BACTERIAL QUALITY OF SHRIMP PROCESSED IN FACTORIES FOR EXPORT TO EUROPEAN UNION

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VERSOVA, MUMBAI - 400 061

BY

P. YESUDHASON
M.F.Sc., POST HARVEST TECHNOLOGY
1998 - 2000

CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY
MATSYAPURI POST, WILLINGDON ISLAND
KOCHI - 682 029

DEDICATED TO
MY BELOVED PARENTS

Telephone } 666846, 666845, 666847, 666848, 666763, 666764, 666765, 666766, 668577, 668578, 668579, 668580
Telex : 0885 6440

Telegram : Matsyaoudyogiki/fishtech.Cochin
Telefax : 0091-484-668212
E.mail : cift@x400.nicgw.nic.in

भाकृ अनुप
ICAR

केन्द्रीय मात्स्यकी प्रौद्योगिकी संस्थान
CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY,
भारतीय कृषि अनुसंधान परिषद
(Indian Council of Agricultural Research)
विल्लिंगडन आईलेंड, मत्स्यपुरी पी. ओ. , कोच्चि – 682 029.
Willingdon Island, Matsyapuri P.O., Kochi - 682 029.

CERTIFICATE

This is to certify that the dissertation entitled **"Bacterial quality of shrimp processed in factories for export to European Union"** submitted by Mr. **P.Yesudhason**, (PHT-02, 1998–2000 Batch) in partial fulfillment of the requirements for the award of ***Master of Fisheries Science in Post Harvest Technology*** of **Central Institute of Fisheries Education (Deemed University) Mumbai 400061,** is a bonafied record of research work done by him during the period of his study at this Institute under our guidance and supervision, and it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

Shri. P.R.G.Varma
Senior Scientist,
QAM Division
Co-chairman and Member,

Dr. T.S.G.Iyer
M.Sc., Ph.D., Dip.Fd.Tech (UK)
A.R.S., F.S.F.T
Principal Scientist and Head
Fish processing Division
Major Advisor & Guide

Countersigned by

DIRECTOR

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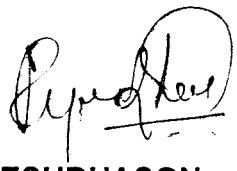
P.Yesudhason

DECLARATION

I here by declare that this dissertation entitled "**Bacterial quality of shrimp processed in factories for export to European Union**" is based on my research and has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship of other similar titles or recognition.



Kochi – 682 029 **P. YESUDHASON**

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Abstract

The purpose of present study is to evaluate the bacterial quality of seafood products processed by EU approved seafood processing plants. Frozen shrimp samples prepared in different styles such as Headless, Peeled and Undeveined, Peeled and Deveined and Cooked and Peeled were collected from different EU approved seafood processing plants around Cochin and the samples were analysed for Total plate count, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* and *Vibrio cholerae*. A total of 82 samples from 12 different factories were collected. All the frozen samples analysed had bacterial load below 6 x 10^5/g, which is below the level permitted by European Union. *E.coli* was present in only one sample and the count was 40/g. 25% of the PUD and 25% of PD samples analysed contained S.*aureus*, and the count was below 100/g. *Salmonella* and *V.cholerae* were absent in all the sample analysed. Bacterial profile analysis of the factories showed that pathogens were absent in all the samples collected from 7 factories, while *S.aureus* was present in few samples collected from 4 factories and both *S.aureus* and *E.coli* were present in one sample collected from a factory.

सारांश

प्रस्तुत अध्ययन का उद्देश्य ई.यू. अनुमोदित समुद्रीखाद्य संसाधन संयंत्रों द्वारा संसाधित समुद्रीखाद्य उत्पादों की जीवाणवीय गुणता का मूल्यांकन है । कोचिन के चारों ओर के विभिन्न ई.यू. अनुमोदित समुद्री खाद्य संसाधन संयंत्रों से विभिन्न रीतियों में तैयारित अवरुद्ध झींगा नमूने जैसे बिना सिर, विशल्कीत और बिना नस, विशल्कीत और नसयुक्त और पके और विशल्कीत को एकत्रीत किया गया और नमूनों को कुल पट्टिका गणना, इसकेरीकीया कोली, स्ट्रपीलोकोकस औरस, सोलमोनिल्ला और विब्रियो क्लोरिया को विश्लेषण किया गया । 12 विभिन्न कारखानों से कुल 82 नमूनों को एकत्रित किया गया । सभी अवरुद्ध नमूनों को विश्लेषित किया गया जीवाणवीय लोड 6 x 105/ ग्रा. से कम था जो यूरोपियन यूनियन द्वारा अनुमोदित स्तर से कम था । ई. कोली केवल एक नमूने में था और गणना 40 ग्रा.। 25% की पी.यू.डी. और पी.डी. के 25% विश्लेषित नमूने एस. औरस से युक्त थे, और गणना 100/ग्रा. से कम है । सभी विश्लेषित नमूनों में सालमोनिल्ला एवं वी. क्लोरिया अनुपस्थित थे । कारखानों के जीवाणवीय रूपरेखा का विश्लेषण दिखता है कि 7 कारखानों से एकत्रीत सभी नमूनों में रोगजनक अनुपस्थित थे, जबकि 4 कारखानों से एकत्रीत कुछ नमूनों में एस. औरस है और एक कारखाने से एकत्रीत एक नमूने में एस.ओरस एवं ई.कोली दोनों उपस्थित थे ।

INTRODUCTION

1. INTRODUCTION

Export trade is one of the important determinants of economic development of a nation. Each country in the world aims at achieving high rates of economic growth through rapid increase in its trade with the developing as well as developed countries. Export of marine products represents emerging new and expanding sources of foreign exchange earning for India. Seafoods have been recognized as one of the thrust areas for accelerating the development process of the Indian economy.

India is strategically located on the Indian ocean with a coastline of 8129 km; a continental shelf of 0.5 million square km; an Exclusive Economic zone (EEZ) of 2.02 million square km; and about 1.4 million hectare of brackish water area offering immense scope for capture and culture fisheries. Of the total fish production of 5.1 million tonnes (1996-1997), 2.87 million tonnes came form marine sources which is about 60 percent of the estimated potential of EEZ. The activity of the traditional and over 90 percent of the mechanized boats is confined to the coastal waters, which form hardly 10 percent of the EEZ.

Seafoods that are to be processed for export pass through several stages before they leave to exporting countries. These include catching by fishing boats, landing at ports, transportation from ports to primary processing factories, processing and packing at factories and storage. Freshness must always be maintained through out this whole process. Each process must be completed in a short time in a clean and hygienic environment keeping the product always under low temperature.

Any incidence of food poisoning from contaminated exported marine products will adversely affect the reputation of the company and the country. Further, the developed as well as developing nations of the world have started demanding introduction of safety-oriented quality systems in the production systems. Therefore, importers will insist on a well-managed quality assurance system giving emphasis to food safety. There would be little use of detecting the problem after wards, as is normally the case.

'Quality' is a highly subjective and relative term. Quality, in general, means wholesomeness or state of excellence of a particular product in terms of its appearance, shape, colour, taste, and competitiveness in price to the buyer. Quality means the fulfillment of the customer's requirements. Fish and fishery products, being highly perishable, deserve special attention through out the distribution chain. The living habitats of fish are completely different from its post- harvest environment and its quality depends on many factors, such as composition, degree of spoilage, damage, deterioration during harvesting, cleaning, washing, handling, preservation, processing, storage, transportation, and distribution. Processing of quality fish and fish products is dependent on various physical factors, such as primary quality of raw material, handling and preservation, transportation and distribution, facilities available at the processing plants; technology used for processing, sanitary and hygienic conditions of the plant; cleanliness of equipment; and better storage facilities.

India has been exporting seafood in dried and canned form for many decades. However, it was in 1953 that the first freezing unit was set up at

Cochin and the first consignment of frozen shrimp (13.26 Mt cost of Rs.57, 000) was exported. Since then growth of the industry had been commendable and marine products have emerged as one of the major foreign exchange earners of the country, contributing a share of about 4 percent of the country's total foreign exchange earnings from exports.

Marine products created a sensation in the world trade because of its health attributes. With the high unit value, seafood has been acclaimed as one of the fast moving commodity. The world market for the seafood has doubled during the last decade reaching US$ 40 billion mark, of which India's share is only 1.2 percent.

Quality control and pre-shipment inspection systems were instrumental for this phenomenal growth of the seafood industry. At the start of the industry, the exporters were free to ship their consignments without any inspection for quality. Later, in the light of certain setbacks, Govt. of India decided that any consignment exported from this country should meet certain predetermined and specified standards of quality. This resulted in the enactment of comprehensive legislation entitled 'Export (Quality control and inspection) Act 1963'. This act came in to force with effect from 1st September 1964. Initially a voluntary pre-shipment inspection system was introduced from 1st September 1964. Subsequently, the export of marine products was brought under compulsory pre-shipment inspection system with effect from 15th march 1965. Later, consequent upon the decision of Govt. of India, inspection of marine products was taken over by the Export Inspection Agency from first May 1969.

In the initial years, inspection carried out by EIA for fishery products were confined to end products. To make the quality control system more meaningful and effective, in addition to the end product inspection, processing plants were encouraged to adopt Good Manufacturing Practices. Accordingly the Govt. of India introduced the In-process Quality Control (IPQC) scheme for processed seafoods with effective from 31.12.1977. Under the scheme, processing of fishery products for export purpose is permitted only in processing units approved by a panel of experts. To qualify for such approval, the processing units must have minimum sanitary and hygienic facilities stipulated by the EIA as per Govt. of India notification s.o.4007 and 4008 dated 31.12.1977.

Over a period of time, there has been considerable change in the concept of fish processing. The world is now moving towards the HACCP concept, a preventive strategy, to combat food problems and above all, a commitment of the management for the production and distribution of safe products. Many of the seafood processing factories in this country have implemented the HACCP system. This HACCP concept can become a part of ISO 9000 series developed by International Standard Organisation.

All processing factories exporting seafoods to European union are now expected to get approval as per EU regulations. The EU fisheries directives have been restructured the way in which seafood should be caught, traded and marketed in single market consisting of over 360 million European consumers. To unify the EC market that came into operation early 1993, a considerable amount of legislation in many fields of activity has been harmonized to become EC legislation. The unified regulations that apply to

fishery products are contained in Council Directive91/493/EC of 22 July 1991, which lays down "the health conditions for the production and the placing on the market of fishery products". Though the primary objective of the legislation is to harmonize practices within the community, it is a principle of the Directive that its provisions should apply to imports from third world countries and that there should be a common import system applied by all member states of the community.

Wide ranges of food commodities are subjected to separate legislation and two directives apply to fishery products. They are council directive of 22 July 1991, pertaining to the sanitary conditions of producing and marketing fishery products (91/493/EEC), and council directive of 15 July 1991, regarding the sanitary conditions of producing and marketing live bivalve mollusks (91/492/ EEC). The thrust of the directive is to ensure the safety of fishery products by controlling the conditions during processing, storage, and distribution and not controlling quality of end products. The directive, however, does have some requirements for testing products.

India's exports of marine products have suffered greatly due to the ban by European union. The EU imposed a ban on imports of Indian fishery products on August 1,1997 as they were not happy with the existing hygiene and infrastructure facilities. As a result of this ban by EU, exports of marine products dropped by 50 percent in value, during 1997-98. However, it was subsequently lifted on December 23, 1997, as our government gave the assurance that we will follow the EU regulations. Export Inspection Council decided to monitor every consignment. Currently only those firms approved by the EU after recommendation of the EIC are allowed to export seafood to

Europe. Firms are accorded approval only if they raise the processing pattern and infrastructure requirements to the level required by EU.

