STUDIES ON THE MICROBIAL PATHOGENS *SALMONELLA* AND *VIBRIO PARAHAEMOLYTICUS* IN SELECTED MARINE PRODUCTS

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C E R T I F I C A T E

This is to certify that the Thesis entitled 'STUDIES ON THE MICROBIAL PATHOGENS, SALMONELLA AND VIBRIO PARAHAEMOLYTICUS IN SELECTED MARINE PRODUCTS' is a bonofide record of the research work carried out by Shri. M. Arul Jyothi, under my supervision and guidance in the Central Institute of Fisheries Technology, Cochin-682029, in partial fulfilment of of the requirements for the award of the Ph.D. degree of the University of Cochin and that no part thereof has been presented for any other degree or diploma.

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C O N T E N T S

STUDIES ON THE MICROBIAL PATHOGENS, SALMONELLA AND VIBRIO PARAHAEMOLYTICUS IN SELECTED MARINE PRODUCTS.

PREFACE

An intensive research programme for good quality processed seafoods is in progress in the Central Institute of Fisheries Technology. Eventhough some of the maritime states like Kerala and Tamilnadu have initiated some fundamental studies on bacteriology, an organised research on the microbiological aspects of food borne pathogens is lacking. In the efforts of the seafood industry to produce quality seafoods, the Central Institute of Fisheries Technology has made valuable contributions for improving the quality of the marine products. The author was actively engaged in the problems dealing with the microbiological aspects of seafoods from 1962 onwards. It is important for seafood industry to develop bacteriologically sound products for the export and internal markets. Its organoleptic qualities must be fully maintainted and it should be free from food poisoning pathogens like Salmonella and Vibrio parahaemolyticus.

Food poisoning through the microbial activity of Salmonella and Vibrio parahaemolyticus is a

regular problem for all those who are involved in the fish processing and in the storage of fish and fishery products. The bacteriological studies are useful in evaluating the quality of fishes and shellfishes during the handling and processing after the catch. The presence of Salmonella, a potential pathogen and Vibrio parahaemolyticus, which constitutes a part of the natural flora of coastal waters and freshly harvested fishes can cause gastroenteritis. These pathogens have therfore of much importance (Beuchat 1975). There is no detailed study on the incidence of Salmonella and Vibrio parahaemolyticus on the seafood products of the West Coast of India except few for a few references. There is also paucity of information on the survival of these pathogens during handling and processing.

In view of the above mentioned factors, the candidate undertook a study on the incidence, distribution, seasonal abundance, survival during various processing methods such as refrigeration, freezing heat processing and their developmental resistance to chemical agents viz: antibiotics, food additives and the sanitisers.

This thesis deals initially with a literature reference survey, taxonomy, their incidence in selected foodfishes and shellfishes, and their incidence and

distribution, their survival during different types of processing, their heat survival at temperaturesof 50° 55° and 60°C, their growth initiation at different low levels of pHs (4.0to 10), and their developmental resistance to various chemical agents. The trials for the study were collected from various landing centers at Cochin and the retail outlets. Based on these data collections the candidate was able to obtain more knowledge of the processing technology and the survival of pathogens like Salmonella and Vibrio parahaemolyticus.

The significance of the work is given below:

1) This is first detailed and systematic investigation along the South West Coast of India on the technological aspects of Salmonella and Vibrio parahaemolyticus.

2) A total of twenty four serotypes of Salmonella and Vibrio parahaemolyticus which are isolated from various fish and fishery products are studied for their survival during different processing treatments viz: refrigeration, freezing and storage and higher temperatures 50°, 55°, and 60°C.

3) Growth iniation studies in low and higher pHs Viz: pH 4.0 and 5.0 and alkaline pH 8 to 10. were carried out.

4) The sensitivity and resistance of different serotypes of Salmonella and Vibrio parahaemolyticus isolated from various marine seafoods were worked for the chemical agents Viz: antibiotics, two preservatives and the sanitiser Chlorine.

It is hoped that these findings and the conclusions drawn herein would appreciably add to our existing knowledge of the bacteriological aspects of Salmonella and Vibrio parahaemolyticus in the Indian tropical conditions.

In the course of his investigation on these pathogens, the candidate has published two research papers and its abstracts are apended in this thesis.

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The author is also greatful to Shri. K. Mahadeva Iyer, Head of Division of Microbiology, Central Institute of Fisheries Technology and to Dr. K.P. Srivastava, Joint Director, Export Inspection Council for the many discussions he had with them and for their personnel encouragement during the course of this study.

During his work on the studies on the pathogens, Salmonella and Vibrio parahaemolyticus, the candidate discussed various aspects with Dr. J.G. Disney, Head of Division of Meat and fish, Tropical products Institute, London, and with Jan Raa, Professor of fishery Chemistry, University of Tromso, Norway during their visits to Cochin.

In the course of this study, the candidate was greatly gained by the correspondance with specialists on Salmonella and Vibrio parahaemolyticus and he would like to thank Professor L.C. Cann, Dr. J.M. Sheeen and

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C H A P T E R O N E

I N T R O D U C T I O N

1.1. Need for the investigation of microbial pathogens.

The production of seafoods in India started in the last fifties. The seafoods constitute an important item of the export from India. The quality of the seafoods has to be of a high standard to make it acceptable in foreign markets. The occurrance of any microbial pathogens is bound to result in the rejection of seafoods in the foreign markets and consequent financial loss to the exporter. So the industrialists and the Export Inspection Agency of the Government of India carefully try to avoid all possibilities of contamination of the seafoods by the pathogenic organisms. The Indian Standards Institution has instituted the standards for several items of food of the marine origins.

The knowledge of the microbial pathogens occurring in seafoods is sufficient for their

quality control. But the most important microbial pathogens of this region deserve intensive investigations. Hence a detailed study of Salmonella and Vibrio parahaemolyticus was undertaken. It is hoped that this study will not only increase our knowledge of these organisms, but also help to suggest suitable measures to avoid their presence in the seafood products.

Fish after catch degrades in quality due to the action of spoilage organisms and its autolytic ensymes. Young (1956) pointed out that the critical peroid is between the time of catch and its processing. It is universally recognised that expeditious handling of perishable fishery products is a prime requisite for maintaining quality. It is however, virtually imposible to prevent the natural contamination of fishes and shellfishes by the normal microflora inhabiting the of capture of culture.

The bacteriological studies are useful in determining the quality of fishes and shellfishes in handling and processing. The presence of Salmonella, a potential food pathogens in seafoods is a result of unsanitory human handling. Vibrio parahaemolyticus is a part of the natural flora of coastal waters. It is well documented that Vibrio parahaemolyticus is widely distributed in inshore waters, sediments and planktons. Salmonella and Vibrio parahaemolyticus have

therefore evoked considerable interest among medical and marine microbiologists.

Salmonella was first isolated by Gaertner in 1888 from meat products which caused food poisoning. Vibrio parahaemolyticus is regarded as an etiological agent for gastroenteritis in Japan (1950) during the warm summer months. Different species of Salmonella were identified in the late 19th century and they established as the wide spread cause of food poisoning to man and animals. The first member of this group was isolated by Gaertner. These organism are widely now distributed in nature and occur in man and animals, they being their primary reservoirs. Salmonella is not saprophytic, and all the species of the genus Salmonella are considered as pathogenic.

Vibrio parahaemolyticus is also known to cause food poisoning. The public health laboratories in several countries generate an increased awareness of these microorganisms as a potential cause for gastroenteritis (Beuchat, 1975). Initially Vibrio parahaemolyticus was considered as a fastidius and mild halophilic organism and it was isolated in a medium containing a low level of sodium chloride.

Privious on the role of these pathogenic organisms and their significance in the marine seafoods were mostly concerned with the qualitative esti-

mation on the export materials to foriegn countries (Bhat and Alberquerque, 1953; Shrivastava, 1974; James and Iyer 1972; Gopalakrishna Iyer et al., 1975; Joseph et al., 1975; Rao and Nandy 1986; Rajagopalan 1978). The variation of these organisms both in quality and quantity depend upon the nature of the marine fishery products and various other ecological parameters, which influence them. Very limited literature is available regarding the occurrance and distribution of these pathogens in marine products. No systematic study on the qualitative aspects in marine seafoods have been under taken so far.

Because of total lack of information on this line, a detailed investigation on the technological aspects has now been under taken in this laboratory. In the present study the emphasis is on the behaviour of the two pathogens Salmonella and Vibrio parahaemolyticus during different types of processing, since they are found to be one of the most important food poisoning organisms in the seafood products.

It is well known fact that Salmonella and V. parahaemolyticus present in fishery products will not only create enteric diseases to those who consume them, but also infect the processors who

handle the products. Hence, in the present study the technological aspects and its survival during different treatments were investigated.

1.2. Review of literature.

1.2.1. Taxonomy of Salmonella.

While the microorganisms of the genus Salmonella are obviously related closely to the other members of the enteric group, the relationship of the varieties of these organisms to one another is not clear. Initially this group of bacteria were separated from one another on the basis of physiological characters. In 1920, emphasis was given on their immunological characters. The commonly occuring varieties of the group Salmonella are having similar biochemical properties and immunological characters.

In the early literature of bacteria, Salmonella was named as Bacterium and later termed as Eberthella, the latter in honour of the Bacteriologist Eberth. The generic name is given in honour of the American bacteriologist, D.E. Salmon by Ligniers in 1900 (Ewing 1963). Later it was known as Salmonella choleraesuis. Different species of Salmonella were later identified in the late 19th century. (Smith 1894; Weldin 1927; Castellni and Chalmers

1919; Schroeter 1886; Warren and Scott 1930; Loeffler 1892) and were established as wide spread cause of food poisoning. The first member of this group Salmonella enteritidis was isolated by Gaertner in 1888, from the diseased beef, responsible for the outbreak of gastroenteritis. All the species and strains of Salmonella are pathogenic for man and the disease syndromes occur differently into several distinct clinical types.

Recent studies (Bissonnette et al., 1975; Cherry et al., 1972) have shown that the Salmonella are widely distributed in the environment and pose a potential threat to the health of man and animals.

The first type is typhoid fever (enteric fever) and is caused by the organism called S. typhi. This produce most severe fever of all the diseases caused by this group. The second type is paratyphoid fevers, caused by S. paratyphoid A, B, and C and other species. The fever produced by these organisms were milder than that of typhoid. In this enteric fever the mortality rate is higher if it is not properly dianized. Of all the species, S. choleraesuis has been found to produce the highest mortality rate about 21% and produce septicaemia.

The thimd type of disease

caused by the other members of Salmonella spp. is called gastroenteritis. This type of disease is entirely different from the enteric fever and having incubation peroid as short as possible about 8 hours only. Generally they are negative in blood cultures and lack of host specificity. Numerous serotypes are capable of producing gastro intestinal irritation with nausea, vomiting , stomach and abdominal pains and cramps diarrhea both mild and severe. These symptoms usually accompanied by prostration, muscular weakness, faintness and restlessness. More than 1600 serotypes of this group cause illness in humanbeings.

The generic term Salmonella was accepted by the international Committee in accordance with the International Rules of nomenclature. This name has been used universally from 1933 onwards.

The taxonomy and nomenclature of Salmonella, which was followed initially indicate the disease and the animal from which they were isolated. Later, new types were given the names of the host species, the town, the region or the country in which the strain was isolated. This resulted in the confusion of overlapping properties. Various workers attempted to classify this genus (Kauffmann 1956; Edwards, Fife and Ramsey 1959; Kauffmann 1963;

and 1960; and Ewing 1963)

The microbiologists attempted to compile numerical and percentage data that could be used toc compare all the reactions given by then members of one genus with those of another so as to provide method for differentiation of different species of Salmonella.

Inspite of tremendous research activities were carried out on its clinical pathology, still no simple system was not evolved. Kauffmann classified this genus Salmonella (1956; 1963; and 1966) but this classification was not widely accepted. Later Ewing (1969 and 1970) recommended the system based on the comparative studies of biochemical reactions by larger members of cultures of each of the species.o Edwards and Ewing (1972) have reviewed the status of the genus Salmonella on the basis of biochemical reactions and used a three species concept for the classification of Salmonella. He also gave allowances that any individual use any system. This concept limits the number of

species of Salmonella to three, (originated from Boreman et al., 1944). This arrangement was also used by Kauffmann and Edwards (1952). The three species recognized are Salmonella choleraesuis (type speicies) Salmonella typhi and Salmonella enteritidis. All the species of, salmonella other than S. choleraesuis and S. typhi are taken as serotypes of S. enteritidis are insubraspecific and they have standing in nomenclature and used in lieu of antigenic formulas. Now more than 1600 serotypes and bioserotypes are added to it. So then serotypes (ser) and bioserotypes (bioser) of enteritidis are written in the manner as S. enteritidis ser Typhimurium, which was previously designated as S. typhimurium. Another example is S. enteritidis bioser Paratyphi-A for S. paratyphi-A. Edwards and Ewing (1972) used bioser -types in connection with certain serotypes of S. enteritidis that possess unique biochemical characteristics. If it is unclassified serotypes are found it can be also be included as S. enteritidis ser 58:a:-

For the biochemical identification of Salmonella, Kauffmann(1960, 1965) divided the genus into four subgenera groups which are named as I,

II, III, and IV. Subgenus I indicate typical biochemical reactions of species, subgenera II and IV included serotypes that yielded aberrant reactions in certain biochemical reactions and the genus Arizona was included as subgenus III. Regarding the subgenus II and IV, Edwards and Ewing (1972, p146) discussed the aberrant reactions in greater details and also reviewed critically for their atypical reactions.

Salmonella generally considered as a nonlactose fermenting organism, this is reflected in the definition of the genus Salmonella in Bergey's Manual of Determinative Bacteriology (1957) and by Edwards and Ewing (1972). Kauffmann (1966) gave a similar definition of the genus except that he included the Arizonae, which usually ferment lactose. In 1966, Ewing and Ball reported that 3 (0.8%) of 371 unselected Salmonella serotypes fermented lactose. A later report by Ewing (1968) indicated that 3(0.3%) out of 787 cultures fermented lactose. Reports of lactose positive Salmonella have been made by Twort (1907), Kristensen (1955), Kauffmann (1937), Seligmann and Saphre (1946), Saphre and Seligmann (1947), Falkow and baron(1962), McCoy (1962), Gonsales (1966), Picket and Agate (1967), Peelma and Romero (1971), Rakey and Mecca (1971), Hedgtnen and Naghski (1972), and Blackburn and Ellis (1973). Twort (1907), Kristensen (1955),

and Blackburn and Ellis (1973) demonstrated that the environment for Salmonella organisms may influence their ability to ferment sugars. Also Blackburn and Ellis, (1973) showed that lactose fermenting ability was limited to three serotypes, S. anatum, S. tennessee, and S. newington.

1.2.2. Taxonomy of Vibrio parahaemolyticus.

The taxonomy of marine bacteria are complicated with problems at all levels of classification. Vibrio parahaemolyticus was first isolated by Fujino et al., (1951) and named as Pasteurella parahaemolytica, while investigating a foodborne outbreak in Osaka Japan. It was once believed that this organism was restricted to the far East. This disease was not known to non-Japanese research workers because of language barrier. Most of the literature in hand about this organism are in Japanese language. V. parahaemolyticus is well documented in Japan and approximately 1200 publications are in circulation a (Smith and Haga, 1971). In 1970 a number of papers published in English gradually increased and the transalations of important contributions, originally published in Japanese language, were also available.

This made the recognition of potential public health hazard and the effort was made to isolate *V. parahaemolyticus* in the environs.

Takikawa (1958) isolated a similar organism and classified it as *Pseudomonas enteritis*. Further Miyamoto *et al*., in 1961, proposed the genus as *Oceanomonas parahaemolytica*. These names were finally placed by Sakazaki and coworkers in the genus *Vibrio* in 1963. At the same time, he separated the two sets with in the species and made group I to be enteropathogenic and group II was nonpathogenic. The exact taxonomic position of *V. parahaemolyticus* later has been deviated from the classification of Sakazaki and coworkers and recommended a change in the current generic name.

V. parahaemolyticus has been remved from the genus and kept in the genus *Beneckea* (Baumann *et al*.1971). It is renamed as *B. parahaemolytica*. The definition proposed for genus *Beneckea* to accommodate peritrichously flagellated members of family Achromobacteriaceae, whichn are Gram negative, chitinoclastic, straight rods of marine origin, facultatively anaerobic and ferment glucose with production of acid but no gas (Combell and Williams 1951). Baumann and his coworkers (1971) enlarged the generic

qualities of Beneckea to embrace Gram negative, fermentative, Na^+ requiring rods, motile by means of mono-polar flagellated sheath in liquid medium, and have DNA base composition of 45-48 moles % Guanine plus Cytosine (G.C). Baumann and his coworkers justified the expulsion from the genus Vibrio on the ecological grounds (1971: 1973:, Reichelt and Baumann 1973, 1974). The type species of Vibrio, and Vibrio cholerae were separated on the different ecological facters, being intestinal and fresh water inhabitants and not required medium level of Na^+ (optimum level 5-15 mM). But Beneckea spp. apparently required higher concentrations of Na^+ (ranging from 100-300 mM) for optimal growth(Reichelt and Baumann, 1974). Several unspecified strains which were excluded from Vibrio were accommodated to the genus Beneckea even though they had higher moles % G.C contents, or uncertainty of the relationship of V. cholerae to Beneckea. Baumann and Baumann (1976) compared the relationship between V. cholerae and Beneckea spp. eventhough low comparison (67-74%) and gave a separate generic status.

Although the arguments put forward by Baumann and Colaboraters to have the genus Beneckea were impressive, many workers did not agree the proposal since the type species of the genus Beneckea

(S. labra) extent in the American Type Culture Collections (Hugh and Sakazaki, 1975), as cited by Martin Abraham in his Ph.D. thesis 1981). The importance of single character emphasis in the genus was become obsolete and confusion arouse with other genus, for example shift in flagellation in Aeromonas and Pseudomonas and chitinolysis in Agarbacterium and Alginobacterium (Kaneko 1973). Similarly Chatterjee (1974) and Chatterjee et al., (1975) found a shift from polar to peritrichous flagellation in the genera Agarbacterium and Chromobacterium and also chitinolysis is manifested by slime bacteria and Alginomonas. This two species misfit in the inclusion of the genus Beneckea (Baumann et al., 1977). A rethinking is required for excluding the Vibrio parahaemolyticus and similar marine vibrios from the genus Vibrio on the ecological grounds. But V. cholerae resident in the brackish waters of Cheasepeake Bay with an optimum salinity of 4-17‰ was taken to be autochthonus bacterial species (Kaper 1979; Kaper et al., 1979). Studies on the evolution (amino acid sequence divergence) of glutamine synthatase and superoxide dimutase (Baumann et al., 1980) have abolished the genus Beneckea and reclassified its constituents species along with

Photobacterium fisheri and P. logei to the genus Vibrio (Baumann, P. , Baumann, L., S.S. Bang, and M.J. Woolkalis, 1980. Current Microbiology. 4. 127-132 as cited by M. Abraham in his thesis 1981).

On the taxonomic assessment of the over all characters of Vibrio parahaemolyticus to the various genera (Pasteurella, Yersnia, and and Actinobacillus), Chatterjee asigned it to the family Brucellaceae. Further Chatterjee et al., (1975) placed the Vibrio parahaemolyticus biotypes to a genus intermediate between Pasteurella and Yersinia under the family Brucellaceae and order Eubacteriale. Antonov and his coworkers(1975) by studying the DNA homology between standards strains of V. cholerae and other representative strains of marine species (V. parahaemolyticus, V. alginolyticus and other allied vibrios of marine origins) marked as Marine Vibrio. Further they recommended to remove the other marine strains from the genus Vibrio. On numerical taxonomy analysis, Vibrio parahaemolyticus and V. cholerae exibited close similarity values of 75% S, and recommended for inclusion of this two species in the same genus (Carney et al., 1975)

From the dichotomous identification scheme and the system of classification stipulated in the Bergey's Manual of Determinative Bacteriology, the taxonomy of Vibrio spp. is still under confusion. Eventhough many standard microbiological laboratories refer the text Bergey's Manual over the past several years there has been a confusion among the bacteriologists with the system of classification, given in the Bergey's Manual. Wood opined (1949) that Bergey's Manual will create more'species' on no grounds other than that they do not fit the described'species'. Moreover another important factors emphasised in Bergey's Manual is polar flagellation for the description of the genus Vibrio spp. (Shewan and Veron 1974). It is well documented that several species of Vibrio are able to produce unsheathed polar flagella when cultured in solid suitable media. In addition a single sheathed polar flagellum was observed in cells harvested in liquid media (Allen and Baumann, 1971; Baumann et al., 1971 and 1973; Reichelt and Baumann 1973; Yabunchi, et al., 1974). Such a variation in the flagellation would eliminate species from the genus Beneckea (including B. para-

haemolytica and B. alginolytica (Baumann et al., 1971). So with out appropriate concessions for the phenomenon of pleomorphism of the cell shapes which under go changes under different physiological and cultural conditions also eliminate the genus Beneckea. If importance and weightage given are given to the cell shpes and flagellation (as in Bergey's Manual), then generic allocation becomes more and more difficult under dissimilar cultural conditions (Baumann and Baumann 1977

Yet another irregular aspect in Bergey's Manual is about the taxonomy status of the V. alginolyticus. But Sakazaki had given a separate identity to V. alginolyticus and this scheme had been accepted by several authors. Stillin Bergey's Manual it is listed as a biotype of V. parahaemolyticus. It is better to remind at this point that the recommendations agreed upon by the Subcommittee on the taxonomy of Vibrio, International Committee on Systematic Bacteriology, (Hugh and Sakazaki 1975): (i) Vibrio parahaemolyticus A.T.C.C. 17802 (type strain) is a species distinct from Vibrio alginolyticus, A.T.C.C. 17749 (type strain); (ii) V. parahaemolyticus should not be excluded from the genus Vibrio and should not be recognised as B. parahaemolyticus as proposed by Baumann et al., (1971); (iii) The type species of the genus Beneckea

is B. labra of which there are no strains known, including type or extent. The relationship of B. labra to V. parahaemolyticus can not be objectively evaluated, determined or established with a type or neotype strains of B. labra (as quoted by M. Abraham 1981)

From the above mentioed controversies about the taxonomy of marine organisms, Vibrio spp. has under gone different classical treatment for identification, by employing lesser of morphological, physiological, cultural and biochemical diagonestic reactions. As suggested by Staley and Colwell 1973, what seems important to one investigator may not be important to another and also not necessarily for microorganisms themselves. Further , some degree of personal expertise and judgement and interpretation of the results is required for the identification of the microorganisms. The strains isolated need not conform to the so called classical or established criteria of a given species (Staley and Colwell 1973).

So the marine organisms are hetrogenus in character and they cannot form one genus unless an integrated approach (Polyphasic taxonomy). The genus Vibrio requires integrated approach and includes all the feasible characters, morpho-

logy, phsiology, biochemical aspects, serology, numerical taxonomy, and genetic compatibility. So these factors must be considered for the taxonomical classification of marine bacteria which are hectrogenous in chafacters.

1.2.3. Distribution.

A. Salmonella.

The primary habitat of Salmonella spp. is the intestinal tract of animals such as birds, reptiles, farm animals, man and occasionally insects. Eventhough their habitat in the intestinal tract, they are also found in other parts of the body from time to time. They are also found in the sewage waters, rivers and backwaters. The intake of polluted water and foods that have contaminated by insects and by other animals produce salmonellosis. This disease is also spread with persons who are suffering mildly or ambulatory form or carriers. These organisms once again shed through the fecal matters, and there by continuation of cycle. By exporting contaminated food products and rapid and fast travelling of persons who suffer from these enteric diseases are largely responsible for the world

wide distribution of salmonellosis.

Survey publications all prove the negative isolations of Salmonella and other enterobacteria of fecal contamination in the open sea and in marine fish. As the sea is the natural recipient of terestial waters from land, it may be polluted by the fecal substances from birds and ships which travel to different countries. The contamination in the sea when taken for all practical purposes, constitute a negligible source. Even though the rivers flow into the sea, it may cause the water surfaces temporarily contaminated highly. So, the contamination of Salmonella in the sea is almost a coastal phenomenon.

Some times the sea may not be diluted through fresh water in the form of discharge from rivers and sewage outlets. Because of lower density and frequently the higher temperature of the incoming fresh water, a thorough mixing is difficult. But on coastal region due to the wave movements the greater mixing is possible. Moore (1954) studied the phenomenon of the transportation of polluted water by surface currents. He found that the thin film of fresh water is carried by the tide and currents. So from Moore finding it is evident that intimate mixing

is not possible and if at all taking it will mix very slowly. Under such conditions cleansing action by sea is not possible. The nonsurvival of Salmonella and other pathogens in sea are due to oxidation, activated by the waves and stream movements, and sedimentation by which the pathogenic organisms are adsorbed by the marine sediments (Rubentschik et al., 1936). However the organisms fixed in the marine sediments are not destroyed and some times they travel with currents.

Stryszak (1949), suggested that the protozoans and other microscopic animals destroy Salmonella organisms in the marine environment. Waksmann and Hotchkiss (1937) showed that the multiplication of protozoans, copepodes and other microscopic animals are proportionately increased by the destruction of bacteria. The bacteriophagic lysis will not possible in the sea because it requires temperature for favourable multilpication.

Various authors studied the bactericidal effect of sea water on enterobacteria (Nicati and Rieten 1885; De Giaxa (1889). They found the sterilised sea water lost its inhibiting effect on Vibrio comma and Salmonella typhi. Krasilnikov (1938), Beard and Meadowcroft (1935) found decrease in the population of Escherichia coli and Salmonella

typhi. Still there is a lot of controversy about the bactericidal effect of sea water but there is no doubt about its thermosensitivity, which was proved beyond doubt by Ketchum et al., (1949) and by Heim de Balsac et al., (1952). But there is suggestion by Heim de Balsac et al., (1952) that sea water contains antibiotic substances, and this has not been proved. Vaccaro et al., (1950) and Rosenfield and Zobell (1947) suggested that marine microorganisms produce a subetance which inhibits the terestrial bacteria.

The survival of Salmonella and other enterobacteria in sea water has been stidied by the several authors as given in the survey by Orlob (1956). But still the survey , according to Buttiaux (1962) is highly inadequate . In vitro experiments on the inoculation of Salmonella cultures in sea water kept in bottles and found the duration of survival is proportional to the concentration of cells inoculated. Gevaudan et al., (1957) found that the concentration of Salmonella typhi 100,000 per ml. survived for three days and with lesser concentration of 25000 per ml. were not survived more than 15 minutes in the sea water. Sherwood (1952) found that the Salmonella typhi disappeared within 24 hours at a concentration of 5450 per ml., and their higher concentration (4.2×10^4 per ml.) survived one week. Beard a

and Meadowcroft (1935) with an artificial inoculation of 3.0×10^8 per ml. in collodion sacs immersed in the bay of San Francisco, reported the survival of Salmonella typhi for 14 to 34 days. Buttiaux and Mours (1953) studied the survival of Sal. typhi, Sal. para typhi B, Sal. enteritidis, and Sal. typhimurium at concentration of 200 to 1800 cells per ml. in water from the North Sea and found the destruction less than 50% in 44 hours. Lafontaine et al., (1956); Gevaudan and Tamalet (1957) found higher destruction (50%) of E. coli. Many investigators (Stainiger, 1951; 1956; Buttiaux and Leurs, 1953) isolated Salmonella serotypes along the coastal area and sea, and proved that the bacteria (Salmonella) can be multiplied by availing themselves the abundant organic matters in the surrounding water. From the review it is found that the Salmonella species can be viable in sea water, contaminated by polluted river waters. But however, Buttiaux in his review (1962) remarked that it is impossible to make viable these organisms along the coastal area which are far away from the polluted zones.

Studies (Bisonnette et al.1975; Cherry et al., 1972) had shown that the Salmonella are widely distributed in the environment and pose a potential threat to the health of human beings and

and other animals. Salmonella had been recovered from rivers and streams in remote areas devoid of any apparant human fecal contaminations, as well as urban stream and recreational lakes subjected to fecal pollution humans and animals. Fair and Morrison (1967) concluded that unpolluted potable surface water sources do not exist. Cherry et al., (1972) demonstrated the ease of recovering salmonellae from urban and rural streams suggested that these organisms may be better indicator of health hazard than the standard coliform counts. It has been suggested also by Cherry et al., (1975) that these organisms , whose habitat has been thought to be intestinal tract, may be free living in nature. Thompson et al.,(1975) conducted a survey of incidence of salmonellae in a harsh and unfavourable environment and showed that the salmonellae survive much a harsh environment.

Salmonella were frequently isolated from various surface waters (Spino 1966). He used for isolation of Salmonella with a selective media at elevated temperature incubation to increase the frequency of isolation. Fair and Morrison (1967) examined the unpolluted water of Podre River in Colarado for Salmonella and Arizona and isolated and found these organisms. They concluded that regardless of the apparant lack of pollution, the existance of natrurally occuring potable

bacteriologically free surface water is a myth.

The presence of Salmonella in relation with general microflora in fishery products will depend upon various parameters like nature of fishery products, environmental conditions of various fish processing factories, catches from environs, depend on the pH and the nutritional requirements. The growth of these organisms depend upon one another directly or indirectly for energy sources and the net results are the toxic formation of substances which are highly harmful when consumed either raw or improperly cooked. Their pathogenicity and association with other fecal indicators like coliforms, streptococci have long been regognised. Various species of Salmonella serotypes survive during processing of the marine fishery products

If the fishes are caught in the polluted waters them they will carry these infectious organisms (Brunner 1949; Steiniger 1951; Reisler 1952; and Moore 1954). Normally fishes do not suffer from salmonellosis. Salmonella infection is more in fresh water than the marine environs. Leigurda et al., in 1950, conducted the survey firm for the mummy presence of Salmonella in fresh water fishes from the River Plate. They found out 97 fishes, comprising 10 species, had 19.6% carried Salmonella spp. Floyd and Jones in 1954, found that 11% of the fishes from the River Nile, on the

Cairo market harboured Salmonella and Shigella and 6% of the fresh water fishes from the Polish rivers and lakes were infected with Salmonella typhimurium (Gengaus and Malwinska, 1957). Jadin et al., in 1956, and 1957 reported that bacillary dysentry has been endemic for a long time in the x region of the Great Lakes in the Central Africa. It has been established that the lake waters and about 20% of the fish caught therein carry Salmonella (Jadin et al., 1956 and 1957). It is clear that the fish infected with these organisms act as intermediate hosts for man and from where it caught, act as immense reservoirs for these organisms. But the fish caught in unpolluted water, lakes and rivers these pathogens have not been isolated (Margolis 1952). Various scientific workers were reported the presence of Salmonella in polluted waters and markets. The eel kept in the polluted waters showed the presence of of Salmonella in the viscera (Van den Broek 1948). Gulasekaram et al., (1956) reported Salmonella in the ocean fish available on the market of Colombo.

In the marine environment these organisms can not survive longer time. Stryszak (1949) suggested that the protozoans in the sea are the major agents for the destruction of Salmonellae. Buttiaux in his review reported that the contaminated fish readily cleaned when they returned to noncontaminated

water. Studies conducted by the Department of Marine Biology at Plymouth showed that the mk artificially contaminated by E. coli in sixty one specimens of Ctenolabrus rupestris were disappeared from the intestines of the fish in 7 days when they kept in noninfected sea water. No similar work has been carried out for Salmonellae.

The fish caught from offshore has been considered free from pathogenic enteric microorganisms. During handling on the deck of the ship and on the shore the contamination by the human sources is likely to arise (Spencer and Georgala 1958). Then fecal contamination of E. coli and fecal streptococci increased during handling and processing. Many investigators (Ross and Thatcher 1958; Larkin et al., 1956) established the increased presence of E.coli and fecal streptococci during handling of fish. But they however could not isolate Salmonellae during handling, processing and at any stage of distribution chain of marine fish in Britain (Shewan et al., unpublished results 1958-59). This may be probably due to the time, temperature conditions during distribution not favouring the growth of pathogens, even if contamination occur at any stage. The problem can arise

only when conditions are suitable, poor sanitory conditions in and around the fish processing factories. Baldiali et al., (1957), reported the isolation of Salmonella paratyphi-B in tuna fish. On reviewing the literature there are sufficient number of recorded incidences of salmonellosis in consuming fishery products and theis disease has not much difference with respect from other foods got (Muller, 1914; Bitter 1920; Kapsenberg, 1938; Kleeman et al., 1942; Kuys 1948; Van den Broek, 1948; Olitzky et al., 1956). In 1951, Pivinick also reported the presence of Salmonella in smoked fish.

In a hot climatic conditions where fishmeal manufactured under primitive conditions salmonellosis will prevail and spread to human via livestock (Bischoff and Rohde, 1956; Rohde and Bischoff , 1956 Adams, 1967; Jacobs et al., 1963; Morehouse and Wedman 1961; Morris et al., 1970). In a study of animal feeds in England for the years 1958 to 1960, and Taylor (1962) isolated Salmonella serotypes from the meat and bone products 855 times from fishery products 100 times. Salmonella senftenberg was more frequently isolated from the fish meal and the next serotypes followed were S. antum and S. cubana.

During the year 1960 the prevalance of Salmonella in fish meal caused more concern.

Several workers indicated that sugh products could be contaminated, if incorporated into animals feeds (Carroll and Ward 1967; Morris et al., 1970; Garrot and Hamilton, 1971; U.S. Department of Agriculture 1965). Fishery products prepared for human consumption appear to be less problem than the animal feeds, which are certainly of more immediate concern of public health (Reference, 5; A.W. Anderson et al., Proc. Tech. Conf. Fish. Inspect. Qual. Cont., Halifax, 1969: B.C. Hobbs, Proc. Natl. Conf. Salmonellosis Atlanta, p 84-93, 1964). It is well understood that Salmonella contamination is a real problem in the widely diversified areas of the fish processing industry. For the solution to this problem it can be tackled properly by finding of the source of infection creaped.

One of the largest reservoirs of Salmonella is the class Aves (Faddoul and Fellows 1966). Although there are extensive reports in the literature on the occurrance of Salmonella in domestic fowl, there is general lack of documentary evidence on the incidence and distribution of these organisms in the free flying wild bird population. Berg and Anderson (1972) studied the incidence of Salmonella and Edwardsella tarda in gull feces on the Oregon Coast and found these birds contaminating the fishery products with these organisms.

Presence of Salmonellae in the fishmeal production had been studied by many workers (Morris, Martin, Shelton, Wells and Brachmann 1970) and reported the survival of Salmonellae during the processing of Menhaden fish caught in the Atlantic Ocean and Gulf of Mexico. Patterson in his review (1972) of the Salmonellae in animal feeding stuffs indicated that marine fresh fish immediately caught from the ocean showed no Salmonellae, but on the boats and ships and at processing plants approximately 50% were contaminated with these pathogens. The question of whether these Salmonella serotypes found in these products of animals feeds, present in the larger numbers to cause infection in animals and poultry had been dicussed by the many scientific investigators. Edwards (1963), summarising the findings of a conference considering the epidemiology of salmonellosis in herbivorous animals) " this incidance of salmonellosis seem to be directly connected with the presence of bacteria in feeds, since Salmonella of the identical serotypes and phage type have been found in the infected animals and in the feeds they consumed" Such a possibility has been suggested by Newell, McClarin, Murdoch and Hutchison (1959) and Kampelmacher, Guinee, Hofstra and Venkeulen

(1962), review of Pomeroy, Siddiqui and Grady (1964) working with animals red/infected with meal. Salmonellae have been found often in animal feeding stuff (Dawkins and Robertson 1967) particularly those protein constituents derived from animals such as meat bone meal and fish meal. Heard , Tennett, and Linton (1969) have also suggested the incidence of various Salmonella serotypes in the excretion of various animal population also found frequently in the animal feeds. Hobbs and Hugh-Jones (1969), have suggested that an outbreak of Salmonella senftenberg infections in human were due to eating turkeys which have been fed with white fishmeal contaminated by this organisms (Patterson 1969 and 1971).

In the semi preserved and fermented seafoods the pH 4.4 is very critical for the growth of these organisms (Hobbs 1970) and pasteurisation temperatures are sufficient to kill them. Exposure to salt before pasteurisation increase the heat resistance of Salmonella (Cortterill and Glauert 1969). In 1970, Hobbs reported ten outbreaks of foodpoisoning of Salmonella in semipreseved fish meat. In U.S.S.R. these organism were isolated from pickled herring (Korovina and Artischeva 1972), and also reported the outbreak of Sal. typhimurium from marrinated herring (Seidel and Muschter 1967).

In Australia 18% of food poisoning caused by the various seafoods(Sutton 1973). The most common type responsible for the out break is Sal. typhimurium (Proudford, personnel communication). 1973). A review of salmonellasis in Australia was given by Atkinson (1965). In that the other serotypes isolated, apart from Sal. typhimurium were Sal. bovis morficans, Sal. anatum, Sal. chester, Sal. derby, Sal. havana, Sal. muenchen and Sal.newport.(Scott (1969) reported the importance of fish and shellfish products on the out break of food poisoning, when he evaluated the geographic distribution of salmonellosis.

Marine fish, which contaminated in the coastal waters, can have greater access to eliminate the pathogens in the clean off-shore waters. But for the edible shellfishes, (oysters and clams) the problem is different. They require for their growth a mixture of salt water and fresh water, with an optimum salinity from 8750 to 30,800 p.p.m. So the mussel and oyster beds are usually located along the coastal areas and river mouths. Gevauden and Gay (1958) reported that the concentration of Sal. typhi cells in mussels were more than the surroundings. This concentrating action in molluscs make more dangerous to the consumer on public health grounds.

During tranportation also Salmonella survived (Hunter and Harrison, 1928; Kelly and Arciss 1954). Several investigators suggested that the cleaning in chlorine water to kill these organisms (Allen et al., 1950; Arciss and Kelly, 1955; Erdmann and Tennant 1956). The sanitary quality of the oysters and mussels will depend on the hygienic condition in the grounds where the edible oysters and other xxxxxx shellfishes were cutivated.

B. Vibrio parahaemolyticus.

This marine bacterium is widely distribured in coastal seawater, sediments and plankton. It can be readily isolated from seawater and coastal fishes and shell fishes. Various investigators studied the distribution, both in tropical and temperate coastal regions of the world.

Recent marine ecological studies have revealed the presence of this organism in Hong Kong, Taiwan, Singapore, Philippines, Hawaii and Germany (Makanishi et al., 1968). The first isolation of V. parahaemolyticus in the U.S. was reported by Barnes and Liston (1968), from the sediments and marine animals of the West Coast. Later Ward (1968),

Gianelli et al., (1970), Martinevski and Kanatov (1973) were isolated similar type of organism in foods, coastal sediments and water.

The incidence of V. parahaemolyticus in off-shore waters is still remain controversial, but in the inshore waters it is well established. Hori et al., (1964) reported the failure of isolationof Vibrio parahaemolyticus in off-shore waters and plankton samples from the pelagic area of the Pacific Ocean. Beckmuhl and Triemer (1974) also found Vibrio species in coastal lagoons and rarely in off-shore waters and sea fishes. Similarly Leistner and Hechelmann (1974) found that the sea water and fish sampled from the open sea of Norht Sea and Baltic Sea did not show the count of V. parahaemolyticus. According to Kaneko and Colwell (1974) the V. parahaemolyticus and other Vibrios decline progressively with increasing distance from towards sea, in the Atlantic Ocean off South Carolina and Georgia, and they showed the V. parahaemolyticus was completely absent in sea water, sediments and plankton s away from 4 to 10 miles from the shore.

The presence of V. parahaemolyticus in nonmarine environs is rather rousing interest, because the Vibrio is generally termed as mild halophilic and require NaCl for their growth and survival. In Nagasaki city, (Japan) Yasunaga (1964) isolated V. parahaemolyticus

from water samples in a river which has no tidal influence. Chatterjee and Neogy (1972) also found the Vibrio parahaemolyticus from the freshwater fishes in Calcutta. De et al., (1977), reported that 1.3% of fresh water fishes, 16.7% of tap water, 25% of sewage water, 30% of the river water and 40% of pond water which contains negligible sodium chloride were positive for Vibrio parahaemolyticus in Calcutta environs. Very recently Natarajan et al., (1979) reported the Vibrio parahaemolyticus (11.3%) in fresh water fishes.

In general this marine bacterium is highly specific for Na^+ requirement for their optimal growth (usually the range from 0.2- 0.3M). MacLeod (1965) reported the failure of the growth on a laboratory media not supplemented with NaCl. Baumann and Baumann (1976) explained the dependance of Na^+ as prerequisites for the marine bacteria to grow and cannot in terrestrial environs.

In seafoods V. parahaemolyticus reportedly positive in various countries, Hong Kong, Singapore Singapore, Baltic Sea of Germany, Spain, Black Sea off Czechoslovakia, U.S.S.R., Vietnam, Israel, Italy, Denmark Sweeden, Indonesia, Canada, Eastern Cape Cod of South Africa, Britain and U.S.A. In marine products V. parahaemolyticus create special interest because most outbreaks caused gastroenteritis by these organisms. At one time it was

considered that V.parahaemolyticus was restricted only to the far East and now it is well documented its distribution in fishery products, including marine fishes in off-shore waters of Mid Pacific Ocean South China Sea, and Indian Ocean (Yasunaga and Kuroda, 1964; Aoki 1967); tuna in Central fish market of Tokyo, (Japan) (Mitsumara et al., 1969); Blue crabs Callinectes sapidus in U.S.A. (Krantz et al., 1969) Fishbein et al., 1970) Colwell 1970); shrimp in Gulf coast of Texas, U.S.A. (Vanderzant et al., 1970 and 1974, Lemary et al., 1970); Pacific oysters, clams, crabs and fishes in marine environs of Washington States, U.S.A. (Barnes and Liston, 1970).; oysters in Newhampshire, U.S.A (Hartly and Slanetz, 1971); pond reared shrimp (Penaeus aztecus and P. setiferus) in Texas, U.S.A. (Vanderzant and Nickelson, 1973); Oysters in North Carolina, U.S.A. (Jonas et al., 1978); Fishbein et al., (1974) did an elaborate survey on marine fishery products of 635 varieties of samples and found V. parahaemolyticus in 440 samples. France et al., (1980) reported the presence of V.parahaemolyticus in Brazilian coastal water fishes.

Chatterjee (1974) and De et al., (1977) reported V. parahaemolyticus in seafoods marketted in Calcutta environs. Recently Natarajan et al., (1979) isolated these organisms in fresh water fishes. Nair et al., (1980) reported the seasonal incidence of

V. parahaemolyticus in freshly caught fish and marketed fishes from Port Novo area. Very recently Palasumtharam et al., (1981) demonstrated the incidence of V. parahaemolyticus in Colombo market and near by environment.

1.3. Scope of the investigation of microbial pathogens.

Considerable interest and increasing awareness have been created on the health and welfare of the people with their environmental impact and utilisation of the fishery products. Greater attention now have been given for exploring the new fishery resources. By the improved fishing and processing technology fresh ocean fishes and fresh waterfishes will be available for consumption. The presence of pathogens like Salmonella and Vibrio parahaemolyticus in the processed fishery products are not only harmful to the consumers but also creating life endangering infection to those handling these marine products in the processing facteries. Fish is a food which rapidly deteriorate and must be handled hgienically from catch till it reaches to the consumer. It is a paramount importance to study and enumerate as the presence of pathogens and trace their origin in fish and fishery products in Cochin region. However most of the work have been concentrated

with the total hetrotrophic microbial activivty of aquatic system and lesser extent focused their attention to the pathogenic microorganisms. Consequently there is little information on the effect of processing of the fish and fishery products.

In order to study the role of pathogens and its significance in the sea foods it is planned to investigate systematically of such organisms in selected marine sea foods. Because of their immense epidemiological implications these pathogenic organisms induce fever and gastroenteritis which transmitted mainly via fish and fishery products in this region.

On cursory glance on the relevent literature, the most of the investigations were concentrated on the ecology of pathogens in the natural habitats and studied for a short period of time. Majority of the literature deal on the incidence of pathogens of Salmonella and V. parahaemolyticus and furnish data rather temporal distribution in the natural marine environs. The presence of absence of these organism in marine products will indicate the sanitary quality under which it is processed. Many investigations were limited to in one way or other to clinical, epidemiological aspects, or ecological look. So the present objectives of this investigations to the technological aspects of micribial pathogens with special refence to

Salmonella and V. parahaemolyticus in selected marine sea foods and its homogenates. An attempt is made to bridge the gap in our knowledge on the technology and survival of these organisms in Indian seafoods are enumerated.

1. To isolate small numbers of Salmonella and V. parahaemolyticus in sea foods and enumarate by comparing different enrichment and selective media and study their nutritional requirments.

2. Incidence and distribution of Salmonella and V. parahaemolyticus in different parts of the fish and shellfish, and ascertain the predominant serotypes of Salmonella encountered in the fish and shellfish products.

3. Behaviour of pathogens during refrigeration, freezing and subsequent storage of fish and shellfish products and ascertain their influence of low temperatures on the survival and injury of Salmonella and V. parahaemolyticus.

4. To illucidate, on a comparative basis on the behaviour of these organisms during cycling freezing and thawing effect of fluctuating temperatures.

5. To study the heat resistance of Salmonella serotypes and V. parahaemolyticus in marine seafoods. Study of thermal death time and injury during heat processing.

6. The effect of pH on the survival of Salmonella and Vibrio parahaemolyticus in fish and shellfish homogenates.

7. To ascertain the sensitivity to chemicals like chlorine, antibiotics, and food additives and study the resistance developed to the above chemicals.

C H A P T E R T W O

MATERIALS AND METHODS

2.1. Review of the methods employed for the isolation of Salmonella and Vibrio parahaemolyticus.

In the seafood industry, it is necessary to control the foodborne diseases because of the self interest on the part of reputable food manufacturers and the strict regulations of importing countries. Salmonella and Vibrio parahaemolyticus can be identified by their cultural and biochemical characters. The numerous variations in the members of the genus Salmonella can be classified by the Kauffman-White scheme (1966) in which the somatic (O) and flagellar (H) antigens are used to distinguish the many types of Salmonella that have similar biochemical reactions.

The range of temperature suitable for the growth of Salmonella in any ordinary laboratory media or in sea foods range from 5° to 47°C, with an optimum temperature at 37°C. Studies

by Floyd and Jones (1954), Alford and Palumbo (1961), Angelotti et al., (1961), and Matches and Liston (1972) observed that the minimum growth temperature tends to increase in foods with the decrease of pH. The growth of Salmonella was inhibited at 5.6°C and pH 6.2 and at 10°C at pH 5.6. They grow well in the range of pH 6.0 to 8.0 according to Prescott and Tanner (1938).

The salmonellae are facultative anaerobes and the growth is rapid under aerobic conditions. There is a paucity of information in the literature on the effects of oxidation and reduction potential on the growth of Salmonella in sea foods.

Most of the Salmonella can grow on a simple medium consisting of glucose as a carbon source, ammonium salts as a nitrogen source and the usual mineral salts. Studies by Stokes and Bayne (1958) revealed that the specific amino acids or vitamins or both are required for growth. They suggested that the heat sensitive growth factors, such as thiamine and cystine may be destroyed or made unavailable during the preparation and autoclaving of culture media. Litchfield (1973) suggested that these heat- sensitive vitamins and amino acids should be present for the isolation of Salmonella.

The methods that are generally

for the isolation of Salmonella involves different steps as follows:

1. Pre-enrichment in a nonselective medium.
2. Enrichment in a selective medium.
3. Isolation on a selective differential agar medium.

Generally the presence of Salmonella in food products is in low numbers and hence the need for detecting them among larger numbers of other micro flora. Huhtanen et al., (1972), studied the effect of sample size in the bone meal and concluded that larger sample size (300 gm) confirmed more Salmonella than the lesser sample size (10 and 30 gm) drawn from the same lots. Similarly Jacobs et al., (1963), were unable to isolate Salmonella from the smaller size samples collected from the different sites of fish meal bags, but they succeeded in isolating them when larger sample sizes of 1000 gm and 500gm were used. In review Litchfield recommended that the sample should be blended well or mixed in a pre-enriched broth in the mildest manner to release the Salmonella from the samples. American Public Health Association recommended methods suggest that a 20 gm sample may be added

to 80 ml. of broth. The Food and Drug Administration and A.O.A.C. methods specify a 25 gm sample in 225 ml. of lactose broth. This ratio is also recommended by the Sub Committee on sampling and methodology for Salmonellae in eggs, egg products and prepared mixes. In seafoods and frog legs samples, the F.D.A. recommends sample size of more than 25 gm for the isolation of Salmonella.

For favouring the growth of smaller numbers of Salmonella in the midst of other competing larger microflora a pre enriched broth is used. Various scientific workers used a nonselective medium for pre enrichment (Thompson 1977; McCoy 1962; Edel and Kampelmacher 1973; Kafel and Bryan 1977). They established good recoveries of Salmonella. The nonselective medium also favours the heat and cold shocked cells during processing of food products.

Selective enrichment media encourage the growth of Salmonella and at the same time suppress or inhibit other Gram negative organisms such as Coliforms, Proteus and Pseudomonas. Thompson (1955) observed that it is difficult to recover Salmonellae of low numbers without selective enrichment.

Three selective media for enrich-

ments that are widely used to recover Salmonellae from food materials and feecal specimens are tetrathionate broth (Mueller 1923), tetrathionate brilliant green broth (Kauffmann 1935) and selinite F broth (Diefson 1936). The efficiency of these broths is influenced considerably by the type of samples to be examined, the proportionate amount of Salmonellae present in the broth, and the period of incubation and temperature. The studies of Jansen (1962; 1963), McCoy (1962); and Galton et al., (1964) revealed that the secondary enrichment of the same medium will increase the yield of Salmonella in food samples

Numerous modifications of these enrichments are developped. Selinite F broth is modified by adding cystine (North and Bartram 1953), brilliant green (Stokes and Osborne, 1955), Sulpha diazine or Sulpha pyridine (Osborne and Stokes 1955), and sterile filterate faeces, (Sillikar et al., 1964). In fatty foods Galton et al.,(1954) used a dispersion and emulsification wetting agent Tergitol No.7 to the tetrathionate broth. Dulcitol in the enrichment media of selinite improved the isolation of Salmonellae in sea foods (Raj 1956). Recently Stott et al.,(1978) studied the incidence of Salmonellae in animal feeds which includes fishmeal and found that the sample

enriched in double strength of mannitol selinite brilliant green broth (M.S.B.G.) was effective in isolating Salmonellae.

During the last ten years the use of higher temperature incubation of the enrichment for the isolation of Salmonellae from different food materials and sea foods had been studied extensively. The increase of temperature from 37° C to 43° C increased the number of serotypes of Salmonella from foods and feeds (Harvey and Price, 1968; van Schothorst and Kampelmacher 1968; Banffer 1971; Carlson and Snoeyenbos 1972). Media based on tetrathionate were found to be toxic when incubated at higher temperature (McCoy 1962; Aleksic et al., 1973; Vassiliadis et al., 1974). Jacobs et al., (1963) compared Rappaport enrichment with tetrathionate broth with and without Brilliant green and selinite green broth and observed no significant difference in the recovery of Salmonella from these enrichments for fishmeal. Kampelmacher (1964) attributed this to the uneven distribution of these organisms in the fishmeal rather than to the quality of enrichment broths.

Despite the claims made by many scientists the literature survey indicates that selinite cystine broth and tetrathionate broth with

minor modifications give the highest recovery of Salmonella in various food samples. It is found that both selinite cystine and tetrathionate broths have to be used simultaneously for the enrichments and the various serotypes respond differently in these media. So the main factors affecting the performance of the enrichments broths are the following:

a) Type of foods.

b) Nature of processing (Fresh, frozen or dehydrated).

c) Time and temperature of incubation.

d) Initial microbial populations.

e) Initial number of Salmonella.

From the enrichment broths the conventional procedure for the isolation of these organisms from foods and feeds is to streak or spread on the differential or a selective plating on agar media. A cursory glance of relevant literature would reveal that numerous selective media are developed and used for isolation. The following plating agars are widely used: Bismuth Sulphite Agar, (Wilson and Blair, 1927; 1931), Brilliant green agar (Kristensen, Lester and Jengens 1935), Salmonella-Shigeela agar, and Desoxy-cholate Citrate agar, developed by Leifson (1935) and modified by Hynes (1942). These media support the growth of Salmonella and Shigella and inhibit the growth

of coliforms and other Gram negative microorganisms.

As regards to the performance of these plating media investigators have different opinions (Cook 1952; McCoy 1962; Hobbs 1963; Galton et al., 1966). The American Public Health Association (1966) recommends the use of both Bismuth Sulphite agar, Salmonella- Shigella agar or Brilliant green agar. The Official A.O.A.C. and Food and Drug Administration methods (1972) specify the streaking on brilliant green agar, SS agar, and BS agar. Some investigators modified the BG agar by including Sulphonamides, particularly, sulphadiazine (Galton et al., 1954; Harrington and Ellis 1970; and Sanderson 1972) to supress Pseudomonas spp and other enteric organisms. Bismuth sulphite agar also allows growth of many organisms which are Gram negative, but also it is very effective in isolating Salmonella typhi. Usually this medium requires an incubation period of 48 hrs to develop the organisms. Cook (1952), McCoy (1962), and Hobbs (1963) observed that this medium inhibits certain Salmonella serotypes in food products and it is necessary to keep medium at 4°C for at least 24 hrs before it is used for isolation. This medium is recommended for the isolation of Sal. typhi.

Taylor (1965) developed Xylose Lysine desoxycholate agar (XLD) for the isolation of

Salmonellae and Shigellae from foods after selective enrichment. This media based on the ability of Salmonellae and Arizonae to ferment Xylose and to decarboxylase Lysine. The Xylose Lysine rate was determined which permit these organisms to grow and exhaust the carbohydrate Xylose and utilise the amino acid and revert to an alkaline pH so that the colonies can be differentiated from E. coli. Lactose and Sucrose are added to the medium so that lysine positive coliform can produce more acid to prevent the pH to the alkline range.

Since there exists a wide choice of media intended for Salmonellae isolation and detection, it is difficult to assess whether media and methods used by different workers on various geographical locations produced comparable results. Raj (1966) found that many of the recommended media failed to isolate these organisms in frozen sea food products. Because of the controversial views in the literature, it is planned to study the efficacy of the common enrichments, viz. Selinite Cystine and Tetrathionate and the newer enrichment medium viz. Dulcitol Selinite enrichment (Raj, 1966) and compare the various selective media for the isolation effectively of small numbers of Salmonella in the presence of high numbers of coliforms and Proteus spp. artificially

artificially inoculated into the fish substrates.

The methods for the isolation and indentification of Vp parahaemolyticus are described by the number of workers (Sakazaki 1965). Selective enrichment broths are necessary to isolate V. parahaemolyticus from seafoods and marine fishes. The most widely used media is polymyxin-Salt enrichment broth (Sakazaki 1973). The following composition is used for the preparation of enrichment: 0.03% yeast extract, 1% peptone, 2% sodium chloride, and 250 ug/ml of Polymyxin B, or 500 ug/ml of colistin methansulphanate with final pH 7.4. Another enrichment broth which is also commonly used is glucose-salt teepol broth of Akiyama et al., (1964), which has the following composition: 3% beef extract, 1% peptone, 3% sodium chloride, 0.5% glucose, 0.0002% methyl violet and 0.4% teepol with final pH 9.4. Most of the enrichment techniques are the modification of methods described by Sakazaki (1965).

