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Evaluation of effect of white spot virus infection on nutritive quality of shrimps and human health from Majmaah province, Saudi Arabia

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Abstract: The white spot disease is highly contagious disease caused by white spot virus (WSSV). It is one of the most damaging viral diseases which causes high morbidity and mortality rates in commercially important edible crustaceans. This disease causes serious economic losses to the shrimp farming industry worldwide. This disease is not transmitted from infected shrimps or other crustaceans to human after their consumption as seafood products. However, little information is available in terms of the evaluation of the nutritive quality of edible muscles of infected organisms. So, the aim of the current study was, therefore, to estimate the concentrations of total protein percentage, level of essential amino acids, protein band electrophoresis and to detect DNA damage induced by WSSV in muscle cells of shrimps by using Comet Assay. In conclusion, regarding the results obtained in the present study in light of nutritional point of view of commercial shrimps, WSSV infection triggers alterations in biochemical components and strongly affects DNA of edible muscles of shrimps. In addition, WSSV is predicted to affect several basic cellular metabolic processes of shrimps. So, WSSV infected shrimps cannot be used as healthy food for human consumption**.**

Keywords: White spot virus, shrimps, Edible muscles, Nutritive quality, DNA damage

INTRODUCTION

Shrimp species is an important popular edible commercial crustacean's species that found in virtually all parts of the world. They are considered popular dietary components of world dishes. Shrimps contribute about 20-22% by volume of the world seafood market[1]. There is a vast scope for the shrimp meat due to its sweat delicate flavor and its high nutritive value. The edible muscles of shrimp species contain beneficial healthy components that play an important role in maintenance of the physiological and biochemical activities of human and other organisms. These biochemical components include protein, carbohydrates, lipids, essential fatty acids, essential amino acids, vitamins and several dietary minerals such as Zn, Na, K, Se, Ca, Fe, etc. [2-6] So, shrimp species as seafood product is considered to be healthy diet choice.

Certain parasites can cause severe diseases in shrimp such as white spot syndrome virus (WSSV). WSSV is a rod shaped double stranded DNA virus. Since this virus first appeared in 1992 in shrimp farms in northern Taiwan, WSSV has become the most devastating viruses infecting penaeid shrimp[7, 8]. All decapod crustaceans (order: Decapoda), including shrimps, lobsters, mantis shrimps and crabs from salt water, brackish water and fresh water environments are considered to be susceptible to WSSV. Furthermore, WSSV has also been detected in a number of non- decapod crustaceans and other species[9]. WSSV affects all life stages of shrimps and is characterized by the rapid onset of high mortalities; mortality rate can reach 100% within 3–9 days of the onset of clinical signs[10].

The WSSV is primarily spread to the local natural environment which constitutes a substantial risk through contaminated water, disposal of infected imported shrimp, unregulated processing or movement of infected shrimps[11, 12]. Furthermore, frozen farmed shrimp sold in the market might be a potential avenue for the introduction of WSSV infection due to shrimp viruses may remain viable in frozen shrimp[13,14]. In the beginning of WSSV infection, the epidermis, antennal gland, gills, gonads, foregut, hindgut, lymphoid organ, cells associated with the nervous system and hematopoietic cells are considered the major targets for WSSV infection. While, the gills, integument and the epithelia of the stomach may become severely damaged in the late stages of infection. This may cause multiple organ dysfunctions and probably lead to death[12, 13]. The most obvious morphological symptom of WSSV infection is represented by the accumulation of calcium salts within the cuticle of cephalothorax and tail part in the form of circular white spots or patches of 0.5-3.0 mm in diameter [14, 15]. In some cases, moribund shrimp may also display reddish-brown / reddish / pinkish / to discoloration over the head and carapace[16]. Rapid reduction in food consumption is also observed in WSSV infection. Furthermore, WSSV provoked some biochemical and hematological changes in infected shrimps[17, 18].

With this background in the present study, an attempt has been made to analyze biochemical composition of edible muscle of infected shrimps to estimate the effect of WSSV infection on the nutritive quality and provide detailed information on nutritional value of the edible muscle tissues of infected shrimps in particular protein, essential amino acids, protein electrophoresis. Additionally, to evaluate DNA damage in muscle tissues to detect the safety of WSSV infected shrimps for human consumption as seafood product.

MATERIAL AND METHODS

Collection of experimental animals: Healthy and infected adult shrimp samples were collected from fishermen from Majmaah province, Saudi Arabia. A clear WSSV-infected shrimp sample collected based on the typical clinical symptoms. An attempt was made to collect consistent size ranges. Samples were washed with deionized water to remove any adhering contamination and drained using filter paper. Samples were put in crushed ice in insulated containers and brought to the laboratory for analysis. The samples on reaching the laboratory were repacked in small quantities (10 samples), in order to avoid repeated thawing and freezing. They were labelled and stored in an Ultra-freezer.

