HISTOPATHOLOGICAL ALTERATIONS IN THE WHITE LEG SHRIMP *L. VENNAMEI* SUPPLEMENTED WITH FARM ISOLATED PROBIOTIC (*LAB*) UPON CHALLENGE WITH WHITE SPOT SYNDROME VIRUS (WSSV)

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Abstract:

A 21-day feeding trial followed by an injection with inoculum of White Spot Syndrome Virus were performed to evaluate the effect of farm isolated feed probiotic (*LAB*) on some tissues of the white leg shrimp *L. vennamei*. The effect of probiotic supplementation on the histopathological changes in *L. vennamei* juveniles (6.0± 0.02 g) challenged with known pathogenic strain of WSSV are reported. The histological observations revealed that probiotic supplementation enhances the thickness of the gut epithelium, number of villi. Hepatopancreas exhibits star shaped lumen with numerous tubules. Gills also exhibited clear gill lamella occluded with numerous haemocytes. But after infection there are significant changes in the hepatopancreas, mid gut and gill tissues of both the experimental and the control groups. These changes are minimal in probiotic supplemented animals compared to controls which were fed without probiotic that indicates the present probiotic bacterium is a safe candidate probiont for the host. Our observations from this study stated that probiotic supplementation improves the histological status of the tissues by lessening the histopathological indices due to WSSV infection in the marine shrimp.

Key words: Histopathological Indices, Probiotics, Marine Shrimp, WSSV

Introduction

Assessment of histopathological indices provides insight into the degree of stress, susceptibility, and adaptive capability of the stressed organism. Hepatopancreas is one of the indicators in the shrimp body that can be used to assess the health condition of the shrimp. As its name reveals it carries out the functions of both liver and pancreas. It is considered as digestive gland of Crustaceans. It is chiefly composed of branched tubules and these tubules are lined by different types of epithelial cells (Wu and Yang, 2011). By observing the shape and colour of the hepatopancreas we can display the health condition and identify the severity of problems that affected the shrimp. If the hepatopancreas is solid triangular shape with brown colouration it can be considered as healthy with optimum appetite. We can suspect that shrimp has infected when the hepatopancreas appeared to be shrinkage in size and mixed blue and brown colouration. This infection may alter the texture of the hepatopancreas making it less solid and turns into mixed texture between solid and slimy and has whitish blue coloration which caused the animal unable to take food. The hepatopancreas consists of numerous blindly ending tubules, held together lightly by basophilic connective tissue strands, which afford an enlarged surface area for digestion and absorption. Each tubule has a lumen in the centre, which is lined by an epithelium of one cell thickness except in the distal blind end.

The histology of hepatopancreas which plays a key role in food assimilation was studied by Dall and Moriarty (1985). Mature male intestine histology shows a layer of connective tissue that cover the longitudinal and circular muscles. The wall of the intestine is lined internally by a thin cuticle. At the centre of the cuticle a layer of columnar epithelial cells presents in the form of ridges. The cytoplasm of these cells is clear with an elongate nucleus, which is located either centrally or basally. The main challenge for shrimp industry is diseases, that can cause annual loss of 22% of shrimp population in the world (Valderrama and Anderson, 2011).

WSSV infected shrimps exhibit symptoms like lethargic behaviour, cessation of feeding, followed by the appearance of moribund shrimp, near the surface at the edge of the pond (Walker and Mohan, 2004; Wang et al., 2008; Afsharnasab, 2012). Severely infected shrimps are reported to exhibit a rapid reduction in feed intake and have a loose cuticle with white spots (hence the name "White spot" disease) of 0.5 to 2.0mm in diameter. These white spots are formed as a result of abnormal deposition of calcium salts by the cuticular epidermis (Anon, 1995; Wang et al.,1995; Lightner,1996a). Symptoms of white spot syndrome in penaeid shrimp are histopathologically characterised as widespread chromatin margination, severe nuclear hypertrophy and the presence of large conspicuous intranuclear eosinophilic Cowdry type-A inclusion bodies in the tissue. (Pazir et al., 2012).