Alkaline peptone water or alkaline buffer of pH8-9.0 is also used for a selective enrichment (Baross and Liston 1970). In this selective medium the longer incubation methods avoided since other intestinal flora may grow at 37°C after 8 hrs. Sakazaki (1973), reported the secondary enrichment of alkaline peptone for 8 hrs incubation at 37°C will result in further growth

of V. parahaemolyticus from the infected patient. Different authors used various selective enrichments for the isolation of V. parahaemolyticus. Fishbein et al., (1970), preferred two enrichments of glucose salt teepol and salt colistin broth (Bacteriological Analytical Manual, 1969) for the isolation of these organisms from the processed meat of Chesapeake Bay blue crabs. Molenda et al., (1972), examined the clinical samples for the enteric V. parahaemolyticus by using peptone water containing 3% sodium chloride, (pH 7.4) as initial selective enrichment. For favouring the growth of smaller numbers of V. parahaemolyticus in the general marine micro flora, these selective enrichment media inhibit other marine microorganisms such as Pseudomonas, Flavobacter, Achromobacter etc. and allow the growth of V. parahaemolyticus.

After the enrichment, several selective media have been formulated for the isolation of these organisms. One of the common media used was Thiosulphate Citrate Bile Salts (TCBS) (Kobayashi et al., 1963) and Bromo thymol blue salt teepol agar, (BTB-ST), (Akiyama et al., 1963), modified by Sakazaki (1969). The composition of modified BTB salt teepol agar is as follows: 1% peptone, 0.3% beef extract, 4% sodium chloride, 2% sucrose, 1.5% agar, 0.02% Sodium heptadecyle sulphate (Tergitol 7), 0.0004% thymol blue and 0.0004% bromothymol blue, with final pH 9.0. Sakazaki

(1973), later found that TCBS (Thiosulphate Citrate bile salt sucrose) agar was much more selective than BTB-teepol brothagar. The composition used by Sakazaki (1973) for TCBS, agar is as follows: yeast extract0.5%, peptone, Sodium citrate, Sodium thiosulphte and Sodium chloride, 1%; Oxgal 0.5%, Sodium cholate 0.3%, sucrose 2%, ferric citrate 0.1%, agar 1.5% and 0.00% of thymol blue and bromothymol blue with final pH 8.6.

Liston (1970), found that the plating media was not worked well for the isolation of V. parahaemolyticus in fish and fishery products, and he developed a plating medium which effectively differentiate the organisms on the ability to hydrolyse the starch anaerobically at higher temperature of 43°C and hemolysis of human blood in 1% sodium chloride used as criteria for selecting V. parahaemolyticus. Later Vanderzant and Nickelson (1972), compared various media and found that twedt et al., (Bacteriol. Proc.,p6. 1970) media was the best for the selective isolation of V. parahaemolyticus. Furhter they modified the medium and called it as M.T. medium (Modified Twedt).

Earliar Sakazaki (1968), recommended media (for the isolation of marine halophilic V. parahaemolyticus containing 10% salt (NaCl) so that the methods will separate V.alginolyticus, which will grow at 10% salt concentration while V. parahaemolyticus

is of negative growth. Twedt et al., (1969), showed that the growth of V. parahaemolyticus in media with 10% salt concentration and cautioned that the characteristic variability should not be used as a key test for identification of V. parahaemolyticus.

Hemolysis has been used to indicate the pathogenicity of V. parahaemolyticus (Miyamoto et al., 1969). Baross and Liston (1970), also used this test as a preliminary screening criteria. Twedt et al., (1969), were unable to separate pathogenic strains in sheep blood. Fishbein et al., (1970), reported that only 50% of Vibrio parahaemolyticus strains isolated from crab meat was haemolytic.

The procedure described by the American Public Health Associations (1962, APHA) and in Bacteriological Analytical Manual (BAM, 1969) is the combination of Baross and Liston's and Japanese workers' methods. By this methodology, after enriching and selective plating, the suspect colonies are screened on triple sugar iron agar slants (TSI slants) reaction, compared with additional biochemical tests of combination will take upto nine days (Vandersant and Nickelson (1972). Reactions in T.S.I. agar slants as a preliminary test for screening (Bacteriological Analy-

tical Manual 1969) are considered useful. But Vanderzant and Nickelson (1972), reported that unaerogenic Aeromonas and some of Enterobacteriaceae produce the same reaction as V. parahaemolyticus.

A cursory glance of literature will show several schemes for the isolation of V. parahaemolyticus and different opinions on the superiority of the media. The relative efficacies of the various selective enrichments and selective media are evaluated, by using reference culture of V. parahaemolyticus (NCIB 1902) and other allied Vibrio spp. In the present investigation, attempts were made to compare the most commonly used media for the isolation and detection of V. parahaemolyticus which are recommended by Food and Drug Administration, (Bacteriological Analytical Manual, 1976) and Vanderzant and Nickelson techniques.

2.2. Standardisation of methods for the isolation and enumeration of Salmonella.

It is very essential to study the efficacy of the various media used for the standardisation of methods on the recovery of pathogens from the seafoods and allied products. As stated above from

the literature review various methodologies were selected for the isolation of Salmonella in different food materials. For each food material, different pre-enrichment, enrichments and selective media were employed. Since each medium is characteristic for own advantages and disadvantages and different methods are also likely to show distorted results, the present investigation employed the methods that are widely used for the recovery of Salmonella in a variety of food products.

In India, apart from clinical studies, the detection of Salmonellae and Vibrios are mainly carried out on the fishery products for exports. The methodology was reviewed and standardised in the Central Institute of Fisheries Technology, Cochin (James and Iyer 1972). The survival of these organisms in sea foods is not studied completely so far. The available information on these organisms in sea foods in India is meagre. For purposes of justification, comparison of the methodologies for the isolation of small numbers of Salmonella in seafodds were carried out.

It is difficult to study all the methods employed in the literature. So attempts were made to study the most commonly employed in the

methods of enrichments and selective media for the isolation and enumeration, which are recommended by FDA, and AOAC. Two pre-enrichment broths, viz: Dulcitol selinite, (LS); Selinite Cystine (SC); and Tetrathionate (Tet) and six plating media, viz: Xylose Lysine deoxycholate agar(XLD), Brilliant green agar (BG), Brilliant green sulphadiazine agar (BGS), Salmonella-Shigella agar (SS), Bismuth sulphite agar (BS), and Hektoen enteric agar(HE), were selected gfor detailed study.

2.2.1. Comparison of different methods for isolation of Salmonella.

a) Comparison of different selective agar media.

Twenty serotypes of pure cultures of Salmonella, Citrobacter spp. and Arizona spp. listed in the table 1. were used in this study. Initially, strains were grown in nutrient broth at 37°C for 24h. Then they were decimally diluted up to 10^8 in sterile 0.1% peptone water and plated in various selective media viz: Hektoen enteric agar (Difco), Salmonella-Shigella agar (Difco), Brilliant green agar (Difco), Brilliant green sulphadiazine agar (Morris and Dunn 1970), Bismuth

sulphite agar (Difco), Xylose Lysine agar deoxycholate agar (Taylor) 1965). The viable counts were taken after the incubation of plates at 37°C and 43°C for 48h. The comparison were made with those simultaneously grown on Triptone yeast extract agar which was taken as hundred percent growth since the medium was non-inhibitory (Table 1).

b) Comparison of enrichment media

Type strains were grown in the nutrient broth for 24hrs at 37°C (tables 2and 3) and decimally diluted with sterile 0.1% peptone water up to 10^{-9}. On this dilution, the concentration of viable cells was less than 50 per ml. This was studied previously by diluting and plating in the non-inhibitory [illegible] of Triptone glucose yeast extract agar. One ml of this decimal dilution was used to inoculate the respective enrichment beoths (9ml) viz; [illegible] Selinite, Selinite cystine and Tetrathionate, so that the final concentration of these cells of the enrichment media will be of 30 to 50 cells per ml approximately. In order to study the efficacy of the enrichments, suitable dilutions of Escherichia coli, Citrobacter and Proteus spps grown in brain heart infusion agar broths (Difco) for 24hrs at 37°C and adjust to the enrichment broths such that the final concentration of

these cells were 10^6, 10^5, and 10^3 per ml respectively. The broths were incubated at 37^oC and 43^oC for 24 to 48 hrs. From the enrichment broths two to three fmix loopful of culture were streaked in different selective media of BS, agar, SS agar, HE agar, BGS agar, BG agar, XLD agar, in duplicate.

e) The appearance of Salmonella and other related organisms in different selective media.

Colonies which appeared in the Bismuth sulphite agar, with black and lustrous surface; convex soft, sometimes brown in colour and with black centres taken as positive presumptive Salmonellae.

Colonies usually colourless, transparent, sometimes light tan or pinkish or yellow with black centres appeared in SS agar and were considered as positive culture. S. arizona and lactose positive Salmonella strain will appeare as pink or orange coloured smooth colonies.

In brilliant green and Brilliant green sulphadizine agars Salmonellae develop into transparent pink to deep fuschia colonies. Sometimes the colour is brown.

In XLD agar the colonies appear deep pink to red and occasionally light brownish.

These colonies are taken as presumptive or suspected Salmonellae.

In Hektoen enteric agar medium the Salmonellae colonies appeared as transparent blue green with or without black centres (H_2S production). This medium inhibits most of the coliforms and other nonlactose fermenters.

Suspected colonies were carefully purified in MacConkey agar by streaking a loopful of culture and incubated at 37°C for 24hrs. The purified culture was streaked and stabed in Triple sugar iron agar slants and incubated for 24hrs at 37°C. The cultures positive in Triple sugar iron agar, were identified as slants give alkaline (red) reaction and Butt gives acid (yellow) with or without gas. These cultures were heavily inoculated in to urea agar, lactose, dulcitol broths, malonate broths, KCN broth and Lysine iron agar. In addition to these biochemical reactions, confirmative serological agglutination tests were carried out with polyvalent 'O' antisera (Difco).

Salmonellae are urease negative (no change in colour in the urea agar). In Lysine iron agar Salmonellae give alkaline reaction of purple colour of the medium. (final colour is slighly darker than the original purple colour through out the medium). Uninoculated control was also kept to identify the difference of colour. When H_2S is produced butt is blackened in

the medium. Negative reaction is taken as the purple or red slant and yellow butt.

In dulcitol broth Salmonellae mostly give positive reaction by showing gas formation (dislpacement of liquid in the inverted Durhams tubes) and acid reaction will be yellow. The negative reaction shows alkaline reaction (red) and no gas formation. In lactose broth Salmonellae show mostly negative reaction.

In malonate broth after incubation of 48hrs at 37°C salmonellae give negative reaction by showing green colour (unchanged). Positive reaction as shown by turning to blue colour (alkaline reaction). Salmonellae showed a negative reaction.

Indole test is negative for Salmonellae when testing the culture grown in tryptone broth for 24hrs at 37°C, by adding kovac'S reagent. Positive test for indole is indicated by the deep red in reagent surface of the broth. Salmonellae are mostly negative.

Along with these biochemical test confirmative tests also were carried out by agglutinating with polyvalent 'O' antisera (Difco). The following procedure was used for confirmation of Salmonella. The growth from TSI slants were emulsified in a drpo of

saline on a glass slide, then mixed with a drop of properly diluted antisera, or mixed directly with diluted antisera with out prior emulsification in saline. Positive reaction was shown by the formation of precipitate or clusters within one minute. The recommended 'O' group antisera are, A, B, C_1, C_2, D, E(E_1, E_2, E_3, E_4) and group II & I and vi (Difco).

In order to study the efficacy of the enrichments employed in this study for isolating low numbers of Salmonella cells were inoculated in fish substrate of jew fish (Pseudosciaenea spp.) and shellfish substrates of Metapenaeus dobsoni (Brown shrimp). Low numbers of six sero types of Salmonella were separately inoculated into 25g. of fresh minced fish muscle and shellfish meat kept in 250 ml conical flask, having general bacterial load of 10^4 to 10^6 per gram. In order to stabilize with general microflora, the flasks were later kept at temperature of 4^0 to 8^0C for 48 hrs. To this was added later 25 ml of sterile standard phosphate buffer of pH 7.2 and shaken thoroughly for 10 minutes. 50 ml of double strength enrichment broth was then added to each flask. In order to study the influence of temperature, one set was kept at 37^0C and another was kept at 43^0C for 24hrs. The higher

incubation was employed to test its reported superiority (Carlson et al., 1967; Harvey and Price, 1968; Morris and Dunn 1970; aleksic et al., 1973; Vassiliadis et al., 1974)

Cultures that were enriched in different enrichment broths were streaked on the six different selective media (qualitatively assessed (table 4).

2.2.2. RESULTS

The data given in tables 1 & 2 compare the different selective media which also include conventional ones. The results presented were the average percentage recoveries as compared with 100% count taken from Triptone yeast extract glucose agar (TGE agar). TGE agar was used because it is a noninhibitory medium for Salmonella. Plate counts were taken after incubation of 24hrs at 37^0C and also 43^0C. The results were the average of six experiments and the percentage recovery. As expected, the selective media employed here for the recovery studies of Salmonellae had varied inhibitory effect. The selective media inhibited differently with different serotypes of Salmonella strains tested. The variations

may be attributed to the ingrredients present in the media. 80% or more recovery of S. anatum, S. newport, and S. enteritidis were isolated in all the media except in Bismuth sulphite agar and Salmonella-Shigella agar was obtained. Most of the serotypes of Salmonella were recovered satisfactorily in Brilliant green agar, Brilliant green sulphadiazine agar and Xylose Lysine Deoxycholate agar. In Bismuth sulphite agar, S. typhi, S. enteritidis and S. anatum were recovered more than any other strains tested. In over all performance of the plating media, significant differences were observed. That is in Brilliant green and Brilliant green sulphadiazine, Xylose Lysine Deoxycholate and Hektoen enteric agars had higher recovery percentage than Bismuth sulphite and Salmonella-Shigella agars. When higher incubation temperature (table 2) was maintained no significant differences in results were obtained. Eventhough Bismuth sulphite agar performance in the recovery of other Salmonella strains was poor, S. typhi was recovered in higher percentage. So in general the recovery of these strains in this study showed Xylose Lysine deoxycholate and the conventional Brilliant green and Brilliant green sulphadiazine and Hektoen enteric agars gave more recovery than in Bismuth sulphite agar and Salmonella-Shigella agar. Citrobacter and Arizona spp. were also recovered in the above

media. The maximum inhibition for citrobacter was in Bismuth sulphite and Hektoen enteric agars.

The growth of Salmonella is readily detected with in 18hrs in Brilliant green and Brilliant green sulphadiazine agars by its characteristic pink to deep fuchsia coloured colonies which can be readily recognised. But if this media, if incubated for more than longer than 24hrs the entire plate became pink and so it was difficult to distinquish the Salmonella from other microflora.

The recovery of Salmonella serotypes in fish and shellfish homogenateswas found to be equally effective in the present experiments (table 3) with a larger number of coliforms. It had been found that the higher incubation temperature at 43°C showed no significant effects (tables 5-8).

The isolation of these organisms in different plating media is summarised in tables 5-8. In fish and shellfish homogenates the recovery of different serotypes of these organisms was quite effective in Xylose Lysine Deoxycholate, Brilliant green, Brilliant green sulphadiazine, Hektoen enteric and Bismuth sulphite agars. Certain serotypes, such as S. typhi, S. enteritidis S. cubana, and S. saintpaul were recovered well in Bismuth sulphite agar. In other selective media, the differnet serotypes tested in this study were recovered from 76-87%.

The superiority of Xylose Lysine Deoxycholate, Brilliant green sulphadiazine and Hektoen enteric agars over Bismuth sulphite and Salmonella-Shigella agars is quite obvious. In this experiment the serotypes tested were the isolated cultures from various fish and marine seafoods and serologically identified by the National Salmonella and Escherichia Centre, Kasauli. H.P.

2.2.3 DISCUSSION

In the isolation of Salmonella in different food products, there an apparent lack of consistancy observed between different scientific workers and sometimes the data analysis seem to be diametrically opposed (Taylor and Schelhart, 1968). These discrepancies and the variations that exist from country to country were tested by Taylor et al., (1963) and (1964), and found that the results were similar and not much variation was observed in using the different media, different methods of analysis and even different diameters of inoculating needles. Tetrathionate broth when compared with Selinite Cystine broth enrichment, gave lesser recovery of 79%. In this series of studies the effectiveness of Brilliant green sulphadiazine and Xylose Lysine Deoxycholate agars in combination with enrichment Selinite cystine broth was more. Jameson (1962, explained the

dynamics of enrichments and his detailed discussion on the complex relationships that exist in mixed cultures and and the effect they exert on lag time, generation time and molar concentration explains many of the variations which are occur frequently than understood in these comparative studies of media of efficacies. McCoy (1962) observed that plating poured at the same time but on different days, frequently produced major variations in the efficiency for detection of Salmonella. Longer periods of refrigeration, more than 96 hrs before use prolongs its selectivity of these organisms, in Bismuth sulphite. This may be due to different manufacturer's brand of prepared media, which different peptones, bile salts, or dyes that are used to produce the discrepancy of results. Sometimes the lot number media preparation to another influence the selectivity of the isolation and small changes in the media chemicals will upset the balance of the sensitivity and selectivity of the media, (either broth or solid media). Georgala and Borthyroyd (1965) found that when Selinite F broth (Leifson formula) was used as diluent for Salmonella cultures, marked toxic effects were noted on the different plating media as compared with 0.1% peptone water. In the present study Xylose Lysine Deoxycholate, Brilliant green s sulphadiazine and Brilliant green agars were able to

isolate the small numbers of Salmonella serotypes from the fish and shellfish muscle homogenates. Harrington and Ellis (1970) reported that Xylose Lysine brilliant green and brilliant green sulphadiazine agars recovered Salmonellae without much differences from the swine tissues.

The enrichment broths Selinite cystine and Dulcitol selinite were effective in recovering more of Salmonella species isolation (89%) than tetrathionate broth which was only 79%.

Factors causing variations in enrichment of selinite broth were discussed by Leifson (1936) who suggested that the substances which particularly influence the enrichment broth are pH, inorganic salts, especially phosphates, meat infusion, beef extract, casein, other proteins, foodproducts and finally microorganisms themselves. Jamson (1962) demonstrated that with pure culture and mixed cultures in the presence of faeces, the enrichment broth selinite with optimum concentration inhibit certain serotypes such as Salmonella choleraesuis and Salmonella gallinarium. North and Bartram (1953) indicated that two commercially prepared selinite broths were not comparablein their ability to recover Salmonella, but addition of cystine to one, made par with each other. They suggested that

the peptones were primarily responsible for these discrepancies. Rappaport (1969) also found that certain peptones produced satisfactory isolations. In his studies tryptone produced good growth for S. paratyphi, without addition of glutamine. Smith (1959) explained the probable mode of action of selinite with sulphur metabolism. He demonstrated that addition of cystine and pantathenate reduced the toxicity of selinite. Selinite , which may bind with peptone, causes toxicity of certain serotypes of Salmonella.

Certain food products which are soluble in enrichment broths, produce noninhibitory effect. Silliker and Taylor (1958) reported that albumin which highly soluble had stopped the inhibitory effect or actions for both tetrathionate and selinite broths when diluted 1000 folds. This phenomenon was also noticed by North (1961) and recommended the use of noninhibitory broths initially for pre-enrichment before inoculation into selinite or tetrathionate broths or dulcitol selinite enrichment.

The presence of sufficient numbers of microbes also influence the isolation through enrichments. Initially they must be present in a viable form and able to initiate growth in the receiving medium. Secondly, the ratio between the relative numbers

of Salmonella and its competitor should not exceed in the plate straked with mixed flora. McCoy (1962) observed that with highly contaminated water, larger volume of sample yielded higher percentages of Salmonella positives. So the noninhibitory medium is very essential to allow the growth of pathogens to the detectable level in the enrichment broths (North Jr. 1961; Taylor 1961; Taylor and Silliker 1961). The relationship will be changed when the competitive microbes were encouraged more than the pathogens. So the relative number of coliforms and other microorganisms with Salmonella will become a determining factor on the successful isolation of Salmonella. Silliker et al., (1964) reported that pre-enrichment gave better results on the traditional enrichments.

It is evident from these data that Dulcitol selinite, and Selinite cystine enrichments behaved almost similar to or even superior than tetrathionate enrichment. However it is obvious that the sole use of one enrichment medium is a risk of missing some positive results. Owing to the great diversity of serotypes found in the marine seafoods it is better use two enrichments for the isolation of Salmonellae. Taylor and Silliker (1961) cautioned the use of either selinite or tetrathionate alone, a calculated risk feeling , that neither by itself is capable of identifying

all the positive samples.

From the fore going discussion the sensitivity of enrichmnets is correlated well with small absolute numbers of Salmonella and its sucessful isolation in marine seafoods and water samples are evident. Eventhough the performance of tetrathionate enrichment broth is lower when compared with selinite enrichment, both enrichments are used for the isolation since some of the serotypes are selectively isolated well. So the sucessful islation of pathogens depends greatly on the absolute numbers in relation to other competitors like coliforms, Proteus, Pseudomonas etc.

In the present study, evidence of false pesitive microbes which occured on the Xylose Lysine Deoxycholate, Brilliant green agars and Hektoen enteric agar, were most frequently not the members of Enterobacteriaceae but Pseudomonas, Proteus, Serratia and Alkaligens. The growth of false positive gave a difficult situation on the methodology for detection of Salmonella. Each selective media has some inherent defects and it is evident that from the data collected on BGA is the least inhibitory, for the isolation of Salmonella and the sole use of this media may loose some positive results. From the analysis of the data, the conclusion is drawn that two or more media (both enrichments and selective media) are essential in enumerating the Salmonella in fish and

fishery products.

Based on the results, the following methodology is recommended as a standard method for the isolation and enumeration of Salmonella in marine seafoods.

1. Pre-enrichment:

The sample is enriched in either lactose or peptone broths, which is a nutritius, non-selective medium to restore injured Salmonella cells to a stable physiological condition.

2. Enrichment:

Salmonella is further enriched in a tetrathionate and selinite cystine broths, which selectively inhibit microbes other than Salmonella and allow it grow continuously.

3. Selective plating media.:

Xylose Lysine Deoxycholate agar, Brilliant green agar, Bismuth sulphite agar and Hektoen enteric agar restrict the growth of non-Salmonella organisms, while allowing the growth of Salmonella microbes to discrete colonies, providing visual recognition.

4. Purification of Salmonella:

Purification allows the Salmonella to grow in pure form and in discrete colonies, so that

the colonies can be readily recognized inp pure form for further biochemical studies.

5. Biochemical studies:

Biochemical screening is used with the following: Triple sugar iron agar, Lysine iron agar, Urea, b-galactosidase, carbohydrates reactions for the tentative identification of Salmonella at genus level.

6. Serological confirmation:

Serological identification for confirmation of Salmonella is carried out by using specific polyvalent 'O' antisera for the positive agglutinations.

2.3. Comparison of standard methods employed for the isolation of Vibrio parahaemolyticus.

Vibrio parahaemolyticus, a marine bacterium, is widely distributed in coastal waters, sediments, and planktons (Sakazaki 1968) and considered to be the vehicle of numarous outbreaks of foodborne gastroenteritis (Fujine et al., 1951) in Japan. Furhter isolation of this halophilic pathogenic organisms in many other countries made itsso occurrance an international problem. Several schemes were formulated

for the isolation of this halophilic pathogenic organism (Sakazaki 1965; Chun et al., 1974). The isolation methods and identification procedures used are lengthy and require various media. Confirmation of suspect V. parahaemolyticus requires numerous biochemical tests and lengthy serological typing. Still there is difficulty in evaluating the results of studies of incidence of V. parahaemolyticus because of the uncertainty on the exact identification of this pathogenic organisms. Sakazaki et al., (1968) had isolated these organisms (pathogens) only from humans infected with this disease and not recovered from marine sea foods or marine environments. Epidemiological studies suggest that marine habitats are the most probable sources of this enteric pathogens. Further , the Japanese literature cites no evidence to show that V. parahaemolyticus may induce infections in man other than the typical enteropathogenesis ascribed to it. However, the epidemiological data and the laboratory isolations gathered by R.R. Weaver of the National Communicable Disease Centre (NCDC) in the United States, indicate that this organism may also be the cause of nonenteric diseases in man (Fishbein et al., 1970)

The major object of this study is to compare the commonly used media for pre-

sumptive identification of Vibrio parahaemolyticus and to determine a few and the most reliable biochemical characteristics for its identification which can be used for a rapid and reliable procedure. Attempts were also made to compare the recommended media for the isolation and enumeration by F.D.A. (Bacteriological Analytical Manual for Foods 1976) and Vandersant and Nickelson techniques.

2.3.1. Comparison of different methods for Vibrio parahaemolyticus.

F.D.A. procedure involves pre-enrichment in glucose salt teepol broth (GSTB) and further streaking on to Thiosulphate citrate bile salts sucrose agar(TCBS). Vandersant and Nickelson (1972) used tripticase soy broth with 7% sodium chloride with pH 7.3 and subsequently streaked on to M.T. medium (Modified Tweet agar).

Method 1.

In order to study the efficacy of Glucose salt teepol broth (GSTB) for isolating V. parahaemolyticus and other allied Vibrios, low numbers of above organisms were inoculated in fish homogenates of Jew fish (Pseudosciaena sp.), in prawn homogenate (Metape-

naeus dobsoni) and in crab meat (Scylla serrata) and in frog legs meat (Rana hexadactyla) also. Low numbers of (100 cells per ml.) V. parahaemolyticus (NCIB 1902) were inoculated in to 50gm amount of meat and blended well with 450ml of 3% sodium chloride solution. One ml portion of three sucessive dilutions were inoculated into each tube containing ten ml of GST broth. In this procedure, nine tubes were used, (three tube most probable number method), three tubes each of the three sucessive dilutions were used. Then the tubes were incubated at 37°C for 18 hrs and loopful of material from each tube was streaked on Thiosulphate citrate bile salts sucrose agar plates, which were preplated and surface ~~streaked~~ dried by keeping it in the incubater at 42°C for 24 hrs Typical colonies were noticed after incubation of 24 hrs at 37°C. The typical colonies of V. parahaemolyticus on TCBS agar plates were easily distinquishable as round colonies 2-4mm in diameter, with green or blue centres, round smooth edged, mucoidal and elevated. These characteristic were considered as characteristic of V. parahaemolyticus. From the results where the tubes were diluted and taken as V. parahaemolyticus pecsitive. Then the ~~95%~~ MPN values were calculated from the standard Most Probable Number Tables.

The enrichment media Glucose Salt

teepol broth were prepared as follows: peptone 1%, beef extract 0.3%, sodium chloride 5%, glucose 5%, teepol 0.1%, methyl violet 0.2 mg. (per 100ml of the media) and the pH is 8.5 (Sakazaki 1965).

The selective TCBS agar contains yeast extract 0.5%, beef extract 0.5%, peptone 1%, sodium chloride 1%, $Na_2S_2O_3.5H_2O$, 1%, dried ox bile 0.5%, Sodium taurocholate 0.3%, sodium citrate 1%, ferric citrate 0.1%, sucrose 2%, Bromothymol blue 4 mg, thymol blue 4mg (both for 100ml of the media) agar 1.5% and final pH 8.6.

Method 2.

Vanderzant and Nickelson (1972) reported that the MF medium (modified twedt) was the selective medium for the isolation and recovery of V. parahaemolyticus in food products. In this method, enrichment broth used, was Tryptone Soy broth(Difco) with 7% sodium chloride, adjusted pH 7.3 and the selective plating medium enumerated was MF medium. The xxxxxxxx recovery efficiency of enrichment and selective plating media were studied by inoculating low numbers of reference culture of V. parahaemolyticus (NCIB, 1902) in various homogenates of fish and shellfishes.

The following homogenates were prepared from Jew fish (Pseudosciaenae sp), prawn (M. dobsoni)

crab meat(Sycila serrata), and frog leg meat (Rana hexadactyla) also by taking 50 gm amount and blended with 450 ml of sterile 7% NaCl broth and inoculated with known cell concentration of V. parahaemolyticus (less than 100 cells per gm approx.) which were previously determined by serial dilutions plating in tripticase soy agar plates with 3% NaCl. In addition to this cells of E.coli or Salmonella enatum or S. typhimurium of concentration less than 1000 cells per gm were used in the homogenates.

The homogenates serially diluted (decimal dilution) in sterile tripticase soy broth with 7% NaCl. In this method, since the low numbers of V. parahaemolyticus were used, the recovery by the direct plate counts were not tried. So the MPN method was used for the recovery. The MPN procedure was followed as described in method 1 using pre-enrichment tripticasesoy broth with 7% NaCl. After incubation at 18 hrs at higher temperature of 42°C aerobically the nine tube were streaked on MT agar medium which was previously prepared and dried over for two hours in the incubator at 45°C and incubated for 24 hrs at 42°C.

Cultures of V. parahaemolyticus on MT medium were white to creamy, circular, smooth and amylase positive (clear zone around the colony). These isolates were further tested for the biochemical and morphologically, by confirming Gram reaction, glucose uti-

listion, presence of cytocrome oxidase, and sensitive to pteridine 0/129 discs and other diagnostic criteria employed for the identifiction are given in page No. 79.

Th e following composition was used for the MT medium (Modified Twedt): peptone 2%, yeast extract 0.2%, cornstarch 1%, NaCl 7% and agar 1.5% with final pH adjusted to 8.0.

2.3.2. RESULTS.

The tables 9 and 10 compare both the selective plating media of Thiosulphate citrate bile sucrose and modified Twedt media for the recovery of both high and low numbers of V. parahaemolyticus. In pure culture isolation studies both the media gave good recovery of 90 to 100% in various cell concentrations, but when the same pure culture strains were added to fish, shellfish and other homogenates showed lower recovery of 75 to 90%, even after longer incubation time of 48 hrs.

Among the two enrichment media under the methods described above, glucose salt teepol broth medium showed lower percentage recovery of V. parahaemolyticus than the tripticase soy broth with 7% NaCl (Table 11). In assessing the sensitivity of the two enrichments, lower inoculum level of 10^2 cells per gram was used, in conjunction with its normal flora of fish, shellfish

Diagnostic criteria employed for the identification of *Vibrio parahaemolyticus* in the present investigation.

Tests	Response
Gram stain	-
Motility	+
Growth in 1% peptone water	
with 0% NaCl	-
3%NaCl	+
7% NaCl	+
10% NaCl	+
Gas from glucose	-
Fermentation	
glucose	+
sucrose	-
Rhamnose	-
Dulcitol	-
Inocitol	-
Sorbitol	-
lactose	-
Mannitol	+
Maltose	+
Voges-Proskaur	-
Methyl red	+
Nitrate reduction	+

Tests	Response
Catalase	+
Citrate utilization	+
Indole	+
Starch hydrolysis (aerobic)	+
Gelatin liquefaction	+
Lysine decarboxylase	+
Arginine decarboxlase	-
H_2S production	-
Urease	-
Growth at 43°C	+
Salicin	-
Phenylalanine deaminase	-
Triple sugar iron agar (ALK/ACID)	+
Cytochrome oxidase	+
Xylose	-

and other homogenates containing Pseudomonas, Escherichia coli, Acenatobacter, and Aeromonas as the major flora. It had been found that by the use of selective enrichment procedure the Vibrio strains could invariably be isolated (table 11). When lesser cell concentrations (less than 10^2 per gm) were used, the isolation by enrichment in tripticase soy broth gave higher recovery than glucose salt teepol broth. The minimum and maximum percentage of recoveries were 33.3 and 66.7% respectively. In the case tripticase soy broth with 7% NaCl the recovery was from 66.7 to 83.0% . The results of higher concentration of cell recovery were equally good in both enrichment media and the ranges of recoveries in various homogenates were 66.7 to 83.0 %.

2.3.3. DISCUSSION.

For the recovery V. parahaemolyticus the tube Most Probable Number (MPN) method was taken as advantages for the following reasons: (a) direct inoculation on highly selective medium like thiosulphate citrate bile salt sucrose agar often inhibits the recovery of low numbers of cells, since the cells were under stressed or injured. (b) The cells in different dilutions mostly may be in clumps or aggraagates and sometimes it is difficult to enumerate in plate counts. (c) Since the cells were in low numbers and they were likely to escape the

enumeration through plate counts unless the enrichment techniques adppted.

Eventhough the MPN technique have certain inherent problems, the method is useful in determining the low numbers of microbial population. Further three tube combination and subsequent plating on to selective plating media will give reliable results of Vibrio parahaemolyticus and Vibrio strains from the general microflora. In an MPN procedure the use of liquid enrichment, glucose salt teepol broth followed by streaking on to selective medium (solid medium) such as thiosulphate citrate bile salt sucrose agar has proved sucessful recovery in low numbers (Kaneko and Colwell, 1973, 1975, 1978; Colwell, 1979). In view of the above features the three tube MPN method is employed in the recovery study of Vibrio parahaemolyticus.

The enrichment media, glucose salt teepol broth and tripticase soy broth with 7% NaCl were able to recover Vibrio strains on subsequent subculturing in the selective plating medium of MT agar and TCBS agar.

On comparing both the methods for the isolation and enumeration of Vibrio strains, it was found that both methods gave false positives reaction on the selective medium of TCBS and MT agars and the range was about 2 to 5%. Vandersant and Nickelson (1972)

also reported that TCBS agar gave more false positives.

The Vibrio parahaemolyticus strains used in this study were morphologically and biochemically similar to the strains reported by Sakazaki et al., (1963) and Twedt et al., (1969). All the false positives Vibrio strains, were separated by its resistance to pteridin 0/129. This substance is known as vibriostatic compound (Collier et al., 1950; Shewan et al., 1954; and Sakazaki et al., 1963).

Regarding the pathogenicity of V. parahaemolyticus, haemolysis has been related to that by Sakazaki et al., (1968) and by Miyamoto et al., (1969). Baross and Liston (1970) used hemolysis as a preliminary screening characteristic for the identification. Several authors (Twedt et al., 1969; Fishbein et al., 1970) reported the failure of pathogenic Vibrio parahaemolyticus from nonpathogens. Vandersant and Nickelson (1972) indicated that hemolysis is dependant on the type of blood used and the NaCl concentrations, and neither a measure of pathogenicity nor an adequate screening test for suspect V. parahaemolyticus. They found no difference could be enumerated between human isolates and marine isolates on blood agar media tested.

Vandersant and Nickelson (1972) reported that the serological identification of these

organisms have little value at present time. With the commercial antisera they were unable to type most of the strains and only they were able to to type Japanese isolates. Similarly fishbein et al., (1970) were able to type only 22 out of 56 isolates from seafoods. So for the at present time serological identification of these organisms is not much of use as a diagnostic tool (Vanderzant and Nickelson 1972).

The procedure used in this study required for about five days for the identification of V. parahaemolyticus. Both selective plating media gave false positive colonial morphology but no problem for the identification of these organisms with biochemical characteristics.. Aeromonas which give false positive reaction in the selective medium can be separated from V. parahaemolyticus by its sensitivity to pteridine 0/129 (Collier et al., 1950; Shewan et al., 1954; and Sakazaki et al., 1963).

Serological identification was not carried out because of nonavailability od antisera and also varied op opinions given by the various workers, (Fishbein et al., 1970; Vanderzant and Nickelson, 1972).

Based on the study carried out the following methodology was used as a standardized method for the isolation of low numbers of V. para-

haemolyticus.

1. Samples of(marine fishery products) 50 gms were blended with 450 ml of sterile 7% NaCl solution for the dispersion of microbes. This was furtherr decimally diluted upto 10^{-4} with sterile 7% NaCl solution.

2. Three tube MPN method was used for the enumeration of Vibrio parahaemolyticus by either TS broth with 7% NaCl or glucose salt teepol broth as enrichment and incubated for 10 to 18 hrs at 37°C.

3. Streaking on selective plating media was carried out either on TCBS agar or MT agar for the presumptive isolation od Vibrio strains. The following colonial morphology was taken as the presumptive Vibrio organisms. In TCBS agar the colonies were bluish green , circular, smooth dark centres with 2 to 4 mm dia., taken as presumptive Vibrio organisms. In MT agar medium colonies which appeared as white or creamy, circular, smooth and starch hydrolysed with a formation of precipitate on a halo around positive colonies. When halos were not distinct, further incubation for additional 6 hrs at 37°C were carried out aerobically allowing the halos to form distinctly so that definite positive cultures identified.

4. Morphological and biochemical were used for the identification.

CHAPTER THREE

INCIDENCE OF SALMONELLA AND VIBRIO PARAHAEMOLYTICUS IN SELECTED MARINE SEA FOODS

3.1. Introduction.

The bacterial quality of freshly caught fish can be rapidly spoiled by subsequent unhygienic handling and processing. The main contributors of contamination at sea are the ship surfaces and the ice. The unhygienic habits of fishermen, equipments and excessive bilge water may also contribute to bacterial contamination. Further contamination may result from the bad handling, exposure to flies, insects, and to birds droppings etc. Environmental pollution, unhygieninic fisherman and workers may introduce pathogens like Salmonella and Vibrios that survive and multiply in sea foods.

Fresh fish muscle is normally free from micro-organisms, but while processing, the fresh cut surfaces quickly harbour the bacteria. It is desirable to remove as much as posible of ~~xxxxxx~~ both

the spoilage and the pathogenic organisms from the surfaces. However, bacteriological studies are useful in determining whether a food is prepared, handled and packed under good sanitary conditions. Bacteriological examinations can indicatee unsanitary handling and the presence of pathogenic organisms. Isolation of these pathogenic organisms from sea foods may cause concern, since these harmful organisms may be a hasard/health to public health.

3.2. MATERIALS AND METHODS.

3.2.1. Collection and transport of samples.

The following group of fishes and shell fishes were regularly sampled in the fresh condition from various landing centres and retail markets in Cochin. The fishes that were used in this study were: Baracudas, (*Sphyraena* spp); Catfishes, (*Tachysurus* spp); Clupeiformes, (*Sardinella longiceps*, *Anchoviella* spp); Macerels, (*Rastrelliger kanagurta*); Sciaenidae, (*Otolithus* spp); Silver bellies, (*Leiognothus* spp); Grey mullets, (*Mugil* spp) Pearl spot, (*Etroplus* spp); and shell fishes, (*Peneaemus indicus*, white shrimp and (*Metapeneeus dobsoni*) brown shrimp and the crab, (*Scylla serrata*).

The samples were collected from diffement places during a peroid of two years (two surveys) from 1979 January to 1980 December at monthly intervals. Samples were asseptically transferred to sterile wide-mouthed bottles from the landing centres and from the retail markets and brought to the laboratory as early as possible for the microbiological analyses. Portions of gills, stomach contents and muscles were sampled aseptically at room temperature and evaluated for the total microbial load and Salmonella.

3.2.2. Evaluation of total microbial load.

The total microbial load of the fish and shell fishe samples of freshly landed and market samples were enumerated by weighing asceptically of 25 g, and blending with sterile 225 ml of 0.1% peptone water and further serially diluted with the same peptone water (0.1%) and evaluated by the plating technique. For the evaluation of the plate technique, triptone, glucose, yeast extract agar was used. The medium was prepared as detailed below.

3.2.2.1. Preparation of triptone glucose yeast extract agar.

Triptone (Oxoid or Difco) per litre .... 5.0g.

Yeast extract powder (Oxoid or Difco) ... 2.5g.

Dextrose powder .....1.0 g.

Agar .....15.0g.

pH 7.0 (approx.)

The inoculated petridishes were incubated at room temperature (28 ± 2°C) for 48 hr. Colonies were and counted and the microbial load were expressed per gram of the respective muscles, or stomach or gill contents.

3.2.3. Isolation of Salmonella.

3.2.3.1. First survey.

Samples of fresh surfaces muscle portions, stomach contents and gill portions were separated asceptically and the weight of 25g. portion was taken. This was placed in 225 ml. of nonselective pre-enrichment broth of either lactose (1% lactose) or peptone (1% peptone). This mixture was well agitated and incubated for 18 to 24 hrs. at 37°C. Further, two enrichments media of tetrathionate and selenite cystine broths were prepared from Difco chemicals and were tempered to 37°C before the addition of pre-enrichment broths. One ml. of the pre-enrichment broth of either lactose or peptone was inoculated to each of the tetrathionate and selenite cystine broths. Pre-enriched samples were mixed thoroughly in enrichment broths by shaking and the both the enrichments were incubated for 24 to 48 hrs. as the case may be at 37°C.

Two selective plating media agars were used for the isolation of Salmonella, viz.,

Xylose Lysine Desoxycholate agar and Brilliant green agar A loopful of enriched culture of each of the two enriched media were spread-plated in the Brilliant greenagar and Xylose Lysine Desoxycholate agar which were previously pour-plated and surface dried. Both the selective plating media were incubated for 24 hrs. at 37°C. The colonies were selected from those showing a pinkish or magenta hue in Brilliant green agar media. However, in cases where none of these colonies were evident, two green colonies were picked. The picked colonies were purified by further spread-plating in MacConkey agar which was previously pour-plated and surface dried in the incubator for about two hours at 42°C. Well separated pure pale colourless and/colonies were taken for further biochemical study. If pale colourless colonies were not seen the pink colonies were picked for the study.

Similarly, the same procedure was used in the Xylose Lysine Desoxycholate agar plates. Colonies which appeared a deep pink to red and slight brown were picked and purified by the above procedure described in the previous paragraph.

The presumptive suspected colonies of Salmonella in both plates were subjected to detailed biochemical studies by inoculating in triple sugar iron agar slants and Lysine iron agar slants and

incubated at 37°C for 24 hrs. The positive Salmonella cultures typically produced an alkaline (red) slant and acid (yellow) butt with and without production of H_2S (blackening of agar) in triple sugar iron agar. In Lysine iron agar Salmonella cultures typically produced alkaline (purple) reaction in the butt of the tubes. The distinct yellow colouration in the butt of the tubes is an acid reaction but it is generally considered as a negative reaction of Salmonella. Some cultures of Salmonella produce hydrogen sulphide in Lysine iron agar. The positive cultures were identified by testing biochemically and then serologically by agglutinating with somatic antisera.

The antisera used were purchased from Difco Laboratories and the following somatic groups were included; A, B, C_1, C_2, D, E_1, E_2, E_3, E_4, F, G, H, I, poly A, poly A-1, poly B, poly C, poly D, poly E, poly F, and Arizona poly dibasic. The cultures that were either positive in Lysine iron agar or in Triple sugar iron agar slants, were tested with polyvalent 'O' antisera and positive agglutination reaction (precipitate formation or clumps) confirmed the culture as Salmonella or Arizona.

3.2.3.2. Second survey.

The procedure described in the section 3.2.3.1. was followed except for the selective plating media of Brilliant green agar. The alternate selective plating media Hektoen enteric agar (Difco) was used. The presumptive suspected colonies in Hektoen enteric agar appeared as bluish green with black centred colonies. They were further subjected to detailed biochemical studies as in the previous section and confirmed by agglutination with polyvalent 'O' antisera.

3.3. RESULTS.

The table 12, and fig. I, summerise the two surveys carried out during the years '79 and '80 of the total viable microbial counts of fresh specimens from the landing centres and those from the retail markets in Cochin. In the present investigation, though much emphasis has not been laid on the total viable bacterial load, an attempt has been made to understand the significance of the microbial population in the environment in relation the pathogenic organisms present in the marine seafoods. Both the fresh fin fishes and the crustaeceans showed no significant correlation in total counts. This may be attributed to the differences in the time taken to transport the catch by the fish vendors.

Both surveys showed that fresh and marketed samples exhibited wide fluctuations on the

total microbial populations. The viable population of the fresh samples from the landing centres varied from $1.2x10^4$ to $6.9x\ 10^6$ per gram on the surface. In the case of the retail market samples the general bacterial load was lower than that of the freshly landed samples in both surveys. This is contrary to the expected results and the decrease may be due to the frequent washings with good potable water in the retail markets by the fish vendors.

Regarding the gills and stomach contents (tables 13 and 14) similar variations were seen. In the gills the bacterial load ranged from $1.8x10^3$ to $4.9x10^5$ per gram and in the stomach contents the variation showed from $2.1x10$ to $5.8x10^6$ per gram. During premonsoon monsoon, and post minsoon periods, the microbial load flutuated and no definite conclusions could be drawn. But in the summer season, the surface counts recorded higher counts of populations ($6.9x10^6$per gram) from the landing centre samples. Coresponding samples of gills and stomach contents did not exhibit any microbial load on the corresponding higher ranges. A similar pattern of flutuation was also seen in the total viable counts in various parts of the fin fishes and crustaceans of the market samples. These data clearly indicate that the total viable microbial load or population did not significantly correalte with different

seasons and with different parts of the fishes, but higher counts were found in the surface samples during the summer season.

The incidence of Salmonella in commercially avaialble selected fresh fishes and crustaceans in the market sample and in the landing places in Cochin area was studied. The result of the first survey are listed in the tables 15 and 16. Out of 238 samples, 38 samples (15.9%) showed positive of Salmonella. Both fresh landed and marketed fishes and crustaceans indicated the presence of Salmonella. Sea foods are contaminated either on the deck of the boat or by the unhygeinic habits of fishermen and the retail sellers. Fresh samples from the landings also recorded the presence of Salmonella (14.05%). This indicated the polluted nature of the water used for cleaning the fishes, and the contamination by some human carriers, while handling the catch, at sea. This survey indicated the occasional presence of Salmonella in fin fishes and crustaceans almost every month(fig.2a and 2b). So the data clearly indicated the unhygeinic natuse and habits of fisherman and fish vendors. The products could become a potenial vehicle for food poisoning (Shewan et al., 1955; Shewan 1962).

In the second survey 23 samples out of 237 samples indicated the presence of Salmonella(9.28%). Similar findings were also noted in the first survey. In this regard the lower incidence of

Salmonella was noted both in the fresh varieties and int the market varieties. In the second survey the lower incidence of Salmonella may be due to the improved methods of handling of fish by the fish handlers and fish vendors.

On studying the distribution of these pathogens it was found that the surface of the fin fishes and crustacaens exhibited more contamination than any other part. The surface contamination was due to the reasons reported in the previous paragraph. Gut contents and gills showed almost free from Salmonella. Generally finfishes and shellfishes of marine origin (off-shore water fishes) will be free of Salmonella (Shewan 1962). This data already shows that the contamination of the surface was after the catch.

The data indicated the incidence of Salmonella can not be correalted with different seasons, as for the total microbial population (tables 15 and 16). Among the groups of finfishes and shellfishes, higher incidence of positive Salmonella was in Mackerel, Clupeiformes and Prawns (15.75%), (table 17 and fig. 2a,&b). The estuarine samples of Pearl spot also recorded higher incidence (11.11). Market samples of crab (Sycila serrata) also exhibited 9.09% pf Salmonella.

Similar findings were also in the second survey. No significant lower incidence of

Salmonella was noted. In general a comparison of both the surveys establish the incidence of these species of pathogens in the selected marine fishes and indicate the polluted nature of the water used for cleaning the catches and the unhygeinic habits of fishermen.

In order to find the frequent occurance of the various serotypes of Salmonella strains isolated from different groups of fishes and shellfishes, were serotyped by the National Salmonella and Escherichia Center, Kausauli, H.P. and the following serotypes occured frequently in the samples tested in the two surveys. The frequency was in the following order: Salmonella anatum, Sal. typhimurium, Sal. typhi, Sal. newport, Sal. saintpaul, Sal. bariely, Sal. arizona, Sal. senftenberg, Sal. roan, Sal. weltevreden, and Sal. waycross.

3.4. DISCUSSION.

3.4.1. Variability of results.

Studies on the incidence of Salmonella species in commercially available fresh and marketed fishes and crustaceans indicate the potential food poisoning organisms. Considering the demand for fish and shellfishes, in the internal markets, it is not surprising that even the contaminated products of

of fish are used for human consumption with out any rejection. The significance of the presence of Salmonella in the consumer fishery products for internal consumption is not looked into ~~promptly~~ as seriouslyas in the products for exports. Because of strict legislation, regarding the quality of seafoods, for export, the hygeinic aspects have to be compiled with processors. Various studies have been carried out to find the methods, to reduce the pathogenic micro-organisms in fresh and frozen materials(Carroll et al., 1966; Williams et al., 1952; Green, 1949; Surkiewicz et al., 1955; Shewan et al., 1955; Shewan 1962). The area or thegrounds from which the fishes and the crustaceans are caught may contribute Salmonella and other food poisoning organisms, if these region are contaminated by the sewage water.

Regarding the distribution of Salmonella strains on the surface and in the gut of the whole and ungutted fish surfacesamples showed higher percentages on the surface than in the gut contents. Usually, as stated before, marine fishes and crustaceans do not contain Salmonella. Geralin (1962) found that adventitious contaminationin fin fishes and crustaceans by the micro-organisms of public health significance occurs during processing and handling.

Further, no detailed work in India concerning the actual potential for the growth of *Salmonella* in marine seafoods is done so far. Matches and Liston (1968), reported that in the crab meat held at 6°C and below, *Salmonella* growth declined.

As stated previously, the incidence of *Salmonella* is undoubtedly due to unhygienic practises during thehandling of the fishes. The surveys of market samples indicated the presence of food poisoning organisms in Cochin markets. Similar findings were also reported by Gulasekaram *et al*., (1959) in the Colombo market of Ceylon. This clearly indicate that the samples were contaminated by the polluted water or by the handlers in processing or by market agencies. Harvey *et al*., (1969), demonstrated that man regularly encountered a wide range of serotypes of *Salmonella* and these organisms contaminated regularly the environment. It is possible that these serotypes of *Salmonella* contaminate the seafoods in the retail markets of Cochin.

In this study comprising ten groups of fin fishes and shellfishes, *Paneaus indicus*, *Metapaneeus affinis* (mature specimens from inshore waters and juveniles from estuaries), and crab meat (*Syclla serrata*), 9 to 18% were contaminated by *Salmonella*. Leigenda *et al*., (1950), reported that

97 samples comprising ten species of finfishes carried 19.6% Salmonella contamination. Floyd and Jones found that 11% of fish marketed in Cairo harboured Salmonella and Shigella. Gangrusz and Malninska (1959), reported that intection by Salmonella of fresh water cray fish from Polis rivers and lakes was about 6%. Studies carried by Jadin et al., (1956 & 1957) showed that the lake waters were infected by Salmonella and Shigella (2% of fishes caught). This study also shows that it is not exceptional to find out viable Salmonella in fish and fishery products contaminated by polluted water or by unhygeinic handling, transport and processing.

3.4.2. Relationship with total viable counts.

The viable counts of the samples carried out during the two surveys did not correlate the presence of Salmonella with different seasons. Both fresh and marketed samples indicated more or less the same flutuating counts. This study did not show any apparent seasonal variations in total bacterial counts, since these materials were already handled by the fishermen and market agencies. In this study there was no correlation between the total bacterial counts and Salmonella counts. But various workers, such as Clise and Swecker (1965), Moyle (1966), Loken et al., (1968), indicated that samples containing Salmonella had high counts of total bacterial load of 10^5 per gram and above.

3.4.3. Distribution of different serotypes.

The serotypes of Salmonella occuring frequently in marine seafoods varied in the two surveys. These variations were due to the contamination by the human handling. Steele and Galton (1967), found that the following species, most common in the seafoods were, Sal. infantis, Sal. oranienberg, Sal. typhimurium, Sal. montivideo and Sal. hiedelberg. Similarly Taylor (1962), isolated Salmonella serotypes from meat and bone meal and from fish products were: Sal. senftenberg, Sal. anatum, Sal. cubana, and Sal. dublin. Of the various serotypes in foodborne out breaks of salmonellosis, Sal. typhimurium, was one of the most frequently encountered throughout the world (Jay, 1970). The results of this study also support this view. In U.S.Public Health Service (1963), reported most prevalent types were: Sal. typhimurium, Sal. heidelberg, Sal. anatum, Sal. choleraesuis Sal. infantis, Sal. montivideo, Sal. derby, Sal. saintpaul and Sal. oranienberg.

Marine seafoods, eventhough contaminated with Salmonella appear to be less of a problem than in animal feeds, but these are certainly, of more immediate public health concern. Anderson et al. (1969) reporeted the same view about the presence of

Salmonella. It is quite clear that low incidence of salmonellosis can be due to the heating (during cooking) which destroy the pathogenic Salmonella species before consumption.

3.5. Collection of samples for the enumerationof Vibrio parahaemolyticus.

Samples described in section 3.2.1. were collected and brought asceptically to the laboratory and the isolation of Vibrio parahaemolyticus was carried out by the following method.

3.5.1. Enumeration of V. parahaemolyticus.

An apprppriate decimal dilution of sterile 7% sodium chloride solution with samples as out lined in the section 2.3.3. of standard methods, was used for this study. The fivestep procedure of MPN (Most Probable Number) was used for both surveys. Bacterial colonies developed on thiosulphate citrate bile salts sucrose agar plates were categorised as described in section 2.3.3., i.e., bacterial colonies developing on TCBS agar plates or on MT agar (Modified Twedt) plates categorised as Vibrio parahaemolyticus and counted appropriately on MPN tables.

3.5.2. Identification of Vibrio parahaemolyticus.

Selected colonies of presumptive Vibrios were picked up from Thiosulphate citrate bile salt agar or Modified Twedt agar, restreaked again into the above medium for purification and maintained in Tripticase soy agar with 3% Na Cl for further characterisation. Identification of Vibrio parahaemolyticus in the present investigation was based on the properties assumed to be of determinative value, as stipulated in section 2.3.2. (page 79), which is based on the identifiaction scheme of Sakazaki (1967) and further extended by Kaneko (1973).

In order to facilitate constant evaluation of the media and methodology used in this study for the isolation and identification, reference culture of Vibrio parahaemolyticus, received from Torry Research Station, Aberdeen, was used. After confirming the identity of Vibrio parahaemolyticus the counts were computed from the appropriate MPN tables.

3.6. RESULTS.

Tables 19 and 20 summerise the percentages of incidence of Vibrio parahaemolyticus in bothe fresh and market samples. The results, show that in the first survey out of 248 samples of fresh

and marketed samples, the average incidence recorded was 45.1% of fresh samples and 43.7% of marketed samples. In the second durvey, the results were slighly lesser, i e., 39.5% in fresh samples, and 41.6% in market samples. On comparing both the surveys, the higher counts of Vibrio parahaemolyticus were detected in the fresh fishes, while coresponding market samples had a lower range.

The distribution and recovery of Vibrio parahaemolyticus in the different region of fishes and shellfishes were examined. In the fresh fishes of selected varieties (tables 19 and 20) and shell fishes of Penaeus indicus, Metapenaeus affinis and in the crab meat of Scylla serrata were taken for this study. It has beenf found medh variations on the surfaces, gills and gut contents of fresh and marketed samples. The monthly survey showed a higer incidence of Vibrio parahaemolyticus on the surfaces and gut contents than on the gills. Both fresh and market samples showed different variable ranges. Similar aspects were found in shrimps and crab meat.