Separation of Muscle tissues away from Exoskeleton: As in most crustaceans, fresh whole bodies of all samples of shrimps were stored at -20^0 C to facilitate peeling process after thawing when needed. After defrosting, the shrimps were separated into the exoskeleton (head and the outer body shell) and the endoskeleton (edible abdominal muscles).

Biochemical Analysis:

(1). The total protein was estimated by method of Lowry *et al*.[19]. The total protein percentage was estimated as per the Folin-Ciocalteu method with bovine serum albumin (BSA) as standard according to the following steps: 1g of wet muscle tissue was homogenized in Homogenizer with 10 ml of 0.1 M phosphate buffer. Take 1 ml of tissue homogenate, 1 ml of 1 N NaOH and keep it for 30 minutes, at room temperature, now add 8 ml of distilled water and centrifuge at 4000 rpm for 30 minutes. Take only 0.1 ml supernatant and add 0.9 ml of distilled water to make volume 1.00 ml. Add 5 ml of alkaline reagent (2 g Na2CO3 in 0.1 N NaOH: 4% Na-K tartarate 2% CuSO4, 200:1:1) leave it for 30 minutes at room temperature. Add 0.5 ml of Folin phenol reagent; leave it for 40 to 45 minutes at room temperature. The color intensity was measured at 750 nm against reagent blank

(2). Amino acids were measured by high performance liquid chromatography (HPLC); Beckman 6300 amino acid analyzer[20]. The profile of amino acids was done following high performance thin layer chromatographic (HPTLC), the muscles were dried (80^0 C for 3 hrs.), digested with 6 M aqueous hydrochloric acid and dried under vacuum. The powdered sample was dissolved in distilled water and 5 µl of sample was loaded on 8 mm thick pre-coated Silica gel. The plate was developed in butane-Ammonia-Pyridine-Water (3.9:1:3.4:2.6) mobile phase. The plate was sprayed with ninhydrin reagent prepared in propan-2-ol and dried. The developed plate was documented using photo-documentation chamber (CAMAG-REPROSTAR 3) at UV 254 nm and UV366 nm lights, the plate was scanned at 500 nm. The peak area of the sample was compared with standard amino-acids and quantified.

(3). Electrophoretic separation of proteins: Polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was carried out using silver stain protocol[21]. Muscle samples (3g) were homogenized with 27 ml of solubilizing agent (2% SDS, 8M urea and 2% β-mercaptoethanol), followed by heating at 85 ^{o} C for 1h. Then the homogenate was centrifuged at 10,000 rpm for 15min at room temperature. The protein concentration of supernatant was obtained by the method of Lowry *et al*.[19]. Protein patterns of different fractions were determined using sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), with 115 12 % running gel and 4 % stacking gel.

(4). Dendrogram analysis: Dendrogram was constructed using UPGMA cluster analysis using simple band match (Tolerance 3.20%) to reveal the relationship between the normal healthy shrimps and WSSV infected shrimps. It determines the degree of similarity between the examined extracts of the muscle of

normal and WSSV infected shrimps according to the position and density of different protein bands in the electrophoretic analysis

(5). The Comet Assay: The Comet Assay is a single cell gel electrophoresis assay for evaluating DNA damage in cells. The premise is that damaged DNA becomes fragmented. Increasing amounts of DNA damage results in increased number of fragments and smaller fragments based on gel electrophoresis. This technique[22] was carried out for 10 infected samples and 10 normal healthy samples according the following steps:

1 gram of crushed muscle samples were transferred to 1 ml ice cold PBS. This suspension was stirred for 5 min and filtered. Cell suspension (100 µl) was mixed with 600 µl of low –melting agarose (0.80% in PBS). 100 µl of this mixture was spread on pre-coated slides were immersed in lysed buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2V/ cm for 2 min and 100 m. A staining with ethidium bromide 20µg / ml at 4°C. The observations was with the samples still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescent microscope (with excitation filter 420-490 nm (issue 510 nm). The comets tail lengths were measured from the middle of the nucleus to the end of the tail with 40 X increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of EtBr- stained DNA using a 40 X although any image analysis system may be suitable for objective on fluorescent microscope. The quantitation of SCGE data, Comet 5 image analysis software was used that developed by Kinetic Image, Ltd (Liverpool. UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates the tail length which is the distance from the comet head to the last visible signal in the tail. Furthermore, the percentage of DNA in the tail is calculated from the fraction of DNA in the tail divided by the amount of DNA in the nucleus multiplied by 100, while tail moment which is the product of the amount of DNA in the tail and mean distance of migration in the tail, also the program calculates tail DNA percent that is represented by the integrated tail intensity x 100 divided by the total integrated cell intensity for a normalized measure of the percent of total cell DNA found in the tail[22]. Generally, 50 to 100 randomly selected cells are analyzed per sample.

