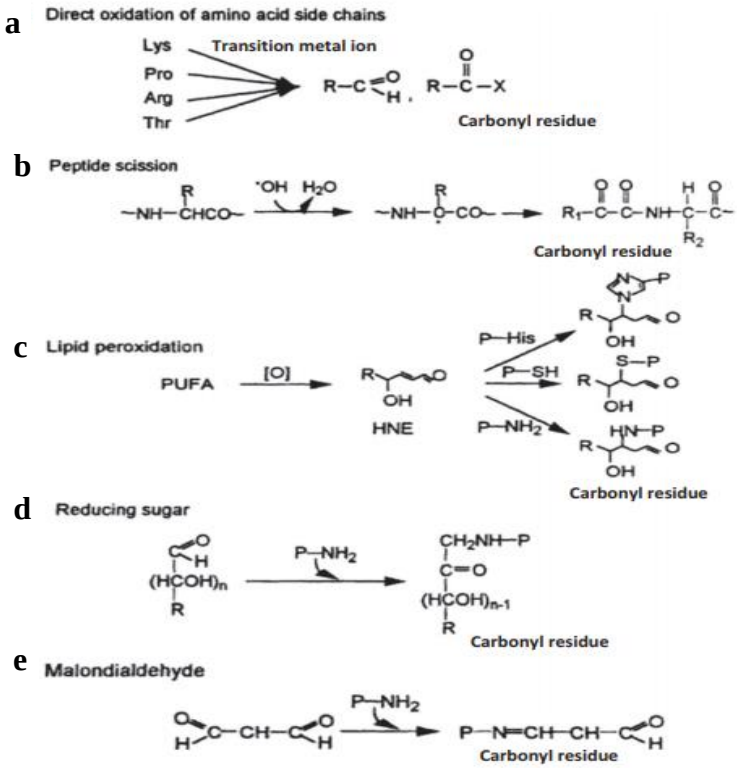

Figure 7. Mechanisms of protein carbonyl formation. Metal catalyzed oxidation of basic amino acid side chains (a), peptide backbone cleavage or peptide scission (b), binding to lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) (c), reaction with reducing sugars (d) and binding to lipid peroxidation products such as malonaldehyde (e).
Source: Xiong (2000)

Protein oxidation in muscle foods impact on flavor due to formation of released carbonyl groups and Schiff bases (Mackie, 1993; Srinivasan and Hultin, 1994). The proposed mechanism was hypothesized by which protein carbonyls might impact flavor and odor, leading to the development of unpleasant odors in meats (Fuentes *et al.*, 2010). Oxidative changes of Protein in mud shrimp (*Solenocera melantho*) are mainly influenced by the freezing duration. Protein denaturation occurred gradually with the prolongation of frozen storage time and protein oxidation mainly occurred after 12 weeks (Zonglin *et al.*, 2018). The sulfhydryl (SH) content of mud shrimp decreased by 18.71%, 15.04%, and 10.11% after 12 weeks of storage at −20, −30, and −40°C, respectively. The increased disulfide bond formation is generally due to the oxidation of SH groups and conformational changes. After 12 weeks of storage at −20, −30, and −40°C, the disulfide bond content of shrimp increased by 46.85%, 51.35%, and

53.15%, respectively (Zonglin *et al.,* 2018). Benjakul *et al.* (2003) reported that the changes of the disulfide bond in tropical fish during frozen storage varied depending on the species, such as in disulfide bond in croaker, lizardfish, threadfin bream, and bigeye snapper increased by 129.5%, 325.4%, 32.9%, and 41.7%, respectively, after 24 weeks of storage. After 16 weeks of storage, protein carbonyls in shrimp stored at −20, −30, and −40°C showed the highest value of 2.97, 2.43, and 2.27 nmol/mg protein (initial value 1.90 nmol/mg protein), respectively (Zonglin *et al.,* 2018).

1.2.8.3 Microbial spoilage

Seafoods are always at high risk to be contaminated by the spoilage and pathogenic microorganisms. Habitat, which is a microbe-rich environment, mostly determines the microbial load of seafood (Ghaly *et al.,* 2010; Kuley *et al.,* 2017). Seafood spoilage is mainly triggered by the metabolic activities of microorganisms producing numerous by products such as organic acids, aldehydes, biogenic amines, alcohols, histamine, putrescine, sulfides and ketones (Kuley *et al.,* 2017). Sivertsvik *et al.* (2002) reported that psychrophilic bacteria identified as aerobic or facultative anaerobic Gram-negative bacteria, such as *Moraxella*, *Shewanella putrefaciens*, *Acinetobacter*, *Pseudomonas*, *Photobacterium*, *Aeromonas*, *Flavobacterium*, and *Vibrio*, are responsible for spoilage in chilled or refrigerated seafood. Specific type of spoilage microbes including *Photobacterium phosphoreum*, *Shewanella* and *Pseudomonas* are referred to as the major micro flora in seafood spoilage (Gram and Dalgaard, 2002). Microorganisms utilize small peptides, carbohydrates and free amino acids from the tissue sources for growth and production of various metabolites such as biogenic amines, histamine and sulfur-containing compounds (Varlet and Fernandez, 2010; Masniyom, 2011).

Lactic acid bacteria (LAB) are Gram-positive bacteria, which have been reported as the major contributors of spoilage in seafood. Dalgaard (2000) reported both Gram-negative bacteria (*P. phosphoreum*) and lactic acid bacteria (LAB) as the major spoilage bacteria in fish. Optimal growth of LAB occurs at the range of pH 5.5–5.8. LAB have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (Mokoena *et al.,* 2017). Based on the production of by-products from various components, they can be classified into

two main types: homofermentative and heterofermentative microorganisms. Homofermentative LAB ferment carbohydrates and produce lactic acid from sugars, whereas heterofermentative LAB produce lactic acid, acetic acid or alcohol (Parada *et al.*, 2007). In addition, some species of LAB produce antimicrobial peptides known as bacteriocins. Several LAB isolates from the *Lactobacillus* genus and their bacteriocins have been applied in food preservation and in the control of pathogens (Ghay *et al.*, 2010). Olatunde *et al.* (2019) reported that the proliferation of LAB in seafood, particularly those packaged under modified atmosphere pacakging, is mediated by the low oxygen or anaerobic environment in the sealed bags.

Additionally, continuous handling and processing or prolonged storage provides favorable chances to other Gram-positive bacteria to cause spoilage under anaerobic conditions (Al-Bulushi *et al.*, 2010). Gram-positive bacteria, such as *Micrococcus, Corynebacterium, Bacillus, Staphylo-coccus, Clostridium, Strepto-coccus* and *Brochothrix thermo-sphacta* were also identified as spoilage microorganisms in seafood (Fall *et al.*, 2010; Al-Bulushi *et al.*, 2010; Lalitha *et al.*, 2005). Moreover, it was presumed that Gram-negative and Gram-positive bacteria are responsible for the spoilage of seafood. However, the type and number of micro flora depended on the sampling patters and geographical location of the capture of seafood (Ghaly *et al.*, 2010).

The enzymatic activity of some other bacteria, such as psychrotolerant Enterobacteria, *Vibrio*spp, *Aeromonas* spp, and *S. putrefaciens* have been reported to reduce trimethylamine oxide (TMAO) in seafood to trimethylamine (TMA), which is responsible for the fishy odor (Arfat *et al.*, 2015; Lidbury *et al.*, 2014). TMA production develops hypoxanthine, which imparts bitter taste in seafood (Tikk *et al.*, 2006). Hypoxanthine in seafoods is produced by the activity of indigenous enzymes or enzymes produced by microbes that aid in decomposition of nucleotides (inosine or inosine monophosphate (Masniyom, 2011; Visciano *et al.*, 2012; Varlet and Fernandez, 2010).

Microbial spoilage of seafood products produce amines, sulfides, alcohols, aldehydes, ketones, and organic acids with unpleasant odors and unacceptable off-flavors (Gram and Dalgaard, 2002). The high content of free amino acids and other soluble non-nitrogenous substances can serve as easily digestible nutrients to facilitate

the rapid growth of microorganisms (Zeng *et al.*, 2005). The spoilage microorganism vary with the type of seafood species that considerably varies depending on the biological, environmental and storage conditions (Gram and Dalgaard, 2002). Generally, pseudomonas, H_2S-producing bacteria and lactic acid bacteria (LAB) are predominant in spoiled fish flora, while *Enterobacteriaceae*, a gram negative bacteria is also frequently present (Sallam, 2007). Pacific white shrimp were also dominated with microbial flora such as *Pseudomonas*, *Enterobacteriaceae* and H_2S-producing bacteria during refrigerated storage (Sae-leaw and Benjakul, 2019). However, the antimicrobial effect of Cashew leaf extract polyphenols was evidenced to arrest the growth of aforementioned microbes significantly during refrigerated storage (Sae-leaw and Benjakul, 2019).

Several other studies has been conducted under frozen, flaked ice and ice-brine slurry storage conditions (Nirmal and Benjakul, 2010; Zhang *et al.*, 2015). The total bacterial load was notably reduced from initial load in fresh fish or shrimp due to cold shock in frozen storage (Nirmal and Benjakul, 2010; Lakshmanan *et al.*, 2002). Furthermore, the storage of shrimp (*Pandalus borealis*) in ice slurry was more effective in microbial inactivation compared to flaked ice or brine-ice storage system (Zeng *et al*., 2005). Black tiger prawns (*Marsupenaeus japonicus*) treated 4-HR (0.1%) along with organic acids such as citric, ascorbic, and acetic acids and other additives including EDTA and disodium dihydrogen pyrophosphate (PPi) had the lower counts of microbial and spoilage bateria such as *pseudomonas*, enterobacteria, H_2S-producing bacteria, and LAB, compared to untreated control and sulfite treated prawn during low temperature storage of 13 days (Martinez-Alvarez *et al.*, 2005). Lopez-caballero *et al.* (2007) reported that thawed deep water pink shrimp (*Parapenaeus longirostris*) treated with different formulations containing 4-HR (0.05 and 0.1 %) in combination with organic acids and chelating agents, showed lowered total bacterial count in comparison with those treated with sulfites, and a mixture of gluconic acid and commercial sulfites during 14 days of chilled storage. Mastromatteo *et al*. (2010) thymol essential oil at different concentrations (500, 1000 and 1500 ppm) have potential antimicrobial effect in ready-to-eat peeled shrimp (*Palaemon serratus*). Fresh white shrimp (*Penaeus vannamei*) coated with antimicrobial film prepared from gelatin obtained from catfish skin, incorporated with sodium tripolyphosphate and potassium sorbate could retard

inhibit delay microbial spoilage up to 10 days of iced storage (Jiang *et al*., 2011). Pacific white shrimp were treated with guava leaf extract prepared via ultrasonication followed by dechlorophyllisation using simple sedimentation process in aqueous medium at different concentrations (0.5 and 1%) showed higher antimicrobial effect against inactivation of microbial and spoilage bacteria compared to those samples treated with 1.25% sodium metabisulphite (SMS-1.25) and the control (without any treatment) during 12 days of storage at 4 °C (Olatunde et al., 2020c).

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CHAPTER 2

EFFECT OF CHAMUANG (*GARCINIA COWA* ROXB.) LEAF EXTRACT ON INHIBITION OF MELANOSIS AND QUALITI CHANGES OF PACIFIC WHITE SHRIMP DURING REFRIGERATED STORAGE

2.1 Abstract

Inhibition of Pacific white shrimp polyphenoloxidase (PPO) with Chamuang leaf extract (CLE) was studied. CLE was rich in polyphenolic glycosides, in which chrysoeriol 6-C-glucoside-8-C-arabinopyranoside and 2-feruloyl-sinapoylgentiobiose were dominant. It also contained organic acids including hydroxycitric acid and oxalosuccinic acid. CLE with copper chelation activity could inhibit PPO in a dose dependent manner. Shrimp treated with 1% CLE had the lower melanosis score than 1.25% sodium metabisulfite (SMS) treated shrimp and the control throughout the refrigerated storage of 12 days at 4 °C ($p<0.05$). Lower total volatile base (TVB) and thiobarbituric acid reactive substances (TBARS) were detected in shrimp treated with 1% CLE, compared to others ($p<0.05$). Lower counts of mesophile, psychrophile, *Pseudomonas*, Enterobacteriaceae and H_2S-producing bacteria were obtained with 1% CLE treatment than the control and SMS treated sample during entire storage. Thus, soaking of shrimps in 1% CLE solution effectively reduced melanosis and quality deterioration.

2.2 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is a vital seafood crustacean with high market potential and is an excellent income of Thailand. Despite their delicacy and popularity, this high value crustacean is highly perishable with short shelf-life. Quality loss in shrimp is due to the onset of melanosis and microbial spoilage (Gokoglu and Yerlikaya, 2008). The blackening or melanosis in shrimps is caused by the formation of melanin (Benjakul *et al.*, 2005). This quality loss in shrimp is induced by a biochemical reaction, associated with phenol oxidation catalyzed by polyphenoloxidase (PPO), with subsequent formation of melanin (Haard and Simpson, 2000). Although the presence of melanin is not harmful, it considerably reduces the market value of the crustacean (Haard and Simpson, 2000). Sulfiting agents such as

sodium metabisulfite (SMS) have been used to prevent browning reaction in foods, including crustaceans. Although sulfites such as SMS have been generally recognized as safe (GRAS) for food applications, they can be allergenic for some sulfite sensitive people (Kronberg, 2008). To avoid aforementioned problem, PPO inhibitor, especially 4-hexylresorcinol, has been employed (Martinez *et al.*, 2008). Nevertheless, it is still costly when used as additive.

Over the years, several studies have been carried out to retard melanosis for shrimp processing industry. Plant extracts rich in polyphenols have been brought into application to inhibit melanosis and quality changes in order to prolong shelf-life of shrimps. However, efficacy in inhibiting PPO varied with type or structure of plant phenols. Sae-Leaw *et al.* (2017) documented that inhibition effect of epigallocatechin gallate (EGCG) on PPO activity was higher than catechin, epicatechin, epicatechin gallate and epigallocatechin. Both green tea and grape seed extracts rich in phenolics were used to treat shrimp for shelf-life extension, especially by lowering melanosis (Gokoglu and Yerlikaya, 2008; Nirmal and Benjakul, 2009a, 2009b).

Chamuang (*Garcinia cowa* Roxb.) is the indigenous plant growing in the southern part of Thailand and tropical countries (Lim, 2012). Its leaves have been used for cooking into several dishes. The leaves are rich in organic compounds such as (-)-hydroxycitric acid and polyphenols (Jena *et al.*, 2002). Additionally, the extract from Chamuang leaves had antimicrobial properties and could retard microbial growth (Sakunpak and Panichayupakaranant, 2012). Nonetheless, no information on the use of extract of Chamuang leaves (CLE) for shrimp treatment exists. The extract of Chamuang leaves rich in polyphenols and organic acids might be used as an alternative PPO inhibitor, in which melanosis in Pacific white shrimps could be prevented. Therefore, this study aimed to investigate the impact of CLE at different concentrations on PPO inhibition and to monitor the quality changes in Pacific white shrimp treated with CLE at various levels during 12 days of refrigerated storage.

2.3 Objectives

To investigate the impact of CLE on the inhibition of PPO activity and retardation of melanosis.

To study the antioxidant and antimicrobial effect of CLE on microbiological and chemical quality of Pacific white shrimp during storage.

2.4 Materials and methods

2.4.1 Chemicals

Malonaldehyde bis (dimethyl acetal) (MDA), L-β-(3,4 dihydroxylphenyl) alanine (L-DOPA), tetramethylmurexide (TMM), thiobarbituric acid (TBA) and Brij-35 were procured from Sigma Aldrich (St. Louis, MO, USA). Sodium metabisulfite (SMS) and copper sulfate were procured from Fisher Scientific (Loughborough, LeicestershireLE11 5RG, UK). Trichloroacetic acid (TCA) was obtained from Merck (Darmstadt, Germany). Potassium carbonate was procured from Ajax Finechem (Auckland, New Zealand). Standard plate count agar (PCA), eosin methylene blue (EMB), *Pseudomonas* isolation agar (PIA), triple sugar iron agar (TSIA), and all media were obtained from Oxoid Ltd. (Hampshire, UK)

2.4.2 Shrimp collection and preparation

Fresh Pacific white shrimp (*Litopenaeus vannamei*) without any treatment were procured from a seafood market, Hat Yai, Songkhla, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and immediately carried in polystyrene boxes to the seafood chemistry and biochemistry laboratory of Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Shrimp on arrival to laboratory were immersed in ice slurry and washed. After washing, shrimp were stored in ice until used (≤1 h).

2.4.3 Preparation and identification of chemical constituents in Chamuang leaf extract (CLE)

2.4.3.1 Extraction

Chamuang leaves (Garcinia cowa Roxb.) were collected from a garden in Hat Yai, Songkhla, Thailand during August and September 2017. The trees were

approximately 5–7 years old. Mature leaves were collected. Chamuang leaves were prepared as described by Chotphruethipong, Benjakul, and Kijroongrojana (2017). The whole leaves were washed with tap water and dried overnight in an air dryer at 50 °C. Moisture content of leaves was below 10%. Dried samples were ground with a blender (Panasonic, Model MX-898N, Berkshire, UK) and sieved (sieve size 80 mesh).

Preparation of Chamuang leaf extract was performed using distilled water with the aid of ultrasound following the method of Chotphruethipong et al. (2017). The CLE was freeze-dried in a freeze dryer (Cool Safe 55, ScanLaf A/S, Lynge, Denmark). The CLE powder prepared was analyzed and used for treatment of shrimp.

2.4.3.2. Liquid chromatography mass spectrometry (LC-MS) profiling and identification

Chromatographic separation and detection of chemical constituents in CLE was carried out using Agilent HPLC (6200 series) coupled with the mass analyzer G6545A MS-Q-TOF LC-MS. Diode Array DetectorDAD (G7117A), an auto sampler (G7129B), column compartment (G7116B) and binary pump 1260 (G7120A) were equipped with the chromatographic instrument. The temperature of chromatographic separation column compartment was set at 40 °C. Injection volume for the auto sampler of 5 µL, binary pump at 55 min with post time of 60 s, rate of flow 0.2 mL/min, max flow gradient of 100 mL/min and maximum pressure of 600 bar were used. Water (solvent A) and 100% acetonitrile (solvent B) at a ratio of 88:12 was used as a mobile phase. Diode array detector set at wavelength of 280 and 352 nm was used to monitor the eluent.

The negatively ionized electrospray (ESI) mode was used with nitrogen as drying gas to obtain mass spectra simultaneously. Online database and TOF LC-MS data processer were used for the identification of constituents. The LC-MS tool system known as ‘Formula calculator’ was used for analyzing the sub-fraction of ESI chromatogram to obtain the Mass value (m/z). On the chromatogram, mass and molecular formula from selected peak were generated from the system, while ESI scan was used to analyze the ratio of mass to charge (m/z) of the selected peaks.

2.4.4 Extraction of polyphenoloxidase (PPO) from Pacific white shrimp cephalothoraxes

The cephalothorax of shrimps was separated, pooled and powdered by grinding in liquid nitrogen. The powder obtained was kept in polyethylene bags, stored at -20 °C and used within 1 week. PPO from the prepared powder was extracted according to the method of Nirmal and Benjakul (2009a). All procedures was performed at 4 °C. The powder (50 g) was mixed with 150 mL of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35). The mixture was stirred continuously for 30 min, followed by centrifugation at 8000×g for 30 min using a refrigerated centrifuge (model CR22 N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation and allowed to stand for 30 min. The precipitate was collected by centrifugation at 12500×g for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume (10 mL) of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 3 changes of 50 volumes of the same buffer for totally 18 h. The insoluble precipitate was removed by centrifugation at 3000×g for 30 min. The solution referred as 'PPO extract' was collected and stored at -40 °C.

2.4.5 Effect of CLE at different levels on PPO inhibition

Firstly, CLE powder with pale yellow color and pH of 3.5 was dissolved in distilled water to obtain various concentrations (0.01, 0.05, 0.1, 0.5 and 1%, w/v). CLE solutions (100 µL) was mixed with PPO extract (100 µL) to obtain the final concentrations of 0.005, 0.025, 0.05, 0.25 and 0.5% (w/v), respectively. The mixtures was incubated for 30 min at room temperature. PPO activity was assayed using L-DOPA as a substrate as per the method of Nirmal and Benjakul (2009a) with a slight modification. To the mixtures, 400 µL of 0.05 M phosphate buffer (pH 6.0) was added. To initiate the reaction, 600 µL of 15 mM L-3,4-dihydroxyphenylalanine (L-DOPA) at 45 °C was added. PPO activity was determined by monitoring the formation of dopachrome at 475 nm after starting reaction for 3 min at 45 °C. A_{475} was measured using a UV-spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as an increase in A_{475} by 0.001/min. Enzyme and substrate blanks was prepared by excluding the substrate and enzyme, respectively, from the reaction

mixture and the deionized water was used instead. The residual PPO activity was determined. The control was run in the same manner, except the deionized water was used instead of CLE.

Inhibitory activity was calculated and expressed as the percentage inhibition as follows:

$$\text{Inhibition (\%)} = \{(A-B)/A\} \times 100$$

where A is PPO activity of the control and B is PPO activity in the presence of CLE.

2.4.6 Effect of CLE at different levels on copper chelation activity

The copper binding capacity of CLE was determined according to the method of Wettasinghe and Shahidi (2002). To 1 ml of CLE solutions, 1 ml of copper sulfate solution (1 mM in 10 mM hexamine-HCl buffer containing 10 mM KCl (pH 5.0) was added. The mixture was allowed to stand at room temperature for 10 min, followed by the addition of 0.1 ml of 1 mM tetramethylmurexide (prepared in the same buffer). Absorbance of the reaction mixtures was recorded at 460 and 530 nm and the ratio of A_{460} to A_{530} nm was calculated. These absorbance ratios was then converted to corresponding free Cu^{2+} concentrations using a standard curve of free Cu^{2+} concentration (50-400 mM) vs absorbance ratio. The difference between total Cu^{2+} and free Cu^{2+} concentrations indicated the concentration of chelated Cu^{2+}. Copper chelating activity was calculated using the following equation:

$$\text{Copper chelation activity (\%)} = \{(\text{Concentration of chelated } Cu^{2+}) / (\text{Concentration of total } Cu^{2+})\} \times 100$$

2.4.7 Effect of CLE treatment on melanosis and quality changes in Pacific white shrimp during refrigerated storage

Whole shrimps were dipped in CLE solutions having various concentrations (0.1, 0.5 and 1%) for 30 min at 4°C. The shrimp/solution ratio was 1:2 (w/v). The sample without treatment (control) and 1.25% sodium metabisulfite treatment (SMS) were also prepared. After soaking, the CLE solution was drained off for 5 min at 4°C. Shrimps placed on polystyrene trays were wrapped with shrink plastic film. Samples were stored at 4 °C, picked randomly and determined for melanosis score, chemical and microbiological quality every 3 days up to 12 days of refrigerated storage.

2.4.8 Analyses

2.4.8.1. Melanosis assessment

Melanosis or blackening of shrimp was evaluated through visual inspection using 10-point scoring test by ten experienced panelists, who was familiar with the scoring or rating of blackspot (melanosis) intensity in raw Pacific white shrimp. Score of 0-10, where 0 = absent; 2 = slight (up to 20% of shrimp surface affected); 4 = moderate (20-40% of shrimp surface affected); 6 = notable (40-60% of shrimp surface affected); 8 = severe (60-80% of shrimp surface affected); 10 = extremely heavy (80-100% of shrimp surface affected) was reported (Montero *et al.*, 2001).

2.4.8.2 Microbiological analyses

Microbiological analyses was performed by the spread plate method (Sallam, 2007). Whole raw shrimp was peeled under aseptic conditions and shrimp meat (10 g) was mixed with 90 mL of 0.85% saline buffer, followed by homogenisation in a Stomacher blender (Mode l400, Seward Ltd. West Sussex, England) for 2 min at 220 rpm. Homogenate was used to prepare ten-fold serial dilutions in 0.85% saline buffer and appropriate dilutions (0.1 mL) was used for the microbiological analyses.

Total viable count (TVC) was determined using plate count agar supplemented with 0.5% NaCl (PCA), in which incubation was done at 35 °C for 2 days (Maturin and Peeler, 1998). Psychrophilic bacteria count (PBC) was measured using PCA containing 0.5% NaCl. Plates was incubated at 4 °C for 10 days (Cousin *et al.*, 1992). *Pseudomonas* count was determined using *Pseudomonas* isolation agar after 48 h of incubation at 25 °C (Salfinger and Tortorello, 2015). H_2S-producing bacteria was enumerated from black colonies appearing on triple sugar iron agar after incubation at 25 °C for 3 days (International Commission on Microbiological Specifications of Foods, 1978). Enumeration of Enterobacteriaceae was performed using eosin methylene blue agar (EMB). Dark colonies with a green metallic sheen was counted upon incubation at 37 °C for 24 h (Salfinger and Tortorello, 2015).

2.4.8.3 Analysis of total volatile base (TVB) content

TVB contents in shrimp meat was determined using the Conway micro-diffusion method (Conway and Byrne, 1933). Peeled shrimp meat (3 g) was added with 8 mL of 4% (w/v) trichloroacetic acid (TCA) solution. The mixture was homogenized at 8,000 rpm for 2 min using an IKA homogenizer model T 25 D (IKA-Werke GmbH and Co. KG, Staufen, Germany). The homogenate was allowed to stand at room temperature for 30 min and filtered through a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrate was collected and the final volume was adjusted to 10 mL using 4% TCA. The inner ring solution (1 mL) and filtrate (1 mL) was added to inner ring and outer ring of the Conway unit, respectively. Saturated K_2CO_3 (1 mL) solution was then added into outer ring. The Conway unit was closed and the solution was mixed slowly. The mixture was incubated at 37 °C for 60 min and the inner ring solution was titrated with 0.02 N HCl using a micro-burette until green color turned into pink. For the blank, TCA solution (4%) was used instead of sample extract. The amounts of TVB was calculated and the results was expressed as mg N/100 g shrimp meat.

2.4.8.4 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS in the samples was determined as described by Benjakul and Bauer (2001) with some modifications. Peeled shrimp meat (0.5 g) was mixed with 4.5 mL of a solution containing 0.375% TBA, 15% TCA and 0.25 N HCl. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4,000×g for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a spectrophotometer. TBARS value was calculated from the standard curve of malonaldehyde (0–5 ppm) and expressed as mg malonaldehyde/kg shrimp meat.

2.4.9 Statistical analyses

Completely randomized design (CRD) was used for entire study. All experiments were carried out in triplicates. The data analysis was done by analysis of variance (ANOVA). Differences between means were determined using the Duncan's multiple range test. Statistical analysis was done using a SPSS statistical package (SPSS 17.0, SPSS Inc., Chicago, IL, USA).

2.5. Results and discussion

2.5.1. Profiling of chemical constituents in Chamuang leaf extract (CLE)

LC-MS coupled with MS-Q-TOF was used in the negative mode for qualitative identification of polyphenolic constituents in CLE. Mena et al. (2012) reported that the negative mode showed the better resolution, in comparison with LC-MS run in the positive mode. Thus, LC-MS run in the negative mode showed higher sensitivity for identification of polyphenolics because of its ability to form negatively charged ions readily, especially in electrospray ionization (ESI) (Hua and Jenke, 2012). Several compounds detected by LC-MS in CLE are listed in Table 3. The chemical constituents were identified on the basis of m/z values. Mostly polyphenolics and their glycosides were found as the abundant compounds. The most dominant compounds detected by LC-MS in CLE were apigenin 7-(3″-ρ-coumaroylglucoside), epioritin-4alpha-ol, veranisatin C, chrysoeriol 6-C-glucoside-8-C-arabinopyranoside and 2-feruloyl-L sinapoylgentio-biose. Apart from polyphenols, especially in the form of glycosides, some naturally occurring organic acids including hydroxycitric acid and oxalosuccinic acid were also detected. The synergistic effect of polyphenols and organic acids more likely contributed to antioxidant and antimicrobial properties of CLE. Several polyphenols have been reported to act as potential antioxidant and antimicrobial agents to retard lipid oxidation and microbial spoilage in shrimp (Sae-leaw, *et al*, 2018). Based on abundance of phytochemical constituents in CLE, the highly abundant flavonoids, chrysoeriol 6-Cglucoside-8-C-arabinopyranoside and other compounds might play a role in quality maintenance of shrimp during the extended storage. Hajji *et al.* (2010) documented that polyphenols in tubers (*Mirabilis jalapa*) with high content of flavonoids exhibited both antimicrobial and antioxidant properties. Therefore, LC-MS analyses indicated the presence of polyphenols and organic acids in CLE, which might have antioxidant, antimicrobial and antimelanosis properties.

Table 3. Proposed structure of chemical constituents based on LC-MS in Chamuang leaf extract (CLE)

Compounds	Diff (ppm)	Molecular Formula	m/z (observed)	m/z (calculated)	Retention Time (min)	Score (%)	Abundance ($x10^6$)
Hydroxycitric acid	-0.2	$C_6 H_8 O_8$	207.01	208.02	1.81	99.61	4.42
Oxalosuccinic acid	-0.12	$C_6 H_6 O_7$	189.00	190.00	2.30	98.63	4.54
3'-Glucosyl-2',4',6'-trihydroxyacetophenone	-0.29	$C_{14} H_{18} O_9$	329.08	330.10	2.60	98.25	0.96
2,6-Dihydroxy-4-methoxytoluene	-2.31	$C_8 H_{10} O_3$	153.05	154.06	3.00	82.95	0.18
Catechin 3'-O-beta-D-glucopyranoside	-0.37	$C_{21} H_{24} O_{11}$	451.12	452.13	4.00	99.55	0.76
Ethotoin	-1.29	$C_{11} H_{12} N_2 O_2$	203.08	204.09	3.65	98.09	0.24
Hesperetin-7-O-glucuronide	-0.42	$C_{21} H_{22} O_{12}$	465.10	466.11	4.61	97.88	1.30
Ethyl 3-O-beta-D-glucopyranosyl-butanoate	-0.6	$C_{12} H_{22} O_8$	293.12	294.13	4.93	90.37	0.18
Enol-phenylpyruvate	-0.96	$C_9 H_8 O_3$	163.04	164.05	5.05	99.02	0.13
cis-β-D-Glucosyl-2-hydroxycinnamate	-0.6	$C_{15} H_{18} O_8$	325.09	326.10	5.16	99.73	2.10
Verbasoside	0.15	$C_{20} H_{30} O_{12}$	461.16	462.17	5.40	97.79	0.36
Apigenin 7-(3"-ρ-coumaroylglucoside)	-0.73	$C_{30} H_{26} O_{12}$	577.13	578.14	5.53	97.15	17.42
Epigallocatechin 3-O-(4-hydroxybenzoate)	-0.04	$C_{22} H_{18} O_9$	425.08	426.10	5.63	94.83	0.13
3-Glucosyl-2,3',4,4',6-pentahydroxybenzophenone	-0.8	$C_{19} H_{20} O_{11}$	423.09	424.10	7.15	98.89	0.85
Ethylvanillin glucoside	-0.38	$C_{15} H_{20} O_8$	327.10	328.12	7.20	97.04	0.10
Epioritin-4alpha-ol	-0.96	$C_{15} H_{14} O_6$	289.07	290.08	7.41	98.36	17.93
Dihydroroseoside	-0.32	$C_{19} H_{32} O_8$	387.20	388.21	7.90	97.37	0.19
Kanokoside A	-0.03	$C_{21} H_{32} O_{12}$	475.18	476.19	8.38	93.09	9.71
Phenylethyl primeveroside	-0.37	$C_{19} H_{28} O_{10}$	415.16	416.17	8.43	99.59	0.28
Veranisatin C	-0.62	$C_{16} H_{20} O_{10}$	371.09	372.11	9.71	98.79	19.82
Norswertianolin	-0.49	$C_{19} H_{18} O_{11}$	421.07	422.09	9.78	99.78	1.30
Fisetin 3-glucoside	-0.47	$C_{21} H_{20} O_{11}$	447.09	448.10	9.95	96.69	1.96
Kaempferol 3-rhamnosyl-(1-2)-galactoside-7-rhamnoside	-0.11	$C_{33} H_{40} O_{19}$	739.20	740.22	10.07	99.53	2.67
Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside	-1.42	$C_{27} H_{30} O_{15}$	593.15	594.16	10.22	97.68	49.42
2-Feruloyl-1-sinapoylgentiobiose	-0.53	$C_{33} H_{40} O_{18}$	723.21	724.22	10.69	98.89	41.47
Lusitanicoside	-0.82	$C_{21} H_{30} O_{10}$	441.17	442.18	11.35	96.88	3.40
Isocytisoside 6"-O-beta-D-apiofuranoside	-0.19	$C_{27} H_{30} O_{14}$	577.16	578.16	11.90	99.23	1.05
Geranyl arabinopyranosyl-glucoside	-0.06	$C_{21} H_{36} O_{10}$	447.22	448.23	12.10	92.69	0.17
Cicerin 7-(6-malonylglucoside)	-0.53	$C_{26} H_{26} O_{15}$	577.12	578.13	13.10	98.73	1.80
Machaerol C	-0.11	$C_{18} H_{20} O_7$	347.11	348.12	14.03	99.70	0.09
Tanacetol A	-1.78	$C_{17} H_{26} O_4$	293.18	294.18	42.17	97.84	0.53

2.5.2. Effect of CLE on inhibition of Pacific white shrimp PPO

PPO inhibitory activity of CLE at varying levels expressed as percentage inhibition is shown in Fig. 8a. The increase in PPO inhibition was observed as CLE increased up to 1% CLE. CLE contained polyphenols and organic acids (Table 3), which acted synergistically to inhibit PPO. Sae-Leaw *et al.* (2017) reported that EGCG was able to inhibit PPO from Pacific white shrimp via the mixed type inhibition kinetics. Polyphenols were documented for the inhibition of PPO activity via their ability to interact with the PPO active sites (Nirmal and Benjakul, 2012a) Ferulic acid (0.1–2%, w/v) effectively inactivated PPO in a concentration dependent manner (Nirmal and Benjakul, 2009b). Furthermore, the hydroxyl group of polyphenols and organic acid, e.g. hydroxycitric acid present in CLE may be involved in the reduction of dopaquinone to DOPA via its ability to donate electrons to intermediate quinone. Catechin and its derivatives were able to inhibit PPO, mainly caused by reduction of quinone, metal chelation, etc. (Nirmal and Benjakul, 2012b).

2.5.3. Effect of CLE at different levels on copper chelation activity

Chelating capacity of copper ions by CLE at different concentrations is depicted in Fig. 8b. The increases in copper chelation activity were obtained with increasing concentrations of CLE up to 0.1% ($p<0.05$). There was no difference in copper chelation activity when CLE concentration ranged from 0.5 to 1% ($p>0.05$). CLE at low concentration was able to exhibit copper chelation activity. Phenolic compounds from the plant source exhibit metal chelation ability, which is governed by maximum number of hydroxyl group at ortho position (Maqsood and Benjakul, 2010; Wettasinghe and Shahidi, 2002). The lowered PPO activity by plant extracts could be due to the chelation of copper (II) localized at active site of PPO. PPO is metallo enzyme containing two copper atoms located in the active site (Jang *et al.,* 2003). The copper chelating activity of CLE in the concentration range of 0–0.1% showed similar trend with PPO inhibitory activity Fig. 8a). At concentration above 0.1%, no correlation was noticeable. This suggested that CLE had other modes of action in inhibiting PPO, apart from chelation of copper in PPO active sites.

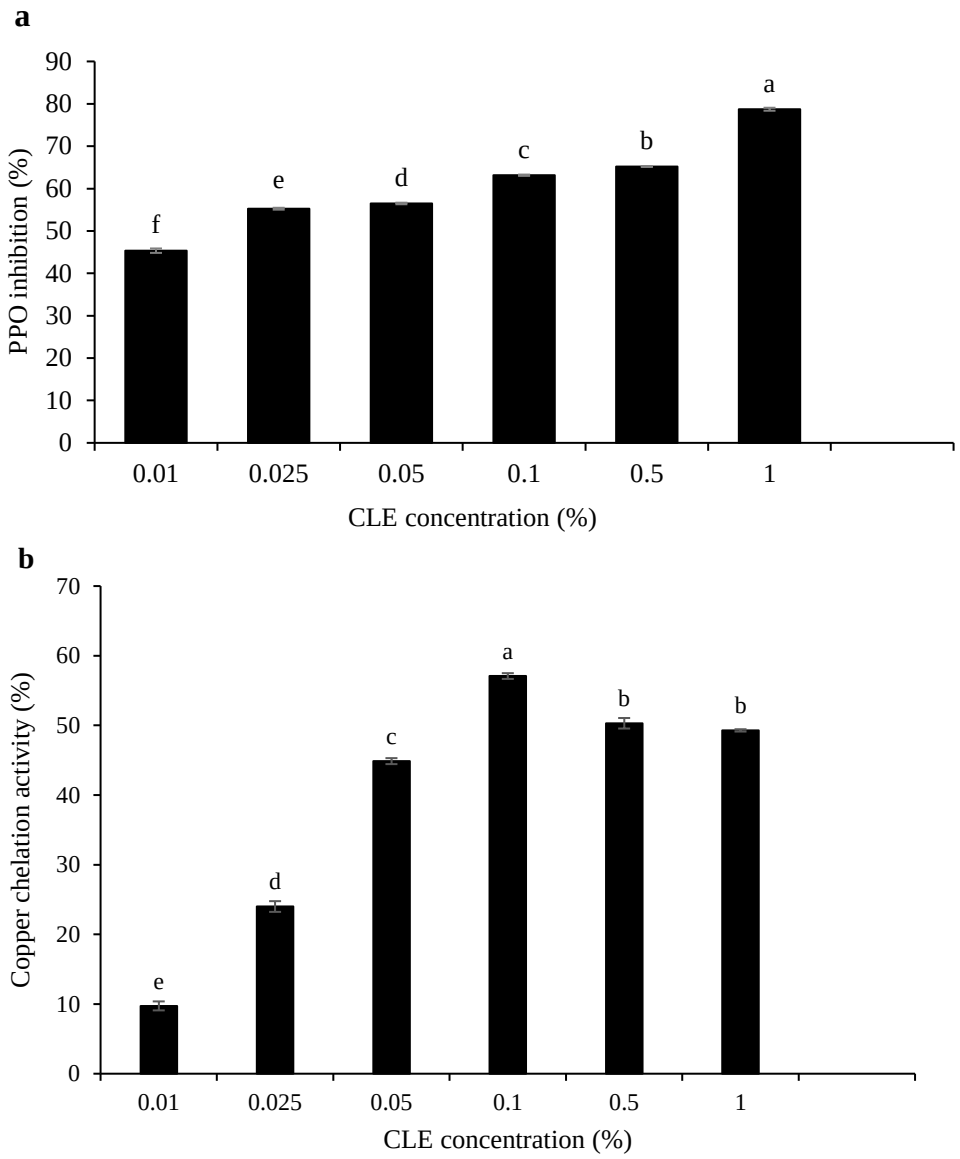

Figure 8. Pacific white shrimp polyphenoloxidase inhibition (a) and copper chelating activity (b) of Chamuang leaf extract at different concentrations. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($p<0.05$). CLE: Chamuang leaf extract.

2.5.4 Effect of CLE treatment on melanosis and quality changes of shrimps during refrigerated storage

2.5.4.1 Changes in melanosis

Melanosis scores of control and shrimps with treatments using 0.1, 0.5, 1% CLE and 1.25% SMS upon the storage are shown in Fig. 9. All samples at day 0

were free of melanosis (score=0). The melanosis scores of the control (without treatment) increased up to the end of storage (12 days) ($p>0.05$). However, shrimps treated with SMS, 0.5 and 1% CLE showed no difference in melanosis until day 6 of storage ($p>0.05$). Generally, shrimp treated with SMS or with 0.5 or 1% CLE had lower melanosis score than 0.1% CLE treated sample within the first 6 days ($p<0.05$). Subsequently, the increased melanosis score of the SMS treated shrimps was found, suggesting that SMS might be unstable and liberated in the form of sulfur dioxide during prolonged storage (Taylor, 1968). Similar results were documented in the SMS treated shrimps during 4 °C storage (Sae-Leaw *et al*., 2017). Thus, shrimps treated with 1% CLE had lower melanosis than SMS treated counterpart ($p<0.05$). During 9–12 days, 1% CLE treated sample showed the lowest melanosis score, followed by those treated with 0.5, 0.1% CLE and SMS, respectively. The lowest melanosis of 1% CLE treated samples was visualized, than other samples at day 12 (Fig. 10). On the other hand, the control showed the highest melanosis score. The lower melanosis score of CLE treated shrimp was related with PPO inhibitory activity of CLE (Fig. 8).

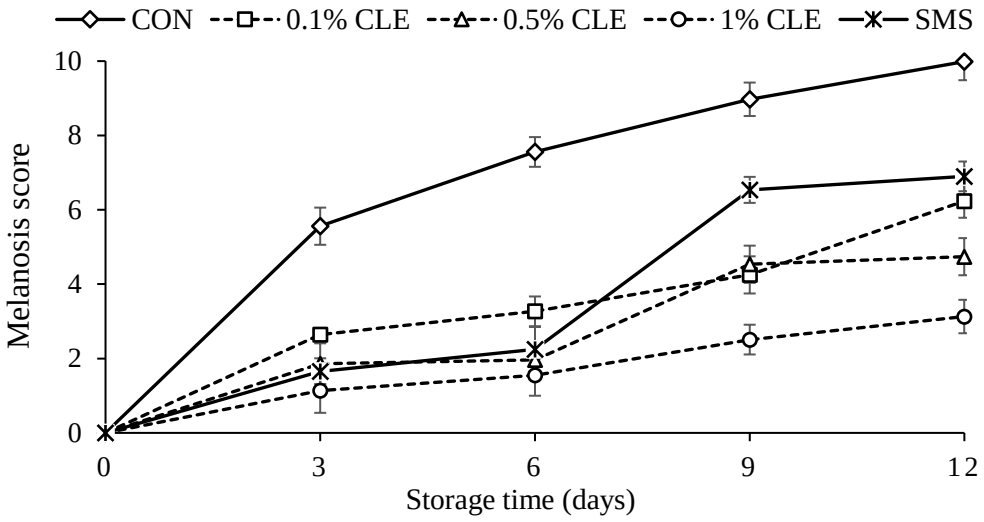

Figure 9. Melanosis score of raw Pacific white shrimp treated with Chamuang leaf extract at different concentrations during 12 days of refrigerated storage. Bars represent the standard deviation (n=10). Control: no treatment, SMS: 1.25% Sodium metabisulfite, 0.1% CLE, 0.5% CLE and 1% CLE: Chamuang leaf extract at levels of 0.1, 0.5 and 1%, respectively.

CLE at 1% level, containing high amount of polyphenols and organic acids (Table 3), might be penetrated toward PPO active sites underneath the shrimp shell during soaking

more effectively. Catechins and derivatives have been employed to reduce black spots in shrimp during refrigerated storage (Nirmal, 2011; Nirmal and Benjakul, 2009b; Sae-Leaw *et al*., 2017). Therefore, CLE was capable of preventing melanosis in raw Pacific white shrimp kept under refrigerated condition.

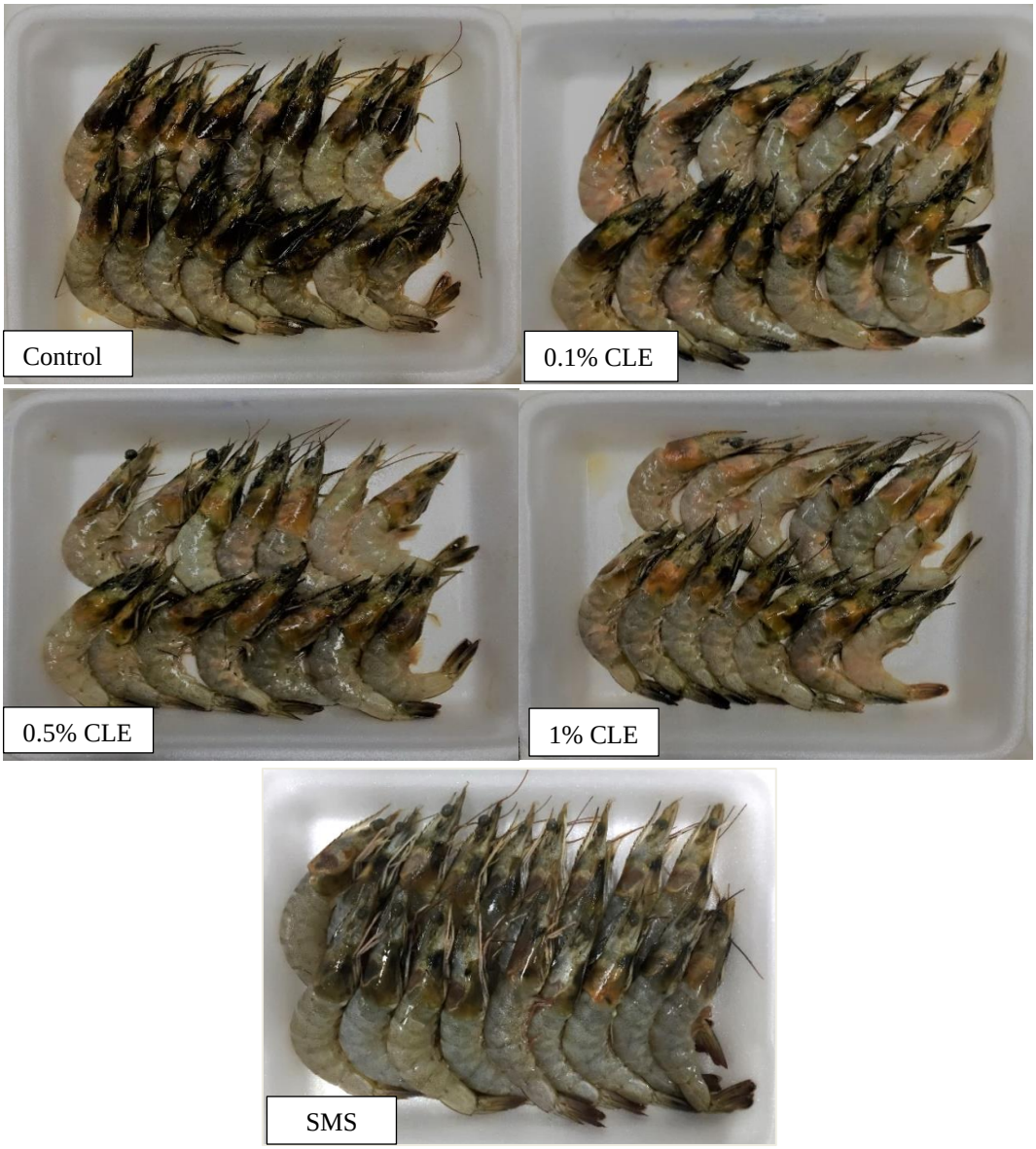

Figure 10. Photographs of raw Pacific white shrimp treated with Chamuang leaf extract at different concentrations after 12 days of refrigerated storage. Control: no treatment, SMS: 1.25% Sodium metabisulfite, 0.1% CLE, 0.5% CLE and 1% CLE: Chamuang leaf extract at levels of 0.1, 0.5 and 1%, respectively.

2.5.4.2. Changes in microbiological and chemical quality

Microbiological changes in shrimp with different treatments during 12 days of storage at 4 °C are presented in Fig. 11. The CLE and SMS treated shrimp had lower TVC than the control ($p<0.05$). TVC of all the samples increased up to 3 days of storage ($p<0.05$). No difference in TVC was observed among all the CLE treated samples ($p>0.05$), while the control showed the higher TVC at day 3 (Fig. 11a). TVC was increased during 3–9 days of storage ($p<0.05$). TVC of 1% CLE treated sample was lowest ($p<0.05$), compared to SMS treated sample and those treated with CLE at lower concentrations ($p<0.05$). Subsequently, lower TVC was obtained at the end of storage. This was plausibly attributed to the intolerance of mesophilic bacteria under refrigerated storage condition. Zeng *et al.* (2005) postulated that the inhibition of mesophilic microorganisms in shrimp meat could be owing to intolerance in cold temperature environment. At day 12, TVC of the control, 0.1, 0.5 and 1% CLE treated samples and SMS treated sample were 4.46, 4.23, 3.92, 3.62 and 4.29 log CFU/g, respectively. Among all the samples, 1% CLE treated sample possessed the lowest TVC at all the storage time ($p<0.05$). The antibacterial activity could be due to polyprenylated benzophenone in Chamuang leaves (Sakunpak and Panichayupakaranant, 2012).

Changes in PBC of control and CLE treated shrimp during refrigerated storage are depicted in Fig. 11b. At the day 0, no difference in PBC was noticeable in all samples ($p>0.05$). Generally, PBC increased from day 3 up to day 12 ($p<0.05$). The highest PBC was obtained in control, compared to SMS and CLE treated shrimps ($p<0.05$). However, 1% CLE treated shrimp meat was lowest in PBC during 12 days of storage, compared to others ($p<0.05$). The control, 0.1, 0.5, 1% CLE and SMS treated shrimps had PBC of 6.10, 5.05, 4.86, 4.63 and 5.25 log CFU/g, respectively, at the end of storage. Thus, the treatment of shrimps with CLE, especially at higher doses, was able to retard the growth of psychrophilic bacteria during the refrigerated storage. The psychrophilic bacteria in raw shrimp remain active at refrigerated environmental conditions and have been referred as a main cause of spoilage during refrigerated storage (Sae-leaw *et al*., 2018). Therefore, the use of CLE for the treatment of shrimps potentially inactivated psychrophilic microorganisms responsible for shrimp spoilage

Pseudomonas count of all samples was in the range of 2.02–2.06 log CFU/g at the first day of storage (Fig. 11c). At day 0, slightly lower count of *Pseudomonas* was noticeable in all the treated samples, compared to the control (without treatment), suggesting the inhibition of Pseudomonas by CLE or SMS. *Pseudomonas* count of all samples increased as the storage time increased ($p<0.05$). Nonetheless, the rate of increase varied, depending on treatments used. The difference in *Pseudomonas* count was noticeable during 3–9 days, in which the lowest *Pseudomonas* count was found in 1% CLE treated shrimps ($p<0.05$). *Pseudomonas* count of the control reached the maximum of 7.08 log CFU/g at day 12 ($p<0.05$). Overall, inhibition ability of CLE towards *Pseudomonas* was dependent on dose used. The result showed that CLE had inhibitory effect on Pseudomonas growth in Pacific white shrimps during storage. *Pseudomonas* have been found dominant in shrimp even at cold temperature and promotes spoilage during storage (Dabadé *et al*., 2015). Thus, the CLE at 1% level in shrimps showed the inhibition effect on Pseudomonas during refrigerated storage.

H_2S-producing bacterial counts monitored in control and shrimp with treatments during refrigerated storage at 4 °C are presented in Fig. 11d. At day 0, no difference was found among CLE treated samples ($p>0.05$). However, the counts were lower than those of control and SMS treated samples ($p<0.05$). The initial H_2S-producing bacterial count at day 0 for all samples was in the range of 1.59–1.92 log CFU/g. During 3–9 days of storage, bacterial counts increased in all the samples. For CLE treatments, shrimps treated at higher doses had the lower count ($p<0.05$). H_2S-producing bacterial count was generally shown to be lower in the CLE treated samples throughout the 12 days of storage ($p<0.05$), compared to SMS treated sample. At the end of storage, H_2S-producing bacterial counts were 7.04, 6.85, 6.72, 6.54 and 6.91 log CFU/g for the control, 0.1, 0.5, 1% CLE and SMS treated samples, respectively. CLE at high level was found to suppress the growth of H_2S-producing bacteria effectively. Morin *et al*. (2015) reported that the growth of H_2S-producing bacteria, especially *Solobacterium moorei*, was inhibited by EGCG treatment. H_2S-producing bacteria identified contribute to seafood spoilage by the production of H_2S gas off-odor development in shrimps (Dabadé *et al*., 2015). Thus, the treatment of shrimps with CLE

at high level could inhibit H_2Sproducing bacteria in Pacific white shrimp during refrigerated storage.

Enterobacteriaceae count was similar among all the samples at the beginning (day 0) of storage at 4 °C ($p>0.05$) (Fig. 11e). Generally, the increased Enterobacteriaceae count was found in all the samples as the storage time proceeded ($p<0.05$). The lowest Enterobacteriaceae counts were obtained in 1% CLE treated raw shrimp at all storage time ($p<0.05$). Lower count of Enterobacteriaceae was noticeable with CLE treatments, indicating its higher inhibition effect than SMS. Production of putrescine and indole are the main indicators of spoilage caused by Enterobacteriaceae detected by off-odor development in shrimps (Dabadé *et al.*, 2015). Catechin treated shrimps were reported to have lower Enterobacteriaceae count (Nirmal, 2011; Nirmal and Benjakul, 2009b). Thus, shrimps treated with 1% CLE had the lowest Enterobacteriaceae counts during refrigerated storage.

TVB contents in shrimps with different treatments kept up to 12 days are depicted in Fig. 12a. Initially, the TVB contents of all the samples ranged from 2.23 to 3.08 mg N/100 g shrimp meat. TVB contents of all samples increased throughout the storage ($p<0.05$). Generally, the CLE treatment at higher concentrations showed the lower TVB contents than those treated with CLE at lower levels at all storage times ($p<0.05$). Nonetheless, the lowest TVB content was found in SMS treated sample, while the control showed the highest value up to 9 days of storage at 4 °C. The lower TVB content of CLE treated samples might be due to the inhibitory effect of polyphenols and organic acids, especially hydroxycitric acid (Table 3) in CLE towards microbes and proteolytic enzymes. Chamuang leaf and extracts from Chamuang fruit rind, were reported to have antimicrobial activity (Negi *et al.*, 2008). TVB is widely used as an indicator to determine the spoilage in seafoods (Gill, 1990). The acceptable level of TVB up to 30 mg/100 g of shrimp meat is indexed for shrimp quality (Cobb *et al.*, 1973). Nevertheless, 1% CLE treated sample had the lowest TVB content (23.24 mg N/100 g shrimp meat), compared to other samples ($p<0.05$). The result was in agreement with the lowest microbial load of 1% CLE treated samples.

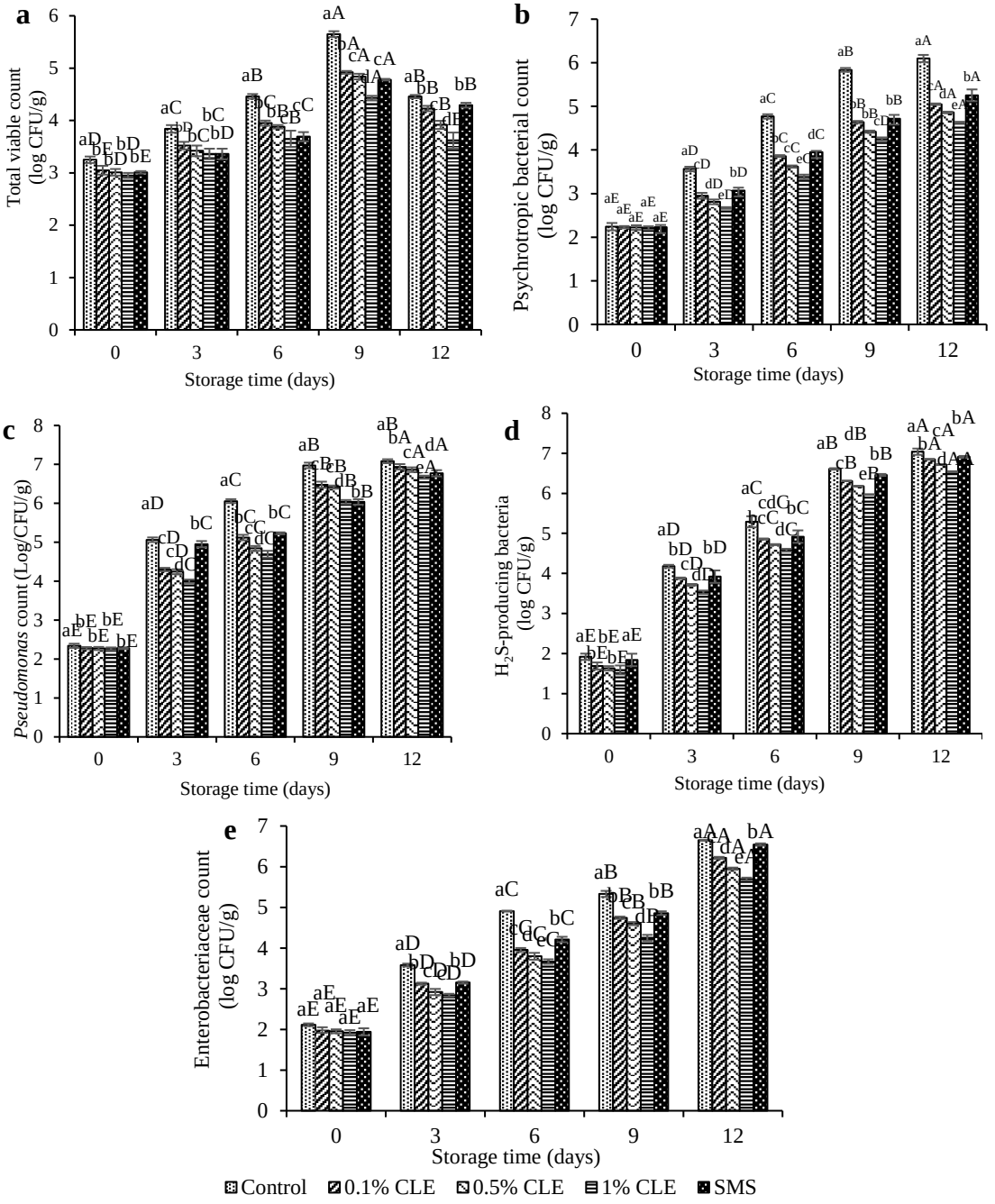

Figure 11. Total viable count (a), psychrophilic bacterial count (b), *Pseudomonas* (c), H_2S-producing bacteria (d) and Enterobacteriaceae (e) counts of raw Pacific white shrimp treated with Chamuang leaf extract at different concentrations during 12 days of refrigerated storage.

Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same treatment indicate the significant differences ($p<0.05$). Control: no treatment, SMS: 1.25% Sodium metabisulfite, 0.1% CLE, 0.5% CLE and 1% CLE: Chamuang leaf extract at levels of 0.1, 0.5 and 1%, respectively.

The TBARS values of untreated and CLE treated shrimp during storage are given in Fig. 12b. Generally, the increment in TBARS of all the samples was observed as the storage time increased ($p<0.05$). The highest increase in TBARS was observed in control, followed by SMS treated sample throughout the storage of 12 days. In contrast, CLE and SMS treated shrimp had the lowest TBARS value, compared to control at all the storage times ($p<0.05$). Lipid oxidation in the control took place at a higher extent. CLE at highest concentration (1%) prevented the oxidation of lipids as shown by the lower TBARS value at day 12 ($p<0.05$) than 0.5, 0.1% CLE and SMS, respectively. Lipid oxidation in crustaceans leads to the formation of unstable hydroperoxide that readily decomposes to malonaldehyde as analyzed by TBARS (Benjakul *et al.*, 2005). Since shrimp treated with CLE had lower TBARS, it indicated that polyphenolics and organic acids (Table 3) in CLE synergistically functioned as antioxidants. Polyphenols from plants have been known as antioxidants, which can terminate oxidation via providing proton or electron to radicals (Ningappa *et al.*, 2008). Higher antioxidant activity was increased as indicated by the lower TBARS found in the samples treated with higher levels of CLE used. Chotphruethipong *et al*. (2017) reported that cashew leaf extract had antioxidative activity as analyzed by 2,2-diphenyl-L-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). Overall, shrimp treated with CLE extract at 1% level, had the lowered lipid oxidation than those treated with SMS and the control ($p<0.05$) at the end of storage.