The counts of Vibrio parahaemolyticus (MPN method) showed (tables 22 and 23) that in both surveys the freshly caught finfishes gave 1.1 to 5.4×10^2 per gram, while market samples recorded

higher values 1.1 to $9.2x10^2$ per gram. Freshly caught prawns had $2.7x10^2$ to $5.4x10^3$, while market samples showed higher detections from 2.8 to $9.2 x10^3$ per gram. Similarly, crab meat of freshly caught specimens showed 3.2 to $8.4x10^3$ per gram, and market samples showed from 3.4 to $9.5x10^3$ per gram. It has been found that in both the surveys, slightly higher Vibrio parahaemolyticus counts were observed in the market samples, than in the freshly caught specimens.

The groupwise detection of Vibrio parahaemolyticus in the two surveys are reported in tables 23 and 24. In the first survey, it is found that in freshly harvested finfishes Clupeiformes registered a higher detection of 43.2%, while Sciaenids, Pearl spot, Baracudas, Mackerels and Cat fishes showed more or less the same ranges of 37 to 40.0%. But on comparing the incidence of Vibrio parahaemolyticus in fresh specimens and in the market samples, the latter showed higher value except in the Pearl spot. Incidence of these organisms, in shrimps; Penaeus indicus, Metapenaeus affinis and the crab, Scylla serrata indicated 62 to 66.7% in fresh samples.

In the second survey Cat fishes and Pearl spot recorded higher amount of Vibrio

parahaemolyticus than the other groups of finfishes. The variations in the other groups were more or less from 29.0 to 40.0%. Similar variations were also seen in the market samples, but the counts were less(table 23). Shellfishes were recorded more or less the same as in the first survey. Freshly harvested samples showed the presence of Vibrio parahaemolyticus from 60.0 to 67.0% and the corresponding market varieties indicated 60.0 to 67.0%.

The seasonal incidence of Vibrio parahaemolyticus(in average total counts) in finfishes, shellfishes; Penaeus indicus, and Metapenaeus affinis are shown in table 24 and fig. 3. From the results, it has been found that no conclusions could be drawn on their seasonal variations. The Vibrio counts varied each month and lower counts were registered in the both surveys in the month of June. In other months the counts were more or less same and fluctuated from 1.7 to 6.4×10^2 per gram in fresh finfishes, and in the market samples, ranged from 2.1 to 7.9×10^3. Fresh shellfishes, registered range of 2.1×10^3 to 7.9×10^3 while corresponding market varieties registered a variation of 1.7 to 9.2×10^3. In general a comparison of the two surveys on prawns and crabs showed higher

counts of Vibrio parahaemolyticus.

Among the freshly harvested seafoods examined during the survey periods '79 and '80, the higher incidence and percentages of Vibrio parahaemolyticus counts, in prawns and crabs than in finfishes, used in this study, were recorded. Similar findings found by various workers, in other countries, (Baross and Liston, 1970; Fishbein et al., 1970; 1971; Hechelmann et al., 1971; Thomson and Trenholm, 1971). Martin(1980), found similar higher incidences of Vibrio parahaemolyticus, in shellfishes and other fresh fishes in the East Coast. The higher incidence on the shellfishes may be due to the abundance source of chitin present in the prawn and crab shells.

The seasonal incidence of Vibrio parahaemolyticus in fresh fishes and shell fishes exhibited variations with different months on recovery. No correlation was observed between the post-monsoon, summer and the premonsoon periods. During rainy seasons low incidence of Vibrio parahaemolyticus, in the two surveys was observed. Baross and Liston(1970), showed similar correlation in the Pacific oysters during rainy season. Similar observation was made by by Krantz et al., (1969) on the Blue crab in Chesapeake Bay.

In studying the recovery of *Vibrio parahaemolyticus* counts, in the different regions, in fresh fishes and prawns, it was found that conspicuous variations occur in the gut contents as well as on the surfaces of the muscles portions of marine seed foods. Such a variation was expected in the case of prawns and crabs because of their food variation and feeding activities at the bottom of sea. It can be also attributed to the environmental conditions and the nutrients present in that region. Usually the proliferation of these organisms in the gut contents takes place when the rich nutrients present in the gastro-intestinal tract of the fish. The gut contents and the surfaces of the fishes showed a higher count these bacteria. The ecological conditions might foster the survival of *Vibrio parahaemolyticus* in the gastro-intestinal tract of fishes and provide a source of enrichments for their proliferation in the stomach, and subsequent dissimination of these micro-organisms along with the fecal matter in the surroundings may increase the surface counts on the fishes, which visit this region for feeding. The symbiotic enteric association of *Vibrio parahaemolyticus*, with other allied chitinoclastic organisms may have an active role in degrading chitin in the gastro-intestinal tract of the

fishes feeding on a chitinous diet.

From the results it is clear that the marine fishes, prawns and crabs caught in the inshore waters and from the back waters of Cochin indicated the presence of Vibrio parahaemolyticus and allied organisms. This is particularly significant in view of the known capability of Vibrio parahaemolyticus to produce gastro-enteritis in human beings. Various authors in Japan and other countries reported the max seasonal incidence of gastroenteritis, due to this micro-organism during summer months (Sakazaki et al., 1962; Yasunaga, 1965; Nakanishi, et al., 1968; Krantz, et al., 1969; Kampelmacher and Mossel, 1970; Chan et lal., 1970; Vandersant and Nickelson, 1972; Bartley and Slanetz, 1971; Van den Brock, Mossel and Eggenkamp, 1979; and Martin, 1980)

Table 19. Incidnece of Vibrio parahaemolyticus in fresh fishes from landings and market fishes around Cochin.

Months	No. of samples examined	Fresh fishfrom landings V.p. +ve %	gills	gut	No. of samples examined	Market samples V.p. +ve %	gills	gut
January	6	2(33.33)	1	2	9	4(44.44)	2	3
February	10	4(40.00)	2	4	11	4(36.37)	3	4
March	15	6(40.00)	4	6	12	5(41.65)	2	5
April	10	5(50.00)	5	5	10	4(40.00)	3	4
May	9	4(33.33)	2	3	8	3(36.99)	2	2
June	6	2(33.34)	1	2	10	4(40.00)	3	3
July	10	3(30.00)	2	2	14	7(50.00)	4	6
August	15	7(42.42)	3	6	7	3(44.84)	3	3
September	10	3(30.00)	3	3	14	6(42.84)	6	5
October	7	4(57.12)	3	4	11	5(45.46)	3	5
November	8	4(50.00)	2	4	7	4(57.12)	2	4
December	16	7(43.75)	3	5	13	6(46.14)	5	6

V.p. = Vibrio parahaemolyticus., +ve = positive.

Table 28. Incidence of *Vibrio parahaemolyticus* in fresh fishes from landings and retail markets around Cochin during the year '80.

Months	Fresh fish from landings				Retail market samples.			
	No. of samples analysed	V.p. +ve %	gills	gut	No. of samples analysed	V. p. +ve %	gills	gut
January	10	4(40.00)	3	4	99	5(55.56)	3	4
February	9	4(44.44)	3	3	11	5(45.46)	3	5
March	14	7(50.00)	3	5	14	6(42.84)	4	6
April	11	5(45.46)	2	4	12	5(41.65)	2	4
May	9	3(33.33)	3	3	11	4(36.37)	2	33
June	7	3(37.50)	2	3	7	4(57.12)	2	3
July	8	3(37.50)	1.	3	8	3(37.50)	1	3
August	9	3(33.33)	1	4	10	3(30.50)	2	3
September	10	4(40.00)	2	3	10	3(30.00)	2	3
October	11	4(36.36)	2	4	10	4(40.00)	2	4
November	9	4(44.45)	2	3	9	3(33.34)	1	2
December	12	3(25.00)	1	2	12	5(41.65)	2	4

V.p = *Vibrio parahaemolyticus*, +ve = positive.

Table 21. Counts of *Vibrio parahaemolyticus* (MPN. Method) from different groups of fin fishes and shellfishes during the year '79.

Groups	Fresh samples Min./gm.	Max./gm.	Retail market samples Min./gm.	Max./gm.
Fin fishes.				
Cat fishes	$1.1x10^2$	$4.3x10^2$	$1.7x10^2$	$5.6x10^2$
Barracudas	$1.7x10^2$	$3.2x10^2$	$2.2x10^2$	$5.4x10^2$
Clupeaformes	$2.4x10^2$	$5.4x10^2$	$1.4x10^2$	$9.2x10^2$
Sciaenids	$1.2x10^2$	$4.3x10^2$	$1.1x10^2$	$5.4x10^2$
Silver bellies	$1.1x10^2$	$2.5x10^2$	$1.7x10^2$	$6.4x10^2$
Mullet	$1.7x10^2$	$4.0x10^2$	$1.4x10^2$	$9.5x10^2$
Pearl spot	$2.2x10^2$	$5.4x10^2$	$2.8x10^2$	$4.3x10^2$
Shell fishes.				
Penaeus indicus.	$4.3x10^2$	$1.6x10^3$	$5.4x10^2$	$6.9x10^3$
Metapenaeus affinis.	$2.4x10^2$	$4.3x10^3$	$2.8x10^2$	$9.2x10^3$
Crab.				
Scylla serrata.	$3.8x10^2$	$8.4x10^3$	$4.3x10^2$	$9.5x10^3$

Table 22. Counts of *Vibrio parahaemolyticus* (MPN. Method) from different groups of finfishes and shellfishes during the year '80.

Groups	Freshly harvested. Min./gm.	Freshly harvested. Max./gm.	Market samples. Min./gm	Market samples. Max./gm.
Cat fishes	2.2×10^2	5.3×10^2	3.5×10^2	4.3×10^2
Baracudas	1.1×10^2	3.5×10^2	2.8×10^2	3.6×10^2
Clupeiformes	1.5×10^2	5.4×10^2	1.1×10^2	5.4×10^2
Sciaenids	2.4×10^2	4.1×10^2	1.3×10^2	4.6×10^2
Silver bellies	1.1×10^2	2.2×10^2	1.7×10^2	3.1×10^2
Mullet	1.2×10^2	2.8×10^2	2.1×10^2	5.4×10^2
Pearl spot	2.8×10^2	4.3×10^2	3.1×10^2	9.2×10^2
Shell fishes				
Penaeus indicus	3.6×10^2	4.3×10^3	5.4×10^2	5.4×10^3
Metapenaeus affinis	2.7×10^2	5.4×10^3	3.6×10^2	9.2×10^3
Crab				
Scylla serrata	3.2×10^2	4.5×10^3	3.6×10^2	6.9×10^3

Table 23. Group wise incidence of *Vibrio parahaemolyticus*.

Survey No. 1.

Groups	Fresh samples		Market samples	
	No. of samples examined	+ve V.p. (%)	No. of samples examined	+ve V.p. (%)
Fin fishes.				
Cat fishes	8	3(37.50)	6	3(50.00)
Baracudas	5	2(40.00)	7	3(42.84)
Clupeiformes	22	9(43.20)	18	8(44.44)
Mackerels	13	5(38.46)	9	4(44.44)
Seisenides	5	2(40.00)	4	1(25.00)
Silver bellies.	9	2(22.22)	9	3(33.33)
Mullet	6	2(33.33)	9	4(44.44)
Pearl spot	10	4(40.00)	9	3(33.33)
Shell fishes				
Penaeus indicus.	23	13(61,88)	14	9(64.26)
Metapenaeus affinis.	16	10(62.50)	15	10(66.70)
Crab				
Scylla serrata	12	8(66.64)	11	7(63.63)

Table 24. Group wise incidence of *Vibrio parahaemolyticus*. Survey No. 2.

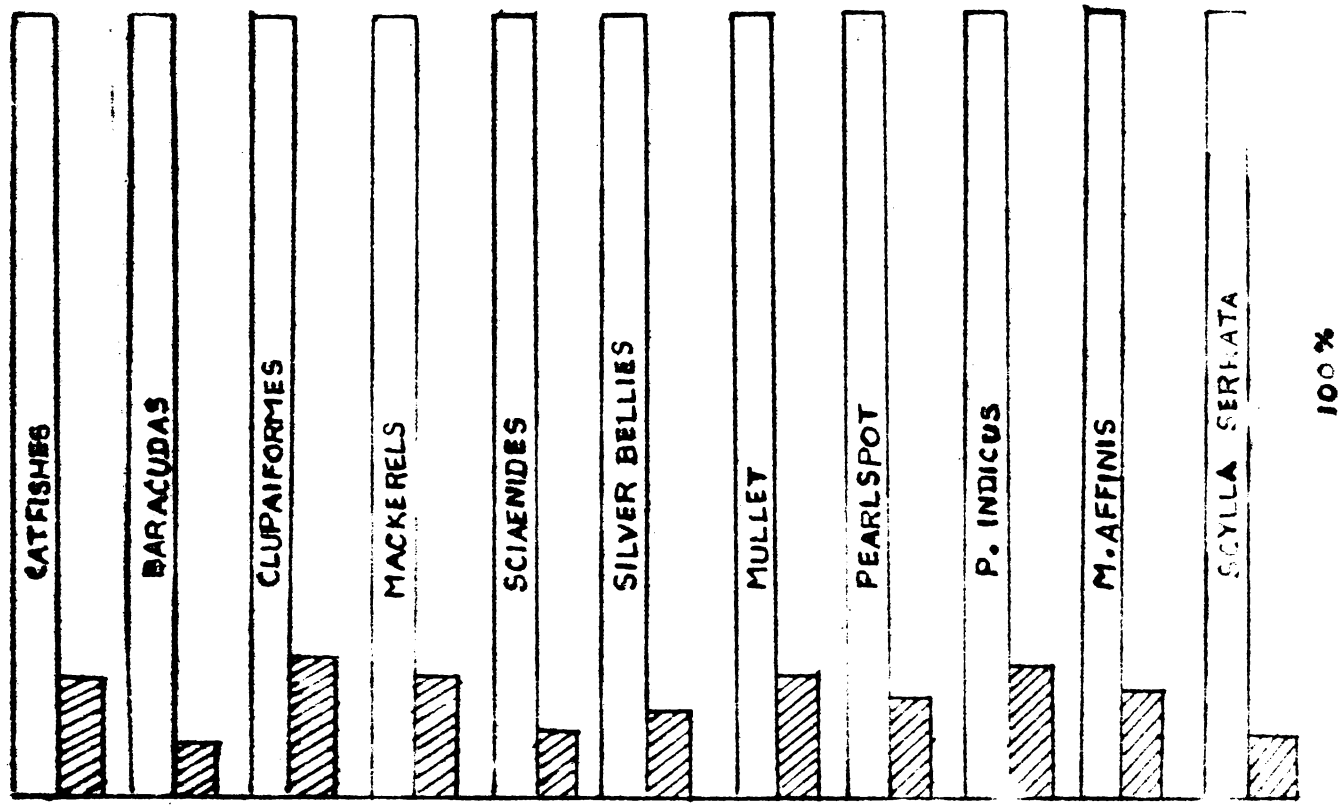
Groups	Fresh samples. No. of samples examined	+ve V.p. (%)	Market samples. No. of samples examined	+ve V.p. (%)
Fin fishes.				
Cat fishes	7	3(43.44)	5	2(40.00)
Barracudas	6	2(33.34)	8	3(37.50)
Clupeiformes	20	8(40.00)	16	5(31.25)
Mackerels	14	5(35.70)	11	3(27.28)
Sciaenids	6	2(33.34)	5	1(20.00)
Silver bellies	8	2(37.50)	10	3(30.00)
Mullet	7	2(28.96)	8	2(25.00)
Pearl spot	9	4(44.44)	11	4(36.37)
Shell fishes				
Penaeus indicus	25	15(67.25)	12	8(68.00)
Metapenaeus affinis	18	12(66.72)	14	9(64.26)
Crab				
Scylla serrata	10	6(60.00)	10	5(50.00)

Table 25. Seasonal counts of *Vibrio parahaemolyticus* in fin fishes and shellfishes in fresh and retail markets. Year '79.

Months	Fin fishes Average counts per gram.		Shell fishes Average counts per gram.	
	Fresh	Retail	Fresh	Retail
January	5.2×10^2	4.5×10^2	5.4×10^3	5.8×10^3
February	2.6×10^2	4.3×10^2	4.5×10^3	6.4×10^3
March	6.4×10^2	6.9×10^2	4.9×10^3	9.2×10^3
April	5.8×10^2	6.2×10^2	7.9×10^3	8.4×10^3
May	4.9×10^2	4.6×10^2	5.8×10^3	6.9×10^3
June	1.7×10^2	2.5×10^2	7.9×10^2	9.2×10^2
July	2.8×10^2	3.5×10^2	2.1×10^3	5.8×10^3
August	2.5×10^2	4.3×10^2	3.5×10^3	5.4×10^3
September	6.2×10^2	3.6×10^2	5.4×10^3	2.8×10^3
October	3.5×10^2	1.3×10^2	4.2×10^3	1.7×10^3
November	5.4×10^2	4.3×10^2	4.9×10^3	2.8×10^3
December	3.6×10^2	3.2×10^2	3.5×10^3	9.2×10^3

Fig.1.a. *Salmonella* percentages in various groups of Fin fishes and Shellfishes samples from the landing centers of Cochin.

FIG. 4a



SAMPLES FROM LANDING CENTERS

■ POSITIVE SALMONELLA □ GROUPS

Fig. 1.b. *Salmonella* **percentages in various groups of Fin fishes and shellfishes samples from the retail market centers of Cochin.**

FIG. 1, b.

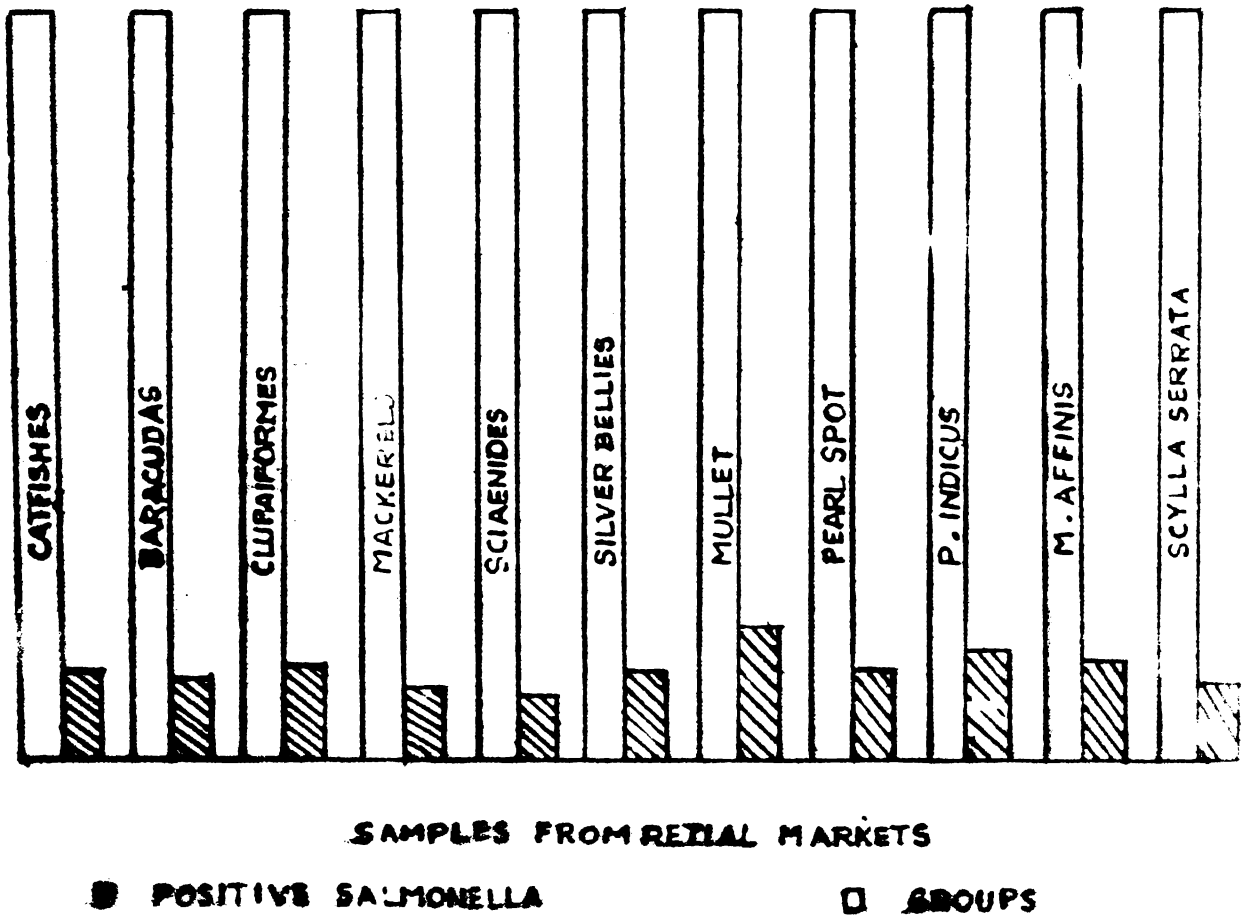

CATFISHES
BARACUDAS
CLUPAIFORMES
MACKEREL
SCIAENIDES
SILVER BELLIES
MULLET
PEARL SPOT
P. INDICUS
M. AFFINIS
SCYLLA SERRATA
100 %
SAMPLES FROM RETAIL MARKETS
POSITIVE SALMONELLA
GROUPS

Fig. 2. Seasonal incidence of Salmonella in fresh and market fish samples.

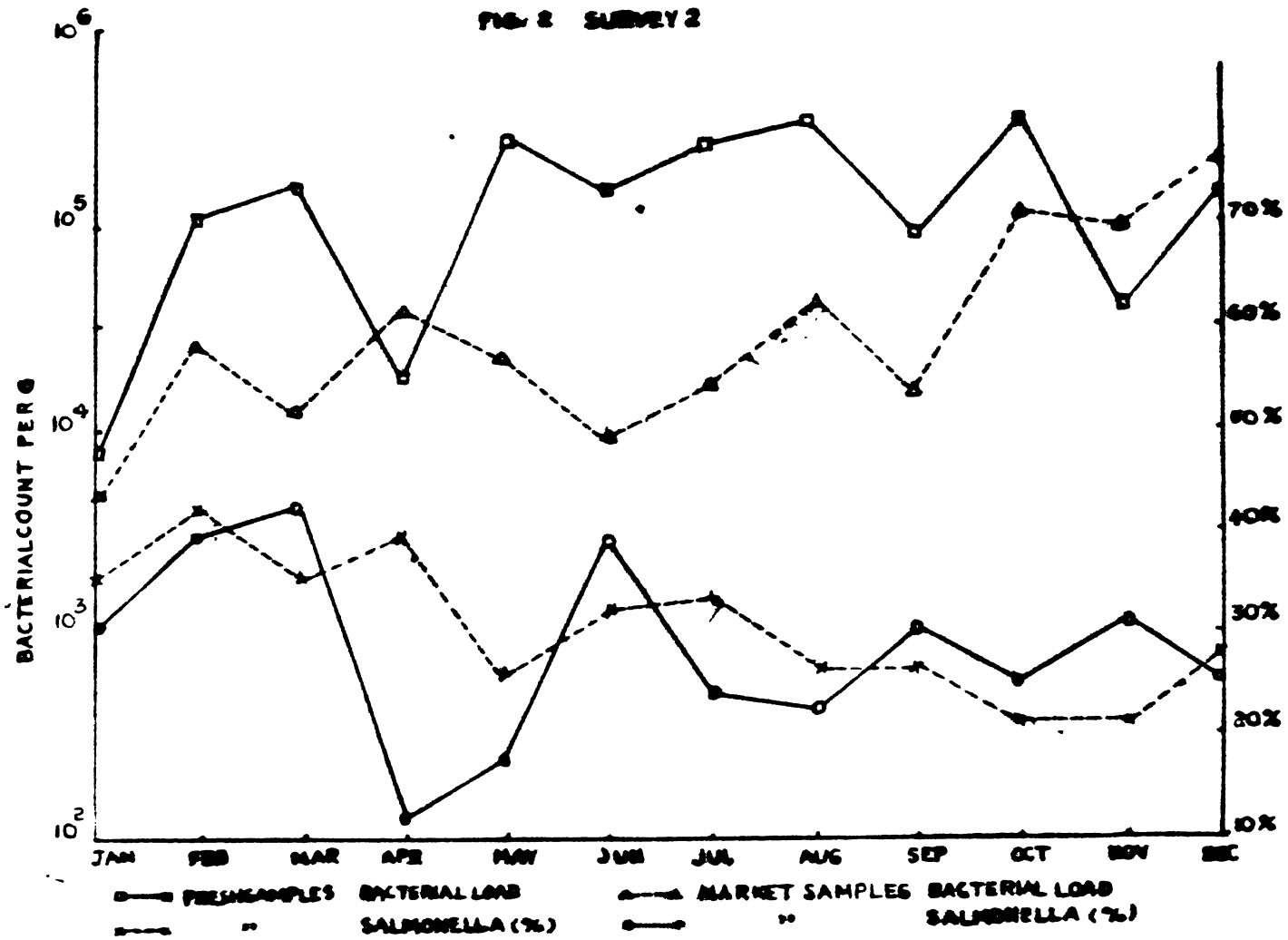
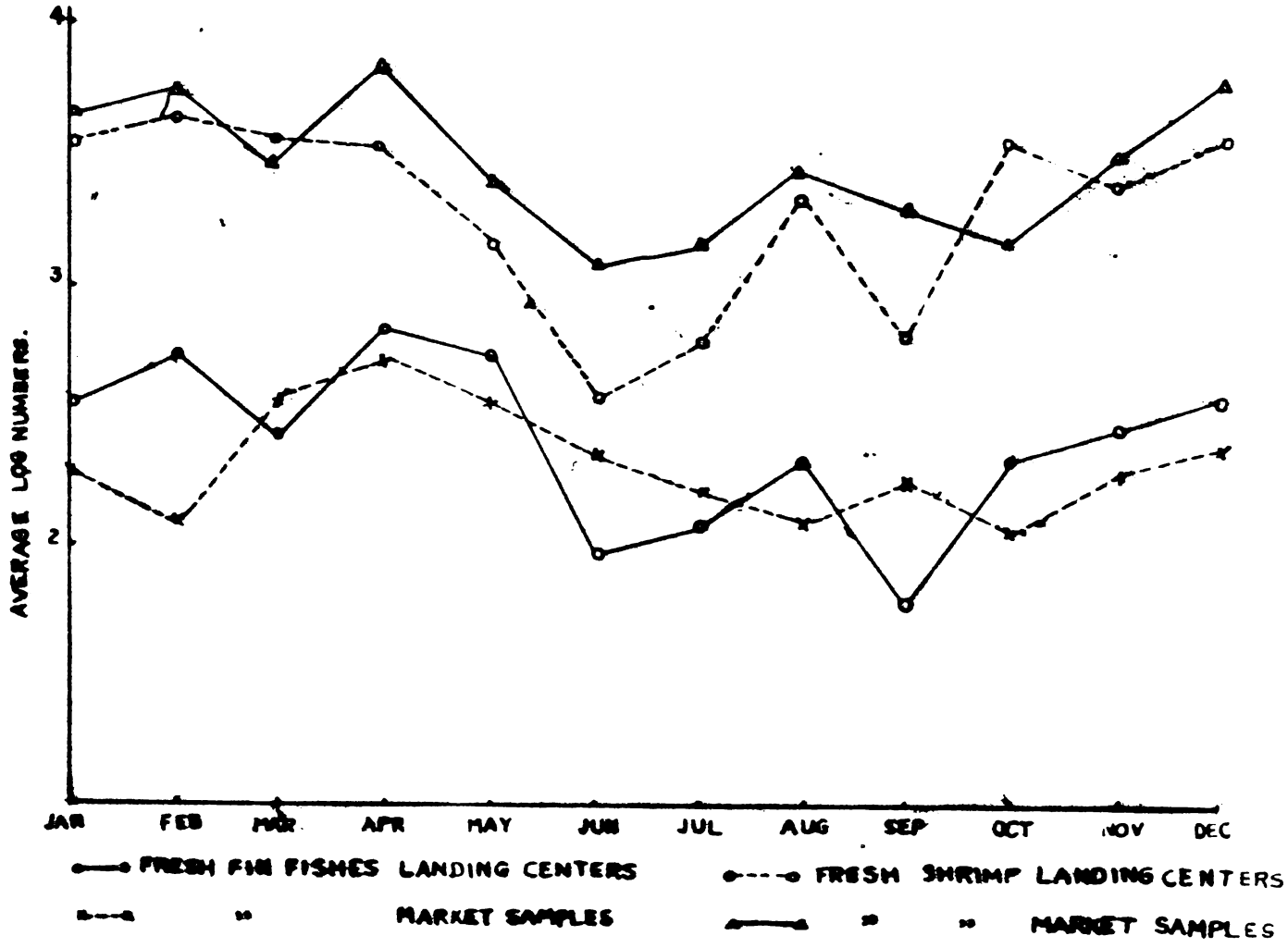

BACTERIAL COUNT PER G
10^6
10^5
10^4
10^3
10^2
70%
60%
50%
40%
30%
20%
10%
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
FRESH SAMPLES BACTERIAL LOAD
" SALMONELLA (%)
MARKET SAMPLES BACTERIAL LOAD
" SALMONELLA (%)

Fig. 3. Seasonal variation on the total vaible counts in various groups of Finfishes and shellfishes samples from landing and retail market centers of Cochin

FIG. 3


4
3
2
AVERAGE LOG NUMBERS.
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
FRESH FIN FISHES LANDING CENTERS
FRESH SHRIMP LANDING CENTERS
MARKET SAMPLES
MARKET SAMPLES

C H A P T E R F O U R

BEHAVIOUR OF SALMONELLA AND VIBRIO PARAHAEMOLYTICUS IN LOW TEMPERATURES

4.1. Introduction.

The tremendous growth of fresh, frozen and precooked frozen marine seafoods in recent years has resulted in the setting up of bacteriological standards for seafoods. Many workers have emphasised the need of additional information on the survival of the pathogenic organisms during various methods of processing of fishery products. Due to increased consumption of precooked seafoods, its potential public health hazard attained paramount importance. Salmonella and Vibrio parahaemolyticus is considered as the vechicle

of numerous out breaks of foodborne gastro-enteritis all over the world(Postgate and Hunter, 1963; Nakanishi, 1968; Baross and Liston, 1968; Ward, 1968; Sakazaki et al., 1968; Ray et al., 1971; Scheusner et al., 1971; Leistner et al., 1971; Kampelmacher et al., 1972; Chatterjee and Neogy 1972; Vanderzant et al., 1973; Sulton, 1974; Chun et lal., 1974; De et al., 1977; Natarajan et al., 1979 a, b, & 1980)

It is weal known that low temperature preservation will induce bacterial death or injury. The literature survey indicated that the exposure of organisms to low temperature resulted in either injury or death (Nagman,1960; Pressnell et al., 1960; Scheusner et al., 1971; Sinesky and Silverman, 1970; Tomlins and Ordal, 1971; Hagan, 1971; Mazur 1966). Pressnell et al.,(1981), noted that Salmonella species in Gulf Coast oysters held at 6°C, showed gradual decrease in numbers(one log less), and survived for six days. Digirolomo et al., (1970), reported that S. typhimurium is very sensitive to freezing and storage at -18°C. He found that 90% of the pathogens were reduced in one week storage. Ray et al., (1972), reported that S. anatum survived during various low temperatures of storage. This study was undertaken to extend the observations of

survival of various Salmonella serotypes in the substrates of fish and shellfishes in refrigerated, frozen and subsequent storage conditions at low temperature of - 20°C.

4.2. Materials and Methods.

4.2.1. Various Salmonella serotypes used in this study.

Various Salmonella serotypes were isolated from the previous study of section 3.3. (vide supra) of fish and shellfish products, and were later serotyped by the courtesy of National Salmonella and Escherichia Center, Kausauli. Nineteen serotypes viz: S. typhimurium, S. typhi, S. anatum, S. enteritidis, S. cubana, S. saintpaul, S. roan, S. arizona (Arizona spp., S. weltevreden, S. paratyphi, A, S. senftenberg, S. bareilly, S. chester, S. poona, S. richmond, S. waycross, S. virchow, S. nchanga, and S. newport were grown and maintain in Brain Heart infusion agar slants and nutrient agar slants. The 24 hr old culture grown in the above broths were used in this study of survival in low temperatures, such as refrigeration, frozen and further storage studies.

4.2.2. Preparation of samples and media.

For the studies of the freezing and low temperature of storage and multiple cyclic freezing and defrosting, Salmonella serotypes mentioned above were used. Each culture was subjected to decimal dilution so that the final cell concentrations of Salmonella strains were approximately of $2x10^6$ per ml or gm, which was evaulated after inoculating with 500ml of samples such as Brain heart infusion broths, Tripticase soy broth (TSB, Difco) with 3% yeast extract, Nutrient peptone (1% peptone with 0.3% beef extract), homogenates of Pearl spot (Etroplus suratensis), Mackerels (Rastrelliger kanakurta), Prawns (Penaeus indicus) and crab (Scylla serrata) which were locally obtained from various landing centres and markets around Cochin. The respective fish, prawn and crab homogenates were prepared by blending 250 gram of muscle with 250 ml of 0.1% peptone water and sterilised and cooled. Suspensions of cultures in broths, and homogenates for each organisms were separately distributed in 50ml or 100 ml quantities into beakers and covered with polythene sheets, so that external contamiation should not creep into the experiments. One set of samples was kept at the refrigerated

temperatuss of 3°to 6°C and another set kept in freezing temperature of -20°C for storage studies.

The initial viable counts at room temperature were estimated by the decimal dilution method by plating in triptone glucose yeast extract agar and incubated at room temperature for 24 hours.

Salmonella counts (artificially inoculated) were decimally diluted samples and esalu-ated by plate counts on either using Brilliant Green agar or Xylose Lysine Desoxycholate agars. In these experiments streaking technique was used by taking 0.1ml of diluted samples streaked on previously plated Xylose Lysine Desoxycholate agar or Brilliant Green agar medium. Minimam two consequtive dilutions of plate counts of the range betweemm 30 to 300 colonies per plate were used. Duplicates were made for each other dilutions. The time of incubation for all plates were 24 hours at 37°C.

Only counts with in practical marigin mmx of ± 20% experimental error were considered. When this margin was not found applicable on certain times because of the larger differences in counts, an average counts were made on all plates taken into consideration . The final thus made for each organism given as the initial count or number of viable cells

present in each preparation before subjected to freezin and low temperature storage.

4.2.3. Effect of refrigeration and freezing.

In order to study the effects of refrigeration and prolonged storage, (up to twelve days), one sample of broth suspension, one sample of respective muscle homogenates of fish and prawn and crab were drawn at different time intervals and test were simultaneously conducted for the total viable coun at room temperature (30°C) and recovery of Salmonella for refrigerated samples were carried out in Xylose Lysine Desoxycholate agar medium by streak plate method. In this case there is no requirement of five steps procedure which described in section 2.2.3. (vide supra). But for the frozen samples the isolation and enumeration were carried out by three tube MPN method, since the number of Salmonella reduced considerably, the streak plate technique using on selective plating media was not sucessful and discontinued. Similarly pour plate method was also not carried out because of the temperature of the moulen agar (around 50°C) may highly determintal for the recovery of stressed cells of microorganisms. Serially diluted samples of 10 ml, 1 ml and 0.1 ml were used

in nonselective medium of nutrient peptone. Then enumerated for the isolation of Salmonella by the standardized procedure described in section 2.2.3. The samples were drawn at from cold storage, at time intervals of 30, 60, 90, 120, 150, 180 and 240 days and in case of refrigerated samples, the time of interval for drawing the samples were 2,6, 8and 10 days.

4.2.4. Behaviour of Salmonella on multible cyclic freezing.

For the behaviour of Salmonella on multiple cyclic defrosting during storage at -20°C, each and frozen specifically labled samples was removed from cold storage and thawed. After the samples were placed back immediately in the freezing followed by prolonged storage and the effects of different times of cyclic freezing followed by defrosting , the study was carried out after 30, 60, 120,150, 210 and 240 days.

The frozen samples were thawed in a water bath at a temperature of 30°C. The broth preparations were thawed with in 10 min. and 15 min. in the case of fish, prawn and crab samples of homogenates with out wastage of time, samples were serially

diluted and plated. The time and temperature of incubation and the counting procedures are already described above for the viable counts. For the enumeration of Salmonella, the three tube MPN method was followed and the counts were taken. The enumeration procedures followed are given in section 2.2.3.

4.2.5. Extend of freeze injury and repair.

To study the extend of repair of the injured cells in broths and homogenates, the following methodology was followed. Four types of broths were used in this study. The minimal broth was prepared according to Ray et al., (1972) having the following composition; K_2HPO_4 0.25%; $MgSO_4$ 0.04%; $(NH_4)_2SO_4$ 0.1%; Sodium citrate 0.01%; Sodium chloride 0.01%; and glucose 0.25%; and the final pH was adjusted to 7.0. For the above broth analytical pure grade chemicals were used . The nutrient broth, Tripticase soy broth, Tripticase soy yeast extract broth were prepared by using Difco or Oxoid grade.

The assessment of the extend of protection, injury and repair was carried out by inoculating Salmonella cells of concentrations

from 1 to $2x10^6$ cells per ml to various broths and homogenates of fish, Sardines, Mackerels, shellfishes, prawns and crabs. Artificially inoculated samples were frozen to -20°C and thawed immediately after freezing. One ml of thawed samples was transferred asceptically after freezing to 9ml of the respective broths for dilution. Similarly thawed homogenates were prepared by carefully tranferred 10g of samples to 90 ml of sterile triptic soy extract broths. Care was taken to avoid the external contamination. The diluted broths of minimal broth, nutrient broth, Triptic soy and Tripticase yeast extract both were diluted and homogenates of fish and shellfishes were incubated at room temperature (30°C) for 3 hours.

The enumeration of injured cells were carried out as per the method of Ray *et al.*, (1972), with slight modifications, by using selective plating medium of Xylose Lysine Desoxycholate agar, (XLD) and nonselective plating medium Xloss Lysine Yeast extract agar, (XLY). Both the plating media were prepared as follows.

Yeast extract	3 g
L-Lysine hydrochloride ...	5 g.
Xylose	3.75 g

Lactose	7.50 g.
Sucrose	7.50 g.
Sodium desoxycholate	2.50 g.
Ferric ammonium citrate ...	0.80 g.
Sodium thiosulphate	6.80 g.
Sodium chloride	5.00 g.
Agar	15.00 g.
Phenol red	0.08 g.
Distilled water	1000 cc.

PH .... 6.9± 0.2.

The above ingredients were dissolved completely by heating and care was taken not to over heat the medium. Over heating may cause precipitation of the ingredients, but the medium reaction was satisfactory. Sometimes over heating made the Salmonella colonies appear slightly smaller in size. The sterilized medium was poured into petridishes and the surfaces were dried in the incubator at temperature of 50°C for 30 min.

The nonselective medium was made by excluding desoxycholate from the Xylose Lysine Desoxycholate agar medium and the plates were prepared and dried in an incubator at 50°C

for 30 min. The unused plates were stored at 4°C in the refrigerator for not more than one week.

Frozen samples which were thawed by the method described above and decimally diluted in peptone water (0.1%) and plated with 0.1 ml portion in triplicate in the nonselective medium of Xylose Lysine agar and Xylose Lysine desoxycholate agar in the seletive plating medium.

The extent of repair of injury and the initiation of growth after freezing and thawing were evaluated for the various serotypes of Salmonella after freezing and thawing were evaluated for the various serotypes of Salmonella after 0, 30, 60, 90, 120 and 180 minutes, of incubation at room temperature (30°C)

The freezing injury and repair by different components of nuecleotides bases such as adenine (6-amino purine) guanine (2-amino- 6-oxypurine) and uracil (2-4 dioxy-pyrimidine), amino acids and vitamins (growth promoting nutrients) after injury was studied. The ability of the individual organic compounds to aid the repair by adding each nutrients with and without in specially made organic media of broths were tested. The following composition of

media were prepared with and without amino acids, (1), vitamins, (2), and nuecleotides bases (3), so that one component is removed in the medium.

(1) Amino acids. Alanine 100 mg; Arginine, 200 mg; Asparagin 5 mg; Aspartic acid 100 mg; Cystine, 200 mg; Glutamic acid 300 mg; Glycine 200 mg; Histidine 200 mg; Hydroxy proline 200 mg; Isoleucine 200mg; Leucine 100 mg; Lysine 100 mg; Methionine 100 mg; Phenyle alanine 100 mg; Proline 200 mg; Serine 100 mg; Threonine 100 mg; Tryptophane 200 mg; Tyrosine 200 mg; Valine 100 mg;

(2) Vitaminss Biotin 0.5 mg; Calcium pantathenate 0.4 mg; Folic acid 0.4 mg; Nicotinic acids 1.0 mg; Para amino benzoic acid 0.04 mg; Pyridoxine 0.4mg Riboflavin 0.2 mg; Thiamin 0.2 mg;

(3) Nucleotides bases. Adenine 30 mg; Guanine 30 mg; Uracil 30 mg;

The following buffer and trace elements were common to the above media. Potassium dihydrogen phosphate 0.44 g; Dipotassium hydrogen phosphate 0.31 g; Ferrous sulphate 10 mg; $MgSO_4 7H_2O$ 200 mg; $MnSO_4 7H_2O$ 200 mg; NaCL 10 mg; and glucose 20 mg; Distilled water 1 litre and final pH was adjusted to 6.9 ± 0.2.

Frozen and thawed samples of minimal broth and tripticase soy broths were centrifuged at

3000 rpm immediately after thawing at a lower temperature (air conditioned room) and the liquid portions of tripticase soy and minimal broths were removed asceptically. To this added the prepared synthetic broths with and without amino acids, vitamins and nucleitides bases. To study the repair of injury one ml portion was inoculated into two 9ml of prepared synthetic broths (with and without 1, 2 and 3) as mentioned above. For a control one ml was inoculated in sterile saline (9 ml) water. These suspensions were incubated at room temperature (30°C) for three hours. Samples were drawn at a definte intervals, decimally diluted and from these 0.5 ml surface plated in triplicate on Xylose Lysine phenolred agar and Xylose Lysine phenolred desoxycholate agar. The streaked plated were incubated at 37°C for 24 hours and the developed colonies were counted. Further incubation was found unnecessary since the colony counts did not increase significantly.

The death rate and percentage of injury were calculated as follows:

$$\text{Percentage of injury} = - \frac{\text{Counts on XLD}}{\text{Counts on XLP}} \times$$

$$\text{Percentage of death} = 1 - \frac{\text{counts on XL treated (freezing sample)}}{\text{counts on XL treated sample}} \times 100$$

The amount of death was determined from the differences in colony forming cells of Salmonella strains on noninhibitory medium of Xylose Lysine phenolred agar before and after freezing. The amount of injury was estimated from the difference in colony forming cells in Xylose Lysine Desoxycholate agar at any particular sampling. The dilution was selected for plating so that approximately 100 to 300 viable cells of Salmonella were isolated in each plate before and after freezing in nonselective medium of Xylose Lysine phenolred agar and selective plating medium of Xylose Lysine Desoxycholate agar.

4.3. RESULTS.

Tables 26 to 29 and fig. 4. show the effect of refrigeration and further storage on the survival of viable serotypes of Salmonella

On a cursory glance, as expected, considerable reduction was found both in broths and homogenates during refrigeration. The reduction of counts were only one log cycle after immediate refrigeration. Further during storage for 12 days, the reduction was about 3 to 4 log cycles in the case of broths. But, the homogenates showed lesser reduction of one to two log cycles. In the homgenates, the serotypes of Salmonella showed little variations. The data show that the survival of Salmonella which were taken for investigation were more in the homogenates than in the broths.

On comparing the survival of Salmonella sero types in fish homogenates, S. typhimurium, S. weltevreden, S. enteritidis, S. roan, S. saintpaul, and S. heidelberg showed higher survival than the other serotypes. During refrigerated storage of 12 days the counts varied from 10^5 to 10^5 per gram. On comparing the data of broths, all the serotypes, taken for the study of this investigation exhibited the similar stress and lower viability. In this case reduction was from 10^5 to 10^2 per gram. Initially in broths after one day refrigeration and storage, the counts was reduced sharply and on subsequent storage, the reduction was slower and reduced to 10^2 per gram after

12 days of storage. However, in the homogenates, the results indicated higher counts of 10^4 per mk gram than that of broths. The data on the reduction of counts were slower after the 4th day onwards during storageat 3°-6°C. A similar trend was also seen in broths.

Figures 5 to 7 and table 30 explain the effect of freezing and low temperature of storage to about three log cycles and further slowed down in homogenates, but in the broths the reduction was at a higher rate and they were almost destroyed in broths. Even after 100 days of storage no Salmonella was isolated in broths of tripticase soy , nutrient and brain heart infusion broths.

So S. typhimurium, S. anatum, S. senftenberg indicated slight reduction during the first 90 days of storage in homogenates. Most of the Salmonella serotypes tested in this investigation showed profound effects in brothsand some what irregular effect in the homogenates stored during that peroid. Broths, especially nutrient peptone, showed a marked decline of S. cubana, S. weltevreden, S. typhi, and S. heidelberg during two months and there after

showed a slow decline in the number. After four months the isolation was not possible because of the stress.

After 150 days of storage the viable numbers of Salmonella reduced to a minimum level at which it was almost impossible to estimate quantitatively in the homogenates also. Further its isolation in selective medium was found difficult because of the stressed condition. In order to evaluate this type of organism only non ihibitory medium such as Brain Heart infusion broth or Tripticase soy broth was used to detect qualitatively and found the presence of Salmonella up to six months. Only S. typhimurium, S. anatum, S. weltevreden were isolated up tp six months. The rest of the serotypes were not detected after six months of storage in the homogenates.

During multiple freezing and thawing (table 31), the viable count of Salmonella strains showed a steady d reduction up to 120 days in the homogenates. Larger reductions i on the viable counts were found in the Brain Heart infusion and Nutrient broths, in the case of Tripticase soy broth a higher survival was found. Since the serotypes of Salmonella behaved similarly in the present investigation, a few examples were taken to elucidate the survival in the broths. S. anatum, S. heidelberg, S. enteritidis, S. roan and S. typhimurium survived more in Tripticase soy broth than in nutrient and

and Brain heart infusion broths. In broths these cultures survived up to 120 days, but the quantitaitve isolation could not be carried out after 90 days of storage.

The rate of injury and daeth during freezing is given in tables 32 and 33 and figures 10 to 12. On comparing the four broths, the minimal broth showed higher percentage of death (30 to 40%) than the Tripticase soy yeast extract, nutrient broth, and Brain heart infusion broth. Among the three broths t of tripticase, soy extract, nutrient broth, and Brain heart infusion broth, the results indicated more or less the same death percentage. The percentage of death of Salmonella strains varied from 25 to 35%. A similar pattern was also seen in the case of injury of cells of various Salmonella strains in broths and homogenates of fish and shell fishes. In broths most of the celàs (90 to98%) were injured while homogenates showed only 50 to 66%. Among homogenates, the same strain indicated varied injury from 50 to 60%. From the data it is found that the strains varied with different substrates of fish and shellfishes. But however,

the injured cells recovered when placed in noninhibitory medium.

The ability of injured cells to repair and grow by the suspending various injured Salmonella strains from the broths to various noninhibitory media of Tripticase soy broth, nutrient broth, Brain heart infusion broth and Minimal broth. Similarly the injured cells of Salmonella organisms from the homogenates were treated with Tripticase soy yeast extract broth. During incubation at room temperature (30°C) the samples were plated on Xylose Lysine phenolred agar and Xylose Lysine Desoxycholate agar at different time intervals. For a control, unfrozen cells were tested in the same manner. It was found that uninjured cells in various broths showed the same counts in Xylose Lysine phenolred agar and Xylose Lysine desoxycholate agar and they multiplied after two hours The frozen cells of Salmonella in various broths and homogenates as indicated previously, showed 50 to 66% reduction of lower counts, on Xylose Lysine Desoxycholate xxxxx agar. However, the counts of Salmonella in the above medium increased further rapidly up to 2 hours and there after the growth slowed down to 3 hours of duration. The control also remained the same during

the period. The increase of counts in Xylose Lysine Desoxycholate agar was attributed to the repair of injured cells of various species of Salmonella in tripticase soy yeast extract broth, minimal broth Brain heart infusion and Nutrient broth.

In comparing the repair of various Salmonella strains in different broths, Tripticase soy yeast extract broth, nutrient broth, Brain heart infusion broth, higher counts of repair for the injured cells were observed in Xylose Lysine Desoxycholate agar than in minimal broth. Because of the higher counts of repair on injured cells by the different broths except minimal broth, the multiplication was proceeded after three hours, while minimal broth showed growth after 3.5 hours.

The data presented showed that the rate of repair from freezing injury repaired rapidly for all the strains tested under this investigation, except S. newport, S.cubana and S. enteritidis. The injury recovery was rapid initially for about one hour and slowed down two hours. The counts on noninhibitory medium of Xylose Lysine phenolred agar in all the suspended media remained closely the same during the two hours test period.

The results of freeze injury repair by different components such as vitamins, amino

acids and nucleosides bases were shown in tables 34 and fig.8and 9. The ability of the individual components to aid the repair was studied in specially made organic media. The table 34 indicated that all components a of amino acids, vitamins,and nucleosides bases of adenine, Guanine and Uracil favour the rapid repairprocess of injured cells. Individual components of amino acids, or vitamins, or nucleosides bases do not significantly increase the repair process. In the repairing process, the above ingredients gave similar results in the other broths used in this study.

When cpmparing the extend of repair from the total amount of injured cells it was foud found that Tripticase soy broth, Brain heart infusion broth and nutrient broth were able to repair 90% of injured cells while minimal broth exhibited only70% after two hours of incubation.

4.4. DISCUSSION.

It was observed from the study that the test organisms of Salmonella survived even after prolonged storage at low temperatures. Sea foods protect these organisms against the lethal

effects of freezing (Liston and Raj 1961,a,b). Survival of Salmonella in marine products in this study strenthen this conclusion and well agree, with the findings of other workers concerning the survival of both psychrophillic and mesophillic microorganisms(Gunderson and Rose 1948; Kerelek and Gunderson 1959; Proctor and Phillip 1949; Larken et al., 1955). Salmonella can survive and grow at low temperatures and this was discussed in the review of Michner et al., (1964). Angelloti et al.(1961), observed that the minimum growth temperature for Salmonella in several foods was between 4.4°C and 10°C. Matches and Liston (1968) found that the growth of Salmonella in perishable foods was prevented at a temperature of 5.6°C. This was previously confirmed by the observation of Prescot et al., 1936 and 1938). Earlier, it was thought that at 5°C or less was required to prevent the growth of Salmonella in refrigerated foods. However Match and Liston (1968) reported from pure culture studies that at low temperature of 5.6°C, Salmonella was able to increase rapidly in numbers.. Further, on the practical point of view, it is of paramount importance to recognise that growth of Salmonella may occur at low temperatures below 6.0°C after relatively long period of time.

The pure culture studies of this investigation yielded basic information on the general sensitivity of these organisms (Salmonella) to low temperature. The protection or survival in homogenates were more profoundly seem than in liquid broths. In liquid broths, that Salmonella was virtually eliminated after 100 days at -20°C. The survival was more in the case of homogenates and this extent of protection might be due to the different substrates of proteins, amino acids, fats, and nucleotides and temperature of freezing and stroage time. Earlier studies of Keith (1913), Haines (1937), Weiser and Osterud (1945) reported the behaviour of pathogens in low temperatures. Different authors (Borgstrom 1955, Gunderson and Ross 1948; Procter and Phillip 1948; Squires and Hurtsell 1955; Woodburn and Strong 1960; Kerelek et al., 1961) confirmed the variability of conditions and effects of foods on survival of these pathogenic organisms. Raj and Liston (1971) studied the effects of survival of microorganisms in seafoods and reported that S. typhimurium survived up to 393 days. However, results of this present investigation could not isolate the various serotypes of Salmonella beyond 240 days in fish and shellfish substrates.

Freezing different homogenates to low temperature of -20°C showed a reduction of 65 to 70% Salmonella orgagnisms. Digirilamo et al., (1970) reported that the Pacific oysters, S. derby and S. typhimurium were highly sensitive to freezing at -18°C. They found that at the end of one week's storage, the the number of Salmonella in oysters was reduced as much as 99%from the original level present in the frozen oysters.

This finding is of public health significance since it clearly indicates that the determination of the low level of Salmonella organisms in the frozen block of fish, prawns and crabs was difficult. After a long storage of 280 days and beyond, in low temperature at -20°C it is rather difficult to estimate quantitatively the low level of serotypes of Salmonella in the whole fish, frozen prawn blocks etc. Larger samples were used to detect Salmonella qualitatively in the frozen samples.

The survival of various serotypes of Salmonella in liquid broths waslower than in the homogenates f of fish and shellfish. The results indicated that the loss of viability in liquid broths

for the various Salmonella serotypes strains was higher than in the homogenates of fish and shellfishes. However, the general trend shown by these experiments was loss of viability and reduction in the number of Salmonella during the entire period of storage at -20°C.

In these studies the Salmonella counts showed regular decline and tended to drop to nil at the end. A similar observation was found by Raj and Liston (1971). Shiflett et al., reported that the loss of viability of Salmonella in blended oysters held at 6.7°C was in three stages; a rapid reduction initially a gradual reduction and a final stage of rapid reduction. In the present study, refrigeration storage temperature of 3-6°C showed only two stages; a rapid reduction at the initial followed by a continuous gradual decline.

Multiple cycle freezing and thawing have been reported to be much more determintal to microorganisms than a single freezing and storage cycle (Borgstrom 1955). Earliar Hilliard and Davis (1988) demonstrated complete elimination of Typhoid bacilli and Serratia marcescens by freezing and thawing four or five times respectively and further

showed similar effects of multiple freezing and thawing on Escherichia coli and to a lesser extent on Bacillus subtilis.

The results of the present d study on broths of Brain heart infusion, Tripticase soy extract and nutrient broths confirmed this observation with test organisms of S. heidelberg, S. weltevreden, S. arizona, S. newport, S. enteritidis, S. saintpaul, S. sainpaul, S. anatum, S. roan, S. cubana, S. richmond and Salmonella strain (unclassified). However, there was profound difference on the survival of these organisms in fish and shellfish homogenates. Multiple freezing and thawing, three or four times extended the protective effect(table 33), as S. nchanga, S. salford, S. chester, S. bredney, and S. newport were more sensitive than other Salmonella species.

The protection mechanism afforded by the seafoods to the sensitive bacterial cells of Salmonella was not understood clearly. Many posible explanations were given and conjectured about the protection. Raj and Liston (1971) explained that fish and shellfish protein molecule which become true colloidcan be expected to interfere with the rate of crstallisation of water from the bacterial cell. The

denaturation of bacterial cell protein is prevented. The extent of protection under such a system would depend on the larger extent of the denaturation of fish protein and thus its ability to hold bound water to maintain the equillibrium. When multiple freezing and thawing process occured, considerable denaturation in fish and shellfish tissue protien might be expected and furhher reduction of their water binding capacity reduces the effectiveness as protecting agents for microorganisms against freezing damage. This largely explains the lethel effect of multiple freezing process on the microorganisms.

The survival of Salmonella of various serotypes in multiple freezing process indicated the health hasard after long storage in frozen state, where the low numbers of Salmonella were found viable. It would not be wise to assumethat freezing per se can provide a safety margin against the effect of unsanitory conditions. Freezing and subsequent thawing of various Salmonella serotypes resulted in death and injury. This has been demonstrated by various scientific workers. (Sinskey and Silverman 1970; Sorrels et al., 1970; Ray et al., 1971 and 1972). A good portion of injured cells were repaired when proper environmental conditions

favoured them.