Previous studies on *M. japanicus* and *P. monodon* that infected with WSSV reported high mortality percentage (70-100%). The intensity of virulence of WSSV differs based on sensitivity of the species and their defensive mechanism and environmental factors of the studied area (Pazir et al., 2012). Cowdry inclusions in WSSV infected shrimp represent an early stage of viral infection. After infection nuclei undergo further degeneration and finally modified into prominent eosinophilic and pale basophilic type inclusions (Perez et al., 2005). Microscopic examination of hepatopancreatic tissue of WSSV infected shrimp indicated vacuolization of tissue due to increased haemolymph from this organ to promote immune system of cell (Afsharnasab et al., 2009a).

Materials and Methods:

Sample collection and preparation of histological sections:

The technique for preparation of histological sections and staining was followed as per the method described by Rajendran (1999). The shrimps were injected with 1ml of Davidson's fixative into hepatopancreas, region anterior to hepatopancreas, cephalothorax, anterior abdominal and posterior abdominal regions. After injecting the fixative, the cuticle was dissected from the sixth abdominal segment to the rostrum with a sharp scissor without damaging the internal organs. The specimen was immersed in 100ml of fixative for 24 hours, and collected the hepatopancreas, intestine and gill tissues carefully.

These tissues were dehydrated through ascending rates of alcohol, clearing of tissue, impregnation with paraffin and embedding. The tissue embedded paraffin blocks were trimmed to suitable size and sections of 5µ thickness were taken with the help of a microtome in the form of a ribbon. The ribbons holding tissue sections were cut into smaller pieces and kept in water bath comprising warm water. The sections were deparaffinized and dehydrated and then stained with hematoxylin and eosin (H & E). The tissue sections were examined under trinocular microscope at 400X magnification for histological changes.

Results and Discussion:

Hepatopancreas:

The hepatopancreas (HP) is a vital and chief organ of a decapod crustacean that performs many of the functions of the liver, pancreas, intestine and other organs in the vertebrates (Boonyaratpalin, 2001).
Hepatopancreas is a huge and physiologically very important gland with many tubules. Normal hepatopancreas in shrimps contain several tubules with star shaped lumen lined with various types of epithelial cells. The wall of the tubule consists of four layers: the inner most layer of epithelial cells; a thin basement membrane on which epithelial cells rest and the outer most layer or tunica propria formed a network of connective tissue and muscle fibers. The hepatopancreas of experimentally infected shrimp showed vacuolization of the whole tissue as stated by Wang et al., (1999a). The four different cells- embryonic cells (E- cells), storage /absorptive cells (R-cells), the large vacuolated excretory cells (B-cells), and the fibrillar cells (F-cells) which line the hepatopancreatic tubules were not distinguishable in the moribund animals.

Our observations (Fig:1-3) shown that the infected hepatopancreas exhibited sloughing, karyomegaly or enlargement of the nuclei, collapse of the hepatopancreas tubules, and degeneration of the tubule lumens, whereas healthy hepatopancreas showed intact E, B, R epithelial cells, and still rounded epithelial tubule instead of degraded or in necrosis conditions. The destruction of hepatopancreas tissue observed may be due to the disease affected the healthy shrimp. Lightner et al., (2012a) studies on both *P. monodon* and *L. vannamei* affected shrimp, showed that the EMS trace limited to the hepatopancreas and that was described as lack of mitotic activity in E cells of hepatopancreas, massive sloughing of central HP tubule epithelial cells and massive inter tubular haemocyte aggregation followed by secondary bacterial infections. Similar histopathological result was also obtained by Prachumwat et al., (2012), who recognized dysfunction of the tubule epithelial cells that progress from proximal to distal ends of hepatopancreatic tubules. However, in treated animal severity of infection is less when compared with control. Hypertrophied nuclei are less in number in treated animal compared to control which may be due to increased resistance through probiotic supplementation.