When the EU prohibited imports of Indian fish and fishery products, the Commerce Ministry revamped the export inspection procedure and system. An inter department panel consisting of Officials of Export Inspection Agency, Marine Products Export Development Authority and Central Institute of Fisheries Technology was set up to asses and recommend the units which could be permitted to export to the EU. Another Team, the Supervisory Audit Team consisting of Officials senior to the member of Inter Departmental Panel, also crosschecked this. The standards in these processing units are exceptionally good and the plants are on par with the processing units of the developed countries. The units have invested heavily to improve the standards of their processing plants for export to EU.

There are four major markets identified for the export of marine products in the world. They are Japan, USA, Western Europe and South East Asia. Our export to European union continued to be the second largest in volume of marine exports mainly owing to their import of cephalopods from this country. Tables 1.1 and 1.2 gave the total marine products export and Table 1.3 to 1.6 give the marine products export to European Union. During the year 1998 the export value was Rs 413.53 crores and it increased to Rs 684.62 crores during 1999. This is mainly due to the increased number of processing units approved for export to European union. Currently there are more than seventy-six approved processing units exporting to EU countries. (Aqua International, 1999) and more factories are in the process of getting approval.

The fishery industry in developing countries is constantly improving the quality of fresh fish destined for export to developed countries. At present, Indian seafood industry is well organized to meet international challenges. Shrimps are processed as and when they are available and serious efforts are made to regulate the purchase or streamline the activities. In general, implementation of EEC regulations into the quality system will pave the way for the success of the industry.

Hence it is felt that time has become ripe to assess the bacterial quality of processed shrimp for export to European Union to ensure quality standards for Indian marine products. No works have been done so far in India after the implementation of European Union regulations. The aim of this study was to investigate the bacterial quality of shrimp processed in EU approved factories and the impact of European Union regulation on the improvement of bacterial quality of processed shrimp exported to European Union. The result of this study is of great value for Indian exporter exporting shrimp to European Union.

Export Statistics

Table 1.1. Export of marine products

Q: Quantity in M.Tons
V: Value Rs. in Crores

Year	Quantity	Value
1988-89	99777	597.85
1989-90	110843	634.99
1990-91	139419	893.37
1991-92	171820	1375.89
1992-93	209025	1768.56
1993-94	243960	2503.62
1994-95	307337	3575.27
1995-96	296277	3501.11
1996-97	378199	4121.36
1997-98	385818	4697.48

Table 1.2.Shrimp export from1993-94 to 1997-98

Q: Quantity in M. tones
V: Value in Rs. Crores

Year		Live	Chilled	Frozen	Total
1993-94	Q:	-	-	86541	86541
	V:	_	-	1770.73	1770.73
1994-95	Q:	2	Neg	102335	102337
	V:	0.02	0.01	2518.06	2518.09
1995-96	Q:	10	213	95724	95947
	V:	0.02	2.17	2356.81	2359.01
1996-97	Q:	1	55	1054.26	105482
	V:	0.02	1.83	2701.76	2703.61
1997-98	Q:	1	126	101318	101445
	V:	0.01	507	3140.56	3145.64

Table 1.3. Export of frozen shrimp to European Union :

Q: Quantity in M. tons
V: Value Rs in Crores

Year	Quantity	Value
1993-94	28417	399.35
1994-95	24653	416.68
1995-96	29397	495.28
1996-97	29330	486.13
1997-98	9195	197.11

Table 1.4. Export of frozen shrimp to different EU member countries

Q:Quantity in M.Tons
V:Value Rs.Crores

Countries		1997-98	1996-97	1995-96
UK	Q:	3584	12469	11970
	V:	82.78	217.49	214.67
Belgium	Q:	1754	5221	3782
	V:	37.59	76.88	53.68
Netherlands	Q:	751	4642	5051
	V:	11.74	54.38	54.96
Italy	Q:	1301	2568	3697
	V:	26.96	47.60	77.95
Spain	Q:	516	1392	1462
	V:	11.22	30.45	32.00
France	Q:	293	1043	1288
	V:	4.29	16.39	17.82
Germany	Q:	498	874	622
	V:	11.18	22.19	11.73
Portugal	Q:	146	425	369
	V:	3.14	7.65	7.09
Greece	Q:	169	271	390
	V:	5.14	6.38	10.06
Denmark	Q:	86	167	345
	V:	1.50	2.72	6.76
Norway	Q:	22	131	305
	V:	0.39	2.14	4.66
Switzerland	Q:	75	85	66
	V:	1.18	1.43	1.58
Ireland	Q:	-	40	46
	V:	-	0.39	1.16

Austria	Q:	-	2	15
	V	-	0.05	0.48
Sweden	Q:	-	-	25
	V:	-	-	0.68
Total	**Q:**	**9195**	**29330**	**29397**
	V:	**197.11**	**486.13**	**495.28**

Table 1.5. Export of frozen shrimp in different product forms to European Union

(M.tons)

Product form	1997-98	1996-97	1995-96
Block Frozen	7611	25048	20985
IQF	1422	3585	4907
AFD	6	16	20
Cultured shrimp	137	602	224
Un classified	19	79	3261
Total	**9195**	**29330**	**29397**

Table 1.6. Various items of shrimps export to European Union (M.tons)

	1997-98	**1996-97**	**1995-96**
Head on:			
White	71	318	544
Tiger	92	401	283
Sea tiger	8	48	13
Brown	167	280	362
Flower	38	23	125
Scampi	1	108	3
Deep sea	74	139	60
Shrimp	-	-	-
Cooked	-	-	-
Sub total	**451**	**1317**	**1396**
Headless:			
White	276	978	1827
Tiger	222	823	554
Sea tiger	104	50	119
Brown	1632	4614	4224
Flower	8	79	261
Scampi	496	1079	1256
Karikadi	26	253	779
Deep sea shrimp	33	40	68
Total	**2797**	**7916**	**9108**

Peeled:

PUD	**4787**	**17453**	**15280**
Tail on	106	75	101
CP	187	288	482
PDC	159	285	534
PD	409	1695	1815
PC	257	132	452
PD tail on	22	55	-
Sub total	**5927**	**19983**	**18763**
AFD	6	16	20
Un classified	14	98	116
Sub total	**20**	**114**	**136**
Total	**9195**	**29330**	**29397**

Source: Marine Products Export Review. 1997-98

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Important Shrimp Species of India

The commercial prawns of India are grouped into penaeid and non-penaeid ones. Prawns are of different sizes and only those, which are in good size and available in plenty, are exploited for commercial purposes. The commercial prawns belong mostly to the families Penaeidae, Pandalidae, Hippolytidae, Sergestidae and Palaemonidae. Important species exploited are *Penaeus indicus, Penaeus merguiensis, Penaeus monodon, Penaeus semisulcatus, Metapenaeus dobsonii, M.affinis, M.monoceros, M.brevicornis* and *Parapenaeopsis stylifera*.

2.2 Prawn Fishing Methods

Various kinds of traditional and mechanized crafts such as Catamaran, vallam, plank built boats, mechanized crafts, shrimp trawler and large deep-sea fishing vessel are in use for capturing prawns

There are several types of devices to catch prawns, like nets traps and hooks. The behavior traits of prawns are taken in to consideration in making nets, traps and other devices to capture them. Nets of various types are used. The trawl net is the most effective gear for capturing bottom dwelling marine species like prawns. In addition to this, cast nets, stake nets, Chinese dip nets, boat seines, shore seine and beach seines are also used.

2.3 Seafood Quality

Frozen shrimp is the most important seafood products in the international market. Annual export of frozen shrimp earns considerable foreign exchange for the country. Since it is highly and easily perishable great care must be taken at all stages to produce premium quality products and the quality of processed shrimp produced for the international market should confirm to world standards. Aspects of quality maintenance begin with harvesting and are carried through the production and marketing system till it reaches the consumer.

The need for a more effective seafood quality control and inspection programme has been repeatedly brought up in national and international consultations. Rejection of products at the importing port denotes non-conformity with product standards.

2.3.1 Factors Affecting Microbial Quality of Fish and Shellfish

Microbiological standards are largely based on observance of good manufacturing practices and proper personal hygiene (Infofish, 1987). The quality changes and spoilage of shrimp is reported to be influenced by post mortem, nucleotide decomposition and other biochemical reaction and microbial growth. Microorganisms play an important role in the spoilage of seafoods (Cann, 1977; Shewan, 1997).

Bacterial flora of fish is more directly related to environmental factors (Lee and Pfierfer, 1977). Shewan (1997) indicated that bacterial flora are a function of the environment. Warm water fishes have more mesophilic and

Gram-positive bacteria while coldwater fishes carry predominantly Gram-negative population (Shewan, 1977).

The method by which fish are harvested is often mentioned as influencing the number and types of bacteria on the raw material (Nickelson and Vanderzant, 1967).

Most of the factors affecting microbial quality previously mentioned are difficult, if not possible to control. Onboard handling is one of the first points where substantial progress is possible. Fish should be cooled down to the temperature of melting ice, as quickly as possible to control bacterial growth and hence spoilage. The unit operation in fish processing should be strictly controlled and monitored to have effective control over bacterial growth. The sanitary conditions of the seafood processing plants do correlate well with the microbial quality of the finished product (Duran *et al.*, 1983; Wentz *et al.*, 1985).

An effective inplant sanitation program could possibly lead to improved product quality and shelf life. But The authors theorized that establishing that effective sanitation would be contingent on decontamination of reducing microbial loads on fish before enter the processing lines. This can be accomplished by effective hygienic practices.

After the removal of shrimp from its natural habitat, a series of standard handling practices begins each of which influence subsequent bacterial population. Improper handling may introduce extraneous microbes and foreign matter. The number and types of bacteria in a shellfish product, therefore reflects the changes that have occured in the initial flora and the degree of

contamination that has taken place in the case of handling on shore (Cann, 1977).

Many operations are followed in handling prawns, which are dependent on the type of products being processed. Each operation has its effect on bacterial quality. E.g. beheading reduces the bacterial counts by about 50-80% (Williams and Campell, 1952). Because the major portion of the shrimp's bacterial load is in the Cephalothoraxes or head. But Cann (1977) did not observe any significant difference in bacterial count between whole and beheaded prawns.

Efficient washing may reduce the initial bacterial count as much as 90% of the surface bacterial flora of prawns (Pillai *et al.*, 1961). Beheading, washing and freezing reduce significantly the total bacterial counts of the final products (Sumner *et al.*, 1982). Chilling is the most important step to contain bacterial growth and delay spoilage.

Each processing operation in the processing plants has effect on the bacterial load and hence these operations should be carefully designed and monitored.

In general, the microbiological quality of frozen fish product is influenced to a great extent by the quality of raw material, the processing treatments adopted by the factory, the sanitary condition of processing factories, the method of freezing and freezing rate (Luyet and Gehenio, 1940; Chen *et al.*, 1990), storage temperature and time (Reilly *et al.*, 1986; Kereluk and Gunderson, 1959; Larkin *et al.* 1955), the initial numbers and type of microorganisms present (Huss, 1934; Record and Taylor, 1953), thawing process and physical protection offered by packaging (Hood,1973).

The high bacterial counts often encounted in cooked frozen products may be due to external contamination (Pillai and Lekshmi, 1961). Major sources of contamination are cooling water, floors, surfaces, utensils, workers and the water used for glazing and re glazing.