2.6 Conclusions

CLE rich in polyphenolic glycosides and organic acids could inhibit PPO from Pacific white shrimp. Lower melanosis score was noticed in 1% CLE treated shrimps than that of control and SMS treated samples. Shrimps treated with 1% CLE had the lowered increase in TVB content, TBARS and microbial growth during refrigerated storage of 12 days. Therefore, CLE was shown to serve as natural additive to control melanosis and prolong the shelf-life of Pacific white shrimps kept under refrigerated condition.

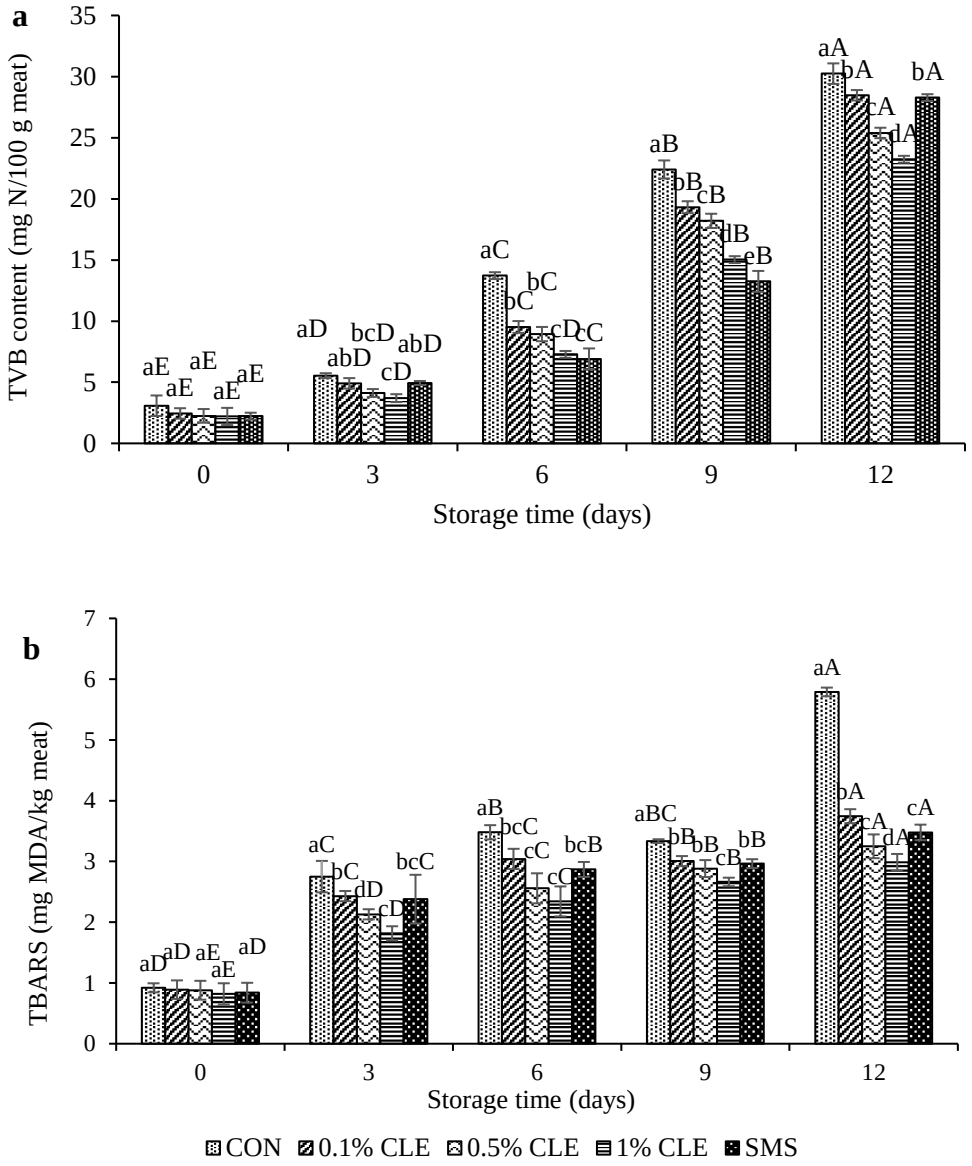

Figure 12. Total volatile base (TVB) content (a) and TBARS values (b) of raw Pacific white shrimp treated with Chamuang leaf extract at different concentrations during 12 days of refrigerated storage.

Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same treatment indicate the significant differences ($p<0.05$). Control: no treatment, SMS: 1.25% Sodium metabisulfite, 0.1% CLE, 0.5% CLE and 1% CLE: Chamuang leaf extract at levels of 0.1, 0.5 and 1%, respectively.

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CHAPTER 3

EFFECT OF PULSED ELECTRIC FIELD TREATMENTS ON MELANOSIS QUALITY CHANGES OF PACIFIC WHITE SHRIMP DURING REFRIGERATED STORAGE

3.1 Abstract

Pacific white shrimps were subjected to the pulsed electric field (PEF) at varying specific energy densities (54–483 kJ/kg) and pulse numbers (200–600). The poly-phenol oxidase (PPO) activity in cephalothorax was decreased as both parameters increased ($p<0.05$). Shrimp treated with PEF at highest level (PEF-T3) (483 kJ/kg, 600 pulses) had lower melanosis score than other samples, packaged in polystyrene trays and wrapped with shrink film, during 10 days of storage at 4°C ($p<0.05$). Highest shear force values were noticed for the PEF-T3-treated sample at Day 10 ($p<0.05$). Microstructural gaping between shrimp muscle fibers was higher in PEF-T3. No protein degradation was observed for all samples, regardless of PEF treatments. Lower mesophilic and psychrophilic microbial counts in shrimp were obtained when PEF-T3 was implemented. After 10 days, higher sensory scores of PEF-T3-treated samples were attained, compared to others ($p<0.05$). Quality deterioration of shrimp was therefore retarded with PEF.

3.2 Introduction

Color is one of the most important quality attribute of any food commodity, not only to appeal but also to enhance consumer acceptability. Pacific white shrimp (*Litopenaeus vannamei*) is a very popular crustacean that undergoes a color change, so-called “melanosis” mediated by polyphenol oxidase (PPO) with atmospheric oxygen. This biochemical process produces a high molecular weight pigment, named melanin (Benjakul *et al.,* 2005; Haard and Simpson, 2000). Melanosis is one of the major hurdles in the economic development of the shrimp processing industry. Several attempts have been employed to prevent melanosis in shrimp by the application of chemicals and natural additives (Iyengar *et al.,* 1991; Sae-leaw and Benjakul, 2019). Chemical treatments including the usage of sodium metabisulfite and 4-hexylresorcinol as melanosis inhibitors have been studied extensively. However, the

former does not show stability during the extended storage period and the latter is still expensive for the commercial application (Martinez *et al.,* 2008). Moreover, the usage of chemical additives in foods has come up with some ethical considerations despite being generally recognized as safe for food applications (Andrade *et al.,* 2015).

Over the past decades, several additives, both natural and synthetic, have been used to tackle melanosis and to retard microbial growth in raw shrimp stored at refrigerated temperature (Nirmal and Benjakul, 2009b, 2010). In addition to the low-temperature storage, thermal processing of shrimps has been carried out to ensure the better quality shrimp (Dabadé *et al.,* 2015). Among the plant extracts, cashew leaf and Chamuang leaf extracts have been proven to be the effective antimelanosis agents for Pacific white shrimp during refrigerated storage (Sae-leaw and Benjakul, 2019; Shiekh *et al.,* 2019). Additionally, plant extracts also exhibit antimicrobial activity toward spoilage microflora, thus maintaining the quality and sensory property of shrimp during the extended refrigerated storage (Shiekh *et al.,* 2019).

Nonthermal processes have gained importance in recent years due to the increasing demand for minimally processed seafood with a high nutritional value and fresh-like characteristics (Olatunde and Benjakul, 2018). These technologies are also eco-friendly and cost-effective in the food system (Kate *et al.,* 2016). Pulsed electric field (PEF) is one of the novel nonthermal technologies that can reduce the microbial load of foods (Lado and Yousef, 2002). PEF is based on electroporation and cell disintegration by the application of repeated pulses when the food is placed between the two parallel electrodes (Gudmundsson and Hafsteinsson, 2001). PEF can inactivate some enzymes potentially by inducing the denaturation associated with conformational change (Zhong *et al.,* 2005). The activities of protease, lipase, and alkaline phosphatase in fresh bovine milk subjected to PEF treatment at electric field strengths ranging from 15 to 35 kV/cm were reduced to 14%, 37%, and 29%, respectively (Riener *et al.,* 2009). Microstructural and textural changes have been reported as induced by electroporation associated with the leakage of cellular fluids in the tissue matrix (Gudmundsson and Hafsteinsson, 2001). Nevertheless, the PEF treatment had no effect on the protein structure in fish and chicken meat (Gudmundsson and Hafsteinsson, 2001). Without heat applied, sensory properties, biochemical and nutritional quality of foods can be maintained after being treated with PEF (Ho and Mittal, 2000). However, no

information on PEF application in shrimp exists. Therefore, this study aimed to investigate the impact of PEF at different electric field intensities and pulse numbers on PPO inhibition and to monitor the physical, microbial and sensorial characteristics of Pacific white shrimp treated with PEF during 10 days of refrigerated storage.

3.3 Objectives

To evaluate the impact of high energy pulsed electric field pulses on the relative inhibition of Pacific white shrimp PPO.

To study the effect of FEF as antimicrobial technology for quality preservation of Pacific white shrimp during storage.

3.4 Materials and methods

3.4.1 Chemicals

L-*β*-(3,4-dihydroxyphenyl) alanine (*l*-DOPA) and Brij-35 were procured from Sigma-Aldrich (St. Louis, MO, USA). Standard plate count agar (PCA) medium was purchased from Oxoid Ltd. (Hampshire, UK). All the chemicals for electrophoresis including Coomassie blue R-250, sodium dodecyl sulfate (SDS), *N,N,N,N′*-tetramethylethylene diamine (TEMED), and polyacrylamide were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

3.4.2 Procurement and preparation of shrimp

Fresh Pacific white shrimp (*L. vannamei*) free of preservatives were procured from a seafood market, Hat Yai, Songkhla, Thailand. The shrimp were handled with care to avoid mechanical damages and placed in polystyrene boxes with a shrimp/ice ratio of 1:2 (w/w). Shrimp were immediately delivered to the seafood chemistry and biochemistry laboratory, Department of Food Technology, Prince of Songkla University, Hat Yai. Immediately on arrival to the laboratory, shrimp were subjected to washing using an ice water slurry to remove extraneous matter. After washing, shrimp were drained and stored in ice until used (<1 hour).

3.4.3 PEF treatment on PPO of shrimp cephalothorax

Shrimp cephalothorax (100 g) free of melanosis was subjected to a lab-scale PEF system with high voltage power supply (PEF LAB-400W, Fenix International Inc. Chiang Mai, Thailand). A square wave pulsed electric waveforms with a frequency of 10 Hz and a pulse width of 100 ms were delivered by the PEF generator. The PEF system consisted of a stainless steel treatment chamber with 1.5 L capacity equipped with a stainless steel electrode 20 × 10 cm, a plexiglass cabinet with aluminium frame and a digital storage oscilloscope (DSO) (UTD2052CEX, UNI-T, Dongguan city, Guangdong, China) with a UT-P03 oscilloscope probe (600 MHz, 10X). About 150 g (10 shrimp) of samples was placed in one layer in a horizontal treatment chamber attached with a perforated plastic mesh at the bottom to avoid direct contact with the bottom electrode. Shrimp placed on the bottom electrode were covered under the surface areas of the top electrode for the effective exposure to PEF. The chamber was filled with distilled water (600 ml) with a mass ratio of material to water (1:4) such that the top of the electrode can be submerged and a uniform electrode gap of 1.5 cm was maintained. The PEF treatment chamber was placed in an ice slurry and the temperature was kept below 1°C such that the temperature of water inside the PEF treatment chamber was not more than 4°C. Different conditions were applied. Those included PEF-T1 (5 kV/cm, 200 pulses), PEF-T2 (10 kV/cm, 400 pulses), and PEF-T3 (15 kV/cm, 600 pulses) with the PEF specific energy of 54, 214, and 483 kJ/kg, respectively. The energy dissipated during PEF treatment was calculated as described by Zhang *et al.* (1995). The PEF specific energy of shrimp was calculated using the following equation:

$$Q = \{V^2 t_{PEF}\}/Rm$$

where Q, V, tPEF, R, and m denote the PEF specific energy (kJ/kg), voltage (kV), process time (s), resistance (Ω), and mass of sample (kg), respectively.

Shrimp with different treatments were collected for analyses in comparison with the control (without PEF treatment).

3.4.3.1 Extraction of PPO from Pacific white shrimp cephalothoraxes

Cephalothorax was ground in liquid nitrogen to obtain a fine powder. PPO from cephalothoraxes was then extracted as described by Nirmal and Benjakul

(2009a). The powder (50 g) was mixed with 150 ml of the extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij-35). The mixture was stirred continuously at 4°C for 30 min, followed by centrifugation at 8,000× g at 4°C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulphate was added to the supernatant to obtain 40% saturation and allowed to stand at 4°C for 30 min. The precipitate was collected by centrifugation at 12,500× g at 4°C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume (10 ml) of 0.05 M sodium phosphate buffer, pH 7.2 and dialyzed against 15 vol of the same buffer at 4 °C with three changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3,000× g at 4 °C for 30 min. The supernatant containing enzyme referred to as "PPO extract" was collected and used for activity assay.

3.4.3.2 PPO activity assay

The activity of PPO was assayed using 1-3,4-dihydroxyphenylalanine (l-DOPA) as the substrate following the method of Nirmal and Benjakul (2009a). To the mixtures, 400 µl of 0.05 M phosphate buffer (pH 6.0) was added. To initiate the reaction, 600 µL of 15 mM l-DOPA at 45°C was added. The PPO activity was determined by monitoring the formation of dopachrome at 475 nm after starting the reaction for 3 min at 45 °C. $Absorbance_{475}$ was measured using a UV-spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The absorbance of all the samples was measured in triplicates (n=3). One unit of PPO activity was defined as the enzyme causing an increase in A_{475} by 0.001/min assayed at pH 6.0 and 45 °C. The residual activity was measured and presented as the relative activity (Nirmal and Benjakul, 2009a).

$$\text{Relative activity (\%)} = (B/A) \times 100$$

where A and B represent the PPO activity of the control (without PEF treatment) and PEF-treated samples, respectively.

3.4.4 Effect of PEF treatments on the quality of Pacific white shrimp during refrigerated storage

Whole shrimp were subjected to PEF under three different conditions as described in Section 2.3. Sample without PEF treatment was used as "control." All the

samples were placed on the polystyrene tray (15 shrimp/ tray) and wrapped with low-density polyethylene shrink film. The samples were stored in the refrigerator (4 °C) with light access for 10 days. Samples were subsequently taken every 2 days for melanosis assessment and microbiological analysis.

3.4.4.1 Melanosis

Black color (melanin) formation of the control (without treatment) and PEF-treated samples were scored based on 10-points scale as tailored by Shiekh *et al.* (2019). Ten experienced panelists were recruited for the assessment.

3.4.4.2 Microbial load

The spread plate method was used for microbiological analyses (Sallam, 2007). Shrimp meat (10 g) was blended with 90 ml of 0.85% sterile saline solution. Subsequently, the mixture was homogenized using a Stomacher blender (Mode l400, Seward Ltd. West Sussex, England) for 2 min at 220 rpm. Homogenate was serially diluted tenfold in 0.85% sterile saline solution.

Total viable count (TVC) and psychrophilic bacterial count (PBC) were enumerated using PCA (Vasavada *et al.,* 2015). Microbiological analyses for all the samples were carried out in triplicate (n=3).

3.4.4.3 Shear force

The shear force of shrimp meat without and with PEF treatment stored at Day 0 and 10 was measured using a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a Warner-Bratzler shear apparatus (Brauer, *et al*., 2003; Nirmal and Benjakul, 2009b). The operating parameters were a cross-head speed of 10 mm/s and a 25 kg load cell. The shear force, perpendicular to the axis of muscle fibers, was measured at the second segment of shrimp. Five samples (n = 5) were determined for each treatment. The peak of the shear force profile was regarded as the shear force value and was expressed in Newton (N).

3.4.4.4 Sensory property

All samples were collected at 0 and 10 days of storage for sensory analysis (Nirmal and Benjakul, 2011). Only samples with the TVC lower than the limit (6 log CFU/g) were used for evaluation. The selected samples were placed on a stainless

steel tray of steaming pot, covered with an aluminum foil and steamed for 5 min, in which the core temperature reached 80 °C (Manheem *et al.*, 2013). The cooked samples were evaluated by panelists, who were familiar with shrimp consumption without having any allergy. The 9-point hedonic scale (9: like extremely; 7: like moderately; 5: neither like or nor dislike; 3: dislike moderately; 1: dislike extremely) was used. Totally 50 untrained panelists with the age between 25 and 35 years old were selected All panelists were asked to evaluate for color, odor, taste, flavor and overall likeness. Samples were presented as unpeeled cooked shrimp in plates coded with three-digit random numbers. Panelists were asked to rinse their mouth during sample evaluation. Sensory analysis was conducted by panelists in an environmentally controlled partitioned booths under white incandescent light in the sensory evaluation laboratory.

3.4.5 Microstructure

At Day 0, shrimp meat samples without and with PEF treatment were immersed in liquid nitrogen. The samples were finely cut into cubical sections (4 mm × 4 mm × 4 mm) using a stainless steel razor blade and immediately fixed in 2.5% glutaraldehyde (0.2 M phosphate buffer, pH 7.2) for overnight at 4°C. All the specimens were washed with distilled water and serially dehydrated with different concentrations of ethanol as described by Rattanasatheirn *et al.* (2008). All specimens were coated with 100% gold (Sputter coater SPI-Module, PA, USA). The microstructure of specimens was analyzed using a scanning electron microscope (SEM) (Quanta 400, FEI, Brno, Czech Republic). The operation was performed at 20 kV with a spot size of 2.5 under an Environmental SEM mode. The microstructure was obtained using a secondary electron image with gaseous secondary electron detector. A standard working distance of 10 mm and pressure of 600 Torr were used for optimal image quality. The samples were visualized at a magnification of 10,000×.

3.4.6 Protein patterns

At Day 0, protein patterns of shrimp meat samples without or with PEF treatments were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Protein samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current

of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, staining of the gel was carried out with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and de-stained with 50% (v/v) methanol and 7.5% (v/v) acetic acid The protein standards (Bio-Rad Laboratories, Inc., Richmond, CA, USA) containing myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), and ovalbumin (45 kDa) were used to estimate the molecular weight of the proteins.

3.4.7 Statistical analyses

Completely randomized design (CRD) was used for entire studies. Experiments were conducted in triplicates. Randomized completely block design (RCB) was implemented for sensory analysis. The data analysis was performed by the analysis of variance (ANOVA). Differences between means were determined using the Duncan's multiple range test. Statistical analysis was performed at a level of significance ($p<0.05$) using an SPSS statistical package (SPSS 17.0, SPSS Inc., Chicago, IL, USA).

3.5 Results and discussion

3.5.1 Effect of PEF on Pacific white shrimp PPO

Relative PPO activities of cephalothoraxes treated with PEF at varying levels of electric field intensities and pulse numbers are shown in Fig. 13. The decrease in the relative PPO activity was observed as the PEF intensity and pulse number increased ($p<0.05$). Among all the samples, the highest PPO inhibition was found in the PEF-T3-treated sample ($p<0.05$). High intensity PEF treatment was documented to be effective for the inactivation of PPO in various foods to avoid undesirable color changes (Giner *et al.*, 2002). The highest PPO inhibition in the PEF-T3-treated sample might be due to higher energy (483 kJ/kg) applied which could induce denaturation of PPO. PPO is metalloenzyme containing two copper atoms located in the active site (Jang *et al.*, 2003). PPO from mushrooms and apples was reported to be inactivated as a result of electrochemical reactions, occurring at the electrode surfaces during prolonged exposure to PEF (Van *et al.*, 2001). The residual PPO activity more likely contributes to melanosis taken place in shrimp during the extended storage. The results were also in line with the PPO extracted from mushrooms and apples subjected to high

intensity PEF, which had a decrease in activity by 30%–40% (Ho and Mittal, 2000). Thus, the PPO in Pacific white shrimp cephalothorax, the anatomical part, which is more prone to melanosis, could be inactivated by PEF in an energy dose-dependent manner (54–483 kJ/kg shrimp).

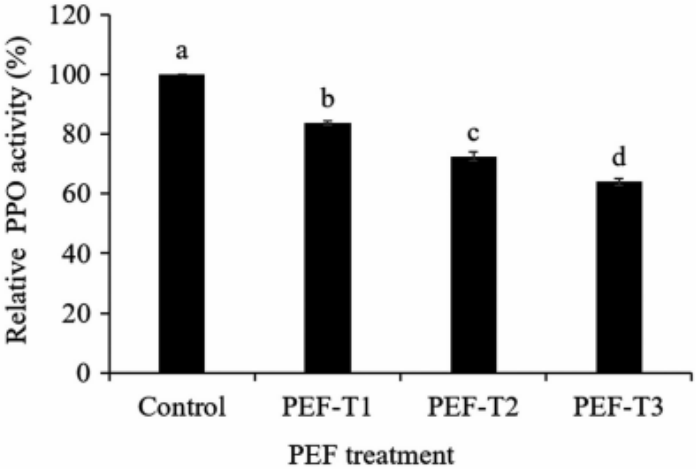

Figure 13. Polyphenoloxidase inhibition of PEF-treated Pacific white shrimp cephalothorax. Bars represent the standard deviation (n=3). Different letters on the bars represent significant differences ($p<0.05$). Control: no treatment; PEF-T1 (5 kV/cm, 200 pulses, 54 kJ/kg); PEF-T2 (10kV/cm, 400 pulses, 214 kJ/kg); PEF-T3 (15kV/cm, 600 pulses, 483 kJ/kg). PEF: pulsed electric field at different voltages of 5, 10, and 15 kV/cm, pulse numbers of 200, 400, and 600 and energy densities of 54, 214, and 483 kJ/kg.

3.5.2 Effect of PEF on melanosis and quality changes of shrimps during refrigerated storage

3.5.2.1 Changes in melanosis

Melanosis scores of control and shrimps with different PEF treatments during 10 days of storage at 4°C are presented in Fig. 14a. All samples at Day 0 were free of melanosis (score = 0). The melanosis scores of the control (without PEF treatment) increased continuously up to the end of storage (10 days) ($p<0.05$). Within the first two days of storage, there was no difference in the melanosis score between the control and PEF-T1-treated sample ($p>0.05$). Generally, the PEF-T3 treated sample had a lower melanosis score than others throughout the storage of 10 days ($p<0.05$). Lowered melanin formation in shrimp, particularly when the PEF at high PEF intensity and pulse number was applied, could be attributed to the decreased PPO activity (Fig. 14a). PPO is known as the major contributor to melanin formation (Haard and Simpson, 2000).

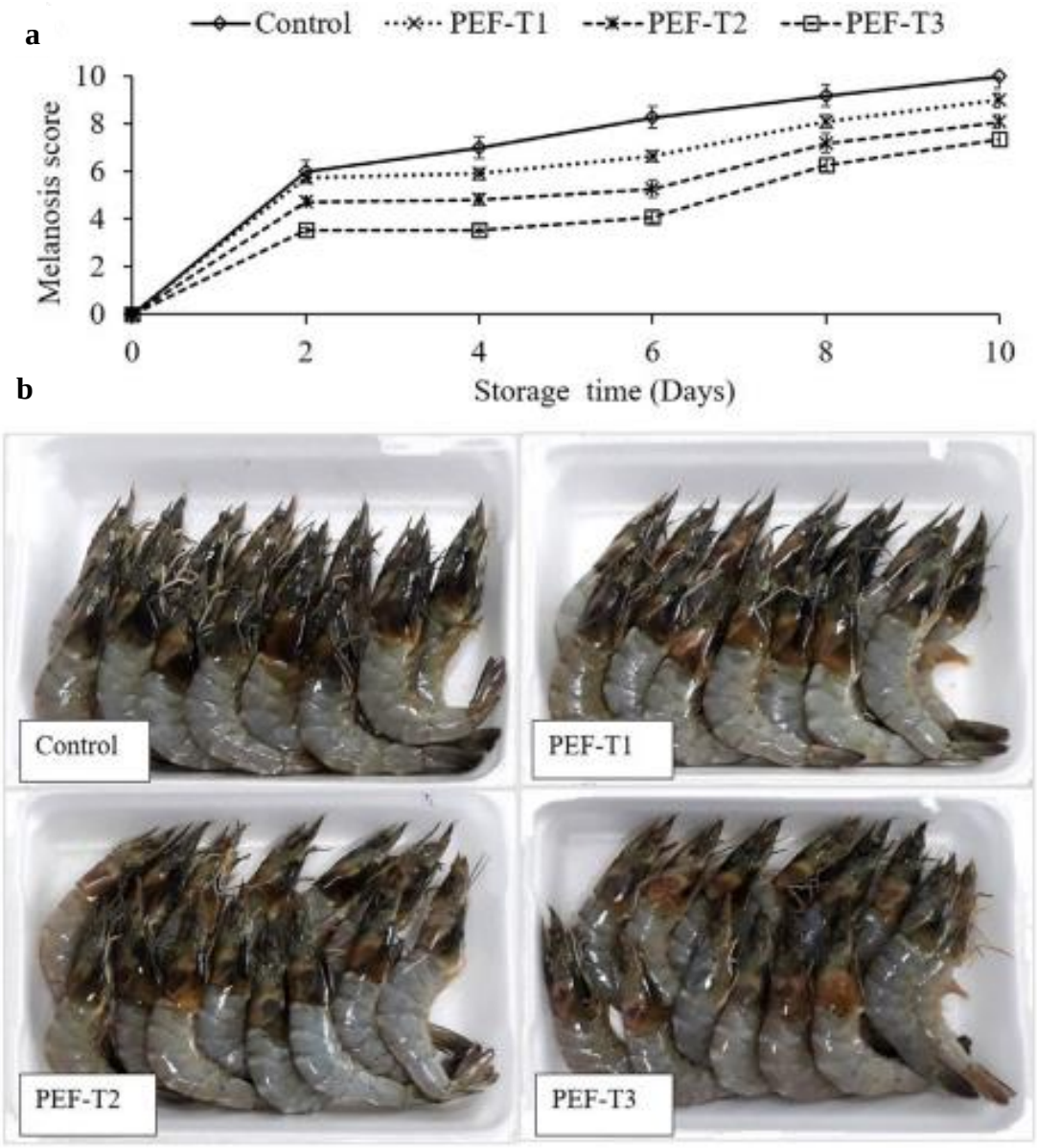

Figure 14. Melanosis score (a) of PEF-treated Pacific white shrimp without and with PEF treatment under various conditions during 10 days of refrigerated storage and photographs (b) of shrimp with different treatments after 10 days of storage.

Bars represent the standard deviation (n = 10). Control: no treatment; PEF-T1 (5 kV/cm, 200 pulses, 54 kJ/kg); PEF-T2 (10 kV/cm, 400 pulses, 214 kJ/kg); PEF-T3 (15 kV/cm, 600 pulses, 483 kJ/kg). PEF: pulsed electric field at different voltages of 5, 10, and 15 kV/cm, pulse numbers of 200, 400, and 600 and energy densities of 54, 214, and 483 kJ/kg, respectively.

The low melanosis score of the PEF-T3-treated sample at the end of storage (Day 10) was in agreement with the lowest black coloration visualized in shrimp at 4°C (Fig 14b). The exposure of shrimp to high intensity electric field pulses could be another means to inactivate PPO, thus lowering melanosis formation. Hence, PEF could

serve as an innovative technology for the prevention of melanosis of Pacific white shrimp during refrigerated storage.

3.5.2.2 Changes in microbiological quality

Microbiological changes in shrimp treated with PEF under various conditions during 10 days of storage at 4°C are presented in Fig. 15a. From Day 0 to Day 10, the PEF-T3-treated sample had lower TVC than others ($p<0.05$). TVC of all the samples increased up to 8 days of storage ($p<0.05$). On Day 10, a slight decrease in TVC was obtained for all the samples ($p<0.05$). This was plausibly attributed to the intolerance of mesophilic bacteria under the refrigerated storage condition (Shiekh *et al.,* 2019). Zeng *et al.* (2005) postulated that the inhibition of mesophilic microorganisms in shrimp meat could be owing to intolerance in a cold temperature environment. On Day 10, TVC of the control, PEF-T1, PEF-T2, and PEF-T3-treated samples was 4.92, 4.76, 4.66, and 4.58 log CFU/g, respectively. Among all the samples, the lowest TVC was obtained in the PEF-T3-treated sample, compared to others during the storage of 10 days at 4°C ($p<0.05$). Inactivation of spoilage microorganisms might be due to the phenomenon of electroporation. The critical field intensity of 10–14 kV/cm or above was reported to decrease the microbial load by the electroporation mechanism (Toepfl *et al.,* 2014). The result suggested that PEF with high electric field intensity and pulse number could lower the growth of mesophile in shrimp.

Changes in PBC of control and PEF-treated samples during refrigerated storage are depicted in Fig. 15b. On Day 0, no difference in PBC was noticeable in all samples ($p>0.05$). Generally, PBC increased from Day 2 to Day 10 ($p<0.05$). The highest PBC was obtained in the control, compared to PEF-treated shrimps ($p<0.05$). However, the PEF-T3-treated sample showed the lowest PBC during 10 days of storage, compared to others ($p<0.05$). The control, PEF-T1, PEF-T2, PEF-T3-treated shrimps had PBC of 6.10, 5.92, 5.80, and 5.65 log CFU/g, respectively, at the end of storage.

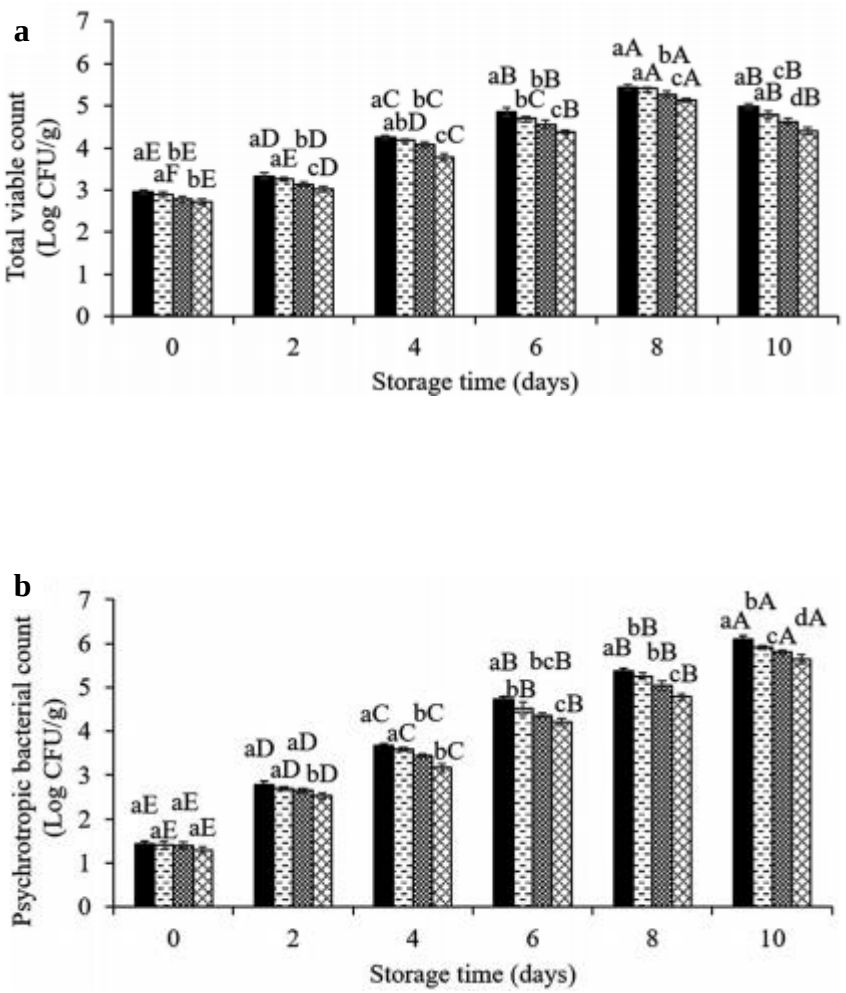

Figure 15. Total viable count (a) and psychrophilic bacterial count (b) of Pacific white shrimp without and with PEF treatment under various conditions during 10 days of refrigerated storage. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Control: no treatment; PEF-T1 (5 kV/cm, 200 pulses, 54 kJ/kg); PEF-T2 (10 kV/cm, 400 pulses, 214 kJ/kg); PEF-T3 (15 kV/cm, 600 pulses, 483 kJ/kg). PEF: pulsed electric field at different voltages of 5, 10, and 15 kV/cm, pulse numbers of 200, 400, and 600 and energy densities of 54, 214, and 483 kJ/kg, respectively.

Thus, the treatment of shrimp with PEF, especially at higher PEF intensity, pulse number (600), and PEF specific energy (483 kJ/kg), was able to retard the growth of psychrophilic bacteria during the refrigerated storage. The psychrophilic bacteria in raw shrimp remain active under refrigerated environmental conditions and have been referred to as the main cause of spoilage during refrigerated storage (Sae-

leaw *et al.,* 2018; Shiekh *et al.,* 2019). PEF at high intensity electric field parameters has been documented to inactivate spoilage and pathogenic microorganisms by the formation of holes in the cell membrane due to electroporation that causes protoplast lysis and leakage of intracellular content, leading to microbial cell death (Castro *et al.*,1993).

Electroporation results in the increased permeability of cell membranes made up of charged ions and proteins that create a gradient referred to as transmembrane potential (TMP) across the cell membrane (Gulzar and Benjakul, 2019). When an external electric field is applied above TMP, polarity-induced charge separation across the cell membrane produces a dipole moment that is parallel to the external field (Zbinden *et al.,* 2013). Due to the presence of this external field with high-intensity short-duration pulses, pores are formed (Teissie *et al.,* 2005). Furthermore, PEF in combination with heat treatment was reported to inactivate pathogenic microorganisms such as Salmonella typhimurium (Yun *et al.,* 2017). Therefore, the use of PEF for the treatment of shrimps potentially inactivated psychrophilic microorganisms responsible for shrimp spoilage.

3.5.2.3 Changes in shear force

The shear force of the meat of Pacific white shrimp without and with different PEF treatments at Day 0 and 10 of storage is depicted in Fig. 16. At Day 0 of storage, all PEF-treated samples showed lower shear force (21.33–19.06 N) than that of control (22.33 N) ($p<0.05$). The decrease in shear force observed in shrimp subjected to PEF, especially with higher electric field intensity and pulse number, might be owing to the disruption of muscle fiber arrangement by high energy dissipation (483 kJ/kg) or pulses. As a result, the looser muscle structure was attained, leading to the softening of muscle. PEF-T3-treated sample showed the lowest value among all the PEF-treated samples and control ($p>0.05$). As the storage time increased (Day 10), different shear forces values were obtained among all the samples ($p<0.05$). The highest shear force was noticeable in the PEF-T3-treated samples, while the control showed the lowest shear force ($p<0.05$). These results revealed that muscle softening of shrimp subjected to PEF during the extended refrigerated storage might be retarded, more likely due to the inactivation of spoilage bacteria and proteolytic enzymes by PEF. For the control (without PEF treatment), indigenous proteases were still retained and microbial

proteases were liberated, leading to the cleavage of peptides. PEF has been reported to inactivate various enzymes including proteases (Van Loey *et al.*, 2001). Thus, the muscle softening of Pacific white shrimp was prevented to some degree when PEF, particularly at high PEF intensity and pulse number, was applied.

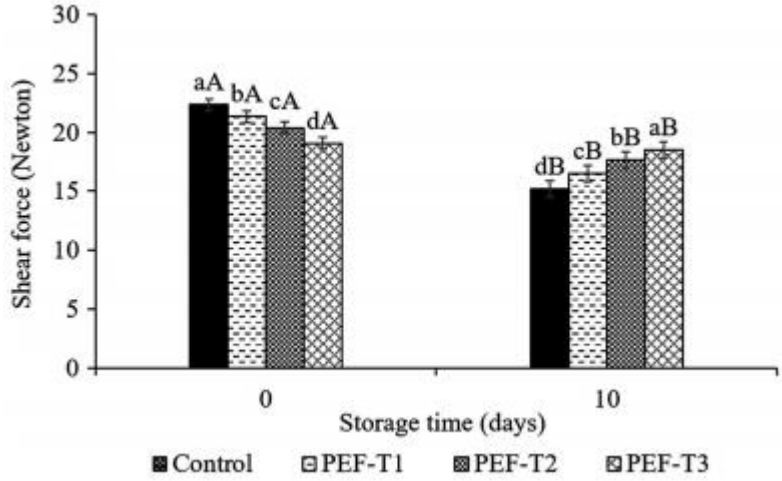

Figure 16. Shear force of Pacific white shrimp without and with PEF treatment under various conditions at Day 0 and 10 of refrigerated storage.

Bars represent the standard deviation (n = 5). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Control: no treatment; PEF-T1 (5 kV/cm, 200 pulses, 54 kJ/kg); PEF-T2 (10 kV/cm, 400 pulses, 214 kJ/kg); PEF-T3 (15 kV/cm, 600 pulses, 483 kJ/kg). PEF: pulsed electric field at different voltages of 5, 10, and 15 kV/cm, pulse numbers of 200, 400, and 600 and energy densities of 54, 214, and 483 kJ/kg, respectively.

3.5.2.4 Changes in sensory acceptance

Likeness scores of PEF-treated shrimps with varying electric field intensities and pulse numbers at Day 0 and 10 of refrigerated storage are presented in Table 4. On Day 0, no differences in likeness scores for all attributes were noticeable among all samples ($p>0.05$). The result suggested that PEF at levels up to 15 kV/cm and 600 pulses with PEF specific energy value of 483 kJ/kg had no adverse effect on the likeness or acceptability of shrimp meat. After 10 days of storage, the decreases in likeness scores for all attributes in all samples were observed ($p<0.05$). However, the higher scores for all attributes were found in the PEF-T3-treated sample, compared to PEF-T1, PEF-T2 treated samples, and the control ($p<0.05$). Control yielded the lowest scores for all attributes tested ($p<0.05$). The higher color likeness score was coincidental with the lower melanosis in PEF-T3-treated shrimp (Figure 14a).

Therefore, the PEF at higher electric field intensity and pulse number had no negative impact on sensorial quality. Moreover, PEF under the aforementioned condition rendered the shrimp with higher likeness scores. Lowered muscle softening in the PEF-T3-treated sample might contribute to higher texture likeness score. For flavor and odor, the higher score of the PEF-T3-treated sample was related to the lower TVC and PBC.

Table 4. Likeness score of Pacific white shrimp without and with PEF treatment under various conditions at Day 0 and 10 of refrigerated storage.

Storage time (days)	Samples	Color	Odor	Texture	Flavor	Taste	Overall
0	Control	8.60±0.63aA	8.60±0.63aA	8.00±0.76aA	8.03±0.58aA	8.07±0.88aA	8.03±0.61aA
	PEF-T1	8.67±0.49aA	8.67±0.49aA	8.10±0.71aA	8.07±0.53aA	8.10±0.81aA	8.12±0.68aA
	PEF-T2	8.67±0.49aA	8.67±0.49aA	8.17±0.88aA	8.20±0.41aA	8.13±0.99aA	8.17±0.59aA
	PEF-T3	8.73±0.59aA	8.73±0.59aA	8.23±0.78aA	8.27±0.59aA	8.17±0.72aA	8.20±0.41aA
10	Control	5.07±0.53cB	5.07±0.53cB	4.00±0.41cB	5.13±0.64cB	4.80±0.30cB	5.00±0.38cB
	PEF-T1	5.37±0.64cB	5.37±0.64cB	4.47±0.52cB	5.27±0.46bcB	5.20±0.41bcB	5.20±0.41cB
	PEF-T2	6.07±0.26bB	6.07±0.26bB	5.53±0.74bB	5.67±0.49bB	5.53±0.52bB	5.53±0.52bB
	PEF-T3	7.03±0.13aB	6.40±0.63aB	6.13±0.83aB	6.50±0.73aB	6.40±0.63aB	6.53±0.44aB

Note: Values represent the mean ± standard deviation (n = 50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: See the caption for Fig.14.

Lower load of the aforementioned microorganisms resulted in the lower formation of off-odor or off-flavor components. Non-thermal processing technologies such as PEF and HPP have been employed for juice processing, in which PEF processed juices appear to have fresh flavor without any detrimental effect on organoleptic quality, compared to HPP and thermally treated juices as perceived by the consumers (Lee *et al.*,2017). The result suggested that non-thermal processes like PEF could aid in maintaining the quality of Pacific white shrimps stored at a refrigerated temperature for an extended time.

3.5.3 Effect of PEF on the property of shrimp meat

3.5.3.1 Microstructure

Microstructures of Pacific white shrimp meat with and with- out PEF treatment at various conditions revealed considerable changes in muscular bundles as illustrated in Fig. 17a. PEF processing resulted in more gaping between the muscle fibers. The control (without PEF treatment) had negligible gaping of muscle fibers, while PEF-treated samples showed the increased gaping, particularly as the energy of PEF applied increased. The highest gaping was marked in the PEF-T3-treated sample. The results were coincidental with the shear force values at Day 0 of refrigerated storage (Fig. 16), in which partial detachment of muscle fibers was more pronounced. The looser structure might be related to the lower shear force (Fig. 16). The similar results were reported in PEF-treated fish and chicken meat (Gudmundsson and Hafsteinsson, 2001). Thus, the PEF-treated shrimp muscle fibers might undergo detachment or dissociation due to electroporation. Furthermore, PEF at high intensity with higher energy value (483 kJ/kg shrimp) might induce the breakage of collagen molecules localized between muscle fibers, causing the looser structure of muscle fibers. The insoluble fraction of collagen was reported to induce networking among the collagen molecules that act as a cementing material for the attachment of shrimp muscle fibers (Sriket *et al.*, 2007). The gaping pattern in fish muscle fibers was also documented to be dependent on insoluble collagen fraction in fish meat (Espe, 2004). Disruption of cross-links in the insoluble collagen network corresponded to a lower shear force (Pilar Montero and Borderias, 1990). Therefore, microstructural changes induced by PEF applied as evidenced by gaping developed had the influence on textural property of shrimp meat.

3.5.3.2 Protein pattern

Protein patterns of PEF-treated samples at different electric field intensities and pulse numbers are shown in Fig. 17b. No differences in the protein patterns were found among all the samples. Myosin heavy chain and actin were found as two major proteins in shrimp muscle. Thus, PEF up to 15 kV/cm or 600 pulses had no influence on shrimp muscle proteins to any extent.

a

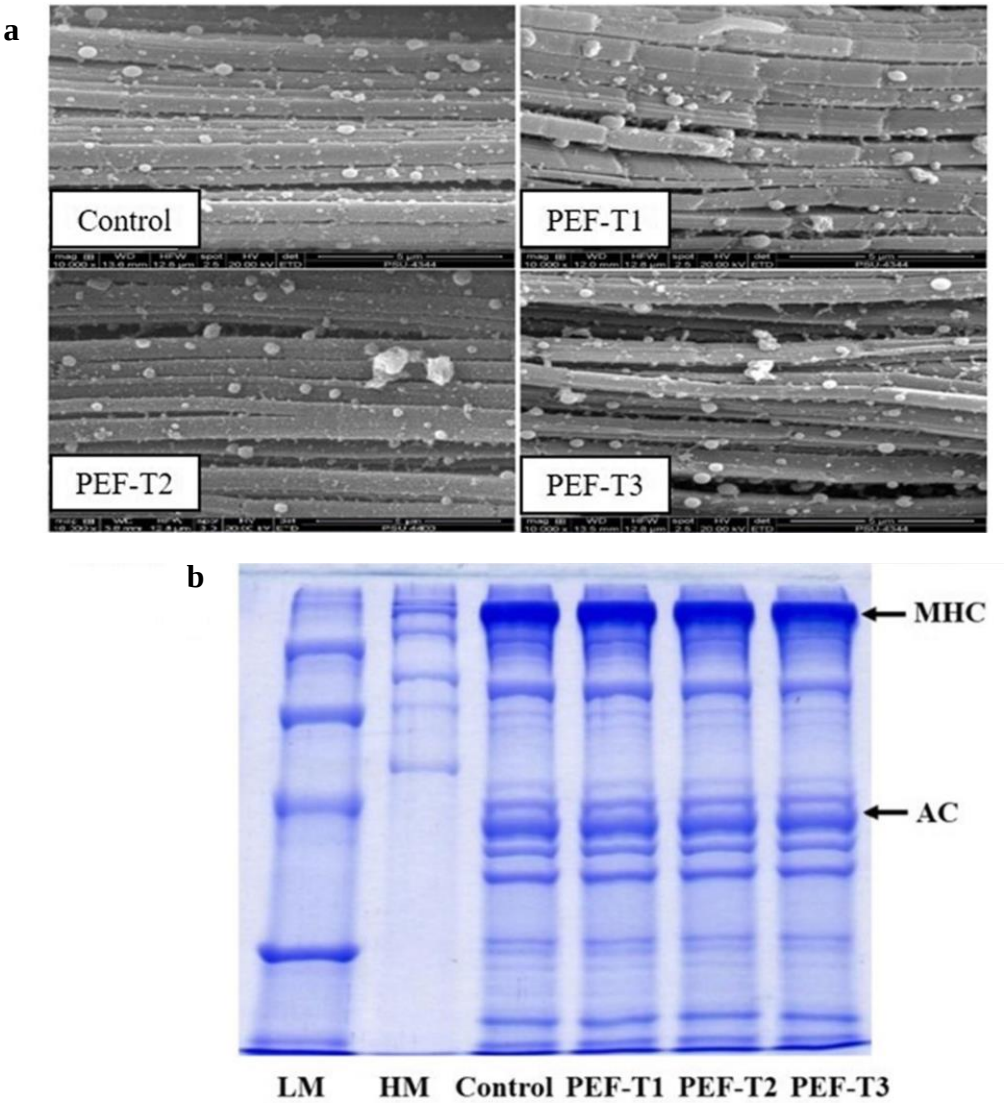

Figure 17. Electron microscopic images (a) and protein pattern (b) of Pacific white shrimp without and with PEF treatment under various conditions.

LM: Low molecular weight marker, HM: High molecular weight marker, MHC: Myosin heavy chain, AC: Actin, Control: no treatment; PEF-T1 (5 kV/cm, 200 pulses, 54 kJ/kg); PEF-T2 (10 kV/cm, 400 pulses, 214 kJ/kg); PEF-T3 (15 kV/cm, 600 pulses, 483 kJ/kg). PEF: pulsed electric field at different voltages of 5, 10, and 15 kV/cm, pulse numbers of 200, 400, and 600 and energy densities of 54, 214, and 483 kJ/kg, respectively.

Protein bands of all the treated samples revealed a similar pattern to the untreated control sample. Similar results were documented for fish proteins subjected to high electric field strength up to 18.6 kV/cm (Gudmundsson and Hafsteinsson, 2001). Furthermore, PEF treatment in the range of 27–33 kV/cm or 50–400 pulses did not notably alter the ovalbumin structure (Fernandez-Diaz *et al.,* 2000). The results suggested that PEF did not cause protein degradation in Pacific white shrimp muscle, regard- less of the aforementioned PEF conditions applied.

3.6 Conclusions

PEF inactivated PPO in an energy-dependent manner. Lower melanosis score and higher PPO inhibition were noticed in the PEF-T3-treated sample. More gaping was visualized in PEF-T3-treated sample, related to the lowered shear force, but PEF had no impact on the protein pattern of shrimp meat. Lowered increases in mesophile and psychrophile were noticeable in the PEF-T3-treated sample during 10 days of refrigeration storage. After 10 days of storage, the PEF-T3-treated sample had the highest likeness score and possessed the highest shear force. Thus, PEF could be used to lower melanosis and retard the quality loss of Pacific white shrimp during the refrigerated storage.

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CHAPTER 4

MELANOSIS AND QUALITY CHANGER DURING REFRIGERATED STORAGE OF PACIFIC WHITE SHRIMP TREATED WITH CHAMUANG (*GARCINIA COWA* ROXB.) LEAF EXTRACT WITH THE AID OF PULSED ELECTRIC FIELD

4.1 Abstract

Pacific white shrimp with prior pulsed electric field (PEF) treatment before soaking in Chamuang leaf extract (CLE) at different concentrations (0.5 and 1%) for 30 min were prepared. Sample pre-treated with PEF and soaked with 1% CLE (PEF-1 CLE) showed lower melanosis score than that with 1.25% sodium metabisulfite treatment, PEF treated sample or those soaked in CLE without prior PEF and the control during storage of 10 days ($p<0.05$). PEF-1 CLE sample showed lower total volatile base content, peroxide value and thiobarbituric acid reactive substances but high sensory scores than others ($p<0.05$). Lower increases in mesophile, psychrophile, Pseudomonas, Enterobacteriaceae and H_2S producing bacterial counts were obtained in PEF-1 CLE, compared to the control and other treated samples. The most abundant compounds from Chamuang leaf extract, including Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside and veranisatin-C were found in PEF-1 CLE sample and were plausibly involved in keeping quality of shrimp.

4.2 Introduction

Melanosis in Pacific white shrimp is mediated by polyphenoloxidase (PPO), which induces phenol oxidation with subsequent melanin formation (Haard and Simpson, 2000). After capture, melanosis can occur within few hours, mainly at carapace (cephalothorax), telson, pereopods and pleopods (Gonçalves and de Oliveira, 2016). Such a discoloration drastically reduces market value of whole raw shrimp, thereby affecting the economic status of farmer, vender as well as shrimp processing industry. To conquer the problem, synthetic and natural additives have been employed as antimelanosis agents to alleviate melanosis in shrimp during chilled or refrigerated storage (Sae-leaw and Benjakul, 2019). Synthetic preservatives such as sodium metabisulfite (SMS) might cause allergic sensation and other health complications in

sulfite sensitive patients (Kronberg, 2008). Natural additives, particularly plant extract rich in polyphenols, can serve as the effective PPO inhibitors, antioxidative and antimicrobial agents (Maqsood *et al.,* 2013).

Pacific white shrimp quality must be well controlled during cold chain distribution (Ndraha *et al.,* 2019). Maintenance of quality of shrimp is of primary concern for seafood processing industries, in which the shelf-life can be prolonged. Refrigeration and frozen storage of shrimp have been demonstrated to reduce microbial counts and to retard physico-chemical changes associated with quality losses (Nirmal and Benjakul, 2010). However, the efficacy of refrigeration or frozen storage without natural additives is insufficient to preserve quality of shrimp. Chemical and biochemical changes still occur even at frozen temperatures (Fennema *et al.,* 2008). Lipids and proteins in the muscle of shrimp undergo oxidation, which can deteriorate the organoleptic quality of the product, such as odor, color, texture, etc. as well as dehydration and weight loss (Tsironi *et al.,* 2009). Shelf-life extension of Pacific white shrimp can be achieved by the addition of natural preservatives that can synergistically prevent quality loss during refrigerated storage (Sae-leaw and Benjakul, 2019).

Pulsed electric field (PEF) is a non-thermal and additive free technology introduced for food processing and preservation without any detrimental effect on nutritional profile (Barbosa-Cánovas and Altunakar, 2006). Without any thermal interference, foods retain high quality (Ho and Mittal, 2000). PEF has been served as an antimicrobial technology, which can reduce microbial load of foods (Lado and Yousef, 2002). PEF in combination with heat treatment was reported to inactivate pathogenic microorganisms such as Salmonella typhimurium (Yun *et al.,* 2017). PEF is based on electroporation and cell disintegration by the application of repeated pulses when the object is inserted between the two parallel electrodes (Gudmundsson and Hafsteinsson, 2001). The main cause of cell destabilization is based on the imbalance in transmembrane potential due to electrochemical gradient maintained across the cell membrane. Moreover, the electroporation becomes well pronounced, if the field strength (E) exceeds the reversible threshold (Ec) with sufficient duration of exposure time that favors reversible electroporation (Čorović *et al.,* 2012). Excessive field strength and level of energy result in an irreversible electroporation (Al-Sakere *et al.,* 2007). PEF also inactivated enzymes localized in several foods (Van Loey *et al.,* 2001).

PEF can inactivate some enzymes potentially by inducing the denaturation via the conformational alteration (Zhong *et al.,* 2005). Activities of lipase, protease and alkaline phosphatase in fresh bovine milk subjected to PEF treatment at electric field strengths (15–35 kV/cm) were reduced to 14%, 37% and 29%, respectively (Riener, Noci *et al.,* 2009).

Plant extracts rich in naturally occurring polyphenolics can function as antioxidant and antimicrobial agents for preservation of seafoods (Olatunde and Benjakul, 2018). Cashew leaf extract (CE) has been documented as a strong antimelanosis agent with antimicrobial and antioxidant potential for preservation of shrimp (Sae-leaw and Benjakul, 2019). Polyphenolic extracts from grape seed and green tea prevented melanosis and deterioration in Pacific white shrimp (Gokoglu and Yerlikaya, 2008; Nirmal and Benjakul, 2009a). Recently, Chamuang leaf extract (CLE) has been reported to prevent melanosis as well as impede chemical and microbial quality changes of refrigerated Pacific white shrimp (Shiekh *et al.,* 2019). CLE possessed antimicrobial properties and was able to retard microbial growth (Sakunpak and Panichayupakaranant, 2012). Since electroporation induced by PEF might loosen structure of shrimp, thus facilitating the migration of the plant extract with melanosis inhibitory activity into shrimp more potentially. This study was conducted to evaluate the combined impact of PEF pre-treatment and CLE soaking to lower melanosis and quality deterioration of Pacific white shrimp during 10 days of storage at 4 °C.

4.3 Objectives

To analyse the abundance of CLE passage via PEF electroporation in shrimp meat.

To investigate the combined effect of PEF and CLE treatments on melanosis and quality loss of Pacific white during 10 days storage.

4.4 Materials and methods

4.4.1 Chemicals

Sodium metabisulfite (SMS) was obtained from Fisher Scientific (Loughborough, Leicestershire LE11 5RG, UK). Rest of the chemicals were bought from Sigma Aldrich (St. Louis, MO, USA). All media for microbial analyses were procured from Oxoid Ltd. (Hampshire, UK).

4.4.2 Shrimp procurement and preparation

Freshly captured Pacific white shrimp free of preservatives were bought from a market, Hat Yai, Thailand. To avoid mechanical damages, shrimp were handled with care by placing in polystyrene boxes, which were filled with ice, twice that of the sample weight. Shrimp were immediately delivered to the seafood chemistry and biochemistry laboratory, Prince of Songkla University, Hat Yai. Immediately on arrival to the laboratory, shrimp were subjected to washing using an ice slurry to remove extraneous matter. Washed shrimp were kept in crushed ice before experiments were conducted (< 1h).

4.4.3. Pre-treatment of shrimp using pulsed electric field (PEF)

Cleaned shrimp were subjected to a Lab scale PEF system with high voltage power supply (PEF LAB-400 W, Febix International Inc., Chiang Mai, Thailand). PEF system consisted of a stainless steel treatment chamber with 1.5 L capacity equipped with a stainless steel electrode 20 cm × 10 cm, a plexiglass cabinet with aluminium frame, a digital storage oscilloscope (DSO) (UTD2052CEX, UNI-T®, Dongguan city, Guangdong, China) and a UT-P03 oscilloscope probe (600 MHz, 10X) connected with a data logger. Scheme of experimental set-up employed for PEF pre-treatment of shrimp is shown in Fig. 18. Samples (10 shrimps) were placed in one layer in a horizontal treatment chamber attached with a perforated plastic mesh at the bottom to avoid direct contact with the bottom electrode. Shrimp were then covered with top electrode for the effective exposure to PEF. The chamber was filled with tap water (600 mL) to ensure that electrode was submerged. A uniform electrode gap of 1.5 cm was maintained. The PEF treatment chamber was placed in ice slurry circulated with cold water to maintain temperature below 1 °C. PEF (15 kV/cm, 600 pulses) with the PEF specific energy of 483 kJ/kg was applied, respectively.

4.4.4 Extraction of Chamuang leaf extract (CLE)

Mature Chamuang leaves were plucked from trees (5−7-year-old) in an orchard in Hat Yai in August 2018 and prepared as described by Shiekh *et al.* (2019). CLE was prepared in distilled water using high intensity power ultrasonic equipment (Amplitude: 70%; Time of sonication: 30 min; pulse on time: 50 s; and pulse off time: 10 s) as reported by Chotphruethipong *et al.* (2019). The CLE was lyophilized using

an equipment (Cool Safe 55, ScanLaf A/S, Lynge, Denmark). Total phenolic content of 546.7 mg gallic acid equivalent (GAE)/g of lyophilized CLE was determined (Benjakul *et al.*, 2014).

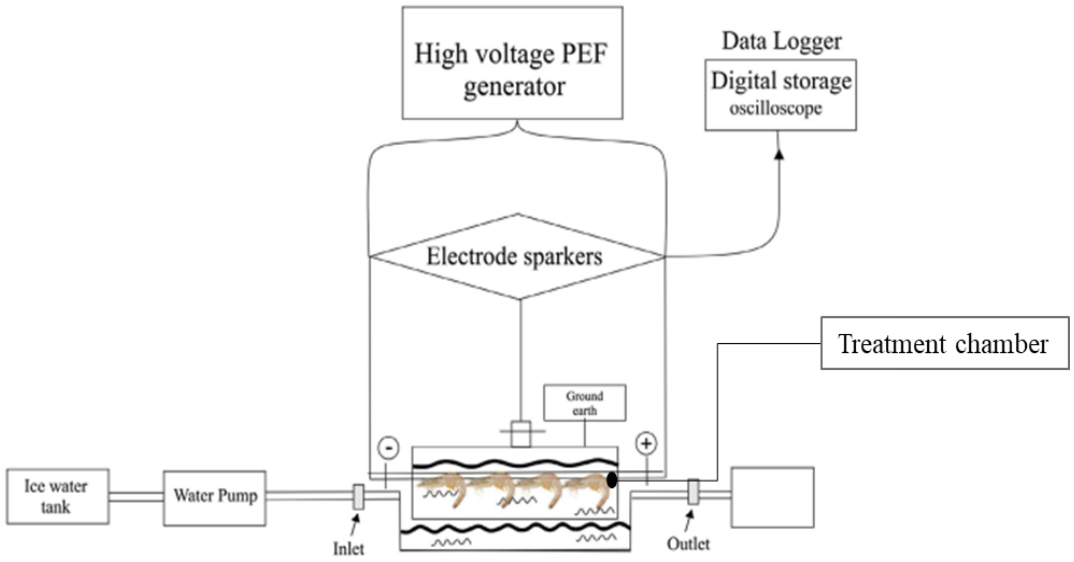

Figure 18. Schematic experimental set-up employed for PEF pre-treatment of Pacific white shrimp.

4.4.5 Effect of CLE treatment without and with PEF pre-treatment on melanosis and quality changes of refrigerated shrimp

Whole shrimp (without and with prior PEF) were immersed in different concentrations of CLE solutions (0.5 and 1%) prepared in distilled water with a shrimp/solution ratio of 1:2 (w/v) for 30 min at 4 °C (Nirmal and Benjakul, 2009b). The samples were named as '0.5-CLE' and '1-CLE' or 'PEF-0.5 CLE' and 'PEF-1 CLE', respectively. The samples without and with 1.25% SMS treatment for 30 min referred to as 'Control' and 'SMS-T' were also prepared. All the treated samples were left on plastic sieves at 4 °C for 5 min. Sample subjected to only PEF named 'PEF-T' was also prepared. Thereafter, shrimps were transferred into foam trays and covered with shrink plastic film and kept at 4 °C. Samples were taken for analyses at an interval of 2 days up to 10 days.