Repair of freeze injured cells of Salmonella sertypes from broths and homogenates was manifested by supplying organic and growth promoting nutrients in the media so that the stress was removed at elevated temperature for growth. In general, the possible effects of cold shock on microorganism may be of two types; a lethal effect of sublethal effect which may be reversible or irreversible. Lethal effects and irreversible sublethal damage may yeild the same result so far as viable counts are concerned. Recovery of ogganisms subjected to reversible sublethal damage, may depend upon the cultivation procedures used (Straka and Stokes 1959, Kohn, 1960). Gunderson and Lee (1948) and Hartsell (1951), reported that pathogens will be inhibited on several selective media and showed that organic complex nutrients, like amino acids, vitamins and nucleotides were required.

In the present study, defrosted cell counts of S. anatum, S. enteritidis, S. roan, S. arizona, S. typhimurium, S. heidelberg and S. senften berg showed lower counts on differential selective media of Brilliant green agar, Xylose Lysine Desoxycholate agar, Hektoen enteric agar, when counts on nonselective medium of Tripticase soy yeast extract agar were com-

Pared. Similar observations were also made by Ray et al. (1971 and 1972), Scheusner et al., (1971) and Hagen (1971). Since it has considerable practical importance for the evaluation of pathogenic organisms in frozen fo foods, the normal procedure could yield misleading result reagarding the number of viable cells of pathogenic bacteria present.

The nature of injury produced by freezing on the Salmonella serotype cells were studied. The repair and recovery mechanism appeared to be the restoration or the requirements of nutrition Ray et al., (1971) explained the common characteristic of the injured cells that required energy synthesis. The serotypes of Salmonella under this study showed injury if freezing and thawing. In broths of nutrient, Brain heart infusion, Tripticase soy agar, 70% were injured. The cells which lost their viability to recover or multiply in nutritionally rich medium such as Tripticase soy yeast extract agar was considered as good. Ray et al., (1972) reported the survival of Salmonella anatum, and its repair of injury in various organic media and in inorganic nutrients during freezing and thawing. 90% of the Salmonellaserotypes were repaired in tripticase soy yeast extract broth, Brain heart infusion broth, Nutrient broths and

compounded broth with adenine, guanine, uracil, amino-acids, and vitamins. Regeneration of ribonucleic acid (RNA) and synthesis of protein (amino acids and peptides) were essential for the repair of the cells (Moss and Spec 1966; Mukerjee and Bhattacharjee 1970; Pierson et al., 1971; Tomlin and Ordal 1971).

One of the manifestation of freezing anf thawing of Salmonella cells, is the impaired permiability (Ray et al., 1972). The ruptured cell allows leakage of cellular components such as protiens, peptides, amino acids and nucleic acids and the entrance of various ions from the environments into the cells (Brata and Kocka 1967; MacLeod et al., 1966; Strange and Postgate 1964; Moss and Speck 1966; Morichi 1969). This causes cell injury and subsequently death occurs in continuous frozen storage. It appears that the damage caused by freezing and thawing (injured cells) is to a certain extent reversible and it can be repaired in the presence of nutrients and nucleotides and phosphates.

Vibrio parahaemolyticus

4.5. PREPARATION OF SAMPLES AND MEDIA FOR VIBRIO PARAHAEMOLYTICUS.

Vibrio parahaemolyticus isolated

from various seafoods such as fresh fishes, shellfishes two strains obtained from the Torry Research Station, Aberdeen, and from Dr. M. Fishbein, Division of Microbiology, Food and Drug Administration, Washington, D.C.satrain No. 1902; and strain No. 3525 respectively, are both kanagava negative. The cultures were maintained in Tripticase soy agar containing 3% sodium chloride.

Homogenates from fresh fishes, Mackereks, Pearlspot, and shellfishes, Prawns (Penaeus indicus) and Scylla serrata (crab) were prepared as described under materials and methods(section 4.2.2.) Fresh Vibrio parahaemolyticus cultures were grown in Triptic soy yeast extract broth with3%% NaCl at room temperature ($30^{o}C$) for 18 to 20 hours and used as inoculum. A known concentration of cellsof $2x10^{6}$ per gram was inoculated in respective homogenates. These samples were stored at 3 to $6^{o}C$ of refrigeration temperature for up to 12 days. The recommended maximum storage period for these type of processed seafoods at this temperature of refrigeration is 10 to 14 days. A similar set of samples described above were prepared and stored at $-20^{o}C$ (frozen temperature), the survival of Vibrio parahaemolyticus was evaluated . All sapmples were kept in sterile plastic bags and sealed well and furhter used in this study.

The inoculated initial viable count of Vibrio parahaemolyticus was determined as control by decimally diluted it in (0.1%) sterile peptone water containing 3% sodium chloride and pH 7.0. The serial dilutions were plated by using direct streak plate technique of taking 0.5 ml quanitities of apprpriate dilutions spread on plates of Modified Twedt starch agar (MT agar) and incubated for 24 hours at 30°C. Farther sampling of refrigerated, stored, and frozen stored materials were carried out as follows: MPN (Most probable Number) counts and direct plate and counts were taken after 1, 2, 3, 5, 7, 10 and 12 days and frozen stored were taken after 1, 5, 10, 20, 30, 45 and 60 days. 20 gram of samples of different homogenates were blended with 450 ml of sterile peptone (0.1%) water with 3% sodium chloride as a dilution medium. Further serially dibuted samples were used for the inoculation for MPN and the streak plate method. all the ~~xxxxxx~~ serial dilutions were used with sterile peptone water containing 3% NaCl and the samples were brought to the room temperature and enumerated. frozen samples were thawed in a minimum time of 30 minutes.

After incubatimn of 24 hours at 37°C , all Vibrio parahaemolyticus-like colonies(VPL)

on Thiosulphate citrate bile salt agar (TCBS) (Fishbein and Wentz 1973) and MT agar (Vandersant and Nickelson 1972) were counted. So the counts taken represented the high inoculated Vibrio parahaemolyticus and the flora containing similar colonial morphology. Suspected VPL colonies were compared with stock cultures and confirmed biochemically.

4.5.1 The effects of multiple cyclic defrosting.

Samples of homogenates were labelled specifically for this purpose, removed from the cold storage and thawed. After thawed sampling were withdrawn for testing, the samples were again put back in the freezer at a temperature -20°C. Comparisons were made between the effects of freezing followed by storage and the effects of multiple cyclic defrosting. The samples were tested after 10, 20, 30, 45, and 60 days by the MPN method. All the frozen samples preparations were thawed in a water bath at 30°C for 10minutes. The homogenates were temperedto room temperature for two to three minutes and during that time serial dilutions were made and inoculated in platings and tubes for MPN procedure. Plates and tube culture counting were already described above (vide supra).

4.5.2. Freezing and thawing effects of Vibrio parahaemolyticus on injury and repair.

Homogenates of fish and shellfish were frozen at -20°C and examined for their injury. The frozen samples were thawed and sampled for the enumeration of injured Vibrio parahaemolyticus by decimally diluted with sterile peptone water containing 3% NaCl and surface plated on Tripticase soy agar with 3% NaCl and on TCBS. The plates were incubated at 37°C for 24 hours and the developed colonies were counted. In this procedure thawed homogenates were immediately transferred to Tripticase soy broth containing %3% salt and on TCBS also. Death and injury of Vibrio parahaemolyticus due to freezing and thawing were estimated from the difference in colony counts (repaired) on a nonselective medium (TSA with 3% salt) and selective medium of TCBS. The percentage of death and injury were calculated as follows:

Percentage of death cells:

$$1- \frac{\text{TSA with salt counts after freezing}}{\text{TSA with salt counts before freezing}} \times 100$$

Percentage of injured cells:

$$1- \frac{\text{TCBS counts after freezing}}{\text{Corresponding TSA with salt medium counts}} \times 100$$

Subsequent growth after freezing and thawing of Vibrio parahaemolyticus cells in their respective homogenates suspended in Tripticase soy broth with %3% salt were determined. Immediately after freezing anf thawing, the thawed samples were e enumerated at intervals of 30 minutes during incubation at 37^0C. The growth of Vibrio parahaemolyticus cells were enumerated by plating Tripticase soy agar with 3% salt and TCBS. Reduction in the percentage of injury during incubation in TS broth of fish and shell fish homogenates were considered to be repair of Vibrio parahaemolyticus cells after freeze injury.

4.6. RESULTS.

The cell populations were enumerated immediately after inoculation in the fish and shellfish homogenates. The results (table 35, fig. 10) indicated that Vibrio parahaemolyticus gradually decline in numbers in the refrigerated storage. After a week of storage the results of enumeration of this organisms were inconsistant. The strain No. 246 which was isolated from fresh prawns of Metapenaeus affinis showed survival for up to 10 days in Mackerel homogenates, while in prawn of P. indicus and of crab

(Scylla serrata) homogenates, a higher level of survival up to 14 days. Low numbers of this organisms ($1x10^2$) in various homogenates recorded a survival maximum up to 13 days of storage. Relatively some Vibrio parahaemolyticus strains such as No. 178, 327 and 341 lost their viability after the 5th day of storage. Most of the Vibrio parahaemolyticus strains were well more than 2 log cycles, within a 48 hour period. The rate of decline in refrigerated storage temperature (3-6°C) in fish homogenates was the same, but in prawn and crab homogenates, the decline was comparatively slowerand extended upto 13 days. The MPN counts showed regular decrease upto 12 days and after that, the results were inconsistant.

The results (table 35) showed that Vibrio parahaemolyticus inoculated in larger numbers in various homogenates, of fish and shellfishes declined gradually after 5 days of storage. The initial level of Vibrio parahaemolyticus in this experiment was taken in the range of $1x10^6$ and during refrigerated storage. The strain No.246 showed survival maximum upto 16 days in shellfish homogenates. All the strain tested under this investigation of refrigerated storage of Vibrio parahaemolyticus survived upto a maximum of

of 14 days and low numbers survived upto seven days only.

During frozen storage at -20°C the decline of these organism was rapid upto five days (table 36) and there after slowed down upto ten days and further declined rapidly. With slow concentration of cells of the Vibrio parahaemolyticus survived only for a month and the extension of survival depends on the initial concentration during frozen storage. All counts in most cases, declined more than 3 log cycles within 24 hours, in the case of higher numbers of cells. The rate of decline was almost similar to the refrigerated storgge and were found to be rapid after 10days. After 40 days of frozen storage the strain tested under this study lost their viability. Even the strain No. 246 which showed maximum survival in refrigeration and storage declined in homgenates of fish and shellfishes similar to other Vibrio parahaemolyticus strains.

During Cyclic defrosting showed lesser survival (table 37) when compared with frozen storage. The viability of Vibrio parahaemolyticus was lost rapidly within 30 days during cyclic freezig and thawing. The result indicat that the cyclic

freezing and thawing had profound effects on the survival i.e., the viability was lost quicker than during refrigeration and freezing. Actually frozen samples showed ten days more survival than the respective cyclic defrosted samples. The reduction of log counts was from the initial value of six to one within 20 days. Since the decline was faster in larger sample sizes, these were used for the quantitative recovery of Vibrio parahaemolyticus. No viable cells were isolated after storage of 30 days and more, even by using enrichment procedure of nonselective medium of Tripticase soy broth with 7% NaCl and incubate them for 48 hours.

Repair of injury and subsequent growth of frozen cells of these organisms were studied by suspending and incubating the cells in Tripticase soy broth with 3% NaCl, A nonselective medium and Thiosulphate citrate bile salt medium, a selective medium. Representative ~~example~~ data showing the rate of death of cells in frozen homogenates indicated that all death as found in the reduction of Tripticase soy broth with 3% salt medium counts occured at a very rapid rate at initial thawing. The difference in counts in two platings (initial and after freezing and thawing) revealed the nonviability of the cells. The comparison of various homogenates show that the death of Vibrio

parahaemolyticus cells ranged from 61 to 75%. Lesser death was found in prawn and crab homogenates. The data (table 38) revealed that the freezing had comparatively greater injury and death (lethal effect) than the refrigeration. The amounts of injured cells among the survivors were represented (table 38 and fig. 11and 12) by the difference in counts between the two platings of non-selective medium of TSA+salt and selective plating medium of TCBS after thawing the frozen samples. In this study the amount of injured cells might be very high 90% or more or the survivers were injured.

Initial cell recovery and repair and initiation of growth of frozen Vibrio parahaemolyticus were studied by growing them in Tripticase soy broth with 3% salt. The representative results of the repair of injury and subsequent multiplication of sublethally stressed cells indicated that a higher number of cells were repaired in homogenates. The detection of injured Vibrio parahaemolyticus by suspending them in TSB+ S was evaluated. It was found that the repairing process was almost completed in two hours of incubation at 37°C.

The results showed that the low population of injured cells (sublethally stressed)

of Vibrio parahaemolyticus can multiply and grow faste after two hours in TSB + salt (3%) concentration.

4.7. DISCUSSION.

From the present study the Vibrio parahaemolyticus cells in marine seafoods were found to be very sensitive to refrigeration temperature of 3-6°C and frozen temperature of -20°C and further storage. Similar results were reported by various authors (Baross and Liston 1970; Matches et al., 1971; Covert et al., 1972; Vandersant and Nickelson 1972; Johnson et al., 1973; Kaneko and Colwell 1973; Bradshaw et al.,1974; Beuchat 1975; Van den Broek and Mossel 1977; Ray et al., 1978). Under both storage conditions (refrigeration and frozen), eventhough large proportions of Vibrio parahaemolyticus cells were killed still the remainder of the cells may grow and cause foodpoisoning, if proper environmental conditions favoured (Liston, 1974). Refrigeration and freezing produced sublethal injury to Vibrio parahaemolyticus cells. The results indicated that 90% or more or the survivars were injured depending upon the low temperatu Due to different kinds of sublethal stress the Vibrio parahaemolyticus cells at low temperatures were sub-

lethally injured and many workers reported this phenomenon (Goatcher et al., 1974; Liston, 1974; Beuchat, 1975; 1977; Ray et al., 1978; Van den Broek et al., 1977).

Relatively low temperatures inoculum level $1.0x10^2$ per gram was used because this range of low numbers usually recovered from various fresh fishes and shellfishes samples. A similar observation was also made in slightly higher numbers usually recovered ($1.0x10^4$) by Baross and Liston (1970). As shown by this study, low numbers of Vibrio parahaemolyticus on fish homogenates and shellfish homogenates were reduced to very low numbers within 48 hours in frozen and within 96 hours in the refrigerated storage. In the case of high numbers of Vibrio parahaemolyticus inoculated samples in refrigerated (3-6°C) as well as frozen samples (-20°C) were reduced to 2 log cycles and 3 log cycles respectively, within 24 hours. Colwert and Woodborne (1972) found that in fish homogenates cells of Vibrio parahaemolyticus tended to stabilise at temperature from -5°C and -18°C. Food poisoning was due to the consumption of raw oysters and fishes. To date there is no confirmed information on the outbreak of Vibrio parahaemolyticus food poisoning in Cochin and neibouring places attributed to the

consumption of fresh fishes and shell fishes. Various authors (Bartley and Slanetz 1971; Thompson and Thatcher 1972) reported that foodpoisonings occured by the consumption of low numbers of Vibrio parahaemolyticus in foods. Since the generation is quicker (Katah, 1965), the possibility of multiplying in large numbers under favourable conditions cannot be dismissed.

In comparing the cells of Vibrio parahaemolyticus in different fish substrates at refrigerated temperature, it was found that the initial reduction in cell numbers was higher up to 48 hours and further reduction was almost linear upto 6 days. The log reduction at -20°C of the strains tested under different substrates showed that the reduction of viable cells was three log cycles. It was found that viability of Vibrio parahaemolyticus upto 40 days. But Matches et al., (1971) found that the survival of these cells in fish homogenates was longer at -18°C. By using different experimental menstrua and different recovery methodology the various investigators obtained analogous results. Vandersant and Nickelson (1972) observed a reduction of 2 logs during the first two days at different storage

temperatures (3°C, 7°C, 10°C) in whole shrimp. Bradshaw et al., (1974), reported in shrimp homogenates an initial slight increase for over 12 hours at all temperatures and then a gradual decline. Johnson and Liston (1973) demonstrated a slow decline in numbers of viable Vibrio parahaemolyticus after 2 to 5 days of storage at 11°C in depurated oysters. Thomson and Thacker (1973) observed the multiplication of these cells inoculated artificially in low numbers ($5x10^3$) in oysters at 10°C and above. Johnson et al., (1973), reported no apparent change of viable cells of Vibrio parahaemolyticus in naturally contaminated oyster shellstock stored at 4°C.

The results of this present study indicate that multiple freezing and thawing has more determintal effect on Vibrio parahaemolyticus The viability was last within 20 days and in some cases extended upto 30 days. The surviving cells are lower than frozen stored cells. Several workers (Borgstrom 1955; Hiliar and Davis 1918) reported that multiple freezing and thawing has a killing effect to microorganisms. Hilliard et al., (1915) showed that four or five times of freezing and thawing completely eliminate Salmonella typhi (typhoid bacilli) and Serratia marcescens (Bacillus prodigious). In this study the results of

freezing and thawing are in aggreement with these findings of the above workers. However there was difference on the survival of Vibrio parahaemolyticus in the homogenates which gave better protection to cells and no proper explanation so far to explain the mechanism of protection exerted by seafoods to the sensitive Vibrio parahaemolyticus. Raj and Liston (1971) conjectured that the protection is due to the fish protein molecule which act as a true colloid and interfered with a rate of crystallisation of water from the bacterial cell and the denaturation of bacterial protein might be prevented. This type of protection would depend upon to a larger extent on the degree of denaturation of fish protein and also its ability to hold bound water to maintain the equillibrium. The multiple freezing and thawing produce considerable damage to the fish tissue protein. This may be reduce the water binding capacity and there by obstruct the effectiveness of protecting the microorganisms from freezing damage.

Three kinds of cells were noted in Vibrio parahaemolyticus after freezing and thawing of fish and shellfish: dead, injured, and uninjured cells. The cells which lost their ability to multiply in a nutritionally rich medium such as Tripticase soy

yeast extract agar, were considered dead. Among the survivers of freezing and thawing, injured cells failed to grow or form colonies on a selective medium of Thiosulphate citrate bile salt agar, but usually form colonies on nonselective medium, such as Tripticase soy extract agar with 3% salt. The growth on nonselective medium was considered as the repaired injured cells. Many workers (Ray et al., 1972; Ray and Speck 1972) studied the effect of the repair of the injured cells in different media and its further isolation in nonselective medium and selective medium. For the isolation of the repaired Vibrio parahaemolyticus in this investigation the methodology recommended by the U.S.F.D.A. was followed. It was found that the lower population of injured cells (probably to be repaired) of Vibrio parahaemolyticus can multiply and reach their maximum population level within 12 hours of growth. By suspending the injured cells in Tripticase soy Yeast extract broth with 3% salt concentration, the injured cells were repaired with in 2 to 3 hours. Several authors (Beuchat 1977; Emswiller et al., 1976; Goatcher et al., 1974; Gray 1977; Ray etc al., 1978) observed while studying the effect of salt concentration on the repair of the injured cells that those which were sublethally injured were sensitive to high salt

concentration. Because of this possibility of sensitivity, the salt concentration was used 3%.

The present study showed that the injures cells of Vibrio parahaemolyticus can repair and multiply if the environmental conditions are favourable for growth. This finding is of public health significance. Survival of pathogenic organisms in seafoods may produce a public health hazard e en after specific peroid offrozen stroage.

Table 26. Effect of refrigerated temperature 3-6°C and storage of the Salmonella strains suspended in nutrient broth. (counts per ml.)

Organisms	Initial counts	One day	Two days	Four days	Six days	Eight days	Ten days	Twelve days
S. typhimurium	2.9×10^5	3.9×10^4	6.8×10^4	5.9×10^3	6.7×10^2	5.4×10^2	4.8×10^2	4.9×10^2
S. anatum	5.1×10^5	3.1×10^4	7.9×10^3	7.7×10^3	6.8×10^2	6.9×10^2	5.9×10^2	6.1×10^2
S. heidelberg	6.7×10^5	4.7×10^3	5.0×10^3	8.6×10^2	6.1×10^2	5.1×10^2	5.8×10^2	5.4×10^2
S. senftenberg	4.1×10^5	6.4×10^4	5.8×10^4	6.1×10^3	6.9×10^2	6.8×10^2	6.1×10^2	5.1×10^2
S. weltevreden	2.9×10^5	3.1×10^4	3.0×10^4	8.2×10^3	7.1×10^3	6.7×10^2	6.1×10^2	6.8×10^2
S. enteritidis	7.7×10^5	3.6×10^4	2.7×10^4	7.3×10^3	7.5×10^3	5.2×10^3	8.9×10^2	8.1×10^2
S. paratyphi A	1.7×10^5	2.9×10^5	2.6×10^4	6.9×10^3	7.1×10^3	7.8×10^2	6.2×10^2	6.4×10^2
S. newport	7.1×10^6	4.7×10^5	2.4×10^4	6.7×10^4	6.3×10^3	6.7×10^2	5.3×10^2	5.9×10^2
S. saintpaul	4.5×10^5	3.3×10^5	2.7×10^4	5.9×10^3	5.3×10^3	5.9×10^2	5.7×10^2	5.1×10^2
S. roan	6.6×10^5	5.3×10^4	3.9×10^3	3.1×10^3	8.6×10^2	8.9×10^2	8.1×10^2	7.9×10^2
S. poona	4.8×10^5	3.6×10^5	3.5×10^3	3.4×10^3	6.9×10^2	5.1×10^2	5.9×10^2	5.4×10^2
S. cubana	8.1×10^5	6.7×10^4	6.4×10^4	4.7×10^3	3.4×10^3	6.3×10^2	6.9×10^2	5.7×10^2
S. chester	7.8×10^5	6.4×10^5	4.3×10^3	4.1×10^3	6.1×10^2	6.8×10^2	6.4×10^2	6.1×10^2
S. arizona	5.2×10^5	6.8×10^4	3.9×10^3	3.6×10^3	6.9×10^2	5.8×10^2	6.3×10^2	5.3×10^2
S. roughstrain (untyped)	6.3×10^5	7.1×10^5	5.7×10^4	4.1×10^3	3.9×10^3	6.1×10^2	6.7×10^2	5.2×10^2

Table 27. Effect of refrigerated temperature 3-6°C and storage of the Salmonella strains suspended in nu Brain heart infusion broth. (counts per ml.)

Organisms	Initial counts	One day	Two days	Four days	Six days	Eight days	Ten days	Twelve days
S. typhimurium	2.7×10^{5}	5.6×10^{4}	3.7×10^{3}	4.6×10^{3}	6.9×10^{2}	6.1×10^{2}	6.9×10^{2}	6.3×10^{2}
S. anatum	4.9×10^{5}	3.9×10^{4}	5.1×10^{4}	4.9×10^{4}	4.3×10^{2}	6.8×10^{2}	6.1×10^{2}	6.4×10^{2}
S. heidelberg	6.8×10^{5}	4.4×10^{4}	6.2×10^{3}	3.4×10^{3}	5.2×10^{3}	5.9×10^{3}	6.3×10^{2}	6.1×10^{2}
S. senftenberg	4.3×10^{5}	3.4×10^{4}	4.7×10^{4}	3.6×10^{3}	3.9×10^{3}	2.9×10^{3}	5.2×10^{2}	5.8×10^{2}
S. weltevreden	5.2×10^{5}	3.7×10^{4}	3.6×10^{4}	4.7×10^{3}	7.1×10^{3}	6.9×10^{2}	5.8×10^{2}	5.3×10^{2}
S. enteritidis	7.9×10^{6}	3.4×10^{5}	3.1×10^{4}	4.7×10^{3}	3.6×10^{3}	7.1×10^{2}	4.7×10^{2}	3.6×10^{2}
S. paratyphi A	1.1×10^{6}	2.7×10^{5}	1.9×10^{4}	3.7×10^{4}	6.9×10^{3}	6.1×10^{2}	5.9×10^{2}	6.2×10^{2}
S. newport	7.2×10^{5}	3.7×10^{5}	1.9×10^{5}	4.4×10^{4}	3.7×10^{3}	7.8×10^{2}	6.8×10^{2}	6.2×10^{2}
S. sainpaul	4.2×10^{5}	2.8×10^{4}	2.7×10^{4}	5.8×10^{3}	2.9×10^{3}	8.2×10^{2}	7.6×10^{2}	5.6×10^{2}
S. roan	6.7×10^{5}	5.1×10^{4}	4.1×10^{3}	3.8×10^{3}	3.9×10^{3}	2.7×10^{3}	2.7×10^{3}	3.2×10
S. poona	4.9×10^{5}	4.3×10^{4}	5.2×10^{3}	6.1×10^{2}	6.7×10^{2}	6.4×10^{2}	6.1×10^{2}	5.7×10^{2}
S. cubana	8.9×10^{5}	7.7×10^{5}	6.1×10^{3}	7.4×10^{2}	7.8×10^{2}	6.9×10^{2}	7.2×10^{2}	6.7×10^{2}
S. chester	7.7×10^{6}	6.2×10^{5}	3.7×10^{4}	4.1×10^{3}	6.1×10^{2}	7.2×10^{2}	5.6×10^{2}	6.2×10^{2}
S. arizona	6.1×10^{5}	5.9×10^{4}	5.8×10^{3}	5.1×10^{3}	7.3×10^{3}	5.8×10^{2}	6.1×10^{2}	5.4×10^{2}
S. roughertrain (untyped)	6.2×10^{6}	4.9×10^{5}	4.3×10^{4}	4.8×10^{4}	5.3×10^{3}	6.1×10^{2}	6.9×10^{2}	6.1×10^{2}

Table 28. Effect of refrigeration temperature 3-6°C and storage of Salmonella strains suspended in Tripticase soy yeast extract broth. (counts/ml.)

Organisms	Initial counts	One day	Four days	Eight days	Ten days	Twelve days
S. typhimurium	2.7×10^5	5.1×10^4	8.6×10^3	7.5×10^2	8.1×10^2	8.3×10^2
S. anatum	4.9×10^5	2.7×10^4	3.9×10^4	8.3×10^2	7.1×10^2	8.0×10^2
S. heidelberg	6.8×10^5	4.8×10^5	4.9×10^3	7.3×10^2	7.2×10^2	6.9×10^2
S. senftenberg	4.3×10^5	3.1×10^4	6.5×10^3	4.8×10^2	6.8×10^2	6.4×10^2
S. weltevreden	4.7×10^5	4.2×10^4	7.1×10^3	5.8×10^2	5.6×10^2	5.2×10^2
S. enteritidis	7.9×10^5	4.8×10^5	6.9×10^4	6.8×10^2	5.9×10^2	5.3×10^2
S. paratyphi A	6.1×10^5	8.9×10^4	5.7×10^3	4.7×10^2	6.1×10^2	5.8×10^2
S. newport	6.1×10^5	8.1×10^4	5.6×10^3	7.3×10^2	7.0×10^2	6.2×10^2
S. saintpaul	5.4×10^5	3.7×10^4	4.9×10^3	6.4×10^2	5.6×10^2	4.9×10^2
S. roan	5.2×10^5	6.9×10^5	3.7×10^4	7.1×10^2	6.3×10^2	5.7×10^2
S. poona	8.9×10^6	7.5×10^5	6.2×10^3	5.8×10^2	6.2×10^2	5.9×10^2
S. eubana	5.3×10^5	4.3×10^4	5.3×10^3	3.4×10^2	5.2×10^2	3.5×10^2
S. chester	6.7×10^5	5.5×10^4	4.4×10^3	7.3×10^2	6.7×10^2	6.4×10^2
S. arizona	6.6×10^5	5.7×10^4	7.8×10^3	6.8×10^2	7.8×10^2	7.1×10^2
S. roughstrain (untyped)	5.6×10^5	4.8×10^5	4.1×10^3	5.6×10^2	4.2×10^2	3.2×10^2

Table 30. Effect of freezing at -20°C and the behaviour of Salmonella during cold storage.

Organisms	Media/ Homogenates.	Initial counts	Days on storage at -20°C. 30	60	90	120	150	210	240
S. typhimurium.	BHI	4.9×10^5	8.2×10^2	1.1×10^2	+	-	-	-	-
	NB	6.2×10^6	5.6×10^2	+	-	-	-	-	-
	TSY	7.1×10^5	4.3×10^2	+	-	-	-	-	-
	M	6.3×10^6	3.1×10^3	4.2×10^2	2.1×10^2	1.9×10^2	+	-	-
	PS	7.6×10^6	5.4×10^3	1.7×10^2	2.3×10^2	1.1×10^2	+	-	-
	Pi	3.6×10^5	6.9×10^3	5.4×10^3	4.1×10^2	1.7×10^2	+	-	-
	C	4.5×10^6	5.9×10^2	4.1×10^2	2.1×10^2	1.1×10^2	+	+	-
S. anatum	BHI	6.2×10^5	4.3×10^2	+	-	-	-	-	-
	NB	4.8×10^5	2.7×10^2	+	-	-	-	-	-
	TSY	4.9×10^5	3.4×10^2	+	-	-	-	-	-
	M	8.1×10^5	5.9×10^3	3.2×10^2	3.8×10^2	1.3×10^2	+	-	-
	PS	4.9×10^6	6.2×10^3	2.8×10^2	1.9×10^2	1.2×10^2	+	-	-
	Pi	6.5×10^5	3.9×10^2	1.9×10^2	1.5×10^2	+	+	-	-
	C	7.3×10^6	4.6×10^3	3.7×10^2	1.1×10^2	+	+	-	-

Contd.....

Contd....

Organisms	Media/ Homogenates.	Initial counts	Days on storage at -20°C. 30	60	90	120	150	210	240
S. heidelberg	BHI	$5.4x10^5$	$6.9x10^2$	$1.7x10^2$	+	-	-	-	-
	MB	$7.2x10^5$	$5.8x10^2$	$1.2x10^2$	+	-	-	-	-
	TSY	$6.8x10^5$	$4.3x10^2$	$1.1x10^2$	-	-	-	-	-
	M	$6.3x10^5$	$5.6x10^3$	$4.7x10^2$	$3.9x10^2$	$1.4x10^2$	+	-	-
	PS	$3.9x10^6$	$2.8x10^3$	$2.4x10^2$	$1.3x10^2$	+	-	-	-
	PA	$5.9x10^5$	$3.7x10^2$	$1.3x10^2$	+	+	-	-	-
	CC	$3.9x10^5$	$4.1x10^2$	$1.7x10^2$	+	-	-	-	-
S. senftenberg	BHI	$4.7x10^5$	$3.5x10^2$	+	+	-	-	-	-
	MB	$8.3x10^5$	$3.5x10^2$	+	-	-	-	-	-
	TSY	$7.9x10^5$	$6.4x10^2$	$1.2x10^2$	-	-	-	-	-
	M	$6.3x10^5$	$4.2x10^2$	$3.3x10^2$	$1.5x10^2$	+	-	-	-
	PS	$8.3x10^5$	$5.2x10^2$	$3.6x10^2$	$1.1x10^2$	+	+	-	-
	PA	$4.7x10^5$	$3.9x10^2$	$2.7x10^2$	$1.4x10^2$	+	-	-	-
	C	$6.5x10^6$	$4.1x10^2$	$3.2x10^2$	$1.1x10^2$	+	+	-	-

Contd....

Contd.....

Organisms	Media/ Homogenates.	Initial counts	Days on storage at -20°C. 30	60	90	120	150	210	240
S. saintpaul	BHI	$7.8x10^5$	$5.8x10^2$	+	+	-	-	-	-
	NB	$6.2x10^5$	$3.3x10^2$	+	-	-	-	-	-
	TSY	$3.7x10^6$	$2.9x10^2$	$1.3x10^2$	+	-	-	-	-
	M	$8.9x10^5$	$6.4x10^2$	$3.2x10^2$	$2.3x10^2$	+	-	-	-
	PS	$9.1x10^6$	$7.2x10^2$	$3.2x10^2$	$2.2x10^2$	+	+	-	-
	Pi	$6.2x10^5$	$5.2x10^3$	$2.8x10^2$	$1.2x10^2$	$1.1x10^2$	+	-	-
	C	$6.9x10^6$	$4.4x10^2$	$3.1x10^2$	$1.7x10^2$	$1.3x10^2$	+	-	-
S. roan	BHI	$3.9x10^5$	$2.6x10^2$	+	-	-	-	-	-
	NB	$4.7x10^5$	$3.1x10^2$	+	-	-	-	-	-
	TSY	$9.1x10^5$	$7.2x10^2$	$1.6x10^2$	+	-	-	-	-
	M	$4.8x10^6$	$7.2x10^2$	$3.2x10^2$	$2.3x10^2$	+	+	-	-
	PS	$7.8x10^5$	$5.8x10^2$	$4.8x10^2$	$2.9x10^2$	+	+	-	-
	Pi	$5.9x10^5$	$4.2x10^2$	$6.9x10^2$	$1.1x10^2$	+	+	-	-
	C	$7.2x10^6$	$6.4x10^2$	$3.0x10^2$	$1.3x10^2$	$1.1x10^2$	+	-	-

BHI= Brain Heart Infusion broth; NB= Nutrient Broth; TSY= Triptiease Soy Yeast extract broth; M= Mackerel (R. kanagurta); PS= Pearl spot (E. suratensis); Pi= P. indicus; C= Crab (Scylla serrata). Counts perm ml/gm.

Contd....

Organisms	Media/ Homogenates	Initial Counts	Days on storage at -20°C. 30	60	90	120	150	210	240
S. weltevreden	BHI	6.4×10^6	3.8×10^2	+	-	-	-	-	-
	NB	3.7×10^6	4.1×10^2	+	+	-	-	-	-
	TSY	7.3×10^5	3.9×10^2	1.1×10^2	+	-	-	-	-
	M	4.5×10^6	6.9×10^2	2.4×10^2	1.1×10^2	+	-	-	-
	PS	6.2×10^5	4.2×10^2	3.1×10^2	1.1×10^2	-	-	-	-
	P1	5.5×10^6	3.2×10^3	1.4×10^2	+	-	-	-	-
	C	4.9×10^6	3.7×10^2	1.5×10^2	+	+	-	-	-
S. typhi	BHI	6.7×10^5	6.2×10^2	+	-	-	-	-	-
	NB	7.2×10^6	5.9×10^2	+	-	-	-	-	-
	TSY	4.8×10^5	2.9×10^2	+	-	-	-	-	-
	M	7.4×10^5	4.8×10^3	2.9×10^2	1.6×10^2	+	-	-	-
	PS	3.7×10^5	2.9×10^2	1.9×10^2	1.1×10^2	-	+	-	-
	P1	7.5×10^5	5.2×10^2	2.3×10^2	+	+	-	-	-
	C	4.3×10^5	3.2×10^2	1.1×10^2	-	+	-	-	-

Contd....

Table 31. Effect of low temperature (-20°C) on multiple cyclic defrosting on various *Salmonella* serotypes.

Suspension medium	Organisms	Initial counts	60 days after	90 days after	120 days after	210 days after	240 days after
Nb	S. typhimurium	5.69	2.29	+	-	-	-
BHI			2.70	1.88	-	-	-
TSY			3.40	+	-	-	-
MH			3.82	2.39	+	-	-
Ps.H			4.15	1.69	+	-	-
Pr.H			3.90	2.17	+	-	-
Cr.H			4.17	2.52	+	-	-
Nb	S. anatum	6.53	3.69	+	-	-	-
BHI			3.90	+	-	-	-
TSY			4.88	2.11	+	-	-
MH			4.39	1.99	+	-	-
Ps.H			3.98	2.33	+	-	-
Pr.H			4.11	2.31	+	-	-
Cr.H			4.56	2.71	+	-	-

Contd....

Contd.....

Suspension medium	Organisms	Initial counts	60 days after	90 days after	120 days after	210 days after	240 days after
Nb	S. heidelberg	6.42	3.19	+	-	-	-
BHI			3.56	+	-	-	-
TSY			3.29	2.17	+	-	-
MH			4.32	1.99	+	-	-
Ps.H			4.62	2.15	+	-	-
Pr.H			4.11	+	+	-	-
Cr.H			3.29	+	-	-	-
Nb	S. roan	5.39	3.49	+	-	-	-
BHI			3.11	-	-	-	-
TSY			3.28	-	-	-	-
MH			4.68	+	+	-	-
Ps.H			4.26	2.13	-	-	-
Pr.H			4.78	2.44	+	-	-
Cr.H			3.27	+	-	-	-

Nb= Nutrient broth; BHI= Brain Heart Infusion broth; TSY= Triptiease Soy Yeast extract broth; MH= Mackerel Homogenate; Ps.H= Pearl spot Homogenate; Pr.H= Prawn (*Penaeus indicus*) Homogenate; Cr.H= Crab (*Neylla serrata*) Homogenate; All counts are expressed in log numbers/ml/gm. (+)= Positive presence of *Salmonella*. (-) Negative(Absence of *Salmonella*).

Table 32. Percentage of dead and injured cells of various *Salmonella* strains in Minimal broth, Nutrient broth, Brain Heart infusion broth, and Trèptiease Soy broth during freezing and thawing.

Organisms	Minimal broth Frozen & thawed		Nutrient broth Frozen & thawed	
	Dead(%)	Injured(%)	Dead(%)	Injured(%)
S. typhimurium	32	61	33	62
S. anatum	36	57	28	64
S. heidelberg	29	61	25	71
S. senftenberg	37	62	32	64
S. veltevredem	28	69	35	58
S. enteritidis	39	58	28	70
S. typhi	34	63	32	67
S. newport	40	58	26	72
S. saintpaul	36	63	29	69
S. roam	40	57	35	62
S. poona	39	59	28	68
S. eubana	32	64	31	66
S. chester	29	68	26	59
S. arizona	34	63	29	68
S. vayeross	31	63	32	64
S. bareilly	36	52	29	58
S. Nchanga vayeross	31	66	36	60
S. rough strain	35	62	29	65

Contd.....

Contd.....

Organisms	NutriBrain Heart infusion broth Frozen &thawed Dead(%)	Injured(%)	Tryptiease soy Yeast extract broth Frozen & thawed Dead(%)	Injured(%)
S. typhimurium	24	65	27	63
S. anatum	28	70	31	61
S. heidelberg	32	62	25	67
S. senftenberg	35	60	28	69
S. veltevredem	27	63	26	70
S. enteritidis	25	71	24	68
S. typhi	29	69	26	61
S. newport	28	68	26	69
S. saintpaul	30	64	35	60
S. roan	33	62	29	68
S. poona	30	64	23	61
S. cubana	29	68	25	65
S. chester	31	63	26	62
S. arizona	33	62	26	69
S. vayereas	27	65	29	67
S. bareilly	30	61	32	64
S. mahanga	26	66	24	71
S. rough strain	29	69	24	67

Table 33. Percentages of dead andinjured cells of various Salmonella strains in various homogenates of Mackerel (R. kanagurta) Pearl spot(E. suratensis), Prawn (P. indicus) and Crab (S. serrata) during freezing and thawing.

Organisms	Mackerel homogenate Frozen and thawed Dead(%)	Injured(%)	Pearl spot homogenate Frozen & thawed Dead(%)	Injured(%)
S. typhimurium	12	64	8	51
S. anatum	19	65	9	45
S. heidelberg	18	53	9	55
S. senftenberg	16	65	12	57
S. weltevreden	14	54	10	65
S. enteritidis	11	53	13	51
S. typhi	9	62	11	63
S. newport	15	65	10	52
S. saintpaul	19	60	12	62
S. roan	18	63	12	60
S. poona	9	53	9	57
S. cubana	10	65	9	42
S. chester	12	58	13	48
S. arizona	16	60	12	69
S. wayeross	10	68	14	51
S. bareilly	11	65	13	60
S. mahanga	10	64	9	54
S. rough strain	12	59	9	59

Contd.....

Contd.....

Organisms	P. indicus homogenate Frozen & thawed Dead (%)	Injured (%)	S. serrata homogenate Frozen & thawed Dead(%)	Injured(%)
S. typhimurium	11	64	10	60
S. anatum	10	61	9	64
S. heidelberg	9	57	11	59
S. senftenberg	12	61	11	62
S. weltevreden	9	52	10	54
S. enteritidis	7	57	12	55
S. typhi	10	61	7	59
S. newport	8	65	10	58
S. saintpaul	12	55	10	57
S. roem	9	61	11	60
S. poona	8	63	8	64
S. cubana	10	57	11	57
S. chester	12	61	9	65
S. arizona	11	53	9	63
S. weyeross	8	65	10	63
S. bareilly	12	61	12	55
S. mchanga	9	55	8	53
S. rough strain	10	62	10	61

Table 34. Effect of organic nutrients on the cell repair process after injury.

Organisms	Amino acids less durations in min.				Vitamin less durations in min.			
*	15	30	60	120	15	30	60	120
S. typhimurium	4.63	4.71	4.86	4.92	4.69	4.78	4.78	4.85
S. anatum	4.54	4.60	4.66	4.70	4.50	4.54	4.59	4.67
S. heidelberg	4.49	4.51	4.61	4.78	4.67	4.78	4.81	4.91
S. senftenberg	3.67	3.89	4.21	4.65	3.53	3.99	4.34	4.67
S. weltevreden	4.34	4.56	4.78	4.89	4.67	4.56	4.88	4.99
S. enteritidis	4.35	4.76	4.98	4.99	4.35	4.86	4.67	4.89
S. typhi	3.61	3.88	4.31	4.56	3.56	3.99	4.36	4.78
S. newport	3.56	3.89	4.34	4.76	3.45	3.97	4.55	4.89
S. saintpaul	3.67	4.12	4.66	4.99	3.34	3.66	4.31	4.76
S. roan	4.32	4.65	4.88	4.91	4.21	4.67	4.88	4.90

* Counts were given in log numbers and expressed per ml.

Contd....

Contd.....

Organisms	Nucleosides bases less. durations in min.				With all the three components. durations in min.			
	15	30	60	120	15	30	60	120
S. typhimurium	4.67	4.70	4.76	4.93	4.66	4.88	5.75	5.78
S. enatum	4.54	4.63	4.79	4.88	4.34	4.99	5.67	5.74
S. heidelberg	4.32	4.53	4.82	4.91	4.38	4.86	5.63	5.33
S. senftenberg	3.66	3.87	4.11	4.21	3.76	3.12	4.69	4.98
S. veltevreden	4.54	4.72	4.81	4.94	4.87	5.34	5.45	5.54
S. enteritidis	4.67	4.63	4.75	4.87	4.65	4.89	5.34	5.47
S. typhi	3.57	3.87	3.91	4.32	3.89	4.34	4.67	4.89
S. newport	3.76	3.86	3.92	4.12	3.94	4.34	4.68	4.89
S. saintpaul	3.87	3.99	4.43	4.53	3.67	4.23	4.56	4.78
S. roam	4.32	4.65	4.78	4.88	4.41	4.98	5.34	5.54

* Counts were given in log numbers and expressed per ml.

Table 35. Survival of Vibrio parahaemolyticus on various homogenates of fish and shellfishes in refrigerated storage at 3-6°C.

Cultures	Initial added level /gm	Days of storage: 1 /gm	2 /gm	3 /gm	5 /gm	7 /gm	14 /gm	16 /gm
Mackerel (R. kanagurta) homogenates								
V.No. 178	5.7×10^{6}	3.7×10^{4}	2.2×10^{3}	1.7×10^{2}	1.0×10^{2}	*100	NC	NC
,, 246	9.2×10^{6}	2.1×10^{4}	4.1×10^{3}	3.7×10^{2}	1.1×10^{2}	1.0×10^{2}	NC	NC
,, 327	8.4×10^{5}	4.7×10^{3}	5.2×10^{3}	3.4×10^{2}	2.4×10^{2}	*100	NC	NC
,, 341	4.6×10^{6}	3.1×10^{3}	3.7×10^{3}	2.1×10^{2}	2.3×10^{2}	*100	NC	NC
,, 1902	5.2×10^{5}	3.8×10^{3}	3.1×10^{3}	2.5×10^{2}	2.1×10^{2}	*100	*30	NC
,, 3525	7.6×10^{6}	4.8×10^{3}	4.1×10^{3}	3.7×10^{2}	1.8×10^{2}	*100	*30	NC
Pearl spot (E. suratensis) homogenates								
V.No. 178	7.3×10^{5}	5.6×10^{3}	4.8×10^{2}	2.5×10^{2}	1.1×10^{2}	NC	NC	NC
,, 246	8.5×10^{6}	5.4×10^{4}	3.9×10^{3}	3.1×10^{2}	1.4×10^{2}	*100	*30	NC
,, 327	6.3×10^{6}	4.8×10^{3}	3.2×10^{2}	2.5×10^{2}	1.3×10^{2}	*100	*30	NC
,, 341	7.1×10^{6}	5.7×10^{4}	4.6×10^{2}	3.6×10^{2}	NC	*100	NC	NC
,, 1902	5.3×10^{5}	4.8×10^{4}	3.2×10^{3}	3.2×10^{2}	1.3×10^{2}	*100	NC	NC
,, 3525	7.4×10^{6}	6.3×10^{4}	4.7×10^{3}	4.2×10^{2}	1.5×10^{2}	NC	NC	NC

NC- No viable microorganisms.

*100- Less than 100 per gram

*30 - Less than 30 per gram.

Contd.....

Cultures	Initial added level level /gm	Days of storage. 1 /gm	2 /gm	3 /gm	5 /gm	7 /gm	14 /gm	16 /gm
Prawns (Penaeus indicus) homogenates								
V.No. 178	7.9×10^{6}	6.3×10^{5}	6.2×10^{4}	3.6×10^{2}	1.4×10^{2}	1.7×10^{2}	*100	NC
,, 246	8.4×10^{6}	7.3×10^{5}	5.2×10^{4}	4.8×10^{3}	1.8×10^{2}	1.5×10^{2}	*100	*30
,, 327	8.1×10^{6}	6.4×10^{5}	8.3×10^{4}	6.1×10^{2}	3.1×10^{2}	1.2×10^{2}	NC	NC
,, 341	7.7×10^{5}	3.6×10^{4}	2.7×10^{3}	2.1×10^{2}	1.1×10^{2}	1.2×10^{2}	*30	NC
,, 1902	4.5×10^{6}	7.1×10^{4}	3.7×10^{3}	2.5×10^{2}	1.2×10^{2}	1.2×10^{2}	*30	NC
,, 3525	7.4×10^{6}	6.3×10^{4}	5.3×10^{3}	4.2×10^{2}	1.4×10^{2}	1.3×10^{2}	*30	NC
Crab (Scylla serrata) homogenates.								
V.No. 178	6.4×10^{5}	8.3×10^{4}	3.8×10^{3}	4.3×10^{2}	2.7×10^{2}	1.9×10^{2}	NC	NC
,, 246	8.4×10^{6}	9.7×10^{4}	5.7×10^{3}	3.8×10^{2}	1.9×10^{2}	1.0×10^{2}	*100	*30
,, 327	6.9×10^{6}	8.3×10^{4}	4.7×10^{3}	3.9×10^{2}	2.7×10^{2}	1.6×10^{2}	NC	NC
,, 341	6.6×10^{6}	5.9×10^{4}	3.7×10^{3}	3.5×10^{2}	NC	*100	NC	NC
,, 1902	8.4×10^{6}	8.6×10^{4}	4.9×10^{3}	3.4×10^{2}	2.6×10^{2}	*100	*30	NC
,, 3525	7.5×10^{6}	6.9×10^{4}	4.8×10^{3}	4.1×10^{2}	3.1×10^{2}	1.7×10^{2}	*30	NC

NC- No viable counts. *100- Less than 100 per gram. *30- Less than 30 per gram.

Contd...

Cultures	Initial added level /gm.	1 /gm	5 Days of storage /gm	10 /gm	16 /gm
Mackerel homogenates.					
V.No. 178	$7.4x10^2$	6.1x10	*30	NC	NC
,, 246	$8.3x10^2$	5.7x10	*30	NC	NC
,, 327	$6.2x10^2$	4.9x10	*30	NC	NC
,, 341	$4.8x10^2$	*100	*30	NC	NC
,, 1902	$8.4x10^2$	3.7x10	*100	NC	NC
,, 3525	$7.9x10^2$	4.8x10	*100	NC	NC
Pearl spot homogenates.					
V.No. 178	$6.9x10^2$	5.8x10	*100	NC	NC
,, 246	$5.7x10^2$	6.2x10	*100	NC	NC
,, 327	$6.2x10^2$	5.0x10	NC	NC	NC
,, 341	$3.9x10^2$	5.2x10	*100	NC	NC
,, 1902	$7.5x10^2$	4.7x10	NC	NC	NC
,, 3525	$7.5x10^2$	3.9x10	*100	NC	NC
Prawns (P. indicus) homogenates.					
V.No. 178	$4.8x10^2$	3.9x10	*100	NC	NC
,, 246	$6.8x10^2$	5.6x10	*100	NC	NC
,, 327	$3.9x10^2$	3.9x10	NC	NC	NC
,, 431	$4.1x10^2$	6.3x10	*100	NC	NC
,, 1902	$6.9x10^2$	140	*100	NC	NC
,, 3525	$8.1x10^2$	1.3x10	NC	NC	NC
Crab (S. serrata) homogenates.					
V.No. 178	$5.2x10^2$	$2.8x10^2$	$1.4x10^2$	NC	NC
,, 246	$7.2x10^2$	$3.8x10^2$	$1.1x10^2$	NC	NC
,, 327	$4.8x10^2$	$2.6x10^2$	*100	NC	NC
,, 341	$5.6x10^2$	$2.1x10^2$	NC	NC	NC
,, 1902	$6.7x10^2$	$4.3x10^2$	*100	NC	NC
,, 3525	$5.9x10^2$	$3.4x10^2$	*100	NC	NC

NC- No viable counts.

Table 36. Survival of *Vibrio parahaemolyticus* in frozen storage at -20°C.

Strains Nos.	Initial level	Storage days. MPN/gm. 1	5	10	20	30	60
Mackerel homogenates.							
V.No. 178	8.4×10^6	*1100	*1100	160	NC	NC	NC
,, 246	7.3×10^6	*1100	*1100	460	150	9.3	NC
,, 327	2.8×10^6	1.6×10^3	*1100	350	240	6.2	NC
,, 341	9.2×10^6	2.1×10^3	*1100	290	150	6.2	NC
,, 1902	1.8×10^6	4.6×10^3	*1100	350	110	7.2	NC
,, 3525	3.2×10^6	2.5×10^3	*1100	240	64	3.0	NC
Pearl spot homogenates.							
V.No. 178	1.8×10^6	3.5×10^3	*1100	240	95	3.0	NC
,, 246	9.5×10^6	1.1×10^3	*1100	420	150	6.0	NC
,, 327	4.3×10^6	1.5×10^3	*1100	280	64	6.2	NC
,, 341	2.8×10^6	3.5×10^3	*1100	350	110	9.2	NC
,, 1902	8.4×10^6	5.6×10^3	*1100	460	95	6.2x1	NC
,, 3525	3.5×10^6	2.1×10^3	*1100	530	120	6.2	NC
Prawn (*Penaeus indicus*) homogenates.							
V.No. 178	2.8×10^6	1.6×10^3	*1100	420	230	3.0	NC
,, 246	9.2×10^6	2.1×10^3	*1100	350	93	6.0	NC
,, 327	4.3×10^6	3.5×10^3	*1100	930	350	6.2	NC
,, 341	2.4×10^6	5.3×10^3	*1100	530	290	7.3	NC
,, 1902	4.3×10^6	1.8×10^3	*1100	430	210	NC	NC
,, 3525	9.2×10^6	5.2×10^3	*1100	240	64	NC	NC
Crab (*Scylla serrata*) homogenates.							
V.No. 178	8.4×10^6	3.5×10^3	*1100	440	95	6.2	NC
,, 246	2.4×10^6	5.6×10^3	*1100	430	64	6.2	NC
,, 32327	3.5×10^6	1.6×10^3	*1100	530	210	7.2	NC
,, 341	1.8×10^6	4.3×10^3	*1100	4400	160	3.0	NC
,, 1902	9.2×10^6	1.7×10^3	*1100	530	210	3.0	NC
,, 3525	3.2×10^6	5.6×10^3	*1100	350	160	6.2	NC

* Greater than; NC= No viable counts.

Table 37. The effect of multicyclic defrosting and freezing on *Vibrio parahaemolyticus*.