2.4 Total bacterial count

This is one of the most commonly used microbiological indicator of food and it is a measure of the total number of bacteria that can grow at 37 °C from each gram of the sample. As a microbiological indicator this test has a two-fold value, i.e. it provides an assessment of the general sanitation level of the plant practices and it serves as an index of the probable shelf life of the product (Bonnel, 1994).

Many authors reported that bacterial load of freshly harvested prawns, from temperate environments range from 10^3 to 10^5/g (Layrisse and Matches, 1984; Lillard *et al.*, 1984; Matches and Layrisse, 1985), but Lannilongue *et al.* (1982) come across counts as high as 10^6 to 10^7/g.

Counts reported from tropical countries range from 10^3–10^6 (Karunasagar, I. *et al.*, 1992; Nirmala *et al.*, 1992). A report by De Silva (1985) how ever, gave counts as high as 10^8/g. With proper washing the bacterial load of prawn can be reduced by as much as 65% (De Silva, 1985). The bacterial loads of brackish water prawn were found to be in the range of 10^3 – 10^4/g immediately after harvesting although the counts increased during handling (Reilly *et al.*, 1984). Soaking the shrimp in 10 PPM chlorine reduce the specific growth rate of TPC (Sunarya *et al.*, 1991). Beheading, washing and freezing reduced the total plate count on final products (Sumner *et al.*,

1982). Peeling and deveining resulted in an increase in bacterial counts (Nirmala and Gopakumar, 1990).

Microbiological evaluation of prawn processing made at 4 different processing stages in a factory (Jayaweera and Subasinghe, 1990), showed that the bacterial counts of raw material ranged from 10^5 to 10^8/g, with majority (45%) of the samples in the range 10^6 to10^7/g and in the end product 58% of samples had the bacterial count in the range 10^5 to 10^6/g. After adopting Good Manufacturing Practices (GMP) the total viable count (TVC) reduced from 10^6 to 10^4 and 10^2 CFU/g in the cooked shrimp (Pongpen *et al.*, 1990).

Iyer *et al.* (1990) conducted a study on shrimp hygiene and quality control and their study showed that 63.6% of the raw shrimp, prior to processing, had higher counts, greater than 10^6/g but in the case of processed shrimp considerable reduction in total bacterial count was noted. Rashid (1992) studied the pathogenic Vibrios and other bacteria in imported frozen samples and found that the total bacterial count were between 2 x10^4 – and 4 x10^6/g. Berry *et al.* (1994) compared the microbial quality of shrimp from different countries at both wholesale and retail level and found that whole sale shrimp products were consistently excellent in quality with respect to aerobic plate count. Higher contamination levels were observed in frozen retail samples. This is most likely due to mishandling once the product has reached the retail level (Berry *et al.*, 1994).

The maximum allowable levels for the total number of bacteria present on seafood's products as per EEC standards are as follows

1. Fresh/frozen products: less than 1000000/g and for cooked produ less than 300000/g.

2.5 Pathogens

2.5.1 Indicator Organisms

2.5.1.1 *Escherichia coli*

Escherichia coli is aerobic, Gram negative, highly motile non sp forming rod shaped bacteria. Coliform groups of bacteria are known to valuable indicators of sanitary quality of foods. This is the most comm aerobic organisms in the intestinal tract of man and warm blooded anim (Huss, 1994) and it is natural to assume that their presence in food indica contamination with faces of human and animal origin and measurement sanitary quality (Geldrich, 1983; Bej *et al.*, 1990; Schardinger, 1892). *E.co* therefore frequently referred to as faecal coli (Paul *et al.*, 1960).

E.coli is chosen as the indicator of faecal contamination becau enumeration of *E.coli* is easy and inexpensive and levels of *E.coli* can easily quantified, where as levels of many pathogens are difficult to quan (USFDA, 1996). Its presence, particularly in small numbers, does necessarily mean that the foods contain faecal matter. It does suggest a l standard of hygiene.

E.coli is a worldwide cause of infection in human and animals result in a wide variety of intestinal and extra intestinal diseases (Salyers *et* 1994). Generally the *E. coli* strains that colonize the gastrointestinal tract harmless commensals, or they play an important role in maintaining intesti

physiology (Huss, 1994). But some of the *E.coli* strains particularly those belonging to the class of Enteropathogenic (EPHC), Enteroinvasive (EIEC), Enterotoxigenic (ETEC), and Enterohemorrhagic (EHEC) are causing diseases. (Doyle, 1990).

E.coli 0157:H7 is recognised as an important human pathogen (Padhye and Doyle, 1992). Though the minimal infective dose of human Enteropathogenic *Escherichia coli* is 107 – 108 cells, detection of low numbers of EEC in food indicates health hazard (ICMSF, 1978). Therefore the number of *E.coli* in food products is to be restricted.

E.coli may be isolated in environments polluted by faecal material or sewage and the organism can multiply and survive for a long time in this environment (Rhodes and Kator, 1988; Jimnez *et al.,* 1989). But, it is also found in unpolluted warm tropical waters, where it can survive indefinitely (Hazen, 1988; Toranzos *et al.*, 1988).

E.coli is commonly found in different seafoods like scallops, mussels, oysters (Gorczuca *et al.*, 1985), frozen shrimp and cuttlefish (Varma *et al.*, 1985), squid (Leejee james *et al.*,1998), and marine fishes, (Stephen *et al.*, 1975). Prevalence of *E.coli* in marine fishes from landing centre and retail market and deck surface of fishing boats are reported by Rao *et al.* (1978).

Freshly caught penaeid shrimp (Cann, 1977) and freshly harvested marine shrimps (Foneska and Widanapthirana, 1990; Iyer *et al.*, 1990), show absence of *E.coli.* Prawns caught in deeper water generally do not carry *E.coli* (Cann, 1977). But smaller in shore shrimp of *Parapeneopsis* spp were contaminated with *E.coli.* Earlier workers have reported *E.coli* in the freshly caught marine shrimps (Sumner *et al.*, 1982; Jauaweere and Subashinghe,

1990). Sahu *et al.* (1998) isolated *E. coli* including enterotoxigenic *E.coli* sero types from shrimp.

It is generally accepted that the hygienic performances of any seafood process (with respect to safety) should be assured on the basis of the enumeration of *E.coli* on the product, because this organism is considered to be the most positive indicator of faecal contamination (Bonnel, 1994) and also is a recognised indicator of possible contamination with enteric pathogens (Liston, 1980; D'Acoust, 1989). But there is no good relation between *E.coli* and enteric pathogens because it dies off faster than the latter (Temple *et al.*, 1988; Burton *et al.*, 1987; Hobbs, 1983).

Presence of these bacteria on fish product indicates a breakdown in the sanitary practices of the plant and is usually due to poor employee hygiene practices, poor clean up procedures and the use of contaminated water (Bonnell, 1994).

Natural water gets contaminated with *E.coli* either by direct contact or by mixing up with terrestrial sewage. When this water used for processing, these organisms enter the product. Process water in the processing area of the factory has been found to be contaminated with *E.coli* (Jayaweera *et al.*, 1990). Similar possibilities occur in the ice used for preservation and in the case of utensils used for processing.

There are reports indicating the presence of *E.coli* from cooked prawns (Green wood, 1985). Varma *et al.* (1985) found that in all the samples of cooked and peeled frozen shrimp *E.coli* was absent.

When once the organism entered into a food product, it is very difficult to get rid of them completely. But significant reduction in bacterial load during

processing stages of prawn has been reported (Jayaweera and Subasinghe, 1990).

Washing with clean water and proper handling techniques will reduce high *E.coli* counts (Pongpen *et al.*, 1990) and they reported that 40 PPM chlorine water was effective and gave a large reduction in *E.coli* counts. Sunarya *et al.* (1992) Sumpeno *et* al. (1990) found that effective inplant chlorination and frozen storage reduce *E.coli* count and *E.coli* was not detected in the final frozen shrimp samples. But Jayaweera and Subasinghe (1990) noticed that the *E.coli* count did not vary significantly during different processing stages and the count declined slightly during the stage just before freezing.

E.coli is very sensitive to sub zero temperature. About 95% reduction in the count *E.coli* takes place during frozen storage at – 20°C in a period of about 4 - 5 months (Iyer, 1989)

Control of enteropathogenic *E.coli* in seafood can be attained by adequate cooking and avoidance of recontamination of cooked seafood from contaminated equipment, water or infected handlers. Monitoring coliform bacteria may be useful in food processing plants where *E.coli* is considered as hazard. The maximum allowable number of *E.coli* in raw fish and fishery products is 20/g and it should be absent in cooked fishery products.

2.5.1.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-motile, facultative anaerobic and nonspore forming cocci belonging to the family Micrococcaceae.

Staphylococci are widely distributed in the environment and the main reservoir of *S.aureus* is man: hands, face, sweats, boils, ulcers, nasal cavities, throat, and post nasal drips of man contain this organisms in considerable number (Bryon, 1976; Minor, 1976). Ahmed (1991) reported that the human carrier rate may be up to 60% of healthy individuals with an average of 25 - 30% of population being positive for entero toxin producing strains.

This organism is important since it produces a toxin that causes the particular food to bring about staphylococcal food poisoning in anyone who ingests it (Bonnel, 1994). *Staphylococcus aureus* can produce several types of entero toxin causing gastroenteritis (Halpin, 1989).

These staphylococcal enterotoxins (SE) are a group of single chain proteins with molecular weights in the range 27500-30000. There are seven serologically distinct enterotoxins, designated SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE (Tranter, 1990) Although these classical food poisoning toxins are thought to be produced mainly by strains of *S.aureus* that are coagulase positive and thermostable nuclease positive (Baird parker, 1974).

A number of comprehensive reviews on staphylococcal food poisoning and the staphylococcal enterotoxins have been published (Baired parker, 1974; Minor *et al.*, 1976; Tranter, 1990). The toxins are very resistant to proteolytic enzymes and heat (Huss, 1994). Less than one micro gram of toxin can result in illness (Tatini *et al.*, 1984).

The disease caused by *S.aureus* is intoxication. The illness starts 2 - 6 hours after eating contaminated food. Common symptoms are acute nausea, vomiting and abdominal pain often followed by diarrhoea. In severe cases

prostration and dehydration can also occur (Huss, 1994). There is high morbidity rate but a low mortality rate. The illness is usually short and sharp with full recovery in most cases with in 24 hour even from a state of collapse. There is no fever (Gilbert, 1974).

A wide variety of foods have been implicated in outbreaks of staphylococcal food poisoning frozen meats, including cured products such as ham, beef and pork pies, and cold poultry are the foods most frequently implicated but some outbreaks are associated with dairy products, canned vegetables, cooked fish and frozen seafoods. (Gilbert and Wieneke, 1973).

There are about 107 cases of staphylococcal intoxication outbreaks in which fish were implicated as vehicle in USA during 1973-75 (Bryon, 1980). Factors that contributed to food borne staphylococcal intoxication are in-adequate cooling, faulty fermentation, inadequate heat processing, infected persons handling the foodstuffs and inadequate cleaning of equipments used for the food processing.

Foods that had been associated with *staphylococcus* enterotoxin include cooked meat, shellfish, tuna and ham (Baired parker, 1987). *Staphylococcus* do not normally present in freshly caught prawn (Reilly *et al*., 1984). In processed prawn it may be present due to insanitary practices during fish product handling (Kvenberg, 1991).