4.4.5.1 Melanosis assessment

Melanosis or blackening of shrimp was analyzed using 10-point melanosis score as tailored by Nirmal and Benjakul (2010). Ten trained panelists were selected for the evaluation.

4.4.5.2. Microbiological analyses

Spread plate method was adopted for the enumeration of total viable count (TVC), psychrophilic bacterial count (PBC), *Pseudomonas,* Enterobacteriaceae and H_2S-producing bacteria (Sallam, 2007).

4.4.5.3 Analysis of total volatile base (TVB) content

TVB content was measured using Conway micro-diffusion method in shrimp meat (Shiekh *et al.*, 2019).

4.4.5.4. Determination of peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

Firstly, lipids were extracted from shrimp meat and analyzed for PV by ferric thiocyanate method (Chaijan *et al.*, 2006). TBARS in shrimp meat was determined as described by Benjakul and Bauer (2001).

4.4.5.5. Sensory evaluation

All samples were prepared and previously described for sensory analysis at day 0 and 10 of storage. Sensory evaluation was carried out via the procedure of Sae-leaw and Benjakul (2019).

4.4.6. Identification of CLE in the treated shrimp

PEF-1 CLE sample (10 shrimps) were collected and peeled to obtain shrimp meat. Shrimp meat was added with distilled water (1:10, w/v) and homogenized at 8000 rpm for 2 min using an IKA homogenizer model T 25 D (IKA-Werke GmbH and Co. KG, Staufen, Germany). The homogenate was then subjected to centrifugation at 8000 × g for 20 min at 4 °C using a Beckman Coulter centrifuge (Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was further filtered to remove suspended particles using a Whatman No. 4 filter paper. Filtrate was further freeze dried using a Model Coolsafe 55, Scanvac freeze dryer (Coolsafe, Lynge, Denmark). Shrimp

extract powder (SEP) obtained was further subjected to LC-MS analysis. CLE constituents in SEP was determined by LC-MS analysis as detailed by Shiekh *et al*. (2019)

4.4.7. Statistical analyses

Completely randomized design (CRD) was used for entire study. Randomized completely block design (RCB) was implemented for sensory analysis study. All experiments were done in triplicates. Analysis of variance (ANOVA) and mean comparison were performed using the Duncan's multiple range test. SPSS statistical package (SPSS 17.0, SPSS Inc., Chicago, IL, USA) was used for analysis.

4.5 Results and discussion

4.5.1 Effect of PEF pre-treatment and CLE on melanosis and quality changes of refrigerated shrimp

4.5.1.1. Changes in melanosis

Melanosis scores of control, shrimp treated with 1.25% SMS and prior PEF shrimp without and with soaking in CLE (0.5 or 1%) during 10 days at 4 °C are depicted in Fig. 19a. No melanosis appeared at day 0. The melanosis score of the control (without any treatment) augmented continuously throughout storage ($p<0.05$). During the storage of 10 days, PEF treated sample (PEF-T) had lower melanosis score than the control ($p<0.05$). The lowered melanosis in PEF-T sample might be due to higher electric field intensity and PEF energy (483 kJ/kg), which could induce denaturation of PPO. The relative activity of PPO was measured to be 40% in Pacific white shrimp cephalothorax after PEF application (Shiekh and Benjakul, 2019). Shrimp treated with SMS alone (SMS-T) had melanosis score similarly to those treated with 1% CLE, regardless of prior PEF within the first 2 days ($p>0.05$). Nevertheless, a sharp increase in melanosis score of SMS-T sample was noticeable during 2−10 days ($p<0.05$) and showed higher score than others except the control after day 6 ($p<0.05$). Instability of SMS associated with sulfur dioxide formation during prolonged storage might cause the loss in its efficacy in melanosis inhibition (Shiekh *et al*., 2019). Generally, melanosis was visualized in PEF-1 CLE sample to the least extent ($p<0.05$), followed by 1-CLE, PEF-0.5 CLE and 0.5-CLE samples, respectively.

Lowered melanin formation in shrimp was attained, particularly when the PEF at high field intensity, pulse number and PEF energy was applied in combination with 1% CLE treatment. This could be attributed to the denaturation of PPO by PEF electroporation and the penetration of CLE, a PPO inhibitor to pass through the underneath tissues as a result of cell disintegration in shrimp cephalothorax. The highest cell disintegration index in cephalothorax of Pacific white shrimp was achieved when PEF at 12 or 16 kV/cm and 240 pulses were applied (Gulzar and Benjakul, 2019). The result indicated that PEF treatment alone (PEF-T) also exhibited melanosis inhibition to some degree ($p<0.05$). Nevertheless, the residual PPO after PEF treatment could cause the progression of melanosis in refrigerated shrimp. Overall, PEF plausibly electropermeabilized shrimp carapace or shell to some extent and facilitated the influx of CLE towards PPO localized sites, particularly underneath the carapace and shell. PPO is known as the major contributor to melanosis in shrimp (Haard and Simpson, 2000). High field intensity PEF treatment itself was documented to inactivate PPO in various foods, especially in mushroom and apple to prevent undesirable color changes (Giner *et al*., 2002; Van Loey *et al*., 2001). With high level of CLE (1%), PPO inhibitors in the extract could penetrate into shrimp to higher extent. Polyphenols from cashew and Chamuang leaves were able to delay melanosis in shrimp (Sae-leaw and Benjakul, 2019; Shiekh *et al*., 2019). Polyphenols with high antioxidative power such as catechin have been used as effective antimelanosis agents (Nirmal and Benjakul, 2009b). Hence, PEF could serve as an innovative technology that facilitated the passage of CLE to the area underneath the shell or carapace via electroporation. Thus, PEF in conjugation with CLE treatment prevented melanosis synergistically in refrigerated shrimp. This was shown by the least melanosis developed in PEF-1 CLE sample (Fig. 19b), while the control had the highest melanosis at day 10 of storage.

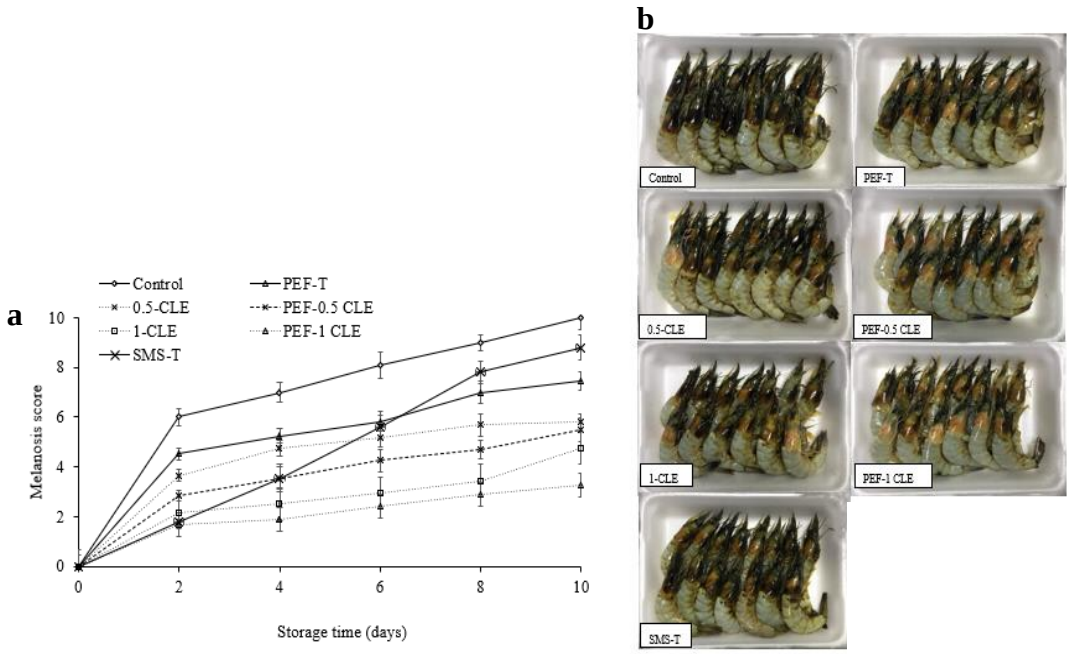

Figure 19. Melanosis score (a) and photographs (b) of Pacific white shrimp pretreated without and with PEF and treated without and with CLE during 10 days of refrigerated storage.
Bars represent the standard deviation (n=10 panelists). Control: no treatment; PEF-T: sample treated with PEF (15 kV/cm, 600 pulses, 483 kJ/ kg). 0.5-CLE or 1-CLE: sample treated with CLE at concentrations of 0.5 and 1%, respectively. PEF-0.5 CLE and PEF-1 CLE: sample pre-treated with PEF, followed by soaking in CLE at concentrations of 0.5 and 1%, respectively. SMS-T: sample treated with sodium metabisulfite (1.25%).

4.5.1.2. Changes in microbiological quality

At day 0, SMS-T sample and those soaked with CLE at both levels, irrespective of prior PEF possessed lower TVC than the control ($p<0.05$) (Fig. 20a). Nevertheless, no difference was attained in TVC between control and PEF-T sample ($p>0.05$). Increases in TVC of all the samples were noticeable up to 8 days of storage ($p<0.05$). Slight decrease in TVC, representing mesophilic bacterial count, was noted at day 10. At day 10, TVC of all the samples including control, PEF-T, 0.5-CLE, 1-CLE, PEF-0.5 CLE, PEF-1 CLE and SMS-T samples was 4.92, 4.58, 4.30, 4.16, 3.98, 3.69 and 4.64 log CFU/g, respectively. This was reasonably the result of intolerance of mesophilic microbes at low temperature (Shiekh *et al.*, 2019). Among all the samples, the lowest TVC was obtained in PEF-1 CLE sample, compared to others during the storage ($p<0.05$) (Fig. 21a). It was noted that samples treated with CLE showed the lowest TVC, regardless of prior PEF. The bactericidal effect of extract from Chamuang leaves could be summarized due to antimicrobial ability of polyprenylated benzophenone, organic acids and polyphenolic glycosides (Sakunpak

and Panichayupakaranant, 2012; Shiekh *et al*., 2019). During 4–10 days, TVC of PEF-T sample was lower than that of control. The critical field intensity of 10 to 14 kV/cm or above was reported to reduce microbial flora (Toepfl *et al.,* 2014). PEF at various levels of high intensity electric field pulses significantly reduced the load of mesophile and psychrophile of Pacific white shrimp during extended refrigerated storage (Shiekh and Benjakul, 2019). Thus, prior PEF (15 kV/cm, 600 pulses, 483 kJ/kg) followed by treatment with CLE (1%) had the combined impact on retarding the growth of mesophiles in shrimp kept for 10 days.

At day 0, PBC exhibited no difference among samples ($p>0.05$), except for those treated with CLE at 1%, regardless of prior PEF, which showed the lower PBC ($p<0.05$) (Fig. 20b). Overall, PBC showed the similar trend to TVC during the storage. PEF-T sample and SMS-T sample had lower PBC, compared to the control($p<0.05$). During the storage, PEF-1 CLE sample showed the lowest PBC than others ($p<0.05$). Nevertheless, increased PBC were obtained in all the samples up to day 10. PBC of control, PEF-T, 0.5-CLE, 1-CLE, PEF-0.5 CLE and PEF-1 CLE and SMS-T samples was 6.10, 5.57, 5.17, 4.79, 4.20, 3.69 and 4.94 log CFU/g, respectively, at day 10. Thus, the treatment of shrimp with PEF was able to inhibit the growth of PBC to some extent. PBC in raw shrimp at low temperature were dominant and actively participated in spoilage of shrimp (Sae-leaw and Benjakul, 2018; Shiekh *et al*., 2019). PEF at high intensity electric field has been documented to kill pathogenic and spoilage microorganisms by damaging the cell membrane due to electroporation and cell disintegration (Castro *et al.,* 1993). Additionally pores of bacterial cell membrane might be formed, thus allowing CLE to penetrate and functioned as antimicrobial agent more effectively. Prior PEF of shrimps along with CLE treatment, particularly at higher level, was capable of retarding PBC during the refrigerated storage potentially. Therefore, PEF and CLE jointly decelerated the growth of psychrophilic microbes, in which shrimp spoilage under refrigeration could be impeded.

At the first day of storage, *Pseudomonas* counts of control and other treated samples were found (2.15–2.18 log CFU/g) (Fig. 20c). Slightly higher counts of *Pseudomonas* were enumerated in the control, PEF-T, 0.5-CLE and PEF-0.5 CLE samples ($p<0.05$) than SMS-T, 1-CLE and PEF-1 CLE samples, at day 0 ($p<0.05$). Increases in *Pseudomonas* count of all samples were obtained with the advancement of

storage time ($p<0.05$). During the storage of 10 days, the lowest count was observed in PEF-1 CLE sample ($p<0.05$). Maximum *Pseudomonas* count of 6.82 log CFU/g was obtained in the control, while the PEF-1 CLE exhibited the lowest value (5.08 log CFU/g) at day 10 ($p<0.05$). Overall, PEF application facilitated the influx of CLE into shrimp and retarded the growth of Pseudomonas by combined impact of high energy electric field pulses and polyphenols with antimicrobial activity. Pseudomonas has been documented to be a major spoilage microbe in shrimp during storage (Dabadé *et al*., 2015).

At the first day of storage, PEF-T sample had similar H_2S producing bacterial count, compared to the control ($p>0.05$). The lowest count was evidenced in PEF-1 CLE sample ($p<0.05$) (Fig. 20d). The initial count for all samples ranged from 1.79 to 2.02 log CFU/g. SMS-T sample had the lower count than the control ($p<0.05$). The lowest count was obtained in PEF-1 CLE samples during storage ($p<0.05$). H_2S-producing bacterial counts of control, PEF-T, 0.5-CLE, PEF-0.5 CLE, 1-CLE, PEF-1 CLE and SMS-T samples were 6.26, 5.90, 5.49, 5.31, 4.85, 4.10 and 5.11 log CFU/g, respectively at day 10. The retarded growth of H_2S-producing bacteria observed in PEF-1 CLE sample might be governed by antimicrobial effect of PEF in conjunction with polyphenols and organic acid, which could migrate underneath the shrimp shell or carapace due to electroporation mediated by PEF. CLE at 1% dose impeded the growth H_2S-producing bacteria in shrimp during refrigerated storage effectively. Morin *et al*. (2015) reported that growth of some species of H_2S-producing bacteria, particularly *Solobacterium moorei*, was suppressed by polyphenolic catechin derivatives. Production of H2S with putrid odor was reported to deteriorate sensorial quality of shrimp stored at 5 °C (Lapin and Koburger, 1974).

At day 0, the control had the similar count to PEF-T, 0.5-CLE and PEF-0.5-CLE samples ($p<0.05$) (Fig. 20e). As the storage proceeded, Enterobacteriaceae count was increased in all the samples ($p<0.05$) and the lowest count was obtained in PEF-1 CLE sample during the storage ($p<0.05$). SMS-T sample showed the similar count to the control at day 10, indicating the inferior potential of SMS in inhibiting Enterobacteriaceae in shrimp. Similar results were documented in 1% CLE treated sample and stored at 4 °C (Shiekh *et al.*, 2019). Development of off odor compounds especially, indole and putrescines, was documented to degenerate quality.

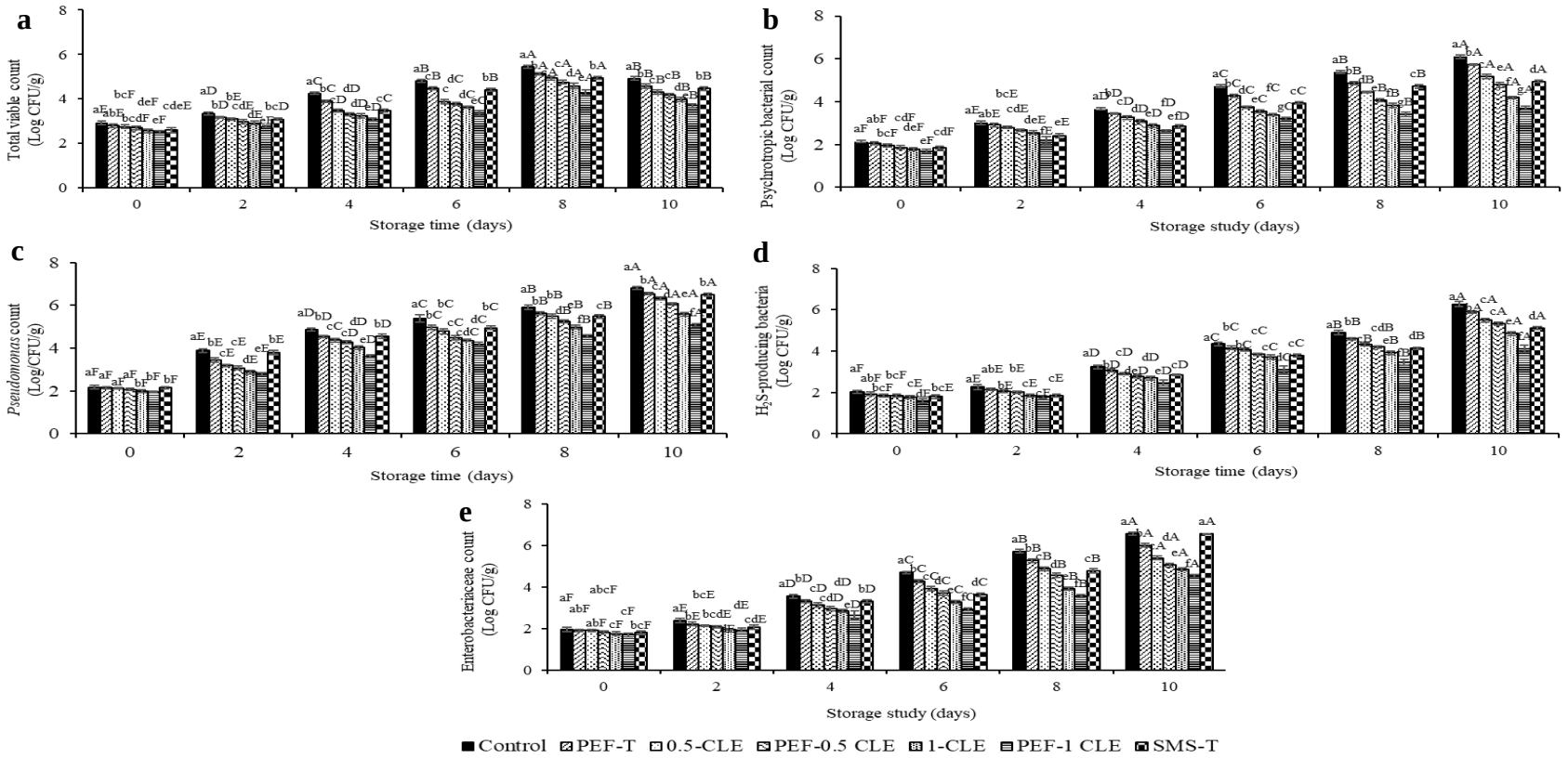

Figure 20. Total viable count (a), psychrophilic bacterial count (b), *Pseudomonas* (c), H_2S-producing bacteria (d) and Enterobacteriaceae (e) Pacific white shrimp pretreated without and with PEF and treated without and with CLE during 10 days of refrigerated storage.

Bars represent the standard deviation (n=3). Different uppercase letters within the same sample indicate the significant difference ($p<0.05$). Different lowercase letters within the storage time indicate the significant difference ($p<0.05$). Key: see the caption for Fig. 19.

Enterobacteriaceae were the major spoilage bacteria in shrimp (Dabadé *et al.*, 2015). Therefore, shrimp pre-treated with PEF followed by soaking in 1% CLE could impede the growth of Enterobacteriaceae counts during the extended storage of 10 days under refrigerated condition.

4.5.1.3 Changes in chemical quality

Initially, TVB contents (2.61−3.08 mg N/100 g shrimp meat) were not different among all the samples ($p>0.05$) (Fig. 21a). During the storage, the highest TVB level was obtained in the control than others ($p<0.05$). Generally, the lower TVB content was detected in PEF-1 CLE sample during storage ($p<0.05$), in which TVB content of 12.96 mg N/100 g meat was detected at day 10. With the treatment of CLE rich in polyphenols, the oxidative changes in protein were also prevented in whole Pacific white shrimp after PEF treatment. Therefore, no protein fragmentation was found when PEF was applied at high electric field intensity (Shiekh and Benjakul, 2019). The minimum TVB level (23.24 mg N/100 g shrimp meat) was reported in 1% CLE treated shrimp at day 12 of storage at 4 °C (Shiekh *et al*., 2019). TVB content of 30 mg/100 g shrimp meat was indexed for safety and quality acceptability (Cobb *et al.*, 1973). The lower increase in TVB content was found in SMS-T sample in comparison with the control during storage. The lower TVB content of sample pre-treated with PEF followed by the immersion in CLE solutions at both concentrations could be caused by the permeation of CLE with the aid of PEF. The results correlated with microbial load, in which PEF-1 CLE sample had the least number of counts, including spoilage bacteria. Inhibition effects of organic acids, including hydroxycitric acid and polyphenolic glycosides in CLE against microorganisms and proteases were more pronounced when the concentration of CLE used for treatment was increased (Shiekh *et al*., 2019). Chamuang leaf and rind extracts had antimicrobial property (Negi *et al.*, 2008). As a result, those antimicrobial agents could act well to inhibit microbial growth in shrimp when they could penetrate into shrimp with the aid of PEF treatment.

PV of the control, PEF-T and 0.5-CLE samples were slightly higher than those of others at day 0 ($p<0.05$) (Fig. 21b). CLE with antioxidant activity could inhibit lipid oxidation of shrimp during preparation to some extent. As storage time

augmented, the increment in PV was noticeable in the control ($p<0.05$), compared to others. Furthermore, SMS-T sample also showed the highest increase in PV. This result suggested that SMS had no marked impact as antioxidant in treated shrimp. Similar PV was found between 1-CLE and PEF-1 CLE samples during the first 8 days, in which PV was lower than others ($p<0.05$). Nevertheless, PV was evidenced in low quantity in PEF-1 CLE sample at day 10 ($p<0.05$). PV quantifies the primary oxidation products in muscle foods (Ladikos and Lougovois, 1990). Onset of oxidation in shrimp muscle was reported to trigger the synthesis of hydroperoxides. Formation of peroxides is mainly associated with the elimination of hydrogen from the fatty acid double bonds with subsequent reaction with oxygen, in which hydroperoxides are produced (Benjakul *et al.,* 2005). PEF treatment might have inactivated oxidative enzymes in whole Pacific white shrimp and also facilitated the migration of CLE with antioxidant activity into shrimp meat. PEF at higher electric field intensity has been documented to lower the lipid oxidation, possibly owing to the inactivation of lipase or lipoxygenase in Pacific white shrimp cephalothoraxes (Gulzar and Benjakul, 2019). As a consequence, lipid oxidation of shrimp meat was prevented with the combined effect of PEF and CLE.

Generally, TBARS values of the control and SMS-T sample increased with the progression of storage up to 10 days at 4 °C ($p<0.05$). The highest TBARS value was obtained in the control, followed by SMS-T sample during the storage (Fig. 21c). PEF-T sample showed lowered TBARS value than the control ($p<0.05$). PEF might inactivate some oxidative enzymes such as lipoxygenase (Van Loey *et al*., 2001). Control possessed the higher degree of lipid oxidation due to rapid conversion of primary oxidation reactive species to secondary oxidation products. With the combined effect of PEF and CLE treatments, the highest suppression of TBARS was observed in PEF-1 CLE sample, indicating the lower rate of lipid oxidation ($p<0.05$), compared to 0.5-CLE, 1-CLE and PEF-0.5 CLE samples. TBARS content in PEF-1 CLE sample was 2.47 mg MDA/kg of shrimp meat at day 10 of storage that was significantly lower than that of shrimp treated with 1% CLE only as reported by Shiekh *et al*. (2019). Lowest TBARS value was found in lipid from shrimp cephalothorax pretreated with PEF up to 16 kV/cm and 240 pulses with higher retention of fatty acids, especially polyunsaturated fatty acids.

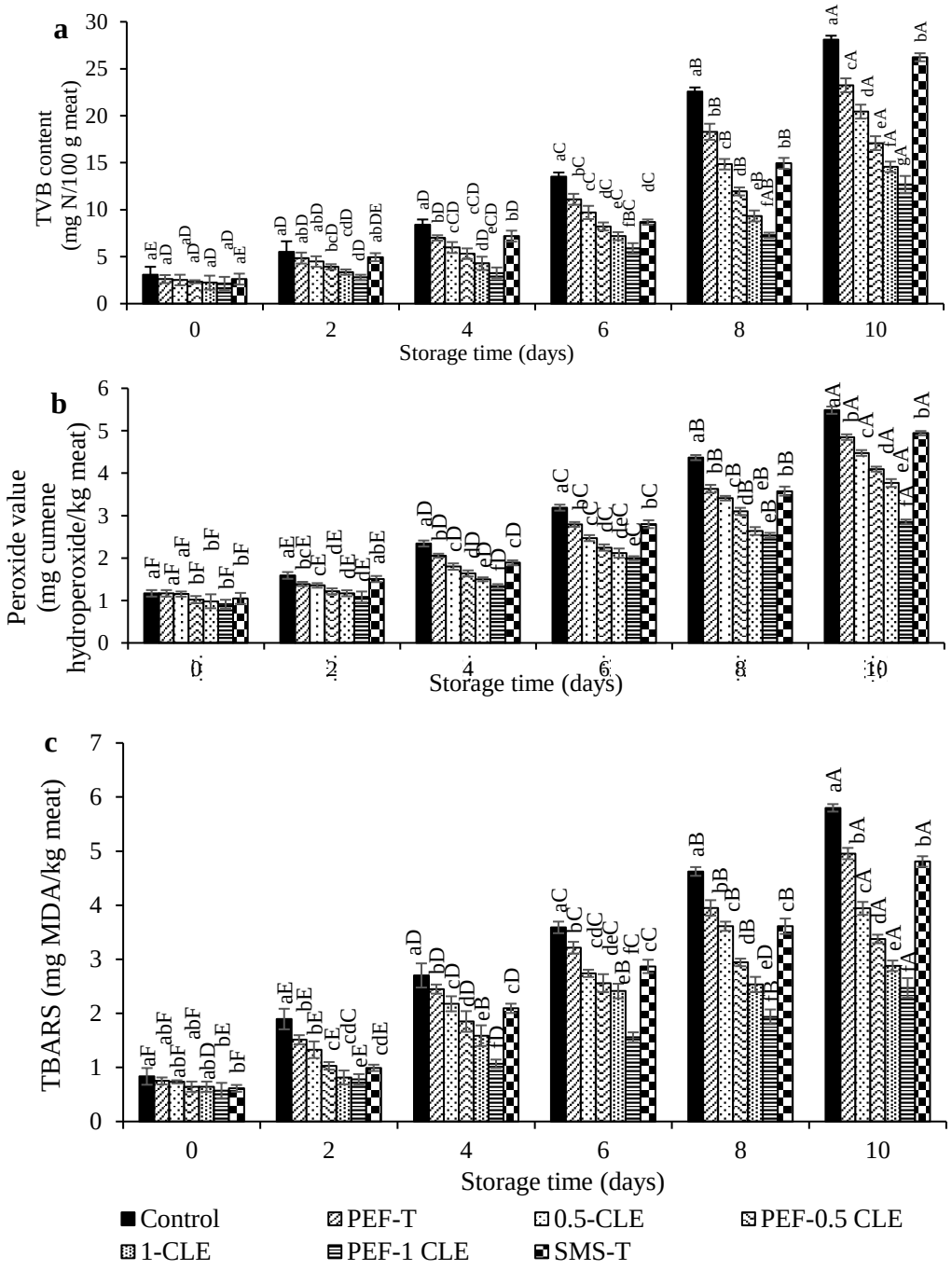

Figure 21. Total volatile base (TVB) Total volatile base (TVB) content (a), peroxide values (PV) (b) and thiobarbituric reactive substances (TBARS) values (c) Pacific white shrimp pretreated without and with PEF and treated without and with CLE during 10 days of refrigerated storage.

Bars represent the standard deviation (n=3). Different uppercase letters within the same sample indicate the significant difference ($p<0.05$). Different lowercase letters within the storage time indicate the significant difference ($p<0.05$). Key: see the caption for Fig. 19.

This was plausibly caused by the inactivation of oxidative enzymes by PEF (Gulzar and Benjakul, 2019). Lipids in crustaceans undergo oxidation, thus forming hydroperoxides that readily decompose to malonaldehyde (Benjakul *et al.*, 2005). Furthermore, shrimps are rich in polyunsaturated fatty acids and high cholesterol also propagates the lipid oxidation (Schnitzer *et al.*, 2007). can act as potential oxidative chain terminators via donating a positive charged specie or electron for oxidative chain reaction termination (Ningappa *et al.*, 2008). Shrimp In addition to PEF treatment, 1% CLE solution containing considerable amount of polyphenolic glycosides and organic acid with antioxidant activity significantly lowered the TBARS in shrimp (Shiekh *et al*., 2019). Natural antioxidants of plant origin immersed in 1% Cashew leaf extract were found to have decreased TBARS value due to strong antioxidant potential of the extract (Chotphruethipong *et al*., 2019; Sae-leaw and Benjakul, 2019). Overall, shrimp pre-treated with PEF followed by emersion in 1% CLE solution, had the significant decrease in lipid oxidation, compared to those treated with SMS, CLE and PEF alone ($p<0.05$).

4.5.1.4. Changes in sensory property

All the samples had similar likeness scores at day 0 ($p>0.05$) (Table 5). The result revealed that PEF pre-treatment followed by soaking in CLE (0.5 or 1%) solutions had no adverse effect on likeness or acceptability of shrimp meat. After 10 days of storage, likeness scores for all attributes were decreased in all samples ($p<0.05$). However, the higher scores for all attributes were found in PEF-1 CLE sample, followed by 1-CLE, PEF-0.5 CLE, 0.5-CLE samples and the control, respectively ($p<0.05$). SMS-T sample was omitted due to the allergenic problem with several panelists. The control yielded the lowest likeness scores for all attributes tested due to chemical and microbial deteriorations ($p<0.05$), plausibly related with the highest microbial load (Fig. 20) as well as highest TVB, PV and TBARS values (Fig. 21). Higher color and firmness scores in PEF-T sample might be due to inactivation of polyphenoloxidase and proteases, respectively. However, combination of PEF and 1% CLE potentially resulted in higher color likeness score, which coincided with the lowered melanosis in PEF-1 CLE sample (Fig. 19). Therefore, the PEF with field intensity and pulse number used in the present study had no negative impact on sensorial quality, including texture. On the other hand, PEF under aforementioned

condition along with 1% CLE solution containing abundant polyphenols and organic acids rendered the shrimp with higher likeness scores. Likeness scores for color, flavor and odor were also highest in PEF-1 CLE sample, mainly due to lower melanosis and microbial loads. Furthermore, higher odor likeness scores of PEF-1 CLE sample were related with the formation of lower off-flavor components. CLE treatment up to 1% in PEF pre-treated shrimps had no negative impact on taste as perceived by panelists. PEF processed juices retained fresh flavor without any detrimental effect on organoleptic quality, compared to HPP and thermally treated juices as perceived by the consumers (Lee *et al.*, 2017).

Table 5. Likeness score of PEF treated Pacific white shrimp without and with CLE at different concentrations at day 0 and 10 of refrigerated storage

Storage time (days)	Samples	Color	Odor	Texture	Flavor	Taste	Overall
0	Control	8.20±0.56aA	8.07±0.26aA	8.13±0.64aA	8.13±0.55cA	8.20±0.77aA	8.13±0.57aA
	0.5-CLE	8.24±0.51aA	8.13±0.35aA	8.27±0.59aA	8.17±0.45bcA	8.27±0.82aA	8.19±0.65aA
	PEF-0.5 CLE	8.27±0.74aA	8.20±0.41aA	8.33±0.72aA	8.23±0.49aA	8.33±0.90aA	8.25±0.58aA
	1-CLE	8.32±0.63aA	8.27±0.46aA	8.37±0.81aA	8.29±0.52aA	8.40±0.71aA	8.29±0.68aA
	PEF-1 CLE	8.35±0.59aA	8.33±0.49aA	8.43±0.82aA	8.36±0.51aA	8.50±0.68aA	8.36±0.51aA
10	Control	4.13±0.35dB	4.00±0.38dB	4.07±0.59dB	4.23±0.26dB	4.20±0.41dB	4.13±0.35dB
	0.5-CLE	4.57±0.46dB	4.25±0.56dB	4.40±0.63dB	4.60±0.63dB	4.47±0.52 dB	4.33±0.62dB
	PEF-0.5 CLE	5.53±0.52cB	5.33±0.59cB	5.60±0.91cB	5.27±0.59cB	5.40±0.74cB	5.27±0.46cB
	1-CLE	6.60±0.83bB	6.42±0.49bB	6.53±0.64bB	6.20±0.41bB	6.27±0.59bB	6.20±0.41bB
	PEF-1 CLE	7.60±0.91aB	7.58±0.83aB	7.6±0.74aB	7.40±0.63aB	7.50±0.68aB	7.27±0.46aB

Values presented the mean ± standard deviation (n=50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 19.

PEF treated shrimp muscle fibers might undergo detachment or dissociation due to electropermeabilization. Furthermore, PEF at high intensity with higher energy value (483 kJ/kg shrimp) was found to induce the breakage of collagen molecules localized between muscle fibers, causing the looser structure of muscle fibers (Shiekh and Benjakul, 2019). The result reconfirmed that PEF in combination with the migration of CLE, particularly at high concentration (1%) through the shrimp shell or

carapace aided in maintaining the quality of shrimps stored at 4 °C, particularly via inhibition of melanosis.

4.5.2 Profiling of chemical constituents in PEF pre-treated shrimp treated with CLE

Constituents of CLE retained in PEF-1 CLE sample as analyzed by LC-MS coupled with MS-Q-TOF are presented in Table 6. LC-MS having negative mode was used for the detection of phenolic compounds in shrimp meat in the present study. Negative mode showed the better resolution and higher sensitivity due to negatively charged ionic species, especially in ESI as compared to positive mode (Mena *et al*., 2012). In the previous study of Shiekh *et al*. (2019), crude CLE powder contained organic acids and polyphenolic glycosides as the abundant compounds. The residual chemical constituents in PEF pre-treated shrimp confirmed the penetration of CLE (1%) into shrimp, more likely due to electroporation, which resulted in gapping among the muscle fibers (Shiekh and Benjakul, 2019). The compounds detected in PEF-1 CLE sample included hydroxycitric acid, oxalosuccinic acid, 2-feruloyl-1-sinapoylgentiobiose, chrysoeriol 6-C-glucoside-8-C arabinopyranoside and veranisatin-C. Among all the compounds detected, chrysoeriol 6-C-glucoside-8-C arabinopyranoside was dominant in shrimp. The result was in accordance with the previous study that this compound was the most prevalent in CLE (Shiekh *et al*., 2019). Furthermore, oxalosuccinic acid and hydroxycitric acid with low molecular weight also penetrated in the shrimp meat. Organic acids such as hydroxycitric acid and oxalosuccinic found in PEF-1 CLE sample were 2.52 and 3.98 × 10^6 in abundance, compared to those detected in crude extract of CLE with the abundance of 4.42 and 4.54 × 10^6, respectively (Shiekh *et al*., 2019). The compounds with large molecular size and complex structure might migrate into shrimp to a lower extent with slower rate than the smaller size counterparts. Nevertheless, pores or looser structure of shrimp shell or carapace formed via electroporation could occur with the aid of PEF. The lower abundance of polyphenolic glycosides, especially 2-feruloyl-1-sinapoylgentiobiose, in meat was plausibly due to its large molecular size, hindering the passage through the pores generated. Moreover, the migration of 1% CLE extract underneath the shell of PEF pre-treated shrimp revealed the potential role of CLE

constituents, which might prevent proteins and lipids from oxidation. Organic acids and polyphenolic glycosides in CLE more likely served to prevent oxidative and microbial changes in shrimp. Cashew leaf extract (CE) at 1% level was proved to be powerful natural preservative against chemical and microbial quality deterioration in shrimp (Sae-leaw and Benjakul, 2019). Phytochemicals present in PEF-1 CLE sample such as chrysoeriol 6-Cglucoside-8-C-arabinopyranoside and rest of the other constituents, probably played a vital role in quality preservation of shrimp. This was in line with the lower increasing rate of microbial load as well as lower oxidation of shrimp pretreated with PEF, followed by soaking in 1% CLE. Therefore, polyphenols and organic acids penetrated into PEF-1 CLE sample more likely contributed to the prevention of microbiological and chemical deterioration in shrimp during refrigerated storage.

Table 6. CLE constituents found in shrimp meat after PEF pre-treatment followed by CLE soaking based on LC-MS

Compounds	Molecular Formula	Mass (g/mol)	m/z	Retention Time (min)	Abundance ($x10^6$)
Hydroxycitric acid	$C_6 H_8 O_8$	208.02	207.01	1.82	2.52
Oxalosuccinic acid	$C_6 H_6 O_7$	190.00	189.00	2.24	3.98
Veranisatin-C	$C_{16} H_{20} O_{10}$	372.11	371.09	9.72	5.72
Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside	$C_{27} H_{30} O_{15}$	594.16	593.15	10.24	9.77
2-Feruloyl-1-sinapoylgentiobiose	$C_{33} H_{40} O_{18}$	724.22	723.21	10.68	1.17

4.6 Conclusions

PEF application based on electroporation in shrimp facilitated the migration of CLE polyphenolic glycosides and organic acids, in which chrysoeriol 6-C-glucoside-8-C-arabinopyranoside was abundant in shrimp as detected by LC-MS. PEF-1 CLE sample exhibited least melanosis score along with the lowered increases in mesophile spoilage microbes (TVC, Enterobacteriaceae, *Pseudomonas*, H2S producing bacteria) and psychrophile during 10 days of refrigeration storage. Decomposition and oxidation measured by TVB, PV and TBARS were lowered in PEF-1 CLE sample. After 10 days of storage, PEF-1 CLE sample had the highest likeness score because of lower melanosis and microbial loads. Therefore, PEF in combination with CLE could be used to lessen melanosis and retard quality loss of refrigerated shrimp.

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CHAPTER 5

EFFECT OF PULSED ELECTRIC FIELD AND MODIFIED ATMOSPHERIC PACKAGING ON MELANOSIS AND QUALITY OF REFRIGERATED PACIFIC WHITE SHRIMP TREATED WITH LEAF EXTRACT OF CHAMUANG (*GARCINIA COWA* ROXB.)

5.1 Abstract

Pacific white shrimp treated with pulsed electric field (PEF) and subsequently soaked in 1% Chamuang leaf extract (CLE) were packed under various modified atmospheres (MAP) including absolute N_2, Ar or CO_2. Lower melanosis score was evidenced in PEF pre-treated shrimp followed by soaking in 1% CLE and packing under CO_2-MAP (PEF-CLE-CO_2) than the control and other treated samples during storage at 4 °C ($p<0.05$). PEF-CLE-CO_2 showed lower pH, protein carbonyl content, total volatile base, peroxide value and thiobarbituric acid reactive substances but exhibited higher likeness scores ($P<0.05$). Psychrophile, Enterobacteriaceae, *Pseudomonas*, lactic acid bacteria and H_2S producing bacterial counts were less than 3 log CFU/g, which was lower than those of other samples at day 10 ($p<0.05$). Oxidation of fatty acids (EPA and DHA) was prevented in PEF-CLE-CO_2 than the control and other treated samples ($p<0.05$). Volatile compounds in PEF-CLE-CO_2 sample were negligible, compared to the control which was abundant in 3-methyl-1-butanol. Therefore, PEF and CLE treatment before MAP, especially under CO_2 exhibited high efficacy in prolonging shelf-life of refrigerated shrimp.

5.2 Introduction

Pacific white shrimp treated with pulsed electric field (PEF) and subsequently soaked in 1% Chamuang leaf extract (CLE) were packed under various modified atmospheres (MAP) including absolute N_2, Ar or CO_2. Lower melanosis score was evidenced in PEF pre-treated shrimp followed by soaking in 1% CLE and packing under CO_2-MAP (PEF-CLE-CO_2) than the control and other treated samples during storage at 4 °C ($p<0.05$). PEF-CLE-CO_2 showed lower pH, carbonyl content, total volatile base, peroxide value and thiobarbituric acid reactive substances but exhibited higher likeness scores ($P<0.05$). Psychrophile, Enterobacteriaceae, *Pseudomonas*,

lactic acid bacteria and H2S producing bacterial counts were less than 3 log CFU/g, which was lower than those of other samples at day 10 ($p<0.05$). Oxidation of fatty acids (EPA and DHA) was prevented in PEF-CLE-CO_2 than the control and other treated samples ($p<0.05$). Volatile compounds in PEF-CLE-CO_2 sample were negligible, compared to the control which was abundant in 3-methyl-1-butanol. Therefore, PEF and CLE treatment before MAP, especially under CO_2 exhibited high efficacy in prolonging shelf-life of refrigerated shrimp.

Non-thermal processing of foods using pulsed electric field (PEF) has gained importance in the food processing sector to retain biochemical, nutritional and sensory properties of foods (Olatunde and Benjakul, 2018). Moreover, the antimicrobial efficacy of PEF is directly correlated with the decreased initial microbial load because of the leakage of cell membrane, releasing out cellular content and eventually inhibiting pathogenic and spoilage microbes (Gulzar and Benjakul, 2020; Yun *et al.,* 2017). Additionally, pores of bacterial cell membrane formed could facilitate penetration of Chamuang leaf extract (CLE) rich in antimicrobial constituents to ensure effective microbial inactivation (Shiekh and Benjakul, 2020a). PEF is a hazard free green technology without imposing any detrimental effect on food quality (Shiekh and Benjakul, 2020b). PEF has been potentially employed to retard chemical, biochemical quality changes in foods by enzyme inactivation possibly due to conformational alteration or denaturation mechanism (Riener *et al.,* 2009; Zhong *et al.,* 2005). The activity of PPO in Pacific white shrimp cephalothorax with the aid of PEF application was notably decreased with lower melanosis during refrigerated storage (Shiekh and Benjakul, 2020b).

Besides PEF application, plant extracts can be combined with MAP to assure quality and extend shelf-life of refrigerated shrimp (Nirmal and Benjakul, 2011). MAP refers to the replacement or alteration in the gaseous composition surrounding the food placed inside a sealed package (McMillin, 2008). MAP at low oxygen content with cold temperature conditions delays melanosis and retards spoilage of shrimp (Kalleda *et al*., 2013). Besides the use of nitrogen, packaging of foods under MAP with noble gases such as argon (Ar) has been reported as important hurdle, which aid in the retention of food quality and safety (Olatunde *et al.,* 2019). However, CO_2-MAP effectively prolonged the shelf-life of deepwater pink shrimp when used together with

several additives (Gonclalves *et al.,* 2003). Natural extracts such as green tea and thymol essential oil with antimelanosis, antimicrobial and antioxidant agents were employed jointly with MAP for keeping *Litopenaeus vannamei* and *Palaemon serratus*, respectively (Mastromatteo *et al.*, 2010; Nirmal and Benjakul, 2011).

Chamuang leaf extract (CLE) with PEF has been recently investigated to inhibit PPO by lowering melanosis, quality losses mediated by several reactions of Pacific white shrimp at 4 °C (Shiekh, Benjakul, and Sae-leaw, 2019). Antimicrobial constituents in CLE retarded microbial growth (Sakunpak and Panichayupakaranant, 2012). High intensity PEF pulses have antimicrobial effect and can inactivate PPO to some degree via denaturation or alteration of enzyme structural conformation (Shiekh and Benjakul, 2020b). Recently PEF has been proven to facilitate the penetration of CLE to position underneath shrimp shell through electroporation in whole Pacific white shrimp (Shiekh and Benjakul, 2020a). This investigation aimed to evaluate the influence of prior PEF and CLE treatments in combination with MAP to impede melanosis and lower quality losses of refrigerated shrimp during storage for 10 days at 4 °C.

5.3 Objectives

To study the combined effects of PEF and CLE treated Pacific white shrimp (PWS) packaged under modified atmosphere for quality preservation

To assess the combined effects of CLE along with PEF and MAP as hurdle technology for quality and shelf-life extension of PWS

5.4 Materials and methods

5.4.1 Chemicals

Chemicals and microbial media were procured from Sigma Aldrich (St. Louis, MO, USA) and Oxoid Ltd. (Hampshire, UK), respectively.

5.4.2 Collection and preparation of shrimp

Fresh shrimp with a total number of 55–60 shrimp per kg and body length of 9−10 cm were harvested from a shrimp farm located in Songkhla province of southern Thailand. Shrimp without any additive were placed in layers and covered with two portions of crushed ice in polystyrene foam boxes and were immediately carried to

the seafood laboratory, Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Shrimps were washed using ice water to remove the extraneous impurities. Eventually, shrimp were transferred carefully in a polystyrene box and covered with layers of crushed ice prior to pre-treatment or packaging.

5.4.3. Pre-treatment of shrimp with PEF and CLE

PEF pre-treatment was implemented on shrimp following the method of Shiekh and Benjakul (2020a). PEF system (Lab scale) with high voltage power supply (PEF LAB-400W, Febix International Inc., Chiang Mai, Thailand) was employed for shrimp treatment. PEF chamber made of stainless steel (1.5 L capacity) was equipped with a stainless steel electrode (20 cm × 10 cm). A plexiglass cabinet con- taining aluminium frame, a UT-P03 oscilloscope probe (600 MHz, 10X), was connected with PEF system together with a digital storage oscil- loscope (DSO) (UTD2052CEX, UNI-T®, Dongguan city, Guangdong, China) for data display. Chamuang leaf extract (CLE) was prepared using distilled water and lyophilized to obtain CLE powder as described by Shiekh *et al*. (2019). Prior PEF treated samples (shrimp) were soaked in 1 % CLE solution for 30 min following the procedure of Shiekh and Benjakul (2020a).

5.4.4 Effect of modified atmospheric packaging on prior PEF and CLE treated Pacific white shrimp during refrigerated storage (4 °C)

Prior PEF and CLE treated shrimps (6 shrimp) were displayed on the tray before placing in nylon/LLDPE bag (29 × 21 cm^2, 80 μm thick) (Asian Foams, Hat Yai, Thailand). Bag had gas permeability of 0.17, 0.01 and 0.04 m^3 mm/cm^2 for CO_2, N_2 and O_2 at 25 °C, 1 atm, respectively. Five volumes of gas were filled in bag with the aid of Henkovac type 1000 (Tecnovac, Italy). Shrimp treated with PEF and CLE treatment and packaged with different atmospheric gases including air, argon (Ar), nitrogen (N_2) and carbon dioxide (CO_2) were named as PEF-CLE-Ar, PEF-CLE-N_2 and PEF-CLE-CO_2, respectively. However, samples only treated with PEF (without CLE treatment) and subjected to MAP were prepared as gas control shrimp for comparison with the CLE treated MAP samples. Those were referred as PEF-Ar, PEF-N_2 and PEF-CO_2, respectively. Control was the shrimp without any treatment and packed in air. Two trays were taken for each treatment every 2 days. Samples were pooled and considered as composite sample. Additionally, fixed sample (20 shrimps/tray) for each

treatment was monitored for melanosis every 2 days for totally 10 days. However, two trays were taken at day 0 and 10 for sensory evaluation.

5.4.4.1. Melanosis assessment

The method of Nirmal and Benjakul (2011) was adopted for melanosis (black spots) assessment using ten trained panelists consisted of seven men and three women with an age group of 25–35 years. Ten-point melanosis score was applied.

5.4.4.2 Microbiological analyses

Psychrophilic bacterial count (PBC), *Pseudomonas*, H_2S-producing bacteria and Enterobacteriaceae counts were enumerated using spread plate technique as detailed by Shiekh *et al*. (2019). Lactic acid bacteria (LAB) and *Clostridium perfringens* counts were determined using de Man Rogosa and Sharpe agar and *C. perfringens* agar medium supplemented with a soft double layer of Tryptic Soy agar and incubated in an anaerobic jar at 35 °C and 37 °C using a CO_2 in flux incubator (Binder Model C 170, Binder Inc., Bohemia, NY, USA), respectively, for 3 days (Olatunde *et al*., 2019). Black colonies grown on Perfringens agar medium indicated *C. perfringens* (Rahman *et al*., 2016).

5.3.5 Physico-chemical analyses

5.4.5.1 pH

Shrimp meat (2 g) was mixed in distilled water (20 ml) before homogenization for 1 min at 10,000 rpm (IKA-Werke GmbH and Co. KG, Staufen, Germany). The homogenate was left for 5 min at ambient temperature and pH was analyzed using a pH-meter (Sartorious North America, Edgewood, NY, USA) (Nirmal and Benjakul, 2011).

5.4.5.2 Total volatile basic nitrogen (TVB-N) and Protein carbonyl content

Conway micro-diffusion method was adopted for measurement of TVB-N content (mg N/100 g shrimp meat) as described by Sae-leaw and Benjakul (2019c). Carbonyl content was determined by derivatization with 2,4-dinitro-phenyl hydrazine (DNPH) and expressed as nmol of DNPH fixed/mg protein as tailored by Nikoo, Benjakul, and Xu (2015).

5.4.5.3 Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

PVs of lipids extracted from shrimp meat were evaluated using ferric thiocyanate method (Arfat *et al.,* 2015). TBARS value in shrimp meat was evaluated as detailed by Benjakul and Bauer (2001).

5.4.6 Sensory evaluation

All samples were collected at 0 and 10 days of storage for sensory analysis as described by Nirmal and Benjakul (2011).

5.4.7 Fatty acid profile

Fatty acid compositions were examined as fatty acid methyl esters (FAMEs) using gas chromatography coupled with flame ionization detector (GC-FID) as per the method of Raju and Benjakul (2020). Fatty acid methyl esters (FAME) were obtained by transmethylation of 10 mg of oil sample with 2 M methanolic sodium hydroxide and 2 M methanolic hydrochloric acid. FAME were analyzed by Agilent 7890B (Santa Clara, CA, USA) gas chromatography system equipped with flame ionization detector (FID). Peaks were identified based on the retention time of standards and the results were expressed as g/100 g lipid.

5.4.8. Volatile compounds

Solid phase micro-extraction (SPME) technique coupled with GC–MS (GC-6890A-MS-5975B VLMSD, Agilent Technologies, Santa Clara, CA, USA) was used for the detection of volatiles in head space atmosphere of control (without any treatment), PEF and CLE pre- treated samples with different MAP at day 10 of storage. GC–MS analysis was conducted using Supelco SPME 85 mm Carboxen/PDMS Stableflex Fiber Assembly (Sigma Aldrich, St. Louis, MO, USA) as described by Lyte *et al*. (2016). Volatile compounds were identified based on mass range m/z of 16–500, and scan rate of 3.0 scans/s using a MS library (Wiley 7th Edition Library for Mass Spectrometer; G1035B; Agilent Technologies) containing 390,000 spectra (Lyte *et al*., 2016).

5.4.8. Statistical analyses

Completely randomized design (CRD) was used for entire studies. Randomized completely block design (RCB) was implemented for sensory analysis. Analysis of variance (ANOVA) was done and mean comparison was performed at a level of significance ($p<0.05$). Duncan's multiple range test in a SPSS statistical software (SPSS 17.0, SPSS Inc., Chicago, IL, USA) was used for data analyses. All the experiments for statistical analyses were conducted in triplicates.

5.5 Results and discussion

5.5.1 Effect of MAP on melanosis and quality changes of PEF and CLE pretreated shrimp during refrigerated storage (4 °C)

5.5.1.1 Changes in melanosis

At the first day of storage, no melanosis was noticed for all the samples (Fig. 22a). The control (without any treatment and packed in air) had the increasing melanosis score up to day 10. Conversely, prior PEF and CLE treated sample packaged under CO_2-MAP (PEF-CLE-CO_2) had lowest melanosis score than others ($p<0.05$) at day 8 and 10 days. In general, PEF-CLE-CO_2 sample showed the lowest melanosis ($p<0.05$) compared to the control. Moreover, prior PEF shrimp (without CLE treatment) such as PEF-CO_2, PEF-Ar, PEF-N_2 in comparison with CLE treated MAP samples showed difference in the inhibition of melanosis. The aforementioned differences were obvious during 8–10 days of storage. Lowered melanin formation in shrimp packaged under CO_2 with prior PEF (15 kV/cm, 600 pulses, 483 kJ/kg) which was able to enhance the penetration of CLE containing polyphenols and organic acids into shrimp as reported by Shiekh and Benjakul (2020a). It was noted that CO_2 showed high efficiency in inhibition of melanosis in prior PEF treated shrimp, although CLE was omitted. CO_2 might impact the PPO by lowering pH to some degree, thus lowering PPO activity.

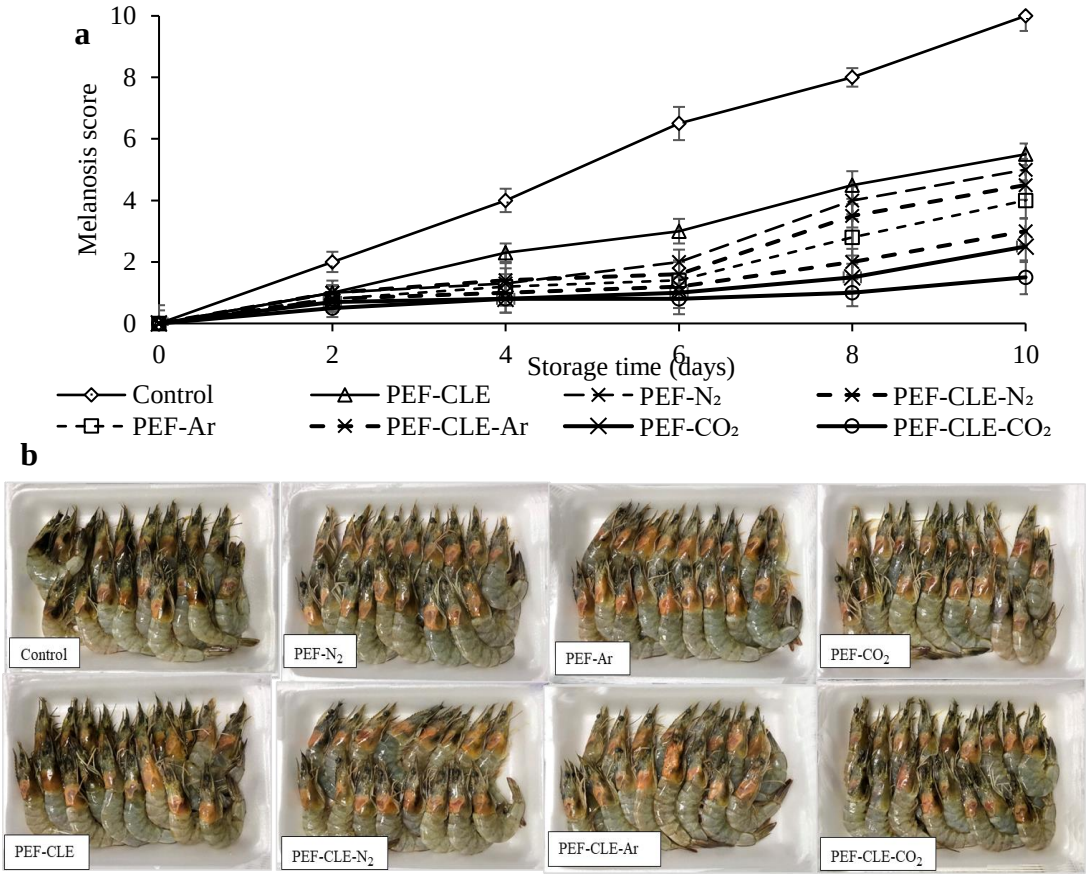

Figure 22. Melanosis score (a) and photographs (b) of Pacific white shrimp with PEF treatment in the absence and presence of CLE under different modified atmosphere packagings during and after 10 days of storage at 4 °C.

Bars represent the standard deviation (n = 10). Control: without any treatment; PEF-CLE: sample pre-treated with PEF (15 kV/cm, 600 pulses, 483 kJ/kg) and 1% CLE. PEF-N_2, PEF-Ar, PEF-CO_2: sample pre-treated with PEF subjected to modified atmosphere packaging (MAP) with absolute nitrogen, argon, carbon dioxide, respectively. PEF-CLE-N_2, PEF-CLE-Ar, PEF-CLE-CO_2: sample pre-treated with PEF and 1% CLE, followed by MAP with absolute nitrogen, argon, carbon dioxide, respectively.

Ar was also shown to effectively retard the melanosis. Ar might replace O_2 in the package. As a result, O_2 was excluded and hydroxylation could not take place. Nevertheless, the CLE treatment of prior PEF treated shrimp showed the combined impact with MAP on retardation of melanosis. CLE contained polyphenols acting as PPO inhibitor (Shiekh *et al*., 2019). Shiekh *et al*. (2019) found that CLE showed the higher melanosis inhibition in Pacific white shrimp during refrigerated storage than sodium metabisulphite. MAP could exclude total O_2 from the package. PEF under the same condition used in the present study was documented to inhibit 40% of PPO activity in shrimp after 10 days of storage (Shiekh and Benjakul, 2020b). Green tea extract (GTE) and ascorbic acid (AA) could prevent melanosis of Pacific white shrimp during

refrigerated storage of 10 days via the combination with MAP (5% O_2; 50% CO_2; 45% N_2) (Nirmal and Benjakul, 2011). MAP with noble gases or their mixtures can be a potential means to tackle melanosis and quality loss of crustaceans (Bono *et al.,* 2012). Thus, PEF and CLE pre-treatments in conjunction with MAP, especially CO_2 retarded melanosis in Pacific white shrimp throughout the storage. The least melanosis was detected in PEF-CLE-CO_2 sample (Fig. 22b), while the highest melanosis occurred in the control at day 10 of refrigerated storage (4 °C).

5.5.1.2 Changes in microbial quality

Changes in PBC of all the samples during 0–10 days are shown in Fig. 23a. At the first day of storage, PBC of control was highest ($p<0.05$). Conversely PEF treated sample packed under CO_2, regard- less of CLE treatment had the lowest PBC than others ($p<0.05$). PEF- CLE sample packed in air or those with prior PEF and packaged in argon and nitrogen exhibited lower PBC at day 0 of refrigerated storage (4 °C), irrespective of CLE treatment ($p>0.05$). PEF-CLE-CO_2 sample, showed decelerated growth of PBC for the entire storage ($p<0.05$). At day 10, PBC of control, PEF-CLE, PEF-N_2, PEF-CLE-N_2, PEF-Ar, PEF-CLE-Ar, PEF-CO_2 and PEF-CLE-CO_2 samples were 6.04, 3.98, 3.64, 3.50, 3.38, 3.24, 3.16 and 2.70 log CFU/g, respectively at day 10. Microbiological load of shrimp for safe consumption under the permissible limits has been reported as less than 6 log CFU/g (ICMSF, 2002). Pacific white shrimp packed in different proportions of CO_2, N_2 and O_2 MAP were documented to inhibit psychrophilic spoilage bacteria due to anti-microbial effect induced by dissolved CO_2 during cold temperature storage (Wang *et al.,* 2016). Numerous seed extracts containing natural antimicrobial compounds comparatively have been reported to act in combination with CO_2 to inhibit psychrophilic bacteria (Udayasoorian *et al.,* 2017). PEF as a non-thermal processing technique of shrimp could prevent the growth of PBC to some levels during 10 days of storage at 4 °C (Shiekh and Benjakul, 2020b). Antimicrobial efficacy of PEF is governed by electroporation, which enhanced transmembrane potential (TMP) across cell membrane (Gulzar and Benjakul, 2020). Moreover, TMP generated augments polarization of cell membrane due to the mobility of free charges across the cell membrane. Such electro-com- pressive forces, causing dielectric rupture of the membrane, lead to the pore formation (Toepfl *et al.,* 2014). Prior

PEF and CLE shrimps along with CO_2 MAP jointly lowered the growth of psychrophilic bacteria, thereby impeding spoilage during refrigerated storage (4 °C).