Statistical Analysis: The obtained data were used for descriptive statistical analysis consisting of means ± standard deviation of 10 separated determinations. In order to test the significance of the differences, one –Sample T Test was used. Means with the same letter for each parameter are not significantly different, otherwise they do ($P < 0.05$). All the analyses were performed by using SPSS statistics, for Windows (Version 15.0).

RESULTS AND DISCUSSION

The total protein percentage of the edible muscle tissues of examined samples is presented in **Fig. 1**. The protein percentage in WSSV infected shrimps was lower (19.24 % ± 1.03) than normal healthy shrimps (45.40% ± 1.32). Statically this decrease was significant ($p < 0.000$). In general shrimps species are considered an extremely good source of protein making them a very healthy choice of edible crustaceans as seafood products for human consumption. Some researchers detected that the protein level varied in shrimps species. Its level started[23, 24] from 39, 45 % to 52, 70 %. In the present study, nutritional analysis of muscle of normal healthy shrimps detected that the presence of a high amount of protein that in turn

indicates their high nutritive value for human. Whereas, the edible muscle of WSSV infected shrimps contained lower percentage of protein. This sharp decrease in total protein due to WSSV infection was also recorded in muscles of WSSV infected different species of shrimps such as *Penaeus indicus* (Indian white shrimp)[17], *Litopenaeus vannamei* (white legged shrimp)[25] and *Penaeus monodon* (black tiger shrimp)[26].

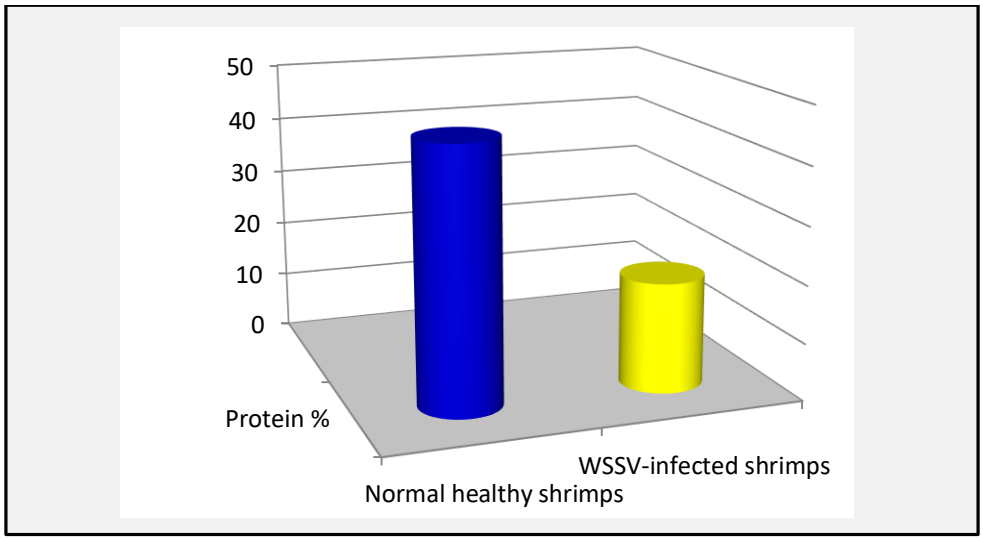

Figure 1: Total protein percentage in edible muscle tissues of normal healthy and WSSV infected shrimps

Analysis of essential amino acids (EAAS) in the present study declare the presence of 10 EAAS (arginine, histidine, methionine, leucine, isoleucine, lysine, valine, threonine, phenylalanine and tyroptophan) in edible muscle portions of studied samples. Furthermore, the present results showed that there was significant differences ($p < 0.000$); the concentrations of determined EAAS had higher mean values in non-diseased shrimps comparable to WSSV infected shrimps (**Fig. 2**).

