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Effects of marine yeast based diet on the histology of *Penaeus indicus* H. Milne Edwards, 1837

P. J. Sarlin[1,2]
[1]Department of Marine Biology, Microbiology and Biochemistry, CUSAT, Fine Arts Avenue, Kochi -16, Kerala, India.
[2]Fatima Mata National College (Autonomous), Kollam, University of Kerala, India.

*Correspondence e-mail: sarlinpoly@yahoo.com

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Original Article

Abstract

Efficacy of three marine yeasts (*Debaryomyces hansenii* S8, *Debaryomyces hansenii* S100 and *Candida tropicalis* S186) as feed supplement for *Penaeus indicus* was estimated in comparison with *Saccharomyces cerevisiae* MTCC 36, a commercial feed (Higashi Maru Feeds, India) and a control feed. The yeast component of control feed was replaced with Carboxy Methyl Cellulose. Biomass of yeast strains in Malt extract agar incorporated into a standard diet was used to prepare the experimental yeast diets. *P. indicus* was fed these diets for a period of 28 days and growth parameters were assessed. Among the three marine yeast diets, *D. hansenii* S8 supported the best bio-growth parameters. Commercial feed was found to be better in efficiency compared to the Baker's yeast (F36) diet and control diet. Histological examination of the hepatopancreas was carried out in an attempt to evaluate the toxic effects, if any, in shrimps fed yeast diets. The cross section of hepatopancreas tubules of the midgut-gland of shrimps that were fed the control feed was taken as reference for the comparative studies. Unlike earlier reports of adverse effects on intestinal function and an intensified immune response in fishes on fish meal replacement diet, no histopathological alterations indicative of toxic effects could be observed in the shrimps fed yeast diets. The three tested marine yeasts could very well be used as feed supplement in aquaculture as they have desirable qualities and no noticeable histological consequences on the shrimps.

Keywords: *Penaeus indicus, Debaryomyces hansenii, saccharomyces cerevisiae, single cell protein, histology*

Introduction

As the aquaculture industry steps in, to relieve the pressure on capture fisheries and to meet the ever-increasing human demand for fishes, functional ingredients in fish feeds and adapted feeding protocols are paramount to the success and sustainability of the practice. With the rapid expansion in the aquaculture industry, the demand for fishmeal (FM), the principal protein source in formulated aqua feeds has risen sharply and the price for the same skyrocketed enhancing the search for alternatives.

Microbial ingredients such as bacterial meal (Vasanth *et al*., 2015; Romarheim *et al.,* 2010 Romarheim *et al*., 2013; Aas *et al*., 2006) and yeast (Couture *et al*., 2019; Meena *et al*., 2013; Micallef *et al*., 2017; Øverland *et al*., 2013), represent potential sustainable alternative single cell protein (SCP) sources in aquafeeds due to their high nutritional value, in addition to

their content of a wide range of bioactive components with potential as functional ingredients. The use of functional feeds to modulate the immune system has received increasing attention in fish farming (Herman and Schmidt, 2016; Kiron, 2012).

Replacement of up to 45% FM with yeast extract in the diet of *Penaeus vannamei* had no adverse effect on the digestibility of the feed or the growth of the shrimp (Zhao *et al*., 2015). Although few studies have investigated bacteria as a SCP in the diet of shrimp, it has been well proven that other types of SCP can have a positive effect on their immune system and growth (Biswas *et al*., 2012; Deng *et al*., 2013). However, 15% of yeast-based SCPs in the diet of Red-Stirling tilapia *Oreochromis niloticus* (Ribeiro *et al*., 2014) and grey mullet *Mugil cephalus* (Luzzana *et al*., 2005) resulted in growth reduction. The adverse effect on growth in several experiments that used SCPs as FM substitutes could be related to differences in target species, sources of SCP, feed formulations, and physico-chemical conditions of the experiments.