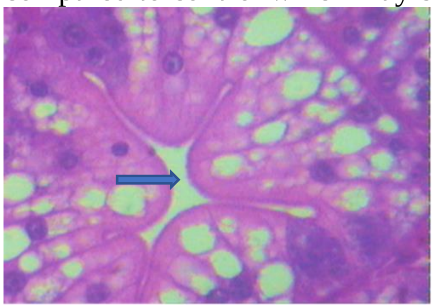


Fig-1 : Cross section of Hepatopancreas of Control animal with star shaped lumen (arrow indicates) (10X) (4X)

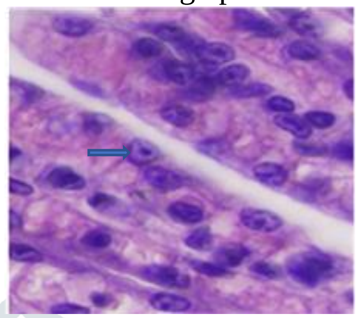


Fig-2: Cross section of WSSV infected hepatopancreatic tissue in high magnification (10X) (40X) showing karyomegaly and Hypertrophied nuclei.

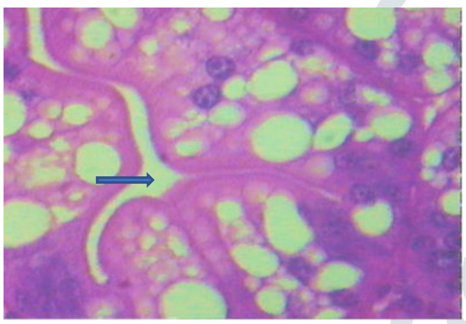


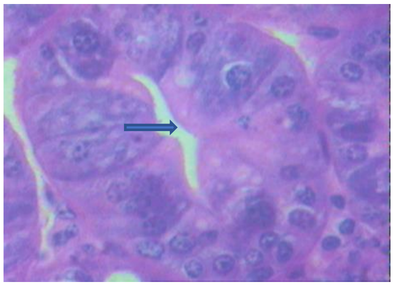
Fig-3: Cross section of Hepatopancreas of treated animal (10X) (4X) with increased lumen and delayed infection (arrow indicates).

Mid Gut:

The gut is regarded as the chief pathogen transmission route (De Schryver and Vadstein, 2014). Consequently, they can penetrate to the mucosal epithelium of the gastrointestinal tract and help to challenge pathogens, by decreasing their numbers in the host (Akhter et al., 2015; Lazado et al., 2015).

Our studies (Fig: 4-6) shown that the midgut included most of the alimentary tract and the widespread damage caused by the virus in this region might be anticipated as the reason, for the cessation of food intake along with the manifestation of the disease. In the infected animals the inner surface of the midgut, with thin columnar epithelial cells forming the internal epithelial lining with small oval nuclei was found to sloughed off from the wall into the lumen. The circular and longitudinal muscle layers, which were situated beneath the epithelial layer, exhibited widespread multi focal necrosis. No hypertrophied nucleus was found in the entire epithelial layer or in any of the underlying tissue. This observation corresponding with that of Villasenor et al., (2015), who stated that the nuclei were not so obviously hypertrophied and remained close to normal size.

One of the roles of probiotics, is the ability to alter the morphology of the digestive tract, this, escalates in villi length and crypt depth (Pelicano et al., 2005).The probiotic bacteria compete with potential pathogenic bacteria for adhesion sites, nutrients and chemical substances in the water and in the gut of the host (Hamza et al., 2016). Samanya and Yamauchi, (2002) confirmed that birds treated dietary *Bacillus subtilis* for 28 days had an ability to show the higher villus extension than the control group. An increase in villi length denotes to high digestion and absorption capability with the presence of good microbial balance and healthy body. Awad et al., (2008) stated that the supplementation of synbiotic Biomin IMBO with diet increased the villus height/crypt depth ratio and villus height in ileum of broiler chickens. Increase in the villus height suggests an increased surface area capable of greater absorption of available nutrients.et al



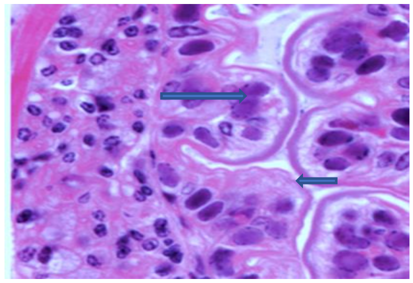


Fig-4: Cross section of Midgut of control (10X) (4X) animal showing distinct epithelium and normal villi

Fig-5: Cross Section of Midgut tissue (10X) (40X) infected with WSSV, showing necrosis with sloughing of Epithelial layer (Short Arrow) and hypertrophied nuclei. (Long Arrow).