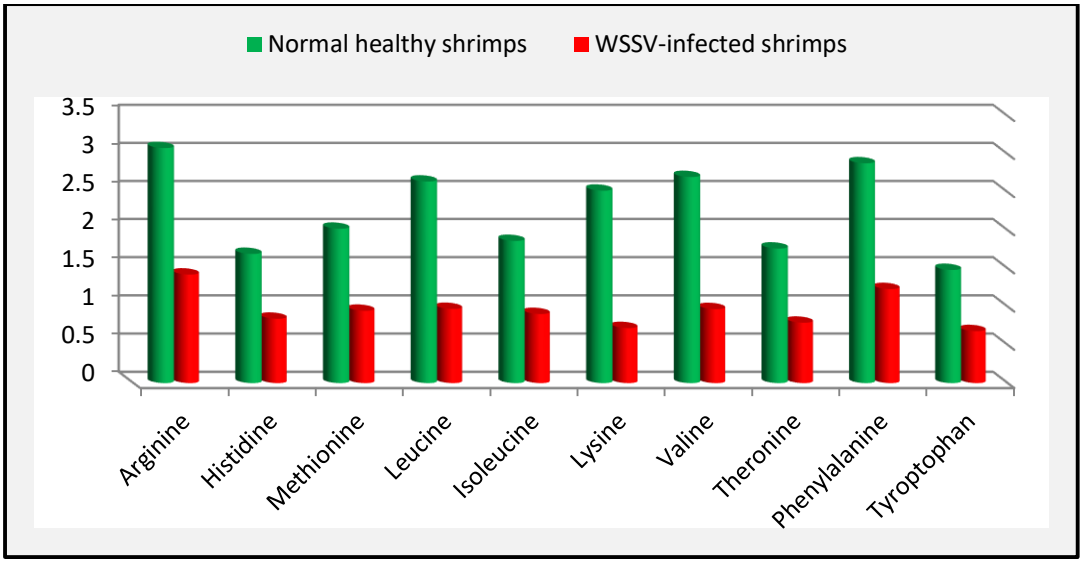

Figure 2: Essential amino acid contents (mg/ 100g) in edible muscle tissues of normal healthy and WSSV infected shrimps

Additionally, according to the concentrations of EAAS in determined muscle tissues, the arrangement of EAAS was recorded according to the following orders: arginine > phenylalanine > valine > leucine > lysine > methionine > isoleucine > theronine > histidine > tyroptophan, and arginine > phenylalanine > leucine > valine > methionine > isoleucine > histidine > threonine > lysine > tyroptophan in normal healthy shrimps and infected shrimps respectively. Generally, amino acids serve as body builders due to they are the units of proteins. They played an important role in physiological functions of aquatic organisms[27, 28].

The nutritive value of any animal used as food product is decided by the presence of EAAS[29]. The EAAS are required for maintenance of growth, isosmotic intracellular regulation, source of energy production, neurotransmission, reproduction, synthesis of vitamins, and sustenance of life[27, 28]. The reduction of protein and EAAS contents in muscle portion of WSSV infected shrimps might be attributed due to WSSV encodes a variety of proteases and other enzymes that dissolve the tissues. Inasmuch as rapid tissue autolysis, the proteins of the "melted" muscle and hepatopancreas cells would be integrated into the shrimp lymph [25, 30].

The protein electrophoretic analysis of edible muscle of normal and WSSV infected shrimps was illustrated in **Fig. 3 and Table 1**. The present data indicate considerable variations in band numbers at molecular weight and intensity of protein band. The electrophoretic bands of normal shrimps included 18 bands with molecular weight ranged from 142.30 to 6.90 KD. Whereas, in infected shrimps 16, 17 and 21 protein bands were observed around molecular weight 189.80 to 9.10, 180.10 to 6.40 KD and 178.20 to 9.00 KD respectively. The dendrogram analysis which showed the similarity between the examined extracts of the muscle of normal and WSSV infected shrimps according to protein bands in the electrophoretic analysis was recorded in **Fig. 4**

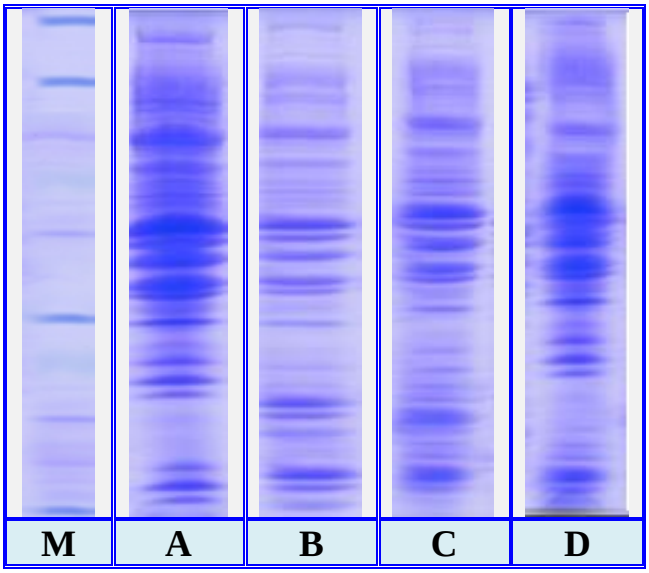

Figure 3: Electrophoretic pattern of muscle proteins of shrimps: M: marker; A:normal healthy shrimp and B, C, D: WSSV infected shrimps