Gunderson *et al.* (1954) examined frozen raw breaded shrimp and found coagulase positive staphylococci in all samples. Silverman *et al.* (1961) examined frozen raw and cooked shrimp and found that uncooked samples generally contained coagulase positive staphylococci, but the organism was absent from most of the cooked samples. Heat processing and normal

cooking temperatures are sufficient to kill the bacterial cells (Kraybill, 1948). Thus the presence of staphylococci in raw naturally contaminated food is of little significance. In contrast, rapid growth and toxin production can take place in precooked shrimp recontaminated with *S.aureus.* Time temperature conditions allow fast growth of the organism (Huss, 1994). Greenwood *et al.* (1985) isolated *S.aureus* from cooked prawn and shrimps. The main methods cause of contamination of cooked shrimp by staphylococci is by handling the food. The organisms can come from nose, hands, or from septic lesions of food handlers. Storage of the food at temperature between 10 and 45°C will encourage both multiplication and production of enterotoxin (Gilbert, 1974). Gorczyea *et al.* (1985) isolated *S.aureus* from seafoods at retail outlets and 97% of them had *S.aureus*.

Fish right from its catch is handled onboard by workers, and from there on, till the final product is prepared, by workers engaged in factory. *Staphylococcus* contamination in the fish and fishery products is considered as an indication of unsatisfactory hygiene condition of workers (Anon, 1996). The carrier rate of the staphylococci among the peeling workers was 32.61% and contamination by peeling tables was 35.19% (Dey, 1994). Delacruz *et al.* (1988) examined the plant workers hands for the presence of *S.aureus* and reported that about 50% of plant workers showed the presence of *S.aureus.* and more than 65% of the factory workers were found to carry *S.aureus* on their fingers (Jayaweera and Subasinghe, 1990).

Staphylococcus count decline during processing although it is also noticed that water used for washing the shrimp in the processing plant is contaminated by *S.aureus* (Sumpeno *et al.*, 1990). But during the process of

beheading and grading the *S.aureus* count increases significantly (Jayaweera and Subasinghe, 1994). The presence of *S.aureus* is detected during the evisceration phase and not at the reception phase (Fernandes *et al.*, 1991). The count increases during processing count ranging from less than 50/g to 10^3/g in the raw material to 10^2 to greater than 10^4/g (Jayaweera and Subasinghe, 1990). The presence of *Staphylococcus aureus* in large numbers indicates poor personal hygiene. Chanda *et al.* (1998) studied the growth kinetics of *S.aureus* and found that *S.aureus* survived up to 10 months of storage in case of liquid nitrogen frozen sample and more than 12 months in case of conventional frozen sample of cooked prawn meat.

Staphylococci are intimately associated with man, and therefore contamination of foods with Staphylococci during preparation is likely. Control of food borne *Staphylococcus* intoxication depends on preventing multiplication of these organisms and the resultant production of their entero toxin in food. The maximum allowable number of *Staphylococcus aureus* in raw and cooked fish and fishery products in India is 100/g.

2.5.2 Other pathogens

2.5.2.1 *Salmonella*

Salmonellae are some of the most important food contaminating bacteria, which can produce life threatening intoxications.

Salmonella are Gram negative rod shaped bacteria mostly motile with the exception of *S.pullorum* and *S.gallinarium.* They do not form spores. *Salmonellae* are enteric organisms belonging to the family Enterobacteriaceae.

These mesophilic organisms are distributed all over the world, but principally occurring in the gut of man and animals and in environments polluted with human or animal excreta (Huss, 1994).

Salmonella as an agent of food borne disease "Salmonellosis" is known all over the world. Two clinical entities caused by *salmonella* are recognised: enteric fever and more common food poisoning syndrome (Flowers, 1988). Enteric fever, commonly referred as typhoid fever, is primarily caused by *S.typhi* and paratyphoid fever is caused by S.*paratyphi* (Ward and Hackney, 1991). The principle symptoms of salmonellosis are non bloody diarrhea, abdominal pain, fever, nausea, vomiting, headache which generally appears within 12 - 48 hours after ingestion (Huss, 1994) and usually persist for several days with the illness being self limiting (Ketchum, 1988; Kvenberg, 1991).

In the genus *Salmonella* more than 2000 serotypes are recognised (Flowers, 1988). The presence of any serotype of *Salmonella* is potentially dangerous as a source of human disease, either directly by consuming the food or indirectly through secondary contamination (Poelma and Sillicker, 1976). and most food borne salmonellosis are caused by non host adopted serotypes (Flowers ,1988).

Human population of all age groups are susceptible to *Salmonella* infection but infants, the elderly, under-nourished and people with illness are known to be more susceptible to the disease (Kvenberg, 1991). The dose of infection may be as low as 15 - 20 viable cells to more than 10^5 depending upon *Salmonella* serotypes, condition, age and the general health status of the person (D'Aoust and Pivinick, 1976). Concerned literature indicates that

seafood is a much less common vehicle for *Salmonella* than other foods and fish and shellfish are responsible for only a small proportion of total number of *Salmonella* cases reported in USA and else where (Ahmed, 1991). But there is a report that consumption of contaminated raw shellfish harvested from sewage polluted water caused Salmonellosis (HEW, 1985). So seafood also can be considered an important source on consumer infection with Salmonellosis (Liston, 1990; Nerker D.P., 1990; D'Aoust, 1989).

Conventional methods for detecting low levels of salmonellae in foods are laborious and time consuming, requiring 3 - 7 days. Rapid, highly sensitive methods such as DNA-DNA hybridization, ELISA, and bacteriophage have been found to be reliable for detection of *Salmonella* in foods (Andrews, 1985, Blackburn, 1993).

Salmonella is widely distributed in the environment. Several authoritative reviews on *Salmonella* associated with fresh water bodies (Roy *et al.*, 1984), sewage (Alcaide *et al.*, 1983) polluted waters (Menon, 1985) brackish water ponds (Reilly, 1992) are available. *Salmonella* is part of the natural micro flora of shrimp culture environment (Reilly *et al.*, 1992). But Dalsgaard *et al.* (1995) reported that *Salmonella* is not commonly found in shrimp production area.

Salmonella is absent in freshly caught seafoods (Cann, 1977; Hobbs, 1982; Karunasagar, 1992). S*almonella* is not found in the marine shrimp naturally and any *Salmonella* in the marine shrimp is probably due to contamination during handling, transportation or processing (Sunarya *et al.*, 1990).

Occurrence of *Salmonella* in seafoods has been reported (Liston *et al.*, 1971; Anon, 1966). *Salmonella* is present in brackish water prawn (Reilly, 1992) frozen white shrimp (Pongpen *et al.*, 1990) and in shrimp before any preprocessing and handling (Iyer *et al.*, 1990; Nayyarahamed, 1995). Normally, the incidence of *Salmonella* in marine fish and shellfish is the result of contamination from the unhygienic surroundings in which they are handled and processed.

In general, the incidence of *Salmonella* was found to be higher in fish samples compared to that in shrimps and cuttlefish (Iyer, 1989 b).

Different *Salmonella* species have been reported from seafoods in India (Narkar and Bendekar, 1990; Iyer, 1991). *S.weltevreden* is the dominating serotypes in frozen seafoods (Iyer and Shrivastava, 1989a)

Varma *et al.* (1991) studied the bacteriological quality of frozen seafoods for export and reported that *Salmonella* was totally absent in samples of cooked and peeled frozen shrimp. Rashid (1992) reported that no *Salmonella* were detected from samples of imported frozen shrimps. Greenwood *et al.* (1985) studied the microbiology of cooked prawns and shrimps and found that *Salmonella* was not present in any of the 148 samples analysed. It was absent through out the plant survey (Delacruz *et al.*, 1990) and any stage during processing (Jayaweera and Subasinghe, 1990).

Contamination with *Salmonella* was investigated by Pongpen *et al.* (1990) and they reported the presence of *Salmonella* in fresh and frozen white shrimp and peeled and cooked marine shrimp. Anderson *et al.* (1971) and Beckers *et al.* (1981) isolated *Salmonella* from cooked frozen shrimps. *Salmonella* is normally found in raw shrimp and not in the freezing stage

(Fernandes, 1991). Iyer *et al.* (1990) reported that 9% of the raw shrimp samples showed the presence of *Salmonella* but after processing only 4% of the shrimp samples showed contamination by *Salmonella*.

Due to decomposition and *Salmonella* contamination, frozen shrimps exported from India were rejected several times (Chiou *et al.*, 1981). Contamination of shrimp by *Salmonella* is not only from India. But from several countries as well (Gecan *et al.*, 1994).

In 1979, FDA imposed ban on Indian shrimps presence of *Salmonella* (Anon, 1980). The main sources of *Salmonella* in shrimps are water from culture ponds, coastal seawater, process water, ice, shrimp contact surface, floor, rodent and lizard droppings (Iyer, 1990). Shrimp from local markets, landing ports and peeling sheds also contributed to contamination with *Salmonella* (Pongpen, 1990).

Salmonella are resistant to freezing at – 40°C and can remain viable for about 7 months during subsequent storage at – 20°C (Iyer, 1989b). Also, *Salmonella* are heat sensitive and ordinary pasteurizing or cooking conditions are generally sufficient to kill *Salmonella* in high moisture foods (Flowers.1988).

All possible measures should taken to avoid contamination of fishery products with *Salmonella* by improving plant sanitation and hygiene and by adhering strictly to the scientifically accepted procedure for handling and processing of the material (Iyer, 1989).

2.5.2.2 *Vibrio cholerae*

Vibrios are Gram negative, motile facultatively anaerobic, non-spore forming curved rod shaped bacteria. The most important member of the group is *Vibrio cholerae*

Man is the natural reservoir of *V.cholerae* and it is transmitted from man to man through the environment.

V.cholerae is usually divided into two groups, serotypes 01 and non-01. Those groups can be further sub divided as toxigenic and non-toxigenic and the 01 serotypes occur in two forms: the classic and the ELTOR. Most cholerae is caused by EL-TOR variant (Huss, 1994).

Vibrio cholerae is the bacterium responsible for cholerae epidemics and outbreaks (Madden, 1988). Symptoms of *V.cholerae* 01 infection can range from mild diarrhoea to severe illness. In severe cases, *V.cholerae* can cause profuse watery diarrhoea, dehydration and death if not promptly treated.

V.cholerae infection has been attributed to the production of toxin by the microorganisms, which adhere to and colonize the small intestine of infected individual. The cholera toxin cause massive fluid loss from cells lining the intestinal tract and, similar rice-water stool (*Farmer et al.*, 1985; Madden *et al.*, 1988). Diarrhoea can last 1-5 days and is usually accompanied by vomiting.

The non-toxigenic *V.cholerae* strains are principally associated with gastrointestinal illness. But it also causes a much wider spectrum of disease (Morries, 1981). Most Vibrios produce powerful entero toxin and as little as 5

micro gram cholera toxin administered orally caused diarrhoea in human volunteers (Varman and Evans, 1991).

A wide variety of foods including soft drinks, fruits and vegetables have been involved in transmission of cholera (Varman and Evans, 1991). How ever, raw uncooked or cooked shellfish have been established as the major vehicle for *Vibrio cholerae* 01 and non-01 (Morris and Black, 1985). Seafood products have been increasingly incriminated in cholera out breaks since the years 1960. An explosive out break of *V.cholerae* ELTOR in the Philippines during 1961 and 1962 was reported by Joseph *et al.* (1965). They suggested that the initial infection was transmitted principally by shrimp that were consumed raw. Dutt *et al.* (1971) investigated the role of shellfish in *Vibrio* infections during 1969 cholerae outbreaks in Kelantan, Malaysia. *Vibrio cholerae* has also been reported in brackish water and fresh water (Black *et al.*, 1980). It has been even reported from marine environment (Kenyon *et al.*, 1984; Mathew, 1986). Lee *et al.* (1982) reported that *V.cholerae* was found in brackish water.