Yeasts are a popular product to use in aquaculture (whole or fractions) as supplements in animal feed as source of amino acids, proteins, and vitamins, principally B-complex with positive effect on shrimp growth and immunity (Chotikachinda *et al.,* 2008; Ferreira *et al*., 2010; Gamboa-Delgado *et al*., 2016; Álvarez Sánchez *et al.,* 2018). Application of yeast species and derivatives as diet supplement, fish meal replacement, probiotics, digestion and growth enhancers in shrimp (*L. vannamei*, *Penaeus monodon*, *Penaeus japonicus*, *P. indicus,* etc.) aquaculture has been demonstrated by Burgents *et al.,* 2004; Sajeevan *et al*., 2006; Zhenming *et al*., 2006; Biswas *et al*., 2012; Babu *et al*., 2013; Zhao *et al*., 2015; Álvarez-Sánchez *et al*., 2018; Xiong *et al*., 2018. Sarlin and Philip (2016) used different species and yeast strains as supplements in shrimp post-larvae (*P. indicus*). Those yeasts displayed protein, lipid, and carbohydrate contents from 22.00 to 30.00, 2.00 to 8.25 and 22.36 to 29.68%, respectively, without any difference in their biochemical composition. The positive effects of yeast or its components on shrimps have been documented in several studies. Marine yeast *D. hansenii* (S8) and *C. tropicalis* (S186) enhanced shrimp (*P. indicus*) immunity (Sarlin and Philip, 2011), yeast products like carotenoid pigments, bioactive products, glucans, lipids, nucleotides, polysaccharides, proteins, and vitamins exhibited immunostimulant property (Aguirre-Guzman *et al*., 2009; Babu *et al*., 2013; Sang *et al*., 2014; Mohan *et al*., 2019).

This study shows the potential of marine yeasts as a feed supplement in aquaculture. Yeasts are nutritionally rich in proteins, vitamins and carbohydrates. Besides being a nutritional source, yeasts serve as an immunostimulant also by virtue of its high carbohydrate (β, 1-3 glucan) and RNA content. Technology for mass production of marine yeasts, storage and incorporation into diet has to be developed for application in culture systems. Present study showed that the three marine yeasts used in the study could very well be used as feed supplement in aquaculture as they demonstrated promising bio growth parameters. Lack of histopathological alterations indicative of toxic effects on shrimps of the above yeast diets elevate the yeast in the hierarchy of shrimp feed ingredient.

Material and methods

Microorganisms used

Based on the results of the preliminary feeding experiment on *P. indicus* post larvae, three marine yeasts were selected for this study. Baker's yeast *S. cerevisiae* (MTCC 36) obtained from Institute of Microbiology (IMTECH) Chandigarh was also included in the study for comparison. The three selected marine yeasts were identified by IMTECH, Chandigarh: (Table.1) and these yeasts are deposited at Microbial Type Culture Collection (MTCC) at IMTECH and the following numbers were (S8- MTCC 4361, S100- MTCC 4363 & S186-MTCC4366) assigned to them.

Biochemical composition of the biomass of 4 yeast cultures (S8, S100, S186 and S36) was analyzed. Protein content of the experimental diets was determined by Micro-kjeldhal method (Barnes, 1959) and lipid by chloroform-methanol extraction (Folch *et al.,* 1957). Ash was determined by incineration at 550^{0}C in a muffle furnace for 5 h and moisture content by drying in an oven at 80^{0}C to constant weight. Fiber content was determined by acid and alkali treatment following AOAC (1990). The nitrogen free extract (NFE) was computed by difference (Crompton and Harris 1969), (NFE = 100–(% protein + % lipid + % fiber + % ash).

Table 1. List of yeast strains used for production of SCP

Culture No	Species	MTCC Number
S8	*Debaryomyces hansenii*	MTCC 4361
S100	*Debaryomyces hansenii*	MTCC 4363
S186	*Candida tropicalis*	MTCC 4366
S36	*Saccharomyces cerevisiae*	MTCC 36

Feeding experiment with P. indicus juveniles

Juveniles of *P. indicus* in the size range 0.10- 0.12 g was brought to the laboratory from a commercial prawn hatchery in Kannamali, Kochi.