Table 1: SDS-PAGE gel electrophoresis of edible muscles of normal healthy and WSSV infected shrimps

Lanes	MARKER		Normal healthy shrimp		WSSV infected shrimps					
Bands	**KD**	**%**	**KD**	**%**	**KD**	**%**	**KD**	**%**	**KD**	**%**
1	200.	10.3	142.3	9.0	189.8	2.8	180.1	1.6	178.2	1.7
2	116.00	11.3	112.4	1.8	171.8	3.4	119.7	2.9	127.5	9.2
3	67.0	19.0	92.7	4.0	119.7	4.9	98.0	4.1	109.0	2.3
4	45.00	6.70	66.2	2.2	72.2	4.6	67.8	8.5	90.4	4.2
5	29.	9.49	56.9	6.7	61.1	7.1	57.1	4.1	79.8	3.1
6	21.	13.7	47.3	12.7	54.3	6.4	50.7	6.2	67.0	2.6
7	12.5	11.88	45.8	6.1	46.8	9.7	44.6	15.2	63.8	1.2
8	6.50	17.7	41.4	9.9	38.7	11.4	37.6	14.8	58.3	5.4
9			37.4	9.3	34.6	10.1	32.1	8.6	54.7	1.9
10			34.8	4.4	30.8	1.9	28.0	11.7	51.9	4.8
11			32.3	8.4	29.0	2.3	24.9	3.2	49.2	4.3
12			30.7	4.9	25.6	3.5	22.9	1.2	45.8	7.8
13			24.3	4.1	22.4	6.5	21.4	5.3	40.4	1.4
14			22.7	2.3	21.4	3.7	18.9	2.3	36.8	7.2
15			16.0	1.5	17.5	4.6	14.8	3.0	33.4	8.4
16			12.8	2.5	9.1	17.3	10.3	5.4	30.7	2.8
17			7.7	6.0			6.4	2.1	27.9	7.2
18			6.9	3.8					24.0	4.2
19									22.7	4.3
20									21.3	1.8
21									9.0	14.2
Sum		100		100		100		100		100
In Lane		100		100		100		100		100

The dendrogram analysis showed the similarity between infected shrimp samples was with 77.78% and 82.35%. While, 66.61% and 52.25% similarity was recorded between them and uninfected shrimps. The variation in protein bands might be explained due to sex variation or to physiological factors such as nutritional state, molting cycle, size of animal, feeding season, environmental conditions, infection, etc.… as reported by[31]. Furthermore, the variations in protein contents in edible muscle tissues of marine organisms might be attributed to the phenotype, genotype and physiological variations [32, 33]. The alternation of biochemical components and variation in protein bands of infected shrimps indicates that how WSSV subverts and exploits the metabolic process and biochemical components of shrimps to benefit its multiplication and life sustenance. In this respect, the previous results of [34] were reported that WSSV relies on certain proteins of shrimp cells related events to facilitate its infection process and its replication in infected cells. Some proteins of infected cells of shrimps are probably being used by the virus to regulate host mitochondrial functions during WSSV pathogenesis. Then, WSSV induced

variations in infected cells in several metabolic pathways that are related to the mitochondria. These metabolic changes, in turn induced variations in infected cells resembling the Warburg effect. The Warburg effect is represented by an abnormal glycolysis response in cancer mammalian cells.

Figs. 5, 6 & 7 illustrated quantitative DNA damage due to WSSV infection through Comet Assay. This quantitative technique is based on the quantification of denaturized DNA fragments. In case of DNA damage, these fragments migrate out of the eukaryotic cell nucleus during electrophoresis. In WSSV infected cells DNA is broken. It forms a tail (broken DNA) that moves away from the unbroken DNA (the head). The estimation of the amount of DNA damage in infected cells was determined from comet tail length as the extent of migration of the genetic material. Furthermore, the level of DNA damage was estimated using an image analysis package and expressed as % tail DNA. Additionally, tail moments (product of distance and normalized intensity integrated over the tail length) was estimated. Regarding, the present results, as shown in **Fig. 5**, mean value of tail length in infected samples (4.37 µm ± 0.33) was increased significantly ($P < 0.000$) comparable with normal healthy shrimps (3.17 µm ± 0.23). Moreover, significant increases ($P < 0.000$) in tail moments of infected shrimps (20.76 µm ± 1.78) were recorded in comparison with normal healthy shrimps (11.72 µm ± 0.96) (**Fig. 6**). Also, the present results in **Fig. 7** reveal that the intensity of tailed percentage (20 % ± 1.58) and tail DNA percentage (4.48 % ± 0.39) of infected shrimps were higher than non-diseased shrimps (14 % ± 1.50 and 3.32% ± 0.1 respectively). Statistically these increases were significant ($P < 0.000$). On the other hand, the DNA head portion percentage (the unbroken DNA) (84.80% ± 3.42) was significantly decreased ($P < 0.000$) comparable with normal healthy shrimps (93% ± 2.42).