Changes in *Pseudomonas* counts of all the samples during 0–10 days are depicted in Fig. 23b. At day 0, Pseudomonas of control and the treated samples were detected (1.40−2.14 log CFU/g). Higher *Pseudomonas* counts were found in the control than others ($p<0.05$), whereas PEF- CLE-CO_2 sample had the lowest count at day 0 ($p<0.05$). Increment in the growth of Pseudomonas was evidenced in all the samples with augmenting storage time ($p<0.05$). High *Pseudomonas* count (6.10 log CFU/g) was obtained in the control, while the PEF-CLE-CO_2 sample exhibited the lowest count (3.02 log CFU/g) after 10 days ($p<0.05$). Combined effect of PEF and 1% CLE as pre-treatment in combination with CO_2 efficiently retarded the growth of *Pseudomonas* during refrigerated storage (4 °C). *Pseudomonas* have been regarded as dominant gram negative spoilage bacteria in seafoods and are highly sensitive to be inactivated in CO_2 atmosphere (DeWitt and Oliveira, 2016). MAP containing 95% CO_2 and 5% O_2 in combination with thymol essential oil decelerated the growth of *Pseudomonas* in shrimp (Mastromatteo *et al*., 2010). Furthermore, PEF in conjunction with CLE was reported to retard the growth of *Pseudomonas* in shrimp (Shiekh and Benjakul, 2020a). Thus, anaerobic conditions also extended the lag phase of Pseudomonas during storage.

H_2S producing bacterial count of all the samples was monitored during 0–10 days (Fig. 23c). At day 0, all the MAP samples had similar H_2S producing bacterial count ($p>0.05$), except PEF-CLE-CO_2, which had the lowest count ($p<0.05$). The initial count of MAP samples with or without CLE was in the range of 1.10–1.93 log CFU/g. Control sample showed the highest H_2S producing bacterial counts than others ($p<0.05$). H_2S producing bacterial count of all the samples increased as storage time augmented ($p<0.05$). At day 10, the control, PEF-CLE, PEF-N_2, PEF-CLE-N_2, PEF-Ar, PEF-CLE-Ar, PEF-CO_2 and PEF-CLE-CO_2 samples had H_2S-producing bacterial counts of 6.11, 4.11, 3.79, 3.48, 3.10, 2.88, 2.72 and 2.53 log CFU/g, respectively. During the entire storage time, PEF-CLE-CO_2 notably retarded the growth of H_2S-producing bacteria ($p<0.05$). PEF as antimicrobial technology in combination with CLE polyphenols and organic acid was able to hinder the growth of H_2S producing bacteria in shrimp during storage at 4 °C (Shiekh and Benjakul, 2020a). MAP with low oxygen content and higher carbon dioxide level (95% CO_2 + 5% O_2) was documented

to decrease the mesophilic bacterial load by 2 log cycle along with the inhibition of H_2S forming bacteria in peeled shrimps (Mastromatteo *et al*., 2010).

Enterobacteriaceae count of all the samples was monitored throughout the storage of 10 days (Fig. 23d). At day 0, the control had higher Enterobacteriaceae count than others ($p<0.05$). Conversely PEF-CLE-CO_2 showed the lowest count ($p<0.05$). Enterobacteriaceae in all the samples augmented with the progression of storage time ($p<0.05$) and the lowest count was noticeable in PEF-CLE-CO_2 sample during 10 days ($p<0.05$). PEF-CLE sample packed in air also exhibited lower Enterobacteriaceae counts than that of control throughout the storage ($p<0.05$). Pacific white shrimp subjected to PEF in conjunction with CLE at 1% was documented to have lower Enterobacteriaceae counts than those treated with PEF and lower CLE concentration during storage at 4 °C (Shiekh and Benjakul, 2020a). GTE and AA in conjunction with MAP were reported to limit the growth of Enterobacteriaceae in Pacific white shrimp more effectively (Nirmal and Benjakul, 2011). Enterobacteriaceae are facultative anaerobic gram negative spoilage bacteria and are highly sensitive to dissolved CO_2 than gram positive bacteria (McMillin, 2008). This could be attributed to the effective passage of CO_2 through the thin layer of peptidoglycan surrounding the cell membrane in gram negative bacteria. Shrimp pre-treated with PEF and 1% CLE before packaging under CO_2-MAP showed the marked reduction of Enterobacteriaceae counts during storage.

Changes in LAB of all the samples during 0–10 days are shown in Fig. 23e. Lower LAB count was found in PEF-CLE-CO_2 sample at day 0 ($P < 0.05$. The decreased LAB count in PEF-CLE-CO_2 obtained at day 0 was plausibly a result of joint actions imposed by CLE polyphenols with antimicrobial activity and the sensitivity of LAB towards dissolved CO_2 in shrimp. Moreover, antimicrobial efficacy was evident with the PEF-CLE pre-treatment, compared to control, thereby inhibiting the growth of LAB, regardless of packaging conditions ($p<0.05$). LAB potentially produced bacteriocins and were illustrated to reduce spoilage microbial flora (Devlieghere and Debevere, 2000). Pathogenic bacterial colonies of *Clostridium perfringens* were not detected even in strictly anaerobic conditions of MAP (free of O_2) used in this study. As a result, no cross contamination was presumed to occur during handling and preparation of shrimp.

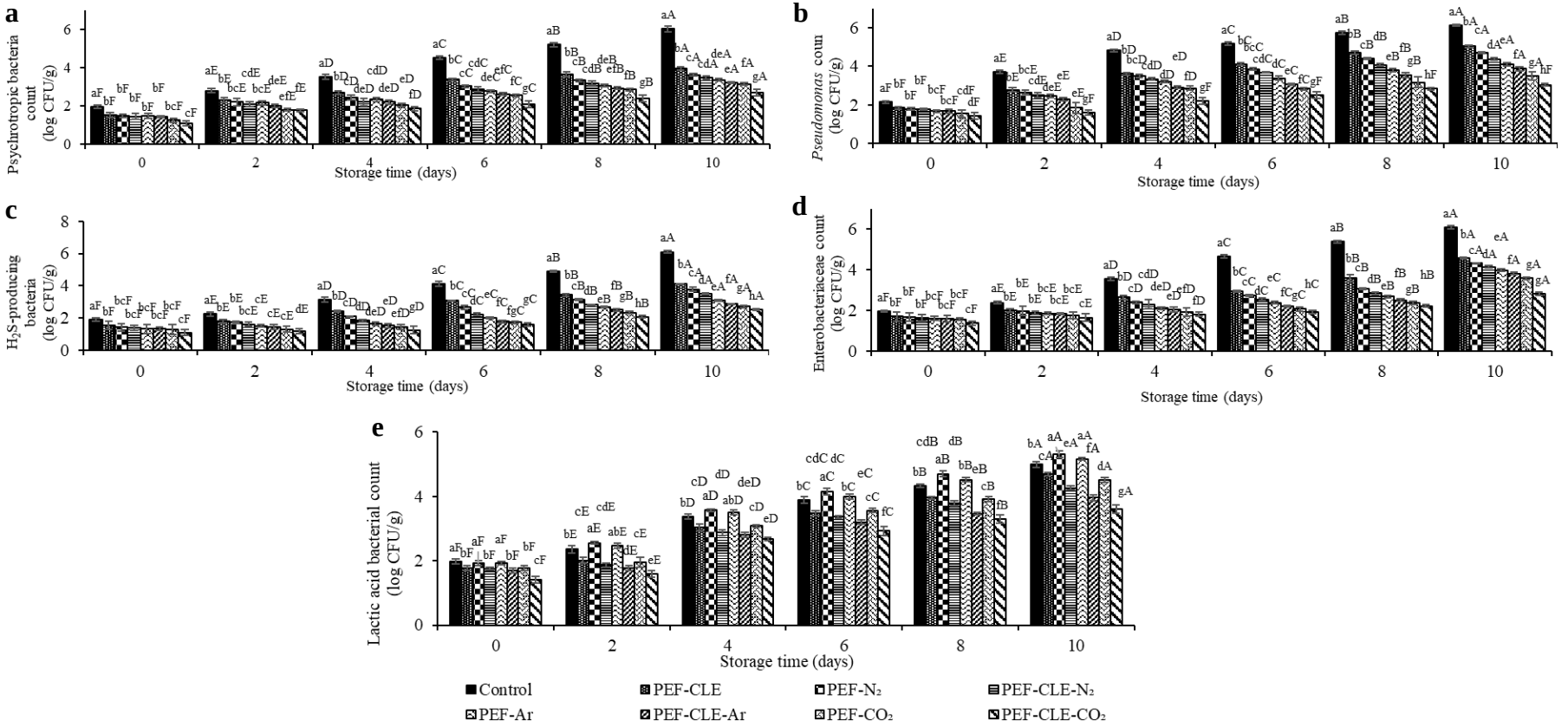

Figure 23. Psychrophilic bacterial count (A), *Pseudomonas* (B), H2S-producing bacteria (C), Enterobacteriaceae (D) and lactic acid bacteria counts (E) of Pacific white shrimp with PEF treatment in the absence and presence of CLE under different gases of modified atmosphere packaging during 10 days of refrigerated storage (4 °C).

Bars represent the standard deviation (n = 3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 22.

5.5.1.3 Changes in chemical quality

Similar pH values were attained for all the sample at day 0 ($p>0.05$) (Fig. 24a). Within the first two days, pH values of all samples were 6.5–6.7 ($p<0.05$). pH augmented in all the samples with increasing storage time ($p<0.05$). Nevertheless, the pH changes were not pronounced in PEF-CLE-CO_2 sample, compared to others during 2–10 days of refrigerated storage (4 °C) ($p<0.05$). The accumulation of basic compounds caused by endogenous enzymes, autolysis and microbial proteases, especially from spoilage bacteria, might contribute to increased pH. Similar trend was reported in Pacific white shrimp pretreated with green tea extract (GTE) and ascorbic acid (AA) under MAP during refrigerated storage (4 °C) (Nirmal and Benjakul, 2011). Combined effect of PEF, CLE and absolute carbon dioxide MAP (PEF-CLE-CO_2) kept the pH under acceptable limits. The acceptable limit for shrimp (*Penaeus merguiensis*) was set at pH 7.6 (Shamshad *et al.*, 1990). Thus, the lowered increase in pH of PEF-CLE-CO_2 sample was more likely due to reduction in microbial load as well as enzyme inhibition during the refrigerated storage (4 °C).

TVB-N contents of control and samples with prior PEF and CLE without and with different MAP ranged from 1.21 to 3.05 mg N/100 g shrimp meat at the first day (Fig. 24b). The highest TVB-N content was obtained in the control throughout the storage ($p<0.05$). TVB-N content of all the samples augmented during storage ($p<0.05$). Generally, the highest TVB-N content was obtained in the control during storage ($p<0.05$). The lowest TVB-N content (4.39 mg N/100 g meat) was detected in PEF-CLE-CO_2 sample at day 10. CLE was shown to have both antioxidant and antimicrobial activities (Shiekh *et al.*, 2019). Therefore, the control (without CLE treatment) showed higher TVB-N, the spoilage products caused by microorganisms. The least changes could be correlated with the lowest pH value obtained in PEF-CLE-CO_2 sample during 10 days, more likely due to dissolved carbon dioxide in the shrimp muscle. TVB-N content (12.96 mg N/100 g shrimp meat) of shrimp treated with high intensity PEF and soaked in 1% CLE solution at 4 °C was documented to maintain freshness in shrimp (Shiekh and Benjakul, 2020a). TVB-N content has been used as a quality indicator for spoilage of shrimp muscle with the different levels of acceptability such as < 12 mg N/100 g for freshly caught shrimp. During postharvest storage, TVB-

N levels of 12−20 mg N/100 g are for edible but the levels of 20–25 and > 25 mg N/100 g are considered as ‘borderline’ and ‘inedible and decomposed’, respectively (Okpala *et al.,* 2014). Additionally, PEF aids in the permeation of 1% CLE in shrimp muscle that served as potential pre-treatment of shrimp prior to the extract soaking or MAP. Low TVB-N content in samples kept in MAP was mainly due to the complete absence of oxygen as reflected by the drastic reduction of spoilage bacteria (Fig. 23). Combination of non-thermal technology (PEF) and natural extract (1% CLE) had the pronounced antimicrobial effect on shrimp during 10 days of storage (Shiekh and Benjakul, 2020a). Moreover, the extract from Chamuang leaves was abundant in organic acids such as hydroxycitric acid and polyphenolic glycosides that retarded the elevation of TVB-N in shrimp during storage (Shiekh *et al*., 2019). TVB-N content of ozone treated Pacific white shrimp in combination with modified atmosphere (100 % CO_2) was very low during refrigerated storage (Gonçalves and Lira Santos, 2019). As a result, PEF and CLE in conjunction with CO_2-MAP was effective means to retain quality of shrimp during storage.

Protein carbonyl content of the control was highest, compared to other samples during storage of 10 days ($p<0.05$) (Fig. 24c). PEF-CLE sample packed in air showed the lowest carbonyl content within the first two days of storage, compared to the control ($p<0.05$). As the storage proceeded, the protein carbonyls were augmented, indicating the triggered protein oxidation. However, the protein carbonyl content in PEF-CLE-CO_2 sample was lower than others at the end of storage ($p<0.05$). In general, PEF application in shrimp had no impact on myofibrillar proteins (Shiekh and Benjakul, 2020b). Moreover, the preservative effect of CO_2-MAP in conjunction with PEF and CLE pretreatment potentially prevented biochemical changes in shrimp during storage. Increased protein carbonyl contents in PEF-CLE-CO_2 were in line with the lowered TVB-N content and microbial counts. Protein carbonyls were remarkably less in the super-chilled swimmer crab packed under MAP (10% O_2 + 60% CO_2 + 30% N_2) (Sun *et al*., 2017).

Control was higher in PV compared to others ($p<0.05$). PEF-CLE-CO_2 sample had the lowest PV at day 0 ($p<0.05$) (Fig. 24d). PEF and CLE as pre-treatment reduced hydroperoxide formation to some degree as evidenced by lower PV at day 0 ($p<0.05$). CLE penetrated in shrimp muscle with the aid of PEF was reported to retard

PV in refrigerated storage of shrimp (Shiekh and Benjakul, 2020a). Furthermore, PV increased notably in the control during storage ($p<0.05$), compared to samples kept under MAP. Nevertheless, PEF-CLE-CO_2 sample exhibited lowest PV at day 10 ($p<0.05$). This could be due to the absence of oxygen and lowered pH that restricted the microbial proliferation and secretion of extracellular enzymes including lipase. Phlorotannins extracted from marine brown seaweed at 5% level with higher antioxidant activity arrested the formation of hydroperoxides in shrimp (Sharifian *et al.*, 2019). PV represents the generation of the primary oxidation product at faster rate rather than degradation at the early stages of storage (Boselli *et al*., 2005). Ar-MAP prevented the formation of hydroperoxides more potentially than the nitrogen MAP (Olatunde *et al*., 2019). Moreover, the synthesis of hydroperoxides might be associated with microbial enzymes produced, liberating free fatty acids, which underwent lipid oxidation easily. Therefore, PEF and CLE pre-treatment jointly worked with CO_2-MAP to restrict the generation of PV during 10 days of storage.

TBARS value of the control was highest among all the samples after 10 days of storage ($p<0.05$) (Fig. 24e). PEF-CLE sample packed in air had lower TBARS value than the control ($p<0.05$). Control sample packed in air possessed the higher content of TBARS, suggesting that hydroperoxides were decomposed to the secondary oxidation products. PEF and CLE pre-treatments along with CO_2 atmosphere helped to suppress the formation of TBARS in PEF-CLE-CO_2 sample. The lowest level (1.65 mg MDA/kg of shrimp meat) was found at day 10. Fatty acids in seafood were degraded to some degree in Ar-MAP without any addition of natural additives during refrigerated storage (4 °C) (Olatunde *et al*., 2019). Additionally, PEF and CLE at 1% level potentially prevented lipid oxidation of shrimp during storage (Shiekh and Benjakul, 2020a).

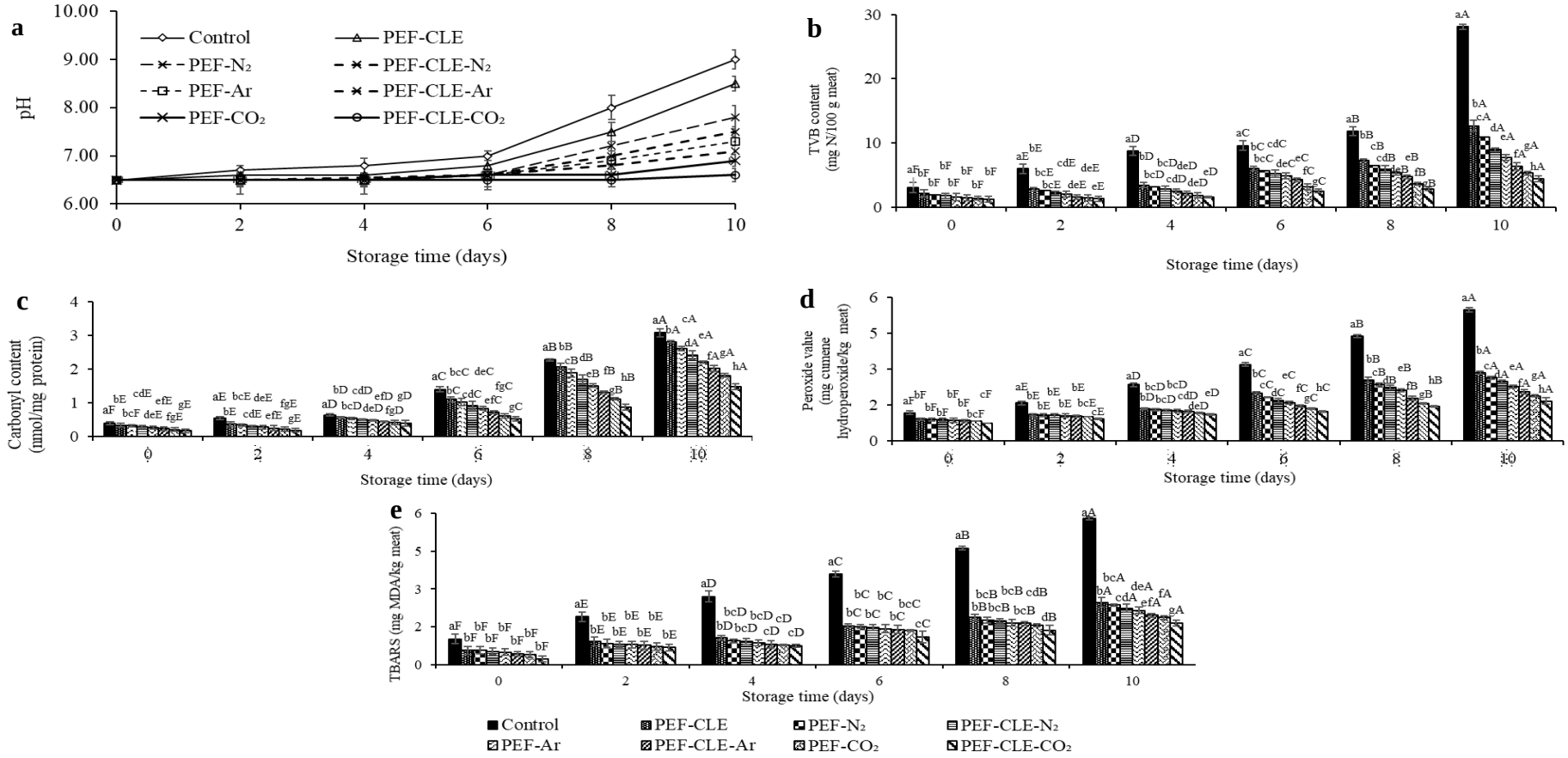

Figure 24. pH (a), total volatile basic nitrogen (TVB-N) (b), carbonyl content (c), peroxide values (d) and thiobarbituric reactive substances (TBARS) values (e) of Pacific white shrimp with PEF treatment in the absence and presence of CLE under different gases of modified atmosphere packaging during 10 days of storage at 4 °C.

Key: see the caption for Fig. 22.

5.5.1.4 Changes in sensory property

At day 0, similar likeness scores were attained for all samples (Table 7). The results reconfirmed that high intensity PEF and 1% CLE level as pre-treatments showed no negative impact on acceptability or likeness of shrimp meat, regardless of the gases used for MAP. The marked decrease in the likeness scores was evidenced at the end of storage ($p<0.05$). However, the likeness scores obtained in PEF-CLE-CO_2 sample were higher for all attributes, followed by PEF-CLE-Ar, PEF-CLE-N_2 and the control, respectively ($p<0.05$). Moreover, PEF treated samples packed under MAP exhibited lower sensory scores than those treated with both PEF and CLE ($p<0.05$). The control possessed the lowest likeness scores for all tested attributes ($p<0.05$). The greater deterioration in the control was related with high microbial load and remarkably higher changes in pH, protein carbonyls, TVB-N, PV and TBARS values (Fig. 24). Higher color likeness score was in accordance with the reduced melanosis in PEF-CLE-CO_2 sample (Fig. 22). Carbon dioxide has the ability to create weak acidic condition, in which carbonic acid could be formed when the gas was dissolved with the water, thus exhibiting preservative effect (Laursen *et al.,* 2006). Shrimp packed in MAP with AA and GTE treatment had higher likeness scores (Nirmal and Benjakul, 2011). Besides the preservative effect of CO_2-MAP, CLE rich in polyphenols has been reported to penetrate deeply through the shrimp shell via pores formed by high energy electric field pulses, in which sensory quality of Pacific white shrimp was enhanced (Shiekh and Benjakul, 2020a).

5.5.1.5. Changes in fatty acids

No difference in fatty acid profile was noticed among all the samples at day 0 ($p>0.05$) (Table 8), in which palmitic acid was predominant ($p<0.05$). PUFA constituted at the higher content than saturated fatty acid and MUFA ($p<0.05$). In addition, EPA, DHA and other n-3 fatty acids were also abundant after storage of 10 days. The result indicated that negligible oxidation of fatty acids took place in all the MAP sample which were completely devoid of oxygen. Shrimp contain a high amount of n-3 fatty acids (Pires *et al*., 2018). With the advancement in storage time, fatty acid content of shrimp samples differed between PEF-CLE-N_2, PEF-CLE-Ar, especially at day 10 ($p<0.05$).

Table 7. Likeness score of Pacific white shrimp with PEF treatment in the absence and presence of CLE under different modified atmosphere packaging at day 0 and 10 of refrigerated storage.

Storage time (days)	Samples	Color	Odor	Texture	Flavor	Taste	Overall
0	Control	8.74±0.45aA	8.50±0.57aA	8.33±0.62aA	8.53±0.52aA	8.47±0.52aA	8.63±0.48aA
	PEF-N_2	8.77±0.37aA	8.57±0.50aA	8.38±0.47aA	8.57±0.50aA	8.53±0.52aA	8.65±0.51aA
	PEF-CLE-N_2	8.78±0.39aA	8.60±0.51aA	8.47±0.64aA	8.60±0.51aA	8.67±0.49aA	8.67±0.49aA
	PEF-Ar	8.80±0.41aA	8.65±0.46aA	8.53±0.64aA	8.67±0.49aA	8.73±0.46aA	8.70±0.46aA
	PEF-CLE-Ar	8.83±0.36aA	8.68±0.47aA	8.57±0.50aA	8.60±0.51aA	8.77±0.42aA	8.73±0.42aA
	PEF-CO_2	8.81±0.41aA	8.70±0.46aA	8.60±0.51aA	8.67±0.49aA	8.81±0.39aA	8.77±0.42aA
	PEF-CLE-CO_2	8.87±0.35aA	8.73±0.46aA	8.67±0.49aA	8.80±0.41aA	8.87±0.35aA	8.83±0.24aA
10	Control	4.53±0.52eB	4.47±4.47eB	4.23±0.68eB	4.18±0.38eB	4.00±4.20eB	4.29±0.45eB
	PEF-N_2	6.87±0.35cdB	6.77±0.50cdB	6.53±0.52cd	6.47±0.52cdB	6.50±0.63cdB	6.59±0.47cdB
	PEF-CLE-N_2	7.33±0.49cB	7.20±0.41cB	6.97±0.40cB	6.91±0.19cB	6.93±0.18cB	7.02±0.33cB
	PEF-Ar	7.20±0.41bcB	7.07±0.46bcB	6.83±0.44bcB	6.77±0.50bcB	6.80±0.56bcB	6.90±0.47bcB
	PEF-CLE-Ar	7.77±0.56bB	7.73±0.46bB	7.49±0.49bB	7.43±0.50bB	7.46±0.51bB	7.55±0.50bB
	PEF-CO_2	7.50±0.50abB	7.37±0.61abB	7.13±0.35abB	7.10±0.28abB	7.13±0.35abB	7.22±0.41abB
	PEF-CLE-CO_2	8.20±0.41aB	8.17±0.70aB	7.93±0.59aB	8.07±0.70aB	8.20±0.37aB	8.23±0.56aB

Values are presented as the mean ± standard deviation (n=50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig. 22

PEF-CLE-CO_2 sample possessed the highest retention of PUFA (42.18 %) with EPA and DHA contents of 14.55 and 14.61 g/100 g, respectively. In contrast, PUFA content of PEF-CLE-N_2 was lowered due to the higher oxidation, probably related with the elevated microbial load, PV and TBARS contents ($p<0.05$). Lipid oxidation generate aldehydes and ketones with unfavorable sensory property in muscle foods and has the negative impact on human health (Pan *et al.*, 2019). Moreover, the extracellular enzymes including lipase produced by different strains of spoilage microorganisms in Pacific white shrimp could also enhance the enzymatic deterioration of fatty acids (Tzuc *et al.*, 2014). Therefore, the higher retention of fatty acids in shrimp stored in CO_2-MAP with prior PEF and CLE at 1% jointly preserved nutritional quality of shrimp as evidenced by the lowered changes in fatty acid profile during 10 days of refrigerated storage (4 °C).

5.5.1.6. Volatile compounds

At day 10, hydrocarbons, aldehydes, ketones and alcohols in PEFCLE-CO_2 sample were very low in abundance and were mostly not detected, compared to the PEF-CLE-N_2, PEF-CLE-Ar and control (Table 9). Numerous compounds at high abundance led to the development of severe off-odor for the control, compared to others. This was related to the lower odor likeness score (Table 7). Some alcoholic compounds were reported in shrimp without and with MAP (50% N_2 and 50% CO_2), resulting in the formation of mixed off-odor inside the package (Jaffrès *et al*., 2011). Aldehydes, ketones and alcohols of PEFCLE-Ar were lower than PEF-CLE-N_2, indicating the lower extent of lipid oxidation. 2-methyl-1-butanal, 2-methyl-1-propanal and pentanal were identified to contribute to the formation of cheesy or nauseous off-odor (Laursen *et al*., 2006). Ketones such as 2-butanone, 2-propanone and 2, 3-heptanedione showed higher abundance in control than others. On the other hand, PEF-CLE-CO_2 sample had the least abundance of ketones. This was related with decreased PV and retention of PUFA values obtained in PEF-CLE-CO_2 (Fig. 24 and Table 8). Aforementioned ketones have been documented to impact the shrimp quality by the generation of buttery or cheesy off-odor (Laursen *et al*., 2006).

Table 8. Fatty acid profiles of Pacific white shrimp with PEF treatment in the absence and presence of CLE under different modified atmosphere packagings at day 0 and day 10 of refrigerated storage.

	Day 0				Day 10		
Fatty acids (g/100 g)	Control	PEF-CLE-N_2	PEF-CLE-Ar	PEF-CLE-CO_2	PEF-CLE-N_2	PEF-CLE-Ar	PEF-CLE-CO_2
C14:0 (Myristic)	1.14±0.02a	1.15±0.01a	1.16±0.02a	1.16±0.01a	0.91±0.06c	1.15±0.01a	1.15±0.01a
C16:0 (Palmitic)	17.97±0.51a	17.99±0.51a	18.41±0.15a	18.52±0.16a	12.33±0.76a	18.58±0.51b	18.39±0.21c
C16:1 (Palmitoleic)	0.53±0.01a	0.55±0.01a	0.57±0.01a	0.58±0.02a	0.32±0.02c	0.56±0.01b	0.60±0.15a
C17:0 (Heptadecanoic)	2.02±0.02a	2.04±0.03a	2.13±0.09a	2.19±0.08a	2.77±0.05a	2.11±0.12b	2.00±0.05c
C17:1 cis 10 (Heptadecanoic)	0.62±0.01a	0.64±0.01a	0.65±0.01a	0.66±0.01a	0.42±0.01b	0.66±0.02a	0.67±0.01a
C18:0 (Stearic)	9.86±0.06a	9.89±0.04a	10.07±0.25a	10.18±0.25a	9.52±0.01c	10.25±0.18b	9.95±0.17a
C18:1 trans 9 (Elaidic)	0.71±0.04a	0.72±0.03a	0.74±0.02a	0.76±0.03a	0.32±0.11b	0.69±0.01ab	0.73±0.03a
C18:1 cis 9 (Oleic)	3.70±0.02a	3.66±0.04a	3.69±0.02a	3.72±0.02a	2.89±0.14c	3.25±0.04b	3.59±0.11a
C18:2 cis 9,12 (Linoleic)	11.83±0.21a	11.88±0.25a	12.00±0.27a	12.09±0.09a	9.62±0.27c	10.74±0.22b	11.59±0.22a
C20:1 cis 11 (Eicosenoic)	0.52±0.02a	0.55±0.02a	0.58±0.02a	0.59±0.01a	0.37±0.02b	0.51±0.02ab	0.57±0.03a
C18:3 cis 9,12,15 (alpha-Linolenic)	0.58±0.03a	0.58±0.03a	0.60±0.01a	0.63±0.03a	0.39±0.03c	0.52±0.03b	0.66±0.02a
C21:0 (Heneicosanoic)	1.06±0.04a	1.07±0.02a	1.11±0.02a	1.19±0.01a	0.82±0.04c	0.94±0.05b	10.20±0.04a
C20:2 cis 11,14 (Eicosadienoic)	1.76±0.03a	1.78±0.04a	1.84±0.03a	1.89±0.06a	1.22±0.07c	1.58±0.06b	1.75±0.06a
C20:0 (Docosanoic)	0.95±0.02a	0.96±0.01a	0.97±0.02a	0.98±0.01a	0.61±0.01c	0.76±0.01b	0.88±0.01a
C22:1 cis 13 (Erucanoic)	0.58±0.01a	0.59±0.01a	0.61±0.04a	0.62±0.01a	0.35±0.01c	0.42±0.04b	0.55±0.02a
C20:4 cis 5,8,11,14 (Eicosatetraenoic)	0.56±0.02a	0.57±0.02a	0.58±0.02a	0.65±0.02a	0.45±0.01b	0.56±0.01ab	0.60±0.02a
C23:0 (Tricosanoic)	3.87±0.02a	3.88±0.03a	3.89±0.03a	3.92±0.03a	3.61±0.09c	3.72±0.11b	3.83±0.09a
C22:2 cis 13,16 (Docosadienoic)	0.57±0.03a	0.58±0.02a	0.59±0.02a	0.67±0.08a	0.44±0.01c	0.61±0.01a	0.59±0.05a
C20:5 cis 5,8,11,14,17 (EPA)	14.59±0.22a	14.69±0.22a	14.85±0.19a	14.93±0.29a	13.83±0.23c	14.18±0.12b	14.60±0.13a
C24:1 cis 15 (Nervonic)	0.67±0.02a	0.68±0.02a	0.69±0.01a	0.76±0.02a	0.50±0.01b	0.59±0.01ab	0.68±0.02a
C22:6 cis 4,710,13,16,19 (DHA)	14.22±0.27a	14.32±0.28a	14.54±0.35a	14.64±0.15a	12.92±0.12c	13.52±0.27b	14.60±0.21a
Saturated fatty acids (SFA)	36.35	36.90	37.02	38.47	34.90	35.12	36.47
Monounsaturated fatty acids (MUFA)	6.69	6.76	6.80	6.88	4.66	5.20	5.28
Polyunsaturated fatty acids (PUFA)	42.68	43.24	43.30	43.58	40.35	41.12	42.18

Values are presented as the mean ± standard deviation (n=3). Different lowercase letters within the same row at the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig 22.

Table 9. Volatile compounds of PEF and CLE pre-treated Pacific white shrimp without or with different MAP stored for 10 days at 4 °C.

Compound	Peak abundance ($\times 10^7$)			
	Control	PEF-CLE-N_2	PEF-CLE-Ar	PEF-CLE-CO_2
Hydrocarbons				
Methylbenzene	4.29	2.83	1.99	ND
Butane	22.2	5.57	3.30	0.26
2,3,4-trimethyl-pentane	11.83	7.85	4.35	ND
3,3-dimethyl-hexane	18.04	12.56	6.25	0.34
3-methyl-heptane	17.24	11.80	4.52	0.53
2,2,4-trimethyl-hexane	8.88	4.27	2.53	ND
Octane	11.02	23.2	5.71	ND
Dodecane	2.49	0.58	0.48	0.20
Aldehydes				
2-methyl-1-butanal	1.97	ND	ND	ND
2-methyl-1-propanal	7.45	5.02	3.54	ND
Pentanal	8.34	6.52	4.25	0.14
Ketones				
2-butanone	20.44	10.52	4.85	1.23
2-propanone	6.52	5.14	ND	ND
2,3-heptanedione	8.22	5.54	2.13	ND
Alcohols				
3-methyl-1-butanol	24.63	11.73	8.54	ND
1-pentanol	11.72	5.87	0.95	0.64
2-octanol	2.20	1.12	0.65	ND
2-propanol	2.50	ND	ND	ND
Others				
Trimethylamine	11.99	7.55	5.42	ND
Methanethiol	10.81	8.99	0.96	ND
Carbonic acid	ND	ND	ND	0.42

ND = not detected; PEF = Pulsed electric field; CLE = Chamuang leaf extract; N_2 = Nitrogen.; Ar = Argon; CO_2 = Carbon dioxide.

Moreover, the off-odor generated by methanethiol and trimethylamine in shrimp was the result of microbial degradation of proteins or amino acids (Laursen *et al*., 2006). Therefore, shrimp placed in CO_2-MAP were free of off-odor, and were related with the retarded quality losses.

5.6 Conclusions

PEF-CLE-CO_2 sample retained the quality most effectively, in which melanosis and microbial growth were retarded. Protein and lipid oxidation was lowered, mainly due to antioxidant activity of CLE. Lower quality changes in PEF-CLE-CO_2 sample were associated with the highest likeness score and coincidentally lower off-odor volatiles. Thus, PEF and CLE pre-treatments in combination with CO_2-

MAP could preserve quality of Pacific white shrimp during 10 days of refrigerated storage (4 °C).

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CHAPTER 6

EFFECT OF HIGH VOLTAGE COLD ATMOSPHERIC PLASMA PROCESS-ING ON THE QUALITY AND SHELF-LIFE OF PACIFIC WHITE SHRIMP TREATED WITH CHAMUANG LIFE EXTRACT

6.1 Abstract

High voltage cold atmospheric plasma (HV-CAP) using dielectric barrier discharge was implemented on Pacific white shrimp (PWS) pre-soaked with 0.5 or 1% Chamuang leaf extract (CLE). PWS were firstly packed under different gas ratios of Ar, O_2 or air and then subjected to HV-CAP for 10 min. Lower melanosis scores were found in the samples treated with 1% CLE and HV-CAP in the presence of Ar and air (80:20) than the control and other samples throughout the storage of 15 days at 4 °C ($p<0.05$). Chemical quality changes in 1% CLE treated shrimp packaged in Ar and Air atmosphere followed by HV-CAP (CP-Ar/Air-1 CLE sample) were not significantly found at day 15. Those were evidenced by lower changes in pH (6.90), lower carbonyl content (2.41 μmol/mg protein), total volatile base (TVB) content (17.81 mgN/100 g meat), peroxide value (4.21 mg cumene hydroxide/kg meat) and thiobarbituric acid reactive substances (TBARS) (2.35 mgMDA/ kg meat) at the end of storage, compared to those of the control and other samples treated with 0.5% CLE and HV-CAP under different gas compositions at day 15 of storage ($p<0.05$). Mesophilic bacteria, Pseudomonas, H_2S-producing bacteria, Enterobacteriaceae, lactic acid bacteria and psychrophilic bacterial counts were less or equal to 5 log CFU/g meat in CP-Ar/Air-1 CLE sample, which were lower than the control and other treated samples at the end of the storage (4 °C) ($p<0.05$). CP-Ar/Air-1 CLE sample also exhibited higher likeness scores for all the tested attributes, mostly associated with the reduced protein and lipid oxidation at the end of storage. After treatment, oxidation of polyunsaturated fatty acids (EPA and DHA) was lowered by 14.9% and 12.4% in CP-Ar/Air-1 CLE sample, compared to that found in the control ($p<0.05$). Protein oxidation of HVCAP treated sample was prevented by 1% CLE, irrespective of gas composition. Therefore, HV-CAP especially under the mixture of Ar and air

along with high level of CLE, exhibited high efficacy in prolonging shelf-life of refrigerated (4 °C) Pacific white shrimp up to 15 days.

6.2 Introduction

Pacific white shrimp (PWS), a valued crustacean, is known for its unique delicacy with appealing characteristics, widely consumed in Thailand and different parts of the world (Gulzar and Benjakul, 2020). Quality of the shrimp is of primary concern during the supply chain management from the capture site to the distribution of shrimp for venders, industries and stock for supermarkets (Shiekh and Benjakul, 2020b). With improper storage, shrimp undergoes discoloration called melanosis that imparts black color (Shiekh and Benjakul, 2020a). Melanosis is triggered by the exposure of copper-containing enzyme named polyphenol oxidase (PPO) to atmospheric oxygen (De Souza Silva *et al*., 2019). Apart from enzymatic deterioration due to PPO, quality changes will continue during extended storage. To curb such a quality loss, different storage systems for maintaining the overall quality of shrimp have been extensively studied (Ndraha *et al*., 2019). In addition, several additives have been employed for the quality control of shrimp, but some of them are not safe for consumption, causing allergy or other health problems. Moreover, the refrigeration or frozen storage is insufficient without the additives to supress the microbiological or biochemical changes in shrimp, respectively (Fennema *et al*., 2008). Without a proper hurdle, lipids and proteins in shrimp was degraded, thus reducing organoleptic quality (Tsironi *et al*., 2009). Recently, the dramatic shift towards exploitation of natural additives, especially from plant sources in conjunction with non-thermal technology has been applied to prolong the shelf-life of shrimp (Shiekh and Benjakul, 2020b). Shelf-life extension of PWS can be achieved by the addition of natural preservatives that can synergistically prevent quality loss during refrigerated storage (Sae-leaw and Benjakul, 2019b).

Plant sources are the rich sources of polyphenols with antioxidant and antimicrobial properties. Their extracts have been applied for lowering the quality losses of seafoods during handling and storage (Olatunde and Benjakul, 2018a). Chamuang leaf extract (CLE) is abundant in polyphenolic glycosides and organic acids that could act as potential PPO inhibitor with antimicrobial and antioxidative characteristics for quality and shelf-life extension of Pacific white shrimp (Shiekh *et*

al., 2019). Chrysoeriol 6-C-glucoside-8-C-arabinopyranoside and 2-feruloyl-1-sinapoylgentiobiose were the most dominant compounds in CLE (Shiekh *et al*., 2019). Antimicrobial constituents in CLE inhibited microbial growth (Sakunpak and Panichayupakaranant, 2012). High intensity pulsed electric field (PEF) also exhibits antimicrobial effect and can inactivate PPO to some degree via denaturation (Shiekh and Benjakul, 2020a). Naturally occurring polyphenols have been employed to reduce protein and lipid oxidation via their free radical-scavenging mechanism in seafoods (Benjakul *et al*., 2014).

Expansion in the use of a synthetic additive-free approach and nonthermal technology in the food sector provides promising results for enhancing shelf-life of products. Several non-thermal and minimal processing techniques have been adopted to prolong shelf-life of shrimp. High-voltage cold plasma is an eco-friendly technology that eliminates spoilage microorganisms and foodborne pathogens in PWS and also aids in the inactivation of PPO (De Souza Silva *et al*., 2019). HV-CAP inactivates microorganisms by creating lesions to the cell membrane and enhanced oxidation of proteins, lipids, and nucleic acids (Laroussi *et al*., 2003; Smet *et al*., 2018). The efficacy of HV-CAP in inactivating both spoilage and pathogenic organisms has been demonstrated in several food products (Lacombe *et al*., 2015; Misra *et al*., 2013; Yong *et al*., 2015). In-bag plasma, gas ionization liberates ionized molecules, atoms and electrons in a strong electromagnetic field (Misra *et al*., 2011). Although ozone and other reactive species generated from HV-CAP treated working gases such as Ar, O_2, N_2 and their mixtures showed the preservative effect, those active species could enhance lipid and protein oxidation in seafoods (Olatunde and Benjakul, 2018b). Nevertheless, the application of HV-CAP has not been extensively employed in extending shelf-life of seafoods because of the pronounced lipid and protein oxidation initiated by the cold plasma reactive species (Papuc *et al*., 2017). Therefore, the combined use of natural extracts with high antioxidative power could mitigate the oxidation process during HV-CAP treatment. This study aimed to investigate the impact of HV-CAP along with CLE for extending the shelf-life of PWS at refrigerated (4 °C) temperatures.

6.3 Objective

To elucidate the impact of HV-CAP along with CLE on melanosis and quality deterioration of Pacific white shrimp during refrigerated storage.

6.4 Materials and methods

6.3.1 Chemicals

Malonaldehyde bis (dimethyl acetal), thiobarbituric acid (TBA) and tetramethylmurexide (TMM) were procured from Sigma Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), chloroform and methanol were obtained from Merck (Darmstadt, Germany). Potassium carbonate was obtained from Fisher Scientific (Loughborough, UK). Standard plate count agar, triple sugar iron agar, *Pseudomonas* isolation agar, eosin methylene blue agar, *Clostridium perfringens* agar, 2, 4-dinitrophenyl hydrazine (DNPH) and guanidine were purchased from Oxoid Ltd. (Hampshire, UK). Chemicals for electrophoresis including Coomassie blue R-250, sodium dodecyl sulfate (SDS), *N,N,N,N′* tetramethyl ethylene diamine (TEMED) and polyacrylamide were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

6.4.2 Collection and preparation of Pacific white shrimp (PWS)

Fresh shrimp of the size 55–60 shrimp per kg were harvested from a shrimp farm located in Songkhla province of southern Thailand. Shrimp without any chemical additive were placed in layers along with crushed ice at two portions in polystyrene foam boxes and were immediately carried to the seafood chemistry and biochemistry laboratory, Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Shrimps were washed using ice water to remove the extraneous impurities. Eventually, shrimp were transferred carefully in a polystyrene box and covered with layers of crushed ice prior to pretreatment or packaging.

6.4.3 Pre-treatment of PWS with CLE

Mature Chamuang leaves (*Garcinia cowa* Roxb.) were plucked from trees (5–7 year-old) from a local garden in Hat Yai during the mid of November 2019 and prepared as described by Shiekh *et al*. (2019). CLE was prepared in distilled water using high intensity power ultrasonic equipment (Vibra-Cell™ VC 750, Sonics and

Materials Inc., Newtown, CT, USA) with an amplitude: 70%; Time of sonication: 30 min; pulse-on time: 50 s; and pulse-off time: 10 s. The CLE was lyophilized in a freeze dryer (Cool Safe 55, ScanLaf A/S, Lynge, Denmark). The lyophilized powder was referred as Chamuang leaf extract (CLE). Shrimp were soaked in 0.5 or 1% CLE solution for 30 min following the procedure of Shiekh and Benjakul (2020b).

6.4.4 HV-CAP system

HV-CAP system (dielectric-barrier discharge) consisted of a high voltage transformer (50 Hz, 230 V input voltage) and a voltage variac (output voltage controlled within 0–120 kV). HV-CAP discharge was spawned between two 15-cm diameter aluminum electrodes separated by two perspex dielectric layers (10-mm and 1.5-mm thickness) (Fig. 25). The system was operated at atmospheric pressure at voltage of 16 kVRMS. A distance of 2.0-cm between two electrodes was retained (Ziuzina *et al*., 2014).

6.4.4.1 Effect of HV-CAP on prior CLE treated PWS during refrigerated (4 °C) storage

Prior CLE-treated shrimp (7 shrimps) were kept in nylon/linear low-density polyethylene (LLDPE) bag (29 × 21 cm^2, 80-µm thickness). Five volumes of gas were filled in bag with the aid of Henkovac type 1000 (Tecnovac, Italy). Shrimp treated with 0.5 or 1% CLE were packaged under different food-grade atmospheric gas mixtures including sterilized zero air (containing < 0.1 hydrocarbon impurities), argon (Ar) and oxygen (O_2). Those included Ar:O_2 (80:20) and Ar: air (80:20). After filling the samples with those gases, the bags were sealed and subjected to dielectric barrier discharge (DBD) for 10 min (Olatunde *et al*., 2019d). Another set of samples were not treated with DBD. The schematic setup for DBD has been provided in Fig. 25. The samples were prepared by the pre-treatment of 0.5 or 1% CLE without and with in-bag HV-CAP using different gas compositions such as Ar/O_2 (80:20) and Ar/Air (80:20). All the sample treatments are listed as follows:

1. Control (without any treatment, packed in atmospheric air)
2. Ar/O_2–0.5 CLE (0.5% CLE treated and packed in Ar/O_2 atmosphere)
3. Ar/Air-0.5 CLE (0.5% CLE treated and packed in Ar/Air atmosphere)
4. Ar/O_2–1 CLE (1% CLE treated and packed in Ar/O_2 atmosphere)

5. Ar/Air-1 CLE (1% CLE treated and packed in Ar/Air atmosphere)
6. CP-Ar/O_2–0.5 CLE (0.5% CLE treated, packed in Ar/O_2 atmosphere, HV-CAP)
7. CP-Ar/Air-0.5 CLE (0.5% CLE treated, packed in Ar/Air atmosphere, HV-CAP)
8. CP-Ar/O_2–1 CLE (1% CLE treated, packed in Ar/O_2 atmosphere, HVCAP)
9. CP-Ar/Air-1 CLE (1% CLE treated, packed in Ar/Air atmosphere, HV-CAP)

Two bags were taken for each treatment every 3 days. Samples were pooled and considered as composite samples. Additionally, fixed samples (20 shrimp) for each treatment were monitored for melanosis every 3 days for totally 15 days. However, the selected bags with different doses of CLE and HV-CAP treatment were taken at day 0 and day 15 for sensory evaluation.

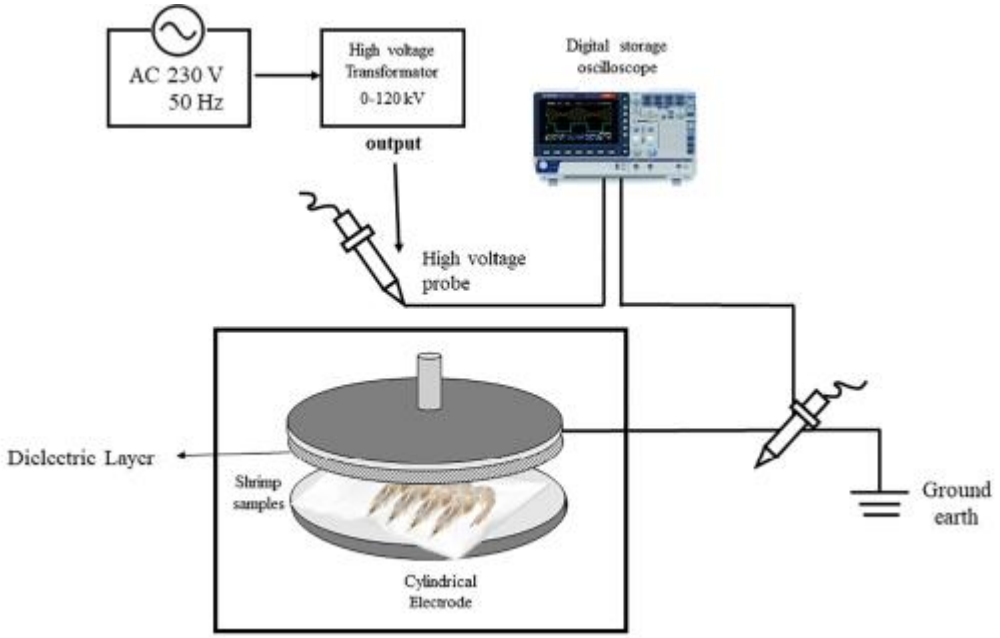

Figure 25. Schematic of experimental set-up of HV-CAP employed for treatment of Pacific white shrimp.

6.4.4.2 Melanosis assessment

The procedure of Nirmal and Benjakul (2010) was adopted for melanosis (black spots) assessment through visual inspection using 10-point scoring test by ten experienced panelists, who were familiar with the scoring or rating of blackspot (melanosis) intensity in raw Pacific white shrimp. Score of 0−10, where 0 = absent; 2 = slight (up to 20% of shrimp surface affected); 4 = moderate (20−40% of

shrimp surface affected); 6 = notable (40−60% of shrimp surface affected); 8 = severe (60−80% of shrimp surface affected); 10 = extremely heavy (80−100% of shrimp surface affected).

6.4.4.3 Microbiological analyses

Total viable counts (TVC) and psychrophilic bacterial counts (PBC), *Pseudomonas*, H_2S-producing bacteria and Enterobacteriaceae counts were enumerated using spread plate technique (Shiekh *et al*., 2019). Shrimp meat (10 g) was blended with 90 ml of 0.85% sterile saline solution. Subsequently, the mixture was homogenized using a Stomacher (Mode l400, Seward Ltd. West Sussex, England) for 2 min at 220 rpm. Homogenate was serially diluted ten-fold in 0.85% sterile saline solution. TVC and PBC counts were determined using plate count agar and incubated at 37 °C for 2 days and 4 °C for 10 days, respectively. Pseudomonas counts displayed as white colonies and hydrogen sulphide (H_2S) producing bacteria counts denoted as black colonies, were enumerated on Triple sugar iron agar and *Pseudomonas* isolation agar, respectively, after incubation at 25 °C for 3 days. Enterobacteriaceae were grown on Eosin Methylene Blue as revealed by the dark colonies with a green metallic sheen upon incubation at 37 °C for 3 days. Lactic acid bacteria (LAB) and *Clostridium perfringens* counts were determined using de Man Rogosa and Sharpe agar and Perfringens agar medium supplemented with a soft double layer of Tryptic Soy agar and incubated in an anaerobic jar at 35 °C and 37 °C using a CO_2 in flux incubator (Binder Model C 170, Binder Inc., Bohemia, NY, USA), respectively, for 3 days (Olatunde *et al*., 2019a). Black colonies grown on *perfringens* agar base indicated *Clostridium perfringens* (Rahman *et al*., 2016).

6.3.5 Physico-chemical analyses

6.4.5.1 pH

Shrimp meat (2 g) was mixed in distilled water (20 ml), homogenized for 1 min at 10000 rpm (IKA-Werke GmbH and Co. KG, Staufen, Germany) and kept for 5 min at room temperature (28 ± 2 °C). The pH of prepared samples was analyzed using a pH meter (Sartorious North America, Edgewood, NY, USA) (Nirmal and Benjakul, 2011). Shrimp meat (2 g) was mixed in distilled water (20 ml), homogenized for 1 min at 10000 rpm (IKA-Werke GmbH and Co. KG, Staufen, Germany) and kept

for 5 min at room temperature (28 ± 2 °C). The pH of prepared samples was analyzed using a pH meter (Sartorious North America, Edgewood, NY, USA) (Nirmal and Benjakul, 2011).

6.4.5.2 Total volatile base (TVB) and protein carbonyl contents

Conway micro-diffusion method was adopted for measurement of TVB content as described by Sae-leaw and Benjakul (2019a). Sample (2 g) was extracted with 8 ml of 4% (w/v) trichloroacetic acid (TCA) solution. The mixture was homogenized at 8000 rpm for 1 min using a PT 2100 homogenizer (Kinematica AG, CH-6014, Littau/Luzern, Switzerland). The homogenate was kept at room temperature for 30 min. The homogenate was filtered through Whatman No. 4 filter paper (Schleicher and Schuell, Maidstone, England). The filtrate was collected and the final volume was adjusted to 10 ml using 4% TCA. The inner ring solution (1 ml) and filtrate (1 ml) were added to inner ring and outer ring of the Conway unit, respectively. Saturated K_2CO_3 solution (1 ml) was then added into outer ring. The Conway unit was closed and the solution was mixed slowly. The mixture was incubated at ambient temperature for 60 min and the inner ring solution was titrated with 0.02 N HCl using a micro-burette until green color turned into pink. For the blank, TCA solution (4%) was used instead of sample extract. The amounts of TVB were calculated and the results were expressed as mg N/100 g shrimp meat.

Carbonyl content was examined via derivatization with 2,4-dinitrophenyl hydrazine (DNPH) and expressed as nmol of DNPH fixed/mg protein as tailored by Nikoo *et al*. (2015). Firstly, samples were extracted for natural actomyosin (NAM) as detailed by Chantarasuwan *et al*. (2011). Shrimp mince (50 g) was homogenized in 10 volumes of chilled 0.6 M KCl, pH 7.0 at a ratio of 1:10 (w/v) at a speed of 10,000 rpm using a homogenizer (IKA, Selangor, Malaysia). To avoid overheating, the sample was placed in ice bath and homogenized for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The extract was centrifuged at 5000 ×g for 30 min at 4 °C using a refrigerated (4 °C) centrifuge (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA, USA). Three volumes of chilled deionized water were added to precipitate NAM. The NAM was collected by centrifuging at 5000 ×g for 20 min at 4 °C. The NAM pellet was stored in ice until use. The prepared NAM solution from the pallet was diluted to obtain a protein concentration

ranging from 2.0 to 2.5 mg/ml and precipitated with 10% (w/v) trichloroacetic acid (TCA) solution. After centrifugation (2000 ×g for 10 min at 4 °C), the obtained sediment was incubated with 0.002% (w/v) DNPH in 2 M HCl in dark for 1 h at room temperature with vortexing every 10 min. A blank was made by adding an equal volume of 2 M HCl instead of adding DNPH solution. The mixture was precipitated with 20% (w/v) TCA solution and centrifuged at 2000 ×g for 10 min at 4 °C. Then, the sediment was washed thrice with 1 ml of ethanol: ethyl acetate (1:1, v/v) containing 10 mM HCl. The ethanol/ethyl acetate pellet was virtually colorless by the third wash, indicating the removal of excess DNPH. The protein sediment was then dissolved in 2 ml of 6 M guanidine-HCl with 20 mM sodium phosphate buffer, and allowed to stand overnight at 4 °C. Finally, the absorbance was measured at 365 nm for the DNPH-treated sample against an HCl control. The amount of carbonyl was expressed as μmol of DNPH fixed/ mg of protein using an absorption coefficient of 22,000 (M^{-1} cm^{-1}) for protein hydrazones

6.4.5.3 Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

Lipids were firstly extracted from shrimp meat using the method of Bligh and Dyer (1959). Sample (2 g) was homogenized with 22 ml of methanol/chloroform (1:2, v/v) at a speed of 13,500 rpm for 2 min. The homogenate was filtered using Whatman No. 1 filter paper. To the filtrate (7 ml), 0.5% NaCl (2 ml) was added. The resulting mixture was vortexed at a moderate speed for 45 s. To separate the mixture into two phases, the mixture was centrifuged at 3000 g at 4 °C for 3 min using a refrigerated (4 °C) centrifuge. Ferric thiocyanate method was adopted for PV measurement (Arfat *et al*., 2015). To the lower phase (3 ml), 25 μL of ammonium thiocyanate (30% w/v) and 25 μL of 20 mM iron (II) chloride were added. The mixture was left for reaction for 20 min at room temperature and the absorbance was taken at 500 nm. In the same manner, the blank was prepared but distilled water was used instead of ferrous chloride. Cumene hydroperoxide (0.5–2 ppm) was used for the preparation of standard curve. PV was expressed as mg cumene hydroperoxide per kg meat after blank subtraction.

TBARS of shrimp meat was evaluated as detailed by Benjakul and Bauer (2001). Shrimp meat (0.5 g) was mixed with 4.5 ml of a solution containing

0.375% TBA, 15% TCA and 0.25 N HCl. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4000 ×g for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a spectrophotometer. TBARS value was calculated from the standard curve of malonaldehyde (0–5 ppm) and expressed as mg malonaldehyde/kg shrimp meat.

6.4.6 Protein pattern

At day 0, protein patterns of the control (without any treatment and packaged in air) and CLE treated samples with HV-CAP treatment were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as detailed by Shiekh and Benjakul (2020a). Protein samples (15 μg protein) were loaded onto polyacrylamide gels comprising a 10% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, staining of the gel was carried out with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid. The protein standards (Bio-Rad Laboratories, Inc., Richmond, CA, USA) containing myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), and ovalbumin (45 kDa) were used to estimate the molecular weight of the proteins.

6.4.7 Fatty acid profile

Fatty acid compositions were examined as fatty acid methyl esters (FAMEs) using gas chromatography coupled with flame ionization detector (GC-FID) as per the method of Raju and Benjakul (2020). Fatty acid methyl esters (FAME) were obtained by transmethylation of 10 mg of oil sample with 2 M methanolic sodium hydroxide and 2 M methanolic hydrochloric acid. FAME were analyzed by Agilent 7890B (Santa Clara, CA, USA) gas chromatography system equipped with flame ionization detector (FID). Peaks were identified based on the retention time of standards and the results were expressed as g/100 g lipid.

6.4.8 Sensory evaluation

All samples were collected at 0 and 15 days of storage for sensory analysis (Nirmal and Benjakul, 2011). Only samples with the TVC lower than the limit (6 log CFU/g) were used for evaluation. The selected samples were placed on a stainless steel tray of steaming pot, covered with an aluminum foil and steamed for 5 min, in which the core temperature reached 80 °C (Manheem *et al*., 2013). The cooked samples were evaluated by panelists, who were familiar with shrimp consumption without having any allergy. The 9-point hedonic scale (9: like extremely; 7: like moderately; 5: neither like or nor dislike; 3: dislike moderately; 1: dislike extremely) was used. Totally 50 untrained panelists with the age between 25 and 35 years old were selected All panelists were asked to evaluate for color, odor, taste, flavor and overall likeness. Samples were presented as unpeeled cooked shrimp in plates coded with three-digit random numbers. Panelists were asked to rinse their mouth during sample evaluation. Sensory analysis was conducted by panelists in an environmentally controlled partitioned booths under white incandescent light in the sensory evaluation laboratory.

6.4.9 Statistical analyses

Completely randomized design (CRD) was used for entire studies. All the experiments were conducted in triplicates. Randomized completely block design (RCB) was implemented for sensory analysis. Analysis of variance (ANOVA) was done and mean comparison was performed by Duncan's multiple range test or t-test using a SPSS statistical software (SPSS 17.0, SPSS Inc., Chicago, IL, USA).