Experimental feed preparation

Powdered ingredients (Table 2) were mixed well into dough with 100 ml water and steamed for 10 minutes in an autoclave

Table 2. Composition of experimental diets

Ingredients	Control diet g	Experimental diet g
Prawn shell powder	10	10
Yeast[a]	-	15
Fish meal	30	30
Ground nut oil cake	8	8
Soybean meal	10	10
Maida	8	8
Rice bran	10	10
Casein	5	-
Vitamin and mineral mix	2	2
Agar	2	2
Carboxy methyl cellulose	10	-
Water	100 ml	100 ml

[a]Biomass of 4 different yeast strains were incorporated in the experimental diets

and pelletised using a laboratory model pelletiser having 1 mm die. Pellets were dried in an oven at 50^{0}C for 18 h and broken into pieces of 4-5 mm size. Four different feeds were prepared incorporating the biomass of 3 marine yeast strains and the baker's yeast (*S. cerevisiae*) plus the control feed (without the yeast biomass). Water stability of feed was checked by immersing pellets in seawater for 15 h and examining stability by visual observation. Feeds were stored in airtight polythene bags at -20^{0}C in a freezer. A fish meal based commercial feed (CF, manufactured by Higashi Maru Feed, India) was also used for the study.

Rearing facility

Fiber reinforced rectangular plastic (FRP) tanks of 30 L capacity with 1 HP compressor providing aeration though air stones were used for the study. 50% of water was exchanged from all the experimental tanks and water quality was maintained daily. Physicochemical parameters like salinity, nitrogen and dissolved oxygen of the rearing water were estimated daily by following standard procedures (APHA, 1995) (Table.3).

Table 3. Rearing conditions and water quality parameters of the experimental system

Initial body weight (Average)	0.10-0.12 g
Stocking density	20 PL/tank
Tank capacity	30 L
Feeding level	10-15% body weight
Feeding frequency	Twice daily
Feeding period	28 days
Water temperature	24-27^{0}C
pH	7.5–8
Salinity (ppt)	15–18ppt
NH_3 (mg/L)	0.01–0.02mg/L
NO_3 (mg/L)	Below detectable
NO_2 (mg/L)	0.00–0.01mg/L
Dissolved O_2	6-7mg/L

Design of experiment

Juveniles of *P. indicus* were maintained on prepared control diet for a period of one week. The shrimps were then stocked into FRP tanks containing 20L seawater with 20 individuals per tank and reared on the experimental diets for 28 days. Feeding trials were conducted using triplicate tanks for each treatment.

Feeding schedule

Six different feeds were given to the prawns including four yeast diet, one commercial feed and one control diet. Pre-weighed experimental diets corresponding to 10-15% of the body weight of the experimental animals were placed in petridishes in the tank. Daily ration was equally divided into two halves and offered at 6 a.m. and 6 p.m. after siphoning off the fecal matter. Leftover feed after 3 h of feeding was collected twice daily by siphoning and washed gently with distilled water to remove salt. It was filtered with a pre-weighed filter paper and dried to a constant weight in an electric oven at 80^{0}C for 24 h.

Data analysis

The data obtained in the feeding experiments were subjected to One-way Analysis of Variance (ANOVA). When a significant difference was found among the various treatments, Duncan's multiple range tests were done to bring out the difference between the treatments means. The statistical analysis was performed using the SPSS 11.0 package for windows.

Histology

To determine the toxic effects, if any, of the yeast incorporated feed on the experimental animals, one animal each was removed from all the six different experimental treatments and the hepatopancreas was dissected out and histological analysis was done as given below. Further studies with histological analysis of a larger number of shrimps from the total experimental setup with representation of at least half a dozen per experimental treatment may provide conclusive results.

Fixation and staining of hepatopancreas sections

The hepatopancreas tissues fixed in Davidson's fixative were transferred to 70% alcohol for post fixation treatment. The tissues were then transferred to two changes of 90% alcohol and two changes of 100% alcohol for 1 h each followed by transfer to a 1:1 solution of absolute alcohol and methyl benzoate for 30 minutes until the tissue became transparent. They were transferred to benzene for 15 minutes and then xylene saturated with paraffin wax for 6 h and then infiltrated

with two changes of paraffin wax at 58-60^{0}C in a hot air oven for 1 h each. The tissues were embedded in paraffin wax at 60-62^{0}C. The blocks with the embedded tissue were sectioned using a microtome at 7.5 μ thickness, heat fixed onto albumin coated glass slides, deparaffinized in xylene, hydrated by passing through a descending series (absolute, 90, 70, 50 and 30%) of alcohol-distilled water solution. The sections were then stained with Haematoxylin (Mayer's) and Eosin (Scott's) stains and then subjected to an ascending series (70, 90, 95% and absolute) of alcohol-distilled water solution cleared in xylene and mounted in DPX (Wilson and Gamble, 2002). The sections were viewed and photographed under a light microscope with 40x lens magnifications.