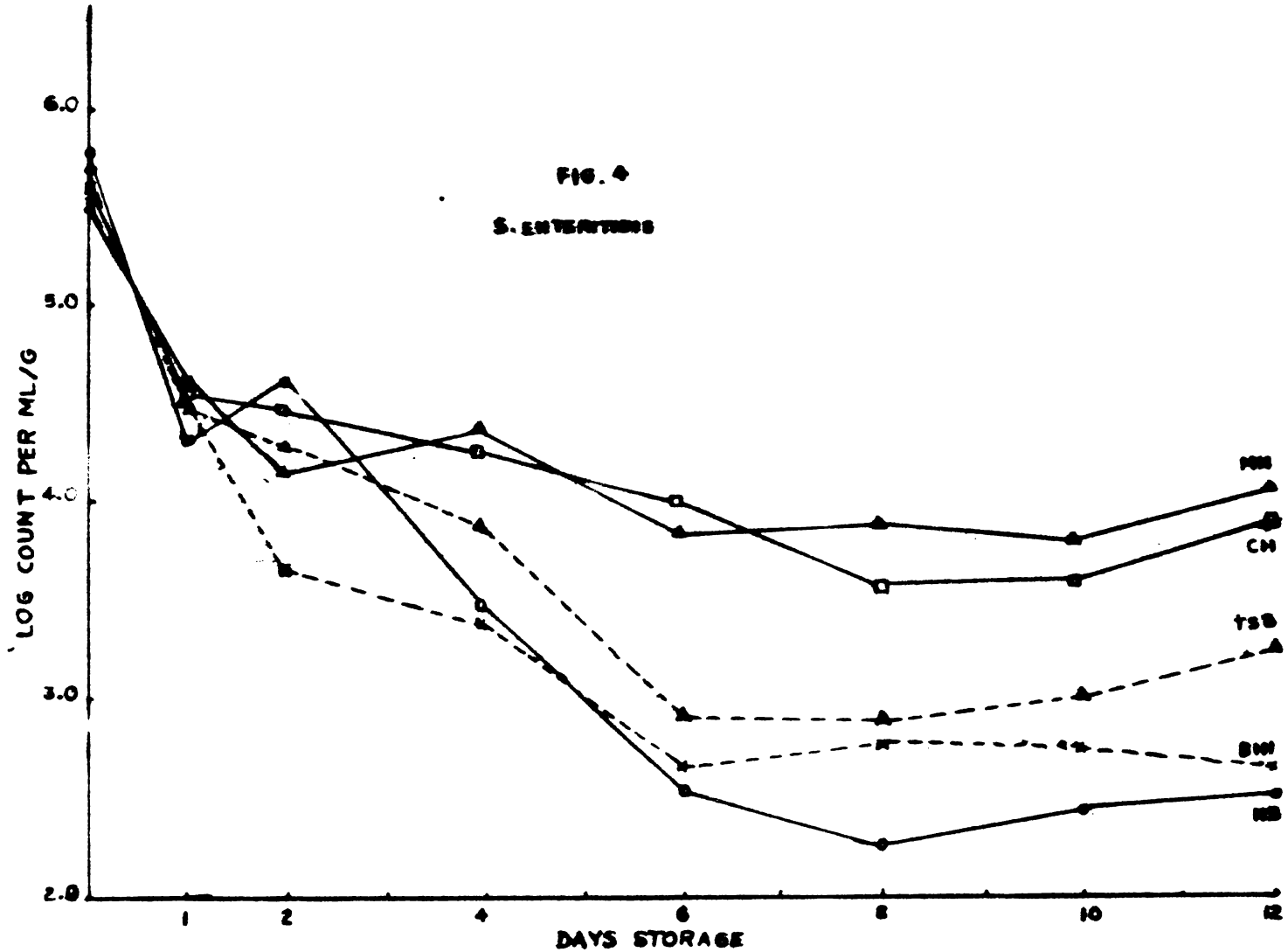
Strains Nos.	Initial counts	MPN counts per gram 1	10	20	30	60
Mackerel homogenates.						
V.No. 178	4.8×10^6	*1100	120	3.0	NC	NC
,, 246	4.6×10^6	*1100	460	6.0	NC	NC
,, 327	6.7×10^6	*1100	210	3.0	NC	NC
,, 341	7.9×10^6	*1100	460	3.0	NC	NC
,, 1902	5.2×10^6	*1100	290	3.0	NC	NC
,, 3525	7.6×10^6	*1100	460	3.00	NC	NC
Pearl spot homogenates.						
V.No. 178	5.1×10^6	*1100	210	3.0	NC	NC
,, 246	3.9×10^6	*1100	290	6.1	NC	NC
,, 327	5.9×10^6	*1100	460	6.2	NC	NC
,, 1902	7.9×10^6	*1100	240	6.1	NC	NC
,, 3525	7.3×10^6	*1100	460	9.3	NC	NC
Prawns (*P.indicus*)homogenates.						
V.No. 178	4.8×10^6	*1100	210	93.0	NC	NC
,, 246	5.9×10^6	*1100	290	75.0	NC	NC
,, 327	3.5×10^6	*1100	240	93.0	NC	NC
,, 1902	7.3×10^6	*1100	210	120.0	3.0	NC
,, 3525	4.8×10^6	*1100	240	75.0	3.0	NC
Crab (*Scylla serrata*) homogenates						
V.No. 178	4.1×10^6	*1100	460	24.0	NC	NC
,, 246	4.7×10^6	*1100	290	43.0	NC	NC
,, 327	8.1×10^6	*1100	210	64.0	3.0	NC
,, 341	6.2×10^6	*1100	460	93.0	3.0	NC
,, 1902	8.3×10^6	*1100	290	75.0	3.0	NC
,, 3525	2.7×10^6	*1100	210	6.2	NC	NC

*Counts always greater than 1100 per gram;
All values were of meanof three experiments.
NC= No viable counts.

Table 38. Effect of freezing and thawing on death and injury of *Vibrio parahaemolyticus*.

Strains Nos.	Initial counts per gm.	% of death after freezing & thawing	% of injury after freezing & thawing
Mackerel homogenates.			
V.No. 178	7.3×10^6	71	82
,, 246	7.4×10^6	65	79
,, 327	5.2×10^6	73	80
,, 341	6.8×10^6	78	85
,, 1902	6.2×10^6	69	81
,, 3525	8.3×10^6	65	79
Pearl spot homogenates.			
V.No. 178	5.8×10^6	74	76
,, 246	6.8×10^6	61	81
,, 327	5.3×10^6	78	82
,, 341	4.8×10^6	68	84
,, 1902	7.9×10^6	65	87
,, 3525	7.1×10^6	73	84
Prawns (*P. indicus*) homogenates.			
V.No. 178	4.7×10^6	62	72
,, 246	6.8×10^6	65	67
,, 327	7.8×10^6	661	74
,, 341	6.3×10^6	63	70
,, 1902	4.9×10^6	65	69
,, 3525	7.1×10^6	62	61
Crab (*scylla serrata*) homogenates.			
V.No. 178	6.3×10^6	60	64
,, 246	7.3×10^6	67	70
,, 327	6.7×10^6	70	71
,, 341	5.6×10^6	65	69
,, 1902	4.5×10^6	68	68
,, 3525	7.6×10^6	71	67

Fig. 4. Effect of refrigeration and storage at 3°-6°C for *Salmonella enteritidis*.



NB - NUTRIENT BROTH, BHI - BRAIN HEART INFUSION, TSB - TRIPTICASE SOY BROTH

CH - CRAB HOMOGENATE, MH - MACKEREL HOMOGENATE

Fig. 5. Effect of frozen storage at -20°C for *Salmonella* newport.

FIG.5

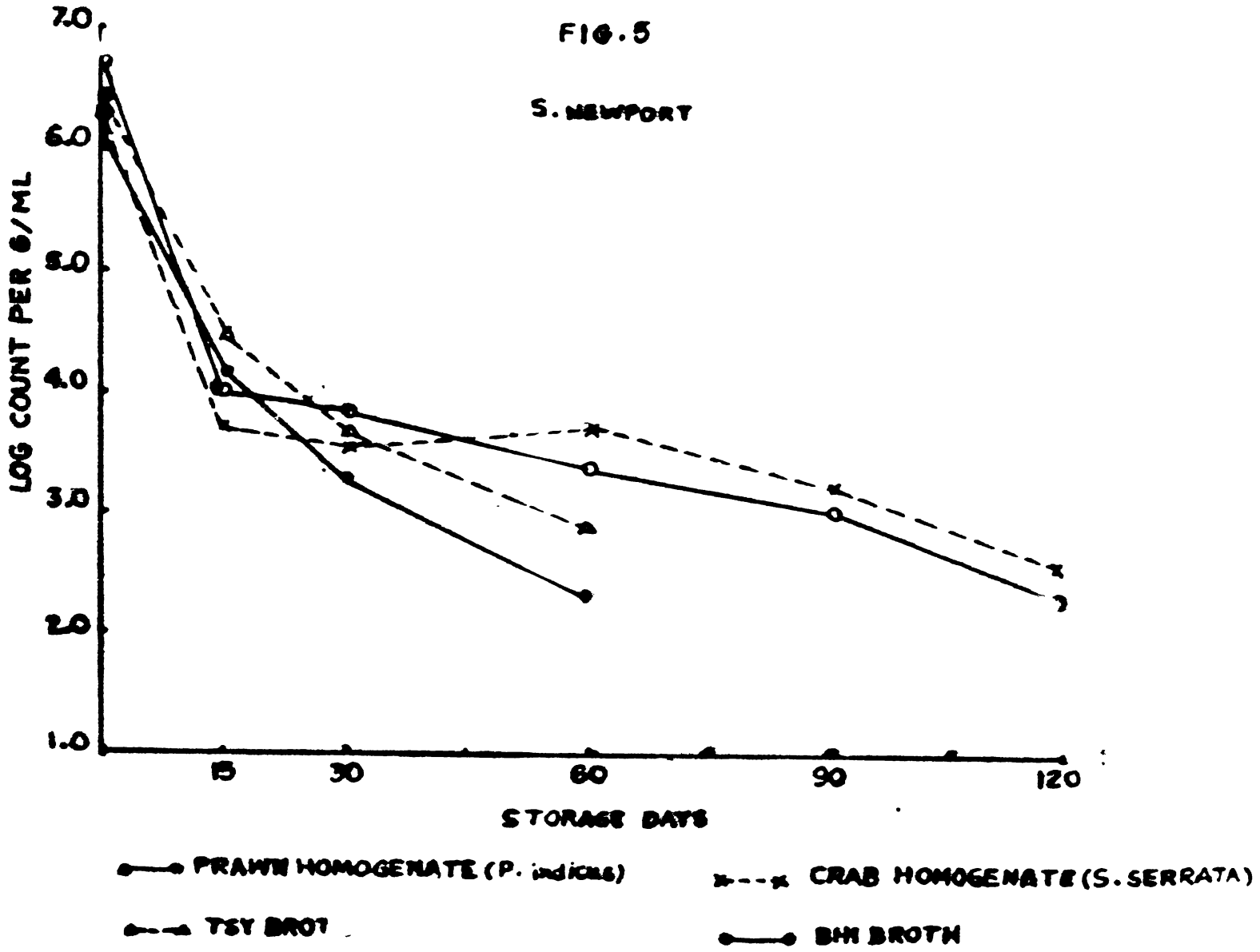

S. NEWPORT
LOG COUNT PER G/ML
7.0
6.0
5.0
4.0
3.0
2.0
1.0
15
30
60
90
120
STORAGE DAYS
PRAWN HOMOGENATE (P. indicus)
CRAB HOMOGENATE (S. SERRATA)
TSY BROT
BHI BROTH

Fig. 6. Effect of frozen storage at -20°C for Samonella arizona spp.

FIG. 6

S. ARIZONA

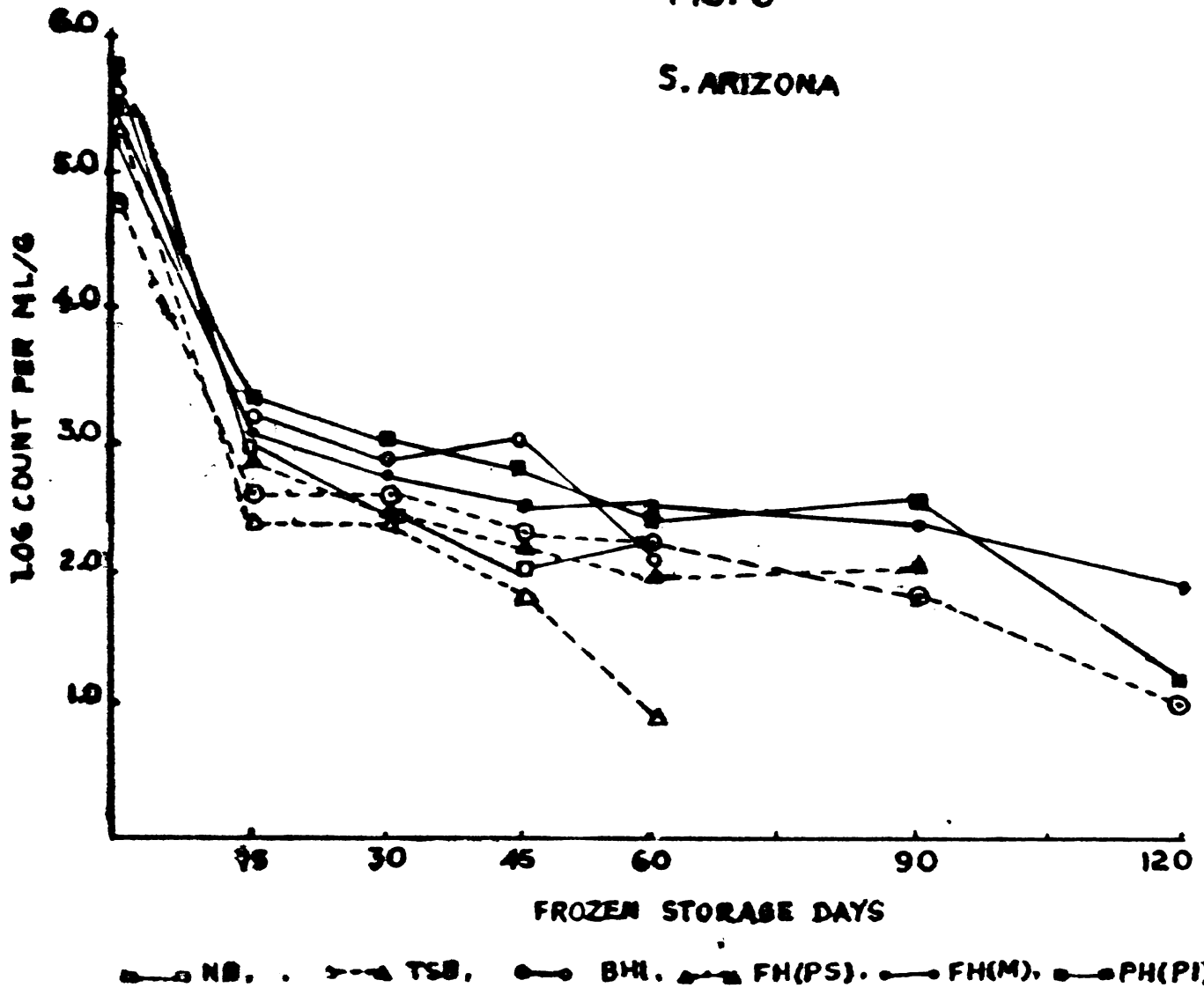


Fig. 7. Effect of frozen storage at -20°C for Salmonella enteritidis.

FIG. 7

S. ENTERITIDIS

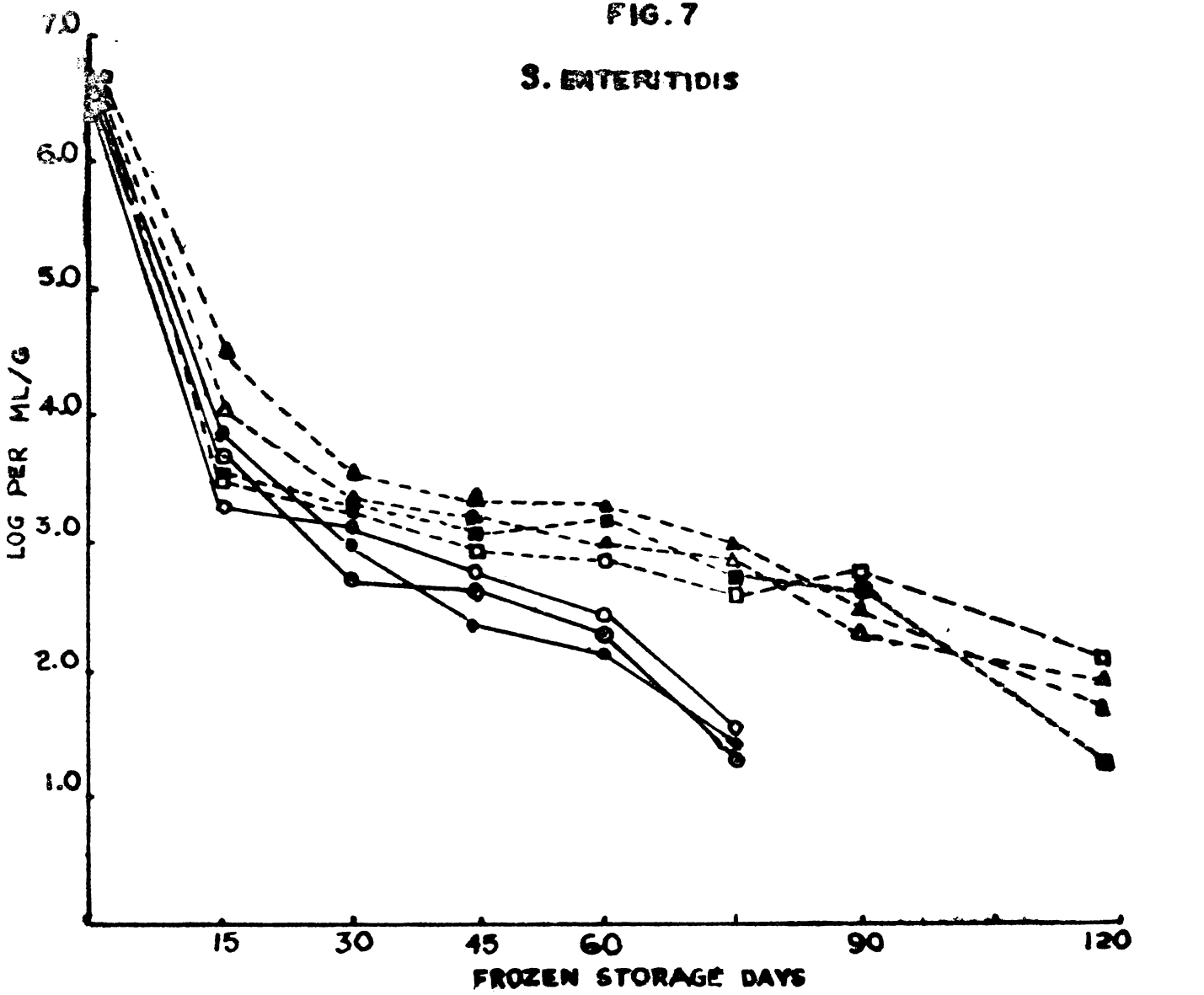


Fig. 8. Cell repair process of different species of Salmonella after freezing.

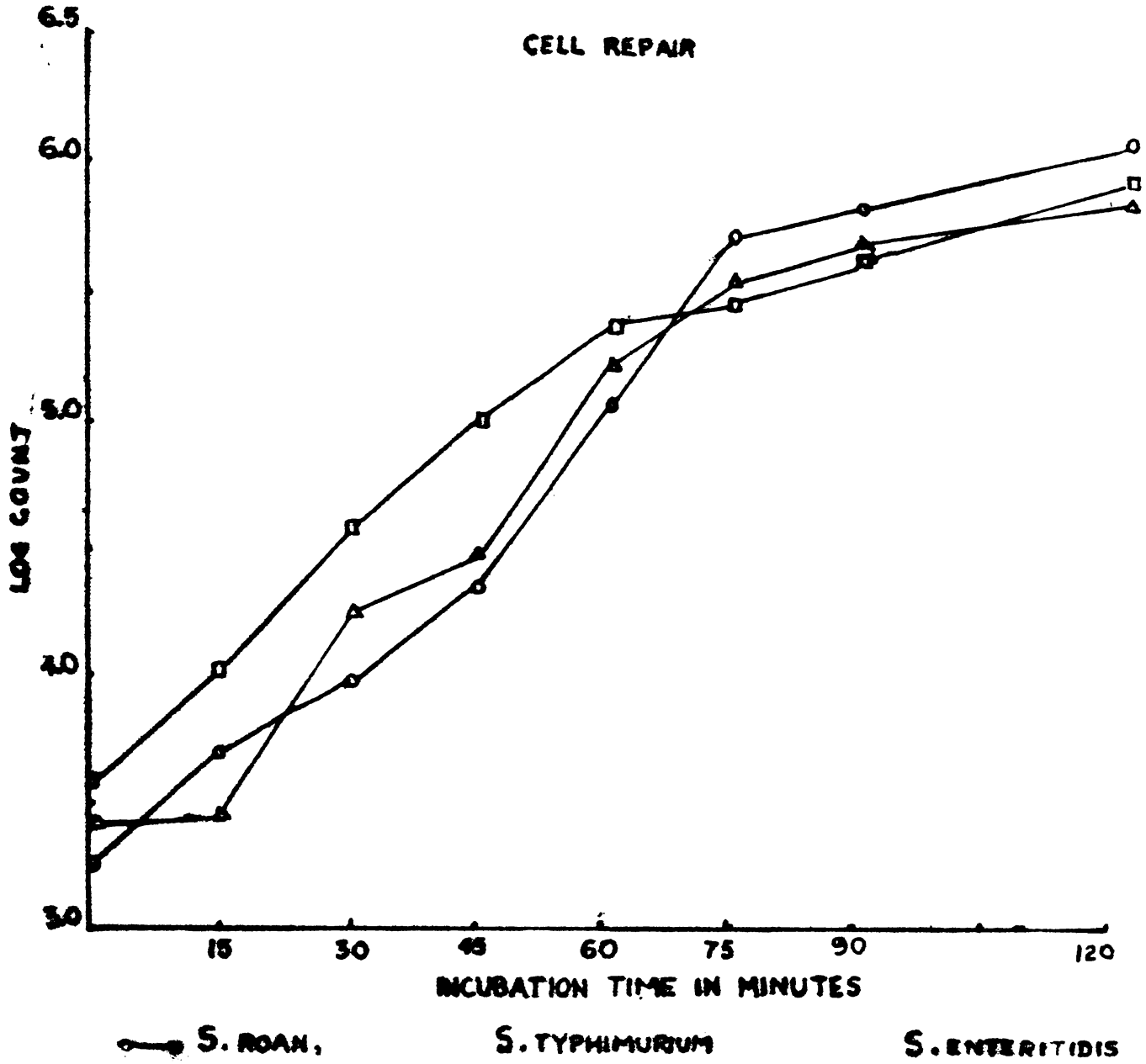

CELL REPAIR
6.5
6.0
5.0
4.0
3.0
LOG COUNT
15
30
45
60
75
90
120
INCUBATION TIME IN MINUTES
S. ROAN,
S. TYPHIMURIUM
S. ENTERITIDIS

Fig. 9. Death and cell repair process in Tripticase soy broth after freezing at -20°C.

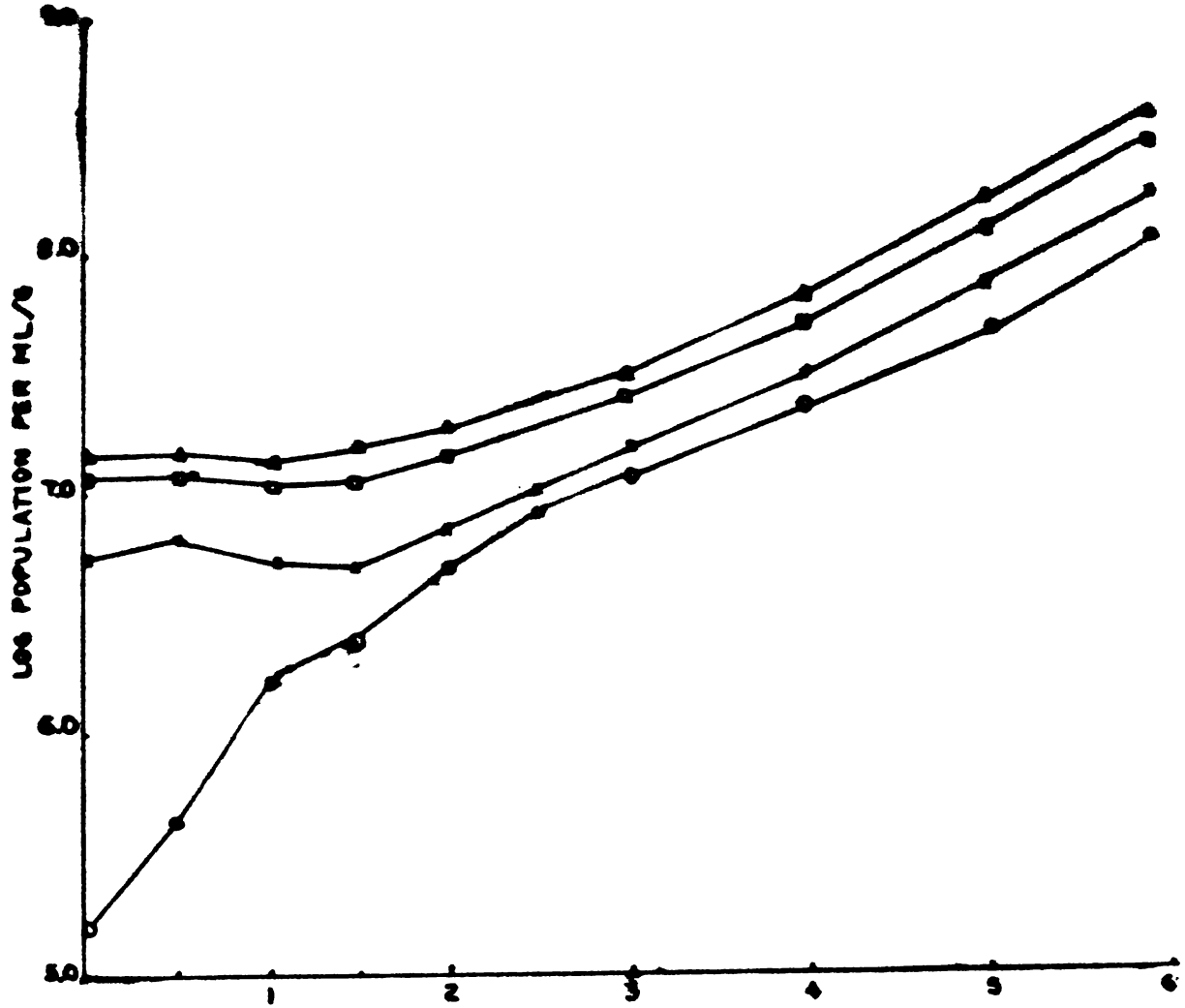


FIG 9 T.S.BROTH

CONTROL UNTREATED XLP. FROZEN CELL XLP

" " XLDP " " XLDP

Fig. 10. Survival of Vibrio parahaemolyticus (strain No. 1902) In variuos finfishes and shellfish homogenates in refrigerated storage at 3^{o}- 6^{o}C.

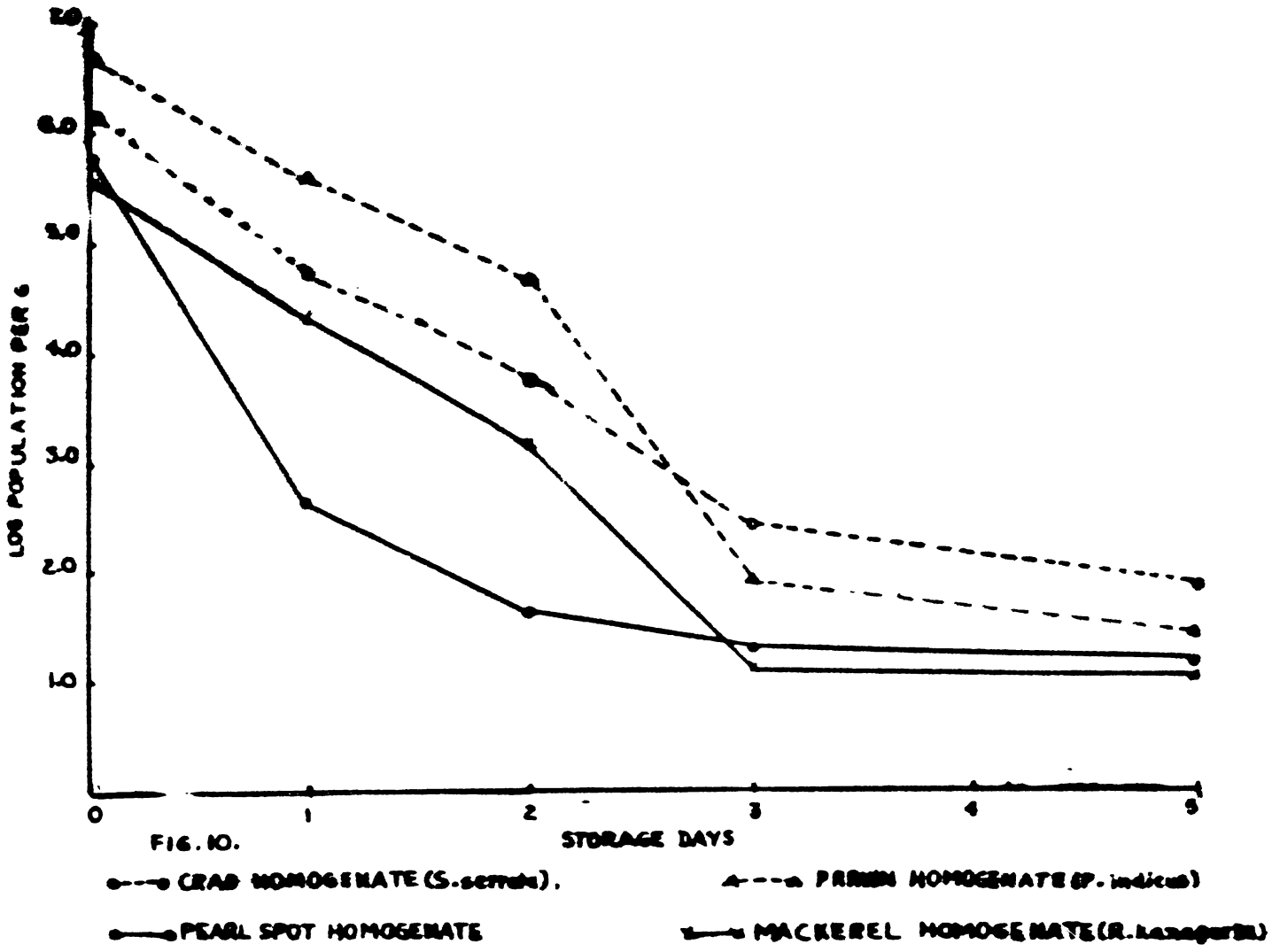

LOG POPULATION PER g
1.0
2.0
3.0
4.0
5.0
6.0
0
1
2
3
4
5
STORAGE DAYS
CRAB HOMOGENATE (S. serrata).
PRAWN HOMOGENATE (P. indicus)
PEARL SPOT HOMOGENATE
MACKEREL HOMOGENATE (R.

FIG. 10.

Fig. 11. Repair of Vibrio parahaemolyticus cells (strain No 1982 NCIB) in different finfish and shellfish homogenates after freezing at -20°C.

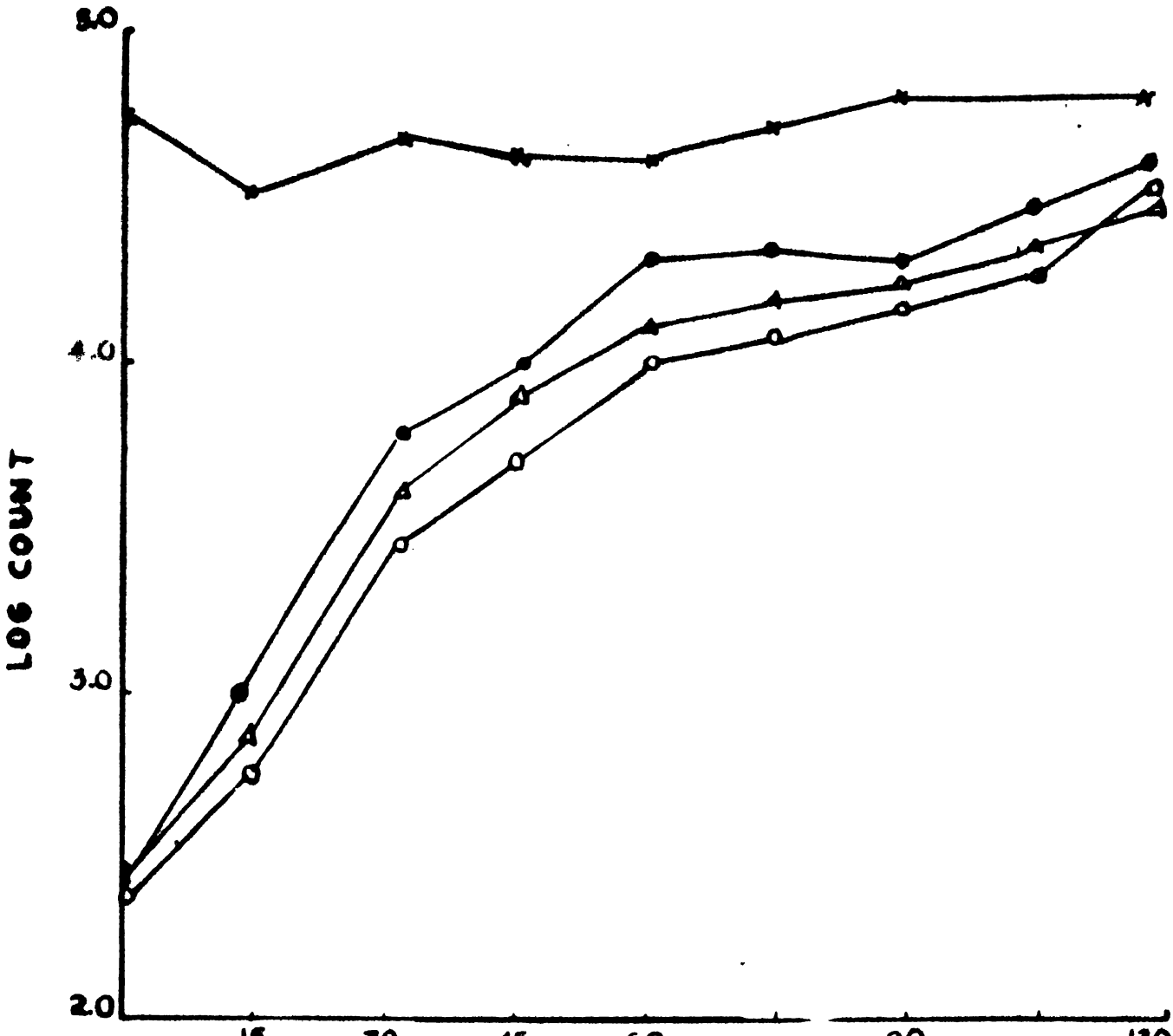

5.0
4.0
3.0
2.0
LOG COUNT
15
30
45
60
90
120
INCUBATION MINUTES
CONTROL T S BROTH
HOMOGENATES OF S. serrata.
" P. indicus.
" E. aurentensis

FIG. 11.

Fig. 12. Repair of *Vibrio parahaemolyticus* cells(No 246) in different fish and shellfish homogenates after freezing at -20°C.

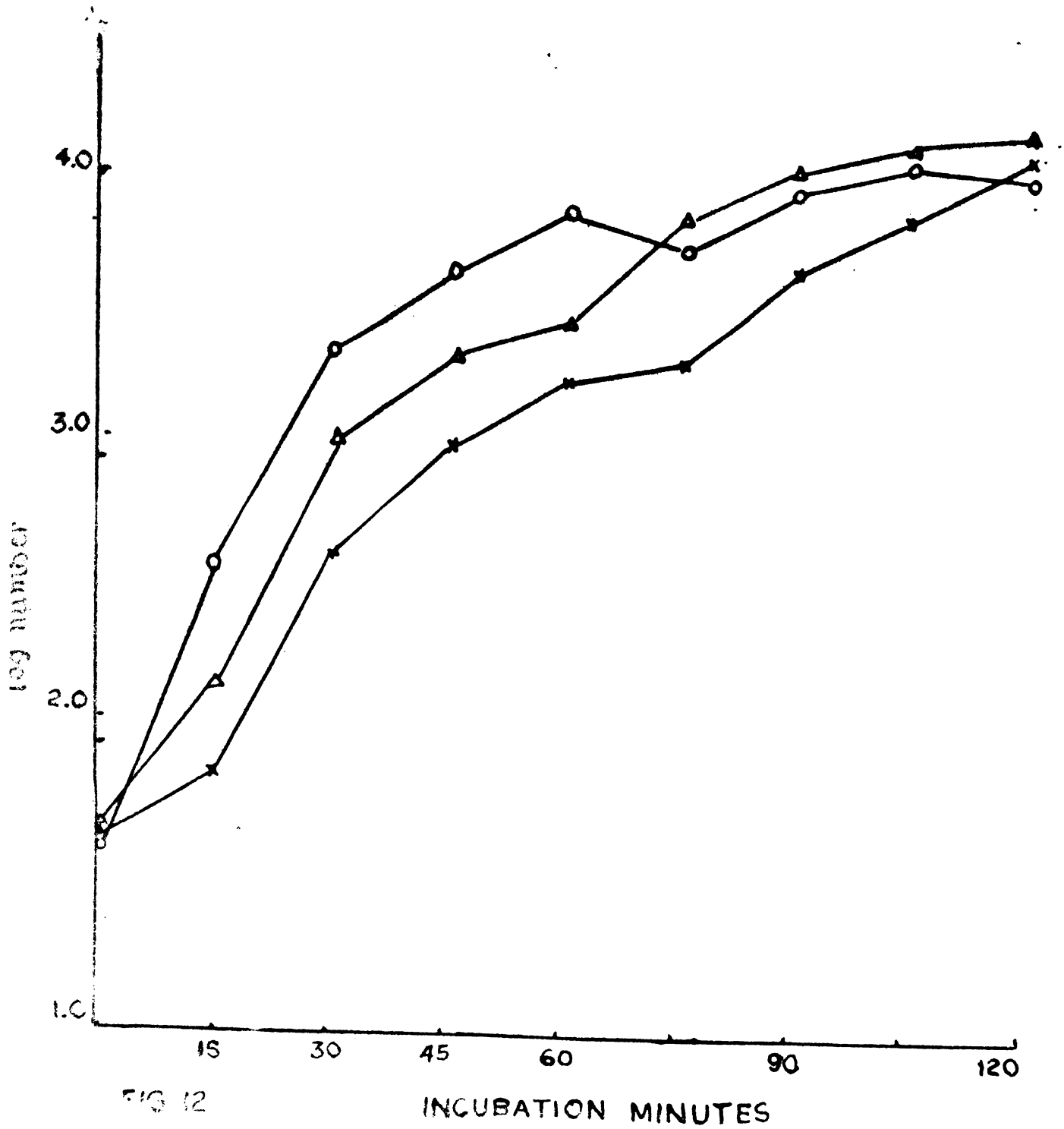


FIG 12

[illegible] BY [illegible]

[illegible] SONATE OF P. [illegible]

[illegible] SONATE OF P. [illegible]

CHAPTER FIVE

5.0. EFFECT OF HEAT ON THE SURVIVAL OF SALMONELLA AND VIBRIO PARAHAEMOLYTICUS.

5.1. Introduction.

The survival of Salmonella and Vibrio parahaemolyticus during heating, in relation to the nature of the heating menstrua was worked by out by many authors (Hansen and Riemann, 1963; Stumbo 1949; Schmidt 1957; Ingram and Baross, 1953; Buttiaux, 1953; Brown et al., 1960; Goepfert et al., 1970; 1972; Beuchat and Worthington, 1976). These studies were mainly carried out by using various parameters such as water concentration, soluble carbohydrates (mainly sucrose)) salts, fats and proteins in the medium which protect the microorganisms or help them to develop thermal resistance. White (1952) found out that Streptococcus lactis and Streptococcus faecalis were resistant to heat in milk than in Ringers solution.

Dabbah et al., (1969) showed that milk whey and Tripticase soy broth(TSB) protected Pseudomonas spp from heat. Luedecke and Harmon (1966) reported that the amount of fat in milk did not affect the survival of Pseudomonas fragi during heating. Cotterill and Glauert (1969) reported that the thermal resistance of Salmonella in egg products increased by the addition of common salt (NaCl). The effect of sugars such as sucrose in egg products was considerablly lowering the resistance. Goepfert et al., (1970) reported that the heat resistance of Salmonella was increased as the water activity of the heating menstruum was reduced. Various workers (Beuchat 1973; Beuchat and Worthington 1976; Covert and Woodburn 1972; Vandersant and Nickelson, 1972; Vandersant et al., 1974; Ray et al., 1971; Simensky 1974; Marr and Ingram 1962), reported the thermal sensitivity of Vibrio parahaemolyticus when heated in either laboratory prepared media or in either food products. Vibrio parahaemolyticus when grown in higher concentration of sodium chloride of about 7.5% and showed higher thermal stability (Beuchat and Worthington 1976). However Vibrio parahaemolyticus is considered to be sensitive to thermal changes when heated in either laboratory medium, or shrimp substrates (Beuchat 1973; Covert and Woodburn 1972; Vanersant and Nickelson 1972). Their recovery after

thermal assaultis greatly influenced by the substrates nutrients in recovery media (Vandersant et al., 1974).

It is well known that bacterial cells are more resistant to dry heat than the moist heat(Baird Parker et al., 1970; Frazier, 1966; Murrell and Scott, 1966; Goepfert et al., 1970; Moats et al., 1971) demonstrated that the vegetative bacterial cells gain more thermal stability as the solute concentration of the heating menstrua increases. This increase has been often interpreted to be the consequnce of reduced water activity (a_w) which is considered to be an important factor. It was found that the chemical nature of the solute was more influencial at the water activity levels above 0.75 on the heat resistance of Salmonellae (Goepfert et al., 1970). The findings of Baird Parker (1970) and Moats et al., (1971) were in agreement with the above influence.

Covert and Woodburn (1972), established the relationship with temperature and NaCl concentration on the survival of Vibrio parahaemolyticus in tripticase soy broth and fish homogenates, and showed that NaCl with optimal range (3 to 12%) protected the cells at 48± 1°C . So the survival of pathogens in fishery products will cause concern since these

organisms are the major cause of gastroenteritis.

In recent years the seafood industries were trying to produce diversified fishery products and cater both the inland markets and the export markets. Restaurents and Cafeteria usually prepare ready to serve foods, especially seafoods whcih may become in the near future. Teh data on the aspect of chemical constituents of seafoods whcih influence heat resistance in pathogens like Salmonellae and Vibrio parahaemolyticus are quite limited. These studies are receiving more importance in India, with the increasing concern about the presence of certain public health-related microorganisms in fish and fishery products. The study on the effect of various fishery products which contain rich organic nutrients capable of increasing the heat resistance of these organisms were carried out.

5.1. METHODS AND MATERIALS.

5.1.1. Different serotypes of Salmonella used.

All the strains of Salmonella spp. used in this study were isolated from various fish and fishery products. Stock cultures were maintained on nutrient agar slants at room temperature and routinely

resultured once in three weeks. Working cultures were transferred daily into nutrient broth of pH 7.0 and incubated at 37°C.

a) Test media: L-Aminoacids used in this experiment were E.Merck & Co. and B.D.H. peptone Tryptone and Yeast extract were obtained from Difco. The sugars used in this study were pure chemicals received from B.D.H. All these nutrients were dissolved in 0.1 M phosphate buffer having a pH 7.0. All the media were stetilised by autoclaving at 15 lbs, for 15 minutes.

b) Homogenates: Fish and shellfish homogenates were prepared from Cat fish (Tachysurus spp) Mackerels (Rastrelliger kanagurta), Shrimp (Penaeus indicus) and Crab (Scylla serrata) as per methodology given in section 4.2.2.

5.1.2. Determination of heat resistance.

All the strains mentioned in table 41 were inoculated from slants to Tripticase soy broth and incubated for 24 hours at 37°C. Teh cells were harvested by centrifuging and washed twice in sterile saline and kept at room temperature till the inoculation studied were carried out.

The heating temperatures chosen for this study, were 50°C, 55°C and 60°C.

since the restaurents and Cafeteria in Metropolitan cities usually keep at these temperatures, the prepared seafoods in advance for catering. It is impossible to measure the heat resistance of organisms in prepared dishes under all levels of bacterial concentrations, but calculation of the thermal resistance based on limited number of experiments is possible.

Two ml. of the grown Salmonella centrifuged culture was added to 200ml of the test medium that had been kept in a water bath and q equilibrated to different temperatures of 50°C, 55°C, and 60°C for these experiments. The heating vessel used in these experiments was a 500ml three necked vessel. During heating the contents of the flask was agitated frequently so as to have a uniform temperature in the test media by using a small mechanical stirrer positioned through the centre neck of the flask. A glass thermometer was mounted through a second port to monitor the temperature during the experiments. The thisd port was used for the introduction of test organisms and for sampling out the material for the evaluation of surviving organism during the course of the heating period.

5.1.3. Enumeration of Salmonella.

The initial inocula level of Salmonella serotypes present in the heating menstrua were simultaneously evaluated by adding 2ml of centrifuged cells to 200 ml of test media and in homogenates of fish and shell fishes and determined the cell counts for various serotypes of Salmonella and they were in the range of $2.1x10^8$ to $8.4x10^8$ per ml.

During heating period one ml of samples were drawn and added to 9 ml of sterile saline as a diluent at room temperature. Sample of 0.5 ml of serially diluted samples, were surfaced streaked in duplicates on prepared prepoured and surface dried Tripticase soy yeast extract agar (Yeast extract 0.25%). The streaked plates were incubated at 37°C for 24 hours. In most trials, streak plates were used.

In the case of homogenates, the three tube M.P.N. technique was employed to enumerate the survivors. In this pre-enrichment procedures one ml samples of appropriate dilution were incubated in nutrient broth for 6 to 8 hours at 37°C and again one ml portion was transferred to tubes containing 9ml Tetrathionate and selenite broths. This enriched media

were incubated at 37°C for 24 hours. A loopful of each broth was streaked either in Brilliant Green agar, or Hektoen enteric agar and incubated at 37°C for 24 hours. Typical colonies were confirmed qualitatively as Salmonella by using polyvalent 'O' antisera. The M.P.N. values were calculated on the basis of the patern of positive dilution in the series.

5.1.4. Evaluation of D values.

D values(the death rate of an organism at given temperature, also termed as death constant or decimal reduction time) were calculated as follows: few various homogenates of fish and shell fishes.

The number of survivors were plotted on semilog paper, on the logrithmic scale against time in minutes on the linear scale and the best straight line was drawn through them. The D values is the time in minutes required for this curve to traverse one logrithmic cycle. It is also the time in minutes at a constant temperature necessary to destroy 90% of the organism present. Trials were conducted for a period of time sufficient toresult in 5 log cycle drop in viable cells. With serotypes of S. anatum, S. typhimurium,

S. cubana, S. chester, S. weltevreden and S. salford, gave diphasic curve, i.e. in the initial phase the plots showed rapid deaths followed by a phase in wbch death proceeded at the slow rate. In such types, curves of the decimal reduction (D) values were obtained from the portion of the curves showing slower rates. D values were calculated by the following equation recommended by National canners and Association Research Laboratories (Laboratory Manual for food canners and Processors, p.190, 1968).

$$L = \frac{U}{\log a - \log b}$$

where

L = death rate in numbers,

U = heating time in minutes,

a = initial numbers of organisms

b = number of survivor organisms which survived time U.

5.1.5. Extent of injury and repair.

The extent of repair of injury and initiation of growth after heat treated microorganisms depends on the isolation and unumeration of the media

employed . Iandolo and Ordal (1966), showed that Staphylococcus aureus are less tolerant to salt for isolating them after heat treatment. Mossel and Ratto (1970), proposed a nonselective enrichment media as prelimary resuscitation. Normally this procedure should allow the recovery of metabolically injured cells, which after, different physical stresses, are unable to multiply in a selectively defined media and require nutrient supplementation.(Straka and Stokes 1959). But however Gomes et al., (1973), showed that the phenomenon of metabolic injury depend on the pretreated growth conditions whcih also has influence on the recovery media.

The enumeration of repair of injury after heating at different temperatures of 50°, 55° and 60°C for twenty minutes and fifteen minutes of various serotypes of Salmonella organisms which were isolated from various seafoods, were used in this study. Organisms of Salmonella, whcih were in late exponential phase were grown in Tripticase soy broth and harvested by centrifugation, washed in sterile saline water and resuspended in sterile saline water.

A known concentration of cells ($1x10^8$ per ml) were inoculated in Tripticase soy broth modified M-9 medium (Adams 1959) whcih contained

Na_2HPO_4 -7.0gm; KH_2PO_4 -3.0gm; NaCl -0.5gm; NH_4Cl -1.0gm; $MgSO_4 7H_2O$ - 0.25gm; Dextrose -2.0gm per litre of distilled water,,and fish homogenates and shellfish homogenates. For the lower temperature of 50°C, time taken for the heat injury study was 30 minutes and for higher temperatures of 55° and 60°C, 15 minutes duration were used. Heated samples were studied for their cell death , injury and repair in Tripticase soy broth as well as in their respective homogenates of fish (E. suratensis spp/ and (Rastrelliger kanakurta) and shell fishes (Penaeus indicus and Scylla serrata) by asceptically weighing 25 gm in 225 gm of the respective homogenates and 10 ml to0 90 ml of respective liquid broths. The serially diluted where ever necessary in phosphate buffer and 0.5 ml of samples were streaked in Xylose lysine agar, and Xylose Lysine Desoxycholate agar and incubated at 37°C for 24 hours.

The control was prepared by taking late exponentially grown cultures of Salmonella strains in tripticase soy yeast extract broths and homogenates and plated in Xylose Lysine agar and in Xylose Lysine Desoxycholate agar at regular intervals. The plate counts were taken after incubation of 24 hours at 37° C.

The stressed cell recovery studies were carried ~~xxxxx~~ out upto 120 minutes of incubation at room temperature with an interval of 15 minutes. In this study, thermal injury and repair by growth promoting compounds such as amino acids, vitamins and nucleosides bases and carbohydrates were evaluated for different serotypes of Salmonella. The preparation of these media schedule is shown below. In this study it is aimed to find out the ability of individual growth promoting compounds to aid the classical metabolic injury of cells in theses organic media.

In the modified broth of M-9, supplemented with the following compositions of nucleosides of bases (adenine, guanine and uracil, 10 µg/ml each); vitamins (biotin, choline, folic acid, inositol nicotinic acid , para amino benzoic acid, pyridoxine, pantothenic acids, thiamin and vitamin B_{12} : 10 µg/ml of each) and aminoacids (alanine, arginine, aspartic acid , cystine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenyl alanine, proline, serine, threonine, tryptophane, tyrosine, valine: 100 µg per ml of each).

Media schedule for the recovery of heat injured or stressed cells of Salmonella.

1) M-9 broth alone.

2) M-9 broth with nucleosides bases.

3) M-9 broth with vitamins of concentration f0 ug/ml each.

4) M-9 broth with all amino acids of concentration of 100 ug/ml.

5) M-9 broth with yeast extract of 0.5%.

6) Tripticase soy broth (Difco)

7) Brain Heart Infusion broth.

5.2.1 RESULTS.

The table 41 gives the details of the survival of Salmonella serotypes exposed to the different temperatures. On a glance of the results the lesser survival in the liquid broths of Tripticase soy broth and in M-9 broth than in the respective homogenates is evident. In this study, initially the bacterial load was taken in the range of 2.1 to 7.6×10^8. At $50^{\circ}C$ heating , Tripticase soy broth showed reduction of about 3 log cycles while M-9 broth showed as reduction to 4 log cycles(figs. 13 a,b). When the temperature was increased to $55^{\circ}C$ and $60^{\circ}C$, the reduction was 6 log cycles in the TSY broth and 7 log cycles in M-9 broth. Similar phenomenon of reductions were noted in the different homogenates of fish and shellfishes also, but

the difference was on the higher survival (Figs 14 a,b). The Salmonella serotypes taken for this study exhibited the phenomenon of reduction except for S. senftenberg, which exhibited the higher resistance to heat at 50°C than at 55° and 60°C. The results indicated more survival diference among the serotypes in the various homogenates(Figs. 15, 16 & 17). It has been noted that the survival of varoius Salmonella strains were not proportional to the time except for the serotypes, S. newport, S. saintpaul, S. weltevreden.

The effects of various amino acids protection at a concentration level of 200 ug per ml were shown in tables 42 and 43. Greater protection was exhibited by the amino acids, alanine, glycine, lysine proline and serine at a temperatures of 55° and 60°C. No significant protection was noted at 60°C. The sulphur amino acids cystine and methionine showed less protedtion, or in other words, it is slighly increased the sensitivity to heat. The protective effect of individual amino acids was compared and found it to be lesser than that of Tripticase soy yeast extract broth. At 60°C, the protection was less in all pure amino acids solutions.

On studying the commercial peptides which are commonly used for the bacterialogical media, it was observed that protection was more than for pure amino acids, used in this study(table 44). At 55°C, yeast extract (1% level) gave more protection than the Tripticase , peptone and triptone. But at 60°C all these peptides showed sensitivity to heat.

The muscle protein of fish and shell fishes gave substancial protection to heat at 55° and 60°C (table 41). The commercial preparation of ready to serve sea foods in Cafeteria and Restaurents where the preparations have the possibility of contamination of these pathogenic organisms and warmed for shorter duration at a temperature of either 55° or 60°C., showed the chances of survival of the Salmonella strains (Figs. 15, 16, & 17). This is a very significant observation that fish muscle proteins need protection from the lethel effects of these organisms.

Regarding the carbohydrates (table 44) no significant protection was seen either at 55° or 60°C. Only sucrose and Rhamnose gave some protection at 55°C, but at 60°C it increased the sensitivity to heat.

The time to kill in minutes of various organisms at a level of 10^8 per gram to undetectable level in various homogenates of fish and shell fishes are presented in table 45. The higher the temperature the lesser the time to kill the organisms e under study. The C_{60} value (minutes of exposure at 60°C required to effect 100% destruction) are as follows: S. senftenberg in fish muscle, crab muscle and prawn muscle homogenates(50%) 80; for other strains of Salmonella the values varied from 25 to 30. The end points of survival-kill at all the test temperatures for both S. senftenberg and other Salmonella spp varied much.

Thermal death times (average D values) of various Salmonella serotypes on different temperatures of exposure showed higher values for homogenates than the liquid broths of Tripticase soy broth, and M-9 broth (tables 46 and 47). At 50°C the average D values of different species of Salmonella in Tripticase soy broth, and in M-9 broth registered value from 61.6 to 86.4 and corresponding homogenates showed higher values of 65.8 to 99.3. Similarly the D values (tables 46 and 47) at higher temperatures showed corresponding lower values. These results are expressed as mean

values of the three or four experiments conducted. The results indicated that S. senftenberg was highly resistant than the other species studied. In other Salmonella spp. the mean D values at 55° and 60°C were more or less the same and no significant higher resistance was noted.

These data indicated that perishable marine seafoods of the types studied in the form of homogenates at 65°C and holding every particle of seafoods at this temperature for at least 15 minutes reduced the Salmonella numbers to an undetectable level. The same degree of destruction was also achieved similarly when homogenates were kept at 60°C for 75 to 80 minutes for S. senftenberg and for other species of Salmonella for 30 minutes.

When the Salmonella organisms were suspended in various homogenates of fish and shellfishes and in Tripticase soy broth after heat treatment the classical metabolic injury recovery was observed (tables 48 and 49). On comparing the homogenates after heat treatment, at 50°C the bacterial load showed that the shell fish (prawn and crab meat) homogenates indicated a higher survival than the fish homogenates. In general it was observed that all the homogenates at

50°C yielded higher recovery of thermally stressed Salmonella than in Tripticase soy broth and M-9 broth. In the case of heat resistant organisms of S. senftenberg, a higher percentage recovery than the rest of the Salmonella. organisms was found.

Heating at 55° and 60°C for 30 minutes and 15 minutes respectively revealed no significant recovery both in homogenates and in liquid broths of Tripticase soy broth and M-9 broth(table 50). Due to the higher temperature of heating, the total bacterial load came down to the range of 100 at 55°C and of 10 at 60°C. Individual serotypes varied accordingly to the initial concentrations inoculated in the heating menstrua. At the higher temperature also S. senftenberg showed higher resistance than the other serotypes studied. At higher temperatures, the recovery of Salmonella serotypes were lesser in the homogenates, but on the contrary, S. senftenberg recovered in higher numbers from the fish homogenated.

When cultures grew in the various homogenates after heating at 50°, 55° and 60°C the thermally injured cells normally recovered within two hours. The post heated organisms in the various homogenates during incubation for 120 minutes at room

temperature, showed the recovery of injured cells when plated on Xylose Lysine Desoxycholate agar (tables 48 and 49). At 50°C, the death cells were less when compared to those of the higher temperatures of 55° and 60°C. All the heat stressed cells were repaired in Tripticase soy broth and their respective homogenates.