6.5 Results and discussion

6.5.1 Effect of HV-CAP on melanosis and quality changes of CLE pre-treated PWS during refrigerated (4 °C) storage

6.5.1.1 Formation of melanosis

PWS were subjected to CLE pre-treatment followed by exposure to HV-CAP in bag with different ratios of mixed gases and kept for 15 days. At the first day, melanosis was invisible in all the CLE treated samples with or without HV-CAP

treatment as well as the control (untreated sample packaged in air) (Fig. 26a). During the storage, 1% CLE pre-treated sample exposed to HV-CAP, packaged under Ar/Air mixture (CP-Ar/Air-1 CLE) had the lowest melanosis than others ($p<0.05$). Intensity of dark color was lowest in the CP-Ar/Air-1 CLE sample ($p<0.05$), followed by Ar/Air-1 CLE, CP-Ar/Air-0.5 CLE, Ar/ Air-0.5 CLE, CP-Ar/O_2-1 CLE, Ar/O_2-1 CLE, Ar/O_2-0.5 CLE, Ar/O_2-0.5 CLE samples, respectively. The differences noticed in the melanosis formation among aforementioned treatments could be due to the varying levels of CLE and gases used. However, the same argon (Ar) portion was equal (80%) in all the packaged samples, proportion of active species, especially those generated by N_2 present in air, might have retarded melanosis formation in PWS during storage. Cluster of active species hydroxyl radicals (-OH) and varieties of nitrogen oxides such as nitrite oxide (NO^{2-}) and nitrate oxide (NO^{3-}) were reported to be generated in non-thermal plasma treated atmospheric air (Charoux *et al*., 2020). The reactive nitrogen species (RNS) and reactive oxygen species (ROS) actively interact with a wide range of compounds in foods, thus changing their properties (Ramazzina *et al*., 2016). Moreover, it was reported that RNS or ROS generated from atmospheric air can attack the cell membranes, causing disintegration and alteration in the protein structure. Those species might destroy sensitive aromatic amino acids that are essential substrates for PPO activity (Surowsky *et al*., 2013).

Lowered melanin formation in CP-Ar/Air-1 CLE sample enhanced the shelf-life of shrimp up to 15 days (Fig. 26a). Highly reactive species generated by HV-CAP might induce conformational changes in PPO. Additionally, the high discharge energy liberated during generation of strong electro-magnetic field could also induce denaturation of PPO. Gas composition especially Ar and low levels of O_2 along with HV-CAP treatment were proved as potential means to tackle melanosis during extended storage. The least melanosis detected in CP-Ar/Air-1 CLE sample indicated the least dark spot formation (Fig. 26a), while the highest melanosis occurred in the control at day 15 of refrigerated (4 °C) storage. Then, HV-CAP for 10 min could help to mitigate the activity of PPO in order to prevent melanin formation in PWS. PPO inactivation mechanism was reported as a result of loss in secondary structure due to breaches in the bonds induced by active species generated by HV-CAP (Zouelm *et al*., 2019).

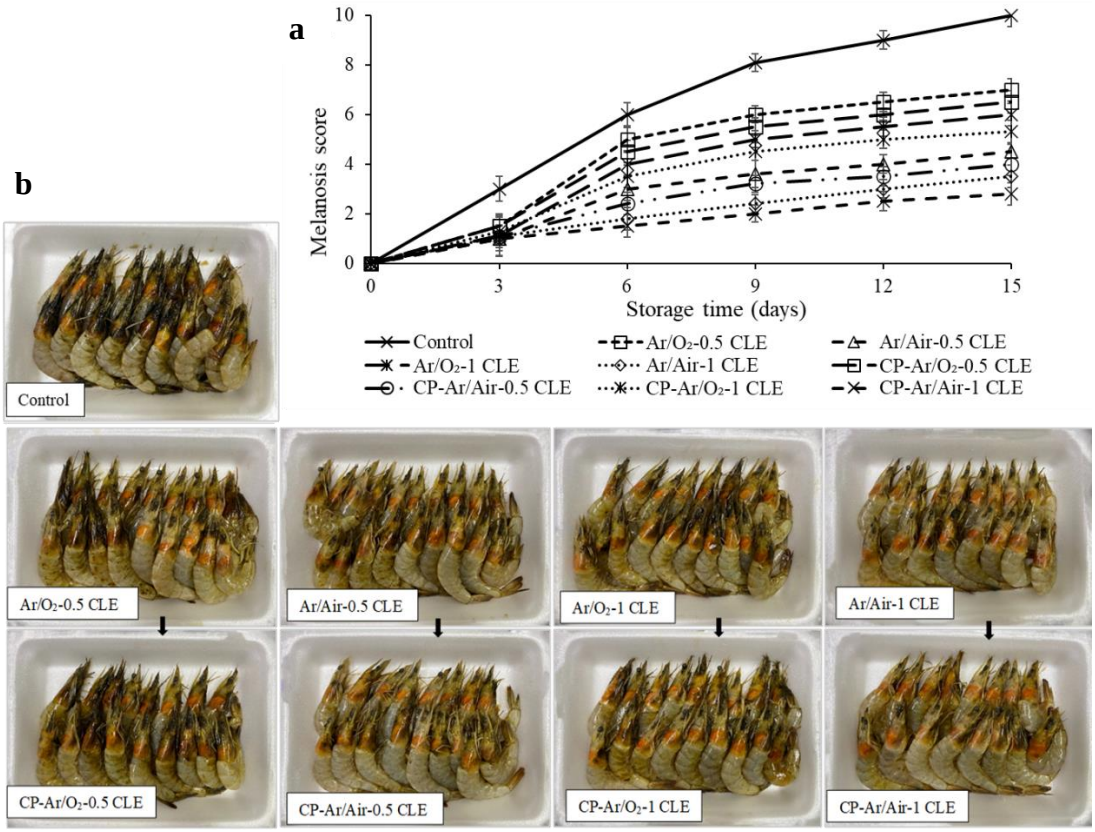

Figure 26. Melanosis score (a)) and photographs (b) of Pacific white shrimp with CLE pretreatment without and with HV-CAP treatment under different gas composition during 15 days of storage at 4 °C.

Bars represent the standard deviation (n = 10). Control: without any treatment; Ar/O_2-0.5 CLE: Treated with 0.5% CLE, packed under Ar/O_2 (80:20); Ar/Air-0.5 CLE: Treated with 0.5% CLE, packed under Ar/Air (80:20); Ar/O_2-1 CLE: Treated with 1% CLE, packed under Ar/O_2 (80:20); Ar/Air-1 CLE: Treated with 1% CLE, packed under Ar/Air (80:20); CP-Ar/O_2-0.5 CLE:Ar/O_2-0.5 CLE, followed by HV-CAP ; CP-Ar/Air-0.5 CLE: Ar/Air-0.5 CLE, followed by HV-CAP; CP-Ar/O_2-1 CLE: Ar/O_2-1 CLE, followed by HV-CAP; CP-Ar/Air-1 CLE: Ar/Air-1 CLE, followed by HV-CAP.

Cold plasma (CP) was applied up to 10 min with frequency of 500 Hz could prevent melanosis in PWS up to 14 days of storage at 4 °C (De Souza Silva *et al*., 2019). Appearance of PWS with different treatments after 15 days of storage at 4 °C are shown in Fig. 26b, in which the control showed the highest melanosis while CP-Ar/Air-1 CLE had the lowest black spot.

6.5.1.2 Changes in microbiological quality

At the first day of storage, TVC of all the treated samples, irrespective of HV-CAP or CLE treatment, had the lower microbial counts than that of control ($p<0.05$). Microbial inactivation was more pronounced due to combined effect of 1% CLE and HV-CAP (Fig. 27a). TVC increased gradually in all the samples all over the storage of 15 days. At day 15, TVC count of the control, Ar/O_2-0.5 CLE, CP-Ar/O_2-0.5 CLE, Ar/O_2-1 CLE, CP-Ar/O_2-1 CLE, Ar/Air-0.5 CLE, CP-Ar/Air-0.5 CLE, Ar/Air-1 CLE and CP-Ar/Air-1 CLE samples was 7.53, 6.77, 6.32,6.57, 5.50, 6.67 6.12, 6.42, and 5.00 log CFU/g, respectively. Eventually, CP-Ar/Air-1 CLE sample had the lowest count than others at day 15 ($p<0.05$). Residual microbial load after CLE treatment was further decreased upon HV-CAP application. HV-CAP decreased the microbial load of all other samples. Additionally, active species during HV-CAP treatment might have occurred more in Ar/air packaged sample in which ozone and reactive nitrogen species produced were able to undergo interaction with bacterial cell wall, leading to cell death. PWS subjected to CP in the presence of N_2 and O_2 had the lowest microbial growth, compared to the control at day 14 of refrigerated (4 °C) storage (De Souza Silva *et al*., 2019). Furthermore, CLE at 1% with pulsed electric field (PEF) treatment could retain the quality of PWS up to 10 days at 4 °C (Shiekh and Benjakul, 2020b).

PBC of the control was highest at day 0 ($p<0.05$). Conversely, treated sample packed under Ar and air and subjected to HV-CAP, regardless of CLE treatment at different doses had the lower PBC than others ($p<0.05$) (Fig. 27b). CP-Ar/O_2-1 CLE and CP-Ar/Air-1 CLE samples had slightly lower PBC, at the first day ($p<0.05$). With the advancement of storage, residual PBC was lowered in CP-Ar/Air-1 CLE.

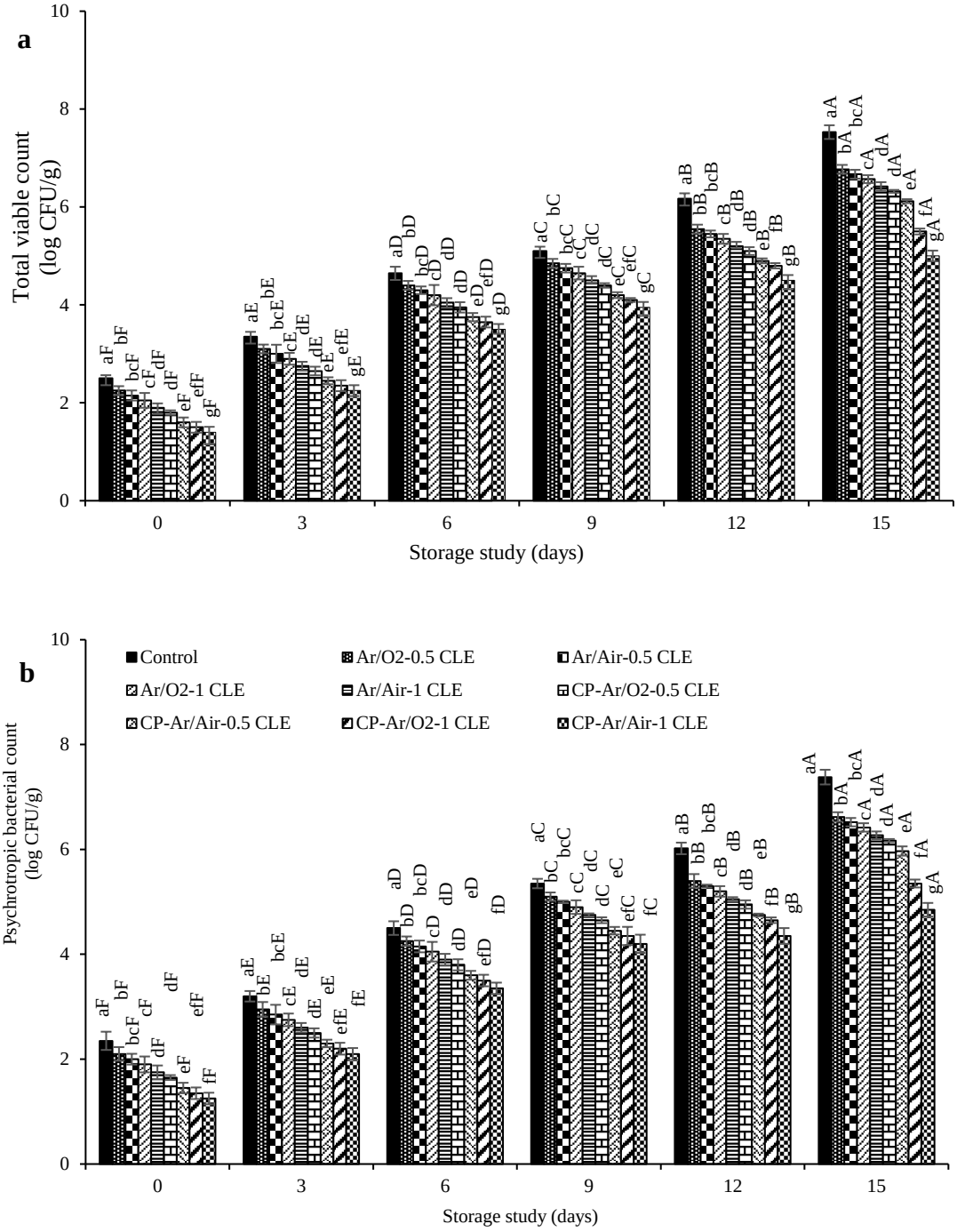

Figure 27. Total viable count (a) and psychrophilic bacterial count (b) of Pacific white shrimp with CLE pre-treatment without and with HV-CAP treatment under different gas composition during 15 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 26.

This might be associated with the less amount of O_2 in the air rather than the absolute O_2 in CP-Ar/O_2 -1 CLE packaged sample. As a result, CP-Ar/Air-1 CLE showed the lower bacterial counts than all others at day 15. PBC of CP treated PWS was markedly lower that of the control (De Souza Silva *et al*., 2019). Besides HV-CAP, CLE at higher doses was demonstrated to be efficient antimicrobial agent to eliminate some population of microbes. CLE has been reported to have strong anti-bacterial effect due to high content of polyphenolics and organic acids content (Shiekh and Benjakul, 2020b).

At day 0, *Pseudomonas* of the control and the treated samples with or without CLE treatment or HV-CAP were detected (1.20–2.30 log CFU/ g) (Fig. 28a). Higher *Pseudomonas* counts were found in the control than others ($p<0.05$), whereas CP-Ar/Air-1 CLE sample showed the lowest count at day 0 ($p<0.05$). Increment in the growth of *Pseudomonas* was evidenced in all the samples as storage time was augmented ($p<0.05$). High *Pseudomonas* count (7.28 log CFU/g) was obtained in the control, while the CP-Ar/Air-1 CLE sample exhibited the lowest count (5.01 log CFU/g) after 15 days ($p<0.05$). Combined effect of HV-CAP in Ar/air atmosphere and 1% CLE as pre-treatment in Ar/air atmosphere efficiently retarded the growth of *Pseudomonas* during refrigerated (4 °C) storage. Combined effect of coconut husk extract rich in polyphenols with HV-CAP revealed shelf-life extension by significant reduction of *Pseudomonas* in seabass slices (Olatunde *et al*., 2019a). *Pseudomonas* have been regarded as dominant gram negative spoilage bacteria in seafoods and are highly sensitive to be inactivated in CO_2 atmosphere (DeWitt and Oliveira, 2016). Furthermore, PEF in conjunction with 1% CLE was reported to retard the growth of *Pseudomonas* in PWS (Shiekh and Benjakul, 2020b). Thus, partially anaerobic conditions found on CP-Ar/Air-1 CLE sample also extended the lag phase of *Pseudomonas* during storage.

The control sample had the highest H_2S producing bacterial count at day 0 ($p<0.05$), whereas CP-Ar/Air-1 CLE sample had the lowest count ($p<0.05$) (Fig. 28b). The initial count of samples with or without CLE and HV-CAP treatment was in the range of 1.36 to 2.40 log CFU/g. H_2S producing bacterial count of all the samples augmented as storage time increased ($p<0.05$). At day 15, the control, Ar/O_2-0.5 CLE, CP- Ar/O_2-0.5 CLE, Ar/O_2-1 CLE, CP- Ar/O_2-1 CLE, Ar/Air-0.5 CLE, CP-

Ar/Air-0.5 CLE, Ar/Air-1 CLE and CP-Ar/Air-1 CLE samples had H_2S-producing bacterial counts of 7.49, 6.73, 6.28, 6.53, 5.46, 6.63, 6.08, 6.38 and 5.02 log CFU/g, respectively. During the entire storage time, CP-Ar/Air-1 CLE sample had notably retarded growth of H_2S-producing bacteria ($p<0.05$). PEF in combination with CLE containing polyphenols and organic acids was able to hinder the growth of H_2S producing bacteria in PWS during storage at 4 °C (Shiekh and Benjakul, 2020b). HV-CAP via DBD was employed for inactivation of gram negative and gram positive bacteria to ensure the safe consumption of stored Asian sea bass slices at refrigerated (4 °C) temperature (Olatunde *et al*., 2019c).

At day 0, the control also had higher Enterobacteriaceae count than others ($p<0.05$) (Fig. 28c). Conversely, CP-Ar/Air-1CLE showed the lowest count ($p<0.05$). Enterobacteriaceae in all the samples augmented with the progression of storage time ($p<0.05$) and the lowest count was noticeable in CP-Ar/Air-1 CLE sample after 15 days ($p<0.05$). CP-Ar/O_2-1 CLE sample packed in Ar and O_2 ratio (80:20) also exhibited lower Enterobacteriaceae counts than that of control throughout the storage ($p<0.05$). However, marked differences in count were visible between CP-Ar/O_2-1 CLE and CP-Ar/Air-1 CLE samples at the end of storage. This could be attributed to the active species based on N_2 present in the air used in the bag generated after HV-CAP. PWS subjected to PEF in conjunction with CLE at 1% was documented to have lower Enterobacteriaceae counts during storage at 4 °C (Shiekh *et al*., 2019). Enterobacteriaceae are facultative anaerobic gram negative spoilage bacteria and are highly sensitive than gram positive counterparts (McMillin, 2008). Effective attachment of polyphenols as well as active species generated from HV-CAP might break down thin layer of peptidoglycan surrounding the cell membrane in gram negative bacteria. Therefore, shrimp pre-treated with 1% CLE under Ar and air (80:20) subjected to HV-CAP showed the marked reduction of Enterobacteriaceae count during storage.

Lower LAB count was noted in the control and those treated with higher levels of CLE in combination with HV-CAP at day 0 ($p<0.05$) (Fig. 28d). The decreased LAB count in CP-Ar/Air-1 CLE and CP-Ar/O-1 CLE obtained at day 0 was plausibly a result of joint actions imposed by CLE polyphenols or organic acids with antimicrobial activities and the sensitivity of LAB towards active species produced by

HV-CAP. At day 0, LAB count of control was higher than others ($p<0.05$). However, LAB count increased from day 3 to the end of storage in all the samples ($p<0.05$). Olatunde *et al*. (2019a) also reported similar result. LAB, which are facultative gram anaerobes and have rigid wall with strong affinity towards phenolic compounds, were reported to be eliminated by HV-CAP (Han *et al*., 2016; Misra *et al*., 2011). Moreover, high antimicrobial efficacy was evident with the CLE pre-treatment at 1% followed by HV-CAP treatment, compared to control, thereby arresting the growth of LAB ($p<0.05$). LAB potentially produce bacteriocins and were illustrated to reduce other spoilage microbial flora (Devlieghere and Debevere, 2000). In the present study, pathogenic bacterial colonies of Clostridium perfringens were not detected. As a result, no cross-contamination occurred during handling, preparation and packaging of shrimp.

6.5.1.3 Changes in chemical quality

pH values attained were similar for all the samples at day 0 ($p>0.05$) (Fig. 29a). Within the first 3 days, pH values of all samples were 6.52–6.75 ($p<0.05$). pH of the control was higher as storage augmented compared to other samples ($p<0.05$). Nevertheless, the pH changes were not obvious in CP-Ar/Air-1 CLE sample, compared to others with or without HV-CAP during 0–15 days of refrigerated (4 °C) storage ($p<0.05$). Similar results were documented by Olatunde *et al*. (2019a). Changes in pH were not noticeable initially, more likely owing to negligible activity of microbial enzymes in shrimp muscle. The accumulation of basic compounds caused by higher activity of microbial enzymes, especially from spoilage bacteria during storage, more likely contributed to increased pH. Similar trend was reported in PWS pre- treated with green tea extract and ascorbic acid under MAP during refrigerated (4 °C) storage (Nirmal and Benjakul, 2011). pH of control and other samples with different treatments ranged between 6.90 and 10, during storage of 15 days. Combined effect of CLE, HV-CAP and in the presence of Ar and air (80:20) retained the pH of shrimp meat under acceptable limits. The acceptable pH limit for shrimp (*Penaeus merguiensis*) was set at 7.6 (Shamshad *et al*., 1990).

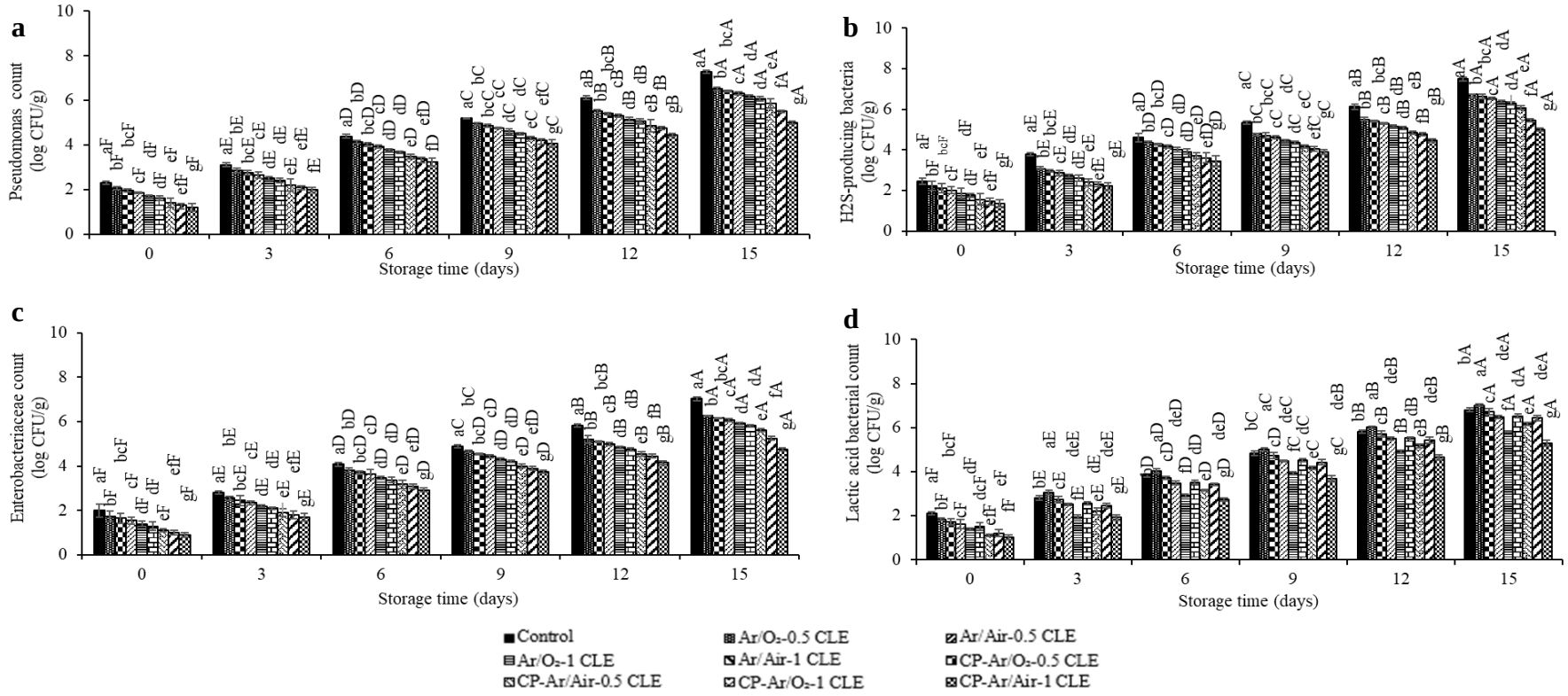

Figure 28. *Pseudomonas* (a), H_2S-producing bacteria (b), Enterobacteriaceae (c) and lactic acid bacteria counts (d) of Pacific white shrimp with CLE pre-treatment without and with HV-CAP treatment under different gas composition during 15 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 26.

Thus, the lowered in- crease in pH of CP-Ar/Air-1 CLE sample was more likely due to the reduction in microbial load as well as enzyme inhibition caused by active species generated by HV-CAP and due to antioxidant and anti- microbial effects of CLE.

TVB contents of the control and samples with prior CLE treatment, without and with HV-CAP under different gas compositions ranged from 2.81 to 5.32 mg N/100 g shrimp meat at the first day (Fig. 29b). TVB content was highest in the control throughout the storage ($p<0.05$). TVB content of all the samples augmented during storage ($p<0.05$). Generally, the highest TVB content was obtained in the control during storage ($p<0.05$). The least TVB content (17.81 mg N/ 100 g meat) was detected in CP-Ar/Air-1 CLE sample at day 15, associated with the lowest microbial load in CP-Ar/Air-1 CLE sample, more likely due to CLE in conjunction with HV-CAP impact. Increased con- tent of TVB was also noticed in CP-Ar/O_2–0.5 CLE and CP-Ar/Air-0.5 CLE, possibly due to the residual microbes after exposure to radicles generated by HV-CAP and low doses of antimicrobial compounds of CLE. TVB content (12.96 mg N/100 g shrimp meat) of shrimp treated with 1% CLE solution at 4 °C was documented to maintain freshness in shrimp for 10 days (Shiekh *et al*., 2019). TVB, a quality indicator for spoilage of shrimp muscle, should be < 12 mg N/100 g for freshly caught shrimp. During post-harvest storage, TVB levels of 12–20 mg N/ 100 g are for consumption but the levels of 20–25 and > 25 mg N/ 100 g are considered as 'borderline' and 'inedible and decomposed', respectively (Okpala *et al*., 2014). Low TVB content in CP-Ar/Air-1 CLE sample kept in Ar and air (80:20) atmosphere was mainly due to the least oxygen level and HV-CAP shock as reflected by the drastic re- duction of spoilage bacteria (Figs. 27 and 28). The use of PEF and natural extract (1% CLE) containing polyphenolic glycosides and organic acids, had the pronounced antimicrobial effect on shrimp during 10 days of storage at 4 °C (Shiekh *et al*., 2019). Antioxidants in cashew leaf extract (CE) were also found to retard the TVB content in shrimp during storage (Sae-leaw and Benjakul, 2019a). CP applied on PWS potentially reduced the TVB content by reduction of spoilage microflora during 12 days of refrigerated (4 °C) storage (Zouelm *et al*., 2019).

Protein carbonyl content of the sample subjected to HV-CAP with a lower dose of CLE treatment ranked highest, compared to control and other samples at day 0 ($p<0.05$) (Fig. 29c). CP-Ar/Air-1 CLE sample packed in Ar and air (80:20)

showed the lowest carbonyl content within the first two days of storage, compared to the control and those treated with HV-CAP and 0.5 CLE ($p<0.05$). As the storage proceeded, the protein carbonyls were augmented, indicating the triggered protein oxidation in lower concentration CLE treated samples. However, the protein carbonyl content of CP-Ar/Air-1 CLE sample remained lowest than others for the entire storage period ($p<0.05$). HV-CAP alone was reported to oxidize Asian sea bass myofibrillar proteins (Olatunde *et al*., 2019d). HV-CAP together with phenolic extract from coconut husk was documented to alleviate the carbonyl formation more potentially (Olatunde *et al*., 2019a). In general, the preservative effect of 1% CLE in conjunction with HV-CAP potentially prevented biochemical changes in shrimp during storage. Decreased protein carbonyl content in CP-Ar/ Air-1 CLE were in line with the lowered TVB content and microbial counts. Protein carbonyls were remarkably less in the super-chilled swimming crab packed under low O_2 content modified atmosphere (Sun *et al*., 2017). Moreover, the highest carbonyl contents were obtained in CP-Ar/O_2-0.5 CLE and CP-Ar/O_2-0.5 CLE samples containing lower levels of CLE. Atmosphere had no marked effect on protein oxidation when HV-CAP was omitted. This reconfirmed the negative impact of active species in inducing protein oxidation of PWS muscle.

Control was higher in PV compared to others followed by CP-Ar/O_2-0.5 CLE and CP-Ar/Air-0.5 CLE ($p<0.05$). Conversely, CP-Ar/Air-1 CLE sample had the lowest PV at day 0 ($p<0.05$) (Fig. 29d). HV-CAP and 1% CLE treatment reduced hydroperoxide formation to some degrees as evidenced by lower PV at day 0 ($p<0.05$). CLE was rich in polyphenolic compounds that retarded PV in refrigerated (4 °C) shrimp (Shiekh and Benjakul, 2020b). Furthermore, PV increased drastically in the control during storage ($p<0.05$), compared to samples kept under different gases with 1% CLE treatment. Nevertheless, CP-Ar/Air-1 CLE sample exhibited lowest PV at day 15 ($p<0.05$). This could be due to lower oxygen levels that might restrict microbial proliferation and secretion of extracellular enzymes including lipase. Air used at 20% in bag contained high portion of N_2 (78%), which did not favor the oxidation of lipids. Phlorotannins from marine brown seaweed at 5% level with higher antioxidant activity arrested the formation of hydroperoxides in shrimp (Sharifian *et al*., 2019). PV represents the generation of the primary oxidation product at faster rate rather than degradation at the early stages of storage (Boselli *et al*., 2005). Seafoods are rich source

of unsaturated fatty acids (Albertos *et al*., 2017). Ar gas filled Asian sea bass slices with natural polyphenols lowered hydroperoxides more potentially than the N_2 MAP (Olatunde *et al*., 2019b). Moreover, the synthesis of hydroperoxides might be associated with microbial enzymes produced, liberating free fatty acids which underwent lipid oxidation easily (Koka and Weimer, 2001). Therefore, HV-CAP and CLE worked in combination to prevent the generation of hydroperoxides during storage.

TBARS value of the control was highest among all the samples for the entire storage time of 15 days ($p<0.05$) (Fig. 29e). CP-Ar/Air-1 CLE sample packed in Ar and air (80:20) possessed lower TBARS value, compared to the control ($p<0.05$). Control sample packed in air showed the higher content of TBARS, suggesting that hydroperoxides were decomposed to the secondary oxidation products. On the other side, CP-Ar/O_2-0.5 CLE and CP-Ar/Air-0.5 CLE samples with lower CLE concentration used for soaking could not withstand HV-CAP, in which shrimp lipids had undergone oxidation to some extent. CP application imposes negative effect on quality of food products, particularly acceleration of lipid oxidation (Pankaj *et al*., 2018). It was clearly indicated that CLE contained sufficient polyphenols at higher level (1%) to prevent oxidative changes in CP-Ar/Air-1 CLE. Moreover, lowered counts of *Pseudomonas* attained in CP-Ar/Air-1 CLE (Fig. 28) that restricted the production of additional microbial lipase and phospholipase. Those enzymes induced the cleavage of ester bond, liberating FFA, which further underwent oxidation with ease (Benjakul and Bauer, 2001; Koka and Weimer, 2001). The lowest level (2.35 mg MDA/kg of shrimp meat) was found in CP-Ar/Air-1 CLE sample at day 15. The findings were in line with the CP treated PWS, in which chemical additives could reduce oxidation with minimum TBARS levels during storage (Zouelm *et al*., 2019). Additionally, PEF and 1% CLE treatment prevented lipid oxidation of PWS during storage (Shiekh *et al*., 2019). Therefore, HV-CAP with CLE had combined effect to prevent lipid oxidation of PWS during 15 days of storage.

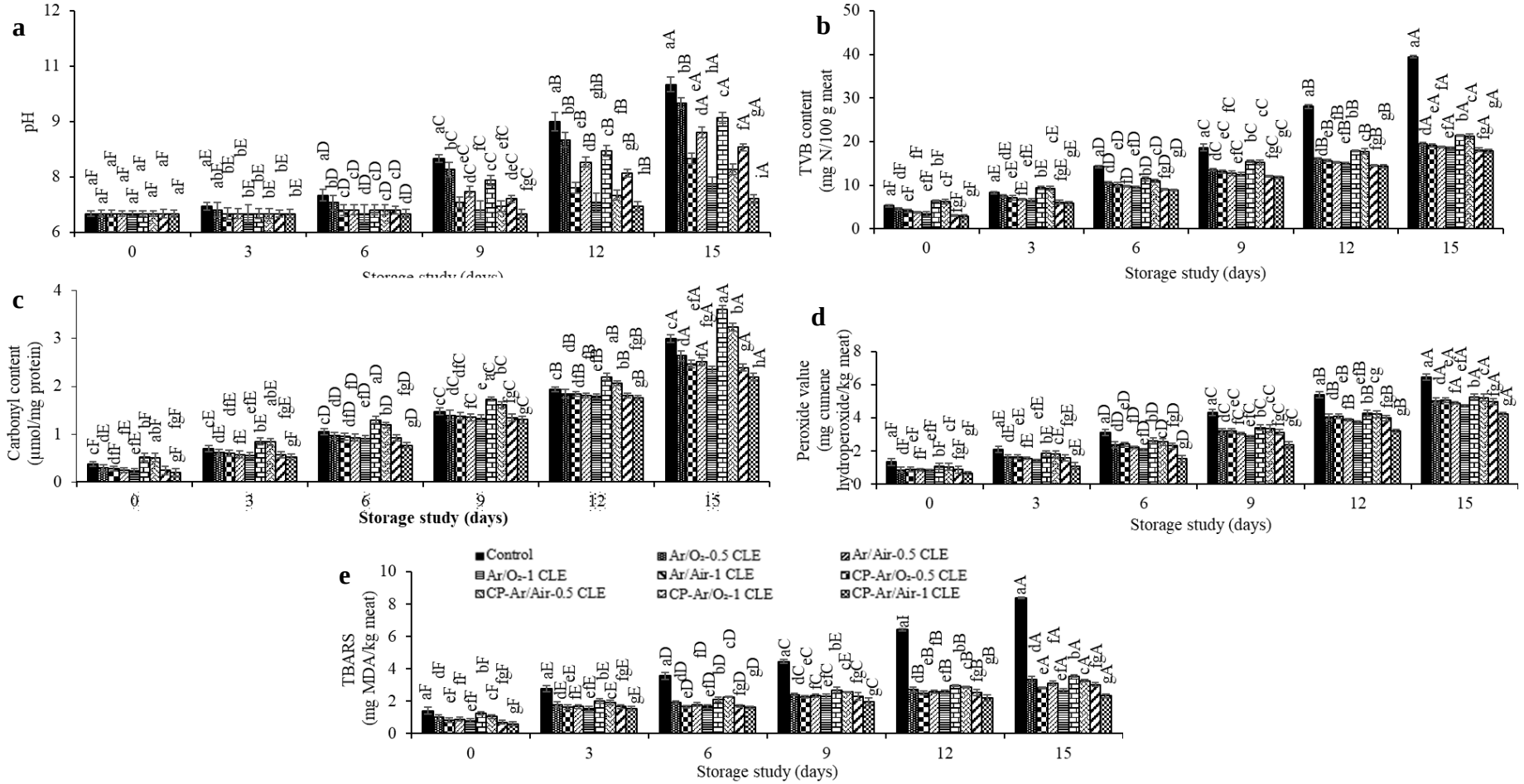

Figure 29. pH (a), total volatile base (TVB) (b), carbonyl contents (c), peroxide values (d) and thiobarbituric reactive substances (TBARS) values (e) of Pacific white shrimp with CLE pre-treatment without and with HV-CAP treatment under different gas compositions during 15 days of storage at 4 °C.
Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$) Key: see the caption for Fig. 26.

6.5.1.4. Changes in fatty acid profile

HV-CAP treated samples without and with CLE treatment showed marked differences in fatty acid profiles at day 0 ($p<0.05$) (Table 10). The control had palmitic acid was predominant, followed by DHA, oleic, linoleic acids and EPA, respectively. Ionized gases with active species during prolonged HV-CAP treatment affected MUFA and PUFA at the first day of storage when CLE at 0.5% was used ($p<0.05$). Similar decreases in MUFA, especially oleic acid (C18:1, n-9) and EPA (C20:5, n-3) were noticed in fresh mackerel (*Scomber scombrus*) fillets after CP treatments (80 kV for 5 min) (Albertos *et al*., 2017). However, CP-Ar/Air-1 CLE sample was still abundant in n-3 fatty acids than other at day 0 ($p<0.05$). The results indicated that negligible oxidation took place in CP-Ar/Air-1 CLE sample due to combined effect of sufficient CLE and low O_2 level in package. It was noted that linoleic acid was drastically decreased in this sample. However, DHA, EPA and oleic acid were increased. For another three samples including CP-Ar/ O_2–0.5 CLE, CP-Ar/O_2–1 CLE and CP-Ar/Air-0.5 CLE, similar changes were attained. Oleic, linoleic acids, EPA and DHA were reduced at day 0. It was documented that degradation of PUFA and MUFA led to the increased saturated fatty acids (SFA) after HV-CAP treatment implemented for 5 min in Asian sea bass slices in the absence of antioxidants (Olatunde *et al*., 2019a).

After storage of 15 days, EPA and DHA of CP-Ar/O_2-1 CLE and CP-Ar/Air-1 CLE were decreased, while oleic and heneicosanoic acids in- creased. However, MUFA and PUFA were still retained at higher level in CP-Ar/Air-1 CLE, compared to those of CP-Ar/O_2-1 CLE sample, irrespective of the CLE treatment, at day 15. This could be attributed to the higher quantity of residual O_2 in the bag that might have triggered the lipid oxidation. The lowered contents of PUFA obtained at day 15 could be attributed to the oxidation of PUFA, in which proportion of MUFA and SFA were augmented. Similar findings were reported in HV-CAP treated Asian sea bass slices, in which high TBARS values were obtained, regardless of antioxidant addition (Olatunde *et al*., 2019a). Sae-leaw and Benjakul (2014) found the decreases in MUFA and PUFA in sea bass skin stored in ice, which was caused by lipid oxidation. Lipid oxidation generates aldehydes and ketones with unfavorable sensory property in muscle foods and has the negative impact on human health (Pan *et al*., 2019).

Table 10. Fatty acid profiles of Pacific white shrimp in the presence of CLE at various levels without and with HV-CAP treatment under different modified atmosphere packaging at day 0 and 15 of refrigerated storage.

	Day 0					Day 15	
	Control	CP-Ar/O_2-0.5 CLE	CP-Ar/O_2-1 CLE	CP-Ar/Air-0.5 CLE	CP-Ar/Air-1 CLE	CP-Ar/O_2-1 CLE	CP-Ar/Air-1 CLE
C14:0 Myristic	1.08±0.02ab	1.01±0.01d	1.05±0.02c	1.06±0.01bc	1.09±0.01a	2.69±0.02b	2.84±0.03a
C15:0 Pentadecanoic	0.78±0.04a	0.68±0.01b	0.71±0.02b	0.72±0.01b	0.80±0.01a	0.68±0.01a	0.69±0.01a
C16:0 Palmitic	24.08±0.03b	17.70±0.01e	18.80±0.02d	21.31±0.01c	29.09±0.01a	15.82±0.03b	16.83±0.02a
C16:1 Palmitoleic	1.32±0.03a	1.12±0.01d	1.08±0.02c	1.22±0.01b	0.53±0.01e	0.66±0.01a	0.64±0.01a
C17:0 Heptadecanoic	2.71±0.03b	2.07±0.01e	2.21±0.02d	2.32±0.01c	3.19±0.01a	1.23±0.01b	1.32±0.02a
C17:1 cis-10-Hepatodecanoic	0.75±0.03a	0.63±0.01c	0.68±0.02b	0.66±0.01bc	0.76±0.01a	0.61±0.01b	0.70±0.01a
C18:0 Stearic	12.43±0.03a	9.17±0.01e	9.59±0.02d	10.69±0.01c	11.75±0.01b	10.97±0.02a	10.99±.02a
C18:1 Oleic	18.63±0.03b	12.66±0.01e	14.10±0.02d	15.81±0.01c	21.21±0.01a	38.31±0.05b	41.66±0.03a
C18:2 Linoleic	18.41±0.03a	12.72±0.01d	13.55±0.02c	15.47±0.01b	1.14±0.01e	15.93±0.02b	16.98±0.01a
C20:0 Arachidic	1.11±0.03b	1.04±0.01c	1.07±0.02c	1.06±0.01c	1.34±0.01a	1.22±0.01a	1.19±0.02a
C21:0 Heneicosanoic	1.27±0.03b	0.98±0.01e	1.12±0.02c	1.08±0.01d	2.76±0.01a	4.48±0.01b	4.86±0.01a
C20:2 Eicosadienoic acid	2.46±0.03a	1.81±0.01d	1.91±0.02c	2.16±0.01b	1.03±0.01e	1.90±0.02a	2.12±0.02a
C20:0 Docosanoic	1.03±0.03a	0.97±0.01b	0.99±0.02b	0.98±0.01b	0.61±0.01c	1.05±0.01a	1.04±0.01a
C20:3 Eicosatrienoic	0.62±0.04a	0.54±0.01b	0.57±0.02b	0.55±0.01b	0.64±0.01a	0.62±0.03a	0.64±0.02a
C20:4 Eicosatetraenoic	0.66±0.04b	0.56±0.01c	0.59±0.02c	0.58±0.01c	6.93±0.01a	0.68±0.01b	0.78±0.03a
C23:0 Tricosanoic	6.08±0.04a	4.36±0.01d	4.68±0.02c	5.35±0.01b	0.63±0.01e	2.16±0.03b	2.23±0.02a
C22:2 Docosadienoic	0.66±0.04b	0.54±0.01c	0.57±0.02c	0.56±0.01c	1.01±0.01a	0.98±0.02b	1.02±0.01a
C20:5 Eicosatrienoic (EPA)	14.44±0.04b	10.35±0.01e	10.80±0.02d	12.93±0.01c	16.60±0.01a	6.50±0.01b	6.89±0.02a
C24:1 Nervonic	0.71±0.04a	0.57±0.01d	0.66±0.02b	0.55±0.01d	0.61±0.01c	0.83±0.01a	0.85±0.01a
C22:6 Docosahexaenoic (DHA)	20.23±0.04b	13.95±0.01e	14.14±0.02d	16.64±0.01c	22.86±0.01a	9.73±0.02b	10.40±0.03a
SFA	51.65	38.00	41.27	45.58	52.29	40.20	41.99
MUFA	22.09	15.58	17.14	18.87	23.11	46.30	50.17
PUFA	57.47	40.48	42.13	48.90	50.21	37.39	39.83

Values are presented as the mean ± standard deviation (n=3). Different lowercase letters within the same row under the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig. 26.

Moreover, the extracellular enzymes including lipase produced by different strains of spoilage microorganisms in PWS could also enhance the enzymatic deterioration of fatty acids (Tzuc *et al*., 2014). The control and other samples were spoiled after 15 days of storage. This result suggested that HV-CAP could initiate the loss of MUFA and PUFA in treated shrimp. However, this phenomenon could be decreased by incorporation of CLE with higher antioxidant potential to mitigate the negative impact.

6.5.1.5 Protein pattern

Protein patterns of CLE pre-treated shrimp samples followed by post treatment with HV-CAP are depicted in Fig. 30. Differences in the protein bands were evident, compared to control (without any treatment) Myosin heavy chain (MHC) and actin in CP-Ar/O_2-0.5 CLE and CP-Ar/Air-0.5 samples were decreased in band intensity, irrespective of the gas composition. Proteins can be denatured by CP reactive species, where the loss in the secondary structure occurred, associated with the destruction of α-helix and β–sheet (Li *et al*., 2014; Segat *et al*., 2016). CP decreased the immobilized water located in the protein-dense myofibrillar network of mackerel (Albertos *et al*., 2017). MHC and actin were two major proteins in shrimp meat (Shiekh and Benjakul, 2020a). In contrast, no degradation of MHC in CP-Ar/O_2-1 CLE and CP-Ar/Air-1 CLE samples was visualized. It can be inferred that disappearance of MHC in aforementioned samples was in CLE dose dependent. This could be attributed to the strong antioxidant potential of polyphenolics in CLE at higher dose (1% level), which could prevent oxidation or fragmentation of proteins during the exposure to the active species generated by HV-CAP. In general, similar patterns were observed between CP-Ar/O_2-1 CLE and CP-Ar/Air-1 CLE and control samples. This indicated the lower protein oxidation when PWS were soaked in 1% CLE solution prior to HV-CAP. Plasma activated water (PAW) ice was employed for preservation of shrimps (*Metapenaeus ensis*). It decreased microbial proliferation and inactivated microbial and indigenous proteases, thus preventing MHC degradation at the first day of refrigerated (4 °C) storage (Liao *et al*., 2018).

Protein fragmentation was retarded in HV-CAP treated Asian seabass slices in combination with natural additives such as ethanolic coconut husk extract and ascorbic acid (Olatunde *et al*., 2019a). During HV-CAP treatment, O_2 readily forms

ozone and other active species. Those compounds or species, could retard the growth of microbes, thus reducing degradation of proteins. Similar results of protein degradation were reported in the HV-CAP treated Asian sea bass slices due to higher ozone formation (Olatunde *et al*., 2019d). Moreover, quercetin-based preservative formulation containing 0.05 g/L quercetin, 0.025 g/L cinnamic acid and 0.025 g/L 4-hexylresorcinol (4-HR) under modified atmosphere (80% CO_2; 10% O_2; 10% and N_2) prevented the degradation of protein in shrimp stored at 4 °C (Qian *et al*., 2015).

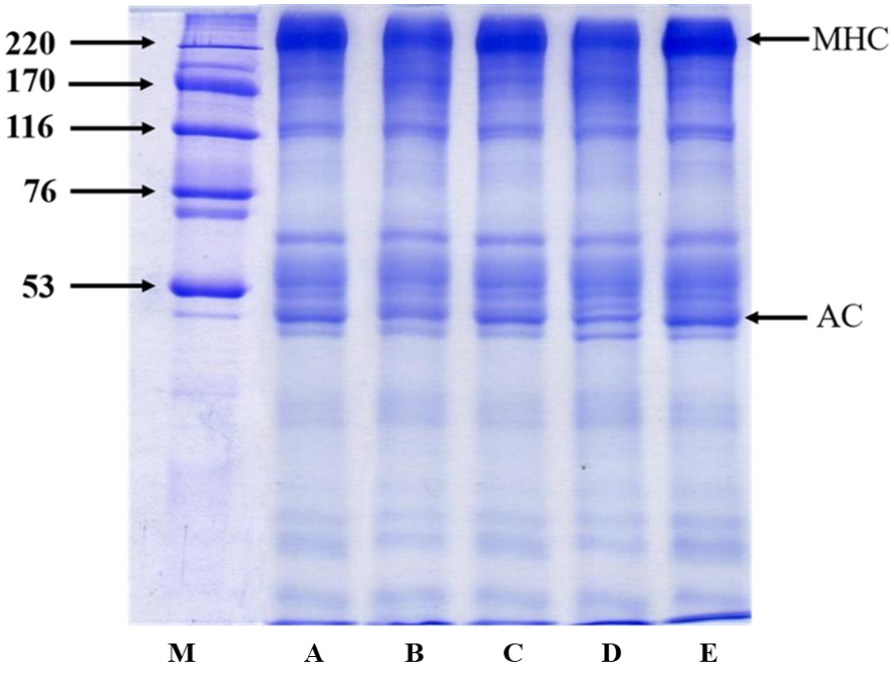

Figure 30. Protein pattern (SDS-PAGE) of Pacific white shrimp with CLE pretreatment without and with HV-CAP treatment under different gas compositions (day 0).
MHC: myosin heavy chain; AC: Actin; M: Marker. A: Control (without any treatment); B:CP-Ar/O_2-0.5 CLE; C: CP-Ar/O_2-1 CLE; D: CP-Ar/Air-0.5 CLE; E: CP-Ar/Air-1 CLE. Key: see the caption for Fig. 26.

6.5.1.6 Changes in sensory quality

At the first day, similar likeness scores were obtained for all the samples (Table 11), except for the odor and flavor of CP-Ar/O_2−0.5 CLE which showed lower score ($p<0.05$). This might be associated with off-flavor caused by lipid oxidation in the sample in the presence of less amount of CLE. The results were in line with lower odor, taste and flavor scores of seabass slices treated with HV-CAP for 5 min, possibly due to generation of oxidation products (Olatunde *et al*., 2019a). HVCAP treated samples with CLE at high dose (1%) received higher color scores ($p<0.05$). At day 15,

marked decrease in likeness scores of samples were attained for CP-Ar/O_2−1 CLE, while microbial load was still below the limit. However, no difference in the likeness score of all attributes was found in CP-Ar/Air-1 CLE between day 0 and day 15 ($p>0.05$). Control and other samples were omitted mainly due to microbial and chemical deterioration and were not safe for panelists After 15 days of storge, the CP-Ar/O_2-1 CLE had lower likeness scores than CP-Ar/Air-1 CLE for all attributes tested ($p<0.05$). CP treatment of chub mackerel (*Scomber japonics*) at optimal voltage level (60 kV) and exposure time of 60 s drastically reduced TVC and TVB-N. This was related with the higher likeness score than the untreated counterpart after 14 days of storage (Chen *et al*., 2019). Control had the higher microbial load exceeding 6 log CFU/g, the average limit for acceptability in seafoods (ICMS, 2002). Higher color likeness score was in accordance with the reduced melanosis in CP-Ar/Air-1 CLE sample (Fig. 26). For odor and flavor likeness, CP-Ar/Air-1 CLE also had lower microbial load and lower oxidation (Figs. 27, 28 and 29). As a consequence, the off-odor caused by the offensive decomposition products was less in CP-Ar/Air-1 CLE. Additionally CLE (1%) was abundant in polyphenolic glycosides and organic acids, especially hydroxycitric acid, which could penetrate deeply through the shrimp shell via pores formed by high energy of PEF, in which sensory quality of Pacific white shrimp was enhanced (Shiekh and Benjakul, 2020b). Optimum exposure time of 90 s CP treatment without any additive inactivated spoilage microorganisms in PWS, rendering the samples with the highest likeness score, compared to those treated with sodium metabisulphite (Zouelm *et al*. 2019). Therefore, CLE in conjunction with HV-CAP treatment in modified gas composition of argon and air (80:20) could preserve quality and shelf-life extension was achieved more potentially.

6.6 Conclusions

HV-CAP in combination with CLE was effective non-thermal treatment for shelf-life extension of shrimp. Melanosis or discoloration was negligible due to combined impact of HV-CAP and 1% CLE. Microbiological proliferation of mesophile (TVC, Enterobacteriaceae, H2S-producing bacteria, *Pseudomonas*, LAB) and psychrophilic bacteria markedly decreased during 15 days of refrigerated (4 °C) storage.

Table 11. Likeness score of Pacific white shrimp in the presence of CLE at various levels without and with HV-CAP treatment under different modified atmosphere packaging at day 0 and 15 of refrigerated storage.

Storage time (days)	Samples	Color	Texture	Odor	Flavor	Taste	Overall
0	Control	7.56±0.26a	7.45±0.47a	7.29±0.55a	7.33±0.28a	7.44±0.35a	7.50±0.28a
	CP-Ar/O_2-0.5 CLE	7.00±0.27a	6.78±0.55a	6.58±0.51b	6.50±0.39b	6.67±0.46ab	6.72±0.52ab
	CP-Ar/O_2-1 CLE	7.06±0.35aA	6.89±0.29aA	6.84±0.53abA	6.79±0.35abA	6.89±0.38abA	6.90±0.46abA
	CP-Ar/Air-0.5 CLE	7.22±0.34a	7.25±0.45a	7.16±0.39ab	7.11±0.56ab	7.11±0.24ab	7.14±0.71ab
	CP-Ar/Air-1 CLE	7.58±0.37aA	7.33±0.27aA	7.32±0.21aA	7.33±0.44aA	7.28±0.35aA	7.42±0.28aA
15	CP-Ar/O_2-1 CLE	5.67±0.64bB	5.22±0.55bB	5.11±0.48bB	5.06±0.48bB	5.22±0.54bB	5.28±0.0.25bB
	CP-Ar/Air-1 CLE	6.83±0.13aB	6.67±0.63aB	6.68±0.54aB	6.51±0.14aB	6.61±0.38aB	6.56±0.53aB

Values are presented as the mean±standard deviation (n=50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig. 26.

Clostridium perfringens was absent in all the samples, thus assuring the safety of PWS. Antioxidants, particularly CLE effectively lowered lipid oxidation, when HV-CAP was implemented. Lipid and protein oxidation was less pronounced in stored shrimp treated with HV-CAP, especially those containing 1% CLE with high antioxidant capacity. PUFA including EPA and DHA were retained in shrimp treated with CLE at 1%, followed by HV-CAP under Ar/Air (80:20) atmosphere. Highest likeness score was attained in shrimp with HV-CAP and 1% CLE under Ar/Air (80:20) gas composition after 15 days. Thus, CLE treated shrimp packaged under Ar/Air (80:20) mixture with subsequent HV-CAP could preserve the quality and extend shelf-life up to 15 days of storage at 4 °C.

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CHAPTER 7

COMBINED EFFECT OF PULSED ELECTRIC FIELD AND CHAMUANG LEAF EXTRACT PRIOR TO HIGH VOLTAGE COLD ATMOSPHERIC PLASMA TREATMENT ON QUALITY AND SHELF-LIFE OF PACIFIC WHITE SHRIMP

7.1 Abstract

Pulsed electric field (PEF) pre-treated Pacific white shrimp (PWS) were soaked in Chamuang leaf extract (CLE) solutions (1 or 2%), followed by high voltage cold atmospheric plasma (HVCAP; Ar + Air at 80:20) for 10 min. Least melanosis scores were attained in the samples pre-treated with PEF-T2 (800 pulses) and CLE (2%) prior to HVCAP (PEFT2-CLE2-Ar/Air-CP), compared to the control, during 18 days at 4 °C ($p<0.05$). The lowest microbial load and spoilage bacteria count (≤ 5 log CFU/g meat) were attained in PEFT2-CLE2-Ar/Air-CP sample ($p<0.05$). PEFT2-CLE2-Ar/Air-CP sample showed lower peroxide value, thiobarbituric acid reactive substances (TBARS), pH, total volatile base (TVB) and protein carbonyl content (PCC) than others ($p<0.05$). Higher likeness scores were noted in PEFT2-CLE2-Ar/Air-CP sample for all the attributes. Oxidation of polyunsaturated fatty acids and proteins was alleviated by CLE treatment. Thus, shelf-life of PWS could be extended by combined processes.

7.2 Introduction

Pacific white shrimp (PWS) is a popular crustacean consumed globally because of its unique delicacy and appealing sensory property (Shiekh and Benjakul, 2020a). PWS were formerly captured from the Pacific ocean and have been farmed commercially in community based ponds to maintain the stock during supply chain management (Kimbuathong *et al*., 2020). Chemical, microbial and appearance especially, color of the shrimp are the important quality attributes to determine their freshness and acceptability (Shiekh *et al*., 2020d). During improper storage, PWS quality deteriorates ascertained by the discoloration so called ‘melanosis’ (Sae-leaw and Benjakul, 2019a). Melanosis is a biochemical process mediated by polyphenol oxidase (PPO), that leads to the formation of black pigment namely melanin (Shiekh and Benjakul, 2020b). PPO (EC 1.14.18.1), also named as tyrosinase, occurs naturally

during molting process and commonly exists as pro-PPO zymogen in PWS (Gonçalves and de Oliveira, 2016). Microbiological and chemical quality deterioration without any proper hurdles continues with the advancement in storage time (Shiekh *et al*., 2019). Moreover, the commercial practice of using chemical additive such as sodium metabisulphite (SMS) to retard microbial, chemical and enzymatic spoilage has been found unethical recently. At low temperature storage, SMS was incompetent for melanosis prevention (Sae-leaw and Benjakul, 2019b).

Pulsed electric field (PEF) is a promising food processing technology due to its additive free and eco-friendly nature (Han *et al*., 2018). Apart from the non-thermal behavior for nutrient quality retention, PEF has been implemented to inactivate pathogens (*Escherichia coli* and *Pseudomonas fluorescens*) and spoilage bacteria to ensure food safety (Walter *et al*., 2016). Microbial inactivation is based on the phenomenon of electroporation, which is achieved by the intermittent delivery of high voltage short pulses that causes permanent permeability of biological lipid bilayer of microbial cell membrane (Delso *et al*., 2020). Thus, the cytosol of the microbial cell leaks out, associated with complete permeability of cell membrane, thus causing cell death.

Natural additives have been brought into application to replace chemical counterparts for shelf-life extension of seafoods (Olatunde and Benjakul, 2018a). Moreover, the coupling of natural extracts with non-thermal technology has been regarded as an advanced practice for quality preservation of seafoods (Olatunde and Benjakul, 2018b). Combination of natural additives have been applied recently with non-thermal technology to provide hurdle effect for quality preservation of PWS during storage (4 °C) (Shiekh and Benjakul, 2020a). Chamuang (*Garcinia cowa* Roxb.) leaf (CLE) extract contains high amount of glycoside bonded polyphenols and organic acids, proven as highly effective antioxidants and antimicrobial agents for quality preservation of PWS (Shiekh *et al*., 2019). PEF has been implemented in PWS in conjunction with CLE for shelf-life extension up to 10 days at 4 °C (Shiekh and Benjakul, 2020a). PEF and CLE have been proven as potential hurdle for the microbial, chemical changes and melanosis occurring during PWS storage. Additionally, PPO of shrimp cephalothoraxes after PEF exposure without addition of inhibitor was inhibited up to 40% (Shiekh and Benjakul, 2020b). PEF electroporation can induce

conformational changes of enzymes (Gulzar and Benjakul, 2020). Pores generated underneath the shrimp shell or cuticle induced by PEF could facilitate the passage of CLE polyphenols and organic acids (Shiekh and Benjakul, 2020a).

In recent years, high voltage cold atmospheric plasma (HVCAP) technology has been employed in food industry considered as non-thermal sanitizing technology and environment friendly. HVCAP generated in a dielectric barrier discharge (DBD) mode has been applied for decontamination to ensure food safety (Bai *et al*., 2020). DBD generates in-bag HVCAP active species by applying an electromagnetic discharge between two electrodes, where one electrode is attached with dielectric layer (Albertos *et al*., 2017). HVCAP reactive species generated via DBD comprise negative and positive ions, radiation energetic ions, electrons, reactive oxygen species (ROS), reactive nitrogen species (RNS) and ultraviolet photons (Coutinho *et al*., 2018). PWS has been also packaged under modified atmosphere packaging (MAP) to retard shrimp quality changes under various gas compositions. MAP with low concentration of oxygen (5-10%) and high carbon dioxide (20-80%) in the presence of nitrogen have been used to preserve quality and inhibit melanosis of PWS for a period of 12 days (Kimbuathong *et al*., 2020). Additionally, prior PEF treated PWS in combination with CLE, packaged in absolute concentration of CO_2 retained organoleptic quality for 10 days at 4 ºC (Shiekh *et al*., 2020d). HVCAP has been implemented along with CLE for shelf-life extension of PWS up to 15 days under refrigerated condition (Shiekh and Benjakul, 2020c). Therefore, the use of PEF to enhance the penetration of CLE into PWS prior to HVCAP, could mitigate oxidation process accelerated by HVCAP. This study aimed to examine the combined impact of pre-treatments (PEF and CLE) on quality and shelf-life of PWS subjected to HVCAP during storage at 4 °C.

7.3 Objective

To elucidate the impact of enhanced penetration of CLE through PEF generated pores followed by HVCAP, on melanosis, chemical and microbial quality of PWS for extended shelf-life.

7.4 Materials and methods

7.4.1 Chemicals

All the microbial media and chemicals obtained from Sigma Aldrich (St. Louis, MO, USA) and Oxoid Ltd. (Hampshire and UK) were of analytical grade.

7.4.2 Collection and preparation of Pacific white shrimp (PWS)

Fresh PWS (55-60 shrimp per kg) were bought from a cultured farm situated in Songkhla, Thailand. PWS placed in crushed ice (ice to sample ratio, 2:1) in polystyrene foam boxes were delivered to PSU-Seafood Laboratory, within 1 h. Extraneous impurities were removed using cold water (≤ 4 °C) and the shrimp antennae were cut with a scissor. After washing properly, whole PWS were placed in ice as mentioned above until pre-treatment or packing was done.