Results

Among the three marine yeast diets *Debaryomyces hansenii* S8 supported the best bio growth parameters (Table. 4). Commercial feed was found to be better in efficiency compared to the Bakers yeast diet and control diet (Sarlin and Philip, 2018). No histopathological alterations indicative of toxic effects could be observed in the shrimps fed yeast diets. Proximate composition of the flesh of shrimps (Fig.1) did not show any remarkable change when fed on various yeast feeds, commercial feed and the control feed. (Table. 5, 6)

Table 4. Relative positions of various feeds with respect to their performance in terms of bio-growth parameters and percentage survival in *P. indicus* juveniles maintained on experimental diets

Parameters	PRO	FCR	SGR	GGE	RGR	PER	CUD
Experimental Feeds	[a]F 8	F 8	F 8	F 8	F 8	F 8	F 8
	[b]F 186	F 186	F 186	F 186	F 186	F 186	F 186
	[c]F 100	F 100	F 100	F 100	F 100	F 100	F 100
	[d]CF	CF	CF	CF	CF	CF	CF
	[e]F 36	F 36	F 36	F 36	F 36	F 36	F 36
	Control	Control	Control	Control	Control	Control	Control

(a D. hansenii (F 8), [b] *C. tropicalis* (F 186), [c] *D. hansenii* (F 100), [d]commercial feed (CF Higashi Maru Feed, India) and Baker's yeast, *S. cerevisiae* (F 36)

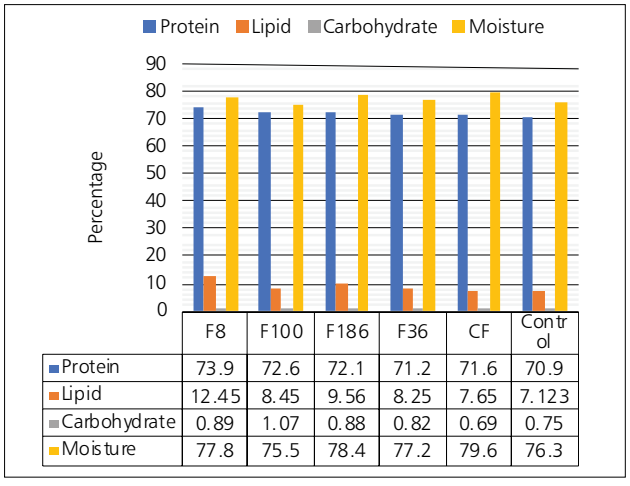

Fig. 1. Proximate composition of flesh of *P. indicus* maintained on different diets

Table 5. Proximate composition of yeasts

Culture No.	Protein	Lipid	Carbohydrate
[a]S8	24.72	3.81	24.8
[b]S100	31.51	7.62	28.8
[c]S186	26.54	3.82	27.9
[d]S36	27.52	4.23	25.8

(aD. hansenii S 8, [b]*D. hansenii* S 100, [c]*C. tropicalis* S 186, *e*Baker's yeast, *S. cerevisiae* MTCC 36.

Table 6. Proximate composition of feeds

Feed no.	Protein	Lipid	Fiber	Ash	Moisture	NFE[f]
[a]F 8	44.3	8.8	2.1	6.7	6.6	31.5
[b]F 100	51.9	10.3	2.0	6.3	4.1	25.4
[c]F 186	48.1	8.3	2.0	7.5	4.6	29.6
[d]F 36	45.2	8.2	1.9	5.2	6.3	33.2
[e]CF	48.5	7.2	2.1	6.2	7.3	28.71
Control	47.2	7.9	2.0	5.8	7.2	29.9