V.cholerae has been reported from brackish water (Black *et al.*, 1980; Lee *et al.*, 1982), fresh water (Black *et al.*, 1980), from marine environment (Kenyon *et al.*, 1984; Mathew, 1986). *V.cholerae* is absent in freshly harvested shrimp (Karunasagar *et al.*, 1992), but is recovered from shrimp production area (Dalsgaard *et al.*, 1995). Reilly *et al.* (1992) have reported that *V.cholerae* is present in brackish water ponds and cultured prawns and they suggested that culturing by intensive methods tend to favour contamination with this pathogen. Potentially pathogenic vibrios could be normal inhabitants of the gut of cultured shrimp (Nayyarahamed *et al.*, 1995).

Abuxapqui (1996) conducted a study to find out the microbiological quality of seafood from Mexico restaurants and *V.cholerae* was not isolated from any of the sample. However, *Vibrio cholerae* was recovered from commercially frozen seafoods including peeled shrimp and fish (Wong *et al.*, 1995), imported seafoods (Akihirominami, 1991) and prawn processing areas (Delacruz *et al.*, 1990). Iyer *et al.* (1990) reported that *V.cholerae* non 01 is not present in marine fish and is introduced into the product during handling.

Berry *et al.* (1994) conducted a study to examine the extent of contamination by *Vibrio* species from major consignments of exported shrimp and found that *V.cholerae* was present in 10% of the sample. Dalsgaard *et al.* (1996) isolated non-01 *V.cholerae* from cooked frozen and raw frozen shrimp products imported into Denmark.

Consignments from India were of frozen raw shrimp, rejected by Japan, during 1980-85 due the presence of *V.cholerae* (Reilly *et al.*, 1986).

The survival of *V.cholerae* under varying conditions has been investigated by a number of researchers. Pollitizer (1959) reviewed the literature on *V.cholerae* and reported a wide range of survival period depending on experimental conditions. Low temperature processing is detrimental to most *Vibrio* spp. Reily and Hackney (1985) showed that mesophilic *V.cholerae* 01 decreased over time, but surviving cells persisted for longer than three weeks at refrigerating or freezing temperature in artificially contaminated seafoods. Cholera vibrios are known to be quite sensitive to low temperature (Singleton *et al.*, 1982). Also vibrios are easily destroyed by heat. Thus cooking is sufficient to eliminate most vibrios.

However, Black *et al.* (1980) found *V.cholerae* 01 to survive boiling for up to 8 min and steaming for up to 25 min in naturally contaminated crab.

Consumption of raw seafoods should be avoided and seafoods should be refrigerated or iced immediately upon harvesting and kept at low temperature until consumption. Adequate cooking and avoidance of recontamination will ensure the safety of consumed foods (Madden, 1988).

Since *V.cholerae* is a pathogen of strong virulence it is expected to be absent from a sample of 25 g fish and fishery products.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1Sources of Samples:

A number of EU approved processing plants around Cochin exporting seafoods to European Union were selected for this study. All important species of prawn viz. Sea Tiger and Black Tiger (*Penaeus monodon*) White (*Penaeus indicus*) Flower (*P.semisulcatus*), Brown (*Metapenaeus dobsonii*), Kazhunthan (*M.affinis*), Choodan (*Metapenaeus monoceros*), *M.brevicornis*, Karukkadi (*Parapenaeus stilifera*), Deep sea shrimp (*Aristus* spp) and Fresh water prawn (*Macrobrachium rosenbergii)* of different size grades were collected.

The following frozen samples from different species mentioned above were collected

Type of sample	Number collected
Headless (HL)	20
Peeled and Deveined (PD)	12
Peeled and Undeveined (PUD)	28
Cooked and peeled (CP)	22

3.2 Sampling of Frozen Shrimps:

At each sampling, different carton frozen shrimp that had-been cold stored (-20°C) for not more than six months were selected at random. Approximately 100 gram of the samples were collected aseptically in a sterile stainless steel dish using a sterile stainless steel scooper. Samples from different cartons were collected separately into sterile sample dishes. These samples were

wrapped in Polythene paper and kept in ice in an insulated box. It was then taken to the laboratory and samples were analyzed on the same day. The samples were kept in the deep freezer at -20°C until they were taken for analysis.

Ten gram of sample was used for enumeration *of Escherichia coli, Staphylococcus aureus* and total bacterial count. For detecting the presence of *Salmonella* and *Vibrio cholera* 25 gram each of samples were used.

3.3 Preparation of Samples:

Samples were aseptically taken from the sample dishes using sterile forceps and scissors. Ten gram samples were homogenized in 90ml of sterile phosphate buffer solution and automatically stomached for 1min using a Stomacher 400 lab blender (Seward medical, London, UK). Appropriate serial dilutions were made in sterile 9 ml phosphate buffer using sterile pipettes and plated in duplicates on specific media shown in the Table 3.1. To enumerate total plate count, *Escherichia coli*, *Staphylococcus aureus* the temperature and incubation time followed in each method are indicated in the Table 3.1.

3.4 Bacteriological analysis

3.4.1 Total bacterial count

One ml of each dilutions viz. 10^2, 10^3,10^4 and 10^5 of shrimp extract were prepared were pipetted in to sterile petridishes in duplicate. 10 -20 ml of sterile molten tryptone glucose beef extract agar cooled to about 40°C was added to each plate. The same medium was added to another two

petridishes, each containing 1 ml sterile phosphate buffer but no inoculam of the sample. These dishes served as control to test the sterility of the media, pipettes and buffer used. Before solidifying, the plates were rotated gently both clock wise and anti clock wise (6 rotation each). The dishes were kept on the laboratory table for 15-20 minutes for solidification of the added agar and then they were incubated in an inverted position at 37°C for 48 hours. After incubation, all the colonies developing on the agar were counted using colony counter and the average count of the duplicate plates were recorded.

Plates containing colonies between 30 and 300 were used for the calculation. Counts are expressed in CFU/g of shrimp sample.

3.4.2 *Escherichia coli*

0.5 ml of 10^2 dilution of shrimp extract was added in duplicate to the sterile Tergitol 7 agar (Oxoid) plates added with 0.4 ml of one % Triphenyl tertazolium chloride (TTC). Then it was spreaded using sterile bent glass rod and incubated at 37 °C for 24 hours. The colonies appear as circular, non-mucoid; flat, yellow with pinkish tinch at the centre were recorded as *Escherichia coli*. The typical yellow colonies were selected and confirmed by the following biochemical tests. Positive colonies were counted, calculated and the results were expressed as number of *E.coli* per gram of shrimp sample.

3.4.2.1. Biochemical tests

The major and important biochemical tests followed for the identification of *Escherichia coli* are

a. Eijkman's Test
b. Indole Test
c. Methyl red Test
d. Voges-proskaver Test
e. Citrate utilization Test

Typical reaction of *E.coli* to IMViC pattern is "positive/ positive/ negative/ negative" respectively.

3.4.2.1.1 Eijkman's Test:

A loopful of culture was inoculated into 5 ml EC broth and incubated at 44.5°C for 48 hours in a water bath. Production of gas in the broth was taken as positive reaction and no gas production in the broth as negative test.

3.4.2.1.2 Indole production Test:

Cultures from nutrient agar (Hi Media Ltd.,) slants were inoculated in to 5 ml Tryptone Broth and incubated at 37°C for 24 hours. To these tubes 0.2-0.3 ml of Kovac's reagent was added and allowed to stand for 10 minutes. Deep red colour in the amyl alcohol surface layer was taken as positive reaction and the development of yellow colour at the surface of the broth as the negative test.

3.4.2.1.3 Methyl Red Test:

A loopful of test culture was inoculated into 5ml sterile MRVP medium and incubated at 37°C for 48 - 72 ± 2 hours. From this 2.5 ml of broth culture was aseptically transferred to a tube and 5-6 drops of methyl red solution was added. Positive reaction was indicated by the development of red colour and a distinct yellow colour indicated methyl red negative.

3.4.2.1.4 Voges-proskauer Test

Inoculated tubes of MRVP medium was incubated at 37°C for 48 hours. From this 2.5 ml of culture broth, 0.6ml of ∝-naphlthol solution and 0.2 ml of potassium hydroxide (KOH) were added to a tube. Positive test is indicated by the development of pink or crimson colour from 2-4 hours after adding the reagent.

3.4.2.1.5 Citrate Utilization Test

A loopful of culture was picked from the nutrient agar slants and inoculated in to Citrate Broth and incubated at 37°C for 3-4 days. Visible growth was recorded as positive.

3.4.3 *Staphylococcus aureus*

0.5 ml of 10^2 dilution of shrimp extract was added in duplicate to the sterile Baired parker agar (oxoid) plates added with 1 ml of 1% potassium tellurate solution and 5 ml of egg yolk emulsion. Then it was spreaded using sterile bent glass rod. The plates were incubated at 37°C for 24-36 hours. The

colonies of *Staphylococcus aureus* were black, convex, narrow white with entire margin and surrounded by a clearing zone of 2-5 mm in width. Suspected colonies were confirmed by coagulase plasma test and microscopic examination. Positive colonies were counted, calculated and the results were expressed as number of *Staphylococcus aureus* per gram of shrimp sample.

3.4.3.1 Coagulase Test

0.5 ml of rabbit plasma was added in to small sterile test tubes and 2 drops of suspected organism 24 hours old in Brine Heart Infusion Broth, was transferred in to the tubes and thoroughly mixed. Tubes were incubated at 37°C and examined periodically over 6 hours period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted was considered positive for *Staphylococcus aureus*

Table 3.1 Media and incubation conditions used for bacteriological sample analysis

Test	Incubation			
	Time (h)	Temperature (°C)	Atmosphere	Medium
TPC	24 – 48	37	Aerobic	TGA
E.coli	24	37	Aerobic	Tergitol 7
S.aureus	24 - 36	37	Aerobic	Baired Parker Medium

3.4.4 *Salmonella*

The method described by Andrews et al. (1984) in the USFDA Bacteriological Analytical Manual (6th ed.) was followed.

25 g of each shrimp sample was taken aseptically and homogenized in 225 ml of pre-enrichment lactose broth (oxoid) contained in a sterile stomacher bags and automatically stomached for 1 min using a Stomacher 400 lab blender (Seward medical, London, UK) and aseptically transferred to a 500 ml sterile conical flask. Each homogenized sample was incubated for 24 hours at 37°C

Enrichment

From the pre-enrichment 1 ml portion each was inoculated into 9 ml of selective enrichment selenite cystine (SC) broth (oxoid) and 9 ml of Tetra thionate broth (TTB) and incubated at 37°C for 24 hours.