7.4.3 Pre-treatment of PWS with PEF and CLE

PEF system (PEF LAB-400W, Febix International Inc., Chiang Mai, Thailand) was implemented on PWS (Shiekh and Benjakul, 2020a). PEF treatment chamber (1.5 L) made of stainless steel was fitted with a steel electrode (20 cm × 10 cm). Treatment cabinet consisted of aluminium frame covered with a plexiglass. A UT-P03 oscilloscope probe (600 MHz, 10X) was linked with a digital storage oscilloscope (DSO) and a PEF generator for data output. The high intensity pulses were varied with the resulting specific energies of 483-697 kJ/kg. Those included PEFT1 (15 kV/cm, 600 pulses, 483 kJ/kg) and PEFT2 (15 kV/cm, 800 pulses, 697 kJ/kg) (Shiekh and Benjakul, 2020a).

Mature leaves of Chamuang trees from a plantation in Hat Yai were collected. During washing, the extraneous matter or damaged leaves were removed. Washed leaves were dried in a tray dryer (24 h) at 50 °C and ground in a high speed blender to obtain Chamuang leaves powder (CLP). Ground CLP (passed through 80 mesh sieve) was mixed with water for ultrasonic extraction to obtain Chamuang leaf extract (CLE) as detailed by Shiekh and Benjakul (2020c). The prepared CLE solution was transferred into zip-lock bags (200 ml) and placed in freezer (-20 °C) for overnight and transferred to -40 °C deep freezer (12 h) prior to freeze drying. The CLE powder was collected after freeze drying for 72 h.

PWS were pre-treated with PEF at different levels (PEFT1 and PEFT2). Thereafter, the PEF pre-treated samples were immersed in 1% or 2% CLE solutions for 30 min (Shiekh and Benjakul, 2020b) and placed on a screen for draining (5 min, 4 °C).

7.4.4 HVCAP for PEF- CLE treated PWS

HVCAP system was equipped with a high voltage transformer (230 V input voltage, 50 Hz) and a voltage variac (0–120 kV output voltage) was connected with dielectric-barrier discharge (DBD) system. DBD was created between the two aluminium electrodes of 15-cm diameter and one electrode was fixed with a dielectric layer as displayed by Shiekh and Benjakul (2020c). HVCAP system was run with a voltage of 16 kVRMS at ambient atmospheric pressure. Two electrodes had a distance of 2 cm for an effective exposure of DBD (Ziuzina *et al.,* 2014).

PWS (7 whole raw shrimp) after PEF and CLE pre-treatment were packed in nylon/linear low-density polyethylene (LLDPE) bag and filled with 5 volumes of gas mixture (Ar + Air = 80:20) using Henkovac type 1000 (Tecnovac, Italy) modified atmosphere packaging (MAP) machine, followed by heat sealing. The gases were of food-grade including sterilized Ar and zero air (containing < 0.1 hydrocarbon impurities). All the bags containing samples were subjected to DBD for 10 min as described by Shiekh and Benjakul (2020c). Some clusters of PWS were treated with 1% or 2% CLE with the aid of PEF without DBD and packed under MAP. Treated samples are defined as follows:

1. Control (untreated, packed in normal air)
2. PEFT1-CLE1-Ar/Air (PEF-600 pulses and 1% CLE, Ar + Air-M)
3. PEFT2-CLE1-Ar/Air (PEF-800 pulses and 1% CLE, Ar + Air-M)
4. PEFT1-CLE2-Ar/Air (PEF-600 pulses and 2% CLE, Ar + Air-M)
5. PEFT2-CLE2-Ar/Air (PEF-800 pulses and 2% CLE, Ar + Air-M)
6. CP-Ar/Air-M (Without any treatment, packed in Ar + Air-M, HVCAP)
7. PEFT1-CLE1-Ar/Air-CP (PEF-600 pulses and 1% CLE, Ar + Air-M, HVCAP)
8. PEFT2-CLE1-Ar/Air-CP (PEF-800 pulses and 1% CLE, Ar + Air-M, HVCAP)
9. PEFT1-CLE2-Ar/Air-CP (PEF-600 pulses and 2% CLE, Ar + Air-M, HVCAP)
10. PEFT2-CLE2-Ar/Air-CP (PEF-800 pulses and 2% CLE, Ar + Air-M, HVCAP)

Analyses of all the samples were done every 3 days up to 18 days.

7.4.5 Analyses

7.4.5.1 Melanosis assessment

The protocol of Nirmal and Benjakul (2010) was used for visual inspection of dark spot formation (melanin) using ten trained panelists, who had experience of the scoring test in PWS. For each treatment, a bag containing around 20 shrimp was fixed for melanosis score test and presented to panelists at 3 day-interval of totally 18 days.

7.4.5.2 Microbiological analyses

Spread plate technique was employed for enumeration of total viable counts (TVC), psychrophilic bacterial counts (PBC), lactic acid bacteria (LAB), *Pseudomonas*, H_2S-producing bacteria, Enterobacteriaceae and *Clostridium perfringens* counts as described by Shiekh and Benjakul (2020d).

7.4.5.3 pH

PWS samples (2 g) were homogenized in deionized water (20 ml) and pH of homogenate was measured with a pH meter (Sartorious North America, Edgewood, NY, USA) (Nirmal and Benjakul, 2011).

7.4.5.4 Protein carbonyl contents (PCC) and total volatile base (TVB)

Natural actomyosin (NAM) from PWS muscle was extracted as described by Chantarasuwan, Benjakul, and Visessanguan (2011). PCC of NAM was determined using 2,4-dinitro-phenyl hydrazine (DNPH) as detailed by Nikoo, Benjakul, and Xu (2015). Measurement of TVB content in PWS via Conway micro-diffusion method was performed (Shiekh and Benjakul, 2020c).

7.4.5.5 Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

PV of shrimp meat was analyzed by ferric thiocyanate method (Arfat *et al*., 2015) using cumene hydroperoxide (0.5–2 ppm) standard. TBARS of shrimp meat was measured (Shiekh and Benjakul, 2020c), in which malonaldehyde (MDA) (0–5 ppm) was used as standard.

7.4.5.6 Protein pattern

At the first day of sample preparation, the selected samples packed under Ar + Air-M without and with PEF and CLE pre-treatment subjected to HVCAP and the control were analyzed for protein pattern using SDS-PAGE as tailored by Shiekh *et al*. (2020c).

7.4.5.7 Fatty acid profile

Fatty acid profiles were determined as fatty acid methyl esters (FAMEs) using gas chromatography with flame ionization detector (GC-FID) following the procedure of Raju and Benjakul (2020). Peaks were evaluated based on the retention time of standards and the samples were quantified.

7.4.5.8 Sensory evaluation

The samples having TVC less than 6 log CFU/g were subjected to sensory analysis after being stored for 18 days 4 °C in comparison with fresh shrimp (Nirmal and Benjakul, 2010). Samples wrapped with aluminum foil were placed in different chambers of steaming pot. Samples were steamed for totally 5 min to reach the core temperature of 80 °C (Manheem *et al*., 2013).

7.4.6 Statistical analyses

Completely randomized design (CRD) was used for the whole study. Analysis of variance (ANOVA) was carried out and mean comparison was performed by Duncan's multiple range test or t-test were used for comparison at a significant level ($p<0.05$) using a SPSS statistical software (SPSS 17.0, SPSS Inc., Chicago, IL, USA). All the experiments were run in triplicates (n=3).

7.5 Results and discussion

7.5.1 Effect of PEF and CLE pre-treatments followed by HVCAP on melanosis and quality of PWS during *refrigerated* storage

7.5.1.1 Melanosis formation

PEF or CLE pre-treatment of PWS was done with subsequent exposure to in-bag HVCAP and the melanosis of PWS was monitored during the storage. Melanosis was not noticed in all samples at day 0 (Fig 31a). During the storage, sample

treated with 2% CLE with the aid of PEF (15 kV, 800 pulses) and high specific energy (697 kJ/kg) with subsequent HVCAP (PEFT2-CLE2-Ar/Air-CP) exhibited the least melanosis ($p<0.05$). Melanosis scores were in the descending order: PEFT1-CLE2-Ar/Air-CP > PEFT2-CLE1-Ar/Air-CP > PEFT1-CLE1-Ar/Air-CP. Lower scores were obtained for the samples treated with HVCAP than those with no HVCAP treatment ($p<0.05$). Different melanosis formation among aforementioned samples was governed by various levels of CLE and PEF pulses used. Ar and Air (80:20) were documented to generate active species, particularly those generated from N_2 in air, might decelerate melanosis, especially in conjunction with 1% CLE treatment (Shiekh and Benjakul, 2020c). In addition to HVCAP, high intensity PEF pulses (600 pulse number) could also inactivate PPO by 40% (Shiekh and Benjakul, 2020b). HVCAP generates a wide range of active species such as hydroxyl radicals ($^{\bullet}OH$), atomic nitrogen ($N^{\bullet}$), atomic oxygen ($O^{\bullet}$), varieties of nitrogen oxides such as nitrite oxide (NO^{2-}) and nitrate oxide (NO^{3-}) and in atmospheric air containing bag (Charoux *et al*., 2020). Moreover, the reactive species nitrogen (RNS) or oxygen (ROS) can cause alteration in protein, especially aromatic amino acids such as tyrosine that act as potential substrate for PPO (Surowsky *et al*., 2013).

Lowered melanosis in PEFT2-CLE2-Ar/Air-CP sample could extend keeping quality of shrimp up to 18 days (Fig. 31a). Apart from PEF and CLE, HVCAP liberates high energy discharge that could denature PPO. Additionally reactive radicles from Ar and O_2 gases induced by HVCAP might inactivate PPO (Shiekh and Benjakul, 2020c). Cold atmospheric plasma (CAP) reactive species were reported to cause alteration of secondary structure of PPO (Zouelm *et al*., 2019).

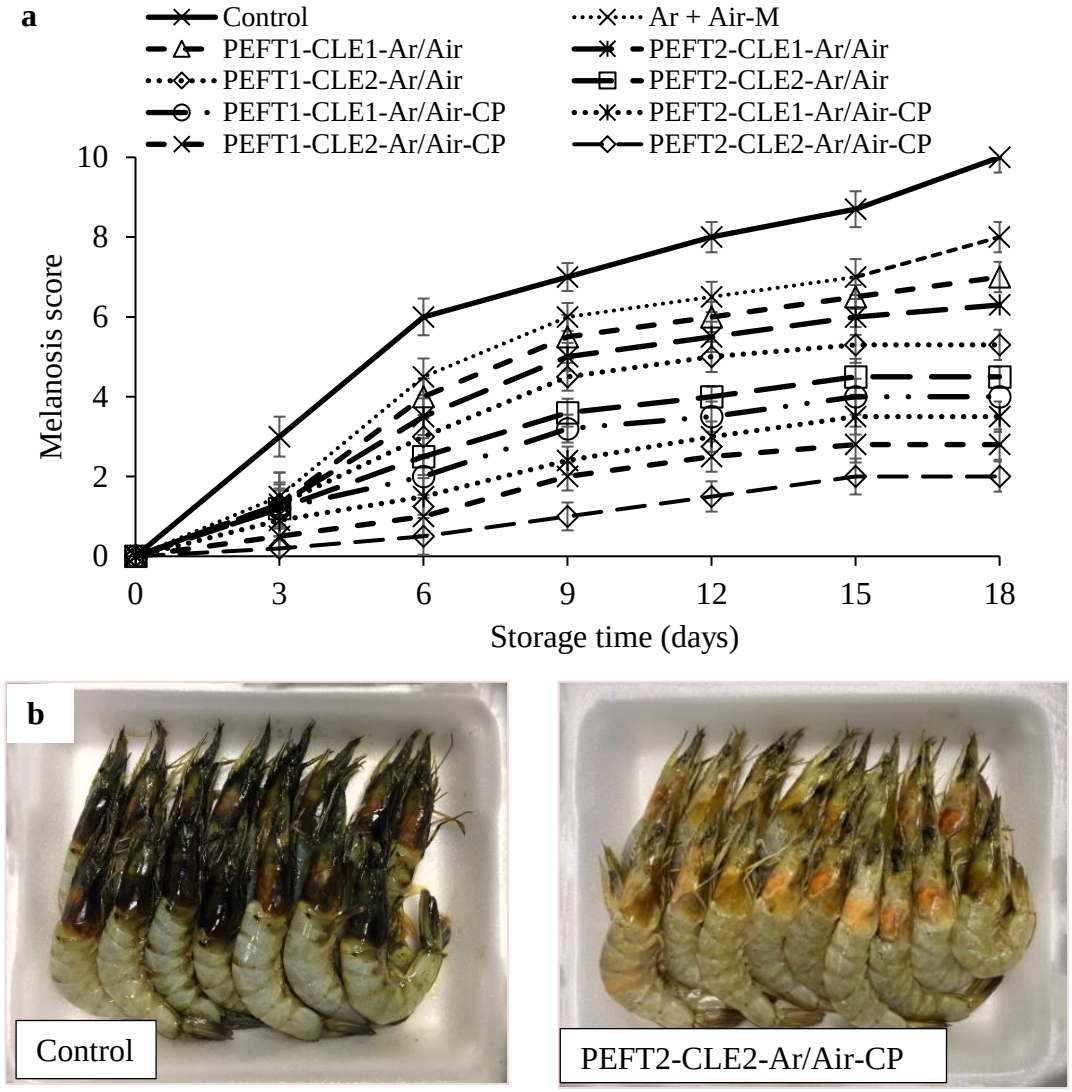

Figure 31. Melanosis score (a) of Pacific white shrimp with PEF and CLE pre-treatment without and with HVCAP treatment during 18 days of storage at 4 °C and photographs of selected samples after 18 days of storage (b).

Bars represent the standard deviation (n=10). Control: without any treatment; CP-Ar/Air-M: Treated with HVCAP, packed under Ar + Air (80:20); PEFT1-CLE1-Ar/Air: Treated with 1% CLE and PEF-600 pulses, packed under Ar + Air (80:20); PEFT2-CLE1-Ar/Air: Treated with 1% CLE, PEF-800 pulses and HVCAP, packed under Ar + Air (80:20); PEFT1-CLE2-Ar/Air: Treated with 2% CLE and PEF-600 pulses, packed under Ar + Air (80:20); PEFT2-CLE2-Ar/Air: Treated with 2% CLE and PEF-600 pulses, packed under Ar + Air (80:20); PEFT1-CLE1-Ar/Air-CP: Treated with 1% CLE PEF-600 pulses and HVCAP, packed under Ar + Air (80:20); PEFT2-CLE1-Ar/Air: Treated with 1% CLE, PEF-800 pulses and HVCAP, packed under Ar + Air (80:20); PEFT1-CLE2-Ar/Air-CP: Treated with 2% CLE PEF-600 pulses and HVCAP, packed under Ar + Air (80:20); PEFT2-CLE2-Ar/Air-CP: Treated with 2% CLE PEF-800 pulses and HVCAP, packed under Ar + Air (80:20).

Moreover, CO_2-MAP in combination with PEF (15 kV, 600 pulses, 483 kJ/kg) and 1% CLE decreased melanosis compared to control (without any treatment) for a period of

10 days (Shiekh and Benjakul, 2020a). Appearance of PWS control (untreated) and PEFT2-CLE2-Ar/Air-CP sample stored for 18 days of storage (4 ºC) is illustrated in Fig. 31b. The control had the pronounced melanosis, whereas PEFT2-CLE2-Ar/Air-CP sample possessed a little black spots.

7.5.1.2 Changes in microbiological quality

At day 0, TVC of all treated samples was lower than that of control ($p<0.05$). Combined impact of PEF with higher pulses and 2% CLE treatment in PWS, followed by HVCAP was more effective in lowering the microbial growth, compared to others. TVC of control and other treated samples increased gradually during the entire storage as given in Fig. 32a. At day 18, TVC counts of the control, CP-Ar/Air-M, PEFT1-CLE1-Ar/Air, PEFT2-CLE1-Ar/Air, PEFT1-CLE2-Ar/Air, PEFT2-CLE2-Ar/Air, PEFT1-CLE1-Ar/Air-CP, PEFT2-CLE1-Ar/Air-CP, PEFT1-CLE2-Ar/Air-CP and PEFT2-CLE2-Ar/Air-CP samples were 8.17, 6.77, 6.29, 5.91, 5.69, 5.49, 5.90, 5.40, 5.25, 5.03 log CFU/g, respectively. At the end of storage (18 days), PEFT2-CLE2-Ar/Air-CP sample ranked lowest in TVC ($p<0.05$). Combined effect of PEF, CLE and HVCAP retarded the bacterial proliferation in PWS. HVCAP was documented to generate active species of O_3 and RNS in PWS packed in Ar or air, resulted in microbiological cell death (Shiekh and Benjakul, 2020c). Additionally, PEF was reported previously to inactivate mesophilic bacteria in PWS due to cell wall disintegration caused by PEF electroporation (Shiekh and Benjakul, 2020b). CAP treated PWS had the lowest microbial load during 14 days of storage (4 ºC) (De Souza Silva *et al*., 2019).

Control had the highest PBC at day 0 ($p<0.05$). PEF or CLE treated samples at different levels followed by HVCAP had the lower PBC ($p<0.05$) (Fig. 32b). At the first day, PBC in PEFT1-CLE2-Ar/Air-CP and PEFT2-CLE2-Ar/Air-CP samples were moderately lower compared to the control and other samples ($p<0.05$). PBC in PEFT2-CLE2-Ar/Air-CP was lower as the storage proceeded.

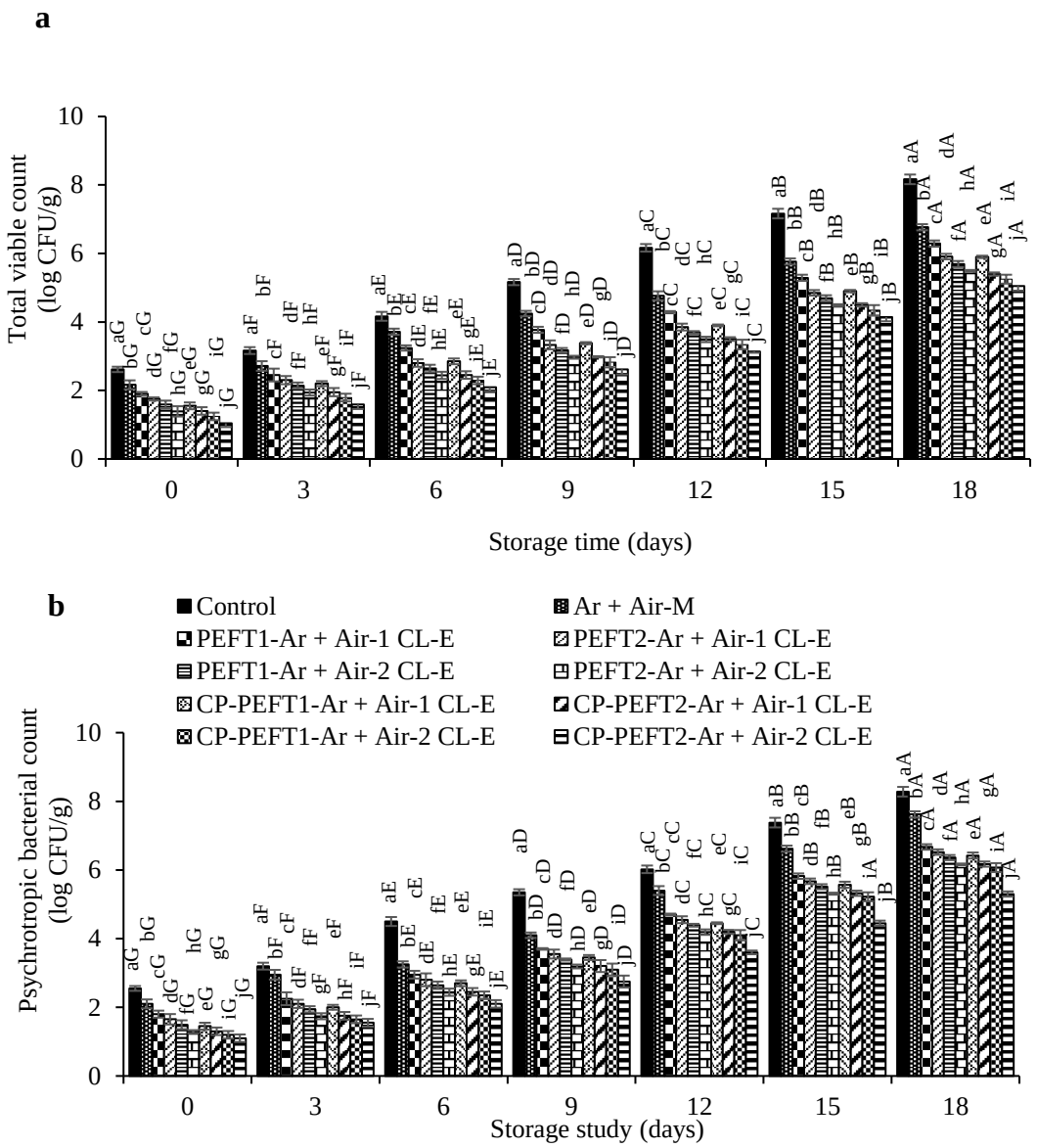

Figure 32. Total viable count (a) and psychrophilic bacterial count (b) of Pacific white shrimp with PEF and CLE pre-treatment without and with HVCAP treatment during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 31.

Generally, the retarded growth of PBC in PEFT2-CLE2-Ar/Air-CP sample throughout storage could be associated with the enhanced CLE passage through the PEF generated pores, especially when PEF at 800 pulses was applied, thereby lowering bacterial growth than others. Besides HVCAP, PEF (15 kV, 600 pulses, 483 kJ/kg) and CLE (1%) were documented to be efficient in inhibiting microbes in PWS (Shiekh and

Benjakul, 2020a). PBC of CP treated PWS was lower than that of control (De Souza Silva *et al*., 2019). PBC were markedly inactivated during in-bag plasma generation by HVCAP in PWS packaged under Ar + Air mixture (80:20) treated with 1% CLE (Shiekh and Benjakul, 2020c).

Pseudomonas of all the samples ranged between 1.20 and 2.30 log CFU/g) at day 0 as depicted in Fig. 33a. Control had the highest *Pseudomonas* than others ($p<0.05$). Conversely, the lowest count was attained in PEFT2-CLE2-Ar/Air-CP sample ($p<0.05$). However, effect of HVCAP as sanitizing non-thermal technology was dominant in the treated sample. Increment in *Pseudomonas* count was noticed during storage of 18 days ($p<0.05$). At day 18, control ranked highest in count (8.09 log CFU/g), whereas the count of PEFT2-CLE2-Ar/Air-CP sample was still under acceptable limits (5.28 log CFU/g) ($p<0.05$). *Pseudomonas* were drastically reduced in coconut husk extract treated sea bass slices (Olatunde *et al*., 2019a). *Pseudomonas* are gram negative spoilage bacteria dominant in seafoods (DeWitt and Oliveira, 2016). Absolute CO_2-MAP along with PEF and 1% CLE inactivated *Pseudomonas* in PWS (Shiekh *et al*., 2020d).

The initial H_2S producing bacterial count ranged from 1.03 to 2.46 log CFU/g for all the samples, and the control had the highest count ($p<0.05$) (Fig. 33b). At day 18, PEFT2-CLE2-Ar/Air-CP samples possessed the lowest H_2S-producing bacterial count (5.33 log CFU/g). PEF and CLE under absolute CO_2-MAP lowered H_2S-producing bacteria notably (Shiekh and Benjakul, 2020d) Olatunde *et al*. (2019c) reported that gram positive and gram negative microbial flora were retarded in HVCAP treated sea bass slices to ensure food safety. Moreover, PWS treated with HVCAP via DBD in the presence of 1% CLE without PEF treatment, inactivated H_2S-producing bacteria notably when stored at 4 ºC (Shiekh and Benjakul, 2020c).

At the first day, Enterobacteriaceae count of control was highest ($p<0.05$) (Fig. 33c). Increases in Enterobacteriaceae were noticeable in all samples as storage time augmented ($p<0.05$). After 18 days, the lowest count was obtained in PEFT2-CLE2-Ar/Air-CP sample ($p<0.05$). PEF with lower pulses along with 2% CLE could also inactivate Enterobacteriaceae, mainly attributed to an effective passage of CLE at 2% level through PEF pores or gapping generated underneath the shell of PWS where PPO was located, when the high energy PEF pulses (800 pulses) were applied.

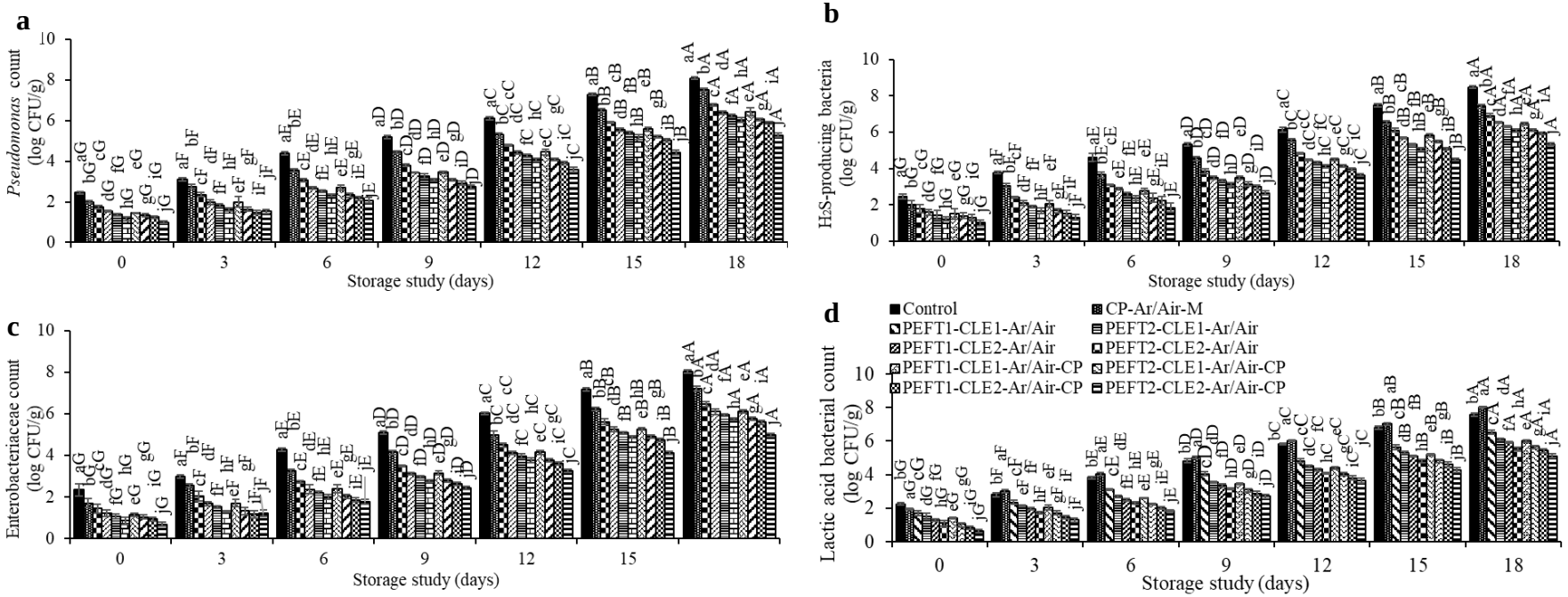

Figure 33. *Pseudomonas* (a), H_2S-producing bacteria (b), Enterobacteriaceae (c) and lactic acid bacteria counts (d) of Pacific white shrimp with PEF and CLE pre-treatment without and with HVCAP treatment during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 31.

HVCAP treated PWS along with 1% CLE drastically reduced Enterobacteriaceae counts compared to control during 15 days of refrigerated storage (Shiekh and Benjakul, 2020c). Enterobacteriaceae are gram negative facultative anaerobes highly susceptible to the damages by polyphenols than gram positive bacteria (McMillin, 2008). Therefore, PEF and 2% CLE treated PWS followed by HVCAP were low in Enterobacteriaceae during 18 days of storage (4°C).

Initially, the control showed the higher LAB count than others ($p<0.05$). LAB count was relatively lower in PEFT2-CLE2-Ar/Air-CP ($p<0.05$) (Fig. 33d), plausibly due to joint effect of PEF electroporation of the bacterial cells that facilitated the passage of CLE antimicrobial agents and migration of HVCAP generated reactive species towards LAB. After day 3 of storage, LAB count remained highest in CP-Ar/Air-M sample than others ($p<0.05$). This could be governed by atmosphere containing Ar and air with less proportion of oxygen in the bag. LAB were reported as facultative anaerobes having sensitivity to phenolic compounds and could be affected by active species of HVCAP (Han *et al*., 2016; Misra *et al*., 2011). Sanitizing effect of PEF along with CLE at 2% prior to HVCAP was pronounced, thus lowering the growth of LAB. *Clostridium perfringens* colonies were not found in all the samples throughout storage. This could be attributed to hygienic practice of sample preparation without any cross-contamination.

7.5.1.3 Changes in chemical quality

The same pH of all the samples were observed at day 0 ($p>0.05$), except PEFT2-CLE2-Ar/Air-CP sample, which showed the lowest pH ($p<0.05$) (Fig. 34a). The pHs of 6.36-6.82 were found within the first 3 days. As storage augmented, pH of the control increased more rapidly than others ($p<0.05$). In contrast, the changes in pH values of PEFT2-CLE2-Ar/Air-CP sample were negligible during 0-18 days ($p<0.05$). Microbial enzymes in PWS might have been inactivated during treatment with high energy PEF pulses. PEF can inactivate enzymes via their denaturation (Shiekh and Benjakul, 2020b). After the end of storage, the pH of control and other samples was in the range of 6.97-11.52. The accumulation of basic compounds in the control sample (without any treatment) could be due to higher activity of microbial enzymes, contributing to the rise in pH. Similar results were documented in HVCAP and CLE treated PWS (without PEF) during 15 days of storage (Shiekh and Benjakul, 2020c).

Lowered increment of pH of PEFT2-CLE2-Ar/Air-CP sample was plausibly owing to the prevention of microbial growth and enzyme inactivation induced by HVCAP as well as antimicrobial effects of CLE.

TVB contents of the control and treated samples (1.63 to 5.34 mg N/100 g) were noted at day 0 (Fig. 34b). During the first 3 days, TVB content of the control was lower than CP-Ar/Air-M sample ($p<0.05$). Gradual increase of TVB content in all the samples was noticed during 18 days ($p<0.05$). The drastic increases in TVB content were observed in the control during 6 to 18 days ($p<0.05$). PEFT2-CLE2-Ar/Air-CP possessed the lowest TVB content (17.81 mg N/100 g meat) at day 18. This was more likely the result of the joint effect between PEF, CLE and HVCAP. TVB levels of 12-20 mg N/100 g in PWS indicate the acceptable limits for safe consumption (Okpala *et al*., 2014). Shiekh and Benjakul (2020c) reported similar range of TVB content in CLE and HVCAP treated PWS without prior PEF during 15 days of storage. Moreover, the use of PEF along with 1% CLE and cashew leaf extract, had the pronounced antimicrobial and antioxidant effects to retard TVB content of refrigerated shrimp (Shiekh *et al*., 2020b, Sae-leaw and Benjakul, 2019a). CAP could potentially preserved quality of PWS by inactivation of spoilage microorganisms at 4 ºC (Zouelm *et al*., 2019).

Protein carbonyl content (PCC) of the sample treated with HVCAP without PEF or CLE treatment (CP-Ar/Air-M) was highest at day 0 ($p<0.05$) (Fig. 34c). PEFT2-CLE2-Ar/Air-CP sample showed the lower PCC during 0-3 days than the control and others ($p<0.05$).With increasing storage time, PCC of the samples were increased, reflecting the augmented protein oxidation due to insufficient concentration of CLE in treated samples. PEF did not impact oxidation of PWS proteins (Shiekh and Benjakul, 2020b). Additionally, the lowest PCC of PEFT2-CLE2-Ar/Air-CP sample was attained throughout storage ($p<0.05$). Myofibrillar proteins of sea bass without antioxidants were oxidized by HVCAP (Olatunde *et al*., 2019d). However, HVCAP with coconut husk extract treatment could retard formation of PCC in sea bass (Olatunde *et al*., 2019a). The effect of 2% CLE towards protein oxidation was evident in HVCAP treated sample.

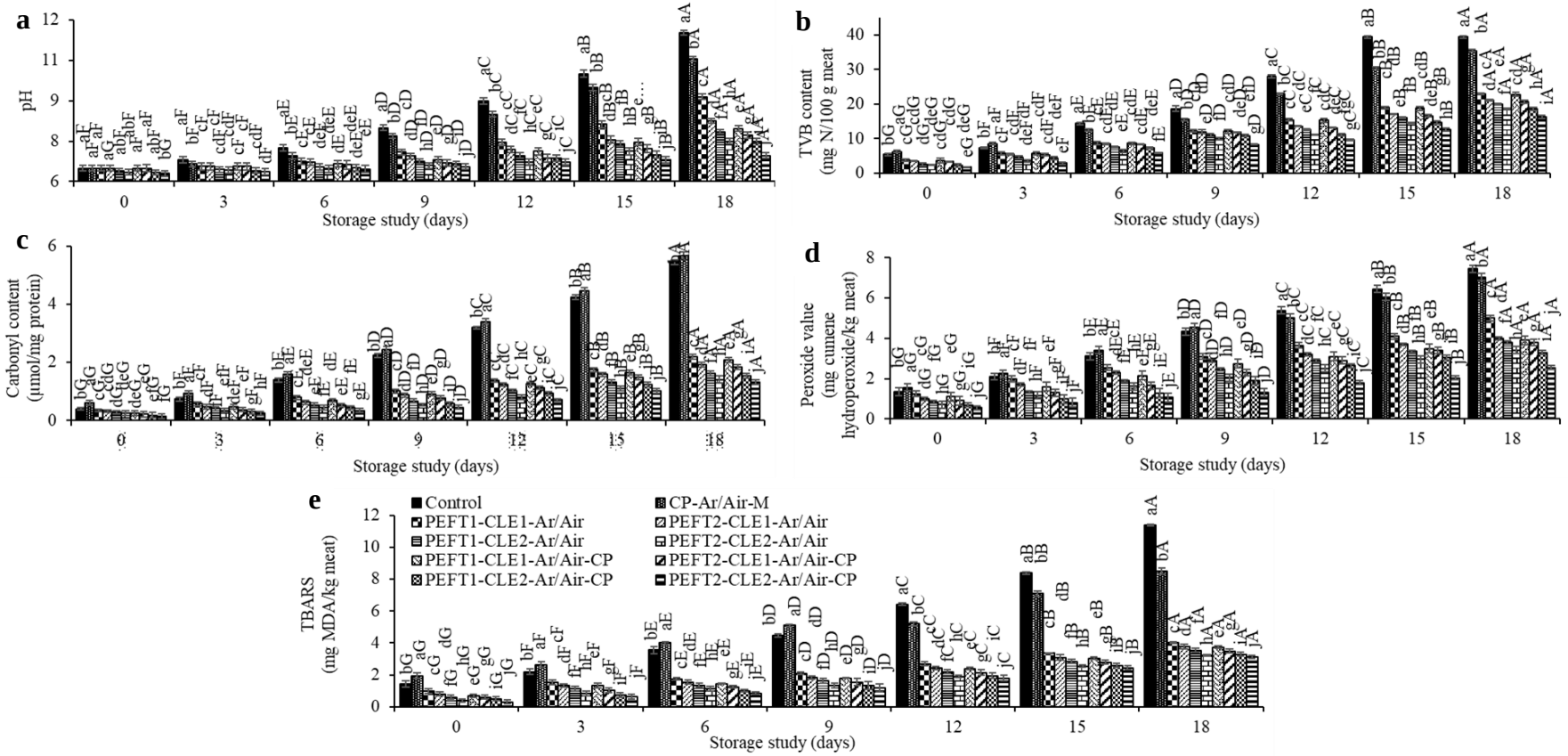

Figure 34. pH (a), total volatile base (TVB) (b), carbonyl content (c), peroxide values (d) and thiobarbituric reactive substances (TBARS) values (e) of Pacific white shrimp with PEF and CLE pre-treatment without and with HVCAP treatment during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$) Key: see the caption for Fig. 31.

Decreased PCC in PEFT2-CLE2-Ar/Air-CP coincided with the lower TVB content and microbial load. PCC in the swimming crab and PWS kept under low O_2 and absolute CO_2 atmosphere along with 1% CLE were remarkably less than the control sample (Shiekh and Benjakul, 2020d; Sun *et al*., 2017). The highest PCC was reported in HVCAP treated PWS containing lower levels of CLE (0.5%) (Shiekh and Benjakul, 2020d). Therefore, higher PEF pulses in presence of sufficient CLE dose prevented protein oxidation during HVCAP treatment. PCC in HVCAP treated PWS was lowered as CLE concentration augmented (Shiekh and Benjakul, 2020c).

PV was higher in CP-Ar/Air-M than the control and others up to 9 days of storage ($p<0.05$). The lowest PV was attained in PEFT2-CLE2-Ar/Air-CP sample at day 0 ($p<0.05$) (Fig. 34d). HVCAP towards PWS with PEF and 2% CLE treatment lowered the formation of hydroperoxide ascertained by lower PV ($p<0.05$). CLE was rich in antioxidants that penetrated through the holes or gaps generated in PWS meat driven by PEF electroporation (Shiekh and Benjakul, 2020a). After 18 days, highest PV was obtained in the control ($p<0.05$), than samples treated with 2% CLE. On the other hand, PEFT2-CLE2-Ar/Air-CP sample showed the lowest PV ($p<0.05$). Lower oxygen levels and effective passage of CLE through shells possibly lowered PV in this sample. It was also the result of combined inhibitory effect of PEF and HVCAP towards microbial and indigenous enzymes including lipase. Seafoods are rich in polyunsaturated fatty acids (Albertos *et al*., 2017). CLE (1%) treatment prevented lipid oxidation of PWS, when HVCAP was applied for 10 min (Shiekh and Benjakul, 2020c). HVCAP treated sea bass with natural antioxidants resulted in lowest hydroperoxide formation (Olatunde *et al*., 2019b). Therefore, PEF and CLE followed by HV-CAP prevented lipid oxidation in PWS during storage.

TBARS values of PWS treated with HVCAP without any antioxidant (CP-Ar/Air-M) were highest within the first 9 days ($p<0.05$) (Fig. 34e). Highest TBARS values were noticed in the control (untreated) up to 18 days of storage. PEFT2-CLE2-Ar/Air-CP sample possessed lower TBARS value than the control and CP-Ar/Air-M samples throughout 18 days ($p<0.05$). The higher TBARS in CP-Ar/Air-M (without PEF or CLE treatment) suggested that hydroperoxides were formed from lipid oxidation induced by HVCAP and further decomposed to the secondary products. After 9 days of storage, the control had highest TBARS, likely correlated with the production

of microbial lipase, which accelerated oxidation. Nevertheless, PEF (800 pulses) treated PWS in conjunction with CLE (2%) alleviated lipid oxidation to some level, in the absence of HVCAP during 18 days of storage. CP alone was reported to trigger lipid oxidation of foods (Pankaj *et al*., 2018). After 18 days, PEFT2-CLE2-Ar/Air-CP was recorded with low amount of TBARS. Similar TBARS levels were found in PWS with added antioxidants during storage (Zouelm *et al*., 2019). PWS with PEF and CLE (1%) treatment along with HVCAP had the lowest TBARS throughout refrigerated storage (Shiekh and Benjakul, 2020c). Therefore, PEF and CLE soaking had combined effect on prevention of lipid oxidation of HVCAP treated PWS during entire storage.

7.5.1.4 Changes in fatty acid profile

Fatty acids of the control and all the treated samples are presented in Table 12. Control and the prior PEF treated samples with 1 or 2% CLE with subsequent HVCAP showed no differences in the fatty acid profiles at day 0 ($p>0.05$). However, shrimp subjected to HVCAP without PEF or CLE had lower fatty acid content, compared to others at the first day ($p<0.05$). Oxidation of PUFA and MUFA was reported to enhance saturated fatty acids (SFA) treated with HVCAP in PWS and sea bass slices (Shiekh and Benjakul, 2020c; Olatunde *et al*., 2019a). The control and other treated samples had higher palmitic acid, followed by DHA, EPA oleic and linoleic acids, respectively. Among all the samples, CP-Ar/Air-M showed the lowest amount of fatty acids. The results were in line with the mackerel (*Scomber scombrus*) fillets subjected to DBD, which had the lower in MUFA, particularly oleic acid (C18:1, n-9) and EPA (C20:5, n-3) with 5 min exposure time (Albertos *et al*., 2017). CLE (1%) was reported to lower fatty acid oxidation during HVCAP processing of PWS (Shiekh and Benjakul, 2020c). Oxidation mediated by the active species produced during gas ionization by HVCAP affected saturated fatty acids, MUFA and PUFA in CP-Ar/Air-M without any antioxidant, at day 0 ($p<0.05$). Additionally PEF (15 kV/cm, 800 pulses, 697 kJ/kg) pre-treatment aided in the passage of 1 or 2% CLE to get into the shrimp shell, thus maintaining the quality of PWS during HVCAP processing more effectively. In general, PEFT2-CLE2-Ar/Air-CP sample had the higher content of retained fatty acids ($p<0.05$). PEF (15 kV/cm, 600 pulses, 483 kJ/kg) was recently implemented together with absolute CO_2 atmosphere for quality preservation of PWS, in which no

impact on fatty acids, regardless of the CLE treatment, was observed (Shiekh *et al*., 2020d). Thus it was presumed that PEF treatment had no negative impact on fatty acids.

After 18 days, EPA and DHA of PEFT1-CLE2-Ar/Air-CP and PEFT2-CLE2-Ar/Air-CP samples were depleted, while myristic acid, oleic acid and linoleic acid augmented. The lowered contents of PUFA could be the result of PUFA oxidation with coincidental increased proportion of MUFA and SFA. Despite of the decreases in fatty acids during prolonged storage (18 days), MUFA and PUFA contents were still higher in PEFT2-CLE2-Ar/Air-CP sample than those of PEFT1-CLE2-Ar/Air-CP sample. Lipid oxidation products (aldehydes or ketones) affect sensory attributes in foods that could impact consumer health (Pan *et al*., 2019). However, this negative impact of oxidation induced by HVCAP could be mitigated by the combined effect of PEF and incorporation of 2% CLE rich in antioxidative polyphenols to retain shrimp quality.

7.5.1.5 Protein pattern

Protein patterns of PEF and CLE treated PWS with augmented HVCAP are shown in Fig. 35. Protein bands of control (without any treatment) and PEF treated shrimp soaked in 1 or 2% CLE were similar. However, myosin heavy chain (MHC) disappeared to greater degree in the CP-Ar/Air-M sample. This signified that MHC and actin (AC) bands were not degraded with pre-treatment of PEF and CLE, regardless of HVCAP. Proteins can be oxidized or fragmented by reactive species, mainly associated with the loss secondary structure and the changes in α-helix and β–sheet (Li *et al.,* 2014; Segat *et al.,* 2016). HVCAP treated sea bass slices suffered protein degradation or fragmentation. Interaction of ozone with the proteins was confirmed by the increased trichloroacetic acid (TCA) soluble peptides (Olatunde *et al*., 2019d). HVCAP along with CLE at 1% level was the potential means to prevent protein oxidation or degradation in PWS under Ar + Air-MAP (Shiekh and Benjakul, 2020c). Polyphenolics in CLE at higher level could prevent oxidation of proteins. Therefore, the retention of MHC and AC indicated the positive effect of CLE at higher doses to curb the oxidation caused by HVCAP. Shrimps (*Metapenaeus ensis*) stored in ice cubes produced from plasma activated water (PAW) inactivated microbes that are the source of indigenous proteases (Liao *et al*., 2018). HVCAP treated Asian seabass slices along with coconut husk extract and ascorbic acid revealed no protein oxidation or fragmentation (Olatunde

et al., 2019a). Moreover, natural and chemical based preservatives such as quercetin, cinnamic acid, and 4-hexylresorcinol in combination with MAP (80% CO_2; 10% O_2; 10% N_2) retarded protein degradation in refrigerated shrimp (Qian *et al*., 2015).

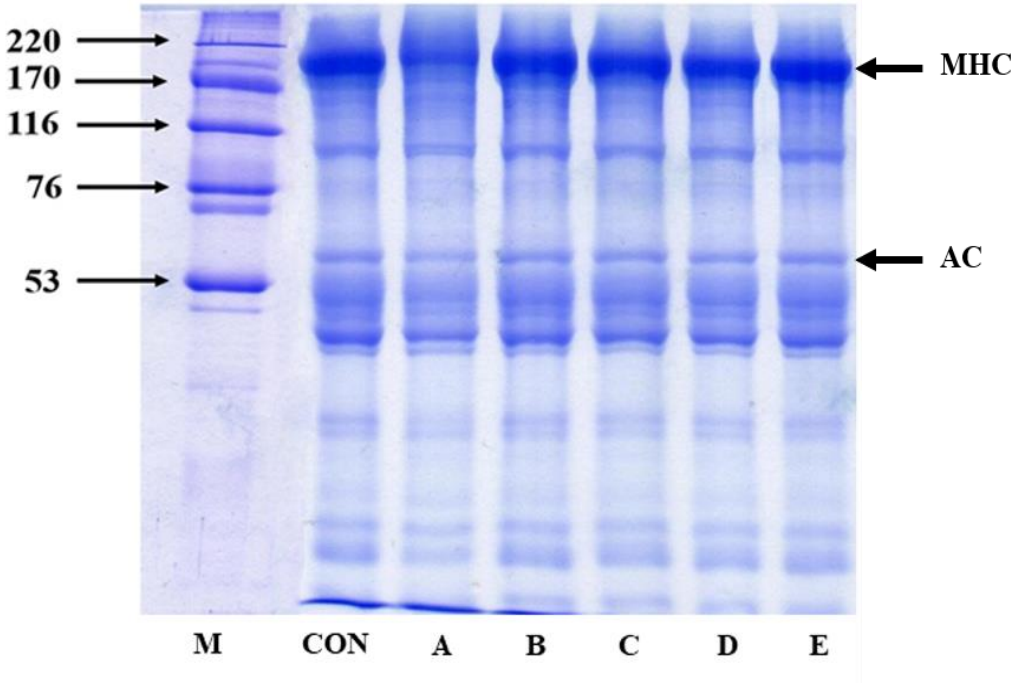

Figure 35. Protein pattern (SDS-PAGE) of Pacific white shrimp with PEF or CLE pretreatment without and with HVCAP treatment at day 0.
MHC: myosin heavy chain; AC: Actin; M: Marker. CON: Control (without any treatment); A: CP-Ar/Air-M; B: PEFT1-CLE1-Ar/Air-CP; C: PEFT2-CLE1-Ar/Air-CP; D: PEFT1-CLE2-Ar/Air-CP; E: PEFT2-CLE2-Ar/Air-CP. Key: see the caption for Fig. 31.

7.5.1.6 Changes in sensory quality

Likeness scores of all the samples are presented in Table 13. At the first day, all the samples with or without treatment had the similar sensory scores ($p>0.05$). Nevertheless odor, flavor and taste of CP-Ar/Air-M had the lower scores ($p<0.05$). This might be associated with the oxidation triggered by HVCAP active species in the absence of CLE, thus causing off-odor and off-flavor development. Shiekh and Benjakul (2020c) reported similar findings, in which lower odor, flavor and taste scores in HVCAP treated PWS were obtained, even though 0.5% CLE was applied. For HVCAP treated seabass slices, oxidation products formed decreased the acceptabily (Olatunde *et al.,* 2019d). HVCAP treated samples with PEF and CLE at high concentration (2%) possessed higher color likeness scores ($p>0.05$).

Table 12. Fatty acid profiles of Pacific white shrimp with prior PEF and CLE at various levels without and with HVCAP treatment under Ar + Air modified atmosphere packaging at day 0 and 18 of refrigerated storage

	Day 0						Day 18	
Fatty acids (g/100g)	Control	CP-Ar/Air-M	PEFT1-CLE1-Ar/Air-CP	PEFT2-CLE1-Ar/Air-CP	PEFT1-CLE2-Ar/Air-CP	PEFT2-CLE2-Ar/Air-CP	PEFT1-CLE2-Ar/Air-CP	PEFT2-CLE2-Ar/Air-CP
C14:0 Myristic	1.03±0.01a	0.56±0.02b	1.03±0.01a	1.04±0.02a	1.05±0.1a	1.06±0.01a	1.22±0.03b	1.35±0.01a
C15:0 Pentadecanoic	0.64±0.01a	0.38±0.01b	0.64±0.01a	0.63±0.01a	0.64±0.1a	0.64±0.01a	0.51±0.01a	0.53±0.02a
C16:0 Palmitic	21.28±0.01a	17.34±0.01b	21.28±0.01a	21.29±0.01a	21.32±0.1a	21.33±0.01a	17.28±0.02b	18.59±0.01a
C16:1 Palmitoleic	1.06±0.01a	0.71±0.01b	1.06±0.03a	1.07±0.01a	1.08±0.3a	1.09±0.01a	1.25±0.01b	1.35±0.01a
C17:0 Heptadecanoic	1.82±0.03a	1.23±0.01b	1.82±0.01a	1.83±0.01a	1.85±0.1a	1.88±0.01a	1.22±0.03b	1.40±0.01a
C17:1 cis-10-Hepatodecanoic	0.56±0.01a	0.35±0.03b	0.56±0.01a	0.56±0.01a	0.56±0.1a	0.57±0.03a	0.57±0.01a	0.56±0.02a
C18:0 Stearic	9.14±0.01a	5.96±0.01b	9.14±0.01a	9.14±0.01a	9.14±0.1a	9.16±0.01a	8..45±0.01b	8.52±0.01a
C18:1 Oleic	11.61±0.01a	7.30±0.02b	11.61±0.01a	11.61±0.01a	11.61±0.1a	11.64±0.01a	24.50±0.01b	25.64±0.01a
C18:2 Linoleic	12.44±0.02a	7.60±0.01b	12.44±0.01ab	12.44±0.01ab	12.44±0.1ab	13.04±0.01a	14.05±0.01b	15.32±0.02a
C20:0 Arachidic	1.04±0.01a	0.95±0.01b	1.04±0.01a	1.04±0.01a	1.04±0.1a	1.05±0.02a	0.84±0.01ab	0.91±0.01a
C21:0 Heneicosanoic	1.14±0.01a	0.97±0.01	1.22±0.02bc	1.35±0.01b	1.45±0.1a	1.50±0.01a	2.54±0.01b	2.86±0.03a
C20:2 Eicosadienoic acid	1.81±0.01bc	1.30±0.01c	1.81±0.02bc	1.85±0.01b	1.94±0.3a	2.01±0.01a	2.25±0.01b	2.45±0.01a
C20:0 Docosanoic	0.95±0.02b	0.51±0.01c	0.95±0.01b	0.98±0.01b	1.12±0.1a	1.15±0.02a	1.21±0.02b	1.33±0.01a
C20:3 Eicosatrienoic	0.64±0.01b	0.41±0.01c	0.65±0.02b	0.72±0.01b	0.86±0.1ab	0.92±0.01a	0.81±0.02b	0.91±0.03a
C20:4 Eicosatetraenoic	0.56±0.01a	0.36±0.01c	0.57±0.01bc	0.61±0.01b	0.65±0.1ab	0.74±0.02a	0.68±0.01b	0.73±0.01a
C23:0 Tricosanoic	3.31±0.01bc	2.15±0.02c	3.32±0.01bc	3.35±0.01b	3.37±0.1ab	3.58±0.01a	4.25±0.02ab	4.31±0.02a
C22:2 Docosadienoic	0.59±0.01b	0.39±0.01c	0.61±0.01b	0.62±0.01b	0.67±0.01ab	0.71±0.01a	0.62±0.01b	0.70±0.01a
C20:5 Eicosapentaenoic (EPA)	12.59±0.01bc	9.65±0.01b	12.62±0.01bc	12.68±0.01b	12.73±0.01ab	12.81±0.01a	7.95±0.01a	7.42±0.01b
C24:1 Nervonic	0.63±0.01ab	0.42±0.01b	0.65±0.01ab	0.67±0.01a	0.68±0.01a	0.73±0.1a	0.81±0.01ab	0.88±0.02a
C22:6 Docosahexaenoic (DHA)	18.22±0.01bc	10.24±0.01b	18.28±0.01bc	18.43±0.01b	18.54±0.01ab	18.63±0.1a	9.45±0.01b	9.64±0.01a
SFA	41.85	31.24	41.95	42.23	42.29	42.31	32.28	40.52
MUFA	13.85	8.78	13.88	13.90	13.92	14.43	27.13	28.40
PUFA	46.85	29.95	46.98	47.35	47.83	48.86	35.90	37.17

Values are presented as the mean ± standard deviation (n=3). Different lowercase letters within the same row under the same storage time indicated significant differences ($p<0.05$). Key: see the caption of Fig 31.

Table 13. Likeness score of Pacific white shrimp with prior PEF and CLE at various levels without and with HVCAP treatment under Ar + Air modified atmosphere packaging at day 0 and 18 of refrigerated storage.

Storage time (days)	Samples	Color	Texture	Odor	Flavor	Taste	Overall
0	Control	8.17±0.26a	8.11±0.32a	8.16±0.51a	8.22±0.28a	8.19±0.31a	8.28±0.26a
	CP-Ar /Air-M	8.27±0.36a	8.1±0.22a	6..52±0.43b	6.42±0.25b	6.45±0.27b	6.48±0.36b
	PEFT1- CLE1-Ar/Air-CP	8.33±0.35a	8.17±0.27a	8.21±0.54ab	8.28±0.45a	8.22±0.32a	8.31±0.36a
	PEFT2- CLE1-Ar/Air-CP	8.43±0.28a	8.33±0.46a	8.26±0.38a	8.33±0.51a	8.25±0.26a	8.34±0.54a
	PEFT1- CLE2-Ar/Air-CP	8.52±0.25aA	8.39±0.49aA	8.32±0.39aA	8.44±0.48aA	8.36±0.25aA	8.45±0.35aA
	PEFT2- CLE2-Ar/Air-CP	8.56±0.37aA	8.44±0.35aA	8.42±0.24aA	8.56±0.41aA	8.50±0.35aA	8.57±0.29aA
18	PEFT1- CLE2-Ar/Air-CP	6.06±0.34bB	6.14±0.55bB	6.11±0.39bB	6.05±0.35bB	6.04±0.46bB	6.12±0.0.35bB
	PEFT2- CLE2-Ar/Air-CP	7.11±0.13aB	7.06±0.52aB	7.16±0.45aB	7.18±0.23aB	7.19±0.32aB	7.22±0.43aB

Values are presented as the mean ± standard deviation (n=50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig. 31.

After 18 days, PEFT1-CLE2-Ar/Air-CP and PEFT2-CLE2-Ar/Air-CP samples had the marked decreases in likeness scores, while microbial load was still below the limit. Differences in likeness score of all attributes were noticeable in PEFT2-CLE2-Ar/Air-CP samples between day 0 and 18 ($p<0.05$). At the end of storge, sample treated with higher PEF pulses (800 pulses) and 2% CLE and subjected to HVCAP had higher likeness scores than that with lower PEF pulses (600 pulses) for all tested attributes ($p<0.05$). Higher likeness score of mackerel fillets was achieved when CP with exposure time of 60 s was used and TVC and TVB-N contents were reduced, compared to the untreated counterpart after storage for 14 days (Chen *et al*., 2019). Higher color likeness score in PEFT2-CLE2-Ar/Air-CP sample was related with the reduced melanosis (Fig. 31). With higher flavor and odor likeness score, PEFT2-CLE2-Ar/Air-CP also showed the lower microbial load and oxidation (Fig. 32, 33 and 34). CP treatment inactivated spoilage microorganisms in PWS more effectively than sodium metabisulphite in terms of retention of sensory quality (Zouelm *et al*., 2019). Therefore, PEF and CLE in conjunction with HVCAP using the mixture of Ar + Air (80:20) was able to maintain quality and shelf-life extension was achieved.

7.6 Conclusions

Prior PEF and CLE treatment in combination with HVCAP as non-thermal sanitizing technology could extend the shelf-life of PWS up to 18 days at 4 °C. Melanosis was negligible as a result of joint impact of PEF, CLE and HVCAP. The growth of mesophiles (TVC, Enterobacteriaceae, H_2S-producing bacteria, *Pseudomonas*, LAB) were drastically declined during 18 days. For safety assurance, *Clostridium perfringens* was not detected in all the samples. Oxidative changes in lipids and proteins were less pronounced, especially when high intensity PEF pulses and CLE (2%) were applied on shrimp before HVCAP treatment. PUFA were not decomposed in the treated shrimp with PEF pulse number (800 pulses) and CLE (2%), which exhibited strong antioxidative potential against HVCAP induced oxidation. PEFT2-CLE2-Ar/Air-CP sample ranked highest in likeness score. Thus, the application of non-thermal technology in association with natural extract in PWS, could act as a promising means for quality control and shelf-life extension of PWS at 4 °C.

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CHAPTER 8

QUALITY AND SHELF-LIFE EXTENSION OF PACIFIC WHITE SHRIMP PRETREATED WITH PULSED ELECTRIC FIELD IN CONJUNCTION WITH VACUUM IMPREGNATION OF CHAMUANG LEAF EXTRACT PRIOR TO MODIFIED ATMOSPHERE PACKAGING

8.1 Abstract

Vacuum impregnation assisted penetration of Chamuang leaf extract (CLE) (1 or 2%) was studied at vacuum pressure of 5 kPa with various vacuum times (VT; 7.5-15 min) and restoration times (15-22.5 min) towards pulsed electric field (PEF) treated pacific white shrimp (PWS) prior to modified atmosphere packaging (MAP) (Ar/Air; 80:20). Least melanosis scores were attained in samples pre-treated with PEF, VT-2 (15 min) and CLE (2%) (PEF-VI2-CLE2-MAP) than the control during 18 days at 4 °C ($p<0.05$). Lower microbial load and spoilage bacteria counts were attained in PEF-VI2-CLE2-MAP sample ($p<0.05$). PEF-VI2-CLE2-MAP sample showed lower lipid oxidation, pH, total volatile base (TVB) and protein carbonyl contents (PCC) than others ($p<0.05$). Higher likeness scores of all attributes were noted in PEF-VI2-CLE2-MAP sample. Oxidation of polyunsaturated fatty acids was alleviated by CLE. Thus, shelf-life of PWS could be extended by CLE with the combined processes.

8.2 Introduction

Pacific white shrimp (PWS) is a popular crustacean consumed globally because of its unique delicacy and appealing sensory property (Shiekh and Benjakul, 2020a). PWS were formerly captured from the Pacific ocean and have been farmed commercially in community based ponds to maintain the stock during supply chain management (Kimbuathong *et al*., 2020). Chemical and microbial qualities, especially color, are the important indices determining acceptability and safety of shrimp (Shiekh *et al*., 2020d). During improper storage condition, PWS quality deteriorates ascertained by the discoloration so called ‘melanosis’ (Sae-leaw and Benjakul, 2019a). Melanosis is a biochemical process mediated by polyphenol oxidase (PPO), causing the formation of black pigment, namely melanin (Shiekh and Benjakul, 2020b). PPO also named as

tyrosinase occurs naturally, especially during molting process and commonly exists as pro-PPO zymogens in PWS (Gonçalves and de Oliveira, 2016). Microbiological and chemical deterioration without any proper hurdles continues with the advancement in storage time (Shiekh *et al*., 2019). Moreover, the commercial practice of using chemical additive such as sodium metabisulphite (SMS) to retard microbial, chemical and enzymatic spoilage had been found unethical recently. At low temperature storage, SMS was incompetent for melanosis prevention (Sae-leaw and Benjakul, 2019b).