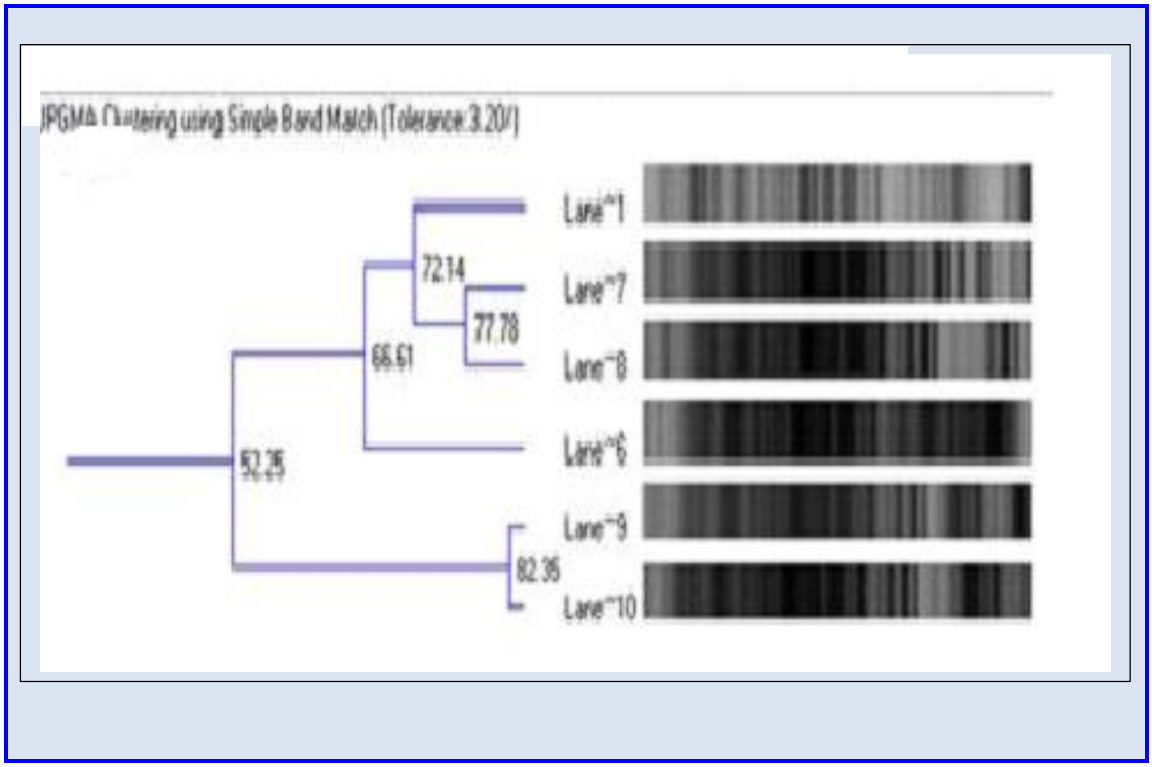

Figure 4: Dendrogram analysis of SDS-PAGE gel electrophoresis of muscle proteins of normal healthy (Lane˜ 1) and WSSV infected shrimps (Lanes˜ 6-10), using UPGMA cluster analysis using simple band match (Tolerance 3.20%)

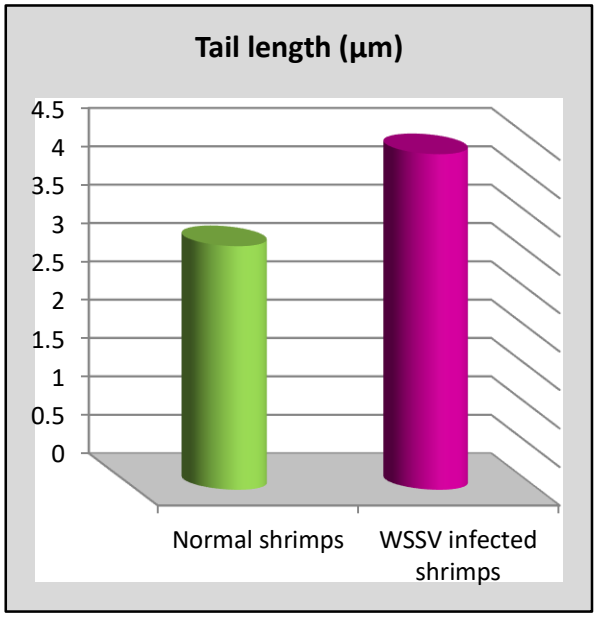

Figure 5: Tail length(µm) (broken DNA) of normal and infected shrimps.

