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Study of the Microbiota in Pacific White Shrimp (*Litopenaeus vannamei*) under varying O_2 Modified Atmosphere Packaging using PCR-DGGE

Qian Yun-Fang, Xie Jing*, Yang Sheng-Ping and Xiong Qing

Shanghai Engineering Research Center of Aquatic Product Processing & Preservation, College of Food Science & Technology, Shanghai Ocean University, Shanghai, 201306, P. R. China.

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Occurrence and importance of spoilage related bacteria in fresh Pacific white shrimp (*Litopenaeus vannamei*) under modified atmosphere were demonstrated in the current study. Culture-independent method including PCR-DGGE (V3 region of 16S rRNA gene), displayed a fingerprint of microbial DNA extracted directly from shrimp. Four batches of raw shrimps packaged under 80% CO_2/5% O_2/15% N_2, 80% CO_2/10% O_2/15% N_2, 80% CO_2/20% O_2, as well as unsealed packages for shrimp used as the control were stored at 4 °C during 10 days of storage. Results showed that the initial dominant bacteria of shrimp were *Vibrio albensis*, *Vibrio* sp., *Photobacterium damselae*, *Shewanella putrefaciens*, *Vibrio harveyi* and *Vibrio parahaemolyticus*. After 10 days of storage, *Vibrio* sp., *Shewanella* sp., *Photobacterium damselae*, *Listonella anguillarum*, *Shewanella baltica*, Uncultured *Aeromonas* sp. and *Aeromonas veronii* dominated in shrimps packaged in air. Modified atmosphere packaging (MAP) showed an inhibitory effect on several bacterial species such as *Shewanella* sp., *Listonella anguillarum*, Uncultured *Aeromonas* sp. and *Aeromonas veronii*. In conclusion, this study showed the benefits of the use of culture-independent method to enhance understanding of the composition of microbial flora in shrimps under different MAP conditions.

Key words: *Litopenaeus vannamei*, modified atmosphere packaging, 16S rRNA, DGGE.

Pacific white shrimp (*Litopenaeus vannamei*) is one of those most important commercial aquatic food all over the world. However, it is very vulnerable for the microbial contamination. The growth of bacteria and melanosis can induce the losing of consumption values. The shrimp can be contaminated by bacteria from marine environments, such as *Vibrio* sp., *Aeromonas*, *Pseudomonas* sp., etc. (Ruangpan *et al*., 1991, Liu *et al*, 2011), or from digestive tract including Enterobacteriaceae and lactic acid bacteria (Ringø *et al*., 1998). These bacteria develop very quickly after post mortem and attribute to[1] the slime of shrimp meat and a high content of volatile low molecular weights compounds emanating off-odor formation.

Modified atmosphere packaging (MAP) combined with low temperature storage method has been developed to contribute a strong barrier, impeding the growth of spoilage bacteria. CO_2 was induced to retard the growth of gram-negative bacteria, while O_2 was employed to inhibit the development of strictly anaerobic bacteria (Kostaki *et al*, 2009). Hence, the use of MAP and chill temperature can influence the spoilage microbial association of the "Specific Spoilage micro-Organisms" (SSO) (Dalgaard, 1995), or an even smaller fraction of SSO named "Ephemeral Spoilage Organisms" (ESO) (Nychas *et al*, 2008;

* To whom all correspondence should be addressed.
Tel.: +86 21 61900353;
E-mail: jxie@shou.edu.cn

Sallam,2007). Their actual contribution to quality deterioration largely depends on storage conditions (La Storia *et al*, 2012; Özoðul *et al*.,2000; Campos *et al*., 2005). Knowledge about the development of spoilage bacteria under different conditions and their activities help to predict more precise shelf life and has led to SSO-targeted inhibition treatments. Hence, characterization of spoilage bacteria communities is needed to understand the quality deterioration of MAP shrimp.

Recently, the study of microbial association in shrimp is typically characterized by identification of colonies plated on solid medium (Nirmal *et al*., 2011, Mejlholm *et al*, 2008, Martínez-Alvarez *et al*, 2009; Noseda *et al*.,2012). This method can provide a countable number of the cultivable community of bacteria and can isolate the colony for identification. However, the method of colony identification is time-consuming and laborious, and may overlook some uncultivable bacteria which exist in shrimp samples. To overcome the limitations, culture-independent method has developed to study the microbial association of food in variable storage conditions. 16S rRNA gene-targeted method, such as temporal temperature gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE) are useful to estimate the bacterial structure of samples. Individual bands represent individual bacterial species, which can be identified by comparison with the gene database . TGGE and DGGE have be applied to several food products including dairy products (Duan *et al*., 2010;Rasolofo *et al*, 2011), fermentation food (Sivertsvik *et al*., 2002; Ruiz *et al*, 2010), and vaccum/MAP-packaged pork (Jiang *et al*, 2010), beef (Ercolini *et al*, 2010; Vijayabaskar *et al*.,2008) and fishy products (Hovda *et al*, 2007, Mace *et al*, 2012;Huis,1996). Though Jaffrès, *et al*. (Jaffrès, *et al*.,2009;Park,1994;Bahmani *et al*.,2011) revealed the development of microbiota in tropical cooked and peeled shrimp stored in MAP by PCR-TTGE, few information has been found about the effect of O_2 on the microbiota changes in raw shrimp under MAP conditions.