Pulsed electric field (PEF) is a promising food processing technology due to its additive free and eco-friendly in nature (Gulzar and Benjakul, 2020). Apart from the non-thermal behavior for nutrient quality retention, PEF has been implemented to inactivate pathogens (*Escherichia coli* and *Pseudomonas fluorescens*) and spoilage bacteria to ensure food safety (Walter *et al*., 2016). Microbial inactivation is based on the phenomenon of electroporation, which is achieved by the intermittent delivery of high voltage short pulses that causes permanent leakage of biological lipid bilayer of microbial cell membrane (Delso *et al*., 2020). Thus, the cytosol of microbial cell leaks out through cell membrane, causing cell death.

Natural additives have been brought into application to replace chemical counterparts for shelf-life extension of seafoods (Olatunde and Benjakul, 2018a). Chamuang (*Garcinia cowa* Roxb.) leaf (CLE) extract contains high amount of glycoside bonded polyphenols and organic acids that have been proven as highly effective antioxidants and antimicrobial agents for quality preservation of PWS (Shiekh *et al*., 2019). Moreover, the coupling of natural extracts with non-thermal technology has been regarded as an advanced practice for quality preservation of seafoods (Olatunde and Benjakul, 2018b). Combination of CLE and PEF have been applied recently to provide hurdle effect for quality preservation of PWS during refrigerated storage (4 °C) (Shiekh and Benjakul, 2020a). Additionally, PPO of shrimp cephalothoxes after PEF treatment without addition of inhibitor was inhibited up to 40% (Shiekh and Benjakul, 2020b). PEF electroporation can induce conformational changes of enzymes (Gulzar and Benjakul, 2019). Pores generated underneath the shrimp shell or cuticle induced by PEF could facilitate the passage of CLE polyphenols and organic acids (Shiekh and Benjakul, 2020a).

Vacuum impregnation (VI) is a promising non-thermal technique to improve food quality by enhancing the penetration of various bioactive compounds through porous tissues rapidly, under low pressure in a solid-liquid food system (Zhao *et al.,* 2019). VI involves the removal of gases or liquid inside pores of solid foods under vacuum condition and then replacing them with VI immersion solutions under restored atmospheric pressure (Yang *et al.,* 2017; Mao *et al.,* 2017). This technology has been proven as an effective means to replace the tradition immersion practices for food preservation. VI has been industrially coupled with antioxidant from natural extracts on fish and seafoods for prevention of chemical and microbial spoilage during storage (Zhao *et al*., 2019). Additionally, high intensity PEF pulses applied on sea bass skin has been reported to facilitate the migration of VI solutions more effectively via pores generated by electroporation (Chotphruethipong *et al.,* 2019). Thus, PEF could aid in the passage of CLE in PWS during VI process, followed by restoration.

Therefore, the application of VI to enhance the penetration of CLE into PWS with the aid of PEF, prior to MAP could mitigate chemical and microbial quality changes. This study aimed to investigate the combined effect of pre-treatments (PEF and CLE) followed by VI for quality maintenance and shelf-life extension of PWS during storage at 4 °C.

8.3 Materials and Methods

To investigate the combined effects of PEF and CLE pretreatments followed by vacuum impregnation for quality maintenance and shelf-life extension of PWS during storage at 4 °C.

8.4 Materials and Methods

8.4.1 Chemicals

All the microbial media and chemicals used in the present study were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) and Oxoid Ltd. (Hampshire and UK).

8.4.2 Collection and preparation of Pacific white shrimp (PWS)

Fresh PWS (55-60 shrimp per kg) were bought from a cultured farm situated in Songkhla, Thailand. PWS placed in crushed ice (ice to sample ratio, 2:1) in

polystyrene foam boxes were delivered to PSU-Seafood Laboratory within 1 h. Extraneous impurities were removed using cold water (≤ 4 °C) and the shrimp antennae were cut with a scissor. After washing properly, whole PWS were placed in ice as mentioned above until pre-treatment or packing was done.

8.4.3 Pre-treatment of PWS with PEF

PEF system (PEF LAB-400W, Febix International Inc., Chiang Mai, Thailand) was implemented on PWS (Shiekh and Benjakul, 2020a). PEF treatment chamber (1.5 L) made of stainless steel was fitted with a steel electrode (20 cm × 10 cm). Treatment cabinet consisted of aluminium frame covered with a plexiglass. A UT-P03 oscilloscope probe (600 MHz, 10X) was linked with a digital storage oscilloscope (DSO) and a PEF generator for data output. The high intensity pulses with the resulting specific energy of 697 kJ/kg and PEF (15 kV/cm, 800 pulses) were obtained (Shiekh and Benjakul, 2020a).

8.4.4 Preparation of CLE from Chamuang leaves

Mature leaves of Chamuang trees from a plantation in Hat Yai were collected. During washing, the extraneous matter or damaged leaves were removed. Washed leaves were dried in a hot air tray dryer (24 h) at 50 ℃ and blended in a high speed blender to obtain Chamuang leaf powder (CLP). Ground CLP (passed through 80 mesh sieve) was mixed with water for ultrasonic extraction to obtain Chamuang leaf extract (CLE) as detailed by Shiekh and Benjakul (2020c). The prepared CLE solution was transferred into zip-lock bags (200 ml) and placed in a freezer (-20 ℃) for overnight and transferred to -40 ℃ deep freezer (12 h) prior to freeze drying. The CLE powder was collected after freeze drying for 72 h.

8.4.5 Impact of vacuum impregnation on PEF pre-treated PWS immersed in CLE solutions followed by MAP

VI system consisted of a closed vessel (5 L capacity) connected with a vacuum pump (VE 125 N, Zhejiang Value Mechanical and Electrical Products Co., Ltd., Wenling, Zhejiang, China) (Fig 36). Prior to VI process, PEF treated PWS (30 shrimps) were immersed in CLE solutions (1 or 2% CLE) with a shrimp to solution ratio of 1:5 (w/v). The vacuum pressure of 5 kPa was implemented on PWS during VI

process (Zhao *et al*., 2019). VI process was conducted at various vacuum times (7.5 and 15 min) using a vacuum pressure of 5 kPa. Thereafter, the vacuum was released to restore an atmospheric pressure. The restoration times (RT) of 22.5 and 15 min were used, in which total operation time of 30 min was achieved. The samples were drained on the screen for 5 min at 4 °C.

PWS (7 whole raw shrimp) after PEF pre-treatment, followed by CLE and VI were packed in linear low density polyethylene (LLDPE) bag and filled with gas mixture (Ar + Air = 80:20) with a sample to gas ratio (3:1, w/v) using Henkovac type 1000 (Tecnovac, Italy) modified atmosphere packaging (MAP) machine, followed by heat sealing. The gases were of food-grade including sterilized Ar and zero air (containing < 0.1% hydrocarbon impurities). All the treatments are defined as follows:

1. Control (untreated, packed in normal air)
2. PEF-CLE1-MAP (PEF-800 pulses and 1% CLE, Ar/Air)
3. PEF-CLE2-MAP (PEF-800 pulses and 2% CLE, Ar/Air)
4. PEF-CLE1-VI1-MAP (PEF-800 pulses, VT-7.5 min or RT-22.5 min and 1% CLE,
5. Ar/Air)
6. PEF-CLE1-VI2-MAP (PEF-800 pulses, VT-15 min or RT-15 min and 1% CLE, Ar/Air)
7. PEF-CLE2-VI1-MAP (PEF-800 pulses, VT-7.5 min or RT-22.5 min and 2% CLE, Ar/Air)
8. PEF-CLE2-VI2-MAP (PEF-800 pulses, VT-15 min or RT-15 min and 2% CLE, Ar/Air)

Analyses of all the samples were done every 3 days up to 18 days of storage at 4 °C

8.4.6 Analyses

8.3.6.1 Melanosis assessment

The method of Nirmal and Benjakul (2010) was employed for visual inspection of dark spot formation (melanin) using ten trained panelists, who had experience in scoring of PWS. For each treatment, a bag containing 20 shrimp was fixed for melanosis score test and presented to panelists every 3 days up to 18 days of storage.

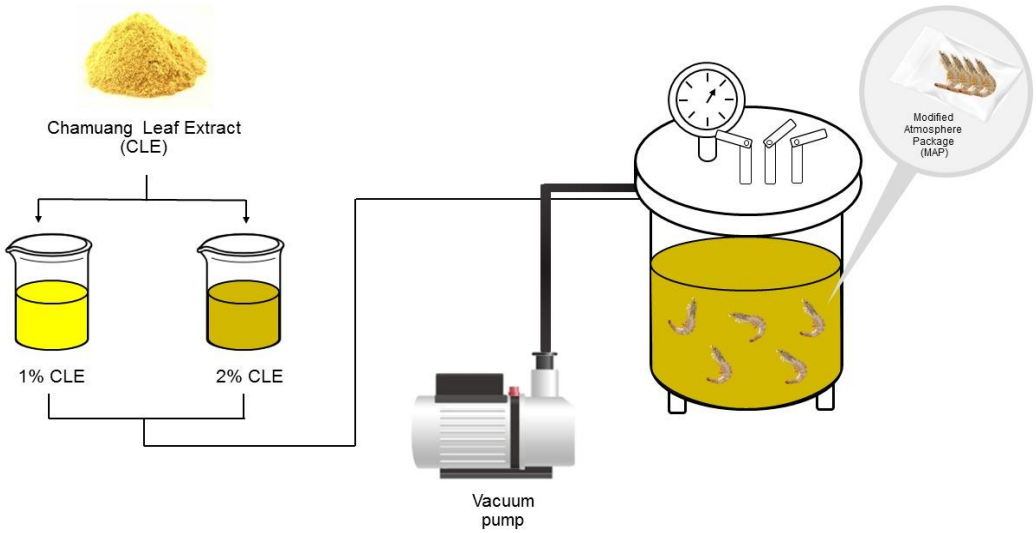

Figure 36. Schematic representation of vacuum impregnation process of CLE penetration in PWS

8.4.6.2 Microbiological analyses

Spread plate technique was adopted for the enumeration of total viable counts (TVC), psychrophilic bacterial counts (PBC), lactic acid bacteria (LAB), *Pseudomonas*, H_2S-producing bacteria, Enterobacteriaceae and *Clostridium perfringens* counts as described by Shiekh and Benjakul (2020d).

8.4.6.3 pH

PWS samples (2 g) were homogenized in deionized water (20 ml) and pH of homogenate was measured with a pH meter (Sartorious North America, Edgewood, NY, USA) (Nirmal and Benjakul, 2011).

8.4.6.4 Protein carbonyl contents (PCC) and total volatile base (TVB)

Natural actomyosin (NAM) from PWS muscle was extracted as described by Chantarasuwan *et al.* (2011). PCC of NAM was determined using 2,4-dinitro-phenyl hydrazine (DNPH) as detailed by Nikoo, Benjakul, and Xu (2015). Measurement of TVB content in PWS via Conway micro-diffusion method was performed (Shiekh and Benjakul, 2020c).

8.4.6.5 Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

PV of shrimp muscle was analyzed by ferric thiocyanate method (Arfat *et al*., 2015) using cumene hydroperoxide (0.5–2 ppm) as standard. TBARS of shrimp meat was measured (Shiekh and Benjakul, 2020c), in which malonaldehyde (MDA) (0–5 ppm) was used as standard.

8.4.6.6 Fatty acid profile

Fatty acid profiles were determined as fatty acid methyl esters (FAMEs) using gas chromatography with flame ionization detector (GC-FID) following the procedure of Raju and Benjakul (2020). Peaks were evaluated based on the retention time of standards and fatty acids in samples were quantified.

8.4.6.7 Sensory evaluation

The samples having TVC less than 6 log CFU/g were subjected to sensory analysis after being stored for 18 days at 4 °C in comparison with fresh shrimp (Nirmal and Benjakul, 2010). Samples were placed in aluminum tray and wrapped with foil. They were placed in steaming pot with perforated chamber, allowing steam to distribute uniformly. Samples were steamed for 5 min, to attain a maximum core temperature of 80 °C (Manheem *et al*., 2013).

8.4.7 Statistical analyses

Completely randomized design (CRD) was used for the whole studies. Randomized completely block design (RCB) was implemented for sensory analysis. Analysis of variance (ANOVA) was done and mean comparison was performed by Duncan's multiple range test or t-test at a significant level ($p<0.05$) by a SPSS statistical software (SPSS 17.0, SPSS Inc., Chicago, IL, USA). All the experiments were run in triplicates (n=3).

8.5 Results and discussion

8.5.1 Effect of PEF and CLE pre-treatments followed by VI on melanosis and quality changes of PWS during refrigerated storage

8.5.1.1 Melanosis formation

PEF and CLE pre-treatment of PWS were done followed by VI and pacakging under Ar + Air (80:20) atmosphere and the melanosis of PWS was monitored during 18 days of storage at 4 °C. Melanosis was not noticed in all the samples at day 0 (Fig 37a). During the storage, sample treated with 2% CLE with the aid of PEF (15 kV, 800 pulses) and high specific energy (697 kJ/kg), followed by VI process with vacuum time of 15 min and restoration time 15 min and packaged under Ar/Air-atmosphere (PEF-CLE2-VI2-MAP) exhibited the least melanosis ($p<0.05$). Melanosis scores were in the descending order: PEF-CLE2-VI1-MAP > PEF-CLE1-VI2-MAP > PEF-CLE1-VI1-MAP. Lower scores were obtained for the samples treated with 2% CLE and VI-2 ($p<0.05$). Different melanosis formation among aforementioned samples was governed by various levels of CLE and PEF pulses used. Ar and Air (80:20) atmosphere with low O_2 concentration was documented to decelerate melanosis, especially in conjunction with CLE treatment (Shiekh and Benjakul, 2020c). In addition to CLE treatment, high intensity PEF pulses (600 pulse number) could also inactivate PPO by 40% (Shiekh and Benjakul, 2020b). MAP containing active species of atomic nitrogen, atomic oxygen , nitrite oxide, nitrates and hydroxyl radicals were reported to retard melanosis in PWS (Charoux *et al*., 2020). Moreover, CO_2-MAP in combination with PEF (600 pulses) and CLE (1%) retarded melanosis, compared to the control (without any treatment) for a period of 10 days (Shiekh and Benjakul, 2020a).

Lowered melanosis in PEF-CLE2-VI2-MAP sample indicated the good keeping quality of shrimp up to 18 days (Fig. 37a). Additionally, a vacuum pressure (5kPa) and vacuum time (15 min) impregnated fish gelatin (FG=3%) and grape seed extract (GSE=0.9%) retained the visual quality of tilapia fillets up to 12 days when stored at 4 ºC (Zhao *et al*., 2019).

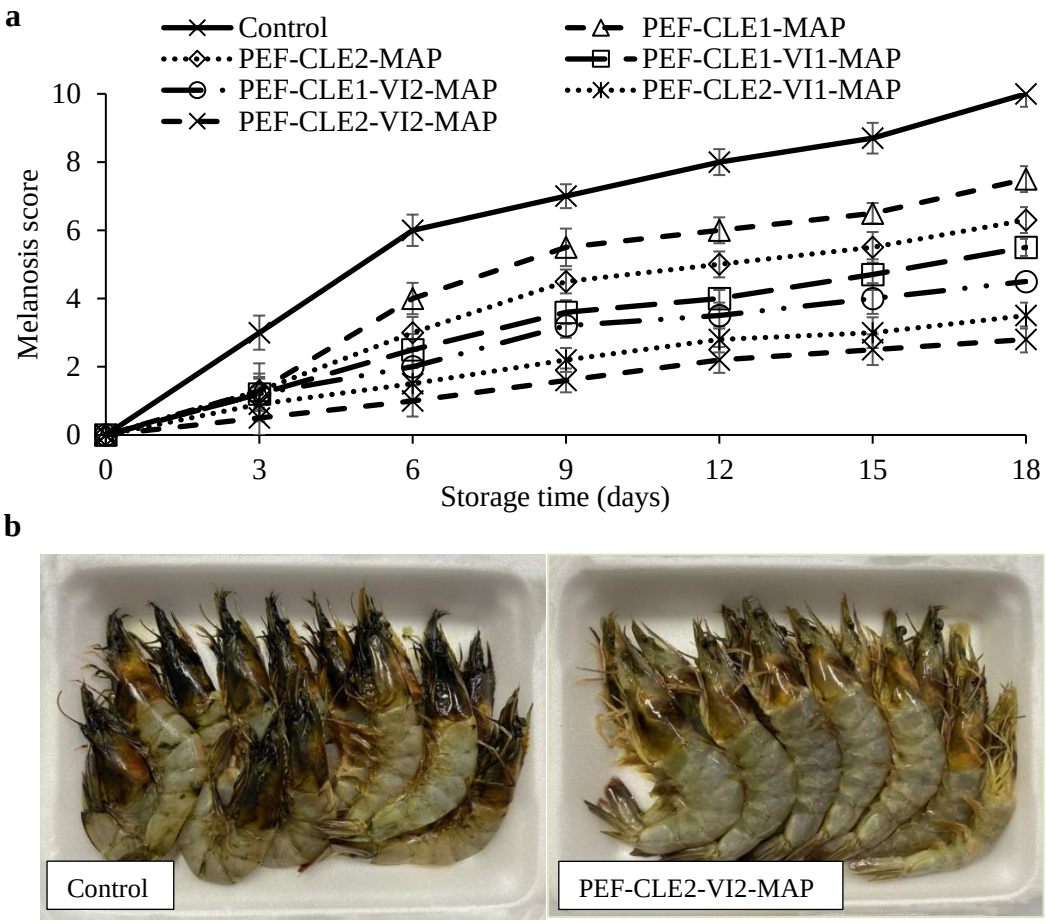

Figure 37. Melanosis score of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C (a) and photographs of selected samples after 18 days of storage (b).

Bars represent the standard deviation (n=10). Control: without any treatment; PEF-CLE1-MAP: Treated with 1% CLE and PEF-800 pulses, packed under Ar + Air (80:20); PEF-CLE2-MAP: Treated with 2% CLE, PEF-800 pulses, packed under Ar + Air (80:20); PEF-CLE1-VI1-MAP: Treated with 1% CLE and PEF-800 pulses, vacuum time (VT-7.5 min) and restoration time (RT-22.5 min), packed under Ar + Air (80:20); PEF-CLE1-VI2-MAP: Treated with 1% CLE and PEF-800 pulses, VT-15 min and RT-15 min, packed under Ar + Air (80:20); PEF-CLE2-VI1-MAP: Treated with 2% CLE and PEF-800 pulses, VT-7.5 min and RT-22.5 min, packed under Ar + Air (80:20); PEF-CLE2-VI2-MAP: Treated with 2% CLE and PEF-800 pulses, VT-15 min and RT-15 min, packed under Ar + Air (80:20).

Chitosan nanoparticle (0.5%) was used to treat peeled shrimp using vacuum impregnation process for quality preservation (Chouljenko *et al.*, 2017). Appearance of PWS control (untreated) and PEF-CLE2-VI2-MAP sample stored for 18 days of storage (4 ºC) is illustrated in Fig. 37b. The control sample had the pronounced melanosis, whereas PEF-CLE2-VI2-MAP sample possessed a few black spots, reconfirming the efficacy of combined treatment to conquer melanosis in PWS.

8.5.1.2 Changes in microbiological quality

At day 0, TVC of all treated samples was lower than that of control ($p<0.05$). Combined impact of PEF with higher pulses and 2% CLE treatment in PWS, followed by VI, was more effective in lowering the microbial growth, compared to others. TVC of control and other treated samples increased gradually during the entire storage as given in Fig. 38a. At day 18, TVC counts of the control, PEF-CLE1-MAP, PEF-CLE2-MAP, PEF-CLE1-VI1-MAP, PEF-CLE1-VI2-MAP, PEF-CLE2-VI1-MAP and PEF-CLE2-VI2-MAP samples were 8.90, 6.80, 6.35, 6.57, 5.30, 6.10, 5.85 log CFU/g, respectively. At the end of storage (18 days), PEF-CLE2-VI2-MAP sample ranked the lowest in TVC ($p<0.05$). Combined effect of PEF, CLE and VI retarded the bacterial proliferation in PWS effectively. Additionally, PEF was reported previously to inactivate mesophilic bacteria in PWS due to cell wall disintegration caused by PEF electroporation (Shiekh and Benjakul, 2020b). Aerobic plate count of chitosan nanoparticles (0.5%) migrated in peeled shrimp using vacuum impregnation process was < 4 log CFU/g at the end of storage (24 days) (Chouljenko *et al*., 2017).

Control ranked highest in PBC at day 0 ($p<0.05$). VI treated samples with prior PEF and CLE at different levels exhibited lower PBC ($p<0.05$) as shown in Fig. 38b. At the first day, PBC in PEF-CLE2-VI2-MAP samples was moderately lower than the control and other samples ($p<0.05$). PBC in PEF-CLE2-VI2-MAP became lower as the storage proceeded ($p<0.05$). Generally, the retarded growth of PBC in PEF-CLE2-VI2-MAP sample throughout storage could be associated with the enhanced CLE passage facilitated by VI to the muscle through the pores generated by PEF, especially when PEF at 800 pulses was applied, thereby lowering bacterial growth than others. Besides VI, PEF (15 kV, 600 pulses, 483 kJ/kg) and CLE (1%) were documented to be efficient in inhibiting microbes in PWS (Shiekh and Benjakul, 2020a). Additionally, PBC were reduced by 1 log CFU/g PBC in fish gelatin and grape seed extract treated tilapia fillets, followed by VI process for 15 min at the end of storage (12 days) (Zhao *et al*., 2019).

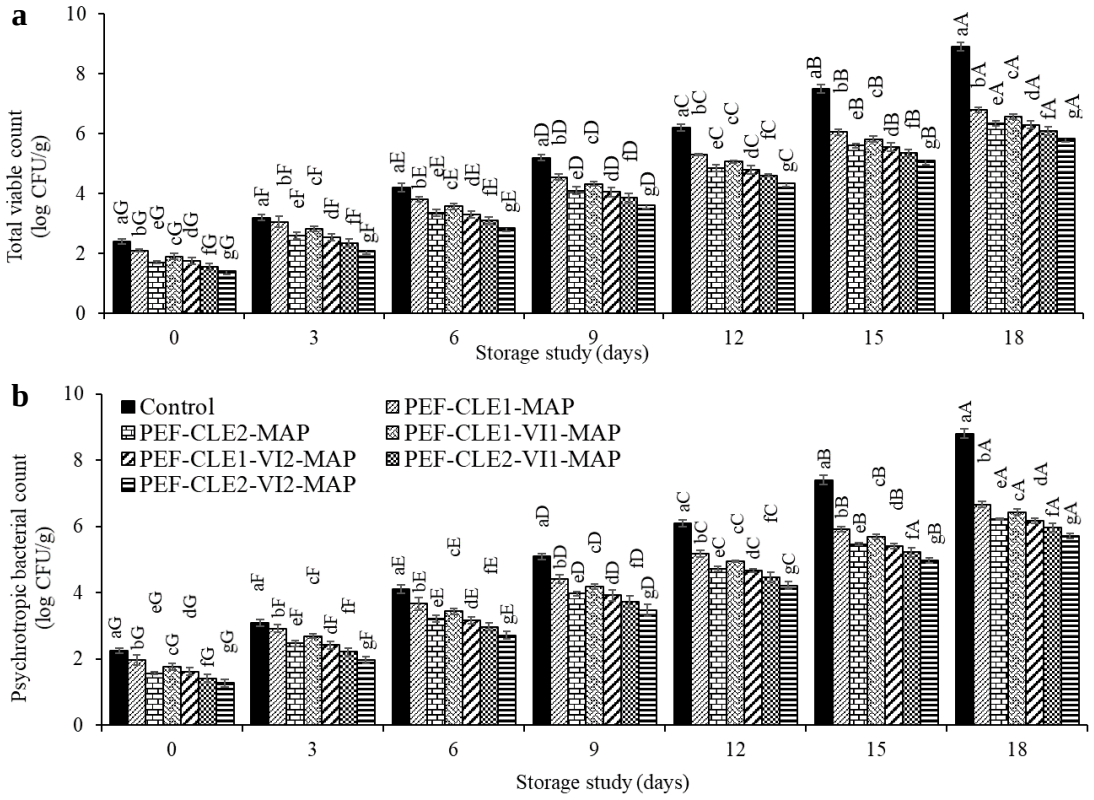

Figure 38. Total viable count (a) and psychrophilic bacterial count (b) of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Key: see the caption for Fig. 37.

Pseudomonas of all the samples ranged between 1.13 and 2.20 log CFU/g) at day 0 as depicted in Fig. 39a. Control sample had the highest *Pseudomonas* than others ($p<0.05$). Conversely, the lowest count was attained in PEF-CLE2-VI2-MAP sample ($p<0.05$). Increment in *Pseudomonas* count was noticed during storage of 18 days ($p<0.05$). At day 18, control sample ranked highest in count (8.10 log CFU/g), whereas the count of PEF-CLE2-VTI-MAP sample was still under acceptable limits (5.55 log CFU/g) ($p<0.05$). VI treated samples immersed in 2% CLE might injure cell membrane during negative pressure generated by vacuum and facilitated the CLE passage for effective inactivation of *Pseudomonas*. *Pseudomonas* were drastically reduced in coconut husk extract treated sea bass slices (Olatunde *et al*., 2019a). Seafoods are prone to spoilage by *Pseudomonas* known as gram negative spoilage

bacteria (DeWitt and Oliveira, 2016). Absolute CO_2-MAP along with PEF and 1% CLE inactivated *Pseudomonas* in PWS (Shiekh *et al*., 2020d).

The initial H_2S producing bacterial count ranged from 1.04 to 2.42 log CFU/g for all the samples, and the control had the highest count ($p<0.05$) (Fig. 39b). At day 18, PEF-CLE2-VI2-MAP samples possessed the lowest H_2S-producing bacterial count (5.47 log CFU/g). PEF and CLE under absolute CO_2 MAP lowered H_2S-producing bacteria notably (Shiekh and Benjakul, 2020d). Olatunde *et al*. (2019b) documented that gram positive and gram negative microbial flora were reduced during non-thermal cold plasma processing of Asian sea bass slices to ensure food safety. Moreover, PWS in the presence of 1% CLE without PEF treatment, inactivated H_2S-producing bacteria markedly when stored at 4 ºC (Shiekh and Benjakul, 2020c).

At the first day, control was highest in Enterobacteriaceae count ($p<0.05$) (Fig. 39c). Increases in Enterobacteriaceae were noticeable in all the samples as storage time augmented ($p<0.05$). After 18 days, the lowest count was obtained in PEF-CLE2-VI2-MAP sample ($p<0.05$). PEF with higher pulses along with 2% CLE, followed by VI could also inactivate Enterobacteriaceae. This could be presumed due to effective penetration of CLE at 2% level through PEF pores or gapping generated the underneath the shell for effective migration of CLE to PWS meat. Enterobacteriaceae counts drastically reduced in PWS treated with 1% CLE along with cold plasma, compared to the control during 15 days of refrigerated storage (Shiekh and Benjakul, 2020c). Enterobacteriaceae are gram negative facultative anaerobes highly susceptible to the damages by polyphenols than gram positive bacteria (McMillin, 2008). Therefore, PEF and 2% CLE treated PWS, followed by VI before Ar-Air MAP were low in Enterobacteriaceae during 18 days of storage (4 °C)

Initially, the control showed the higher LAB count than others ($p<0.05$). LAB count was relatively lower in PEF-CLE2-VI2-MAP ($p<0.05$) (Fig. 39d), plausibly due to joint effect of PEF electroporation of the bacterial cells followed by VI that facilitated the passage of CLE antimicrobial agents towards LAB. After 3 days of storage, LAB count of the control kept increasing, compared to treated samples, in which the rate of increase was lower ($p<0.05$). This could be governed by the absence of CLE polyphenols with antimicrobial properties in the control.

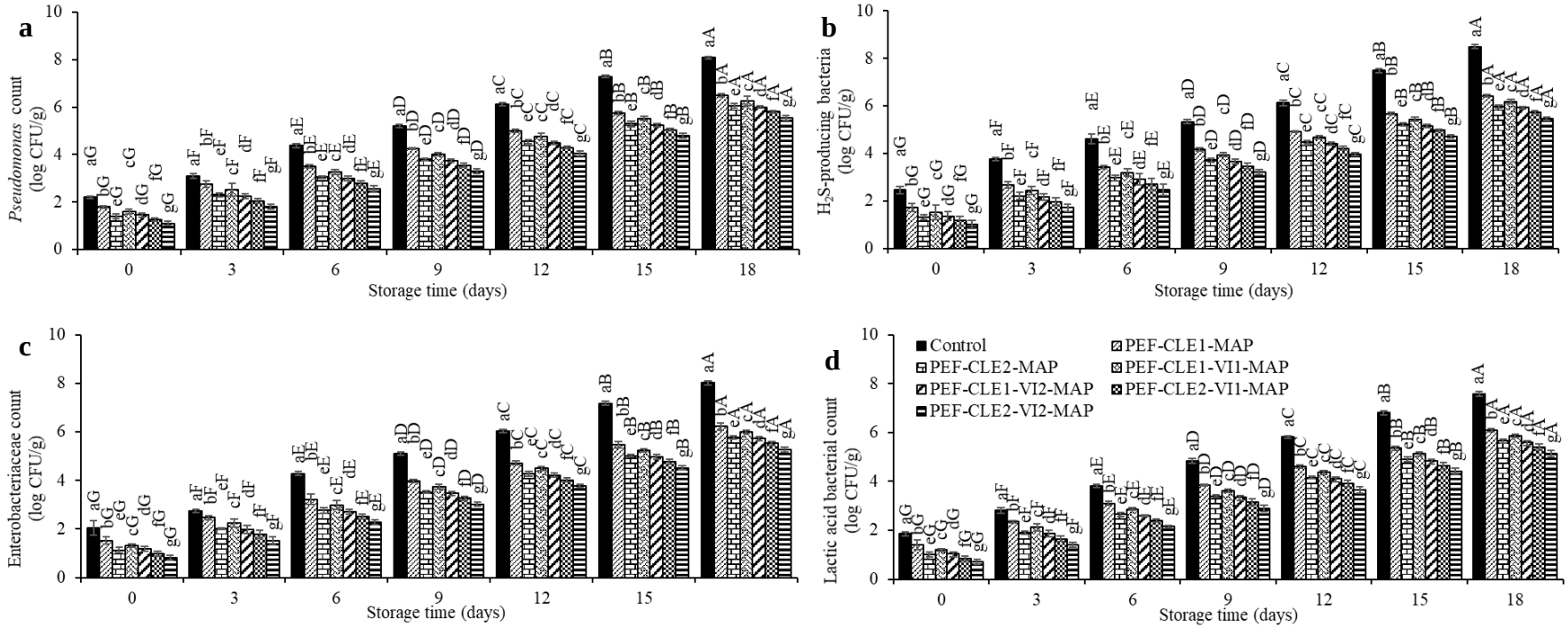

Figure 39. pH (a), total volatile base (TVB) (b), carbonyl content (c), peroxide values (d) and thiobarbituric reactive substances (TBARS) values (e) of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key caption Fig 37.

LAB were inactivated by combination of PEF and CLE 1% under CO_2-MAP effectively (Shiekh and Benjakul, 2020d). Additionally, LAB were reported as facultative anaerobes having sensitivity to phenolic compounds (Shiekh and Benjakul, 2020c). Sanitizing effect of PEF along with CLE at 2%, followed by VI processing was pronounced, thus lowering the growth of LAB. *Clostridium perfringens* colonies were not found in all the samples throughout storage. This could be attributed to hygienic practice of sample preparation without any cross-contamination.

8.5.1.3 Changes in chemical quality

The similar pHs of control and treated samples were observed at day 0 ($p>0.05$), except for PEF-CLE2-VI1-MAP and PEF-CLE2-VI2-MAP sample, which had the lower pHs ($p<0.05$) (Fig. 40a). The pHs of 6.25-6.68 were found within the first 3 days. As storage time augmented, pH of the control increased more rapidly than others ($p<0.05$), while the changes in pH values of PEF-CLE2-VI2-MAP sample were negligible during 0-18 days ($p<0.05$). At the end of storage, the pHs of control and other samples were in the range of 7.11-11.52. The accumulation of basic compounds in the control sample (without any treatment) could be due to higher enzyme activity triggered by microbes, contributing to the raised pH. Microbial enzymes in PWS might have been inactivated during treatment with high energy PEF pulses. PEF can inactivate enzymes by inducing their denaturation (Shiekh and Benjakul, 2020b). Similar results were documented in PEF and CLE treated PWS in CO_2-MAP stored for 10 days (Shiekh and Benjakul, 2020d). Lowered increment of pH of PEF-CLE2-VI2-MAP sample was possibly caused by the prevention of microbial growth and enzyme inactivation as described previously.

TVB contents of the control and treated samples (2.10 to 6.51 mg N/100 g) were noted at day 0 (Fig. 40b). During the first 3 days, TVB content of the control was higher than PEF-CLE2-VI2-MAP sample ($p<0.05$). All the samples showed gradual increases in TVB content of PWS stored for 18 days ($p<0.05$). The drastic increases in TVB content were observed in the control during 6 and 18 days ($p<0.05$). PEF-CLE2-VI2-MAP possessed the lowest TVB content (16.24 mg N/100 g meat) at day 18. This was more likely the result of the combined effect between PEF, CLE and VI. TVB content (12-20 mg N/100 g) in PWS indicates the acceptable limits for safe consumption (Okpala *et al*., 2014). Shiekh and Benjakul (2020d) reported similar range

of TVB content in CLE treated PWS without prior PEF under CO_2-MAP during 10 days of storage. Cashew leaf extract, had the pronounced antimicrobial and antioxidant effects to retard TVB content of refrigerated shrimp (Sae-leaw and Benjakul, 2019a). Therefore, VI aided in maintaining freshness of shrimp via removal of residual gases through the PEF pore spaces of PWS shell and facilitated influx of high dose of CLE to prevent protein degradation caused by microorganisms.

At day 0, control sample had the highest PCC ($p<0.05$) (Fig. 40c). PEF-CLE2-VI2-MAP sample showed the lower PCC during 0-3 days than the control and others ($p<0.05$). With increasing storage time, PCC of the samples increased, reflecting the induction of protein oxidation due to insufficient concentration of CLE in some samples. It was reported that PEF did not impact oxidation of PWS proteins (Shiekh and Benjakul, 2020b). Additionally, the lowest PCC of PEF-CLE2-VI2-MAP sample was attained throughout storage ($p<0.05$). The effect of 2% CLE towards protein oxidation was evident in vacuum impregnated sample. Decreased PCC in PEF-CLE2-VI2-MAP coincided with the lower TVB content and microbial load. PCC in the swimming crab and PWS kept under low O_2 and absolute CO_2 atmosphere along with PEF and 1% CLE were remarkably less than the control sample (Shiekh and Benjakul, 2020d; Sun *et al*., 2017). Therefore, higher PEF pulses in the presence of sufficient CLE dose prevented protein oxidation associated with the enhanced penetration of 2% CLE via PEF pores under VI process. PCC were lowered in tilapia fillets coated with fish gelatin and grape seed extract during VI process (Zhao *et al*., 2019). Therefore, the use of antioxidants is an effective strategy to control the formation of carbonyl groups during protein oxidation (Zhang *et al*., 2018). Moreover, natural and chemical based preservatives such as quercetin, cinnamic acid, and 4-hexylresorcinol in combination with MAP (80% CO_2; 10% O_2; 10% N_2) retarded protein oxidation in refrigerated shrimp (Qian *et al*., 2015).

PV was higher in control than treated samples during the whole storage ($p<0.05$). At day 0, PEF-CLE2-VI2-MAP sample attained lowest PV ($p<0.05$) (Fig. 40d). VI towards PWS with PEF and 2% CLE treatment lowered the formation of hydroperoxide ascertained by the lower PV ($p<0.05$). Antioxidants from CLE were documented to penetrate through the holes or gaps generated in PWS meat driven by PEF electroporation (Shiekh and Benjakul, 2020a). After 18 days, higher PV was

obtained in the control ($p<0.05$) than samples treated with 2% CLE. On the other hand, PEF-CLE2-VI2-MAP sample showed the lowest PV ($p<0.05$). Lower oxygen levels and effective passage of CLE through shells with the aid of prolonged vacuum time possibly loaded higher amount of CLE through the PEF pores, thereby lowering lipid oxidation in this sample. Combined inhibitory effect of PEF and CLE towards microbial and indigenous enzymes including lipase was also postulated. Seafoods are rich in polyunsaturated fatty acids and prone to oxidation (Albertos *et al*., 2017). CLE (1%) treatment prevented lipid oxidation of PWS, when PEF was implemented at 600 pulses and packaged under absolute CO_2-MAP (Shiekh *et al*., 2020d). Therefore, PEF and CLE followed by VI process effectively prevented lipid oxidation in PWS during storage.

TBARS values of PEF and CLE (2%) treated PWS samples, followed by VI was relatively lower during storage of 18 days ($p<0.05$) (Fig. 40e). Highest TBARS values were noticed in the control (untreated) up to 18 days. PEF-CLE2-VI2-MAP sample had the lower TBARS value than the control and treated samples without VI at day 18 ($p<0.05$). After 6 days of storage, the control had highest TBARS, likely correlated with the production of microbial lipase, which accelerated hydrolysis with subsequent oxidation. Nevertheless, PEF (800 pulses) treated PWS in conjunction with CLE (2%) alleviated lipid oxidation to some level, when VI process was applied during 18 days of storage. After 18 days, PEF-CLE2-VI2-MAP sample had low amount of TBARS. Low TBARS levels were found in stored PWS added with antioxidants (Zouelm *et al*., 2019). PEF and CLE (1%) treated PWS under CO_2-MAP had the lowest TBARS throughout refrigerated storage (Shiekh *et al*., 2020d). Therefore, prior PEF treated PWS immersed in CLE (2%) solution along with vacuum impregnation prevented lipid oxidation during the entire storage.

8.5.2 Changes in fatty acid profile

Fatty acids of all the treated samples and the control are presented in Table 14. At the first day, control and treated samples (PEF-800 pulses and 1 or 2% CLE) with subsequent VI process showed similar fatty acid contents ($p>0.05$).

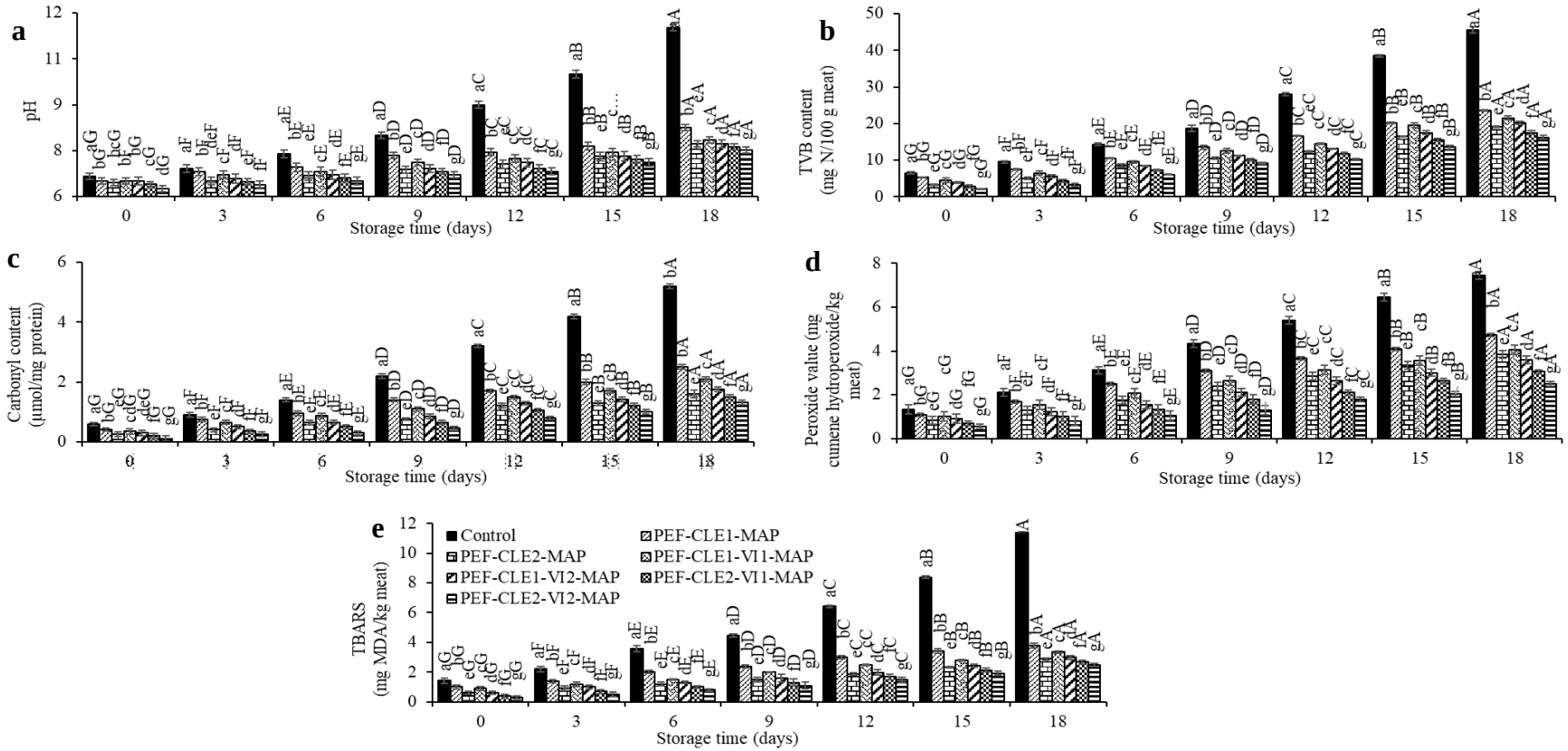

Figure 40. pH (a), total volatile base (TVB) (b), carbonyl content (c), peroxide values (d) and thiobarbituric reactive substances (TBARS) values (e) of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Key caption Fig 37.

Table 14. Fatty acid profiles of Pacific white shrimp with prior PEF and CLE at various levels without and with vacuum impregnation process under Ar and Air modified atmosphere packaging at day 0 and 18 of refrigerated storage (4 °C).

	Day 0					Day 18	
	Control	PEF-CLE1-VI1-MAP	PEF-CLE1-VI2-MAP	PEF-CLE2-VI1-MAP	PEF-CLE2-VI2-MAP	PEF-CLE2-VI1-MAP	PEF-CLE2-VI2-MAP
C14:0 Myristic	1.12±0.01a	1.13±0.03a	1.14±0.01a	1.15±0.02a	1.17±0.01a	1.25±0.02b	1.38±0.01a
C15:0 Pentadecanoic	0.61±0.02a	0.63±0.04a	0.65±0.03a	0.65±0.04a	0.65±0.02a	0.55±0.01a	0.57±0.02a
C16:0 Palmitic	18.24±0.01a	18.26±0.01a	18.28±0.02a	18.31±0.01a	18.33±0.03a	18.21±0.02b	19.14±0.01a
C16:1 Palmitoleic	1.06±0.03a	1.06±0.01a	1.07±0.01a	1.08±0.02a	1.13±0.01a	1.26±0.03b	1.38±0.01a
C17:0 Heptadecanoic	1.75±0.01b	1.76±0.02b	1.78±0.02b	1.81±0.04ab	1.83±0.05a	1.22±0.04b	1.40±0.02a
C17:1 cis-10-Hepatodecanoic	0.55±0.01a	0.56±0.07a	0.56±0.04a	0.56±0.05a	0.57± 0.01a	0.52±0.01	0.54±0.04
C18:0 Stearic	9.12±0.03a	9.14±0.02a	9.14±0.02a	9.14±0.03a	9.16±0.02a	8.52±0.02	8.61±0.01
C18:1 Oleic	11.85±0.4c	11.88±0.01bc	11.89±0.04bc	11.92±0.01b	12.10±0.01a	25.21±0.01ab	25.74±0.01a
C18:2 Linoleic	12.15±0.2b	12.17±0.03b	12.18±0.03b	12.19±0.02b	12.31±0.02a	13.25±0.01b	14.48±0.02a
C20:0 Arachidic	1.06±0.01a	1.07±0.04a	1.07±0.05a	1.08±0.03a	1.09±0.01a	0.86±0.03b	0.93±0.04a
C21:0 Heneicosanoic	1.14±0.01b	1.18±0.01ab	1.20±0.07ab	1.20±0.01ab	1.25±0.02a	2.54±0.02b	2.68±0.05a
C20:2 Eicosadienoic acid	1.92±0.03b	1.94±0.03b	1.95±0.01b	1.98±0.01b	2.15±0.03a	2.26±0.01b	2.43±0.02a
C20:0 Docosanoic	0.95±0.04b	0.95±0.02b	0.98±0.02b	1.12±0.02a	1.15±0.04a	1.21±0.02b	1.33±0.03a
C20:3 Eicosatrienoic	0.76±0.01c	0.84±0.04b	0.85±0.01b	0.86±0.03b	0.92±0.01a	0.85±0.01ab	0.91±0.02a
C20:4 Eicosatetraenoic	0.58±0.03b	0.59±0.05b	0.61±0.01b	0.64±0.05b	0.74±0.01a	0.78±0.01ab	0.82±0.01a
C23:0 Tricosanoic	3.42±0.05a	3.44±0.01a	3.45±0.03a	3.45±0.02a	3.46±0.03a	4.24±0.03b	4.33±0.01a
C22:2 Docosadienoic	0.61±0.04b	0.63±0.02ab	0.64±0.01ab	0.68±0.01ab	0.71±0.02a	0.67±0.05ab	0.70±0.01a
C20:5 Eicosapentaenoic (EPA)	13.12±0.03b	13.14±0.01b	13.15±0.02b	13.17±0.04b	13.30±0.01a	7.85±0.02b	8.35±0.03a
C24:1 Nervonic	0.63±0.02b	0.65±0.01ab	0.67±0.05ab	0.67±0.02ab	0.71±0.01a	0.82±0.01ab	0.86±0.02a
C22:6 Docosahexaenoic (DHA)	19.25±0.01b	19.26±0.03b	19.28±0.01b	19.31±0.01b	19.41±0.01a	9.45±0.06b	11.24±0.01a
SFA	39.14	39.39	39.51	39.63	39.66	41.43	41.45
MUFA	14.09	14.15	14.19	14.23	14.51	27.81	28.52
PUFA	48.39	48.57	48.66	48.83	49.54	35.11	38.93

Values are presented as the mean ± standard deviation (n=3). Different lowercase letters within the same row under the same storage time indicated significant differences ($p<0.05$). Key: see the caption of Fig 37.

PEF-CLE2-VI2-MAP sample which had higher fatty acid content, especially MUFA and PUFA, compared to others at the first day ($p<0.05$). At day 0, PEF-CLE2-VI2-MAP sample had higher DHA, EPA, oleic, linoleic and eicosadienoic acids, compared to other samples. CLE (1%) was reported to lower fatty acid oxidation of PWS under Ar and Air (80:20) atmosphere during 15 days of storage. Thus it was presumed that PEF treatment had no negative impact on fatty acids.

After 18 days, EPA and DHA contents of PEF-CLE2-VI1-MAP and PEF-CLE2-VI2-MAP samples were depleted, while contents of oleic acid, linoleic acid and myristic acid were augmented. The lowered contents of PUFA could be the result of PUFA oxidation with coincidental increased proportion of MUFA. Despite of the decreases in fatty acids during prolonged storage (18 days), MUFA and PUFA contents were still higher in PEF-CLE2-VI2-MAP sample than those of PEF-CLE2-VI1-MAP sample. Lipid oxidation products (aldehydes or ketones) affect sensory attributes of foods and consumer health (Pan *et al*., 2019). However, this negative impact of oxidation could be mitigated by the combined effect of PEF and vacuum impregnation of 2% CLE rich in antioxidative polyphenols to lower lipid oxidation.

8.5.3 Changes in sensory quality

Likeness scores of all the selected samples are presented in Table 15. At the first day, all the samples with or without treatment had the similar sensory scores ($p>0.05$). High likeness scores at the first day could be attributed to the lower TVB contents, reflecting the freshness and negligible spoilage of all the samples. Shiekh *et al*. (2020d) reported similar findings, in which lower odor, flavor and taste likeness scores in PEF (600 pulses) and CLE (1%) treated PWS packaged under CO_2-MAP were found as the storage time increased. After 18 days, PEF-CLE2-VI1-MAP and PEF-CLE2-VI2-MAP samples had the marked decreases in likeness scores, in which microbial counts were still less than 6 log CFU/g. Differences in the likeness of sample attributes were noticeable in PEF-CLE2-VI2-MAP samples between day 0 and 18 ($p<0.05$). At the end of storge, sample treated with higher PEF pulses (800 pulses) and 2% CLE and subjected to vacuum impregnation had higher likeness scores than those with lower vacuum time (7.5 min) for all tested attributes ($p<0.05$).

Table 15. Likeness score of Pacific white shrimp with prior PEF and CLE treatments subjected to vacuum impregnation and packaged in modified atmosphere (Ar + Air = 80:20) at day 0 and 18 of refrigerated storage.

Storage time (days)	Samples	Color	Texture	Odor	Flavor	Taste	Overall
0	Control	8.11±0.76a	8.06±0.73a	8.05±0.52a	8.06±0.42a	8.03±0.70a	8.14±0.33a
	PEF-CLE1-VI1-MAP	8.17±0.71a	8.11±0.76a	8.16±0.50a	8.14±0.41a	8.06±0.42a	8.22±0.43a
	PEF-CLE1-VI2-MAP	8.22±0.43a	8.17±0.71a	8.21±0.42a	8.17±0.51a	8.08±0.49a	8.28±0.57a
	PEF-CLE2-VI1-MAP	8.33±0.49aA	8.28±0.46aA	8.26±0.45aA	8.22±0.43aA	8.14±0.41aA	8.33±0.49aA
	PEF-CLE2-VI2-MAP	8.39±0.50aA	8.33±0.59aA	8.37±0.50aA	8.33±0.49aA	8.22±0.65aA	8.36±0.54aA
18	PEF-CLE2-VI1-MAP	6.22±0.43bB	6.11±0.47bB	6.05±0.23bB	6.06±0.54bB	6.03±0.12bB	6.17±0.51bB
	PEF-CLE2-VI2-MAP	7.19±0.62aB	7.06±0.64aB	7.05±0.52aB	7.08±0.26aB	7.06±0.42aB	7.22±0.55aB

Values are represented as the mean ± standard deviation (n=50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig. 37.

Moreover, the extended vacuum time (15 min) might allow higher quantity of CLE to penetrate through the pores generated by PEF, when 800 pulses were implemented. Higher color likeness score in PEF-CLE2-VI2-MAP sample was related with the reduced melanosis (Fig. 37). With higher flavor and odor likeness score, PEF-CLE2-VI2-MAP also showed the lower microbial load and oxidation (Fig. 38, 39 and 40). Similar findings were documented in 1% CLE treated PWS along with cold plasma treatment under Ar + Air MAP during 15 days of storage (Shiekh and Benjakul, 2020c). Therefore, PEF and CLE in conjunction with VI process using the mixture of Ar and Air (80:20) MAP was able to maintain quality and prolonged s
life of PWS with combined treatments.

8.6 Conclusions

Prior PEF and vacuum impregnation of CLE and stored under Ar + Air MAP could extend the shelf-life of PWS up to 18 days at 4 °C. Melanosis was negligible as a result of joint impact of PEF, vacuum impregnation, CLE treatment and low O_2 proportion in MAP. The growth of mesophiles (TVC, Enterobacteriaceae, H_2S-producing bacteria, *Pseudomonas*, LAB) were under acceptable limits during 18 days. For safety assurance, *Clostridium perfringens* was not detected in all the samples. Oxidative changes in lipids and proteins were less pronounced, especially when high intensity PEF pulses and CLE (2%) and vacuum impregnation time (15 min) were applied on shrimp before MAP. PUFA were not decomposed in the shrimp treated with 800 PEF pulse number and 2% CLE, which exhibited strong antioxidative potential against oxidation. PEF-CLE2-VI2-MAP sample ranked highest in likeness score. Thus, the application of non-thermal technologies (PEF and VI) in association with natural extract could act as a promising means for quality control and shelf-life extension of PWS at 4 °C.

8.7 References

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CHAPTER 9

COMBINED HURDLE EFFECTS OF VACUUM IMPREGNATION OF CHAMUANG LEAF EXTRACT ON QUALITY AND SHELF-LIFE OF PACIFIC WHITE SHRIMP SUBJECTED TO HIGH VOLTAGE COLD ATMOSPHERIC PLASMA

9.1 Abstract

Combined effects of Chamuang leaf extract (CLE) (1 or 2%) penetration at vacuum pressure of 5 kPa with a vacuum time (VT; 15 min) and restoration time (15 min) toward pulsed electric field (PEF) treated Pacific white shrimp (PWS) followed by high voltage cold atmosphere plasma (HVCAP) using Ar/Air (80:20) for 10 min. Least melanosis scores and microbial load were attained in sample 2% CLE with the aid of PEF and VI and exposed to HVCAP (PEF-VI-CLE2-HVCAP) than the control during 18 days at 4 °C ($p<0.05$). This sample also showed lower lipid oxidation, pH, total volatile base (TVB) and protein carbonyl contents (PCC) than others ($p<0.05$). Higher likeness scores of all attributes were noted in aforementioned sample. Oxidation of polyunsaturated fatty acids was alleviated by CLE. Thus, shelf-life of PWS could be extended by the combination of non-thermal processes along with CLE up to 18 days.

9.2 Introduction

Pacific white shrimp (PWS) is a popular crustacean consumed globally. It had been formerly captured from the Pacific ocean and is nowadays farmed commercially in community based ponds to maintain the stock for increasing supply chain (Kimbuathong *et al.,* 2020; Shiekh and Benjakul, 2020a). Chemical and microbial qualities, especially color, are the important indices determining acceptability and safety of shrimp (Shiekh *et al*., 2020d). Melanosis is a biochemical process mediated by polyphenol oxidase (PPO), causing the formation of black pigment, namely melanin (Sae-leaw and Benjakul, 2019a; Shiekh and Benjakul, 2020b). PPO also named as tyrosinase occurs naturally, especially during molting process and commonly exists as pro-PPO zymogens in PWS (Gonçalves and de Oliveira, 2016). Microbiological and chemical deteriorations without any proper hurdles continue with longer storage time (Shiekh *et al.,* 2019). Moreover, the commercial practice of using chemical additive

such as sodium metabisulphite (SMS) to retard post-harvest quality changes in PWS has been found unethical and incompetent for melanosis prevention during storage (Sae-leaw and Benjakul, 2019b).

Nonthermal processes have been employed for seafood quality maintenance and shelf-life extension. Pulsed electric field (PEF) is a promising food processing technology due to its additive free and eco-friendly nature. It can induce conformational changes of some enzymes, thus inactivating their activity (Gulzar and Benjakul, 2020). PPO of shrimp cephalothoxes after PEF treatment without addition of inhibitor was inhibited by 40% (Shiekh and Benjakul, 2020b). Apart from the non-thermal behavior for nutrient quality retention, PEF has been implemented to inactivate pathogens (*Escherichia coli* and *Pseudomonas fluorescens*) and spoilage bacteria (Walter *et al*., 2016). Microbial inactivation is based on the phenomenon of electroporation, which is achieved by the intermittent delivery of high voltage short pulses that causes permanent leakage of biological lipid bilayer of microbial cell membrane (Delso *et al*., 2020). Thus, the cytosol of microbial cell is leached through cell membrane, resulting in cell death.

Chamuang (*Garcinia cowa* Roxb.) leaf (CLE) extract contains high amount of glycoside bonded polyphenols and organic acids that have been proven as highly effective antioxidants and antimicrobial agents (Shiekh *et al.,* 2019). Application of non-thermal technologies such as PEF and high voltage cold atmospheric plasma for quality and shelf-life extension of PWS was achieved more potentially in the presence of CLE (Shiekh and Benjakul, 2020a,c). Moreover, the coupling of natural extracts with non-thermal technology has been regarded as an advanced practice for quality preservation of seafoods (Olatunde and Benjakul, 2018b). Additionally, PWS pretreated by PEF in combination with CLE and packaged in absolute concentration of CO_2 retained organoleptic quality for 10 days at 4 ºC (Shiekh *et al.,* 2020d). Pores generated underneath the shrimp shell or cuticle induced by PEF could facilitate the passage of CLE polyphenols and organic acids into shrimp (Shiekh and Benjakul, 2020a).

Vacuum impregnation (VI) is a promising technique to enhance the penetration of various bioactive compounds through porous tissues under low pressure in a solid-liquid food system (Zhao *et al.,* 2019). VI involves the removal of gases or

liquid inside pores of solid foods under vacuum condition and then replacing them with VI immersion solutions during restoration to atmospheric pressure (Yang *et al.,* 2017; Mao *et al.,* 2017). VI has been industrially coupled with antioxidant from natural extracts on fish and seafoods for prevention of chemical deterioration and microbial spoilage during storage (Zhao *et al.,* 2019). Additionally, high intensity PEF pulses applied on sea bass skin was reported to facilitate the migration of VI solutions more effectively via pores generated by electroporation (Chotphruethipong *et al.,* 2019). Thus, PEF could aid in the passage of CLE in PWS during VI process, followed by restoration.

High voltage cold atmospheric plasma (HVCAP) technology coupled with MAP has been implemented in foods as a non-thermal sanitizing and environment friendly technology. HVCAP in a dielectric barrier discharge (DBD) mode has been applied for decontamination to ensure food safety (Bai *et al.,* 2020). DBD generates in-bag HVCAP active species by applying an electromagnetic discharge between two electrodes, where one electrode is attached with dielectric layer (Albertos *et al.,* 2017). HVCAP reactive species generated via DBD comprise negative and positive ions, radiation energetic ions, electrons, reactive oxygen species (ROS), reactive nitrogen species (RNS) and ultraviolet photons (Coutinho *et al.,* 2018). HVCAP has been implemented along with CLE on PWS, in which shelf-life could be extended up to 15 days under refrigerated condition (Shiekh and Benjakul, 2020c).

Therefore, CLE treatment with the aid of PEF and VI prior to HVCAP of PWS could collectively mitigate chemical and microbial changes associated with quality losses. This study aimed to investigate the combined effect of pre-treatments of CLE with the aid of PEF and VI and subsequent HVCAP for quality maintenance and shelf-life extension of PWS during storage at 4 °C.

9.3 Objectives

To study the combined hurdle effects of pretreatments of CLE with the aid of PEF and vacuum impregnation along HVCAP on PWS.

To investigate the effects of combined hurdles on spoilage bacteria and oxidative stability of lipids and proteins of PWS during prolonged shelf-life at refrigerated storage conditions.

9.4 Materials and methods

9.4.1 Chemicals

All the microbial media and chemicals used were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) and Oxoid Ltd. (Hampshire and UK).

9.4.2 Collection and preparation of Pacific white shrimp (PWS)

Fresh PWS (55-60 shrimp per kg) were bought from a cultured farm situated in Songkhla, Thailand. PWS placed in crushed ice (ice to sample ratio of 2:1) in polystyrene foam boxes were delivered to PSU-Seafood Laboratory within 1 h. Extraneous impurities were removed using cold water (≤ 4 °C) and the shrimp antennae were cut with a scissor. After washing properly, whole PWS were placed in ice as mentioned above until pre-treatment or packing was carried out.

9.4.3 Pre-treatment of PWS with PEF

PEF system (PEF LAB-400W, Febix International Inc., Chiang Mai, Thailand) was implemented on PWS (Shiekh and Benjakul, 2020a). PEF treatment chamber (1.5 L) made of stainless steel was fitted with a steel electrode (20 cm × 10 cm). Treatment cabinet consisted of aluminium frame covered with a plexiglass. A UT-P03 oscilloscope probe (600 MHz, 10X) was linked with a digital storage oscilloscope (DSO) and a PEF generator for data output. The high intensity pulses with the resulting specific energy of 697 kJ/kg and PEF (15 kV/cm, 800 pulses) were obtained (Shiekh and Benjakul, 2020a).