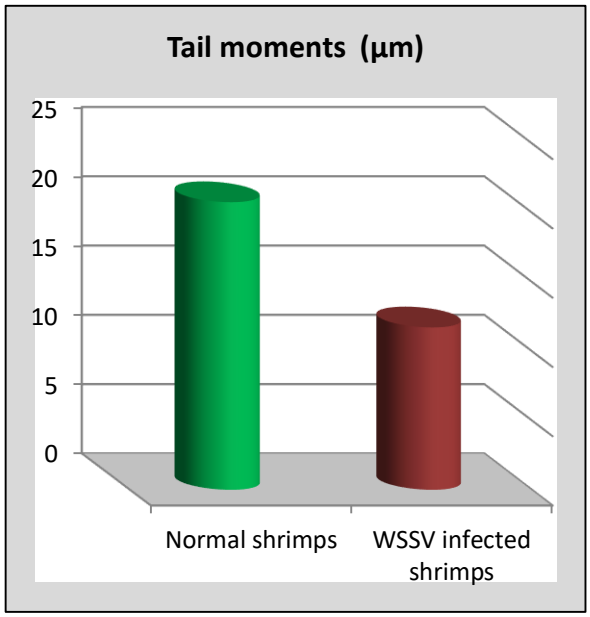

Figure 6: tail moments (µm) (product of distance and normalized intensity integrated over the tail length) of normal and infected shrimps.

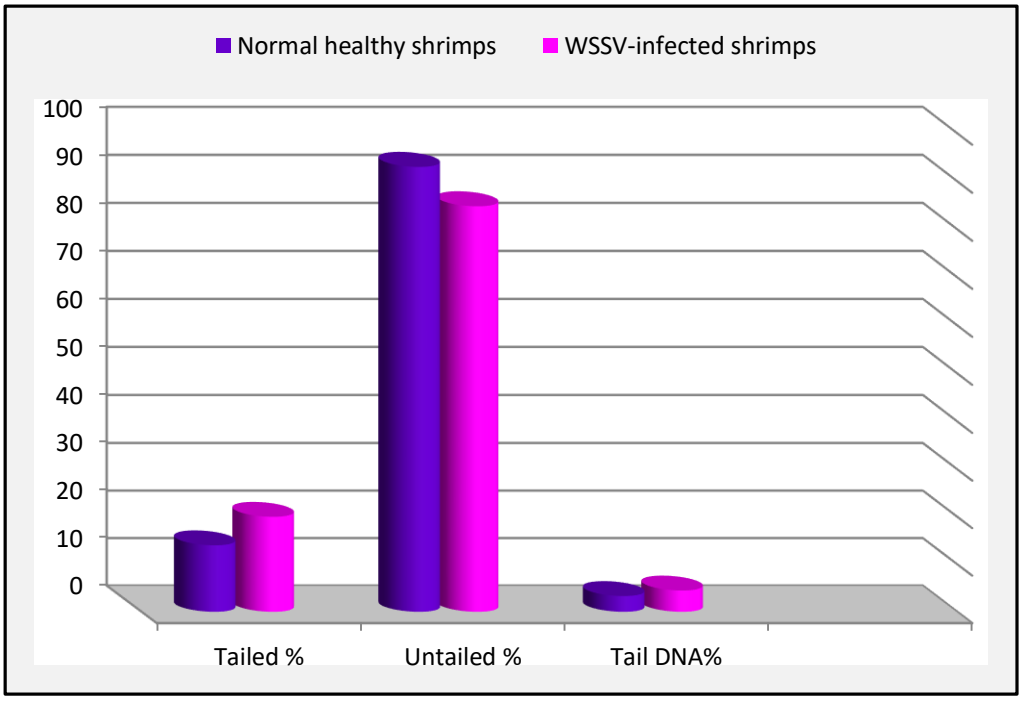

Figure 7: Intensity percentages of tail, untailed (head) and tail DNA in normal and WSSV infected shrimps