Colony analysis after enrichment of culture:

After enrichment, a loopful of enriched sample was streaked on to Brilliant Green Agar plates, Xylose Lysine Desoxycholate Agar plates, Hektoen Enteric Agar plates and Bismuth Sulphite agar plates (all Oxoid media) that were pre dried for 1 hour at 45°C and these plates were incubated at 37°C for 24 - 48 hours. After incubation the plates were checked for the following colony morphology

On BSA plates : Brown grey to black, with slivery metalic sheen

On BGA plates : High pink transparent colonies with surrounding medium pink to red

On XLDA plates : Red colonies with black centre

On HEA plates : Green-blue colonies with black centre

Suspicious colonies were streaked on Triple sugar iron (TSI) agar slants and stabbed into the butt of slants with a sterile inoculating needle. Followed by inoculation of Lysine Iron agar (LIA) slants with the same loop. The TSI and LIA slants were incubated at 37°C for 24 hours. In TSI slants, *Salmonella* spp. typically produces an alkaline (red) slant and acid (yellow) butt, with or with out producing H_2S (blackening of agar). Similarly the typical colonies showed an alkaline (purple) reaction in the butt of LIA with or without H_2S. Suspected colonies on both TSI and LIA slants were analysed further with biochemical tests for the identification of Salmonella.

3.4.4.1 Biochemical Tests for *Salmonella*

The minimal biochemical characteristics that are required to identify the genus *salmonella* are given in the Table 3.2

Table 3 .2 Biochemical profile of *Salmonella*

Test		Typical reaction
1	TSI	Acid/Alkaline with or without H_2S
2	LIA	Deep purple colour in the butt, with or without H_2S
3	Urease	Negative
4	IMViC Tests Indole test M.Red test V.P. test Citrate Utilization	 Negative Positive Negative Positive
5	Sugar fermentation Lactose Sucrose Salicin Dulcitol	 No acid, no gas No acid, no gas No acid, no gas Acid and gas
6	Amino acid decorboxylase test Lysine Ornithine Arginine	 Positive Negative Negative
7	Melanate test	No colour change
8	KCN broth	No turbidity, No yellow colour
9	Agglutination with *Salmonella* polyvalent O antiserum	 Positive

Source: USFDA Bacteriological Analytical Manual, 1984

3.4.4.1.1IMViC Tests

The cultures were tested for the four IMViC tests as detailed in the sections 3.1

3.4.4.1.2 Sugar Fermentation Test:

Basal sugars fermentation broth containing 1% of the sugars of Lactose, Sucrose, Salicin, Dulcitol and control (with out sugar) were prepared separately in different test tubes. All these tubes were inoculated with a loopful of culture from TSI and LIA. Tubes were incubated at 37°C and were then examined for 4 subsequent days. The pink coloration of the sugar solution was read as positive.

3.4.4.1.3 Amino acid decorboxylase Test

This was conducted for Lysine, Ornithine, and Arginine. Basal decorboxylase broth containing 0.5 % lysine, 0.5% ornithine, 0.5% arginine and control (with out amino acid) were prepared separately in different tubes. All the tubes were inoculated with a loopful of LIA and TSI culture. After the inoculation sterile liquid paraffin was overlaid as a thin layer in all the tubes. Further the tubes were incubated at 37°C for 4 days. The reaction was recorded as positive when the control tubes turned yellow and the tubes with amino acid turned yellow first and then purple after 48 hours or more of incubation. The reaction of *Salmonella* for these tests are detailed in Table 3.2

3.4.4.1.4 Urease Test:

Two 3mm loopful of growth from positive TSI agar slants culture was transferred into rapid urea broth and these tubes were incubated at 37 ± 0.5°C for 2 hours in water bath. No change in colour of medium indicated negative test.

3.4.4.1.5 Malonate Test:

A loopful of TSI agar positive culture was transferred into tryptone broth and incubated at 37°C for 24 hour. 3 mm loopful of 24 hours old tryptone broth culture was transferred to malonate broth with a uninoculated broth as control. These tubes were incubated at 37°C for 48 ± 2 hours. Most salmonella culture react negatively to this test, with green or unchanged broth.

3.4.4.1.6 KCN broth:

3 mm loopful of tryptone broth culture was transferred to KCN broth. Rim of the tube was heated to get good sealing when the tube was stoppered with wax-coated cork. This was incubated at 35°C for 48 ± 2 hours the tube was, also, examined interruption of after 24 hours. Growth indicated positive results. Most Salmonella species do not grow in this medium, as indicated by lack of turbidity.

3.4.4.1.7 ONPG Test:

For each suspected colony cell suspension was prepared in 0.25 ml of physiological saline solution from TSI slants. One drop of toluene was added into each tube and shacked well. These tubes were allowed to stand for about 5 minutes at 37°C. After that 0.2 ml of buffered 0.75 ONPG solution was added to each tubes and incubated in a water bath at 37°C. Tubes were

examined after 30 minutes, 1 hour and 24 hours of incubation. Development of yellow colour indicated positive results.

3.4.4.1.8 *Salmonella* polyvalent O antisera:

Loopful of positive culture was placed on to a clean glass slide marked into two sections. One drop of saline solution was added to lower part of one section and 1 drop of *Salmonella* polyvalent O antisera was added to other section. With clean sterile needle the culture was emulsified in saline solution in one section and also repeated with other section containing antiserum. The mixture was tilted and observed against dark back round. Any degree of agglutinin was considered positive reaction.

3.4.5 Vibrio cholerae

25 g portion of each sample was transferred aseptically to a stomacher bag and homogenized in stomacher with 225 ml sterile alkaline peptone water and aseptically transferred into 500 ml sterile conical flask and incubated for 24 hours at 37°C. From the enrichment a loopful of surface growth was streaked in duplicate on TCBS agar plates that were predried for 1 hour at 45°C and also 1 ml portion of enriched sample was inoculated into 9ml of sterile alkaline peptone water as a secondary enrichment and incubated for 6 hour at 37° C . After incubation one loopful of secondary enrichment was streaked on another TCBS agar (oxoid) plates. Then both the TCBS plates were incubated at 37°C for 18-24 hours. After the incubation period TCBS

plates were observed for typical colonies. Yellow flat smooth colonies with opaque centre and transparent peripheries of 2-3 mm diameter. Suspected colonies were inoculated in to Kligler Iron Agar (Difco) slants and incubated at 37° C for 18 hours. In KIA slants *V.cholerae* produces Acid (yellow) butt and alkaline (pink) slant with no gas and no H_2S (No black colour). Typical positive reaction in KIA is confirmed by the biochemical tests shown in Table 3.3

Table 3.3

3.4.5.1 Biochemical profile of *Vibrio cholerae*

	Test	Typical reaction
1	Motility test	Motile
2	Oxidase test	Positive
3	Sugar fermentation test	
	Glucose	Acid, no gas
	Sucrose	Acid, no gas
	Mannitol	Acid, no gas
	Inositol	No acid, no gas
	Arabinase	No acid, no gas
4	Amino acid decorboxylase test	
	Lysine	Positive
	Ornithine	Positive
	Arginine	Negative

3.4.5.1.1 Motility Test:

Semisolid medium:

Culture from KIA was inoculated in to motility medium by stabbing it into the top of a tube of the semi-solid medium to a depth of about 5mm. and incubated at 35-37°C for 48 hours and spreading of the growth through the medium indicated as motile.

3.4.5.1.2 Oxidase Test:

Piece of filter paper was placed into an empty petridish and 3 drops of freshly prepared 1% aqueous solution of Tetramethyl para phenylene diamine dihydro chloride solution was added to its centre and with a sterile platinum wire, the culture was smeared thoroughly into the reagent impregnated paper. The transferred culture turn dark purple in 5-10 seconds indicated the Oxidase test is positive.

3.4.5.1.3 Sugar fermentation Tests:

Basal Sugar fermentation broth containing 0.5 % various sugars such as Glucose, Sucrose, Mannitol, Arabinose, Inositol and control (with out sugar) were prepared separately in different tubes. All these tubes were inoculated with a loopful of culture from KIA. Tubes were incubated at 37°C and examined for 4-5 subsequent days. Yellow coloration of the sugar solution was read as positive.

3.4.5.1.4 Amino acid Decarboxylase Test:

Basal decorboxylase broth containing o.5 % Lysine, 0.5% Ornithine, 0.5% Arginine and control (without aminoacid) were prepared separately in different tubes. All the tubes were inoculated with a loopful of KIA culture.

After the inoculation, sterile liquid paraffin was over laid as a thin layer in all the tubes. Further, the tubes were incubated at 37°C for 4 days. The reaction was recorded as positive when the control tubes turned yellow and the tubes with amino acid turned yellow first and then purple or alkaline after 48 hours or more of incubation.

3.4.5.1.5 Serological test:

Cell suspension was prepared from the nutrient agar slant and small amount of cell suspensions was transferred into a clean glass slide marked into two sections. A drop of polyvalent *V.cholerae* 'O' antiserum was added to the cell suspension in one section only and mixed it with suspension using a sterile loop of the needle. A rapid strong agglutination indicates is positive reaction. And these positive cultures are tested with Ogawa and Inaba and Hikojima for confirming identity and for specific typing of isolates.

RESULTS

4. RESULTS

The results obtained on bacteriological analysis of 82 samples of frozen prawns collected from different EU approved seafood processing factories are shown in Table 4.1 to 4.8

Table 4.1 gives the bacteriological quality of frozen headless shrimp

In Headless shrimp the bacterial level varied from a minimum of 6×10^4 organisms/g to a maximum of 2.9×10^5 organisms/g. Out of total 20samples 5% samples had a bacterial count of less than 1×10^5 and in 45% samples the bacterial load was between1.1×10^5 and 2.5×10^5. Most of the samples (50%) had a bacterial above 2.5×10^5. No sample had the total bacterial count more than 5×10^5. (Table 4.5)

Table 4.2 gives the results of the bacteriological analysis of frozen PUD shrimp.

The total plate count for PUD ranged from 45000 to 620000 organisms/g.14.28% of PUD shrimp had the total bacterial count less than 1 $\times 10^5$ and 60.71% samples showed the bacterial count between $1 \times 10^5 - 2.5 \times 10^5$. In 21.42% of the samples the bacterial count fell with in the range of 2.5×10^5 to 5×10^5.and only one sample had the count in 6×10^5. Table (4.5)

Table 4.3 gives the results of the bacteriological analysis of PD shrimp

Total plate count of PD shrimps were from 6.3×10^4 to 2.4×10^5. The bacterial load of the PD samples were mainly with in $1.0 \times 10^5 - 2.5 \times 10^5$ (66.66%) range. and the rest had the counts less than 1.0×10^5 (Table 4.5).

Tables 4.4 gives the results of the bacteriological analysis of cooked and peeled shrimp.

Results showed that the total bacterial count was between 1.0 x 10^3 to 2.5 x 10^3 in 27.27% of the samples and 22.72% of the samples contained counts in the range of 1 x 10^4 - 2.5 x 10^4, 18.18 % of the samples showed bacterial count between 5.0 x 10^3 to 10 x 10^3 and 18.18% fell in the range 2.5 x 10^3 to 5.0 x 10^3 13.68% samples had bacterial count less than 1 x 10^3 (Table 4.6).

In this study Out of 82 different frozen prawn samples analysed only one PUD sample *E.coli* was detected and the count was 40 *E.coli*/g (Table 4.2).

Staphylococcus aureus was not detected in any of the frozen headless sample (Table 4.1) But 25% of the PUD sample contained *S.aureus* and the counts were between 20 and 60, well with in the permitted limit (Table 4.2). 25 % of the PD samples also showed the presence of S.aureus (Table 4.3) and in one sample the count was 100/g.

Staphylococcus aureus was not detected in any of the cooked and peeled frozen shrimps (Table 4.4).

Neither *salmonella* nor *Vibrio cholerae* was detected in any of the 82 samples analysed.