9.4.4 Preparation of CLE from Chamuang leaves

Mature leaves of Chamuang trees from a plantation in Hat Yai were collected. During washing, the extraneous matters or damaged leaves were removed. Washed leaves were dried in a hot air tray dryer (24 h) at 50 °C and blended in a high speed blender to obtain Chamuang leaf powder (CLP). Ground CLP (passed through 80 mesh sieve) was mixed with water for ultrasonic extraction to obtain Chamuang leaf extract (CLE) as tailored by Shiekh and Benjakul (2020c). The prepared CLE solution was transferred into zip-lock bags (200 ml) and placed in a freezer (-20

°C) for overnight and transferred to -40 °C deep freezer (12 h) prior to freeze drying. The CLE powder was collected after freeze drying for 72 h.

9.4.5 Impact of vacuum impregnation on PEF pretreated PWS immersed in CLE solutions, followed by HVCAP

VI system consisted of a closed vessel (5 L capacity) connected with a vacuum pump (VE 125 N, Zhejiang Value Mechanical and Electrical Products Co., Ltd., Wenling, Zhejiang, China). Prior to VI process, PEF treated PWS (30 shrimps) were immersed in CLE solutions (1 or 2% CLE) with a shrimp to solution ratio of 1:5 (w/v). The vacuum pressure of 5 kPa was implemented on PWS during VI process (Zhao *et al.*, 2019). VI process was conducted at vacuum time of 15 min using a vacuum pressure of 5 kPa. Thereafter, the vacuum was released to restore an atmospheric pressure with restoration times (RT) of 15 min. The samples were drained on the screen for 5 min at 4 °C.

PWS (7 whole raw shrimp) treated with CLE using the aid of PEF and VI were packed in linear low density polyethylene (LLDPE) bag and filled with gas mixture (Ar + Air = 80:20) with a sample to gas ratio of 3:1 (w/v) using Henkovac type 1000 (Tecnovac, Italy) modified atmosphere packaging (MAP) machine, followed by heat sealing prior to HVCAP treatment. The gases were of food-grade including sterilized Ar and zero air (containing < 0.1% hydrocarbon impurities). Pretreated PWS samples packaged under Ar/Air-MAP were subjected to HVCAP for 10 min. HVCAP system was equipped with a high voltage transformer (230 V input voltage, 50 Hz) and a voltage variac (0–120 kV output voltage) was connected with dielectric-barrier discharge (DBD) system. DBD was created between two aluminium electrodes of 15-cm diameter and one electrode was fixed with a dielectric layer as displayed by Shiekh and Benjakul (2020c). HVCAP system was run with a voltage of 16 kVRMS at ambient atmospheric pressure. Two electrodes had a distance of 2 cm for an effective exposure of DBD (Ziuzina *et al.*, 2014).

All the treatments are defined as follows:

1. Control (untreated, packed in normal air)
2. PEF-VI-CLE1-MAP (PEF-800 pulses; VT-15min, RT-15 min; 1% CLE; packed under Ar/Air)
3. PEF-VI-CLE2-MAP (PEF-800 pulses; VT-15 min, RT-15 min; 2% CLE; packed under Ar/Air)
4. PEF-VI-CLE1-HVCAP (PEF-800 pulses; VT-15 min, RT-15 min; 1% CLE; packed under Ar/Air and exposed to HVCAP)
5. PEF-VI-CLE2-HVCAP (PEF-800 pulses; VT-15 min, RT-15 min; 2% CLE; packed under Ar/Air and exposed to HVCAP)

Analyses of all the samples were done every 3 days up to 18 days of storage at 4 °C.

9.4.6 Analyses

9.4.6.1 Melanosis assessment

The method of Nirmal and Benjakul (2010) was adopted for visual inspection of dark spot formation (melanin) using ten trained panelists, who had experience in scoring of PWS. For each treatment, a bag containing 20 shrimp was fixed for melanosis score test and presented to panelists every 3 days up to 18 days of storage.

9.4.6.2 Microbiological analyses

Spread plate technique was used for the enumeration of total viable count (TVC), psychrophilic bacterial count (PBC), lactic acid bacteria (LAB), *Pseudomonas*, H_2S-producing bacteria, Enterobacteriaceae and *Clostridium perfringens* counts as described by Shiekh and Benjakul (2020d).

9.4.6.3 pH

PWS samples (2 g) were homogenized in deionized water (20 ml) and pH of homogenate was measured with a pH meter (Sartorious North America, Edgewood, NY, USA) (Nirmal and Benjakul, 2011).

9.4.6.4 Protein carbonyl contents (PCC) and total volatile base (TVB)

Natural actomyosin (NAM) from PWS muscle was extracted as described by Chantarasuwan, Benjakul, and Visessanguan (2011). PCC of NAM was

determined using 2,4-dinitro-phenyl hydrazine (DNPH) as detailed by Nikoo, Benjakul, and Xu (2015). TVB content in PWS via Conway micro-diffusion method was measured (Shiekh and Benjakul, 2020c).

9.4.6.5 Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

PV of shrimp meat was analyzed by ferric thiocyanate method (Arafat *et al.,* 2015) using cumene hydroperoxide (0.5–2 ppm) as standard. TBARS of shrimp meat was measured (Shiekh and Benjakul, 2020c), in which malonaldehyde (MDA) (0–5 ppm) was used as standard.

9.4.6.6 Fatty acid profile

Fatty acid profiles were determined as fatty acid methyl esters (FAMEs) using gas chromatography with flame ionization detector (GC-FID) following the procedure of Raju and Benjakul (2020). Peaks were evaluated based on the retention time of standards and fatty acids in samples were quantified.

9.4.6.7 Sensory evaluation

The samples having TVC less than 6 log CFU/g were subjected to sensory analysis after being stored for 18 days at 4 °C in comparison with fresh shrimp (Nirmal and Benjakul, 2010). Samples were placed in aluminum tray and wrapped with foil. They were placed in steaming pot with perforated chamber, allowing steam to distribute uniformly. Samples were steamed for 5 min, to attain a maximum core temperature of 80 °C (Manheem *et al.,* 2013).

9.4.7 Statistical analyses

Completely randomized design (CRD) was used for the whole studies. Randomized completely block design (RCB) was implemented for sensory analysis. Analysis of variance (ANOVA) was done and mean comparison was performed by Duncan's multiple range test or t-test at a significant level ($p<0.05$) by a SPSS statistical software (SPSS 17.0, SPSS Inc., Chicago, IL, USA). All the experiments were run in triplicates (n=3).

9.5 Results and discussion

9.5.1 Effect of CLE pretreatment with the aid of PEF and VI, followed HVCAP on melanosis and quality changes of PWS during refrigerated storage

9.5.1.1 Changes in melanosis

Melanosis of PWS was monitored during 18 days of storage at 4 °C. Melanosis was not noticed in all the samples at day 0 (Fig 41a). During the storage, HVCAP was implemented on sample treated with 2% CLE with the aid of PEF (15 kV, 800 pulses and high specific energy, 697 kJ/kg) and VI process with vacuum time of 15 min and restoration time 15 min followed by HVCAP (PEF-VI-CLE2-HVCAP) exhibited the least melanosis ($p<0.05$). Melanosis scores were in the descending order: PEF-VI-CLE2-HVCAP > PEF-VI-CLE1-HVCAP > PEF-VI-CLE2-MAP > PEF-VI-CLE1-MAP. Lower scores were obtained for the samples treated with 2% CLE with the aid of both PEF and VI ($p<0.05$) than those treated with 1% CLE, regardless of HVCAP treatment. A vacuum pressure (5kPa) and vacuum time (15 min) impregnated fish gelatin (FG=3%) and grape seed extract (GSE=0.9%) retained the visual quality of tilapia fillets up to 12 days when stored at 4 ºC (Zhao *et al.*, 2019). Chitosan nanoparticle (0.5%) was used to treat peeled shrimp using vacuum impregnation process for quality preservation (Chouljenko *et al.*, 2017). Electroporation induced by PEF might generate the pores, in which CLE could penetrate more effectively with the aid of VI, especially during restoration process. This could help lower melanosis of shrimp. It was noted that the preservative effect towards melanosis depended on CLE level used. Ar and air (80:20) atmosphere with low O_2 concentration was documented to decelerate melanosis, especially in conjunction with CLE and active species generated by HVCAP treatment for PPO inactivation (Shiekh and Benjakul, 2020c). In addition to HVCAP and CLE treatment, high intensity PEF pulses (600 pulse number) could also inactivate PPO (Shiekh and Benjakul, 2020b).

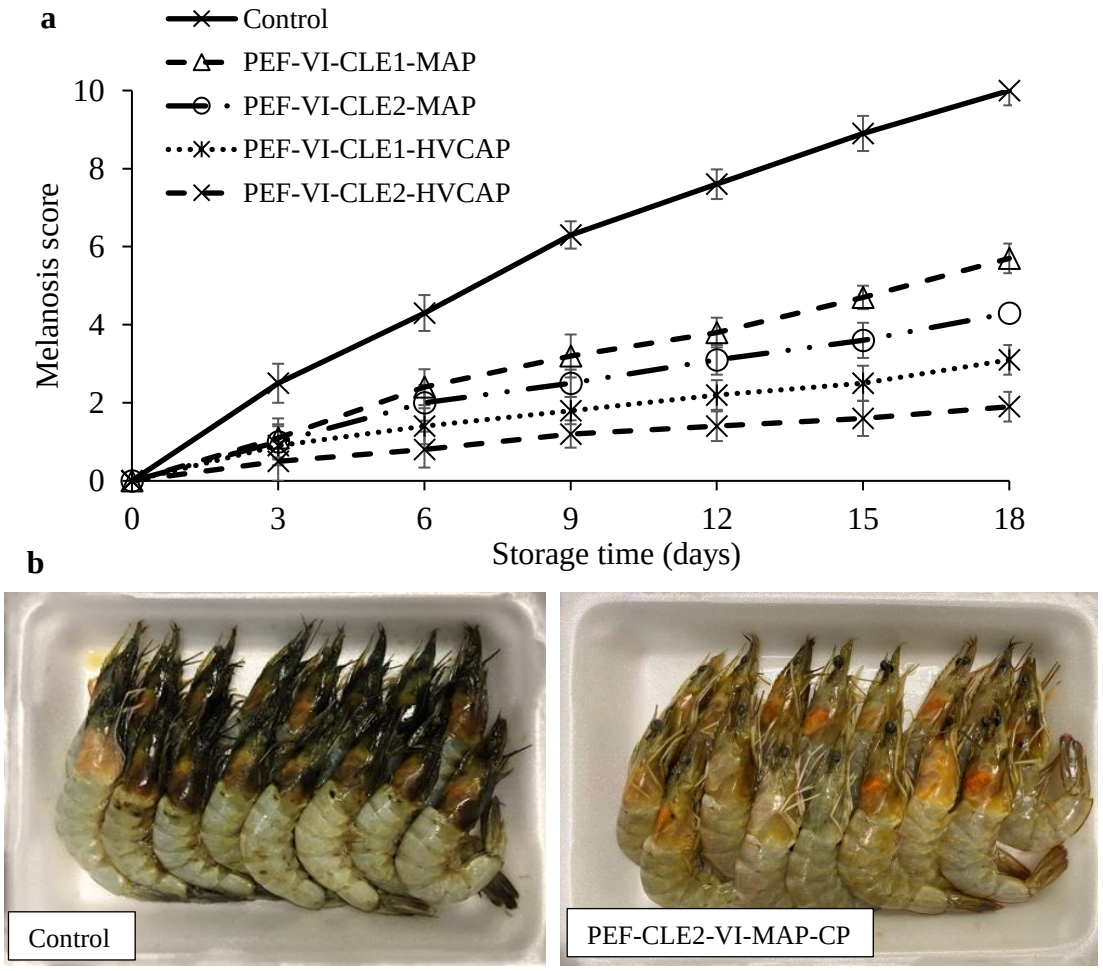

Figure 41. Melanosis score of Pacific white shrimp treated with CLE with the aid of PEF and vacuum impregnation followed by modified atmosphere packaging or in-bag high voltage cold atmosphere plasma (HVCAP) during 18 days of storage at 4 °C (a) and photographs of selected samples after 18 days of storage (b).

Bars represent the standard deviation (n=10). Control: without any treatment; PEF-VI-CLE1-MAP: Treated with 1% CLE and PEF-800 pulses, vacuum time (VT-15 min) and restoration time (RT-15 min), packed under Ar + Air (80:20) atmosphere; PEF-VI-CLE2-MAP: Treated with 2% CLE, PEF-800 pulses, vacuum time (VT-15 min) and restoration time (RT-15 min), packed under Ar + Air (80:20) atmosphere; PEF-VI-CLE1-HVCAP: Treated with 1% CLE and PEF-800 pulses, vacuum time (VT-15 min) and restoration time (RT-15 min), HVCAP under Ar + Air (80:20) atmosphere; PEF-VI-CLE2-HVCAP: Treated with 2% CLE and PEF-800 pulses, vacuum time (VT-15 min) and restoration time (RT-15 min), HVCAP under Ar + Air (80:20) atmosphere.

Moreover, CO_2-MAP in combination with PEF (600 pulses) and CLE (1%) retarded melanosis, compared to the control (without any treatment) for a period of 10 days (Shiekh and Benjakul, 2020a). Lower melanosis in PEF-VI-CLE2-HVCAP sample indicated the good keeping quality of shrimp up to 18 days (Fig. 41a). Appearance of PWS control (untreated) and PEF-VI-CLE2-HVCAP sample stored for 18 days of storage (4 ºC) is illustrated in Fig. 41b. Cold plasma treatment at 30 kV for

5 min, generated active species including atomic nitrogen, atomic oxygen, nitrite oxide, nitrates and hydroxyl radicals and were detected by optical emission spectroscopy during processing of black pepper grains (Charoux *et al.,* 2020). Moreover, the reactive species nitrogen (RNS) or oxygen (ROS) can cause alteration in protein, especially aromatic amino acids such as tyrosine that act as potential substrate for PPO (Surowsky *et al.,* 2013). Cold atmospheric plasma (CAP) reactive species were documented to cause alteration of structure of PPO (Zouelm *et al.,* 2019). The control sample had the pronounced melanosis, whereas PEF-VI-CLE2-HVCAP sample possessed a few black spots, reconfirming the efficacy of HVCAP along with CLE as well as PEF in conquering melanosis in PWS.

9.5.1.2 Changes in microbiological quality

At day 0, TVC of all treated samples was lower than that of control ($p<0.05$). Combined impact of 2% CLE treatment with the aid of PEF and VI, followed by HVCAP under Ar/Air atmosphere, was more effective in lowering the microbial growth, compared to others. This confirmed the combined effect of plant extracts and HVCAP on inhibition of microorganisms. TVC of control and other treated samples increased gradually during the entire storage as given in Fig. 42a. At day 18, TVC counts of the control, PEF-VI-CLE1-MAP, PEF-VI-CLE2-MAP, PEF-VI-CLE1-HVCAP, PEF-VI-CLE2-HVCAP samples were 8.90, 6.81, 6.33, 5.74 and 5.12 log CFU/g, respectively. The last two samples had TVC lower than the limit (6 log CFU/g). At the end of storage (18 days), PEF-CLE2-VI-MAP-CP sample ranked the lowest in TVC ($p<0.05$). Combined effect of PEF, CLE and HVCAP retarded the bacterial proliferation in PWS effectively. Additionally, PEF was reported previously to inactivate mesophilic bacteria in PWS due to cell wall disintegration caused by PEF electroporation (Shiekh and Benjakul, 2020b). VI could help in the migration of CLE into the shrimp through pores generated by PEF. This resulted in augmented efficacy in inhibition of microorganisms. Aerobic plate count of chitosan nanoparticles (0.5%) migrated in peeled shrimp using vacuum impregnation process was < 4 log CFU/g at the end of storage (24 days) (Chouljenko *et al.,* 2017).

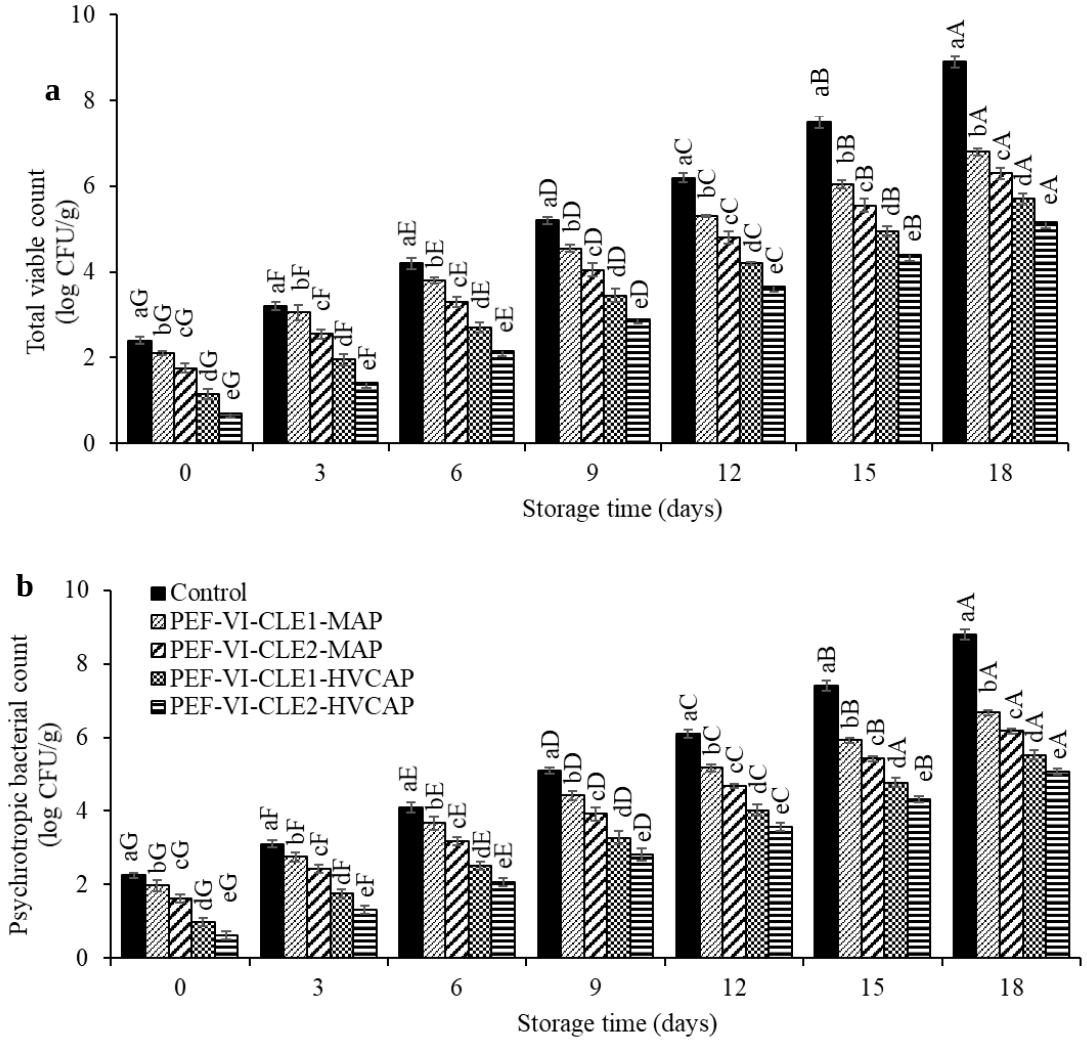

Figure 42. Total viable count (a) and psychrophilic bacterial count (b) of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Key: see the caption for Fig. 41.

Control ranked highest in PBC at day 0 ($p<0.05$). CLE treatment, especially at higher concentration (2%), showed lower count ($p<0.05$) as shown in Fig. 42b. When HVCAP was applied, the lower count was obtained, particularly for the sample treated with 2% CLE. This suggested the combined effect of the phenolic compounds in CLE and active species generated from HVCAP. At day 0, PBC in PEF-VI-CLE2-HVCAP samples was lower than the control and other samples ($p<0.05$). All the samples had the increase in PBC during the storage up to 18 days. Nevertheless, PBC in PEF-VI-CLE2-HVCAP showed the lower rate of increase as the storage

proceeded ($p<0.05$). Generally, the retarded growth of PBC in PEF-VI-CLE2-HVCAP sample throughout storage could be associated with the enhanced CLE at high level passage facilitated by VI to the muscle through the pores generated by PEF. Subsequently when HVCAP was applied, bacterial growth was further inhibited. Besides HVCAP, PEF (15 kV, 600 pulses, 483 kJ/kg) and CLE (1%) were documented to be efficient in inhibiting microbes in PWS (Shiekh and Benjakul, 2020a).

Additionally, PBC were reduced by 1 log CFU/g PBC in fish gelatin and grape seed extract treated tilapia fillets, followed by VI process for 15 min at the end of storage (12 days) (Zhao *et al.*, 2019). PBC were markedly inactivated during in-bag plasma generation processing by HVCAP in PWS packaged under Ar + Air mixture (80:20) treated with 1% CLE (Shiekh and Benjakul, 2020c). With the aid of VI, CLE especially at 2% could penetrate into shrimp to high level and functioned as antimicrobial agent. Moreover, PBC of CP treated PWS was lower than that of control (De Souza Silva *et al.*, 2019). Thus, the growth of psychrophilic bacteria could be effectively reduced with the combined processes during cold temperature storage.

Pseudomonas of all the samples ranged between 1.01 and 2.21 log CFU/g at day 0 as depicted in Fig. 43a. Control sample had the highest *Pseudomonas* than others ($p<0.05$). Conversely, the lowest count was attained in PEF-VI-CLE2-HVCAP sample ($p<0.05$). Increment in *Pseudomonas* count was noticed during storage of 18 days ($p<0.05$). At day 18, control sample ranked highest in count (8.10 log CFU/g), whereas the count of PEF-VI-CLE2-HVCAP sample was 4.95 log CFU/g ($p<0.05$). Samples immersed in 2% CLE with prior PEF and aid of VI might injure cell membrane during vacuum and facilitated the CLE passage for effective inactivation of *Pseudomonas*. Absolute CO_2-MAP along with PEF and 1% CLE inactivated *Pseudomonas* in PWS (Shiekh *et al.*, 2020d). *Pseudomonas* were drastically reduced in coconut husk extract and HVCAP treated sea bass slices (Olatunde *et al*., 2019a). HVCAP treatment (without PEF or VI) along with 1% CLE could lower the growth of *Pseudomonas* in refrigerated PWS stored for 15 days (Shiekh and Benjakul, 2020c). Seafoods are prone to spoilage by *Pseudomonas* known as gram negative spoilage bacteria (DeWitt and Oliveira, 2016).

Initial H_2S producing bacterial count ranged from 1.02 to 2.42 log CFU/g for all the samples, and the control had the highest count ($p<0.05$) (Fig. 43b).

H_2S counts were augmented for all the samples up to 18 days. At day 18, PEF-VI-CLE2-HVCAP samples possessed the lowest H_2S-producing bacterial count (5.07 log CFU/g). PEF and CLE under absolute CO_2-MAP lowered H_2S-producing bacteria notably (Shiekh and Benjakul, 2020d). Moreover, 1% CLE without PEF treatment could inactivate H_2S-producing bacteria of PWS markedly when stored at 4 ºC (Shiekh and Benjakul, 2020c). Olatunde *et al*. (2019b) documented that gram positive and gram negative microbial flora were reduced during non-thermal HVCAP of Asian sea bass slices to ensure food safety. Therefore, combined effects of CLE with the aid of PEF and VI, followed by HVCAP aided in the reduction of H_2S-producing bacterial counts in PWS during storage at 4 ºC.

The highest in Enterobacteriaceae count was found in the control (8.02 log CFU/g) ($p<0.05$) (Fig. 43c). Increases in Enterobacteriaceae were noticeable in all the samples as storage time augmented ($p<0.05$). After 18 days, the lowest count was obtained in PEF-VI-CLE2-HVCAP sample (4.87 log CFU/g) ($p<0.05$). Due to the effective penetration of CLE at 2% level through PEF pores or gapping generated the underneath the shell for effective migration of CLE to PWS meat along with the sanitizing effect of active species generated by HVCAP, the lowest Enterobacteriaceae count was gained. Enterobacteriaceae counts drastically reduced in PWS treated with 1% CLE along with HVCAP, compared to the control during 15 days of refrigerated storage (Shiekh and Benjakul, 2020c). Enterobacteriaceae are gram negative facultative anaerobes highly susceptible to the damages by polyphenols than gram positive bacteria (McMillin, 2008). Therefore, 2% CLE treated PWS with the aforementioned combined processes could reduce the growth of Enterobacteriaceae during 18 days of storage (4 °C)

Initially, the control showed the higher LAB count than others ($p<0.05$). LAB count was relatively lower in PEF-VI-CLE2-HVCAP ($p<0.05$) (Fig. 43d), plausibly due to joint effect of PEF electroporation of the bacterial cells followed by VI that facilitated the passage of antimicrobial agents in CLE towards LAB. Additionally, in-bag HVCAP could generate active species which showed, inhibitory effect on LAB. During storage, LAB count of the control kept increasing to higher extent, compared to samples treated with combined processes in the presence of CLE, in which the rate of increase was lower ($p<0.05$). This could be governed by the absence of CLE

polyphenols with antimicrobial properties in the control. LAB were inactivated by combination of PEF and at 1% CLE under CO_2-MAP effectively (Shiekh and Benjakul, 2020d). LAB were reported as facultative anaerobes having sensitivity to phenolic compounds and could be affected by active species of HVCAP (Han *et al*., 2016; Shiekh and Benjakul, 2020c). *Clostridium perfringens* colonies were not found in all the samples throughout storage. This could be attributed to hygienic practice of sample preparation without any cross-contamination. Therefore, the sanitizing effect of CLE (2%) with the aid of PEF and VI, followed by HVCAP treatment was pronounced, thus lowering the growth of microorganisms in PWS during refrigerated storage of 18 days.

9.5.1.3 Changes in chemical quality

Highest pH of control was observed at day 0 ($p>0.05$), while PEF-VI-CLE2-HVCAP sample had the lower pH ($p<0.05$) (Fig. 44a). The pHs of all the samples were found in the range 6.30-6.68 within the first 3 days. As storage time augmented, pH of the control increased more rapidly than others ($p<0.05$), while the changes in pH values of PEF-VI-CLE2-HVCAP sample were lowest during 0-18 days ($p<0.05$). At the end of storage, the pH of the control was 11.55. The accumulation of basic compounds in the control sample (without any treatment) could be due to higher enzyme activity triggered by microbes, contributing to the raised pH. Microbial enzymes in PWS might have been inactivated during treatment with high energy PEF pulses. PEF can inactivate enzymes by inducing their denaturation (Shiekh and Benjakul, 2020b) Furthermore, phenolic compounds in CLE also showed antimicrobial activity. Similar results were documented in PEF and CLE treated PWS in CO_2-MAP stored for 10 days (Shiekh and Benjakul, 2020d). Lowered increment of pH of PEF-VI-CLE2-HVCAP sample was possibly owing to the prevention of microbial growth and enzyme inactivation induced by HVCAP as well as antimicrobial effects of migrated CLE through the PEF pores underneath the shrimp shell towards PWS meat lowering spoilage.

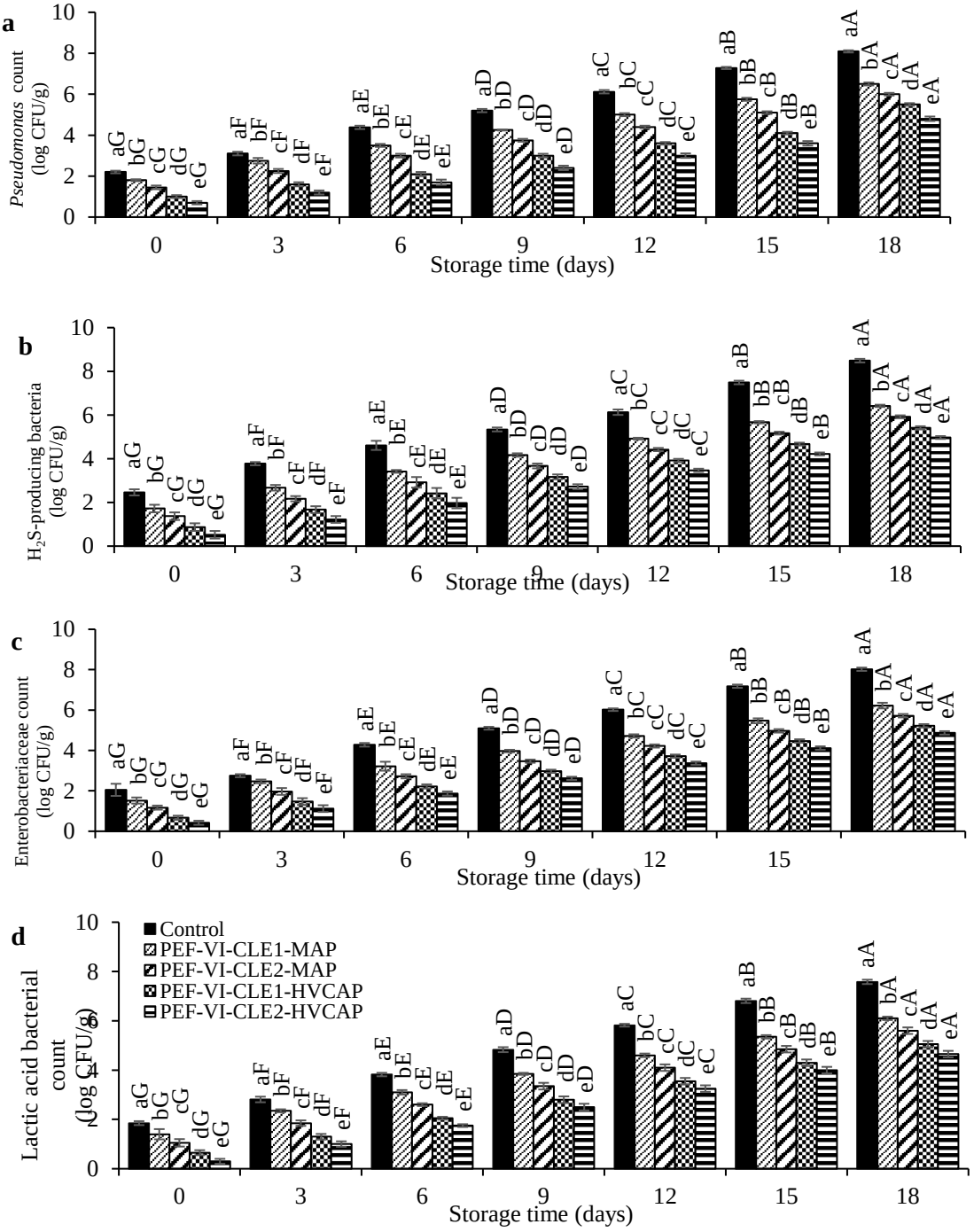

Figure 43. *Pseudomonas* (a), H_2S-producing bacteria (b), Enterobacteriaceae (c) and lactic acid bacteria counts (d) of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($p<0.05$). Key: see the caption for Fig. 41.

TVB contents of the control and treated samples (2.21 to 6.51 mg N/100 g) were noted at day 0 (Fig. 44a). TVB content of the control was higher than others

throughout storage ($p<0.05$). All the samples showed gradual increases in TVB content up to 18 days ($p<0.05$). The drastic increases in TVB content were observed in the control during 6 and 18 days ($p<0.05$). PEF-VI-CLE2-HVCAP possessed the lowest TVB content (14.25 mg N/100 g meat) at day 18. This was more likely the result of the combined effect between PEF, CLE, VI and HVCAP treatments. TVB content (12-20 mg N/100 g) in PWS indicates the acceptable limits for safe consumption (Okpala *et al.,* 2014). Shiekh and Benjakul (2020d) reported similar range of TVB content in CLE treated PWS without prior PEF under CO_2-MAP during 10 days of storage. CLE had the pronounced antimicrobial and antioxidant effect which could to retard TVB content of refrigerated shrimp (Sae-leaw and Benjakul, 2019a). HVCAP could potentially preserve quality of PWS by inactivation of spoilage microorganisms that produces proteases during storage at 4 ºC (Zouelm *et al.,* 2019). Additionally, VI aided in maintaining freshness of shrimp via removal of residual gases through the PEF pore spaces of PWS shell and facilitated influx of high dose of CLE by mitigating the negative impact of HVCAP active species on sanitization of PWS.

At day 0, the control sample had the highest PCC ($p<0.05$) (Fig. 44c). PEF-VI-CLE2-HVCAP sample showed the lowest PCC during 18 days than the control and others ($p<0.05$). With increasing storage time, PCC of the samples increased, reflecting the induction of protein oxidation induced by the active species, especially when treated by HVCAP. It was noted that sufficient concentration of CLE or high concentration could decrease PCC in the samples. It was reported that PEF did not impact oxidation of PWS proteins (Shiekh and Benjakul, 2020b). Additionally, the lowest PCC of PEF-VI-CLE2-HVCAP sample was attained throughout storage ($p<0.05$). The effect of 2% CLE towards protein oxidation was evident in vacuum impregnated and HVCAP treated sample. Decreased PCC in PEF-VI-CLE2-HVCAP coincided with the lower TVB content and microbial load. PCC in the swimming crab and PWS kept under low O_2 and absolute CO_2 atmosphere along with PEF and 1% CLE were remarkably less than the control sample, respectively (Sun *et al.,* 2017; Shiekh and Benjakul, 2020d). PCC were lowered in tilapia fillets coated with fish gelatin and grape seed extract during VI process (Zhao *et al.,* 2019). Myofibrillar proteins of sea bass without antioxidants were oxidized by HVCAP to high degree (Olatunde et al., 2019c). The highest PCC was found in HVCAP treated PWS containing lower levels

of CL-E (0.5%) (Shiekh and Benjakul, 2020c). Additionally, the use of antioxidants at sufficient quantity is an effective strategy to control the formation of carbonyl groups during protein oxidation (Zhang *et al*., 2018). Moreover, natural and chemical based preservatives such as quercetin, cinnamic acid, and 4-hexylresorcinol in combination with MAP (80% CO_2/10% O_2/10% N_2) retarded protein oxidation in refrigerated shrimp (Qian *et al*., 2015). Therefore, higher PEF pulses in the presence of sufficient CLE dose prevented protein oxidation associated with the enhanced penetration of 2% CLE via PEF pores with the aid of VI process, although HVCAP was subsequently applied. Thus, the combined processes with CLE containing antioxidants safeguarded proteins from oxidation during HVCAP treatment.

PV was higher in the control than treated samples during the whole storage ($p<0.05$). At day 0, PEF-VI-CLE2-HVCAP sample attained the lowest PV ($p<0.05$) (Fig. 44d). HVCAP of 2% CLE treated sample with the aid of PEF and VI, lowered the formation of hydroperoxide ascertained by the lower PV ($p<0.05$). Antioxidants from CLE were documented to penetrate through the holes or gaps generated in PWS meat driven by PEF electroporation (Shiekh and Benjakul, 2020a). After 18 days, highest PV was obtained in the control ($p<0.05$) than sample treated with 2% CLE with combined processes (PEF + VI) followed by HVCAP. Lower oxygen levels and effective passage of CLE through shells with the aid of prolonged vacuum time possibly loaded higher amount of CLE through the PEF pores, thereby lowering lipid oxidation in this sample. CLE (1%) treatment prevented lipid oxidation of PWS, when PEF was implemented at 600 pulses and packaged under absolute CO_2-MAP (Shiekh *et al*., 2020d). Additionally, combined inhibitory effect of PEF and CLE towards microbial and indigenous enzymes including lipase was also postulated. Seafoods are rich in polyunsaturated fatty acids and prone to oxidation (Albertos *et al*., 2017).

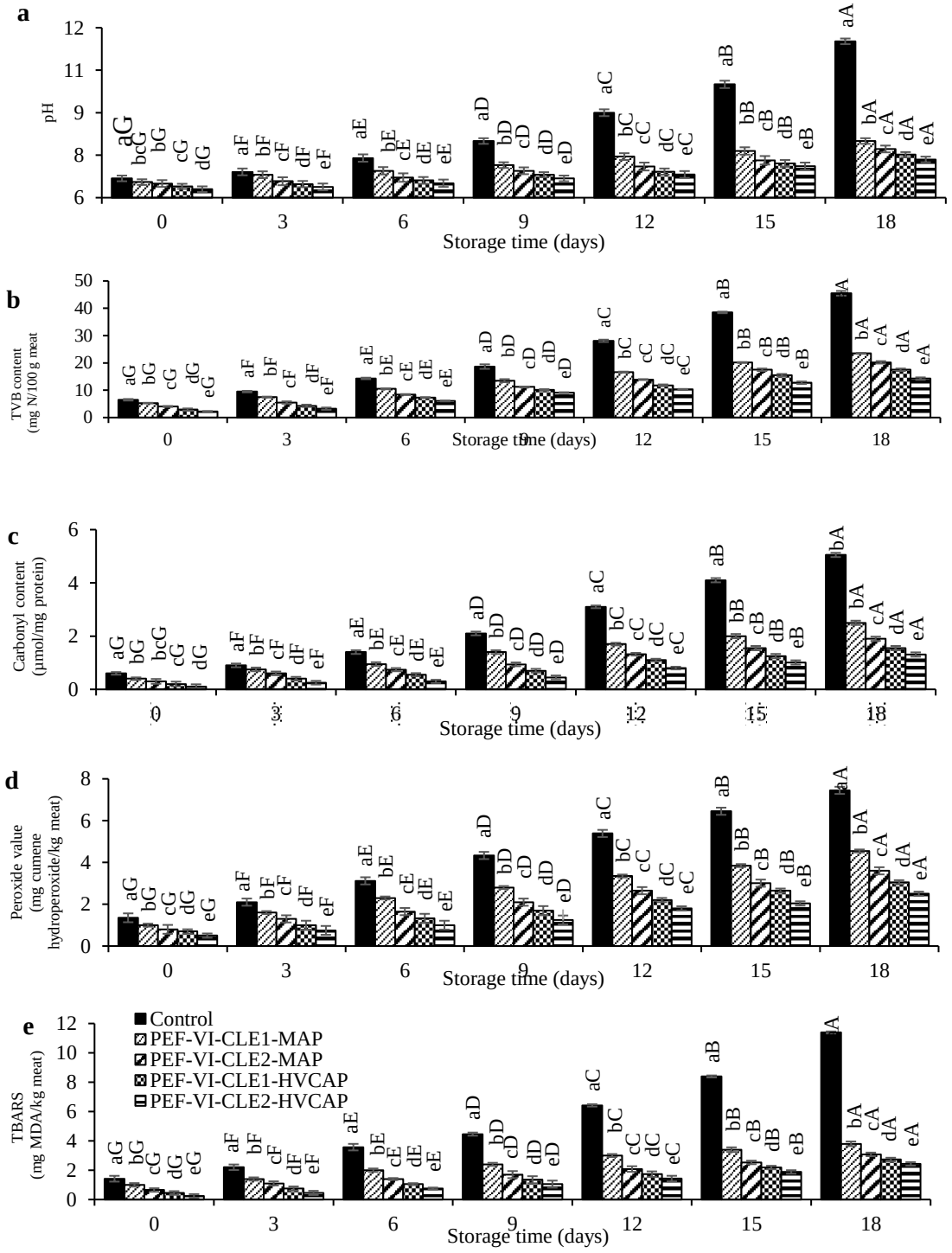

Figure 44. pH (a), total volatile base (TVB) (b), carbonyl content (c), peroxide values (d) and thiobarbituric reactive substances (TBARS) values (E) of Pacific white shrimp with PEF and CLE pre-treatment without and with vacuum impregnation process, followed by MAP during 18 days of storage at 4 °C.

Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same treatment indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Key caption 40.

HVCAP treated sea bass slices with natural antioxidants resulted in lower hydroperoxide formation (Olatunde *et al*., 2019b). Moreover, CLE (1%) treatment prevented lipid oxidation of PWS, when HVCAP was applied for 10 min (Shiekh and Benjakul, 2020c). Therefore, CLE treatment with the aid of PEF and VI process effectively prevented lipid oxidation and lowered the oxidation induced by HVCAP in PWS.TBARS values of samples treated with CLE (2%) with the aid of PEF and VI, followed by HVCAP treatment was relatively lower during storage of 18 days ($p<0.05$) (Fig. 44e). Highest TBARS values were noticed in the control (untreated) up to 18 days. PEF-VI-CLE2-HVCAP sample had the lower TBARS value than the control and those without HVCAP treatment at day 18 ($p<0.05$). After 6 days of storage, the control had highest TBARS, likely correlated with the production of microbial lipase, which accelerated hydrolysis to free fatty acids which were prone to subsequent oxidation. Nevertheless, PEF (800 pulses) treated PWS in conjunction with CLE (2%) alleviated lipid oxidation to some level. PWS with PEF and CLE (1%) treatment along with HVCAP had the lowest TBARS throughout refrigerated storage (Shiekh and Benjakul, 2020c), indicating that phenolic compounds with antioxidant activity could retard lipid oxidation. After 18 days, PEF-VI-CLE2-HVCAP sample had low amount of TBARS as a result of combined effects. Low TBARS levels were found in stored PWS added with antioxidants (Zouelm *et al*., 2019). Moreover, absence of O_2 in PEF and CLE (1%) treated PWS kept under absolute CO_2-MAP had the lowest TBARS throughout refrigerated storage (Shiekh *et al*., 2020d). HVCAP alone was reported to trigger lipid oxidation of foods (Pankaj *et al*., 2018). Therefore, CLE at high level with the aid of PEF and VI could mitigated the oxidation caused by HVCAP due to antioxidants in CLE.

9.5.2 Changes in fatty acid profile

Fatty acids of all the treated samples and the control at day 0 and 17 are presented in Table 16. At day 0, the control and treated samples (PEF-800 pulses and 1 or 2% CLE) with subsequent VI process followed by HVCAP showed difference in fatty acid contents ($p<0.05$). PEF-VI-CLE2-HVCAP sample had higher fatty acid content, especially MUFA and PUFA, compared to others at the first day ($p<0.05$).

Table 16. Fatty acid profiles of Pacific white shrimp without any treatment and with prior PEF and vacuum impregnation of CLE treatments packaged in Modified atmosphere (Ar + Air = 80:20) followed by HVCAP at day 0 and 18 of refrigerated storage.

Fatty acids g/100g	Day 0			Day 18	
	Control	PEF-VI-CLE1-HVCAP	PEF-VI-CLE2-HVCAP	PEF-VI-CLE1-HVCAP	PEF-VI-CLE2-HVCAP
C14:0 Myristic	1.10±0.02c	1.10±0.01c	1.11±0.01c	1.22±0.03b	1.35±0.01a
C15:0 Pentadecanoic	0.76±0.01a	0.79±0.03a	0.80±0.02a	0.51±0.01b	0.53±0.02b
C16:0 Palmitic	20.62±0.01b	20.62±0.01b	21.29±0.02a	17.28±0.02d	18.59±0.01c
C16:1 Palmitoleic	0.51±0.03c	0.53±0.05c	0.54±0.01c	1.25±0.01b	1.35±0.01a
C17:0 Heptadecanoic	2.40±0.01b	2.44±0.03b	2.72±0.02a	1.22±0.03d	1.40±0.01c
C17:1 Hepatodecanoic	0.56±0.04b	0.61±0.02a	0.64±0.01a	0.57±0.01b	0.56±0.02b
C18:0 Stearic	9.59±0.02b	9.62±0.01b	10.82±0.01a	8..45±0.01cd	8.52±0.01c
C18:1 Oleic	12.81±0.01e	12.92±0.03d	13.14±0.05c	14.05±0.01b	15.32±0.02a
C18:1 Elaidic	0.57±0.01c	0.65±0.02b	0.67±0.03b	0.84±0.01ab	0.91±0.01a
C18:2 Linoleic	13.22±0.01e	13.78±0.01d	14.95±0.02a	13.88±0.01c	14.75±0.02b
C20:0 Arachidic	1.02±0.02b	1.08±0.01b	1.09±0.01b	1.15±0.01a	1.18±0.01a
C20:1 Eicosenoic	0.48±0.02b	0.52±0.02b	0.52±0.04b	0.68±0.01ab	0.75±0.01a
C21:0 Heneicosanoic	0.59±0.01b	0.61±0.03b	0.62±0.03b	0.65±0.01b	0.86±0.03a
C20:2 Eicosadienoic acid	2.02±0.03d	2.05±0.01d	2.13±0.02c	2.25±0.01b	2.45±0.01a
C20:0 Docosanoic	1.01±0.01c	1.01±0.01c	1.01±0.01c	1.21±0.02b	1.33±0.01a
C20:3 Eicosatrienoic	0.62±0.05c	0.62±0.01c	0.62±0.01c	0.81±0.02b	0.91±0.03a
C20:4 Eicosatetraenoic	0.61±0.01ab	0.65±0.02ab	0.66±0.02a	0.68±0.01ba	0.73±0.01a
C23:0 Tricosanoic	4.82±0.02b	4.39±0.03c	4.39±0.04c	4.85±0.02b	4.95±0.02a
C22:2 Docosadienoic	0.61±0.04b	0.67±0.02a	0.71±0.01a	0.62±0.01b	0.70±0.01a
C20:5 Eicosapentaenoic (EPA)	14.43±0.03b	13.43±0.01c	15.76±0.03a	8.95±0.01e	10.42±0.01d
C24:1 Nervonic	0.70±0.01b	0.70±0.02b	0.71±0.02b	0.81±0.01a	0.88±0.02a
C22:6 Docosahexaenoic (DHA)	13.01±0.05b	12.63±0.01c	14.37±0.01a	8.45±0.01e	11.64±0.01d
SFA	41.90	41.75	43.86	32.28	40.52
MUFA	15.62	15.92	16.21	27.13	28.40
PUFA	44.52	43.83	49.19	35.90	39.07

Values are presented as the mean ± standard deviation (n=3). Different lowercase letters within the same row indicated significant differences ($p<0.05$). Key caption Fig 41.

At day 0, PEF-VI-CLE2-HVCAP sample had higher DHA, EPA, oleic, linoleic and eicosadienoic acids, compared to other samples. CLE (1%) was reported to lower fatty acid oxidation of HVCAP treated PWS under Ar and Air (80:20) atmosphere during 15 days of storage (Shiekh and Benjakul, 2020c). Thus, it was presumed that PEF and HVCAP treatment had no negative impact on fatty acids in the presence of sufficient CLE which was migrated into muscle with the aid of PEF and VI.

After 18 days, EPA and DHA contents of PEF-VI-CLE1-HVCAP and PEF-VI-CLE2-HVCAP samples were depleted, while contents of oleic acid, linoleic acid and myristic acid were augmented. The lowered contents of PUFA could be the result of PUFA oxidation with coincidental increased proportion of MUFA. Despite of the decreases in fatty acids during prolonged storage (18 days), MUFA and PUFA contents were still higher in PEF-VI-CLE2-HVCAP sample than those of PEF-VI-CLE1-HVCAP sample indicating the major role of antioxidant compounds in CLE. Lipid oxidation products (aldehydes or ketones) affect sensory attributes of foods and consumer health (Pan *et al*., 2019). However, this negative impact of oxidation could be mitigated by the combined effect of PEF in conjunction with vacuum impregnation of 2% CLE rich in antioxidative polyphenols to lower lipid oxidation during HVCAP sanitization of PWS.

9.5.3 Changes in sensory quality

Likeness scores of the selected samples are presented in Table 17. At the first day, all the samples with or without treatment had the similar sensory scores ($p>0.05$). High likeness scores at day 0 could be attributed to the lower TVB contents, reflecting the freshness and negligible spoilage of all the samples. Shiekh *et al*. (2020d) reported similar findings, in which lower odor, flavor and taste likeness scores in PEF (600 pulses) and CLE (1%) treated PWS packaged under CO_2-MAP were found as the storage time increased. After 18 days, PEF-VI-CLE1-HVCAP and PEF-VI-CLE2-HVCAP samples had the noticeable decreases in likeness scores, while microbial counts were still < 6 log CFU/g. Decreases in the likeness of sample attributes were noticeable in PEF-VI-CLE2-HVCAP samples after 18 days ($p<0.05$).

Table 17. Likeness score of Pacific white shrimp without any treatment and with prior PEF and vacuum impregnation of CLE treatments packaged in modified atmosphere (Ar + Air = 80:20) followed by HVCAP at day 0 and 18 of refrigerated storage.

Storage time (days)	Samples	Color	Texture	Odor	Flavor	Taste	Overall
0	Control	8.56±0.41a	8.44±0.35a	8.42±0.37a	8.39±0.51a	8.39±0.48a	8.36±0.45a
	PEF-CLE1-VI-MAP-CP	8.61±0.45aA	8.50±0.44aA	8.45±0.34aA	8.44±0.55aA	8.42±0.35aA	8.39±0.42aA
	PEF-CLE2-VI-MAP-CP	8.64±0.25aA	8.53±0.53aA	8.47±0.46aA	8.47±0.45aA	8.44±0.55aA	8.42±0.55aA
18	PEF-CLE1-VI-MAP-CP	6.06±0.36bB	6.03±0.34bB	5.95±0.48bB	5.94±0.41bB	5.92±0.45bB	6.00±0.25bB
	PEF-CLE2-VI-MAP-CP	7.61±0.32aB	7.56±0.38aB	7.45±0.41aB	7.42±0.35aB	7.39±0.43aB	7.42±0.35aB

Values are presented as the mean ± standard deviation (n=50). Different uppercase letters in the same column within the same treatment indicate significant differences ($p<0.05$). Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Key: see the caption of Fig. 41.

At the end of storge, sample treated with 2% CLE with the aid of PEF and VI, had higher likeness scores than those with lower CLE concentration (1%) for all tested attributes ($p<0.05$). Moreover, the extended vacuum time (15 min) might allow higher quantity of CLE to penetrate through the pores generated by PEF, when 800 pulses were implemented. HVCAP active species might have also sanitized PWS as indicated by reduction in microbial load (Fig 42 and 43). Higher color likeness score in PEF-VI-CLE2-HVCAP sample was related with the reduced melanosis (Fig. 41). With higher flavor and odor likeness score, PEF-CLE2-VI-MAP-CP also showed the lower lipid oxidation (Fig. 44). HVCAP treatment inactivated spoilage microorganisms in PWS more effectively than chemical additives such as sodium metabisulphite (SMS) in terms of retention of sensory quality (Zouelm *et al*., 2019). In comparison with SMS, 1% CLE as natural additive was more effective along with HVCAP treatment under Ar + Air MAP for retaining the sensorial quality of PWS during 15 days of storage (Shiekh and Benjakul, 2020c). Therefore, CLE in conjunction with PEF and VI with subsequent HVCAP using the mixture of Ar and Air (80:20) atmosphere were able to maintain quality and prolonged shelf-life of PWS potentially.

9.6 Conclusions

Prior CLE treatment with the aid of PEF and vacuum impregnation prior to HVCAP treatment under Ar/Air modified atmosphere could extend the shelf-life of PWS up to 18 days at 4 °C. Melanosis was negligible as a result of joint impact

of CLE and all the processes used. The growth of mesophiles (TVC, Enterobacteriaceae, H_2S-producing bacteria, *Pseudomonas*, LAB) were under acceptable limits during 18 days. For safety assurance, *Clostridium perfringens* was not detected in all the samples. Oxidative changes in lipids and proteins were less pronounced, especially when CLE (2%) along with high intensity PEF pulses and vacuum impregnation time (15 min) were applied on shrimp followed by HVCAP. PUFA were not decomposed in the HVCAP treated shrimp with 800 PEF pulse number and 2% CLE, which exhibited strong antioxidative potential against oxidation. PEF-VI-CLE2-HVCAP sample ranked highest in likeness score. Thus, the application of non-thermal technologies (PEF and VI) in association with natural extract for treatment of PWS prior to in-bag HVCAP, could act as a promising means for quality control and shelf-life extension of PWS at 4 °C.

9.7 References

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CHAPTER 10
SUMMARY AND SUGGESTION

10.1 Summary

Chamuang leaf extract was rich in polyphenolic glycosides and organic acids that could inhibit PPO from Pacific white shrimp. Lower melanosis score, lipid oxidation and spoilage bacteria were attained in 1% CLE treated sample during refrigerated storage of 12 days.

PEF inactivated PPO in an energy-dependent manner with lower melanosis score. More gaping was visualized in PEF-T3-treated sample, related to the lowered shear force, but PEF had no impact on the protein pattern of shrimp meat. After 10 days of storage, lowered increases in microbial load in PEF-T3 samples resulted in highest likeness score with augmented shear force.

PEF application based on electroporation in shrimp facilitated the migration of CLE polyphenolic glycosides and organic acids, in which chrysoeriol 6-C-glucoside-8-C-arabinopyranoside was abundant in shrimp as detected by LC-MS. Melanosis and spoilage microbes (TVC, psychrophile, Enterobacteriaceae, *Pseudomonas*, H_2S producing bacteria) along with lipid oxidation were retarded during 10 days of refrigeration storage. PEF in combination with CLE retained freshness of refrigerated shrimp.

PEF in presence of CLE 1% followed by CO_2-MAP (PEF-CLE-CO_2) sample retained the quality most effectively, in which melanosis and microbial growth were retarded. Protein and lipid oxidation was lowered, mainly due to antioxidant activity of CLE and antibacterial effect of CO_2-MAP. Lower quality changes in PEF-CLE-CO_2 sample were associated with the highest likeness score and coincidentally lower off-odor volatiles extended shelf-life up to 10 days of refrigerated storage (4 °C).

HVCAP in combination with CLE was effective non-thermal treatment for shelf-life extension of shrimp. Melanosis was negligible and spoilage bacteria including LAB were decreased due to combined impact of HVCAP and 1% CLE during 15 days of refrigerated (4 °C) storage. Antioxidants, particularly CLE effectively lowered lipid oxidation, when HVCAP was implemented. Lipid and protein oxidation was less pronounced in stored shrimp treated with HVCAP, especially those containing 1% CLE with high antioxidant capacity. PUFA including EPA and DHA were retained

in shrimp treated with CLE at 1%, followed by HVCAP under Ar/Air (80:20) atmosphere. Highest likeness score was attained in shrimp with HVCAP and 1% CLE under Ar/Air (80:20) gas composition after 15 days.

Prior PEF and CLE treatment in combination with HVCAP as non-thermal sanitizing technology could extend the shelf-life of PWS up to 18 days at 4 °C. Melanosis was less pronounced by the joint impact of PEF, CLE and HVCAP. The growth of spoilage microbes were drastically declined during 18 days. Oxidative changes in lipids and proteins were negligible, especially when high intensity PEF pulses (800 pulses) and CLE (2%) were applied on shrimp before HVCAP treatment. PUFA were not decomposed in the treated shrimp with PEF pulse number (800 pulses) and CLE (2%), which exhibited strong antioxidative potential against HVCAP induced oxidation. PEFT2-CLE2-Ar/Air-CP sample ranked highest in likeness score and shelf-life of PWS was extended up to 18 days at 4 °C.

Vacuum impregnation (VI) of CLE in Prior PEF PWS samples and stored under Ar + Air MAP could extend the shelf-life of PWS up to 18 days at 4 °C. Melanosis alleviated by the joint impact of PEF, VI and CLE under low O_2 proportion in MAP. The growth of spoilage microorganisms were under acceptable limits during 18 days. Oxidative changes in lipids and proteins were less pronounced, especially when high intensity PEF pulses and CLE (2%) and vacuum impregnation time (15 min) were applied on shrimp before MAP. PUFA were not decomposed in the shrimp treated with 800 PEF pulse number and 2% CLE, which exhibited strong antioxidative potential against oxidation. PEF-CLE2-VI2-MAP sample ranked highest in likeness score with the combined processes.

Combined hurdle effects of CLE treated PWS with the aid of PEF and vacuum impregnation prior to HVCAP treatment under Ar/Air modified atmosphere could extend the shelf-life of PWS up to 18 days at 4 °C. Melanosis was not noticed as a result of joint impact of CLE and all the processes used. The growth of microbial and spoilage bacteria were less than 6 log CFU/g meat during 18 days. *Clostridium perfringens* was not detected in all the samples. PWS rendered with less protein and lipid oxidation with the retention of PUFA (EPA and DHA), especially when CLE (2%) along with high intensity PEF pulses and vacuum impregnation time (15 min) were applied on shrimp followed by HVCAP. Thus, the application of non-thermal

technologies (PEF and VI) in association with natural extract for treatment of PWS prior to in-bag HVCAP, could act as a promising means for quality control and shelf-life extension of PWS at 4 °C.

10.2 Suggestions

Studies on inactivation of lipoxygenase and protease from shrimp should be investigated separately by nonthermal technologies such as pulsed electric field and high voltage cold atmospheric plasma.

Stability of astaxanthin should be monitored after PEF and HVCAP treatment during storage of whole raw Pacific white shrimp.

APPENDIX

Chamuang leaves (*Garcinia cowa* Roxb.)

Chamuang leaf powder

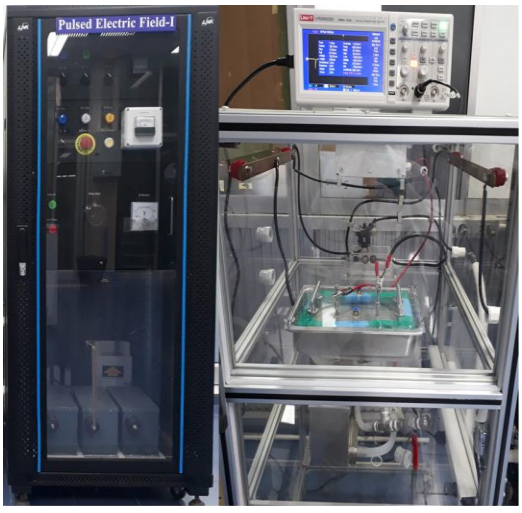
Ultrasonic extraction using water as solvent

Chamuang leaf extract (CLE)

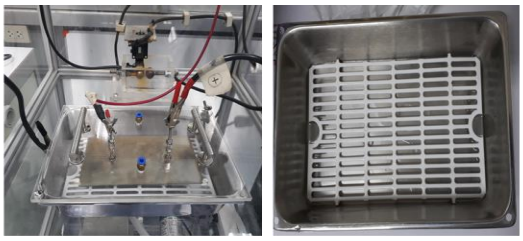
CLE lyophilized powder

Appendix-A. Preparation of Chamuang leaf extract.

Source: Shiekh *et al.* (2019)



Setup for pulsed electric field (PEF)

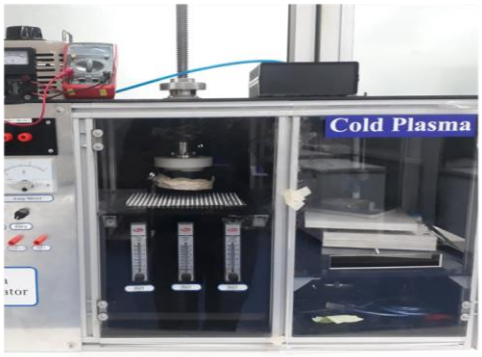


PEF treatment chamber

Shrimps place in one layer in a PEF treatment chamber

Appendix-B. PEF treatment of Pacific white shrimp.

Source: Shiekh and Benjakul (2020a)



High voltage cold atmospheric plasma (HVCAP)

Modified atmosphere packaging of Pacific white shrimp (Ar/Air-MAP; 80:20)

HVCAP treatment of Pacific white shrimp

Appendix-C. HVCAP processing of Pacific white shrimp.

Source: Shiekh and Benjakul (2020b)

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List of Publication and Proceeding

Publications

Shiekh, K. A., Benjakul, S. and Sae-leaw, T. 2019. Effect of Chamuang (*Garcinia cowa* Roxb.) leaf extract on inhibition of melanosis and quality changes of Pacific white shrimp during refrigerated storage. Food Chemistry. 270: 554-561.

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Proceeding

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