(a D. hansenii (F 8), [b] *D. hansenii* (F 100), [c] *C. tropicalis* (F 186), [d] Baker's yeast, *S. cerevisiae* (F 36). [e] commercial feed (CF Higashi Maru Feed, India) and [f] Nitrogen Free Extract

Histological examination of hepatopancreas

Histological analysis of the hepatopancreas did not show any structural or functional abnormalities with feeds *D. hansenii* S8 (F8), *D. hansenii* S100 (F100), *Candida tropicalis* S186, (F186) a Baker's yeast, *Saccharomyces cerevisiae* MTCC 36, (F36) and a commercial feed (CF Higashi Maru Feed, India). Fig. 2 shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed the control feed taken as reference for comparative studies to evaluate the toxic effects of the experimental diets. The mature B cells are seen in the tubules which are compactly arranged. The healthy tubules are intact and possess a characteristic stellate

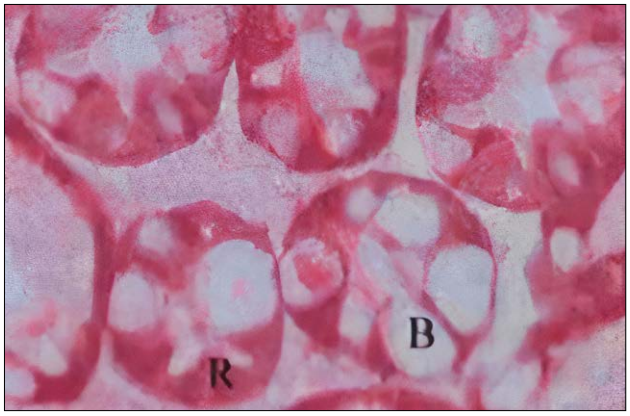

Fig. 2. C.S. of heptopancreas of *P. indicus* maintained on control feed. Haematoxylin-Eosin stain 40X

luminal space. Large numbers of R and F (pyramidal or cylindrical shape) cells are visible, indicating that the hepatopancreas is in a healthy condition.

Fig. 3. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F36. Large number of vacuolated cells occupies the epithelium these could be both R or B cells. Obliteration of the lumen of the tubules was found which was mainly the result of excessive enlargement and vacuolation of B cells.

Fig. 4. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed commercial feed CF. The B cells are more in number and larger suggesting an active and healthy condition of digestive process. Active B cells which are comparable to those fed on the control feed was found. Compression of lumen space was noticed in some tubules and sloughing off of the cells in some tubules.

Fig. 5. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F8. The B cells are more in number and larger suggesting an active and healthy condition of digestive process. The normal healthy structure comparable to that of the control is observed and no degenerative changes are noticeable in the tubules.

Fig. 6. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F100. Almost normal

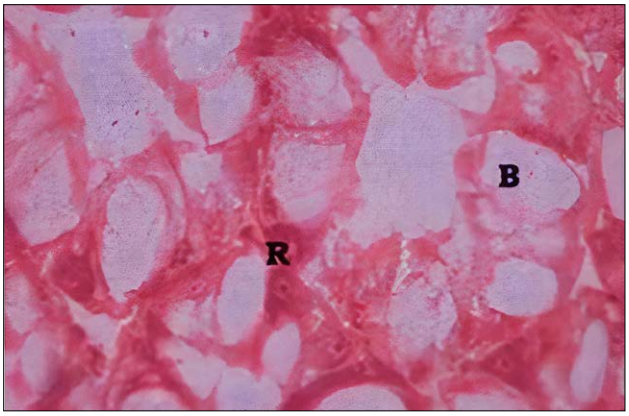

Fig. 5. C.S. of heptopancreas of *P. indicus* maintained on experimental feed F8. Haematoxylin-Eosin stain 40X