Table 4.8 gives factory wise bacterial profile. Out of the 12 factories from where the samples were collected, few of the samples collected from 5 factories showed the presence of *S.aureus*, even though the counts were far below the permitted limit.

Table 4.1

The bacteriological quality of frozen headless shrimp collected from different factories

S.No	TBC/g	*S.aureus*	*E.coli*	*Salmonella*	*V.cholerae*
1	6×10^4	*	*	*	*
2	2.2×10^5	*	*	*	*
3	2×10^5	*	*	*	*
4	2.45×10^5	*	*	*	*
5	2.85×10^5	*	*	*	*
6	2.88×10^5	*	*	*	*
7	2×10^5	*	*	*	*
8	1.8×10^5	*	*	*	*
9	2.6×10^5	*	*	*	*
10	2.0×10^5	*	*	*	*
11	2.85×10^5	*	*	*	*
12	2.8×10^5	*	*	*	*
13	2.8×10^5	*	*	*	*
14	2.65×10^5	*	*	*	*
15	2.2×10^5	*	*	*	*
16	2.2×10^5	*	*	*	*
17	2.8×10^5	*	*	*	*
18	2.4×10^5	*	*	*	*
19	2.6×10^5	*	*	*	*
20	2.9×10^5	*	*	*	*

*: not detected

Table 4.2

Bacteriological quality of frozen PUD shrimp collected from different factories

S.No	TBC/g	*E.coli/g*	*S.aureus/g*	*salmonella*	*V.cholerae*
1	2.1×10^5	*	*	*	*
2	2.7×10^5	*	*	*	*
3	2.45×10^5	*	*	*	*
4	2.26×10^5	*	*	*	*
5	2.65×10^5	*	*	*	*
6	1.02×10^5	40	*	*	*
7	7×10^4	*	*	*	*
8	7.6×10^4	*	*	*	*
9	1.68×10^5	*	*	*	*
10	2.1×10^5	*	*	*	*
11	2.26×10^5	*	*	*	*
12	4.5×10^4	*	60	*	*
13	2.25×10^5	*	*	*	*
14	1.28×10^5	*	*	*	*
15	2.92×10^5	*	20	*	*
16	3.74×10^5	*	60	*	*
17	1.80×10^5	*	20	*	*
18	1.02×10^5	*	20	*	*
19	6.2×10^5	*	*	*	*
20	1.04×10^5	*	20	*	*
21	1.70×10^5	*	*	*	*
22	1.12×10^5	*	40	*	*
23	2.6×10^5	*	*	*	*
24	3.5×10^5	*	*	*	*
25	2.45×10^5	*	*	*	*
26	2.1×10^5	*	*	*	*
27	9.9×10^4	*	*	*	*
28	2.1×10^5	*	*	*	*

* not detected

Table 4.3
Results of the bacteriological analysis of frozen PD shrimp collected from different factories

S.No	TBC/g	*E.coli/g*	*S.aureus/g*	*Salmonella*	*V.cholerae*
1	1.70×10^5	*	*	*	*
2	1.02×10^5	*	*	*	*
3	7×10^4	*	*	*	*
4	1.17×10^5	*	*	*	*
5	2.40×10^5	*	100	*	*
6	1.83×10^5	*	*	*	*
7	1.10×10^5	*	80	*	*
8	2.10×10^5	*	*	*	*
9	1.18×10^5	*	40	*	*
10	6.8×10^4	*	*	*	*
11	7.2×10^4	*	*	*	*
12	6.3×10^4	*	*	*	*

* not detected

Table 4.4

Results of Bacteriological analysis of cooked and peeled shrimp

S.No	TBC/g	*E.coli/g*	*S.aureus/g*	*Salmonella*	*V.cholerae*
1	1.6×10^4	*	*	*	*
2	1.2×10^4	*	*	*	*
3	2000	*	*	*	*
4	1.2×10^4	*	*	*	*
5	4000	*	*	*	*
6	360	*	*	*	*
7	320	*	*	*	*
8	400	*	*	*	*
9	9800	*	*	*	*
10	1.04×10^4	*	*	*	*
11	6000	*	*	*	*
12	8900	*	*	*	*
13	1200	*	*	*	*
14	2700	*	*	*	*
15	3500	*	*	*	*
16	9800	*	*	*	*
17	4000	*	*	*	*
18	1800	*	*	*	*
19	2200	*	*	*	*
20	2000	*	*	*	*
21	2200	*	*	*	*
22	1800	*	*	*	*

* : not detected

Table 4.5

Number and percentage of frozen prawn samples under different TPC range

Type of product	< 1.0 x 10^5	1.0x10^5-2.5x10^5	2.5 x10^5-5x10^5	5.0x10^5-9.9x10^5
HL	1 (5)	9 (45)	10 (50)	*
PUD	4 (14.28)	17 (60.71)	6 (21.42)	1 (3.57)
PD	4 (33.33)	8 (66.66)	* *	* *

Figures in paranthesis indicate percentage.
* not detected

Table 4.6

Number and percentage of cooked and peeled shrimp under different TPC range

<1 x 10^3	1.0 x10^3-2.5x10^3	2.5 x10^3-5x10^3	5.0x10^3-9.9x10^3	1x10^4-2.5x10^4
3 (13.63)	6 (27.27)	4 (18.18)	4 (18.18)	5 (22.72)

Table 4.7

Percentage of different frozen prawn under different range of *S.aureus* load

Range of bacterial count	HL Total No.20	PUD Total No.28	PD Total No.12	CP Total No.22
Nil	20 (100)	21 (75)	9 (75)	22 (100)
1 - 5	*	*	*	*
5.1 - 20	*	4 (14.28)	*	*
20.1 - 40	*	1 (3.57)	1 (8.33)	*
40.1 –60	*	2 (7.140)	*	*
60.1 _80	*	*	1 (8.33)	*
80.1 -100	*	*	1 (8.33)	*
Over 100	*	*	*	*

*: Nil

Table 4.8
Factory wise Bacterial profile

Source	Nature of sample	No. of sample	TPC/g	*S.aureus/g*	*E.coli/g*
Factory A	HL	5	$2.2x10^5$-$2.9x10^5$	-	-
	PUD	5	$2.1x10^5$-$2.7x10^5$	-	-
	PD	*			
	Cooked	*			
Factory B	HL	1	$6x10^4$	-	-
	PUD	1	$7x10^4$-$1.02x10^5$	-	-
	PD	3	$1.70x10^5$	-	-
	Cooked	*			
Factory C	HL	5	$2x10^5$-$2.85x10^5$	-	-
	PUD	*			
	PD	*			
	Cooked	5	$2x10^3$-$1.6x10^4$	-	-
Factory D	HL	6	$1.8x10^5$-$2.8x10^5$	-	-
	PUD	*			
	PD	*			
	Cooked	*			
Factory E	HL	*			
	PUD	*			
	PD	5	$7x10^4$-$3x10^5$	100	-
	Cooked	*			
Factory F	HL	*			
	PUD	2	$1.68x10^5$-$2.1x10^5$	-	-
	PD	3	$9.8x10^4$-$1.18x10^5$	40-80	-
	Cooked	*			
Factory G	HL	*			
	PUD	2	$4.5x10^4$-$2.26x10^5$	60	40
	PD	3	$6.3x10^4$-$7.2x10^4$	-	-
	Cooked	*			
Factory H	HL	*			
	PUD	5	$1.28x10^5$-	20-60	-
	PD	*	$3.74x10^5$		
	Cooked	*			

Factory I	HL	*			
	PUD	5	$1.02x10^5$-$6.2x10^5$	20-40	-
	PD	*			
	Cooked	5	$1.01x10^4$	-	-
Factory J	HL	3	$2.2x10^5$-$2.8x10^5$	-	-
	PUD	*			
	PD	*			
	Cooked	8	$1.2x10^3$-$6x10^3$	-	-
Factory K	HL	*			
	PUD	*			
	PD	*			
	Cooked	4	$1.8x10^3$-$2.2x10^3$	-	-
Factory L	HL	*			
	PUD	6	$9.9x10^4$-$3.5x10^5$	-	-
	PD	*			
	Cooked	*			

- : not detected
*: not collected

DISCUSSION

5. DISCUSSION

The principle interest of this investigation was to find out the bacterial quality of shrimp processed in factories for export to European union after the EU regulation. This discussion is presented under following headings

1.Total bacterial count

2.*Escherichia coli*

3.*Staphylococcus aureus*

4.*Salmonella*

5.*Vibrio cholerae*

6.Seafood processing factories

5.1 Total bacterial count

The total bacterial count of frozen raw shrimp falls in the range of 10^4 to 10^5/g and 10^3 to 10^4/g in the case of cooked and peeled frozen shrimp. All the samples have TPC below the permitted limit i.e. less than 1 x 10^6/g for frozen raw shrimp and 3 x10^5/g for cooked prawns. The lower count in the present study can be attributed mainly to Goof Manufacturing Practices adopted during post harvest handling, transportation, processing, preservation and storage. It also indicates that temperature is controlled and maintained near 0°C throughout the various processing operations.

In the present study the maximum count reported are 2.9 x 10^5 organisms per gram for headless, 6.2 x 10^5 /g for PUD, 2.4 x 10^5 /g in PD and 1.6 x 10^4/g for CP (Tables 4.1 – 4.4).

Most of the earlier studies on frozen prawn samples showed very high bacterial count in seafood samples analysed in different parts of the world. Gunderson *et al.* (1961) noticed bacterial count between 9.5 x 10^5 and 1.4 x 10^7/g in raw breaded shrimp. Lakshmy and Pillai (1964) observed 7.2 x 10^5organisms/g for PD, 3.1 x 10^6 organisms/g for headless and 2 x 10^5 organisms/g for cooked frozen prawns

In contrast, the present results showed that the bacterial count of frozen shrimp and frozen cooked shrimps are very low and this study indicates that adoption of Good Manufacturing Practices based on accumulated scientific knowledge and stringent quality requirements from the importing countries can substantially improve the quality of processed prawns.

This reasonably low count in HL may be due to be beheading in addition to other factors. Beheading reduces the bacterial counts by about 50-80 % (Williams and Campell, 1952). Effect of thorough washing on the reduction of microbial load has been documented by Pillai *et al.* (1982). In general beheading, washing and freezing reduce the bacterial load considerably.

The microbial quality of frozen fish product is greatly influenced by the quality of raw materials and various operations adopted by the factory. These possibilities are well documented by Iyer *et al.* (1990) and Delacurz *et al.* (1994).

Low TPC value in cooked and peeled shrimp indicates that cooking and further handling practices are carriedout with great care and external contamination from other sources including human and working environment are strictly controlled. A through effort was made by the processors to avoid all these contamination and it provides an assessment of the general sanitation level of the plant.

5.2 *Eschericha coli*

In the present study incidence of *E.coli* was noticed only in one sample, viz in one from frozen PUD sample. All other samples were free from *E.coli*.

Prawn caught in deeper water does not contain *E.coli* and it is reported to be present that in prawns collected from inshore waters (Cann, 1977). When contaminated water is used for fish processing this organism may enter into the product. In certain cases tap water in the processing area of the factory is found contaminated with *E.coli* (Jayaweera *et al.*, 1990). Ice used for preservation and utensils used for processing may also be another possibility. Which ever may be the source; the presence of *E.coli* is used as a microbiological indicator of faecal contamination and as a measurement of sanitary quality (Geldrich, 1983; Bej *et al.,* 1990). Presence of these bacteria in fish products indicates breakdown in the sanitary practices of the plant. But its presence, particularly in small numbers, does not necessarily mean that the foods contains faecal matter, but suggests a low standard of hygiene.