DNA is the hereditary material that carries the genetic information for the survival and life sustenance of living organism due to it used in the functioning, growth, development, protein synthesis and reproduction of all known living organisms. For detecting and analyzing DNA strand damage in various cells, Comet Assay is used. This technique is considered simple, sensitive, visual, uncomplicated and rapid technique. Furthermore, the Comet Assay is now widely used in researches of DNA damage processes to routine estimation of damage to genetic materials (genotoxicity) [35, 36]. In the Comet Assay process, when a charge is applied to a cell, the breaks in DNA strands due to DNA damage allow DNA fragments to move from the nucleus towards the anode. The amount of DNA that leaves is represented an indication of the amount of DNA damage. Which in turn resulting in (Comet) formation[22]. Additionally, the frequency of DNA strand breaks has been proportional to tail intensity which is represented by percentage of DNA in the tail[34]. The migration tail length as well as the fraction of DNA migrated in the tail is estimated through tail moment which is considered a simple descriptor calculated by the computerized image analysis system[35]. According to Comet Assay in the present study, it was detected that WSSV infection induced severe damage of DNA of edible muscle tissues of infected shrimps. This finding is in agreement with results of previous study[36] which was reported that WSSV infection modulates expression of various kinds of genes in different tissues (cuticular epidermis, muscles, hepatopancreas, stomach and eyestalk) of shrimp species. The researchers found that at the late stage of WSSV infection, the cuticular epidermis of *Penaeus monodon* is heavily infected with WSSV; becomes necrotic and characterized by presence of white spots. These white spots are represented by abnormal deposits of calcium salts as recorded previously by[37]. The appearance of white spots in the cuticle of WSSV infected was explained by[36] as the result of the WSSV controls the regulation of gene expressions of various cuticular proteins in infected *Penaeus monodon* shrimp cells. Their results showed that WSSV infection induces abnormal production of certain cuticular protein known as crayfish calcification-associated peptide-1 (CAP-1). It has been found that CAP-1 protein has most importantly anti-calcification activity and the abnormal production of it leading to the formation of white spots in the cuticle of infected shrimps as described by[38]. Furthermore, the same authors[36] attributed the reduction of food consumption in WSSV infected *Penaeus monodon* shrimps due to WSSV mainly infects the myoepithelial cells of the hepatopancreatic sheath and subvert their physiological functions (such as gene transcription), i.e.. The RNAs of the digestive enzymes reduced after WSSV infection that in turn leads to abnormal expression of digestive enzymes in hepatopancreas then this, together with the presence of WSSV in stomach explain why WSSV-infected shrimp reduce their food consumption. Additionally, these authors recorded that in WSSV infection in *Penaeus monodon*, however, their muscles are only lightly infected by WSSV, the infection affects strongly on transcription of several genes which are particularly and/or highly expressed in the muscles. This reduction of gene transcription leads to alternation of metabolic processes of muscles including oxidative phosphorylation, glycolysis, and protein synthesis. This variation of physiological functions of muscles of infected shrimps due to WSSV infection in turn explains the decrease of protein level, essential amino acids concentrations and variation in electrophoretic protein bands comparable to normal healthy shrimps which are recorded in the current study.

CONCLUSION

WSSV is considered the fatal virus among the lethal viruses infecting penaeid shrimp. It is a fast proliferating that causes massive mortalities resulting in a rapid decline in the shrimp production and great economic losses to the shrimp industry. The present study quantified the concentration of protein percentage, essential amino acids, protein bands of edible muscles of WSSV infected shrimps to evaluate the nutritional quality of infected shrimps as seafood product for human consumption. Furthermore, the estimation of damage of DNA in edible muscles of infected shrimps was taken place to determine the effect of WSSV infection on genetic materials in infected edible muscle portions of shrimp. The results of the current study showed significant differences in the biochemical components of infected shrimps comparable to normal healthy shrimps. There was sharp reduction of total protein and EAAS of infected muscles, also variations in protein band numbers was observed. Furthermore, according to Comet Assay analysis, it was detected that WSSV infection triggers alterations in DNA of muscle portions and induced severe damage to genetic materials of infected muscle cells comparable to normal healthy shrimps. The biochemical and DNA alternations indicated that WSSV subverts and exploits the metabolic process and biochemical components of shrimps to benefit its multiplication and life sustenance. Thus, in accordance with the above results it can be concluded that WSSV infected shrimps cannot be used as a healthy seafood products for humans. The current work can thus be taken as a basis for further WSSV researches on the estimation of active components in WSSV infected shrimps to evaluate the alternation in these components which resulted from DNA damage that in turn will illustrate also, the consumer health risks associated with eating imported farmed WSSV infected shrimps.

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