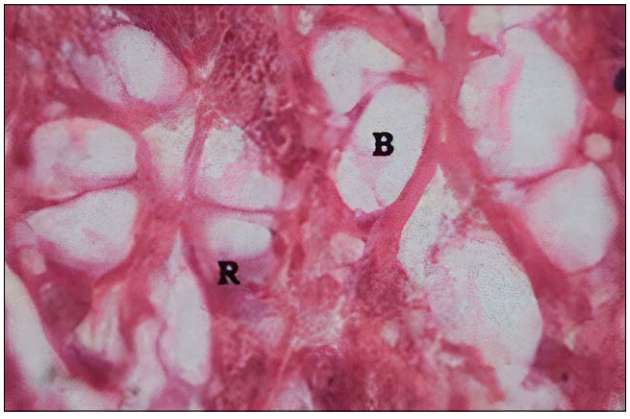

Fig. 3. C.S. of heptopancreas of *P. indicus* maintained on experimental feed F36. Haematoxylin-Eosin stain 40X

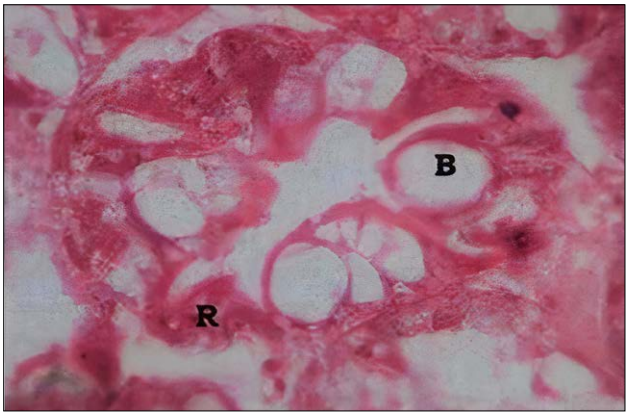

Fig. 6. C.S. of heptopancreas of *P. indicus* maintained on experimental feed F100. Haematoxylin-Eosin stain 40X

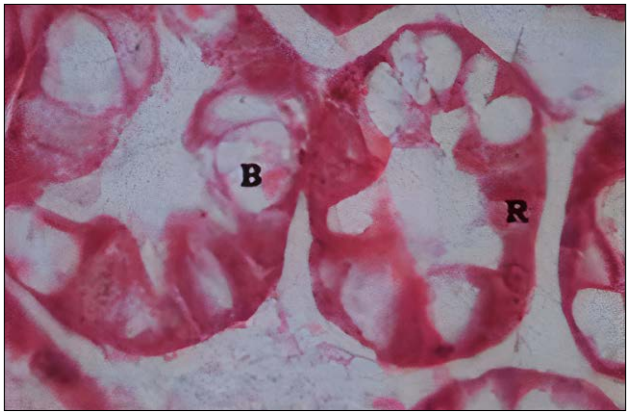

Fig. 4. C.S. of heptopancreas of *P. indicus* maintained on commercial feed CF. Haematoxylin-Eosin stain 40X

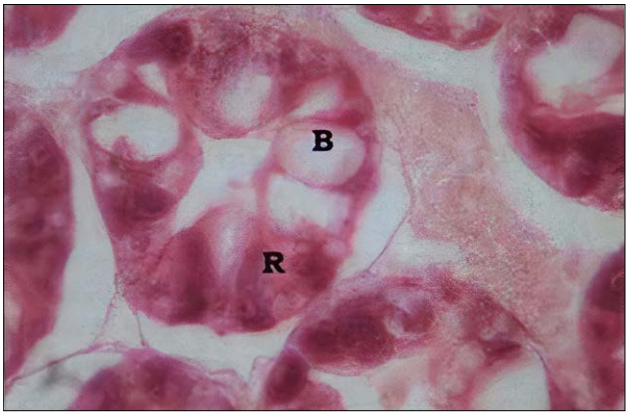

Fig. 7. C.S. of heptopancreas of *P. indicus* maintained on experimental feed F186. Haematoxylin-Eosin stain 40X

condition was noticed in the tubules. They were not completely obliterated or damaged. Slight disruption of the basal membrane was observed.

Fig. 7. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F186. Normal structure of B cells and F cells were noticed. The homogeneity of inclusions on the basal vacuoles was found to be similar to those found in control tubules. Slight disruption of the basal membrane was also observed no possible reason can be found for this at this point of study. There was no shrinkage in the size of the tubules. F cells retained their pyramidal shape, an indication of their healthy condition.