In this study, we describe the structural changes of microbial communities of raw *L. vannamei*. The microbial flora of three batches of shrimps packaged in different O_2-concentrations was investigated in comparison with the batch of shrimp stored in air. Sensory evaluation was employed to identify the spoiled point of shrimp. PCR-DGGE were used to monitor the microbiota changes and bacterial population during storage.

MATERIAL AND METHOD

Preparation and storage of raw material

Pacific white shrimps (*L. vannamei*) with the size of 12~16g for each one were transferred from the local aquatic market nearby (Shanghai, P. R. China) to the laboratory in water. The alive shrimps were washed in ice water, then drained. Each PA/PE bags (180 ìm, 25cm×17cm; O_2 transmission 10~20 ml/m^2•d at 23~25 °C, 1 atm preasure) produced by Eno-Packaging (Shanghai, P. R. China) were filled with 30 shrimps. A food grade gas (A: packaged in 80% CO_2/ 15% N_2/ 5% O_2; B: packaged in 80% CO_2/ 10% N_2/10% O_2; C: packaged in 80% CO_2/0% N_2/ 20% O_2) was introduced into the packages before heat sealing with a heat sealing machine (model DQB-360W, Packing Machine factory of Qingpu, Shanghai, P.R. China).

Sensory evaluation

The shrimp samples were placed on a cleaned white porcelain plate after steamed with aluminum foil for 5 min. 30 panelists, the graduate students of College of Food Science and Technology, Shanghai Ocean University, Shanghai, P.R. China were selected to evaluate the organoleptic quality of the samples. Before the experiment, the panelists were trained to be familiar with the rating scales. The samples were scored by 9-point hedonic scales, from 9 = like extremely to 1 = dislike extremely.

Extraction of total DNA from shrimp

The headless shrimp samples (25 g) were homogenized for 5 min in 225 ml of saline water (0.85% NaCl), and agitated for 30 min at room temperature. The bacterial cells were obtained by centrifugation method according to Jiang *et al*. (Jiang *et al*.,2010).

PCR protocol

DNA of the shrimp sample was amplified with the universal primers F338 (5'- ACT CCT ACG GGA GGC AGC AG - 3') (Ampe *et al*, 1999) including a 40 base GC clamp (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG CCA CGG GGG G - 3')

(Muyzer *et al*, 1993) at the 5' end and R518 (5' - ATT ACC GCG GCT GG - 3') (Muyzer *et al*, 1993;Antonacopoulos *et al*.,1989) spanning the variable V3-region on 16S rRNA gene. PCR reaction was composed of 1 ìl template, 2 ìl each primer (10 ìM), 25 ìl Taq PCR Master Mix (1×, Sangon, China) and 20 ìl ddH_2O with the final volume of 50 ìl. The reaction was carried out on a Mastercycler personal (Eppendorf, Germany) using the following program: 30 cycles of 94 °C for 30s, 55 °C for 30s, 72 °C for 30s. PCR products were analysed and verified by 1.2% agarose gel electrophoresis, and then visualized by ethidum bromide staining.

DGGE analysis

The DGGE apparatus (DCode, Bio-Rad Ltd., USA) was used for DGGE analysis of the PCR V3 region of 16S rRNA products of bacteria. The procedure was performed according to (Hovda *et al*, 2007) with a little modification. A 0.75mm thick 20% (w/v) polyacryl-amide gel (37.5:1 acrylamide: bisacrylamide) containing a denaturation gradient of 40% ~ 65% urea-formamide was electrophoresed in 1× TAE (40mM Tris-Acetate and 1mM EDTA, pH 8.3) at 80 V for 30min, and than at 60 V for 15 h with 15 ìl of the PCR products. The DGGE gel was stained with 1 mg/l SYBR Green I (Solarbio Co., Ltd., China) for 20 min, rinsed ddH_2O and photographed under UV light using the GelDoc 2000 system (Bio-Rad, USA).