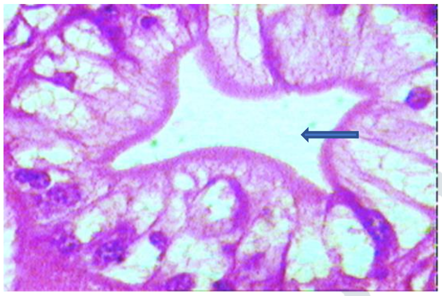


Fig-6: Cross Section of Midgut tissue (10X) (4X) of probiotic treated showing early stage of infection without lumen collapse and a smaller number of hypertrophied nuclei.

Gill:

The histological sections of the dendrobranchiate gill (Fig: 7-9) comprising of a median gill axis and the gill filaments showed numerous lacunae occluded by haemocytes in control. During infection they showed massive degeneration, generalized vacuolization and the hypertrophied nuclei of the epithelial cells. Hypertrophied nuclei showed lightly to deeply basophilic central inclusions surrounded by marginated chromatin in gill tissue after staining with haematoxylin and eosin. Similar interpretations were reported by Yoganandhan et al., (2003), who observed cellular disintegration, nuclear hypertrophy with intranuclear inclusion bodies in gill tissue, eye stalk, head muscle tissue, connective tissue and stomach epithelium in WSSV infected *Penaeus indicus*. The epithelial pillar cells of secondary lamella of WSSV infected gill tissue showed large clear intranuclear basophilic cow dry type-A inclusion bodies. (Afsharnasab, 2009a). In addition to that infected gills exhibited diffused lamellar fusion with basal epithelial necrosis and epithelial lifting of some filaments. These observations are in accordance with Wongteerasupaya et al., (1995) and Inouye et al., (1996). But probiotic treated shrimps exhibit minimal pathological alterations compared to controls. The gills of probiotic fed shrimp showed proliferation of granular cells, of secondary lamellae and infiltration of leukocytes and slight necrotic change was observed. This indicates the effectiveness of probiotic in improvement of immune response and disease resistance.

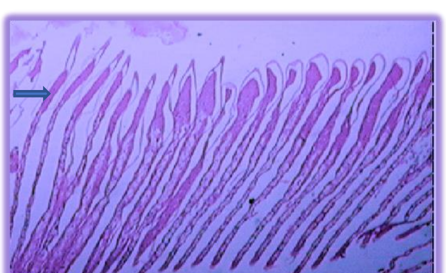


Fig-7: Cross section of Gill tissue (10X) (4X) of control showing primary and secondary lamella (Arrow).

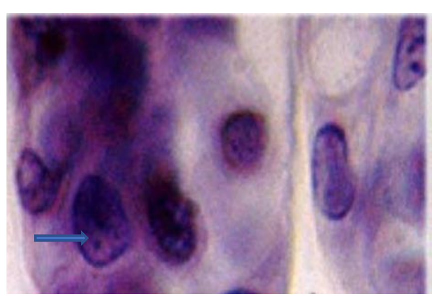


Fig-8: Cross section of WSSV infected cow-dry type gill tissue in high magnificence (10X) (40X) showing basophilic inclusion bodies.

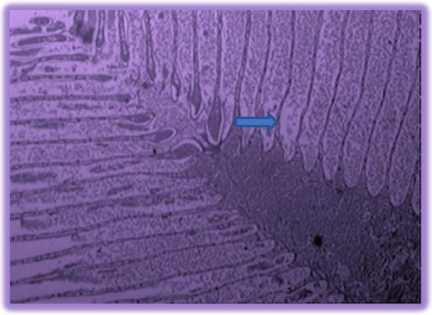


Fig-9: Cross section of probiotic treated gill tissue showing evenly distributed secondary lamellae with uniform length (10X) (4X) (Arrow)

Conclusion:

The histopathological observations in the selected tissues (Hepatopancreas, Midgut and Gill) of *L. vennamei* indicate that the probiotic treated shrimp exhibited minimal pathological alterations compared to controls. Observation of less karyomegaly, and decreased hypertrophied nuclei in hepatopancreas, increased lumen and villi in midgut and presence of numerous lacunae occluded by hemocytes in the gill filaments indicate the effectiveness of the probiotic in improving immune response and disease resistance.

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