Discussion

Proximate composition of the flesh of shrimps did not show any remarkable change when fed on different yeast feeds, commercial feed and the control feed indicating that the nutritional components were in the required proportion and amounts. In the present study three marine yeasts (*D. hansenii* S 8, *D. hansenii* S 100, *C. tropicalis* S 186), a Baker's yeast, *S. cerevisiae* MTCC 36, a commercial feed (CF, Higashi Maru Feed, India) and a control feed were used for feeding trials. The performance of marine yeast incorporated feeds was superior compared to other feeds.

Among SCP, yeasts have been the most used within aqua feeds (Tacon, 1994). As a protein source, single cell proteins (SCP) of yeast or bacterial origin appear especially attractive because the protein content and amino acid composition of these organisms compare well with those of fish meal (Spinelli *et al*., 1979). Most of the studies performed so far on the use of yeast as food source for crustaceans and fishes were related to the baker's yeast as main source. Studies on the use of marine yeast as main source of protein are limited. Substantial replacement of fishmeal with 40-60% SCPs with varying degrees of success have been reported by Viola and Zohar (1984) Davies and Wareham, 1988; Oliva-Teles and Goncalves, 2001; Rosales *et al*., 2017. Gamboa-Delgado *et al.* (2016) successfully replaced 60% FM with torula yeast *C. utilis* in the white leg shrimp. Replacement of up to 45% FM with yeast extract in the diet of *P. vannamei* had no adverse effects on the digestibility of the feed or the growth of the shrimp (Zhao *et al*., 2015). It has been well proven that SCP can have a positive effect on the immune system and growth of shrimp (Sarlin and Philip, 2011; Biswas *et al*., 2012; Deng *et al*., 2013). However, 15% of yeast-based SCPs in the diet of Red-Stirling tilapia *O. niloticus* (Ribeiro *et al*., 2014) and gray mullet *M. cephalus* (Luzzana *et al*., 2005) resulted in growth reduction. The adverse effects on growth in several experiments that used SCPs as FM substitutes could be related to differences in target species, sources of SCP, feed formulations, and physico-chemical conditions of the experiments. Adverse effects on intestinal function and an intensified immune response in fishes on plant-based protein di*et alo*ng with sub-optimal environmental condition were reported by Mosberian-Tanha *et al*., 2018.

The positive effects of yeast or its components have been documented in several studies (Øverland and Skrede, 2017; Hauptman *et al*., 2014; Morales-Lange *et al*., 2015; Salah *et al*., 2017; Meena *et al*., 2013).

In the present study, the growth and survival of shrimps maintained on yeast diets were much higher when compared to the control diet and the commercial feed (Sarlin and Philip, 2018) Yeast products (primarily brewer's yeast and baker's yeast) are frequently used as feed ingredients in aquaculture because of the nutritional value of these products, which include protein, lipids, B-vitamins etc. (Mahnken, 1991; van der Meeren, 1991).

Among the three marine yeasts, *D. hansenii* S8 supported the best biogrowth parameters, in terms of production, FCR, SGR, GGE, PER and CUD followed by S186 (*C. tropicalis*) and S100 (*D. hansenii*) (Sarlin and Philip, 2018).

Histopathological studies envisaged assessment of cellular damage, variations in the nature of inclusions, proliferation of specific types of cells and total damage of epithelia or any connected structure of this important organ. Hepatopancreas is essentially an organ, which constantly produces new cells, the growth taking place from the basic embryonic cells (Embryonalenzellen) situated at the distal end of the tubules.

The R cells (Restzellen) which are absorptive in nature undergo variation, as a result of exposure to toxicants or when subjected to starvation. The histological examination of the hepatopancreas was carried out in an attempt to evaluate the toxic effects, if any, in shrimps fed yeast diets. No histopathological alterations indicative of toxic effects could be observed in the shrimps fed yeast diets.

This study shows the potential of marine yeasts as a feed supplement in aquaculture with less histological consequences. Yeasts are nutritionally rich with proteins, vitamins and carbohydrates. Besides being a nutritional source, yeasts serve as an immunostimulant also by virtue of its high carbohydrate (β, 1-3 glucan) and RNA content. Technology for mass production of the marine yeasts, storage and incorporation into diet has to be developed for application in culture systems.

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