Statistic analysis

To study the effect of modified atmosphere packaging and time on sensory score, two factors such as time (0, 2, 4, 6, 8 and 10 days) and design (CK, A, B, C) were used for a factorial design. Three replicates were performed for each experiment. ANOVA analysis was performed by using SPSS computer package (SPSS Version 15.0, Inc, Chicago, IL, USA). Significance of differences was defined at ≤ 0.05.

RESULTS

Sensory evaluation

The changes of likeness score of shrimp in MAP conditions are shown in Table 1. In general, the sensory properties decreased during storage. The differences of sensory likeness scores between shrimp in MAP and in air became significant ($P < 0.05$) after 4 days storage. The color likeness scores of shrimp in 80% CO_2/5% O_2 and 80% CO_2/10% O_2 were higher than that in 80% CO_2/ 20% O_2 concentration (data not shown). However, the differences of sensory quality between 80% CO_2/5% O_2 and 80% CO_2/10% O_2 were not significant ($P > 0.05$).

Table 1. Changes in sensory scores of raw shrimp samples during refrigerated storage

Samples	Storage time (Days)					
	0	2	4	6	8	10
Air	8.85±0.13^a	7.88±0.15^a	5.25±0.26^a	3.88±0.28^a	3.23±0.38^a	1.75±0.65^a
5% O_2	8.85±0.13^a	8.20±0.29^a	7.13±0.22^b	6.43±0.21^b	5.85±0.26^b	4.93±0.19^b
10% O_2	8.85±0.13^a	8.23±0.34^a	7.53±0.13^b	6.68±0.28^b	5.75±0.26^b	5.13±0.25^b
20% O_2	8.85±0.13^a	7.98±0.30^a	6.43±0.38^c	5.33±0.25^c	4.93±0.35^c	3.78±0.22^c

*Different letters in the same column within the same treatment indicate the significant differences ($P < 0.05$)

Bacterial profiles of shrimp using DGGE

Bands a, b, d, f, g, h, j, k, l and m were visible on day 0; bands a, b, d, h, j and m faded out, while bands c, l, n and p became intense and bands e and q appeared after 10 days of storage when shrimp packaged in air. After 10 days of storage, bands a, b and d became intense in shrimp samples packaged in 5% and 10% O_2 atmosphere, and band i appeared while bands c, e, n, p and q faded out. The bacterial fingerprint profiles of shrimp under 20% O_2 atmosphere were unlike that of shrimp sample in air or in 5% and 10% O_2 atmosphere. Band e only could be visualized in the sample packaged in 20% O_2 atmosphere, while bands a and m faded out.

DGGE analysis enabled to visualize the evolutionary dynamics of microbiota of shrimps under MAP conditions during storage by examining fingerprints of dominant bacterial groups. 16S rRNA gene of bacteria obtained from

shrimp samples were amplified by PCR approach. A high bacterial diversity of shrimps were observed, evidenced by the presence of multiple bands (Fig. 1).

Individual bands showed in acrylamide gel were excised and re-amplified to sequencing. Partial sequencing detected *Vibrio albensis, Vibrio* sp., *Shewanella* sp., *Vibrio cholerae*, Uncultured

Table 2. Microbial species identification after sequencing of the variable V3 region of 16S rRNA gene purified from PCR-DGGE profiles of samples of shrimp (Fig.3)

Band no.	Closest relative in GenBank database	Accession number	Similarity (%)
a	*Vibrio albensis*	AB681432	99
b	*Vibrio* sp.	AM989317	100
c	*Shewanella* sp.	AB300600	99
d	*Vibrio cholerae*	AY494843	100
e	*Vibrio cholerae*	JN003627	97
f	Uncultured *Acinetobacter* sp.	JX301558	100
g	*Photobacterium damselae*	FJ161309	100
h	*Shewanella putrefaciens*	GQ372872	100
i	Uncultured bacterium	JQ480740	99
j	*Vibrio harveyi*	JX290081	99
k	*Listonella anguillarum*	JN601477	100
l	*Shewanella baltica*	AY771739	100
m	*Vibrio parahaemolyticus*	JX290081	99
n	Uncultured *Aeromonas* sp.	EF679187	100
o	*Shewanella putrefaciens*	AB680168	100
p	*Aeromonas veronii*	AY987778	100
q	Uncultured *Aeromonas* sp.	AB745445	97

Table 3. Sequencing of dominant bands in DGGE profiles obtained from direct extraction on DNA from the shrimp matrix