5.3. DISCUSSION.

The results of the present study on the various homogenates present in foods which protect the organisms of Salmonella of various serotypes from heat. Fish and shellfish homogenates and Tripticase soy broth gave more protection than M-9 broth which is more chemically defined media. However, the protection was more in the semisolid media than in the liquid media. The earlier reports (vide supra) suggest that the total solid contents of homogenates are higher than in the chemically defined media and that they protect the cells from the heat of reduced water activity (a_w) in foods (Calhoun and Frazier 1966; Hansen and Reimann, 1963; Moats et al., 1971; Pierson et al., 1971; Leudeke and Hansen 1966). Earlier Scott (1957) showed that the water activity (a_w) greater

than 0.99 in all media gave less protection during heating. Goepfert et al., (1970), concluded that the chemical nature of the suspending medium was more important than the water activity of the range 0.75 to 1.00. However, the explanation of Moats et al., (1971), suggested that the compounds giving the protective effects to the heat sensitive protein molecules in the cells became stable to heat. And further, they have stated that enzyme molecules are protected by heat. Still further, Read et al., (1968), showed that S.anatum S. hinga, S. cubana, S. tennese and S. senftenberg 775W survived during pasteurisation when the cell concentration exceed $3x10^{12}$ per ml of the milk.

Of the three temperatures studied, the protection was less at 60°C than at the other temperatures of 55° and 50°C. It is also true that a high concentration of fish muscle homogenates protect Salmonella cells from heat.

It was found that the carbohydrates also have little control on the relative protecting effects of the organisms (Goepfert et al., 1970). On the analysis of carbohydrates at a temperature of 55°C, large differences in the protective effect of heat were observed . Xylose, Rhamnose and glucose protect

the S. weltevreden, S. enteritidis, S. senftenberg, S. saintpaul and S. cubana to some extent. Since other Salmonella also behaved similarly the data are not presented here. Calhoun and Frazier, (1966) observed similar protection by glucose for Escherichia coli and Pseudomonas fluorescens. At a higher temperature of 60°C, no protection was observed.

Among the amino acid parameters studied, sulphur amino acids likemethionine and cystine showed increased sensitivity to heat at 55° and 60°C. Similarly, casein and triptone also showed lesser protection to heat. This may be due to sulpha-hydryl groups which might spilit S-S bonds in proteins thtem more sensitive to heat. Moats et al., (1971), also that casein, cystein, glutathione and sodium citrate increased the sensitivity to heat for S. anatum. They further explained that the sodium citrate may pull Ca^{++} and Mg^{++} ions out of the cells and thereby increase the sensitivity to heat.. Strange and Shon (1959), reported that Mg^{++} ions decreased thermal death rate and that chelating(compound) agent ethyline diamino tetraacetic acid which bonds Ca^{2+} and Mg^{2+} ions increased the thermal death rate.

Individual amino acids, alanine, aspatic acid, glutamic acid, glycine, hydroxy proline,

lysine, proline and serine showed better protecting effect than the other amino acids. At lower temperature of 50°C, almost all amino acids protected the Salmonella organisms. Moats et al., (1971), reported that protein had little effect although peptides and aminao acids play substancial role i protection. Earlier in 1964, Gerhardt and Judge gave an explanation that the bacterial cell wall act as hetreporus molecular sieves to exclude macromolecules. Since proteins are macro molecules and unable to penetrate the the heat sensitive sites in the cell wall, the bacterial cell becomes insensitive to heat.

The effect of time and temperature in killing various serotypes of Salmonella were studied in different homogenates of fish and shell fishes. The thermal death studies showed that by increasing the temperature from 60°C to 65°, the death rate was increased eventhough the time taken was less. In fact the end points survival-kill at all the test temperatures for the organisms tested were considerably less than for the other foods studied (Angelotti et al., 1961). It must be remembered here that these data indicate that heat perishable foods of the types studied here in the form of homogenates maintained at

temperature of 65°C for at least 10 minutes, recorded a reduction from 10^8 Salmonella per gram, to an undetectable level. The lesser temperature of 60°C for 20 minutes reduced safely the same bacterial numbers to undetectable level.

It is observed from the results obtained with heat resistant S. senftenberg, that a large difference in the heat resistance was not found. S. senftenberg 775W (Soloway, Sutton and Calesmick, 1948; Osborne et al., 1954; Angelotti et al., 1959; Angelotti et al., 1961) is exceptionally tolerant to heat The results of this study are unexpected in view of the earliar work above. They found greater resistance in the custard and chicken à la king. Their C_{60} values in custard and in chicken à la king were 78.0 and 81.5 respectively. In this context it is important to note these organisms were originally isolated from egg products: therefore it is reasonable to assume to have greater resistance to heat, while organisms for this study were isolated from fishery products.

The other serotypes of Slamonella organisms were the least heat resistance studied and yielded low C_{60} values in keeping withthose generally recognised for Salmonella (Solomey et al., 1948; Anellis et al., 1954; Osbourne et al., 1954).

In these experiments the thermal death time varied for the same pure culture, because of culture manipulation. It may be due to the number of variables which cannot be controled such as intial numbers of viable cells taken for the inoculation and the growth conditions and their different growth phases. Similar variations of thermal death time of pure culture were reported by Olsen et al., (1952), on E. coli and by Moats et al., (1971), on S. anatum. So far as possible the experiments were conducted with various media simultaneously with single culture. Moats et al., (1971) recommended and showed that the D values can be satisfactorily compared (survival) in different media at a given temperatures of 50°, 55° and 60°C were selected because little work was carried out on the survival of various serotypes of Salmonella isolated from marine seafoods. The required temperatures of most of the ready to serve seafoodsare maintained in advance for cattering in various metropolitan city hotels and restaurents. Moreover, the survival differences are more at that time and temperature combinations. The D_{60} values (table 47) emphasise the relative short period of time from that of the whole treatment required to kill 90% of the microbial population and

the values are independant of the initial load of the organisms (Youland and Stumbo 1953; Pfleg and Esleen 1954; Schmidt, Angellotti et al., 1961). Although some evidences differ with this inference, (Reed et al., 1951; El-bisi and Ordal 1956 a,b), a greater flexibility is available for interpreting heat resistance data than by thermal death time determination alone. Earliar reports of Bigelow and Esty (1920) recognised that heat resistance of organisms was independant of the initial concentration. The C values for different suspensions were not comparable, unless the initial inoculum levels were identical. By studying the D values, it is posible to determine the heat resistance of an organisms and make a direct comparison possible between data in whcih various amounts of inocula of Salmonella organisms used for the study. In order to avoid the dependance on inoculum size, Winter et al., (1951), and Osborne et al., (1954) followed methods in which the thermal resistance can be determined in given substrates under various conditions of microbial load. This fact is more important particularly in a study of this type presented here. From the practical stand point, it is impossible to measure the heat resistance of Salmonella serotypes in prepared foods and dishes under all levels of microbial load. However, the calculated D values can be used to predict the thermal resistance.

Based on the limited number of experiments, it is possible to give D values for many bacterial concentrations which are attainalble seafoods.

The C_{65} values of other Salmonella strains reported here are in agreement with values reported by the other workers (Winter et al., 1951; Osborne etal., 1954 and Anallis et al., 1954) and the values are of the order of 3-5. But these values were considerably less than the values of S. senftenberg. The extended time of 10 minutes will kill the organisms to an undetectable level and thus become advantageous also. The results indicated here that perishable foods like marine seafoods of the type employed in this study kept at 65°C for less than 12 minutes safely reduced Salmonella organisms to an undetectable level.

It was intersting to note that S. barielly showed extraordinary heat resistance in prawn and crab homogenates and this was isolated from fishmeal and prawn shell wastes. This would be easy to speculate because this resistance enabled the S. barielly strain to survive the processing treatment. This aspect of correlation beween resistance to environmental conditions and frequency of isolation has been suggested (Enkiri and Alford 1971). Cotteril and

Glaubert reported similar behaviour for S. oranienberg held in either in Tripticase soy broth or egg white containing sodium chloride prior to heating. Lega, et al., (1972) reported that concomitant growth by the microflora on milk was influenced by the heat resistance of Salmonella.

The recovery of heat treated Salmonella organisms depended upon the isolation of the enumeration of the media employed. Mossell and Ratto (1970), suggested its use for the resuscitation in non-selective medium or enrichment broth. This procedure should allow the recovery of metabollically injured cells (Straka and Stokes, 1959) which after a variety of physical stresses, are unable to multiply on a single defined media or selective media and require rich nutrients to be supplemented for the repair of the injured cells. The metabolic injury will depend upon the pretreatment growth conditions (Gomez et al., 1973).

The heat treated Salmonella cells were studied for their cell repair by incubating on the respective homogenates and Tripticase soy broth and M-9 broth. It was observed that metabolically injures cells showed growth. On comparing the liquid broth of Tripticase soy and M-9 broth, the former yielded

higher recovery of injured cells. This result is in agreement with the findings of Gomes et al., (1973). This suggest that the lower recovery may be due to the nonavailability of the special nutrients such as amino acids, nucleosides and carbohydrates.

When the organisms were suspended in various homogenates of fish and shellfishes, the initial growth was upto 15 minutes and in 60 minutes the repair was rapid and become ineffective to the selective inhibitors such as desoxycholate. The rate of injury of cells and death will depend upon the temperatures to which it was exposed. At 50°C the rate of death of Salmonella serotypes studied showed about 2 log cycles reduction. At 55°C and 60°C, the r reductions were very high up to 5 log cycles respectively. The injured surviving cells started growing up after one houf. A similar observation was found in Tripticase soy broth . The higher temperature of 60°C showed considerable percentages of death and the majority of cells could not recover in a noninhibitory media (Nelson 1943; Allwood and Russell, 1966; Harries and Russell, 1966 ans Russell and Harries, 1968).

The effect of thermal injury repair by the growth promoting compounds such as amino

acids, vitamins, nucleosides and carbohydrates showed that individual compounds alone could not give the proper boost for the cell repair. All these in combination in M-9 broth reached almost the same extent as in Tripticase soy broth growth repair.

These findings substantiate the inference of Allwood and Russell (1966), Harries and Russell (1966), and Gomez et al., (1973), who had reported that the addition of yeast extract , 20 amino acids, casamino acids, vitamins and nucleosides will repair the sublethally heated cells of coliforms and Salmonella. Mukerjee and Bhattacharjee (1970), had also found an increase in counts in nutrient agar during incubation in phosphate buffers of heat treated E. coli. Gansen and Smith (1968; 1971) extensively studied the recovery of lethally injured cellsof E. coli by radiation. They concluded that the injury phenomenon is due to molecular fast hitting during heating which injures the periperal cell membrane. When these cells (injured) were subjected to the complex media, this require the synthesis of deoxyribonucleic acids (DNA), and resulted in minimal medium recovery. Woodcock and Grigg (1972) and s Sedjwick and Briggs (1972), showed the presence of single and double strand and breaks in the DNA mole-

cule of *E. coli*, after heat treatment and the former group showed the restitution of these organisms during incubation in phosphate buffer.

Indola and Ordal (1966), showed that heating of *Staphylococcus aureus* to 55° C stopped the leakage of intracellular constituents and at a higher temperature of 60°C, the intra cellular leakage and loss of viability of microorganisms were more. They further reported that the presence of sucrose gave some protection against the thermally induced death but sucrose failed to prevent the leakage at 60°C, eventhough the cell membrane damage appeared to be reduced . The greater reduction of *Salmonella* survival at 60°C may be explained in the light of the above facts that intracellular proteins of the cells coagulate and emulsified with RNA like materials and thus further prevents leakage. At this temperature, the muscle proteins of fish and shellfishes could also reduce the protein coagulation so that increased leakage could result. At 50°C, the partial protection against leakage was afforded by the muscle proteins.

5.4. PREPARATION OF SAMPLES AND MEDIA FOR VIBRIO PARAHAEMOLYTICUS.

The strain of Vibrio parahaemolyticus for this study was the same as the one described in the section 4.4. on the effect of freezing. Pure cultures were grown in Tripticase soy broth with addition of 3% sodium chloride (pH 7.0) at room temperature for 18 to 24 hours. The cells were harvested and washed in sterile 1% sodium chloride solution and suspended in sterile 1% sodium chloride solution.

The effect of Vibrio parahaemolyticus on the thermal stability in variuos concentrations of sodium chloride in Tripticase soy broth and in various homogenates of fish and shell fishes, various amino acids mixtures, yeast extract, vitamins and nucleosides in phosphates buffers at temperatures of 50°C and 55°C were carried out.

Heating menstrua such as Tripticase soy broth (pH 7.2, containing various salt concentrations of sodium chloride (0.5%, 3%, and 7.5%); Fish and shellfishes homogenates having the above salt concentrations, L-aminoacids, vitamins, peptones and tryptones in phosphate buffers (vide section 5.2.5.)

described elsewhere with above salt concentrations were sterilized and used for this study.

5.4.1. Determination of heat resistance.

For the heat treatment, the washed Vibrio parahaemolyticus cells of concentration used in the heating menstrua of the range of 1.0×10^8 per gram or ml was predetermined. Heating menstrua were kept in a water bath at different temperatures of 50°C and 55°C. When the required temperature was attained, 5 ml of washed Vibrio parahaemolyticus cells were added to 200 ml or gram of heated menstrua and frequently agitated so as to makee the temperature uniform. At regular intervals of 15 minutes, one ml of gram as the case may of sample was withdrawn asceptically and serially diluted with 0.1M K_2HPO_4 (7.2) containing 3% NaCl The assay medium used for the recovery was Tripticase soy agar containing 3% sodium chloride. Serially diluted samples of 0.5 ml was surface streaked in T.S agar containing 3% Na Cl. The streaked plates were incubated at room temperature (30± 2°C) for 18 to 24 hours.

In the case of homogenates of fish and shellfishes three tube MPN method was used for enumeration of survivers. In this case pre-enrichment

procedure, one ml of appropriate sample was added to Tripticase soy broth and kept for 12 hours at room temperature and then transferred one nl portion to 9 ml Tripticase soy broth. This enrichment was incubated at room temperature. Further, a loopful of each broth was streaked in Thiosulphate citrate bile salt agar.

5.4.2. Enumeration of thermal death time values (D values).

The death rate of Vibrio para haemolyticus organisms at temperatures of 50°C and 55°C wass studied in the above liquid broth and in different homogenates of fish and shellfishes. The number of heat surviving viable cells (log 10) were plotted in semilog graph on the logrithmic scale with time in minutes at 50°C and 55°C. Decimal reduction times of D_{50} and D_{55} (time in minutes required to destroy 90% of the viable cells of Vibrio parahaemolyticus were from the graph. The values represented were the mean values of three or more independent trials.

5.4.3. Evaluation of injury and repair of Vibrio parahaemolyticus.

Preliminary experiments on the recovery of stressed cells of V. parahaemolyticus

showed no appreciable difference between nutrient agar with 3% NaCl and Tripticase soy agar containing 3% sodium chloride. So far the total recovery of injured cells, Tripticase soy agar with 3% sodium chloride was used in this study.

Washed cells were resuspended in 3% NaCl and 5 ml inoculated in phosphate buffer (pH 7.0) containing 3% Na Cl, T S broth, homogenates of fish and shell fishes as described in the section 5.2.1. of methods and materials and aminoacids media. At the desired temperatures of 50°C and 55°C the cells of concentration of 1.0×10^8 per ml/gm were inoculated and heated for 30 minutes, with frequent agitation. At the termination of heating period, samples of 1 ml were immediately withdrawn and serially diluted and streaked in prepoured Tripticase soy agar with 3% NaCl and in Thiosulphate citrate bile salts agar for the recovery of Vibrio parahaemolyticus. The plated of Tripticase soy agar and TCBS were incubated in duplicates at room temperature for 24 hours or 48 hours as the case may be before counting. When metabolic inhibitor like TCBS used, the injured cells could not be recovered. The difference of the recovery between TSA and TCBS indicates the injury level.

5.5. RESULTS.

The results of the study of the survival of Vibrio parahaemolyticus strains in various heated menstrua containing different concentrations of sodium chloride (low, medium and high) are given in tables 49 and 50. A relatively high inoculum of cells of Vibrio parahaemolyticus ($1x10^8$ per ml or gram) was used. In comparing the different broths and homogenates of fish and shellfishes (vide supra) the latter gave greater protection than the former. Homogenates of fish and shellfishes with increasing concentrations of NaCl significantly increased the surviving numbers of Vibrio parahaemolyticus. The cells were rapidly decreasing at 55°C, due to the higher temperature. The data presented herewith four strains and the two type strains (table 49) showed the lower counts when heating proceeds. T S broth, chemically defined aminoacids media, peptone and tryptone registered lesser survival than the homogenates. A very sharp drop was noted in the lower concentrations of sodium chloride (0.5%) in broths and homogenates through out these experiments. T S broth heated at 50°C showed a higher survival in 3% and 7.5% sodium chloride concentrations.

On comparing the increasing salt concentration of 3% and 7.5%, the rate of survival of Vibrio parahaemolyticus was enhanced in greater proportions in the homogenates than in the liquid broths. Chemically added amino acids, vitamins and nucleosides of bases in phosphates buffer at pH 7.0 did not enhance the survival as in the homogenates. A similar pattern of results was obtained with peptones and triptone. The data of the present investigation indicated that 3% and 7.5% have greater resistance to the stressed conditions and enhance the survival of cells at 50° and 55°C.

Decimal reduction time D values were calculated from the thermal survivors curves. The curves did not show a proper smooth slope and it was often diphasic and of irregular form. D value calculatio were taken from the inactivation curves which had the greatest slopes and trancended at least 5 log reductions of the viable cells of Vibrio parahaemolyticus strains. At 55°C , the D values were reduced and the cells recorded lesser value of heat survival eventhough more sodium chloride was added. This was true for all the tests carried where in the percentage of sodium chloride was increased (7.5%). The D 55°C values are given in table 53. The D values were pronounced when cells of Vibrio parahaemolyticus were heated in homogenates of

fish and shell fishes. Chemically defined media of aminoacids, vitamins, nucleosidesof bases, peptone and tryptone have lesser values than the TSB and these homogenates.

The effect of isolating the injured cells during heating at 50°C and 55°C are shown in table 54. Heating at 50°C and 55°C for ten minutes in different substrates, such as TS broth, homogenates of fish and shellfishes and in chemically compounded amino acids, vitamins, peptone and tryptone broths indicated different rates of injury. At 50° C in TS broth the percentage of injury was morein lesser concentrations of salt (0.5%). The same trend was also seen in all the media where the salt concentrations were less (0.5%). With increased salt concentrations, the injury was less with 3% and 7.5% for the strains of Vibrio parahaemolyticus including type strain of NCIB 1902 and 3525.

Heat injured cells is placed in Tripticase soy broth with 3% sodium chloride recovered within two to three hours (table 55). Slow recovery within 30 minutes was reached by different salt concentrations of tripticase soy broth. Further they recovered fully within 30 minutes to 90 minutes. On comparing growth, the recovery in homogenates and

In comparing growth, the recovery in homogenates and Tripticase broth, no significant increase have seen even after adding 7.5% sodium chloride.

5.6. DISCUSSION.

Heat resistance of both isolated strains and type strains of Vibrio parahaemolyticus increased when salt concentration was increased. This observations are in agreement with the findings of Beuchat and Worthington (1976). In general the Vibrio parahaemolyticus organisms were more heat sensitive than the other pathogens such as Salmonella, Staphylococcus aureus, and Clostridia. By thermal heat, these organisms were inactivated in either laboratory media or in homogenates of fish and shrimp substrates (beuchat 1973; Covert and Woodburn 1972; Vanderzant and Nickelson 1972). But, these organisms can resuscitate after thremal assault by the influence of different chemical substrates and rich nutrients provided in the recovery media (Vanderzant et al., 1974; Covert and Woodburn 1972; Russel and Harries 1967; Beuchat and Worthington 1976; Enswiller, Pierson and Shoemaker 1976; Benis, Beuchat and Jones 1977). This is also in agreement on the observation on the

influence of temperature on the growth of Vibrio parahaemolyticus and on the nutrient supply in the growth media on the survival of these organisms during heating (Hienan et al., 1970; Kito et al., 1973; Merr and Ingram 1962; Ray et al., 1971; Sinensky 1976; Beuchat and Worthington 1976).

In this investigation the isolated and type strains of Vibrio parahaemolyticus survived in 3% and 7.5% salt concentrations in various media, and especially in substrates of fish and shrimps. These substrates influence the protection of these cells during heating to 50°C and 55°C. Obviously, Vibrio parahaemolyticus is halophillic and it requires salt for their growth and the salt protect them during heating. Christian and Wolbe (1974) also noted that the salt concentrations and temperatures affectedthe rate of growth of Vibrio spp. In thermal survival studies, the cells of the late exponential growth were used as suspensions. In recent years, different research workers, (Russell and Harles 1967; Covert and Woodburn 1972; Beuchat and Worthington 1976) studied the effect at lower temperatures on the survival of Vibrio parahaemolyticus and therefore this study was made on the survival on these at higher temperatures of 50° and 55°C in various broths and substrates with different

salt concentrations. Increased thermal resistance was found when a menstrum containing higher percentage of salts (Na Cl) was used. These data showed that the addition of sodium chloride to the heating menstrua had a protective effect against destruction. Calhoun and Frazier (1966) showed similar behaviour for E. coli. Enhanced heat resistance was reported for cells heated in media where in water activity(a_w) was reduced by controlled addition of sodium chloride (Beuchat and Worthington 1976). The protective effect of sodium chloride in heating media against destruction of sensitive Salmonella had also reported (Baird-Parker et al., 1970).

Resistance to heat stress of microorganisms is dependent on the chemical make up of the cell as well as the physiological nature of the heating environment. Several investigators (Beuchat and Worthington 1976, Baird-Parker et al.,1970. Calhoun and Frazier 1966, Christian and Wiebe 1974) showed co-relation between cellular nutrient composition and heat resistance. The changes of fatty acids content in the cellular components have marked influence on the heat resistance of Vibrio parahaemolyticus cells, (Beuchat and Worthington 1976). Covert and Woodburn

(1972) reported the protective effect and stabilisation effect at temperature of 48± 1°C with the optimal concentration of salt at 3% to 6%. A similar result was reported by Matches et al., (1970) and Tenmyo (1966)

D values of Vibrio parahaemolyticus were increased with increasing concentration of sodium chloride at50° and 55°C (Table 53). Beuchat and Worthington (1976), in their studies reported D values of these organisms that were higher than this study. The lower values may be due to the heat tolerance of these strains, and they may also dependant on the chemical make up or the nature of the heating environments. Moreover, it was found that maintaining the same heating environment for the study of D_{50} and D_{55} values was difficult since the substrates of different fish and shell fishes were caught in different environments and seasons which cannot be controlled physically. However, the resistance of Vibrio parahaemolyticus to varying concentrations of sodium chloride was significantly higher in all substrates of fish and shell fishes than in the broths under study.

Recently Emswiler et al., (1976) found that the heat injured Vibrio parahaemolyticus cells at 41°C when heated for 30 minutes in phosphate

buffer (100 mM) with 3% sodium chloride recovered in higher number in Tripticase soy broth. Similar results were also obtained by them in glucose salt medium (GSM) which was used to recover these cells injured at 45°C. So the injured Vibrio parahaemolyticus cells can be recovered either plating them in Tripticase soy broth containing 3% sodium chloride or glucose salt medium (GSM) with 3% salt (NaCl) for 3 hours. The recovery was independant on the proteins, ribonucleic acid and deoxyribonucleic acid synthesis (Emswiler et al., 1976)

The alteration of metabolic mechanism of injured cells is related to an increased lag time taken when the injured cells are placed in nutrient broth and Tripticase soy broth. Much work was carried out on the study of the effects of sublethal heat stress on microorganisms and the determination of these cells of biosynthetic process involved in the recovery of injured cells. Many research workers had found that the thermal injury in Staphylococcus aureus, Salmonella, Streptococcus faecalis, E. coli, Pseudomonas fluorescence, Vibrio marinus, Clostridium perfrigens involved alterations in their structural permiability and bio synthetic characters (Emswiler et al., 1976)

Cell membrane damage had been reported to be due to increased salt concentration sensitivity of these organisms (Stiles and Witter 1965; Iandolo and Ordal 1966; Clark and Ordal 1969; Clark et al., 1969). The leakage of the damaged intracellular compounds such as proteins, amino acids and metal ions etc., had been demonstrated by various scientific workers (Sogis and Ordal 1967; Strange and Shen 1964; Haight and Morita 1966; Kenise and Morita 1968; Allwood and Russell 1969,. The repair of these thermally injured cells was found to occur during recovery in the rich nutritional media.

The fish processing technology used in the manufacture of fishery products cause stress or injury to Vibrio parahaemolyticus cells that might be present. Sublethal stress on these cells can affect the metabolic and the reproductive functions which hamper the growth in the selective media which are commonly used to detect the presence of these organisms in foods.

Table 40. Relative effects of L- Aminoacids on the various serotypes of Salmonella by heating at 55°C for 30 minutes.

Heating Menstrua	S.weltevreden	S.enteritidis	S.senftenberg	S.saint paul	S.cubana
		Cunts per ml.			
Control TSB*	2.3×10^{8}	8.4×10^{8}	4.7×10^{8}	3.8×10^{8}	8.8×10^{8}
Heated	8.2×10^{3}	4.2×10^{3}	3.8×10^{5}	1.9×10^{2}	3.9×10^{2}
PO_4 buffer	2.1×10^{2}	6.4×10^{2}	4.4×10^{2}	3.6×10^{2}	7.5×10^{2}
L-Aminoacids in 0.1M buffer					
Alanine	8.3×10^{3}	3.3×10^{4}	2.4×10^{3}	1.9×10^{3}	4.9×10^{3}
Arginine	1.2×10^{2}	8.0×10^{4}	3.4×10^{4}	1.5×10^{3}	7.8×10^{2}
Asparagine	3.6×10^{2}	1.4×10^{2}	1.8×10^{4}	4.9×10^{2}	4.2×10^{2}
Aspartic acid	4.7×10^{2}	8.9×10^{2}	1.2×10^{4}	8.3×10^{2}	6.3×10^{2}
Cystine HCl	3.1×10^{2}	1.9×10^{3}	3.6×10^{4}	6.8×10^{2}	3.4×10^{2}
glutamicacid	1.3×10^{3}	2.3×10^{3}	2.4×10^{5}	4.8×10^{3}	7.1×10^{3}
Glycine	8.2×10^{3}	4.6×10^{3}	8.4×10^{5}	3.6×10^{3}	4.7×10^{3}
Hydroxyproline	5.2×10^{3}	3.9×10^{3}	3.7×10^{5}	6.1×10^{3}	1.9×10^{3}
Histidine	3.3×10^{2}	4.7×10^{2}	2.8×10^{3}	3.7×10^{2}	7.2×10^{2}
Isoleucine	4.7×10^{2}	5.7×10^{2}	2.1×10^{3}	8.1×10^{2}	6.3×10^{2}
Leucine	6.8×10^{2}	8.1×10^{2}	4.8×10^{3}	4.5×10^{2}	3.7×10^{2}
Lysine	4.8×10^{4}	3.6×10^{3}	7.9×10^{5}	3.9×10^{2}	4.8×10^{2}
Methionine	1.5×10^{3}	6.2×10^{1}	2.7×10^{3}	4.8×10^{1}	3.7×10^{3}
Phenylalanine	1.6×10^{3}	6.2×10^{1}	3.9×10^{3}	3.6×10^{3}	4.9×10^{2}
Proline	3.4×10^{3}	4.6×10^{3}	7.7×10^{4}	7.3×10^{3}	8.3×10^{3}
Serine	7.4×10^{4}	6.7×10^{3}	5.8×10^{5}	4.9×10^{3}	6.7×10^{4}
Threonine	2.3×10^{3}	3.8×10^{3}	4.7×10^{3}	6.7×10^{1}	7.8×10^{2}
Valine	2.7×10^{3}	8.3×10^{1}	4.9×10^{3}	6.8×10^{1}	9.4×10^{2}

Amino acids concentrations used in this are 200 ug/ml.

* Triptiease soy agarbroth

Table 41. Relative effect of L-Amino acids on the various serotypes of Salmonella by heating at 60°C. for 15 min.

Heating menstrua	S.veltev-reden	S.enteri-tidis	S.senft-enberg	S.saint-paul	S.cubana
	Counts per ml.				
Control					
TSB*	$4.3x10^8$	$6.4x10^8$	$5.4x10^8$	$4.1x10^8$	$7.8x10^8$
Heated	$8.6x10^1$	$9.5x10^1$	$4.7x10^1$	$5.9x10^1$	$9.2x10^1$
PO_4	30**	30**	30**	30**	30**
L- Amino acids in buffer?					
Alanine	$4.9x10^1$	$7.9x10^1$	$8.5x10^1$	$6.9x10^1$	$6.3x10^1$
Arginine	NC	NC	30**	NC	NC
Aspargine	30**	30**	$6.9x10^1$	30**	$5.7x10^1$
Aspartic acids	$7.1x10^1$	$5.3x10^1$	$6.1x10^1$	30**	$5.9x10^1$
Cystine HCl	NC	NC	NC	NC	NC
Glutamic acid	$5.7x10^1$	$6.8x10^1$	$8.9x10^1$	$6.4x10^1$	$5.9x10^1$
Glycine	$5.5x10^1$	30**	$5.6x10^1$	30**	30**
Hydroxy proline	30**	$4.9x10^1$	$6.2x10^1$	30**	30**
Histidine	NC	NC	NC	NC	NC
Isoleucine	NC	NC	NC	NC	NC
Leucine	30**	NC	30**	NC	NC
Lysine	NC	NC	NC	NC	NC
Methionine	NC	NC	NC	NC	NC
Phenyl alanine	NNC	NC	NC	NC	NC
Proline	$5.9x10^1$	$7.3x10^1$	$9.0x10^1$	$4.7x10^1$	$5.9x10^1$
Serine	$8.1x10^1$	$9.1x10^1$	$9.8x10^1$	$8.7x10^1$	$7.4x10^1$
Threonine	NC	NC	NC	NC	NC
Valine	30**	30**	$4.9x10^1$	NC	30**

* Amino acids concentrations 200 ug per ml., * Triptiease soy broth., ** Count less them., NC No count as such plated without dilution.

Table 42. The protecting effect of simple proteins and carbohydrates by heating at 55°C for 15 minutes.

Heating menstrua	S.weltev Seden	S.enteritidis	S.senftenberg	S.saint-paul	S.cubana
		Counts per ml.			
Control					
TSB*	$3.8x10^8$	$6.9x10^8$	$5.2x10^8$	$6.1x10^8$	$4.7x10^8$
Heated					
TSB	$2.7x10^3$	$4.9x10^3$	$3.5x10^4$	$1.7x10^3$	$3.9x10^3$
Yeast extract (1%)	$7.9x10^3$	$6.8x10^3$	$7.3x10^4$	$3.9x10^3$	$6.5x10^3$
Peptone (1%)	$2.1x10^3$	$3.7x10^3$	$1.8x10^4$	$3.4x10^3$	$2.9x10^3$
Tryptone (1%)	$2.4x10^3$	$4.1x10^3$	$2.9x10^4$	$2.7x10^2$	$3.5x10^2$
Carbihydrates in 0.1M phosphate buffer					
Glucose (4%)	$3.7x10^3$	$8.9x10^3$	$7.8x10^4$	$4.8x10^3$	$4.6x10^3$
Sucrose (4%)	30**	30**	30**	30**	30**
Lactose (4%)	$6.2x10^2$	$5.6x10^1$	$3.9x10^2$	$6.5x10^1$	$5.9x10^1$
Xylose (4%)	30**	$6.1x10^1$	$6.9x10^1$	30**	30**
Rhamnose(4%)	$1.7x10^2$	$6.1x10^1$	$5.6x10^1$	$4.9x10^1$	$5.6x10^1$

*TSB Tripticase soy broth,. ** Count is less than.

Contd.....

Heating Menstrua	S.weltev-reden	S.enteri-tidis	S.senf-tenberg	S.saint-paul	S.cubana
		Counts per ml. Heating at 60°C		for 15 minutes.	
Control					
TSB*	$6.8x10^8$	$5.7x10^8$	$6.2x10^8$	$5.8x10^8$	$3.9x10^8$
Heated					
TSB	$7.9x10^1$	$8.1x10^1$	$6.7x10^2$	$5.6x10^1$	$7.3x10^1$
Yeast extract (1%)	$5.4x10^1$	$4.6x10^1$	$4.9x10^1$	$5.3x10^1$	$6.8x10^1$
Peptone (1%)	$4.6x10^1$	$5.3x10^1$	$4.7x10^1$	$6.2x10^1$	$3.9x10^1$
Tryptone(1%)	30**	30**	30**	30**	30**
Carbohydrates in 0.1M phosphate buffer					
Glucose (4%)	NC	NC	NC	NC	NC
Sucrose (4%)	30**	NC	30**	NC	NC
Lactose (4%)	30**	30**	30**	NC	NC
Xylose (4%)	NC	NC	NC	NC	NC
Rhamnose(4%)	NC	NC	NC	NC	NC

*TSB Triptiease soy broth., ** Count is less than.,
NC No viable count as such plated without dilution.

Table 43. Minutes taken to kill the various Salmonella serotypes in different substrates of fish and shellfishes.

Organisms	Ps H.	M H	P H	C H
S. weltevreden				
50°C	490	480	490	500
55°C	200	210	210	210
60°C	25	30	30	30
65°C	5*	5*	5*	5*
S. roam				
50°C	530	490	450	480
55°C	210	200	220	250
60°C	15	20	15	20
65°C	5**	5*	5*	5*
S. enteritidis				
50°C	500	500	490	490
55°C	220	200	210	210
60°C	20	20	20	20
65°C	5*	5*	5*	5*
S. typhimurium				
50°C	520	500	510	510
55°C	210	200	220	200
60°C	25	30	25	25
65°C	5*	5*	5*	5*
S. senftenberg				
50°C	680	680	680	680
55°C	500	490	490	490
60°C	70	65	70	75
65°C	10	10	10	10
S. anatum				
50°C	500	510	510	500
55°C	210	210	200	210
60°C	15	20	15	20
65°C	5	5*	5	5

Contd.....

Organisms	Ps H	M H	P H	C H
S. arizona spp				
50°C	490	500	500	500
55°C	190	200	190	190
60°C	25	30	30	25
65°C	5*	5*	5*	5*
S. chester				
50°C	500	480	490	500
55°C	190	180	190	190
60°C	25	30	30	30
65°C	5	5	5	5
S. vayorana				
50°C	480	490	480	490
55°C	190	190	180	180
60°C	30	25	30	30
65°C	5	5*	5*	5
S. heidelberg				
50°C	490	510	500	500
55°C	190	190	190	190
60°C	25	30	30	30
65°C	5*	5*	5*	5*
S. salford				
50°C	490	500	500	500
55°C	180	180	180	180
60°C	30	30	30	30
65°C	5*	5*	5*	5*

Results expressed as a mean value of three experiments
* less than. Ps H. Pearl spot homogenates; M H. Mackerel homogenates; P H. Prawn homogenates(P. indicus); C H. Crab homogenates (Scylla serrata).

Table 44. Variation od D values during heat treatment of *Salmonella* at various temperatures in TSY broth and M-9 broth.

Organisms	Mean D values in TSY broths			Mean D values M-9 broths		
	50°C	55°C	60°C	50°C	55°C	60°C
S. typhimurium	64.4	12.3	1.5	61.9	10.4	1.3
S. saintpaul	62.6	14.7	1.7	61.6	11.2	1.4
S. veltevreden	69.3	11.5	1.8	64.3	10.1	1.5
S. senftenberg	86.4	22.8	7.6	79.6	16.3	5.9
S. anatum	68.5	11.3	1.4	63.4	11.3	1.4
S. enteritidis	65.4	10.5	1.5	61.9	10.3	1.3
S. heidelberg	62.4	11.5	1.8	62.2	11.6	1.4
S. typhi	65.8	12.6	1.8	63.4	12.1	1.6
S. cubana	66.5	11.4	1.5	62.7	10.3	1.2
S. newport	68.4	10.3	1.4	63.5	11.4	1.5
S. waycross	69.2	11.4	1.6	64.2	10.5	1.6
S. roan	70.5	10.3	1.4	61.5	9.2	1.3

TSY= Triptiease Soy Yeast extract broth.

Table 45. Influence of thermal resistance of various serotypes of Salmonella in different homogenates.

Organisms	Temp. °C	M H D value	Ps.H Dvalue	P H D value	C H D value
S. typhimurium	50	80.5	80.2	84.3	75.7
	55	15.9	14.3	16.1	13.7
	60	2.9	2.7	2.8	2.4
S. roan	50	72.4	70.6	75.3	69.4
	55	12.4	12.8	13.5	11.6
	60	2.0	2.3	2.6	2.2
S. saintpaul	50	69.7	71.5	77.4	70.3
	55	11.8	11.4	12.8	12.3
	60	2.1	2.3	2.4	2.4
S. veltevreden	50	90.5	97.3	99.3	96.4
	55	15.8	16.7	17.4	16.7
	60	3.0	2.8	2.6	2.6
S. senftenberg	50	110.4	118.6	110.6	104.5
	55	44.5	55.7	57.2	59.4
	60	8.3	9.7	8.7	9.3
S. amatum	50	87.3	82.7	89.6	86.5
	55	19.7	21.6	23.1	20.4
	60	3.9	4.5	4.2	4.8

D values were of average of three experiments.
MH= Mackerel homogenates; Ps.H.= Pearl spot homogenates; PH= Prawns(P. indicus) homogenates; CH= Crab (S. serrata) homogenates.

Contd.....

Organisms	Temp. °C	M H D values	Ps H D values	P H D values	C H D values
S. enteritidis	50	86.3	81.2	78.4	77.3
	55	11.5	10.7	11.8	11.0
	60	3.2	3.4	3.9	3.5
S. heidelberg	50	65.8	73.8	78.3	77.4
	55	17.4	18.4	17.6	18.5
	60	3.6	2.9	4.2	3.8
S. typhi	50	76.3	79.2	80.4	78.6
	55	19.2	18.6	19.7	18.9
	60	4.7	5.3	5.5	6.1
S. cubana	50	79.6	89.4	91.4	89.8
	55	11.8	16.8	17.4	18.9
	60	4.1	3.7	3.5	3.7
S. newport	50	89.6	90.4	91.6	88.9
	55	18.9	19.7	20.2	19.5
	60	5.9	4.8	5.3	5.6
S. wayoross	50	89.2	92.3	92.8	88.3
	55	18.9	19.7	20.7	19.8
	60	2.7	3.1	3.4	3.7

Table 46. Repair of injured cells of different strains of *Salmonella* after thermal stress at 50°C 55°C and 60°C in fish homogenates of Pearl spot (*E. suratensis*).

Organisms	Initial	Recovery plate count in XLD agar at 50°C.			
		30 min.	60 min.	90 min.	120 min.
	$x10^3$	$x10^4$	$x10^5$	$x10^6$	$x10^6$
S. heidelberg	4.1	5.3	6.6	1.3	1.7
S. weltevreden	5.9	6.8	7.3	3.7	3.9
S. barielly	2.1	1.4	1.7	3.6	4.6
S. newport	4.9	5.8	6.7	7.2	7.6
S. arizona spp.	2.9	3.6	5.7	3.6	4.2
S. vayerous	1.4	1.9	2.5	5.8	5.9
S. saintpaul	4.3	4.8	5.6	1.8	3.9
S. bresdney	4.8	5.6	1.9	3.6	5.3
S. anatum	4.5	4.9	7.1	5.3	4.9
S. roan	2.1	3.7	6.7	2.5	4.9
S. senftenberg	1.1[a]	1.7[b]	2.7[b]	4.6[b]	5.1[b]
S. chester	5.1	6.3	7.1	5.6	4.9
S. salford	3.3	3.6	6.4	4.3	5.1

a= $x10^4$; b = $x10^6$; c= $x10^7$; d = $x10^5$

Table 46 cont'd....

Organisms	Initial	Recovery of plate counts in XLD agar. 30 min	60 min	90 min	120 min	Initial	30 min	60 min	90 min	120 min
	x10^2	x10^2	x10^3	x10^5	x10^5	x10	x10	x10^2	x10^3	x10^3
	55°C.					60°C.				
S. heidelberg	3.7	7.2	1.5	2.1	3.1	7.3	4.5	4.7	5.1	4.9
S. weltevreden	3.5	5.7	6.3	3.5	4.2	6.9	6.6	6.4	5.1	4.1
S. barielly	1.9	3.9	4.6	3.1	4.3	3.8	7.9	6.8	4.5	5.3
S. newport	2.8	6.5	5.8	4.3	5.1	4.9	7.8	5.1	3.7	4.3
S. arizona spp	6.3	9.2	3.4	4.3	5.6	4.9	6.7	4.7	5.9	4.8
S. wayeross	3.3	7.2	4.2	5.3	5.9	4.8	8.6	6.4	6.1	6.3
S. saintpaul	4.5	6.8	8.9	3.9	3.4	6.4	7.8	7.1	3.1	443
S. bredaney	6.5	9.2	4.6	7.1	7.6	4.7	8.4	3.7	4.7	5.3
S. anatum	4.8	8.3	5.7	3.2	3.5	6.4	7.3	5.7	6.3	6.2
S. roan	3.9	7.6	6.4	5.8	5.1	5.6	8.8	4.7	3.7	3.8
S. senftenberg	1.9[a]	7.4[a]	4.5[b]	8.5[b]	8.2[b]	9.3[e]	4.7[a]	3.8[d]	4.1[d]	4.9[d]
S. chester	4.7	8.9	6.3	3.4	3.2	4.8	5.6	4.2	5.1	4.7
S. salford	4.9	6.8	4.7	5.1	5.3	7.1	9.9	7.3	5.3	5.1

a = x10^4; b = x10^6; c = x10^7; d = x10^5; e = x10^2.

Table 47. Repair of injured cells of different strains of *Salmonella* after thermal stress at 50°, 55° and 60°C in crab homogenates (*S. serrata*).

Organisms	Initial x10^3	30 min. x10^4	60 min. x10^5	90 min. x10^6	120 min. x10^6
	Recovery of plate counts in XLD Agar 50°C.				
S. heidelberg	1.4	2.9	4.3	6.1	5.8
S. weltevreden	3.2	5.2	5.6	2.7	2.9
S. barielly	4.2	5.6	6.2	3.1	3.6
S. newport	2.1	4.7	4.3	3.9	4.1
S. arizona spp	3.7	4.1	5.6	3.2	3.4
S. waycross	4.5	3.9	5.8	2.3	2.7
S. saintpaul	3.4	4.8	6.1	5.8	5.1
S. broadney	5.2	6.1	6.8	3.4	3.5
S. anatum	4.3	7.8	4.2	2.1	3.2
S. roan	5.1	6.7	4.1	6.3	5.3
S. senftenberg	2.4[b]	1.6[c]	8.9[c]	1.2[d]	1.5[d]
S. chester	3.9	4.8	6.1	5.3	6.1
S. salford	7.3	6.2	5.4	7.3	7.9

a= x10^4; b= x10^5; c= x10^6; d= x10^7; e= x10^3; f= x10^2

Contd.....

Organisms	Initial x10^2	30 min. x10^2	60 min. x10^3	90 min. x10^5	120 min. x10^5	Initial x10	30 min. x10	60 min. x10^2	90 min. x10^3	120 min. x10^3
	Recovery of plate counts in XLD agar.									
	55°C.					60°C.				
S. heidelberg	4.7	6.4	6.9	5.4	6.1	3.9	6.7	5.4	4.8	5.6
S. veltevredon	2.3	3.5	4.8	6.7	5.3	7.2	9.3	6.5	4.3	4.9
S. barielly	2.1	6.7	6.4	3.6	4.3	4.8	6.9	3.1	5.5	4.7
S. newport	3.5	6.8	3.1	4.4	5.9	4.3	5.7	6.2	5.6	6.1
S. arizona spp	5.1	7.8	8.4	4.3	5.1	7.7	6.2	3.6	4.2	3.8
S. wayeross	1.8	6.6	7.2	4.9	3.8	6.9	7.7	6.2	3.8	4.6
S. saintpaul	4.6	8.9	4.8	3.7	5.4	5.1	4.9	7.2	6.8	5.9
S. brodney	3.2	6.3	6.2	4.9	6.1	4.9	7.8	4.9	5.5	6.1
S. anatum	4.6	7.9	4.1	3.6	5.2	4.8	7.3	4.3	5.9	6.7
S. roan	1.9	6.7	5.3	4.7	6.4	5.7	8.3	4.7	2.7	3.8
S. senftenberg	1.4[a]	7.4[a]	2.7[b]	1.9[c]	2.7[c]	3.2[f]	7.7[f]	3.9[e]	7.2[e]	6.7[e]
S. chester	1.8	6.4	5.4	5.2	3.8	5.1	6.7	6.3	5.2	6.7
S. salford	3.1	6.6	6.2	3.9	4.7	4.4	8.8	6.3	6.8	6.1

a= x10^4; b= x10^5; c= x10^6; d= x10^7; e= x10^3; f= x10^2.

Table 48. Effect of thermal injury repair by growth promoting compounds. Plated in XLD agar initially and after one hour (heated at 50°C for 30 min.).*

Organisms	Heated cell in media Initial $x10^2$	M-9 broth alone after one hr. $x10^2$	M-9 with nucleosides 10 ug per ml. $x10^2$	M-9 with amino acids 100ug per ml. $x10^2$	M-9 with vitamins 10 ug per ml. $x10^2$	M-9 broth with yeast extract(1%) $x10^4$	M-9 broth with Tripticase soy (2%) $x10^4$	Brain-Heart infusion broth $x10^4$
S. heidelberg	1.6x	3.2	2.7	3.9	3.6	3.9	4.8	2.9
S. weltevreden	2.5	3.3	2.9	3.7	2.6	3.5	3.9	3.1
S. bariolly	1.3	2.1	1.9	2.7	1.8	2.3	2.9	3.8
S. newport	2.4	3.6	1.7	2.3	2.9	3.1	3.4	3.6
S. arizona spp	1.1	3.1	1.6	1.9	2.7	2.4	3.9	2.7
S. wayeross	3.2	3.8	3.4	3.5	2.9	4.6	3.4	4.1
S. saintpaul	2.7	3.1	2.8	2.9	2.4	2.9	3.1	2.9
S. bredney	4.2	4.8	4.3	3.9	4.8	2.8	4.2	3.1
S. anatum	2.1	2.6	2.8	2.6	2.5	3.1	4.3	2.8
S. senftenbegg	3.9	4.3	3.8	3.6	4.6	8.9	6.7	5.7
S. chester	4.2	4.8	4.6	4.8	4.5	2.1	3.4	2.7
S. salford	2.3	3.1	2.8	2.9	3.0	2.7	3.1	3.2

* Initial inoculated counts were ranged from 2.6 to $8.7x10^6$ per ml.

Table 50. Number of viable cells that survived at higher temperature of 55°C.

Sampling time Min.	V.178 % NaCl 0.5%	3%	7.5%	V. 1902 % NaCl 0.5%	3%	7.5%
	Plate counts/ml/gm.			Plate counts/ml/gm.		
Tryptiease soy broth.						
Initial	$1.6x10^8$	$1.9x10^8$	$2.1x10^8$	$1.6x10^8$	$1.7x10^8$	$2.2x10^8$
30	$1.2x10^2$	$1.6x10^2$	$3.5x10^3$	$2.1x10^2$	$2.5x10^3$	$1.3x10^3$
60	100*	$1.1x10^2$	$1.3x10^2$	100*	$1.4x10^2$	$2.1x10^2$
Homogenates Pearl spot (*E. suratensis*).						
Initial	$2.7x10^8$	$2.6x10^8$	$3.1x10^8$	$2.5x10^8$	$2.7x10^8$	$2.9x10^8$
30	$3.7x10^2$	$1.9x10^3$	$4.2x10^3$	$3.4x10^2$	$1.6x10^3$	$2.8x10^3$
60	$1.1x10^2$	$1.6x10^2$	$1.3x10^2$	$1.9x10^2$	$1.1x10^3$	$1.7x10^3$
Homogenates of Prawns (*P. indicus*).						
Initial	$2.3x10^8$	$2.9x10^8$	$3.4x10^8$	$2.3x10^8$	$2.7x10^8$	$3.3x10^8$
30	$4.2x10^2$	$6.3x10^3$	$3.4x10^3$	$3.9x10^2$	$2.9x10^3$	$7.8x10^3$
60	$1.6x10^2$	$4.2x10^3$	$2.1x10^3$	$2.5x10^2$	$1.1x10^3$	$2.6x10^3$
Homogenates of crab (*S. serrata*).						
Initial	$3.1x10^8$	$4.2x10^8$	$3.1x10^8$	$2.9x10^8$	$3.5x10^8$	$3.7x10^3$
30	$4.1x10^2$	$6.1x10^3$	$6.9x10^2$	$8.1x10^2$	$3.9x10^3$	$5.2x10^3$
60	$2.1x10^2$	$2.7x10^3$	$3.7x10^2$	$4.2x10^2$	$1.9x10^3$	$3.5x10^3$

* Less than.

Contd.....

Sampling time Min.	V. 178 %NaCl 0.5%	3%	7.5%	V. 1902 %NaCl 0.5%	3%	7.5%
	Plate counts/ml.			Plate counts/ml.		
Amino acids chemically defined medium.						
Initial	$1.3x10^8$	$2.6x10^8$	$1.8x10^8$	$1.8x10^8$	$3.4x10^8$	$1.7x10^8$
30	$5.3x10^2$	$6.2x10^3$	$6.5x10^3$	$3.7x10^2$	$4.8x10^3$	$5.8x10^3$
60	$3.1x10^2$	$2.5x10^3$	$2.1x10^3$	$1.6x10^2$	$1.9x10^3$	$2.8x10^3$
Tryptone 2%						
Initial	$2.1x10^8$	$1.8x10^8$	$2.6x10^8$	$3.1x10^8$	$2.8x10^8$	$4.1x10^8$
30	$4.2x10^2$	$4.8x10^2$	$6.1x10^3$	$3.6x10^2$	$5.7x10^3$	$6.3x10^3$
60	$1.1x10^2$	$1.1x10^2$	$2.2x10^2$	$1.9x10^2$	$2.7x10^2$	$2.4x10^2$
Peptone 2%						
Initial	$2.3x10^8$	$2.8x10^8$	$3.1x10^8$	$2.5x10^8$	$3.3x10^8$	$3.7x10^8$
30	$6.7x10^2$	$4.8x10^3$	$2.3x10^3$	$5.4x10^2$	$4.2x10^2$	$5.1x10^2$
60	$1.2x10^2$	$1.9x10^2$	$1.4x10^2$	$1.2x10^2$	$2.1x10^2$	$2.3x10^2$

Table 51. Effect of different salt concentrations on the injury (%) of cells of V. parahaemolyticus in different heating menstrua at 50°C.

Heating menstrua	V. 178. 0.5%	3.0% % NaCl	7.5%	V. 1902. 0.5%	3.0% % NaCl	7.5%
	% injury			% injury		
TSB	97.4	96.1	95.7	97.5	96.3	96.2
Homogenates						
Peakspot (E. suratensis)	97.1	96.0	95.1	95.4	94.3	96.2
Penaeus indicus	96.8	95.5	95.3	97.6	97.6	95.4
Crab (S.serrata)	94.2	96.4	93.2	96.4	95.3	94.3
Amino acids mixtures & vitamins	98.3	97.4	96.4	98.5	97.1	96.5
Tryptone 2%	98.7	97.3	96.7	98.1	97.2	96.3
Peptone 2%	98.1	97.5	96.5	98.4	97.2	96.6

TSB= Triptiease soy broth.

Table 52. Effect of salt on the protection of injury of *V. parahaemolyticus* cells in different heating menstrum at 55°C.

Heating menstrum	V. 178 % Na Cl 0.5%	3%	7.5%	V. 1902. % Na Cl 0.5%	3%	7.5%
	% injury.			% injury.		
TSB	98.4	97.7	96.2	98.1	97.2	96.4
Homogenates						
Pearlspot (*E. suratensis*)	98.1	97.6	97.4	98.4	97.5	96.1
Penaeus indicus	98.0	97.4	96.8	99.1	97.6	96.7
Crab (*S. serrata*)	97.8	97.1	96.2	98.4	98.4	97.7
Amino acids mixtures & vitamins	99.1	98.4	98.2	98.6	98.1	97.6
Tryptone 2%	99.4	98.6	98.0	99.0	98.4	97.4
Peptone 2%	99.1	98.7	98.1	99.1	98.5	98.0

Table 53. D values of *Vibrio parahaemolyticus* at 50°C and 55°C.

Heating menstrum	V. 178 % Na Cl			V. 1902 % Na Cl		
	0.5%	3%	7.5%	0.5%	3%	7.5%
TSB	0.8	1.1	1.8	0.6	0.9	2.5
Homogenates						
Pearlspot (*E.suratensis*)	1.1	2.7	5.4	0.1	2.9	5.8x1
Penaeus indicus	1.2	2.9	6.4	0.9	3.4	6.3
Crab (*S. serrata*).	1.2	3.2	6.3	1.1	3.5	6.6
Amino acids mixtures & vitamins	0.5	1.1	3.3	0.6	1.2	2.7
Tryptone 2%	0.3	0.9	1.6	0.4	1.0	1.7
Peptone 2%	0.2	0.7	1.7	0.3	0.9	1.9

Contd.....

Heating menstrua	V. 178 % NaCl 0.5%	3%	7.5%	V. 1902 %NaCl 0.5%	3%	7.5%
	D values at 55°C					
TSB	0.4	1.2	1.7	0.2	0.8	1.5
Homogenates						
Pearlspot (*E.suratensis*)	0.6	0.9	2.1	0.6	1.3	1.9
Penaeus indicus	0.6	1.1	1.9	0.5	0.7	2.1
Crab (*S. serrata*)	0.4	1.8	2.1	0.3	0.8	1.7
Aminoacids mixtures & vitamins	0.5	0.6	0.8	0.2	0.4	0.8
Tryptone 2%	0.3	0.6	0.7	0.2	0.5	0.9
Peptone 2%	0.3	0.5	0.8	0.2	0.8	0.9

Table 54. Recovery of injured *Vibrio parahaemolyticus* cells isolated from substrates of fish and shell fishes after thermal stress at 55°C. Recovery carried out in TSB and TCBS with 3% salt.

Sampling time min.	V. 178. % NaCl			V. 1902. % NaCl.		
	0.5%	3.0%	7.5%	0.5%	3.0%	7.5%
	Plate counts/ml/gm			Plate counts/ml/gm		
T S broth						
Initial	1.8×10^3	3.4×10^4	2.8×10^4	2.3×10^3	2.5×10^4	4.3×10^4
30	5.6×10^3	7.8×10^4	6.5×10^4	6.9×10^3	8.6×10^4	8.8×10^4
60	2.4×10^5	4.1×10^5	4.6×10^5	2.3×10^5	3.6×10^5	2.9×10^5
90	4.3×10^5	6.3×10^5	5.3×10^5	3.9×10^5	7.1×10^5	6.7×10^5
120	5.6×10^5	7.9×10^5	6.8×10^5	6.2×10^5	8.1×10^5	7.1×10^5
Homogenates						
Pearlspot (*Etroplus suratensis*)						
Initial	1.6×10^4	2.7×10^4	3.2×10^4	3.6×10^3	2.6×10^4	3.6×10^4
30	5.2×10^4	4.9×10^4	6.2×10^4	5.9×10^3	6.2×10^4	4.9×10^4
60	7.6×10^5	5.8×10^5	6.5×10^5	3.4×10^5	4.3×10^5	4.6×10^5
90	8.1×10^5	6.4×10^5	7.8×10^5	5.8×10^5	5.6×10^5	6.8×10^5
120	8.5×10^5	7.3×10^5	8.1×10^5	6.6×10^5	7.8×10^5	7.1×10^5

Contd.....