There are reports indicating that the presence of *E.coli* in cooked prawns (Greenwood, 1985) and frozen shrimp (Varma *et al.*, 1985). Varma *et al.* (1985) found that it was absent in all the samples of cooked and peeled

frozen shrimp. In the present study also it is found that all the samples of cooked and peeled frozen shrimp contained no *E.coli.* One frozen PUD sample had 40 *E.coli/g*, which is above the permitted limit of 20/g prescribed in the EEC standards.

When once the organisms enter into a food product it is very difficult to get rid of them completely. But Jayaweera and Subasinghe (1990) pointed out significant reduction in bacterial load during various processing operations.

Effective use of chlorine water used for washing glazing and frozen storage reduce *E.coli* and final products are found free from *E.coli* (Sumpeno *et al.*, 1988). Since *E.coli* is very sensitive to sub zero temperature about 95% reduction in the count takes place during frozen storage.

In the present study incidence of *E.coli* is found only in one sample Presence of *E.coli* in one shrimp sample is not sufficient to confirm that the food material is contaminated with faecal material

Cooked and peeled shrimp contained no *E.coli.* This clearly shows that recontamination of cooked shrimp is carefully avoided by using hygienic equipment, water, utensils and adopting hygienic processing.

5.3 *Staphylococcus aureus*

All the samples of frozen headless, cooked and peeled shrimps analyzed showed the absence of *Staphylococcus aureus* (Table 4.1 and 4.4) 25 % of both PD and PUD samples showed the presence of *S.aureus* (Table 4.2 and 4.3)

S.aureus can grow better than other bacteria in a food in which competing microorganisms are less (Iyer *et al.*, 1995). Rapid growth and toxin production can take place in precooked shrimp if recontaminated with

S.aureus (Huss, 1994). Since man is the fundamental source of *S.aureus* (Bryon, 1976;Minor, 1976; Ahmed, 1991) Its contamination in fish and fishery products is considered as an indication of unsatisfactory hygienic level of workers (Anon, 1996). *S.aureus* is an inhabitant of skin and it is estimated that more than 50% of healthy human adults carry these organisms. Prawns are susceptible to contamination because of the amount of handling that it under goes. Personnel hygiene becomes even more critical in the case of cooked prawns which are handled after cooking and normally do not receive further treatment before consumption.

S.aureus is not normally present in prawn (Reilly *et al.*, 1984) and is often found in frozen raw breaded shrimp (Gunderson *et al.*, 1954) uncooked shrimp (Silverman *et al.*, 1961) and cooked shrimp (Greenwood *et al.*, 1985) and seafoods at retail outlets (Gorczyla *et al.*, 1985).

Even in this study, over 20% of the samples were positive to *S.aureus*. Presence of *S.aureus* in large number indicates poor personnel and plant hygiene and improper sanitary procedure practiced in the processing plant.

Previous studies have demonstrated the presence of *S.aureus* during the evisceration phase and not at the reception phase (Fernandes *et al.*, 1991) and count increased during processing ranging from <50/g to 10^3/g in raw material to 10^2 to >10^4/g during processing (Jayaweera and Subasinghe, 1994)

In this present study it is found that *Staphylococcus* count was more in PD (40-100/g) than PUD (20-60) and it was absent in the case of headless. No *S.aureus* was found in cooked and peeled frozen shrimp. The absence of *S.aureus* in HL and presence in 25% of PUD and PD prawn samples point to

the possibility that the product may be contaminated with this organism during the peeling operation from workers. It is necessary to take more care in peeling operations too so as to completely eliminate this organism from all the frozen products.

5.4 *Salmonella*

. From Table 4.1 to 4.4 it is clear that *Salmonella* is absent in all the frozen shrimps. Generally, *Salmonella* or any other bacteria of enteric origin is absent in the sea. *Salmonella* is absent in freshly caught seafoods (Cann, 1977;Hobbs, 1982; Karunasagar, 1992).

Salmonella is not found in marine shrimp naturally and any *Salmonella* in the marine shrimp is probably due to external contamination (Synarya *et al.*, 1990). The main sources of contamination with *Salmonella* during processing of shrimps are water from culture ponds, washing the catch with coastal seawater, dumping the day's catch on the sea beach for preliminary sorting, process water, ice, shrimp contact surfaces, floor of processing hall, utensils, rodents, lizard drippings and shrimp peeling sheds (Iyer, 1986;Pongpen, 1990).

Total absence of *Salmonella* in the frozen shrimps indicates improved plant sanitation, hygiene and adherence strictly to technically accepted procedure for handling and processing the material.

The total absence of *salmonella* in the frozen shrimp products is in full agreement with most of the earlier reports. Varma *et al.* (1991) studied the bacteriological quality of frozen seafoods for export and they found that *Salmonella* was totally absent in cooked and peeled shrimp. Rashid (1992)

reported that no *Salmonella* were detected from samples of imported frozen shrimps. Green wood *et al.* (1985) found that *Salmonella* was not present in any one of 148 samples of cooked prawns and shrimps analyzed. *Salmonella* was not found in any processing stages (Jayaweera and Subasinghe, 1990).

Numerous studies have been under taken in recent years in an attempt to investigate *Salmonella* contamination. Anderson *et al.* (1971) and Backers *et al.* (1981) reported the isolation of *Salmonella* from cooked frozen shrimps. Pongpen *et al.* (1990) found that fresh and frozen white shrimp and peeled and cooked marine shrimps were contaminated with *Salmonella*. Iyer *et al.* (1990) found *Salmonella* in raw and processed shrimp. Fernandes (1991) found *Salmonella* in shrimp in the reception phase and not in the evisceration and freezing stage. Nayyarahamed (1995) reported the presence of *Salmonella* in shrimp before any pre processing and handling. *S.weltevreden* is the most common species isolated (Iyer and Shrivastava, 1989). However, no *Salmonella* is detected in any of the samples analyzed in the present study.

Evidences showed that exported frozen shrimps were rejected in high proportion due to *Salmonella* contamination (Chiou *et al.*, 1981). In 1979 FDA imposed ban on Indian shrimp imports on the ground of presence of *Salmonella* and other forms of contamination (Anon, 1980). Presence of any serotypes of *Salmonella* at any level in food material is to be regarded as a potential hazard (Iyer, 1986).

So, in order to produce good quality shrimp free from *Salmonella* prevention of any contamination during handling and processing of shrimp are

needed. Improvement of hygienic conditions and adoption of GMP are the main requirements for prevention of contamination.

5.5 *Vibrio cholerae*

In the present study, *Vibrio cholerae* is absent in all the frozen shrimp samples. Man is the natural reservoir of *V.cholerae*. Presence of *V.cholerae* has been reported in the marine environment (Kenya *et al.*, 1984; Mathew, 1986). Water used for processing can be a major source of contamination of *Vibrio cholerae* (ICMSF, 1978).

None of the 82 shrimp samples were positive for *V.cholerae*. This is in agreement with the results of Varma *et al.* (1991) who found that *V.cholerae* was absent in all the frozen samples tested. The water used in the processing factories should be chlorinated to a residual level of 10 PPM to get full protection against *V.cholerae*.

Seafood have often been reported to be contaminated with *V.cholerae*. *V.cholerae* is isolated from commercially frozen seafoods including peeled shrimp and fish (Wong *et al.*, 1995) imported seafoods (Akihirominami, 1991) and prawn processing areas (Delacruz *et al.*, 1990)

Berry *et al.* (1994) conducted a study to examine the frequency of contamination by *Vibrio* sp. from major exporters of shrimp and found that *V.cholerae* was present in 10% of the total samples.

Dalsgaard *et al.* (1996) isolated *V.cholerae* non-01 from cooked frozen shrimp product and raw frozen shrimp products imported into Denmark. The contamination of shrimp by *V.cholerae* is a world wide one.

Samples of frozen and raw shrimp was rejected and destroyed by health authorities in Japan due to the presence of pathogenic *V.cholerae* (Reilly, 1986). However none of the shrimp samples analysed in the present study were positive for *V.cholerae*.

The occurrence of *V.cholerae* in the environment is a serious concern for both processors and exporters. There is high risk that contaminated prawns will be accepted in to processing plants and this pathogen will be detected in exported frozen shrimp.

The main sources of these organisms may be due the natural waters, working surfaces, utensils, contact surfaces, water, ice and floor of processing hall (Iyer *et al.*, 1988)

Low temperature processing is detrimental to most *Vibrio* Sp. Usually lower levels of these organisms are present in frozen foods compared to corresponding unprocessed products (Wong, 1992).

In the present study *V.cholerae* are absent in all the samples analysed. Seafoods have been increasingly incriminated in cholerae out breaks. Adequate cooking and avoidance of contamination will ensure the safety of consumed foods (Madden, 1988).

Care must be taken in seafood processing environments as *Vibrio* may be brought into the factory through the raw product. *Vibrio* can also be introduced into processing areas by a number different routes.

5.6 Seafood Processing Factories

From Table 4.8 it is seen that few of the PD/PUD samples collected from 5 factories contain *S.aureus*. Though the count of *S.aureus* is well below

the permitted level, the presence indicates the need for further improvement in the hygienic condition. All HL samples are free from *S.aureus*, but it is noticed in PD and PUD samples. The CP also is free from this organism. The present results, then, point to this strong possibility that the samples an contaminated with the organism during peeling operations and it may be mainly from the peeling workers or from peeling premises. It is necessary that these factories may take additional care during primary processing of the samples so as to make the samples free from *S.aureus*.

One PUD sample from a factory contained 40/g of *E.coli*. Though the count is above the permitted level, it can be taken as an isolated case. There is all the possibility that the sample is contaminated with organism during the primary processing and a processing worker may be the contributor to this. More stringent measures are needed to check the health condition of the workers.

However, the absence of organisms like *V.cholerae* and *Salmonella* clearly indicates that the factories are in general maintaining very good sanitary and hygienic conditions

SUMMARY

6. Summary

The purpose of the present study was to evaluate the bacteriological quality of seafood products processed by EU approved seafood processing plants and to compare the changes in quality with those reported by earlier workers.

All the frozen samples analysed have low bacterial load significantly below the level permitted by European Union.

E.coli was absent in all the samples except one. It was present in one PUD sample. The sample might have contaminated from the primary processing area. This indicates more stringent steps to be taken to monitor and control the health and hygienic conditions of the workers and further improvement in plant sanitation in that factory.

S. aureus was absent in all HL and CP samples, but it was present in 25% of PUD and PD samples, with in the permitted level. These samples seemed to contaminate during primary processing and indicated the necessity for frequent health check up and maintenance of Good sanitary conditions to prevent the contamination.

Salmonella and *V.cholerae* were totally absent in all the products of frozen shrimp analysed.

The significant improvement in the bacterial quality of frozen products from EU approved plants showed stringent implementation of processing procedure as stipulated by European Union.

Conclusions

The low values of total plate count i.e. below 6 x 10^5 /g, absence of *Salmonella* and *V.cholerae*, low level of *S.aureus* and the absence of *E.coli* in all samples except one pointed out the general improvement in the bacterial quality of shrimp products processed in India. The presence of *S.aureus* and *E.coli* in 25% of PUD and PD, and 3.57% of PUD respectively, though below the permitted level, brings out the necessity to monitor regularly the raw material, personnel hygiene of workers, sanitary conditions of the primary processing areas and strict implementation of Good Manufacturing Practices to further improve the quality of processed seafoods.

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7. REFERENCES

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