Day	Day 0	Day 10			
Bacteria/storage	X	Air	5%O_2	10% O_2	20% O_2
Vibrio albensis (#a)	×		×	×	
Vibrio sp. (#b)	×		×	×	×
Vibrio sp. (#c)		×			
Vibrio cholerae (#d)	×		×	×	
Vibrio cholerae (#e)		×			×
Uncultured *Acinetobacter* sp. (#f)	×	×	×	×	×
Photobacterium damselae (#g)	×	×	×	×	×
Shewanella putrefaciens (#h)	×		×	×	×
Uncultured bacterium (#i)			×	×	×
Vibrio harveyi (#j)	×		×	×	
Listonella anguillarum (#k)	×	×	×	×	×
Shewanella baltica (#l)		×	×	×	×
Vibrio parahaemolyticus (#m)	×	×	×	×	
Uncultured *Aeromonas* sp. (#n, q)		×			
Shewanella putrefaciens (#o)		×	×	×	×
Aeromonas veronii (#p)		×			

"×" represent to visible band existed in DGGE profile.

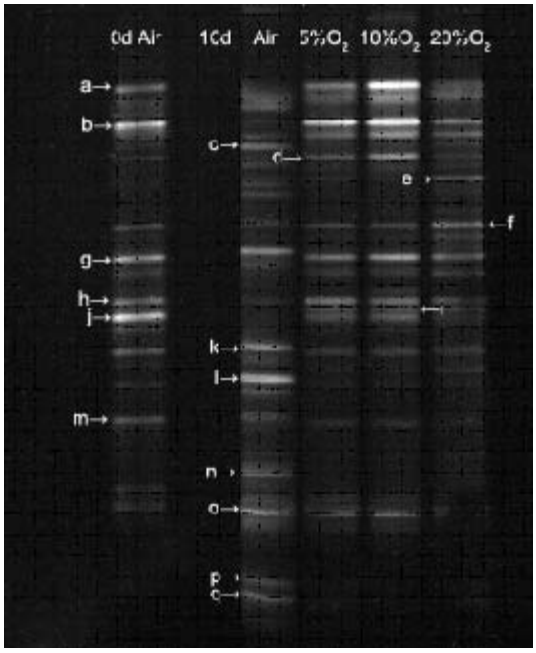

Fig. 1. DGGE profiles of 16S rRNA gene V3 regions obtained by PCR amplification from shrimp samples stored on day 0 and day 10. Bands indicated by numbers from a to t were subjected to sequencing

Acinetobacter sp., *Photobacterium damselae*, *Shewanella putrefaciens*, Uncultured bacterium, *Vibrio harveyi*, *Listonella anguillarum*, *Vibrio parahaemolyticus*, Uncultured *Aeromonas* sp. and *Aeromonas veronii*, respectively (Table 2). The bacteria flora changes were observed towards bands shown in Table 3.

DISCUSSION

In this study, the sensory scores were used to determine the quality of shrimp stored aerobically or under modified atmosphere.

Regarding to the DGGE profiles, *Vibrio albensis*, *Vibrio* sp., *Photobacterium damselae*, *Shewanella putrefaciens*, *Vibrio harveyi* and *Vibrio parahaemolyticus* were predominate bacteria in fresh shrimp. These *Vibrio* bacteria are mainly pathogenic bacteria to fish and shrimp (Rajeswari *et al*, 2012; Gram,2002;Cadun *et al*.,2005;Souza *et al*.,2010). *Vibrio* sp., *Shewanella* sp., *Photobacterium damselae*, *Listonella anguillarum*, *Shewanella baltica*, Uncultured *Aeromonas* sp. and *Aeromonas veronii* were able to grow in refrigerated temperature in shrimp samples packaged in air, while MAP condition could inhibit the growth of *Listonella anguillarum*, *Shewanella baltica*, Uncultured *Aeromonas* sp. and *Aeromonas veronii*. However, *Vibrio albensis*, *Vibrio* sp., *Vibrio cholerae* (#e) and *Shewanella putrefaciens* (#q) could still proliferate in MAP-shrimps and became dominant bacteria.

Our research indicated that MAP could retard the growth of spoilage bacteria and deterioration of shrimp during storage at 4 °C. In samples from 80% CO_2/ 20% O_2, the growth of *Vibrio albensis*, *Vibrio cholerae* and *Vibrio harveyi* were inhibited. The inhibitory effect of 80% CO_2/5% O_2 and 80% CO_2/10% O_2 atmosphere was targeted to *Aeromonas veronii* and Uncultured *Aeromonas* sp.. Considering the effect of MAP on sensory quality indicators, low O_2-concentraion atmosphere packaging was recommended. In further study, a combination method of antibacterial packaging should be employed to enhance the inhibitory effect on spoilage bacteria.

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