Sampling time min.	V. 178. % Na Cl			V. 1902. %NaCl.		
	0.5%	3.0%	7.5%	0.5%	3.0%	7.5%
	Plate counts per gram			Plate counts per gram.		
Homogenates						
Penaeus indicus						
Initial	$1.3x10^3$	$1.9x10^4$	$2.1x10^4$	$3.2x10^3$	$3.7x10^4$	$2.7x10^4$
30	$4.5x10^3$	$3.6x10^4$	$5.6x10^4$	$6.7x10^3$	$7.6x10^4$	$8.3x10^4$
60	$3.3x10^5$	$4.5x10^5$	$6.2x10^5$	$6.1x10^5$	$4.5x10^5$	$3.4x10^5$
90	$8.1x10^5$	$6.4x10^5$	$7.8x10^5$	$7.6x10^5$	$6.9x10^5$	$6.4x10^5$
120	$7.3x10^5$	$7.6x10^5$	$8.2x10^5$	$7.4x10^5$	$6.9x10^5$	$7.1x10^5$
Crab (Scylla serrata).						
Inital	$2.1x10^3$	$2.9x10^4$	$2.4x10^4$	$4.3x10^3$	$2.9x10^4$	$3.5x10^4$
30	$4.1x10^4$	$3.6x10^4$	$5.7x10^4$	$7.8x10^3$	$3.6x10^4$	$8.9x10^4$
60	$2.6x10^5$	$4.5x10^5$	$6.2x10^5$	$7.2x10^4$	$5.2x10^5$	$6.7x10^5$
90	$4.3x10^5$	$5.6x10^5$	$7.3x10^5$	$6.7x10^5$	$6.1x10^5$	$7.3x10^5$
120	$6.7x10^5$	$7.8x10^5$	$7.7x10^5$	$8.1x10^5$	$7.1x10^5$	$8.2x10^5$

Fig. 13. a. Effect of M-9 broth on the protection of different *Salmonella* sertypes at 50°C.

Fig. 13. b. Survival of different *Salmonella* serotypes on heating at 50°C in Tripticase soy broth.

FIG 13,a

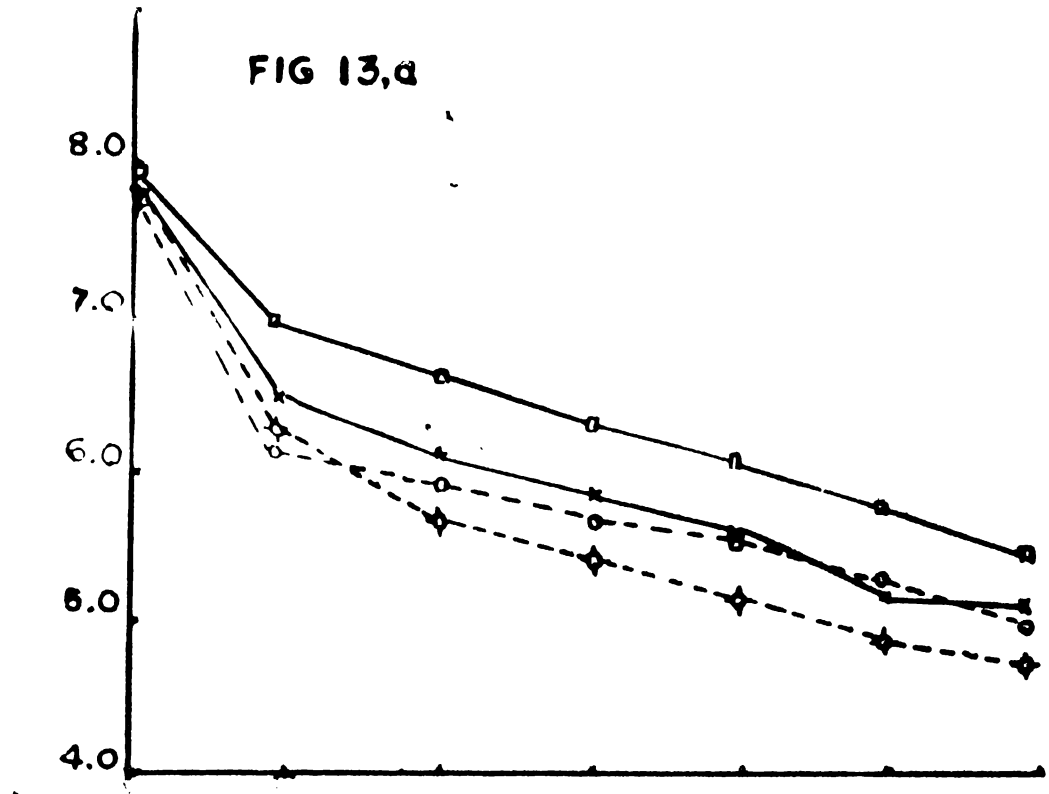


FIG 13,b

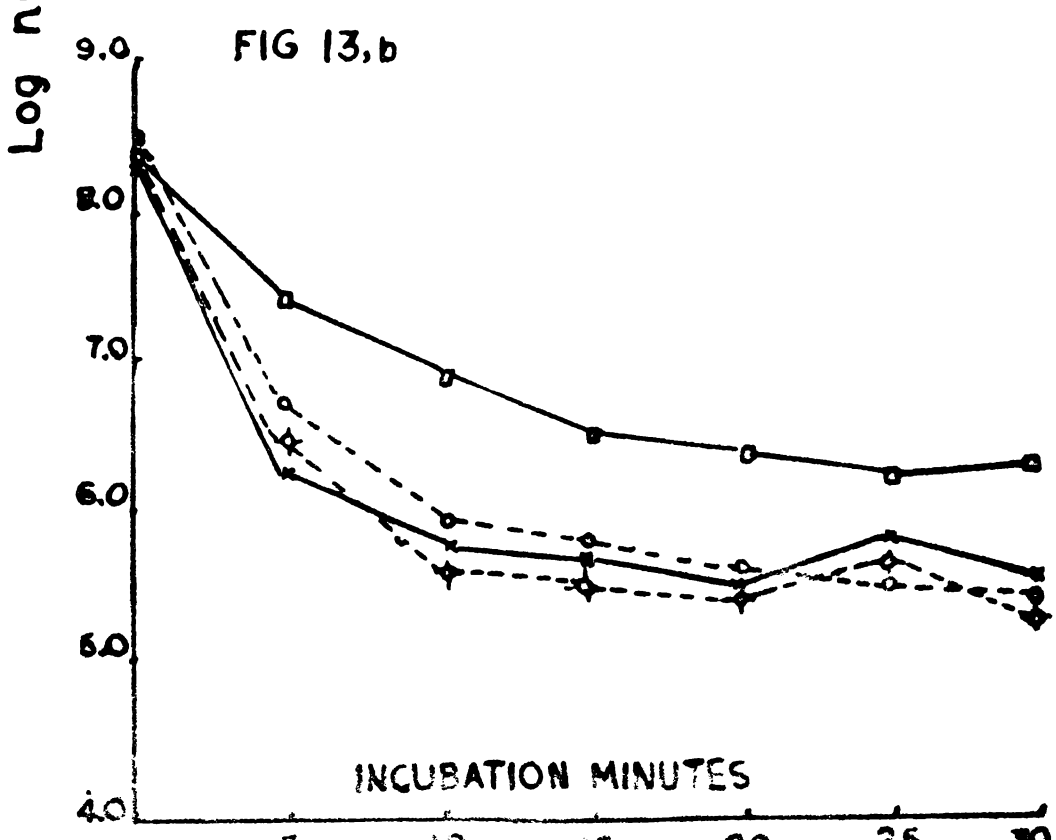


Fig. 14. a. Survival of Salmonella enteritidis in different finfish and shell fish homogenates on heating at 50°C.

Fig. 14. b. Survivak of Salmonella senftenberg in different finfish and shellfish homogenates on heating at 50°C.

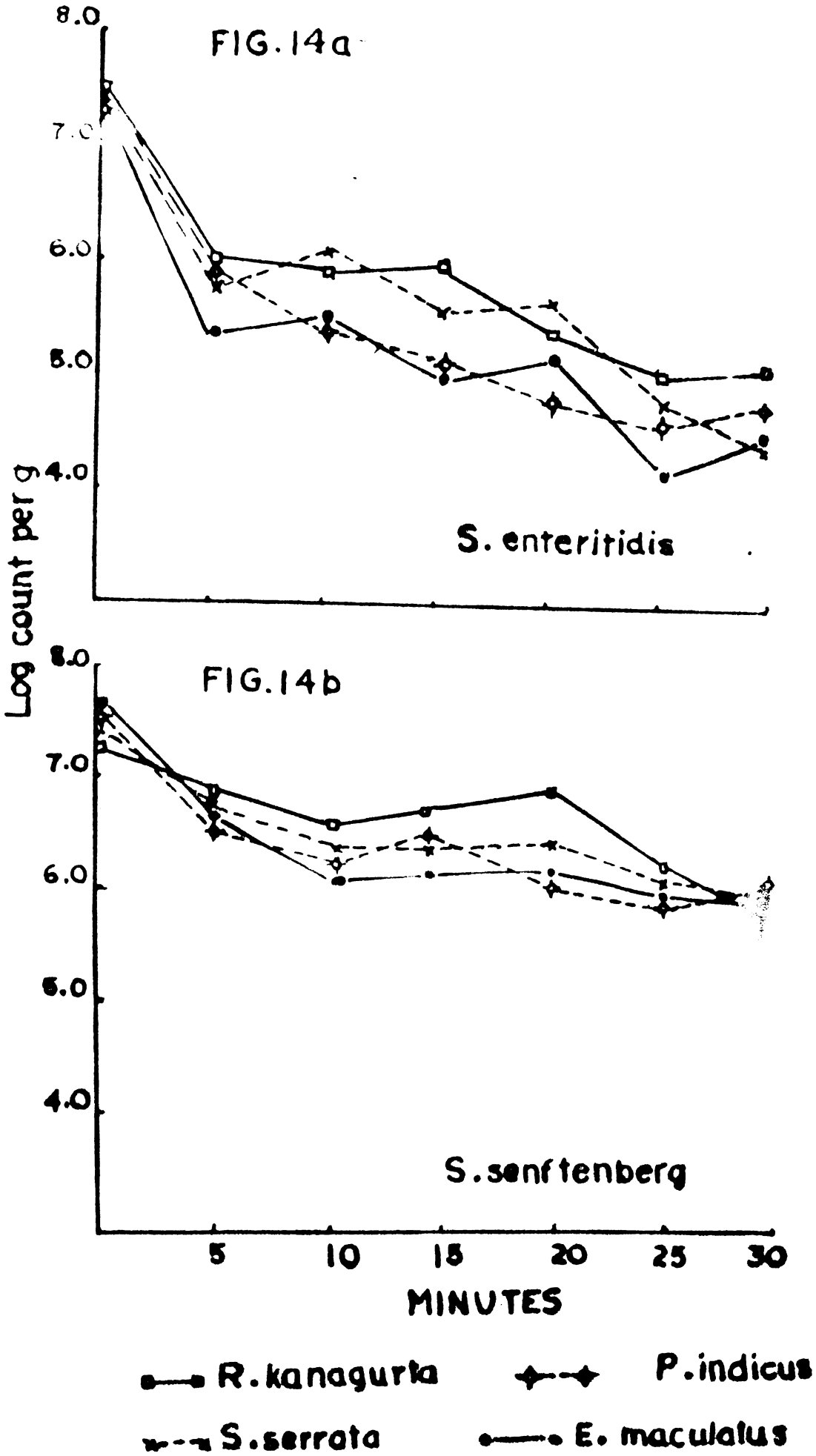


FIG. 14a
8.0
7.0
6.0
5.0
4.0
S. enteritidis
FIG. 14b
8.0
7.0
6.0
5.0
4.0
S. senftenberg
Log count per g
5
10
15
20
25
30
MINUTES
R. kanagurta
P. indicus
S. serrata
E. maculatus

Fig. 15.a. Survival of Salmonella typhimurium in different finfishes and shellfish homogenates on heating at 55°C.

Fig. 15.b. Survival of Salmonella senftenberg in different finfish and shellfish homogenates on heating at 55°C.

FIG.15a

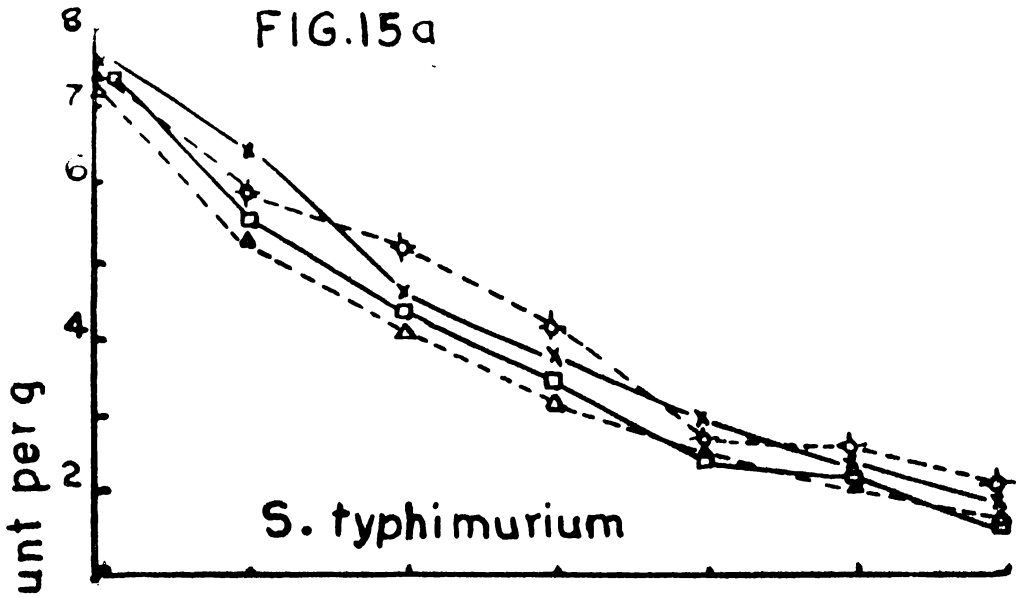


FIG.15b

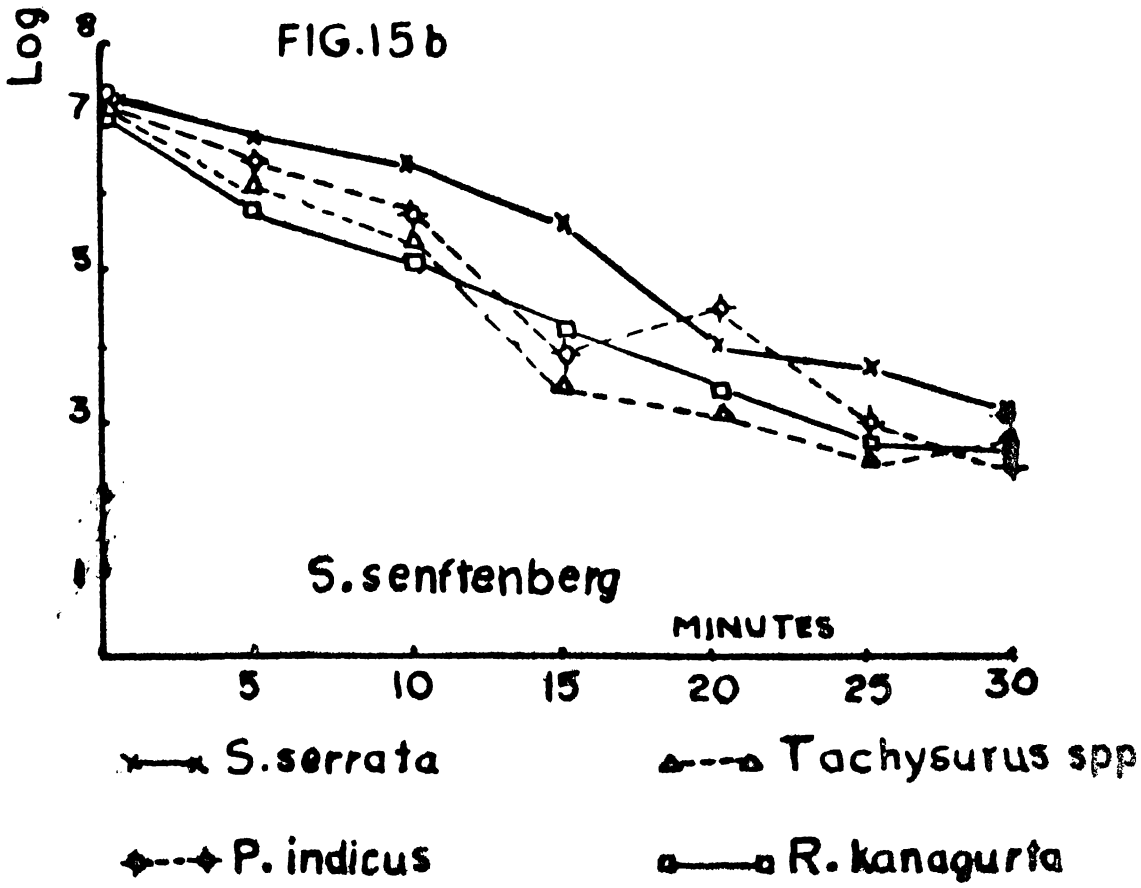


Fig. 16. a. Lethal effect of different Salmonella serotypes in M-9 broth on heating at temperature at 55°C.

Fig. 16. b. Lethal effect of different Salmonella serotypes in Tripticase soy broth on heating at temperature at 55°C.

FIG. 16a

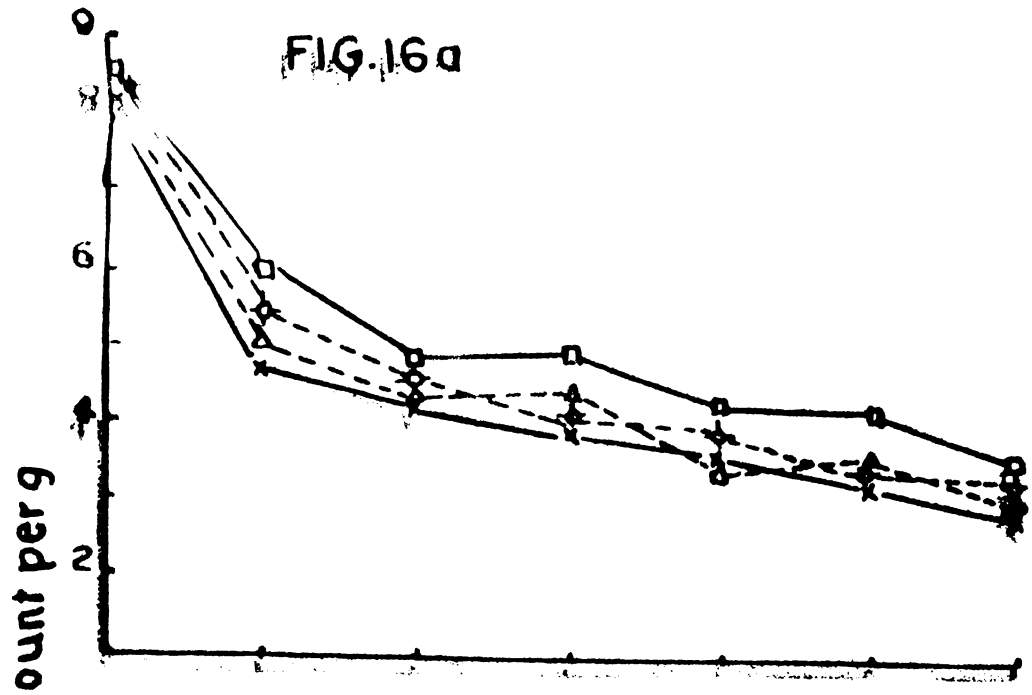
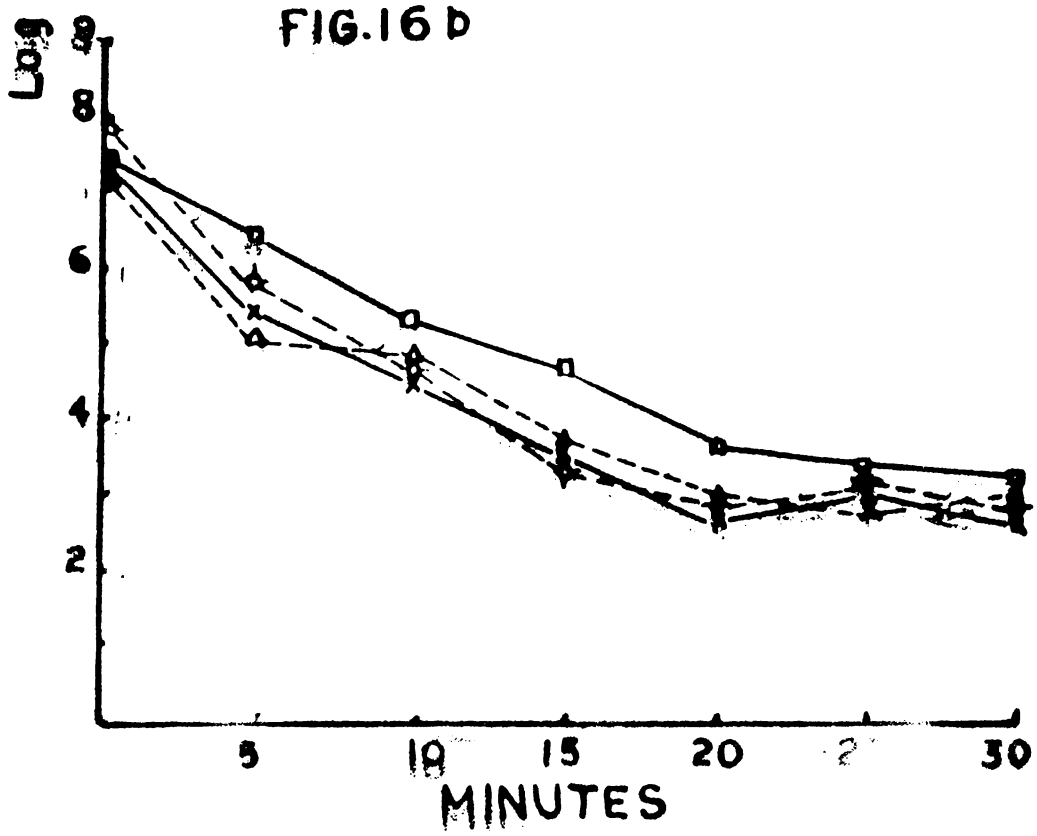


FIG. 16b



S. SENFTENBERG

S. TYPHIMURIUM

S. ROAN

S. ENTERITIDIS

Fig. 17. a. Lethal effect of different Salmonella serotypes in various broths and in fish and shellfish homogenates on heating at a temperature of 60°C.

Fig. 17. b, c, d, are all same as 17a.

FIG. 17 a

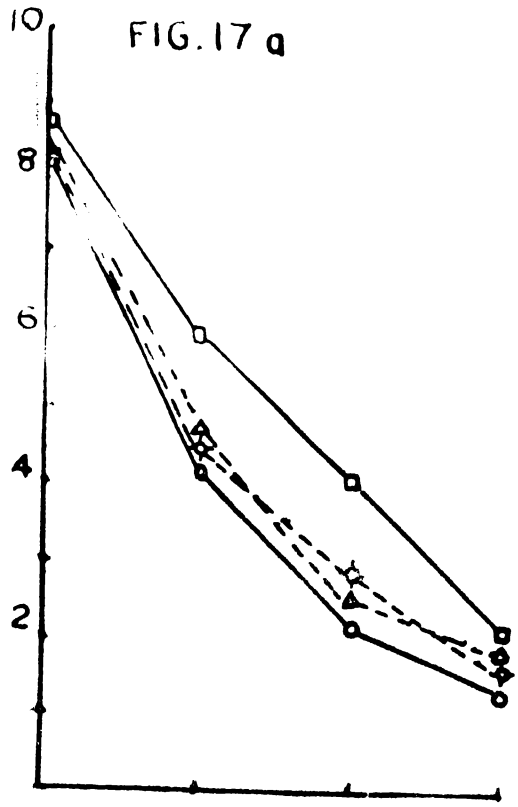
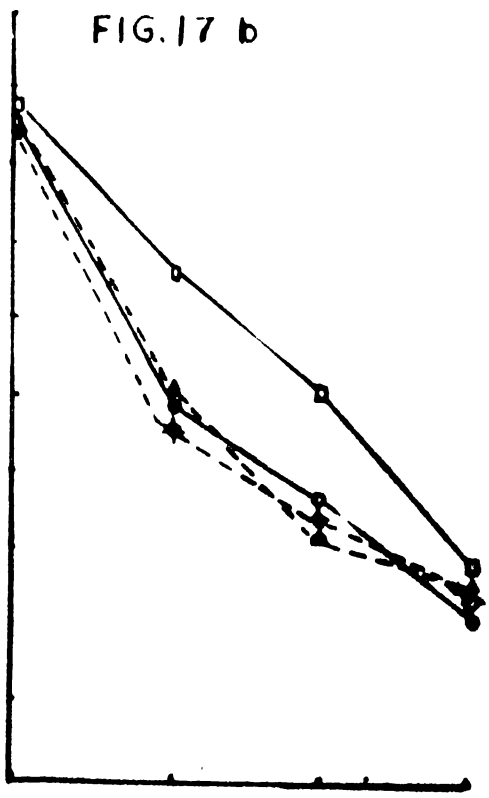


FIG. 17 b



S. SENFTENBERG

S. ENTERITIDIS

S. TYPHIMURIUM

S. ROAN

FIG. 17 c

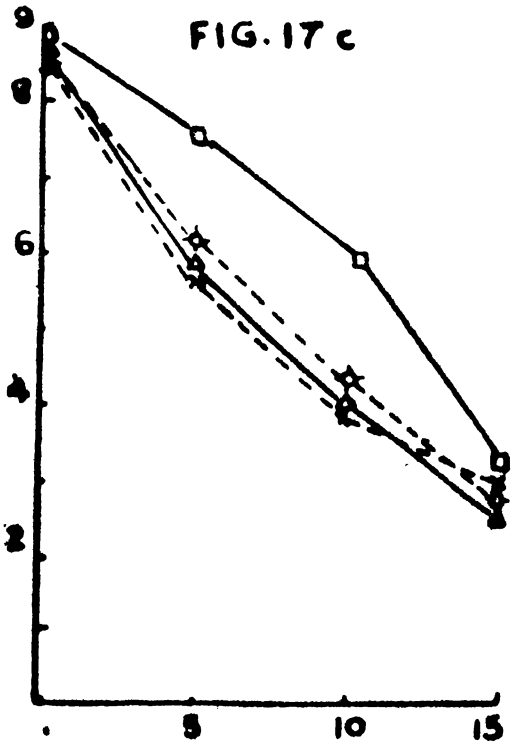
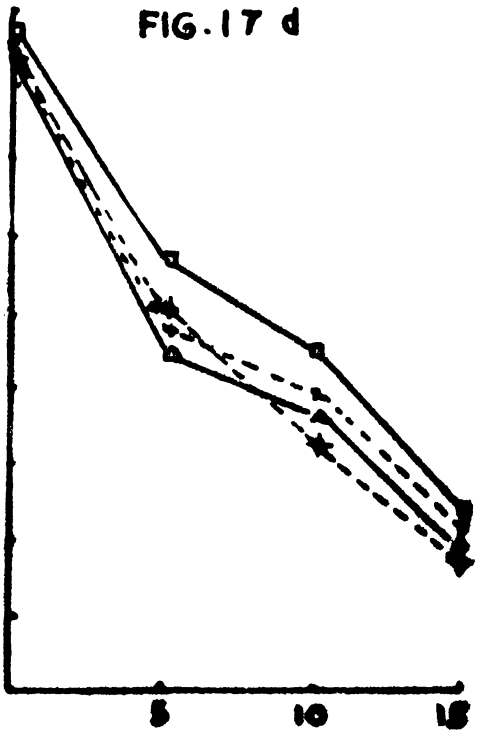


FIG. 17 d



MINUTES

P. INDICUS

R. KANAGURTA

S. SERRATA

TACHYSURUS SPP.

C H A P T E R S I X

EFFECT OF PH ON THE SURVIVAL OF SALMONELLA SEROTYPES AND VIBRIO PARAHAEMOLYTICUS.

6.1. Introduction.

Generally Salmonella organisms rapidly proliferate well at pH 6.5 to 7.5 and they are also able to grow readily in more acidic environments. The minimum pH at which these organisms are able to initiate growth, has not been studied properly in seafoods. It will vary depending on the serotypes, temperature of incubation, nature and composition of the growth medium. Dernby (1921), studied the effect of three serotypes, viz. S. typosa, S.paratyphi-B, and S. paratyphi-A at pH levels of 6.2 and 4.5 and stated that the minimum pH value for initiating growth is 4.5. Levine et al., (1940) cited that S. aertryeke, grow in broth having pH 4.5 with lactic acid, and pH 4.0 with hydrochloric acids. Stokes et al., (1957), explained the inability of Salmonella to grow below pH 5.0. Frost et al., (1967) reported that the pH value below 4.5

had lethal effect. Chang and Geopfert (1970), observed the growth at pH value below 4.5 had lethal effect. Ayers studied the lethal effect of higher concentration (10^6-10^7) of cells of six strains in lemon juice (2.5). But in tomato juice of pH the Salmonellae survived up to 30 days depending on the storage temperature. Vanderzant and Nickelson (1972), reported the lethality of Vibrio parahaemolyticus at lower pH 1 to 4 in shrimp tissues. Geopfert et al., (1969) reported the sensitivity of Salmonellae to volatile fatty acids. Furhter they studied the behaviour of three serotypes of S. anatum, S. senftenberg, and S.tennessee in different organic acids i and in hydrochloric acid which are commonly used as acidulant in food industries and found them very sensitive to organic acids. On scaning the literature, it has been found that little work has been carried out on the behaviour of Salmonella organisms in sea foods at lower pH .

6.2. Materials and methods.

6.2.1. Test organisms.

Twenty three serotypes of Salmonella isolated from fish and fishery products were used in this study (table 55). Stock cultures were maintained on nutrient agar slants at room temperature

after growing them at 37°C. They were transferred once in a month. Working cultures were maintained daily in Tripticase soy broth or Brain heart infusion broth and incubated at 37°C.

Similarly Vibrio parahaemolyticus cultures were maintained in Tripticase soy agar with 3% sodium chloride slants after growing at 37°C and stocked at room temperature. Cultures under use were maintained in Tripticase soy broth regularly every day incubating at 37°C.

6.2.2. Samples.

For the survival studies in lower pH values, the following homogenates were prepared from catfish (Tachysurus spp), Mackerel (R. kanagurta) Prawn (Penaeus indicus) and Crab (Scylla serrata) as described in section 4.2.2.

The pH of the homogenates were adjusted to various pH levels with various acids: Hydrochloric acid, Acetic acid, Propionic acid, Citric acid and Formic acid. The lower pH 4.5 and the higher pH up to 10 were used in this study. The excess acids were neutralised by sodium hydroxide for correct pH values.

The above homogenates were inoculated with an appropriate dilution of 24 hours old cultures of Salmonella and Vibrio parahaemolyticus so that the given cell concentrations were of the range from 10^4 to 10^6 per gram.

6.1.3. Enumeration of viable counts of Salmonella and Vibrio parahaemolyticus.

Samples were taken from the respective homogenates at various time intervals of 15, 30, 60, 120 min. and 8 hours. One ml of the sample (homogenates) was added to 9 ml of the sterile peptone broth or Tripticase soy broth and incubated at 37°C for 6 hours and then an appropriate serial dilution was made with 0.1% sterile peptone water. The toal viable counts of Salmonella were made in either Xylose Lysine Desoxycholate agar or Brilliant agar by incubating at 37°C for 24 hours. Developed colonies were counted by the methodology described in section 2.2.3.

Similarly for the enumeration of Vibrio parahaemolyticus one ml samples of homogenates were enriched in Tripticase soy broth with 3% sodium chloride and incubated at 37°C for 6 hours and plated in Thiosulphate citrate bile salt agar. Colonies were counted as per description given in section 2.3.2.

6.2. RESULTS.

Table 55 summarises the pH values at which the Salmonella serotypes and Vibrio parahaemolyticus could initiate growth in homogenates. Since all the homogenates of Cat fish (Tachysurus spp.) Mackerel (R. kanagurta), Prawns (Penaeus indicus) and Crab (Scylla serrata) exhibited similar results, only one set of data was presented. Among the serotypes, the magnitude of these pH values were not very different. Among found pH 5.0 the cells showed the initiation of growth . At pH 4.5 and below the Salmonella organisms were almost killed in 120 minutes (table 56).

The effect of the viable population of Salmonella and Vibrio parahaemolyticus (table 56) by the acids used in this study showed a hundred fold decrease with in 30 minutes, followed by another 10 fold decrease in 60 minutes. Beyond 120 minutes the organisms were completely eliminated.

The influence of temperatures on the varied concentrations of cells (low and high) 10^2 and 10^6 per gram of homogenates at different pH levels showed that Salmonella species were sensitive at pH 4.5 and below of incubation at 42°C for 8 hours. No significant difference of the cell concentrations

both and low, showed any survival at this temperature. Further frequent shaking during incubation had no influence on these organisms. The level of inoculum and incubation temperatures is shown in table 57. It can be seen that Salmonella and Vibrio parahaemolyticus were not tolerent of low pH 4.5 at room temperature $30^{\circ} \pm 2^{\circ}$ C and higher temperatures of 37°C and 42°C. It was found that at this pH values, the growth of these organismswas not initiated but killed. The rate of killing was more rapid at a higher temperature of 42°C.

In the case of Vibrio parahaemolyticus cultures, the viability was lost after 120 minutes at pH 4.5 and below, in both low and higher concentrations used in this study. At this pH 4.5, recovery of viable cells showed a drastic decrease after 60 minutes of incubation. In order to test whether a small fraction of the cell population may develop acid tolerant than the remainder studies were conducted by the enrichment technique and used MPN method for the enumeration of results. The data indicated that both Salmonella spp. and V. parahaemolyticus lost their viability (killed) after 120 minutes. In another other words, no survivors was found.

At higher pH of 8.0, 9.0 and 10.00 (alkaline range) at 37°C Salmonella spp and

V. parahaemolyticus strains behaved differently (table 58). At pH 10 Salmonella serotypes lost their viability to 120 minutes, while V. parahaemolyticus showed viability. But in both species the total viable populations decreased rapidly. At pH 8 and 9, Salmonella species decreased to a level of 10^2 per gram. In contrast V. parahaemolyticus population remained more or less same up to 120 minutes. These data indicated that higher pH value of 10 was lethal to Salmonella and V. parahaemolyticus and no viable cells were enumerated by the MPN method after 120 minutes exposure to pH 10 at 37°C.

Survival at room temperature and elevated temperature of 42°C did not give any significant change. The trend of data were almost the same as at 37°C.

Additionally, attempts were made to tollerate or grow in lower pH level the serotypes of Salmonella and V. parahaemolyticus, by numerous sequential subcultures. But the cultures tried in this study failed to grow.

6.3. DISCUSSION.

In various fermented seafoods such as fish ensilage and fish pickles, various fishfood acidulants are either used or produced by the micro-

organisms (fermentation). The minimum pH at which the various serotypes would indicate growth with food acidulants in various homogenates, showed that below pH 4.5 was not favourable for the growth of Salmonellae and Vibrios. At lower pH after 2 hours these organisms were almost wiped out and no viable cells were isolated even after longer incubation in the MPN procedure. But Chung and Goepfert (1970) found out the nutritional medium like triptone yeast extract glucose favoured growth of S. anatum, S. senftenberg and S. tennessee at pH 4.05 ± 0.05. The natural flora present in the homogenates didnot show any significant inhibition in the lower pH and in the higher pH (alkaline range).

The behaviour of Salmonella and Vibrio parahaemolyticus strains in fish and shellfish muscle homogenates indicated that at pH 4.5 and below and at pH 9 and above they cannot survive. Vanderzant and Nickelson (1972) studied on the survival of Vibrio parahaemolyticus at various pH values (1-10) in shrimp tissue and found that below pH 5.0, no organisms survived and above pH 8 to 10 they were static only at pH 9.0 and pH 10.0 the viability was lost after two hours. For favourable growth, the temperature of incubation and the water activity (a_w) required were

higher. If one of the parameters is less the growth was limited.

In this study then growth behaviour of Salmonella serotypes and Vibrio parahaemolyticus in various acids tested was restricted to the enumeration of survival of the growth. Further, the inocula of cultures used for this study were at stationary phase cultures of cells and give greater challenge for the survival or growth than the actively metobolising organisms (young logrithmic growth phase cells).

Almost all serotypes of Salmonella (in this study) showed more or less same pH values for their growth limit. Chung and Geopfert (1970), reported variance of pH values for the different acids tested. Since their values were in laboratory media, it is impossible to extrapolate the behaviour in food products.

These findings were not unexpected in view of the reports by other workers (Levine et al., 1940; Mossel 1963) that the pathogenic organisms can tolerate the adverse environmental parameters only when the other parameters are optimum. These serotypes of Salmonellae and Vibrio parahaemolyticus strains showed a lower range of pH tolerance of 4.5 and higher pH tolerance of 9.0

The present study provides

information concerning the survival of various serotypes of Salmonella and Vibrio parahaemolyticus strains isolated from different seafoods at lower pH and at higher pH.

Table 55. Minimum pH at which various serotypes of *Salmonella* and *Vibrio parahaemolyticus* were grown in Cat fish homogenates (Tachysurus spp).

Organisms	Hydrochloric acid	Acetic acid	Propionic acid	Lactic acid	Citric acid	Formic acid
S. heidelberg	5.0	5.0	5.0	5.0	5.0	5.0
S. arizona spp.	5.0	4.5	5.0	5.0	4.5	5.0
S. veltevreden	4.5	4.5	4.5	4.5	5.0	5.0
S. barielly	4.5	5.0	4.5	4.5	5.0	5.0
S. wayeross	5.0	4.5	5.0	4.5	5.0	5.0
S. newport	4.5	5.0	5.0	4.5	5.0	4.5
S. saintpaul	5.0	5.0	5.0	5.0	5.0	5.0
S. matopenmi	5.0	5.0	5.0	5.0	5.0	5.0
S. chingola	4.5	4.5	5.0	5.0	5.0	5.0
S. bredeney	4.5	5.0	5.0	5.0	5.0	5.0
S. anatum	4.5	5.0	5.0	5.0	5.0	5.0
S. stanley	4.5	4.5	4.5	5.0	5.0	5.0
S. hvittingfoss	5.0	5.0	5.0	5.0	5.0	5.0
S. nchanga	5.0	5.0	5.0	5.0	5.0	5.0
S. salford	4.5	5.0	5.0	5.0	5.0	5.0

Contd.....

Organisms	Hydro-chloric acid	Acetic acid	Propionic acid	Lactic acid	Citric acid	Formic acid
S. roan	5.0	4.5	5.0	4.5	5.0	5.0
S. chester	4.5	5.0	5.0	5.0	5.0	4.5
S. poona	5.0	5.0	5.0	5.0	5.0	4.5
S. cubana	4.5	5.0	5.0	5.0	5.0	5.0
S. senftenberg	5.0	4.5	5.0	5.0	5.0	5.0
S. typhimurium	4.5	5.0	5.0	5.0	5.0	5.0
S. enteritidis	4.5	4.5	5.0	5.0	5.0	5.0
S. typhi	5.0	5.0	5.0	5.0	5.0	5.0
Vibrio parahaemolyticus strains						
V. No. 178	5.0	5.0	5.0	5.0	5.0	5.0
.. 246	5.0	5.0	5.0	5.0	5.0	5.0
.. 327	5.0	4.5	5.0	5.0	5.0	5.0
.. 341	5.0	5.0	5.0	5.0	5.0	5.0
.. 1902	5.0	5.0	5.0	5.0	5.0	5.0
.. 3525	5.0	5.0	5.0	5.0	5.0	5.0

Table 56. Growth at pH 4.0 for Salmonella and Vibrio parahaemolyticus in Cat fish (Tachysurus spp) homogenates.

Oraganisms	15 min.	30 min.	60 min.	120 min.	8 hrs.
S. heidelberg	$2.1x10^4$	$4.2x10^2$	$1.7x10^2$	*	-
S. arizona spp	$1.7x10^4$	$1.8x10^2$	100	*	-
S. veltevreden	$6.1x10^4$	$2.4x10^2$	80	*	-
S. barielly	$4.5x10^4$	$3.1x10^2$	$1.3x10^2$	100	-
S. wayeross	$4.3x10^4$	$2.3x10^2$	210	-	-
S. saintpaul	$1.9x10^4$	$2.1x10^2$	100	*	-
S. matopenni	$3.6x10^4$	$1.9x10^2$	179	*	-
S. chingola	$3.9x10^4$	$2.9x10^2$	114	*	-
S. bredeney	$4.3x10^4$	$3.1x10^3$	610	*	-
S. anatum	$4.1x10^5$	$2.8x10^2$	180	*	-
S. stanley	$3.6x10^4$	$1.8x10^2$	110	*	-
S. hvittingfoss	$4.3x10^4$	$2.7x10^2$	$1.1x10^2$	*	-
S. nchanga	$3.7x10^5$	$4.3x10^2$	$1.7x10^2$	*	-
S. salford	$4.1x10^5$	$2.8x10^2$	$1.1x10^2$	*	-

* Salmonella detected. (-) absent.

Contd.....

Organisms	15 min.	30 min.	60 min.	120 min.	8 hrs.
S. roan	$3.9x10^5$	$2.1x10^2$	$1.3x10^2$	*	-
S. chester	$4.8x10^5$	$1.2x10^2$	$1.1x10^2$	*	-
S. poona	$5.1x10^4$	$1.3x10^2$	110	*	-
S. cubana	$2.8x10^5$	$1.8x10^2$	158	*	-
S. senftenberg	$5.1x10^5$	$2.8x10^2$	$1.2x10^2$	*	-
S. typhimurium	$4.3x10^4$	$1.7x10^2$	$1.1x10^2$	*	-
S. enteritidis	$3.9x10^4$	$2.1x10^2$	$1.0x10^2$	*	-
S. typhi	$3.8x10^5$	$1.7x10^2$	$1.1x10^2$	*	-
Vibrio parahaemolyticus.					
V. No. 178	$3.7x10^5$	$1.1x10^2$	*	-	-
.. 246	$3.1x10^5$	$1.1x10^2$	*	-	-
.. 327	$4.9x10^5$	$1.2x10^2$	*	-	-
.. 341	$5.9x10^5$	$2.1x10^2$	*	-	-
.. 1902	$4.1x10^2$	$1.4x10^2$	*	-	-
.. 3525	$5.6x10^5$	$1.1x10^2$	*	-	-

* Salmonella detected. (-) absent. * Vibrios detected. All values expressed per gram of homogenates

Table 57. Influence of temperatures at pH 4.5 on the growth of Salmonella and Vibrio parahaemolyticus after 120 minutes.

Organisms	Temperatures 30±2°C		37°C		42°C	
	L	H	L	H	L	H
S. heidelberg	-	*100	-	-	-	-
S. arizona spp.	-	*100	-	*100	-	-
S. weltevreden	-	*100	-	*100	-	-
S. barielly	-	-	-	-	-	-
S. newport	-	*100	-	*100	-	-
S. wayeross	-	*100	-	*100	-	-
S. metapeuni	-	*100	-	-	-	-
S. saintpaul	-	*100	-	-	-	-
S. chingola	-	*100	-	*100	-	-
S. bredeney	-	*100	-	-	-	-
S. anatum	-	* 30	-	-	-	-
S. stanley	-	*30	-	* 30	-	-
S. salford	-	* 30	-	*30	-	-
S. roan	-	*100	-	*100	-	-
S. senftenberg	-	*100	-	*100	-	-
S. typhimurium	-	* 30	-	* 30	-	-
S. enteritidis	-	* 30	-	* 30	-	-
Vibrio parahaemolyticus.						
V. No 178	-	-	-	-	-	-
V. no 246	-	-	-	-	-	-
.. 341	-	-	-	-	-	-
.. 1902	-	-	-	-	-	-
.. 3525	-	-	-	-	-	-

* less than; L- low level of inoculum (x10^2 per gram) H- High level of inoculum (x10^6per gm.) in Catfish homogenate (Tachysurus spp).

Table 58. Effect of higher pH ranges[+] on the survival of various Salmonella strains and Vibrio parahaemolyticus.

Organisms	pH range exposed to 120minutes. 8	9	10
S. heidelberg	$6.9x10^2$	$3.2x10^2$	*
S. arizona spp	$4.5x10^2$	$1.1x10^2$	*
S. veltevreden	$1.8x10^2$	$1.3x10^3$	*
S. bariolly	$2.2x10^2$	$1.4x10^2$	*
S. newport	$4.5x10^2$	$1.2x10^2$	-
S. wayeross	$5.2x10^2$	$1.3x10^2$	-
S. saintpaul	$3.8x10^2$	$1.2x10^2$	-
S. anatum	$3.7x10^2$	$1.1x10^2$	-
S. salford	$5.9x10^2$	$1.4x10^2$	-
S. roam	$3.9x10^2$	$1.2x10^2$	-
S. senftenberg	$4.7x10^2$	$1.7x10^2$	-
S. typhimurium	$2.7x10^2$	$1.2x10^2$	-
S. enteritidis	$4.8x10^2$	$2.5x10^2$	*
S. metapenni	$3.6x10^2$	$1.3x10^2$	-
S. chingola	$3.7x10^2$	$2.4x10^2$	-
S. bredeney	$4.9x10^2$	$1.1x10^2$	*
S. stanley	$3.7x10^2$	$2.5x10^2$	-
Vibrio parahaemolyticus.			
V. No. 178	$6.1x10^4$	$5.6x10^4$	*
.. 327	5.7x10	$5.9x10^4$	*
.. 246	$8.1x10^4$	$6.2x10^4$	*
.. 341	$8.3x10^4$	$5.6x10^4$	*
.. 1902	$4.8x10^4$	$5.7x10^4$	*
.. 3525	$6.9x10^4$	$5.8x10^4$	*

* Salmonella/ Vibrio detected; (-) No viable cells

[+]Catfish homogenates (Tachysurus spp.).

C H A P T E R S E V E N

SENSITIVETY OF SALMONELLAE AND VIBRIO PARAHAELYTICUS TO VARIOUS CHEMICAL AGENTS.

7.0. Introduction.

Information about the sensitivity of pathogenic organisms as Salmonella and Vibrio parahaemolyticus to various chemical agents is needed. Antibiotics are chemical agents or substances which have ability to act on particular cellular structures, interfere uniquely with metabolic path ways and remove co-ordinated biochemical systems by selectively affecting one component (Bryson, Liberfarb and Danielson 1970, Antimicrobial Agents and Chemotherpy, 1969, Ed. G.C. Hobby).

On reviewing the literature there is clearly a burgeoning clinical importance to the strains of Salmonella, and Vibrio parahaemolyticus that are resistant to various antibiotics, detergents

disinfectants and food preserving chemicals (Harvey and Edwards 1958; Ramsey and Edwards 1961; Yanagiwesa 1967; Lee 1973). In recent years studies are made on the resistense developed by various species of Salmonella and Vibrio parahaemolyticus to various chemical agents isolated from different fishes and processed products which were unhygenically handled during processing by the infected workers and carriers

Salmonella surveillance reports of U.S.A. (1970), indicated that Salmonella strains developed resistance to certain antibiotics such as Tetracyclines and Amphicillin. Many of the antibiotics substances had been examined for their developed resistance to Salmonellae. Various workers have studied and shown the resistant developed by S. typhimurium to tetracyclines (Harvey and Edwards 1958; Ramsey and Edwards 1961; McWhorter et al., 1963). The

In view of the wide spread use of these antibiotic materials as therapeutic agents for treatment of various bacterial infections necessiated the development of methods for their detection and assay study. Also antimicrobial agents are used extensively outside the medical field and so it is extremely important to realise the possible effects of these substances when in the water system from where fishes and shellfishes

are caught. For this reason, some of the typical representataive Salmonella serotypes and Vibrio parahaemolyticus were selected to determine their susceptibility to different antimicrobial agents. The work was mainly the study of the resistance developed by various serotypes of Salmonella and Vibrio pagahaemolyticus spp. isolated from fishes and processed fishery products.

In the present study, 210 isolates of Salmonella spp., comprising 24 serotypes and 180 isolates of Vibrio parahaemolyticus isolated from selected marine fishes and sea foods. Reference culture Vibrio parahaemolyticus NCIB 1902 also had been used for its sensitivity to various chemical agents, such as antibiotics, potassium sorbate, sodium benzoate and different concentrations of chlorine which is used as a disinfectant.

7.1. MATERIALS AND METHODS.

7.1.1. Preparation of test organisms and antibiotics

Two hundreds and ten strains of Salmonella during 1979-'81, were maintained regularly in nutrient agar slants by subculturing regularly once in two weeks and keeping at room temperature.

One hundred and eighty of Vibrio parahaemolyticus were maintained as stab cultures in

nutrient agar (0.8% agar) at room temperature.

All antibiotics mentioned in table 59 were obtained from varoius Pharmaceuticals firms that market these products. The desired concentrations of 100 ug/ml or lowerconcentrations were prepared from aquous suspension (neutral buffer) of the active subetances. The concentrated antibiotic solutions were prepared and sterilised by filteration by using Sieta filter. Further dilutions were prepared by sterile neutral buffer and kept in sterile glasswares and kept at frozen temperature of $\div 10^{\circ}C$. The concentrated antibiotic solutions were also kept in the frozen temperature of -10° C. The solutions kept in the frozen temperature had good stability.

7.1.2. Agar diffusion technique with cylinder method.

The cylinder plate method for the assay of antibiotics, described by Abraham et al., (1941) and later modified by Foster and Woodroff (1943) and aagain modified by Schmidt and Moyer (1984), was used in this study. This method depends on the diffusion of the antibiotic materials from steel or porcelain cups placed upon agar plates which are seeded with test organisms. Inhibition of growth of organisms occur in the proximity of the cup portions and the diameter of the inhibited zones vary with the concentration of the antibiotics.

This study was carefully designed

This study was carefully designed to control the variables such as preparation of bacterial suspensions, preparation of assay solutions, maintanance and inoculation of culture media, constant volume of nutrient agar poured into petridishes (assay plates), application of the antibiotics solutions to the petridishes, time of inoculation and readingof the inhibited zones in the petriplates and calculating the results, since these paramaters exert influence on the sensitivity of the organisms assayed for antimicrobial agaents.

Salmonella organism were grown in either nutrient agar of trypticase soy agar (Difco) for 12 hours at 37°C and centrifuged and further serially diluted up to 10^{-4} in sterile phosphate buffer (pH 7.0). One ml of the inoculum poured into petridishes and 20 ml exactly moulten nutrient agar (temperature approximately 50°C) was added and rotated for uniform distribution and allowed to solidify on a leveled surface so that the agar solidify uniformly. Then the plates were kept at refrigerated temperature (4°C) and left inverted for one hour to avoid excessive condensation. On each plates six cups were made with sterile hard steel punch so that each cup made at a distance which will not interfere with the zone of inhibition.

These petridishes were held for a peroid of two to three hours approximately at room temperature. A longer holding was done because of erratic inhibiting zones or some times poor zones development.

Fresh dilutions were made from the concentrated antibiotic solutions so that when diluted the antibiotic solutions of one ml will give a series of concentration of 0.25, 0.5, 1.0, 10.0, 25.0 50.0 100.0 and 200.0 ug. In addidion, higher concentrations were prepared for sulpha diazine as 80.0, 160.0, 320.0 and 640.0 ug per ml.

Salmonella spp. and Vibrio parahaemolyticus were tested for antimicrobial sensitivity according to Bauer et al., (1966). The levels of antibiotics (Bauer et al., 1966) were used for this investigation: ampicillin 10 ug/ml, chlorophenicol 30 ug/ml, chlorotetracycline 30 ug/ml, dihyro streptomycin 10 ug/ml, neomycin 30 ug/ml, nalidixic acid 30 ug/ml, oxytetracycline, 30 ug/ml, penicillin G 10 i.u., polymyxin B 300 units and kanamycin 30 ug/ml.

The set of seeded were carefully held in upright positions and with the help of standard sterile pipette, the antibiotic solutions were applied on the cup side, which was made by sterile steel punch

of diameter 3/8" (ca 0.95 mm). After the antibiotics were added to the plates were kept for 60 to 120 minutes in the incubator in inverted position so that any condensed water vapour will not hamper the zone of inhibition of 24 hours at 37°C and at times 48 hours were used for reading of the assay. The zone of inhibition was measured and compared with the table 60 reproduced from Bauer (1966) and Ryan et al., (1970).

Simultaneously agar dilution susceptibility tests were performed for comparison with the same suspension of Salmonella serotypes and Vibrio parahaemolyticus as prepared in the cylinder plate method and higher dilutions of antibiotics were incorporated in the Tripticase soy agar medium (Difco or Himedia). The average culture of inoculum used for seeding the antibiotics plates was around of 10^2 per ml. After 24 hours of incubation at 37°C, the results were recorded as complete inhibition (No visible growth), partial inhibition (isolated colonies), or no inhibition (growth comparable to the antibiotic free control plates). In other words, the number of colony forming units (CFU) capable of growing in the presence of antibiotic concentration of the drug equal to the untreated antibiotic plates' growth. Similar colony formations in the treated

plates, were treated as treated as resistant to the assayed antibiotic. Minimum inhibitory concentration (MIC) was considered as the least amount of antibiotic concentration ug/ml od medium that resulted in no visible growth. So antibiotic resistance was defined as a MIC greater than 10ug/ml. The Minimum inhibitory concentrations (MIC) were determined for the strains of Salmonella and Vibrio parahaemolyticus which showed different sensitivity in the diffusion technique, by the conventional tube dilution method with the medium of Tripticase soy broth. In the case of Vibrio parahaemolyticus, 3% sodium chloride was used in tripticase soy broth. To each tube was added (inoculated) one drop of standardised suspension of Salmonella spp (various serotypes) or Vibrio parahaemolyticus cultures having concentration ranges from 10^2 to 10^3 per ml. In this way all the tubes, were inoculated with bacterial concentrations in uniform numbers of assaying organisms. The same pipette was used for one series of experiment. A control was also carried out and the viable cell counts was made immediately. In this method , a slightly modified concentrations of antibiotics, in various tubes, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 50.0 and 100.0 ug/ml were used. In this investigation for serial dilution Tripticase soy broth

was used and further 3% sodium chloride was incorporated for the assay of Vibrio parahaemolyticus, at pH 7.4. The minimum inhibitory concentration end points were determined after 24 and 48 hours incubation. The extent of bacteriostatic or bacterial activity of the antibiotics was taken in the change of viable cell counts or in other words, the lowest concentration at which the inhibitory development of visible growth was taken. With Tripticase soy agar as plating medium, one ml of sample dilutionseries from each tube of MIC series were plated . Where the tube series was not diluted before plating, 0.25 ml of the solution was added to 20ml of moulten agar which is kept at 50°C (1:80) dilution) and plated . The usual dilutions of 10^{-1} and 10^{-2} were made before plating.. The lowest concentration of antibiotics showing no single colony formation of growth or growth in the plates of tripticase soy agar, after 48 hours were taken as the minimum bacterial concentration or in other words, minimum inhibitory concentration (Drew Bury, Otoole and Sherries 1972).

7.1.3. Food additives.

The effectiveness of food additives of potassium sorbate and sodium benzoate on Salmonella serotypes and Vibrio paranaemolyticus cont-

ained in fish homogenates and shell homogenates in controling the microbial activity were investigated. Salmonella cultures and Vibrio parahaemolyticus cultures including type cultures NCIB 1902 were grown in Tripticase soy broth at 37°C for 24 hours. For growth of Vibrio parahaemolyticus TSbroth was used. Cultures of known concentrations of $1x10^5$ per ml were inoculated in the following homogenates, viz: Cat fish (Tachysurus s Mackerel (R. kanagurta), Prawns (Penaeus indicus), and crab (Syclla serrata) (vide section 4.2.2.). The inoculated samples were kept in the refrigerated temperature of 4 to 8°C for ten days and contrlo also was carried out with out adding food additives of potassium sorbate and sodium benzoate (0.1% level).

For the enumeration of viable counts of Salmonella and Vibrio parahaemolyticus, samples were taken (25 grams) and blended with 225 ml of sterile phosphate buffer (0.003 M of K_2HPO_4) of pH 7.0 for one minute. With appropriate serial dilution, the viable counts were evaluated and confirmed as described in section 22 2.2.3 and 2.3.2 (vide supra).

7.1.4. Chlorine as disinfectant.

Chlorine is used commonly as a disinfectant to control various microorganisms in

different environmental conditions and in food products. The action of chlorine on various microorganisms was studied by many workers and it was explained as the free chlorine atom chlorinating the outer components of cells and breaking the peptides bonds as do other halogens (McKee et al., 1960; Fetner and Ingols 1956).

Despite the wide spread use of chlorinating agents as disinfectants in the fish processing industry and in the control of water supplies, little attention has been paid to their effect on the viability of these pathogens. This investigation was carried out to study the effect of chlorine on various serotypes of Salmonella and Vibrio cultures present in fish and fishery products.

Known low and high cell concentrations ($1x10^2$ to 3 and $1x10^6$ to 7 per gram) of Salmonella spp. and Vibrio parahaemolyticus were inoculated in sterilef fish and shell fish homogenates described above (vide supra). The homogenates were treated with 0.5% ppm (mg per liter) to 10 ppm of free available chlorine at pH 7.0 with definite time up to 15 minutes at room temperature (30°- 32°C).

Commercial chlorine bleach was purchased and kept in the refrigerated before use. Initially the available chlorine was determined from the commercial hypochlorite solution by pipetting out

10 ml in to 250 ml graduated flask containing pure glass- distilled water (chlorine free). The pipetted solution was added carefully by keeping the nosle of the pipette beneath the surface of the distilled water. With the glass distilled water, the solution was made up to the mark. 20 ml of the aliquqt solution was added with 20 ml potassium iodide solution and titrated with N/10 sodium thiosulphate. On nearing the end point (straw yellow colour) 5 ml of freshly prepared starch solution was added and titrated further with N/10 $Na_2S_2O_3$ until the blue colour appeared.

Available chlorine in NaOCl solution =

$$\frac{\text{ml N/10 } Na_2S_2O_3 \times 0.3546}{\text{ml of the sample}} = \% \text{ W/V}$$

Desired level of 0.5 ppm to 10 ppm of available chlorine (free) solution were prepared in fish and shellfish homogenates which contain the inoculated organisms of Salmonella serotypes and Vibrio parahaemolyticus cultures separately (concentration 10^6 per ml). The homogenates were stirred well and after exposure for one minute 5 minutes, 10 minutes and 15 minutes the homogenates were neutralised by adding one ml to 9 ml of sterile Tripticase soy broth

and further serially diluting with 0.1% sterile solution and plating in Xylose Lysine desoxychloate agar for *Salmonella* serotypes and Thiosulphate citrate bile salt agar for *V. parahaemolyticus*.

The number of dead organisms were determined from the difference in the counts on TS agar before and after exposure to the chlorine soultion (sanitizer). The metabolic injury was determined fmx by plating on differential plating media such as XLD agar for *Salmonella* species and TCBS agar for *Vibrio parahaemolyticus* and TS agar (Difco). The difference in TS agar and selective plating medium will give number of injured organisms.

7.3. RESULTS.

The twenty four serotypes of *Salmonella* tested for sensitivity to different antimicrobial agents were represented in tables 59 and 61 and showes the different types of resistances. The resistances were catagorised into three forms, resistnace intermediate, and sensitive in accordance with Bauer *et al*., (1966) and later by Ryan *et al*., (1970) and presented in table 60 along with standard inhibition zones of diameters in parenthesis.

In table 62 as expected, there were wide differences among its serotypes in the percentage of resistance. This was illustrated by the resistance level CTC in different serotypes of Salmonella and showed a percentage of 11.9%. Similarly the same serotypes showed resistance to different antibiotic agents. For examples S. typhymurium exhibited resistances to various antibiotics tested: three were resistant to chlorotetracycline, one was resistant to chloramphenicol, four were resistant to Neomycin, three were resistnat to Kanamycin and one was resistant to Oxytetracycline and six were resistant to penicillin.

On examination of the antibiotic sensitivity, results showed that 78% cultures of Salmonella were completely sensitive to the spectrum , leaving the antibiotic of sulpha diazine. Among the remaining cultures were resistant to three or more drugs. Among the serotypes of Salmonella, S. typhymurium, S. S. anatum, S.cubana, and S. saintpaul showed higher multible drug resistance. Of the 210 isolates of Salmonella serotypes, 11.9% were resistant to CTC, 17.14% were resistant Neomycin, 11.0% to Kanamycin, 10.0% to Oxytetracycline, 28.0% to pennicillin, 26.7% to Streptomycin and almost all were resistant to Sulphadiazine. None of the isolates showed resistance to Ampicillin.

Nalidixic acid, Polymyxin B, and only two isolates, S. typhi and S. enteritidis were resistant to Chloramphenicol.

Cultures of Vibrio parahaemolyticus also showed variable sensitivity to different antibiotics tested in this investigation and they are classified as resistant, intermediate, and sensitive as classified for the Salmonella species (table 64). It was evident that 88% were sensitive to chloramphenicol, 57.9% Neomycin and further 61.7% sensitive to Nalidixic acid, 82.8% to streptomycin and 50.6% to Polymyxin B. The strains were resistant to chlorotetracycline(63.3%), Penicillin (77.2%), and Sulphadiazine (94.4%). To other antibiotics, the organisms were moderately sensitive.

The minimum inhibitory concentrations of different antibiotics tested were shown in table 63. For this purpose dilution method and the cup plate diffusion method were comparatively studied. The diffusion plate method (steel cylinder cup) and dilution procedures are well accepted procedures for evaluation of the antibiotics(Wick 1964). The antibiotics used in this investigation are clinically sufficiently stable to remain unchanged during usual 24 to 48 hours incubation period. Experimental results of tube dilution

and diffusion technique exhibited similar results and only tube dilution is presented (table 63). The minimum inhibitory concentration study showed that the reduction number of viable cells of organisms increased with higher concentrations of antibiotics. It is reasonable to conclude that gradation of antibiotic concentrations was achieved in the zone of inhibition by the diffusion plate method (cup diffusion) corresponding to the individual MIC tube dilution mehtod. Thus a bactericidal antibiotic will produce a decrease in the number of colonies from the edge of zone of inhibition in the cup plate method(diffusion). When there was no colony present around the zone of inhibition, the antibiotic tested gave complete bactericidal action.

For the study of MIC, five or six cultures of Salmonella serotypes from these same species at random were taken and examined. The tables 63, and 65 list different serotypes of Salmonella cultures and Vibrio parahaemolyticus cultures, isolated from fish and shellfishes species. On examination, it was revealed that minimum inhibitory concentration varied with serotypes for different antibiotics. The MIC was worked out for the strains whcih were sensitive to the various antibiotics tested (table 63). It is evident

that most of the Salmonella serotypes and Vibrio cultures were well below the level of sensitivity to Chloramphenicol, Nalidixic acid, Polymyxin B, and Neomycin (fig. 18). To other antibiotics they are moderately sensitive, but completely resistant to sulphadiazine and penicillin G. In the case of Vibrio parahaemolyticus cultures also showed resistant to ampicillin.

On comparing the diffusion plate technique with the tube dilution method, the concentration of antibiotics for MIC study showed slightly higher values than the tube technique. On tube dilution method on that concentration (table 63 and 65) it was found that Salmonella and V. parahaemolyticus cultures showed static growth in the low level of ~~the~~ each of the antibiotic examined, a higher level either 5 to 10 ug or more was selected for all the cultures under study. It was also found that the plate count of Salmonella and Vibrio parahaemolyticus cultures showed a change in numbers of viable cells over 48 hours peroid which indicates an uprupt rise in MIC value. The increase in the viable cells indicated in the antibiotics that Penicillin and Streptomycin under test was degraded after 48 hours. Further experimental results indicated that MIC test reduced the number of viable cells when the concentration of antibiotics was increased to a

point where the antibiotic is almost 100% bactericidal.

Food additives or preservatives such as Potassium sorbate and sodium benzoate were used in foods to prolong the out growth of spoilage organisms. The effect of these food additives on Salmonella and V. parahaemolyticus cultures are listed in table 66. This study was carried out with all serotypes of Salmonella and Vibrio parahaemolyticus ~~xxxxxx~~ cultures. The percentage populations of Salmonella and Vibrio parahaemolyticus cultures decrease initially in both homogenates (fresh) of inoculated one and the cooked homogenates (to eliminate the native flora) stored at 4-8°C. ~~xx~~ There were three steps of decrease in percentages of the microbial load. The initial reduction (on two days) on early stages of storage, was rapid. This was followed by gradual reduction from the third day to the 66th day and further the loss of viability after this period was again rapid.

In examining the data for the effect of sodium benzoate (0.1%) and potassium sorbate, it has been found that no conclusive evidences on the reduction of the total microbial load even though the homogenates showed higher recovery of Salmonella species.

In the case of autoclaved samples, the total bacterial land (natural flora) was elimi-

nated and the higher percentages of redovery of these organisms were found in the presence of additives. The data of the present investigation indicated that the presence of additives of potassium sorbate and sodium benzoate resulted in these no substantial reduction of Salmonella species and Vibrio parahaemolitticus (table 66) but maintain only a static level for a period of three days.

Table 67 summarizes the percentages of survival of different Salmonella serotypes and cultures of Vibrio parahaemolyticus. At the concentration of 0.5 ppm Salmonella organisms survived up to 20 minutes of exposure but at 1.0 ppm rapidly vanished within ten minutes of exposure. On increasing concentrations of available free chlorine at 10 ppm and 20 ppm the higher population ($1x10^7$ per gram) lost its viability. All the serotypes under study behaved in the same manner. So only cultures at random were taken and presented in table 69.

Regarding Vibrio parahaemolyticus the viability was lost within 10 minutes at a concentrationof 0.5 ppm to 1 ppm . In the higher concentration rapidly lost its viability. The Vibrio species were more sensitive to lower concentration of available free chlorine. The present investigation explains that the

exposures of these organisms to 1 ppm to 5 ppm for 10 minutes caused death almost lost its viability and could not isolate even after enrichment techniques.

Exposure to lower concentration of 0.5 ppm and 1.0 ppm for 1 to 5 minutes, caused injury and death. The action of chlorine on cells was almost rapid in the initial exposure of time (less than 1 minute), although injury occured during the time of exposure. The injury of cells were detected by the selective medium. Experiments with Salmonella serotypes and Vibrio parahaemolyticus organisms showed similar injury when exposed to 0.5 ppm for 5 minutes. The injury counts on Tripticase soy agar and minimal agar did not differ significantly (data were not given). Figures 19&20 indicate death and injury of cells occured during exposure of chlorine. Higher concentrations of chlorine caused the death (table 69). V. parahaemolyticus cultures also behaved in the same manner as Salmonella.

Ampicillin. No resistant strains against these drugs were isolated.

Inferences based on the in vitro sensitivity to clinical effectiveness can mislead and may even be erratic. The study of Adams and Nelson (1968) and others, Daikos et al., (1960); Nelson and Maltalin (1966), showed that kanamycin and polymyxin B showed that they are highly effective in vitro, but failed in the treatment of salmonellosis and typhoid fever(Nelson and Maltalin 1966; Riley and Ryan (1961). Similar clinical results have been reported on the relative high toxicity of polymyxin B make these drugs undesirable for the therapy of Salmonella infections. Eventhough the reports showed some negative results, in vitro studies were carried out by many workers in recent years(Yanagisawa 1967; Sanayal et al., 1973; Sen et al., 1977; Lee 1973).

With specified criteria, the survey showed a incidence of multiple drug resistance for the cultures of Salmonella serotypes among which S. typhimurium, S. anatum, S. bredeney and S. typhi indicated more resistance. These pathogenic Salmonella organisms with infectious multiple drug resistance may be present in environment and possibly contaminate the fishes around.

Adams and Nelson (1968), reported that strains of S. typhimurium became resistant to ampicillin (21%), to tetracycline(36%) compared to other types of Salmonella. A similar phenomenon was also noted by Kaye, Merselis and Hook (1963) of 20% resistant. Since this strain S. typhimurium was commonly isolated in fish and fishery products and in human infections, it is likely to get resistant. The result of this study are in agreement with the findings of Adams and Nelson (1968) and showed resistant to tetracyline. Almost all the strains tested under this study showed resistant to sulphadiazine according to Baue et al., (1966) and Ryan et al., (1970).

The data reported here are from the techniques of diffusion and tube dilution plate methods and results are reiable for the detection of MIC to various antibiotics (Barber and Waterworth 1962; Parker and Jevans 1964; Sutherland 1964; Sutherland and Robinson 1964; Hallender et al., 1968). On a comparative study of MIC, both methods showed lowest concentrations of antibiotics. Drew et al., (1972) also stated similar findings by studying the resistance of Staphylococcus aureus to methicillin and related drugs. He found that the results were favourable at 35°C or below and resistance was lost at 37°C.

In view of the high incidence of gastroenteritis caused by Vibrio parahaemolyticus reported (Sen et al., 1977), it has become necessary to determine the antibiotics sensitivity to various drugs which are commonly used for clinical therapy. Out of 180 isolates of Vibrio parahaemolyticus from fish and fishery products, 170 were resistant to sulphadiazine, 107 to ampicillin, 114 to chlorotetracycline, and 139 to penicillin. All the strains were sensitive to tetracyline chloramphenicol, neomycin (MIC ranging gfrom 5.0 to 10 ug per ml). Sakazaki et al., (1963) reported that different type strains were sensitive to tetracycline and chloramphenicol. In this study, the isolates of Vibrio parahaemolyticus showed a mild resistance (intermediate) to chloramphenicol according to the standard of Bauer (1966). But Japanese workers used different concentrations to study the resistance and it is higher than these used by Bauer and Kirby (1965). Bauer standard is widely used by various authors (Barber 1964; Hewitt et al., 1969; Mallander et al., 1969; Chatterjee et al., 1970; Poursel et al., 1971; Muschel and Gustagson 1968; Drew et al., 1972; Gilardi 1971; Sanyal et al., 1973; Banang et al., 1974; Macphee 1977; Kelch and Lee 1978 Sen et al., 1977). The present d study has followed the recommended concentrations of antibiotics by Bauer et al., (1966).

Chatterjee et al., (1970), tested Vibrio parahaemolyticus cultures isolated from human gastroenteritis cases during 1969. Similarly Sanyal et al., (1973), studied the antibiotics sensitivity of 111 cultures isolated in Calcutta from cases of diarrhea and gastroenteritis in 1969, and grouped the results into three categories, viz; resistant, intermediate, and sensitive depending upon diameters of zones of inhibitions (Bauer 1966).

The Vibrio parahaemolyticus strains were sensitive to the Chloramphenicol, Polymyxin B, and Streptomycin and none was found to be resistant to the antibiotics tested under this study. To Oxytetracycline these organisms were sensitive as p per method employed by Sanyal et al., (1973). A similar behaviour was seen with Chlorotetracycline also. These result are in agreement with the findings of Sanyal et al., (1973). 39.4% of the strains were resistant to ampicillin and only 8% were sensitive and the rest were intermediate.

Minimum inhibitory concentration of different antibiotics in general with the two methods agreed well. All the strains tested, showed the range from 0.5 to 20 ug per ml. to various antibiotics. The present study indicated that except ampicillin, chlorotetracycline, oxy tetracyline without citrate, and penicillin and sulphadiazine are effective in the control.

The results of previous workers Chaterjee et al., (1970) Sircar et al., (1975); Sanyal et al., (1973); and Somang et al., (1974) showed gentamycin, chlorampenicol were most effective against this halophillic organisms, whereas ampicillin, kanamycin and streptomycin clinically were ineffective Sen et al., (1977) recommended chloramphenicol for the treatment of gastroenteritis rather tetracycline.

In the study of food additives both potassium sorbate and sodium benzoate had little effect in both raw and cooked muscle homogenates of fish and shell fishes on the reduction of Salmonella. Tomkin, et al., (1974) in cooked sausage reported that the spoilage flora was delayed by one day when treated with potassium sorbate. A similar phenomenon was showed by Ennard and Vaugher (1952) earliar. But in this study no inhibition of spoilage flora and pathogenic organisms of Salmonella and hallophilic V. parahaemolyticus was seem but only delay in their growth. Both additives were not effective in reducing the bacterial growth. On comparing both additives, potassium sorbate had better delaying effect than sodium benzoate. In thecase of autoclaved samples where thee natural flora was eliminated the inoculated pathogenic organisms increased in the presence of either potassium sorbate or sodium benzoate.

They recovered in greater percentages. Recently Roback and Hickey (1978) and Beuchat (1980) reported the delayed initiation of growth of Vibrio parahaemolyticus in the sea food homogenates.

Hypochlorite solution of different concentrations were used as a sanitizer and higher concentrations appeared to be needed to cause death and injury. The injury of organisms can be intrepted as an inability to obtain nutrients from various inorganic and organic sources or increased sensitivity to inhibit the growth in the media. Many research workers are engaged in the study of injury of microorganisms after exposure to freezing temperatures (Moss and Speck 1966; Nakamura and Dawson 1962; Postgate and Hunter 1963; Streka and Stokes 1959); higher temperatures, (Greenberg and Silliker 1961; Edwards and Busta and Specks 1965); irradiation (Roepke and Mercer 1947); and to chemicals such as phenol (Jacobs and Harris 1961). In recent years injury to microorganisms by environmental stresses has been received much attention as a possibe problem of extensive public health interest(Garvie 1955; Hurwitz, Rosano and Blattberg 1957; Scheusner , Busta, and Speck 1971; Camper and McFeters 1979).

The earliest research on the physiology of bacterial injury and death by chlorine was

.eported by Green and Strumpf in 1946 (Hoyano et al., 1973). They suggested that chlorine acted as inhibitor of glucose oxidation in cells. This work along with that of Knox et al., (1948) attempted to demonstrate that chlorine specifically a oxidised sulphahydryl groups of certain enzymes important in carbohydrate metabolism. They proposed that aldolase was the main site of action due to its essential nature in matabolism (Ingžols et al., 1953; Skidals'kaya 1969). Venkobachor et al., 1975; investigated the effect of chlorine in bacterial dehydrogenases. Electron microscopic examination of E. coli (Haringmann 1953) cells exposed to chlorine showed no morphological alteration.

This investigation included the study of the extent of chlorine injury to Salmonella species and Vibrio parahaemolyticus organisms in different concentrations. The recovery of experiments showed that the lower concentration of 0.5 ppm showed injury and was reversible under suitable conditions. The results in this study clearly indicated that Salmonella cells surviving in chlorination were capable of repairing from the resultant injury (figs 19 and 20). The cells were capable of repairing after a period of two to four hours inenrichment media of Tripticase soy broth. Furhter evidence of repair was shown by identical rate of growth of chlorine treated and untreated control

cells growth after 2 to 4 hours of nonselective nurtient media of Tripticase soy broth (fig 20). Similar effects were noted with Vibrio parahaemolyticus cells but higher concentration of 5 ppm to 10 ppm were lethal even by one minute exposure at concentration of cells 10^{5-7} per ml. The injury of cells usually enumerated by using increased nutritional media such as Tripticase soy broth , Brain heart infusion broth etc., (Straka and stokes 1959; Moss and specks 1963; Scheusner et al.,1971).

The injury now demonstrated as a result of chemical sanitisers adds to the information on those causing subletral impairment. Significantly, the injury was evident when lower concentrations of chlorine (sanitisers) used for the sanitising treatment and requires the need to measure insure the proper concentrations to effect the death of cells of Salmonella and V. parahaemolyticus.

The inability of selective media such as thiosulphate citrate bile salts agar and Xylose Lysine Desoxycholate agar for Vibrio parahaemolyticus and Salmonella species respectively used for growth, may lead to,erratic conclusions since these tests were normally employed in media for the monitoring of the sanitiser treatments. Since sanitising agents are used in the food industries and they are capable of causing injury to

pathogens like *Salmonella* and *Vibrio parahaemolyticus* organisms and may contaminate the fish and processed fishery products. This study indicates that higher concentrations cause death and larger percentages of survivors were injured . This suggests the necessity of maintaining the proper concentration of available chlorine in sanitizers which will be lethal to the pathogens.

Table 59. Antibiotics dissolved in neutral buffer (pH 7.0) tested in this investigation.

1) Ampicillin	aqueous
2) Chloramphenicol	aqueous
3) Chlorotetracycline	aqueous
4) Dihydro streptomycin	aqueous
5) Kanamycin	aqueous
6) Nalidixic acid	aqueous
7) Neomycin	aqueous
8) Oxytetracycline	aqueous
9) Penicillin G.	aqueous
10) Polymyxin B.	aqueous
11) Sulpha diazine	aqueous

Table 60. Zones sizes and their intrepretation for the sensitivity to antibiotics.

Antibiotics or Chemotherapeutic agents	Cup potency	Inhibition zone diameter Resistant mm	Intermediate mm	Sensitive mm
Ampicillin	10 ug/ml	11 or less	12-13	14 or more
Chloramphenicol	30 ug/ml	12 or less	13-17	18 or more
Chlorotetracycline	30 ug/ml	14 or less	15-18	19 or more
Dihydro streptomycin	10 ug/ml	11 or less	12-14	15 or more
Kanamycin	30 ug/ml	13 or less	14-17	18 or more
Nalidixic acid	30 ug/ml	13 or less	14-18	19 or more
Neomycin	30 ug/ml	12 or less	13-16	17 or more
Oxytetracycline	30 ug/ml	14osr less	15-18	19 or more
Penicillin G	10 iu/ml	20 or less	21-28	29 or more
Polymyxin B	300 units	8 or less	9-11	12 or more
Sulphadiazine	30 ug/ml	9 or less	10-11	12 or more

As per Bauer (9966) and Ryan et al., (9970)

Table 61. Zone of inhibition of various serotypes of Salmonella to different antibiotics.

Serotypes	CTC 30[a]	CP 30	KM 30	AM 10	NN 30	PM-B 300[b]	PN 10[c]	SM 10	NA 30	OTC 30	SD 30
S. heidelberg	26	24	23	24	21	16	21	14	20	16	10
S. arizona spp.	25	26	24	24	20	17	24	16	19	15	9
S. veltevreden	26	25	23	22	22	18	25	19	18	14	10
S. bareilly	24	26	25	23	22	19	24	16	19	16	9
S. newport	25	24	24	24	20	16	23	18	19	14	9
S. anatum	25	24	24	23	20	17	22	15	20	15	9
S. roan	23	22	21	20	21	17	23	14	21	15	9
S. bredeney	26	25	24	23	21	19	24	16	20	14	10
S. saintpaul	25	26	25	24	20	17	22	17	19	14	10
S. saintpaul	25	26	25	24	21	17	20	16	19	14	10
S. wayeross	23	26	25	24	19	16	24	19	18	16	10
S. chester	25	24	24	23	20	18	25	19	17	15	9
S. poona	24	23	23	24	22	19	22	16	16	16	10
S. senftenberg	26	24	23	23	21	16	24	17	18	16	10
S. typhimurium	24	22	22	22	20	18	23	17	17	15	9
S. typhi	25	23	24	23	22	15	21	18	20	14	9
S. eubana	25	24	23	22	19	15	22	20	20	16	10

(a) Indicates ug per ml. (b) Expressed in units per cup.
(c) in i.u. units. (d) Zone size diameter expressed in nearest mm of mean average of three readings.

CTC- Chlorotetracycline; CP- Chloropheniccl; KM- Kanamycin AM- Ampicillin; NM- Neomycin; PM-B. Polymyxin -B; PN- Penicillin; SM- Streptomycin; NA- Nalidixic acid; OTC- Oxytetracycline; SD- Sulphadiazine.

Table 62. Antibiotic resistance of Salmonella serotypes isolated from various fishes and fishery products.

Serotypes	Nos.	AMP	CTC	CP	NM	NA	KM	OTC	PM	PM-B	SM	SD
S. heidelberg	7	0	2	0	2	0	0	2	2	0	2	6
S. enteritidis	13	0	1	8	3	9	0	3	3	9	2	12
S. weltevreden	9	0	1	0	2	0	0	2	2	0	2	7
S. barielly	6	0	0	0	1	0	0	1	1	0	1	3
S. newport	12	0	2	0	3	0	1	1	2	0	3	6
S. wayerose	7	0	1	0	2	0	1	2	2	0	2	7
S. metapenni	4	0	0	0	0	0	0	0	2	0	0	3
S. saintpaul	10	0	3	0	4	0	2	2	4	0	4	3
S. chingola	5	0	0	0	0	0	0	0	3	0	1	3
S. bredeney	12	0	3	0	2	0	0	3	2	0	2	9
S. anatum	19	0	4	0	2	0	1	0	4	0	3	12
S. stanley	4	0	0	0	1	0	0	0	1	0	1	3
S. hvitingfoss	6	0	0	0	0	0	0	0	3	0	1	3

Contd.....

Serotypes	Nos.	AMP	CTC	CP	NM	NA	KM	OTC	PN	PM-B	SM	SD
S. nchanga	5	0	0	0	2	0	0	0	2	0	1	4
S. salford	12	0	0	0	1	0	0	0	2	0	2	8
S. roan	6	0	0	0	1	0	0	0	3	0	4	4
S. chester	7	0	1	0	1	0	1	0	3	0	2	5
S. poona	10	0	0	0	0	0	0	0	2	0	2	6
S. eubana	13	0	1	0	2	0	0	1	3	0	4	9
S. richmond	7	0	0	0	0	0	0	0	2	0	2	5
S. virchow	4	0	0	0	0	0	0	0	1	0	1	2
S. senftenberg	8	0	1	0	1	0	1	1	2	0	3	4
S. typhimurium	20	0	3	1	4	0	3	1	6	0	8	14
S. typhi	6	0	2	0	1	0	0	2	2	0	1	3
	210	0	25	2	36	0	11	21	58	0	56	141

AMP- Ampicillin; CTC- Chlorotetracycline; CP- Chlorphenicol; NM- Neomycin KM- Kanamycin; OTC- Oxytetracycline; Pn Penicillin; PM-B. Polymyxin -B; SM- Streptomycin; SD- Sulphadiazine.

Table 64. Antibiotic sensitivity pattern of 180 isolates of *Vibrio parahaemolyticus*.

Antibiotics	Resistant	Intermediate	Sensitive
Ampicillin	107 (59.44)	59(32.78)	14(7.78)
Chloratetracycline	114 (63.33)	63(34.99)	3(1.67)
Chloramphenicol	-	14(11.78)	166(88.10)
Kanamycin	-	87(48.33)	93(51.66)
Nalidixic acid	4 (2.22)	65(36.11)	111(61.66)
Neomycin	6 (3.31)	69(38.32)	105(58.22)
Oxytetracycline (containing Na citrate	6 (3.31)	97(53.91)	77(42.88)
Penicillin	139 (77.21)	41(22.79)	-
Streptomycin	-	22(12.22)	168 (87.78)
Sulphadiazine	170 (94.43)	10(5.57)	-

The sensitivity interpretation according to Bauer *et al*.,1966.

Table 65. Minimum inhibitory concentration(MIC) of different antimicrobial substances for *Vibrio parahaemolyticus*.

Antimicrobial substance	MIC in ug/ml	Nos. of strains studied
Ampicillin	10-20	10
Chlorotetracycline	5-10	15
Chloramphenicol	0.5-10	15
Oxytetracycline	5-10	10
Kanamycin	10-20	10
Nalidixic acid	5-10	
Penicillin	20-30 i.u.	15
Polymyxin B	80-100 u.	10
Streptomycin	10-20	10

Table 66. Percentage of population survival of various Salmonella serotypes and V. parahaemolyticus in various fish and shellfish homogenates stored at 4°C.

Serotypes	MH			Ca.H			PH			CH		
	2	4	6	2	4	6	2	4	6	2	4	6
	Days of storage.											
S. heidelberg	61	43	28	69	53	39	63	51	31	68	58	41
Homogenate												
Homo+ 0.1% K												
Sorbate	55	41	33	62	39	31	57	47	39	58	46	39
Homo+ 0.1% Na												
benzoate	64	51	35	61	50	37	68	57	41	68	54	37
Autoclaved+												
0.1% K sorbate	91	78	67	92	80	72	93	75	61	90	79	62
Autoclaved+												
0.1% Na benzoate	94	85	80	90	83	73	91	83	76	93	81	76
S. typhimurium												
Homogenate	68	47	32	71	49	36	60	44	31	62	50	42
Homo+ 0.1% K												
Sorbate	62	41	27	68	42	34	67	41	34	59	49	44
Homo+ 0.1% Na												
benzoate	63	47	31	70	51	38	61	52	40	62	51	36
Autoclaved+												
Homo+0.1% K												
sorbate	92	74	69	90	81	74	91	76	71	94	83	76
Autoclaved+												
0.1% Na benzoate	93	85	81	92	82	79	93	82	76	96	80	71

Contd....

Serotypes	MH			Ca.H			PH			CH		
	2	4	6	2	4	6	2	4	6	2	4	6
	Days of storage.											
S. roan												
Homogenate	63	41	30	73	51	38	63	47	34	60	52	44
Homo+ 0.1% K sorbate	57	43	28	62	32	29	59	49	35	59	49	41
Homo+ 0.1% Na benzoate	61	49	35	78	54	39	68	51	37	68	47	30
Autoclaved + 0.1%K sorbate	94	81	76	91	79	71	93	80	70	93	84	76
Autoclaved + 0.1% Na benzoate	93	87	80	92	81	76	90	84	73	92	81	72
S. anatum												
Homogenate	61	47	36	62	57	43	61	55	39	62	54	49
Homo+ 0.1% K sorbate	56	39	21	51	41	29	49	31	32	51	38	30
Homo+ 0.1% Na benzoate	62	50	31	59	48	31	64	52	39	63	51	33
Autoclaved + 0.1% K sorbate	91	71	63	93	81	73	91	73	61	94	79	71
Autoclaved + 0.1% Na benzoate	92	74	68	94	84	79	92	80	76	93	81	73

Contd....

Organisms	MH			Ca.H			PH			CH		
	2	4	6	2	4	6	2	4	6	2	4	6
	Days of storage.											
V. p. (NCIB)1902 Homogenates	71	31	29	61	29	21	63	33	24	67	30	22
Homo+ 0.1% K sorbate	68	37	30	63	31	27	67	34	27	61	29	21
Homo+ 0.1% Na benzoate	61	34	31	66	30	24	61	31	26	60	31	27
Autoclaved+ 0.1%K sorbate	92	42	39	91	47	38	94	41	36	92	39	34
Autoclaved + 0.1% Na benzoate	91	43	37	93	41	32	91	42	33	90	41	35
V. p. No 246 Homogenate	73	34	29	63	31	28	67	36	27	61	29	24
Homo+ 0.1% K sorbate	69	31	28	66	29	27	69	38	24	69	34	28
Homo+ 0.1% Na benzoate	63	34	32	61	31	28	68	38	28	61	32	29
Autoclaved+ 0.1% K sorbate	89	51	43	91	50	39	90	40	31	92	46	33
Autoclaved+ 0.1% Na benzoate	92	47	38	91	43	33	89	41	37	91	43	38
V. p. No. 341 Homogenate	75	29	27	73	39	27	76	39	89	79	43	31
Homo+ 0.1%K sorbate	73	37	31	70	41	36	71	43	34	81	46	36
Homo+ 0.1% Na benzoate	71	36	26	66	39	30	69	34	26	80	39	31
Autoclaved + 0.1% K sorbate	93	51	48	91	50	41	93	50	39	94	49	42
Autoclaved + 0.1% Na benzoate	91	49	41	89	49	39	91	51	30	90	41	36

Table 67. Percentage survivors at different concentrations of chlorine [illegible] for different times.

	MH				Ca.H				PH				CH			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
S. heidelberg																
Cons. Cl.																
0.5 ppm	89	35	10	4	83	37	12	6	81	31	9	3	81	33	9	7
0.0 ppm	51	21	3	-	47	19	6	-	39	21	4	-	49	19	3	-
5.0 ppm	32	10	-	-	28	7	-	-	41	6	-	-	29	3	-	-
10.0 ppm	5	-	-	-	6	-	-	-	2	-	-	-	7	-	-	-
20.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. typhimurium																
Cons. Cl.																
0.5 ppm	73	29	14	7	79	31	20	11	81	26	11	4	76	37	11	7
0.0 ppm	56	19	6	-	51	29	4	-	57	23	7	-	55	19	8	-
5.0 ppm	21	3	-	-	29	6	-	-	31	11	-	-	27	2	-	-
10.0 ppm	3	-	-	-	10	-	-	-	8	-	-	-	12	-	-	-
20.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MH - Mackerel homogenate (R. kanagurta); Cat fish homogenate (Tachysurus spp.); Prawn haemogenate (Penaeus indicus); Crab homogenate (S. serrata);

Contd ....

	MH				Ca.H				PH				CH			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	20 min	20 min
S. roam																
Cons. Cl.	91	37	12	3	86	31	7	-	89	32	5	-	86	29	11	-
0.5 ppm																
1.0 ppm	53	19	-	-	50	27	9	-	56	21	4	2	51	21	7	-
5.0 ppm	29	3	-	-	21	7	-	-	24	8	4	-	23	11	5	-
1000 ppm	-	-	-	-	8	-	-	-	12	-	-	-	9	-	-	-
20.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. salford																
Cons. Cl.																
0.5 ppm	81	33	9	6	87	27	11	-	84	29	14	6	65	31	11	4
1.0 ppm	47	25	4	-	46	31	7	2	51	22	5	2	43	37	6	2
5.0 ppm	23	7	-	-	31	11	-	2	39	5	-	-	36	7	6	4
10.0 ppm	9	-	-	-	10	-	-	-	6	-	-	-	4	-	-	-
20.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Contd....

	MH				Ca. H				PH				CH			
	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min	1 min	5 min	10 min	20 min
S. anatum																
Cons. Cl.																
0.5 ppm	88	37	12	6	84	31	7	-	80	41	19	11	87	39	17	7
1.0 ppm	51	20	6	-	49	21	3	-	43	19	12	-	53	21	12	3
5.0 ppm	24	4	-	-	27	11	-	-	24	8	-	-	2	4	-	-
10.0 ppm	4	-	-	-	7	-	-	-	7	-	-	-	12	-	-	-
20.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V.p. NCIB 1902.																
Cons. Cl.																
0.5 ppm	56	10	7	-	51	14	5	-	59	11	3	-	61	17	6	-
1.0 ppm	21	8	-	-	24	11	-	-	26	4	-	-	20	8	-	-
5.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V.p. spp No. 246																
Cons. Cl.																
0.5 ppm	53	12	3	-	49	7	-	-	46	6	-	-	57	11	-	-
1.0 ppm	19	4	-	-	24	6	-	-	18	4	-	-	14	2	-	-
5.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V.p. spp No. 341																
Cons. Cl.																
0.5 ppm	49	17	10	-	54	19	7	-	51	16	4	-	48	12	-	-
1.0 ppm	16	2	-	-	17	4	-	-	21	11	7	-	15	3	-	-
5.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.0 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MH- Mackerel homogenate (R. kanagurta); Ca. H.- Cat fish homogenate (Tachysurus)spp);
Ph- Prawn homogenate (P. indicus); Crab homogenate (S. serrata).
- indicates no viable organisms.

Fig.18. *In vitro* susceptiblity of various *Salmonella* serotypes to different antibiotics.

FIG. 18

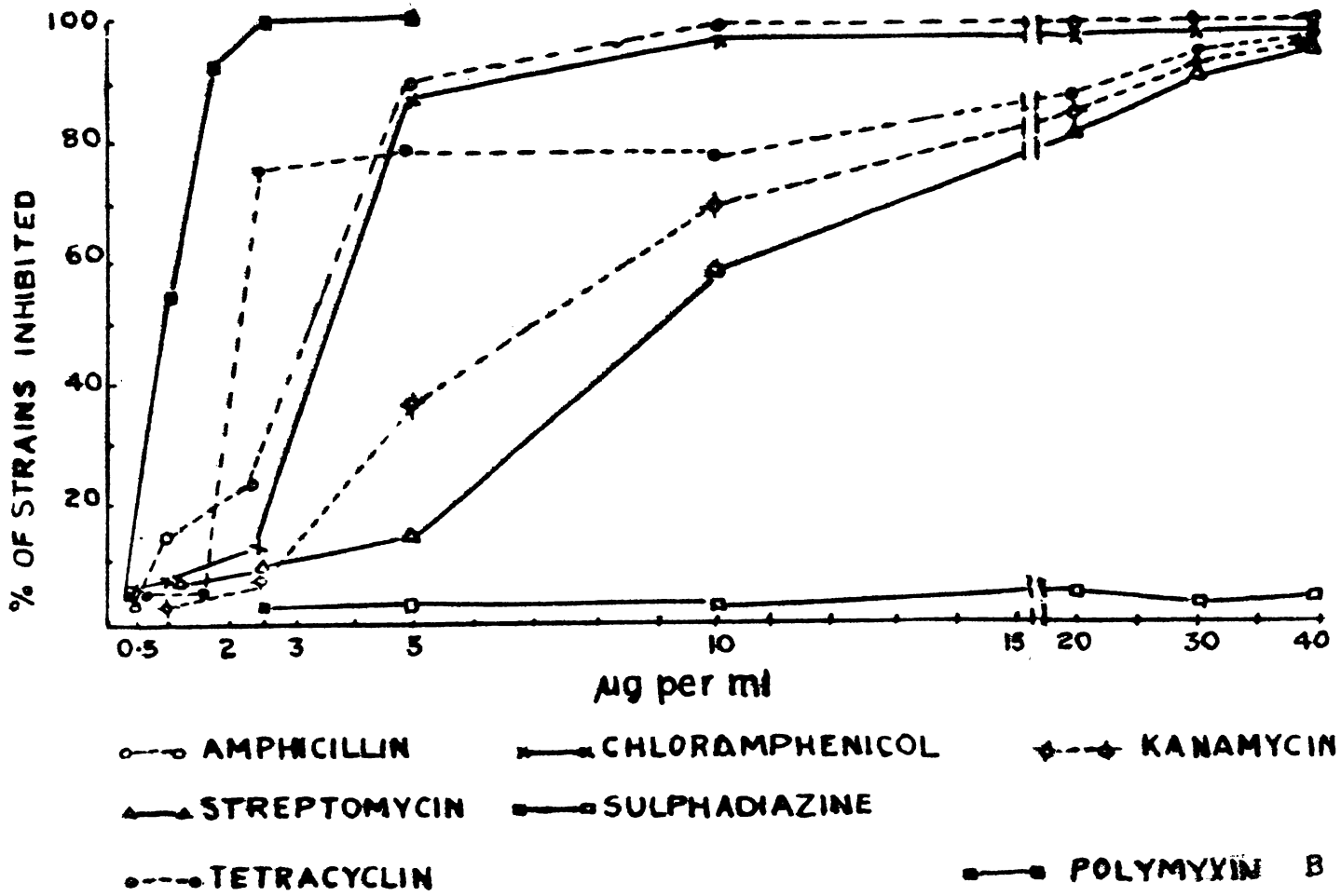

100
80
60
40
20
% OF STRAINS INHIBITED
0·5
2
3
5
10
15
20
30
40
μg per ml
AMPHICILLIN
CHLORAMPHENICOL
KANAMYCIN
STREPTOMYCIN
SULPHADIAZINE
TETRACYCLIN
POLYMYXIN B

Fig. 19. Injury and repair of different *Salmonella* serotypes that were exposed to chlorine (disinfectant) of concentration 0.5 ppm.

Fig. 20. Effect of exposure time for death and injury of various serotypes of *Salmonella* isolated from various fish and fishery products.

FIG.19

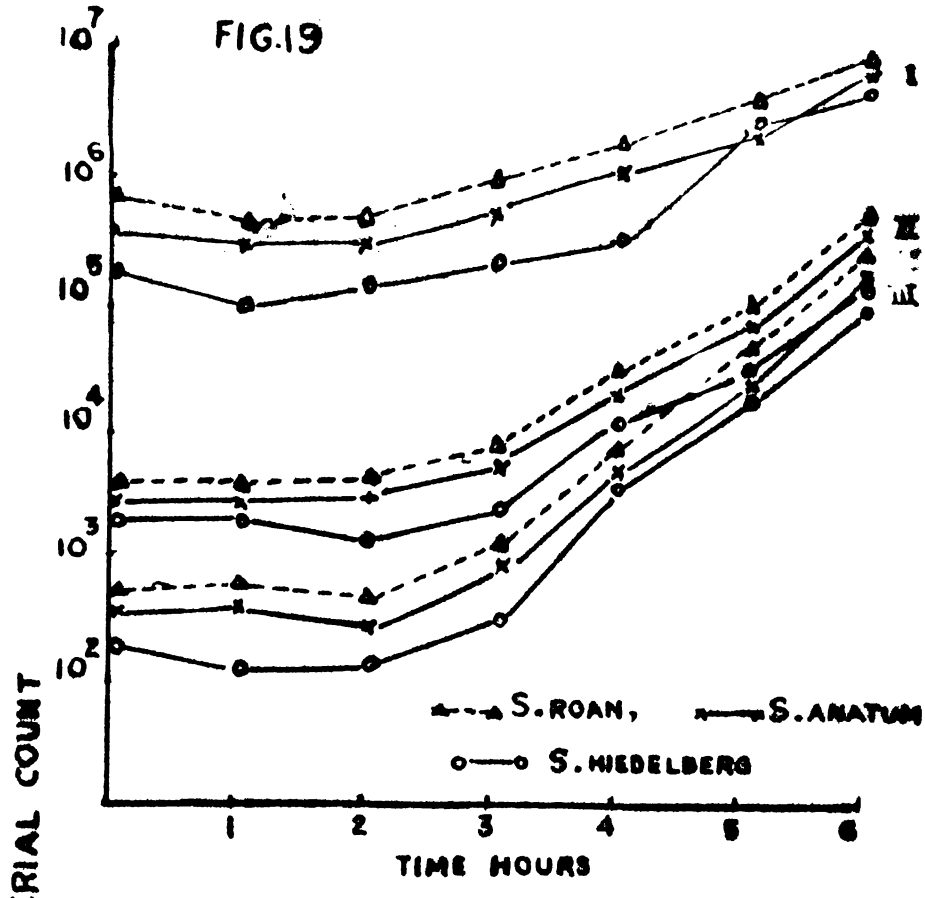

10^7
10^6
10^5
10^4
10^3
10^2
I
II
III
S.ROAN,
S.ANATUM
S.HIEDELBERG
1
2
3
4
5
6
TIME HOURS
BACTERIAL COUNT

FIG. 20

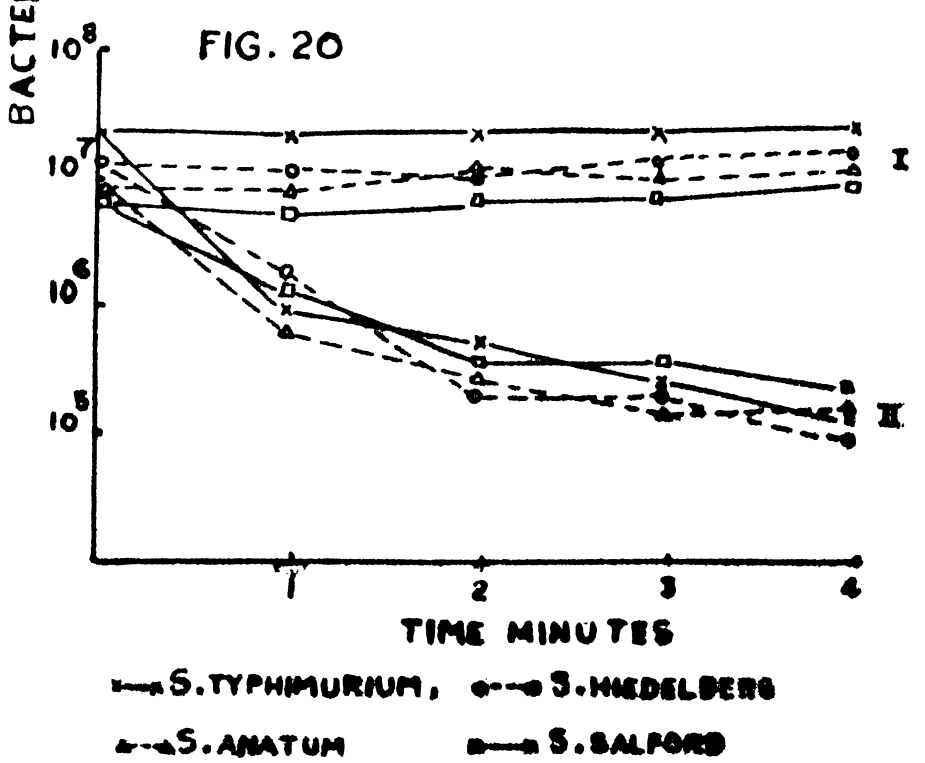
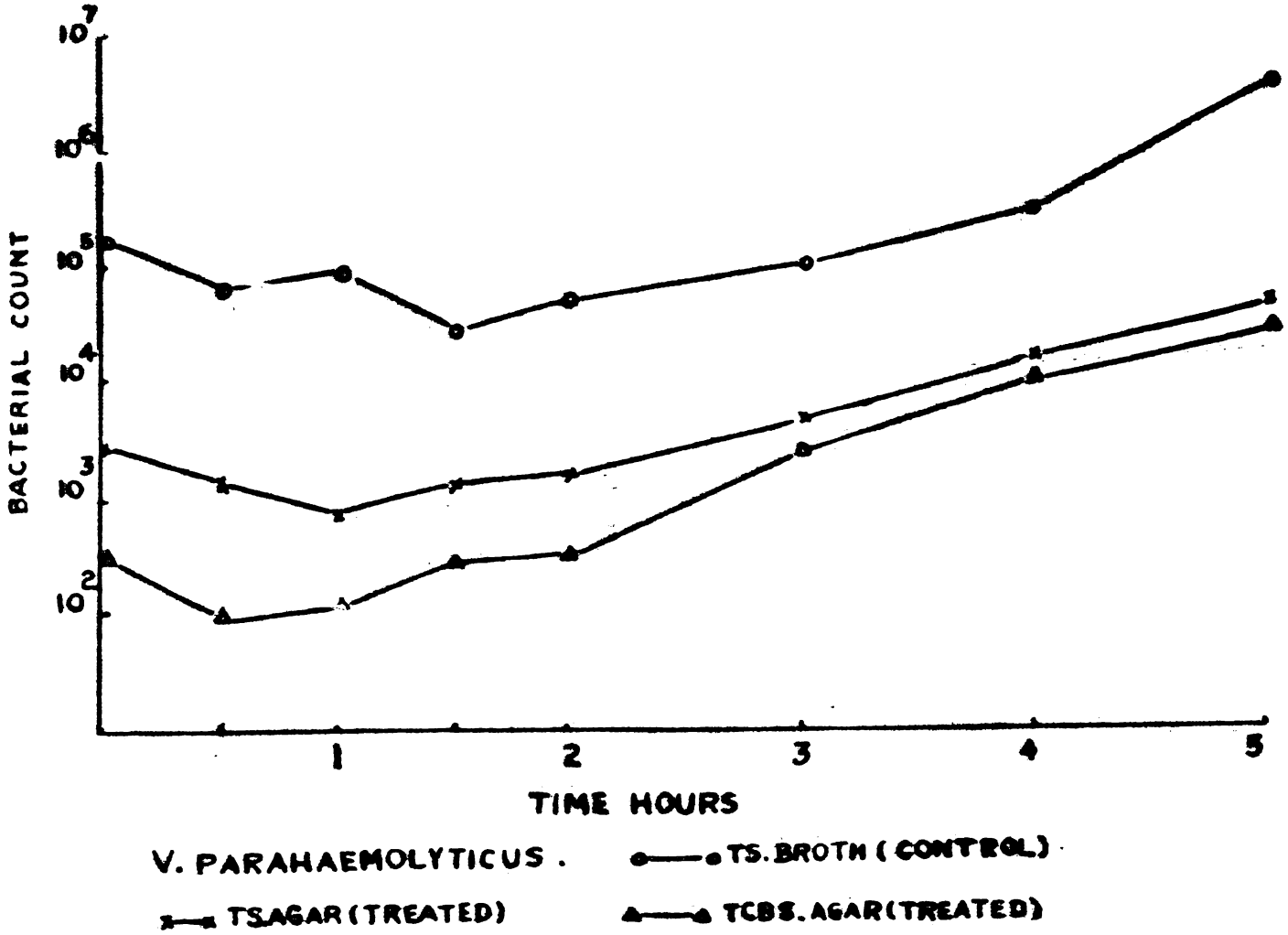

10^8
10^7
10^6
10^5
I
II
1
2
3
4
TIME MINUTES
S.TYPHIMURIUM,
S.HIEDELBERG
S.ANATUM
S.SALFORD

Fig. 20. Injury repair of Vibrio parahaemolyticus cells that were exposed to chlorineof concentration 0.5 ppm.

FIG. 21


BACTERIAL COUNT
10^7
10^6
10^5
10^4
10^3
10^2
1
2
3
4
5
TIME HOURS
V. PARAHAEMOLYTICUS .
TS. BROTH (CONTROL)
TS AGAR (TREATED)
TCBS. AGAR (TREATED)

S U M M A R Y

The present study provides information on the following facets of *Salmonella* and *Vibrio parahaemolyticus*, marine foodborne enteropathogens involved in out breaks of gastroenteritis: i) A general review of the relevant literature with special emphasis on the taxonomy of *Salmonella* and *Vibrio parahaemolyticus*; ii) the isolation of small numbers of *Salmonella* and *Vibrio parahaemolyticus* and enumeration by comparing different enrichment media and selective plating medium (agar media) with emphasis on the nutritional requirements; iii) incidence and distribution of *Salmonella* and *V. parahaemolyticus* in selected marine fishes and shellfishes from catches and from retail outlets in and around Cochin; iv) The influence and the behaviour of these organisms to lower temperatures during the processing phase, refrigerated 3°C to 6°C, and freezing -20°C ; v) the lethal effect of these organisms by cyclic freezing and thawing; vi) study of thermal death times (D values) and injury during freezing and heating of marine seafoods; vii) influence of different acids pHs (4.0-5.0) using different organic acids and also influenceof alkaline pHs (8-10) on the growth initiation of different sero-

types of Salmonella and V. parahaemolyticus; viii) sensitivity to chemical agents like antibiotics, food preservatives, (Sodium benzoate and potassium sorbate) and chlorine and their resistance developed by these organisms.

Some impotant aspects of the investigation are given below.:

Both surveys(79 and 80) indicated thart fresh and marketed samples of selected marine fishes and shellfishes exhibited wide fluctuations of the total viable bacterial population. In fresh harvested samples, the total bacterial population varied from $1.2x10^4$ to $6.9x10^6$ per gram. Among the retail market samples, the viable bacterial load was from $1.7x10^4$ to $7.6x10^6$ per gram. The incidence of Salmonella in commercial available selected marine fresh fin fishes and shellfishes in the retail markets and landing places indicated the presence of Salmonella and Vibrio parahaemolyticus. Both the landing centres and the retail outlets showed 22.2% of Salmonella. The V. parahaemolyticus registered 45.1% in fresh landing samples and 43.7% in market samples in the first survey. The second survey indicated 22.2% of Salmonella in the retail outlets samples while fresh landing samples recorded a lesser value of 20.0%. V. parahaemolyticus was detected in slighly lesser

numbers in fresh samples; 39.5% and 41.6% in market samples. The occurrance of Salmonellae was more on the surface region of the fin fishes and the shellfishes than on the gills and in the stomach regions. In the case of V. parahaemolyticus higher incidences were also found on the surface and in the stomach regions. In both surveys the fresh landed finfishes exhibited $1.1x10^2$ to $5.4x10^2$ per gram, while retail outlet samples recorded on higher value of $9.2x10^2$ per gram . Higher values were exhibited in the prawns (Penaeus indicus) and Crab (Syn Sp Scylla serrata) both in freshly harvested condition and in retail markets. The occurrance of various serotypes of Salmonella in different groups of fin fishes and shellfishes was as given below: S. anatum, S. typhimurium, S. typhi, S. newport, S. saintpaul, S. barielly, S. arizona Spp., S. senftenberg and S.weverose. The higher incidence of V. parahaemolyticus was found in both fresh and outlets of shellfishes as compared with finfishes. From the trends recorded with frequency of recovery of Salmonella from different regions of fish examined in finfishes and shellfishes it was recognised that the unhygienic nature and habits of fisher men and fish vendors made the fishery products a potential vehicle for food poisoning. The higher

percentage detection of *V. parahaemolyticus* in the prawns and crabs (*Scylla serrata*) may be due to food and feeding habits or activities at the bottom of the sea and the back waters. Further association of *V. parahaemolyticus* with other *Vibrios* may have the active role of degradation of chitin in the gastro-intestinal tract of the fishes and shellfishes, feeding on chitinous diets. Considerable proportions of fresh and retail marine fishes were examined during the two surveys and showed the presence of *Salmonella* and *V. parahaemolyticus*. The former was indicating the contamination by the handlers and vendors during the landings and at the retail outlet centers and the latter indicated the incidence of natural contamination from the environs. But normally this organism harbour only in low levels and the high incidental counts probably stemmed up from the cummulative effect of unhygienic practices in transportation and handling.

Both refrigerated (3°-6°C) and frozen (-20°C) temperatures registered sizable proportion of reduction of counts of *Salmonella* and *V. parahaemolyticus* in liquid broths and homogenates of fin fishes and prawns crabs when artificially inoculated. The counts of *Salmonella* reduced to one log cycle immediately after

refrigeration and still further during storage of 12 days of refrigeration registered reduction of 3 to 4 log cycles in broths and one to 2 log cycles the homogenates. At -20°C and further storage at the same temperature, the viable count of Salmonella was reduced to considerable extent in broths and homogenates. During 90 days of storage, the reduction came down to about 3 log cycles. Conversely, the broth showed high rate of mortality and were almost destroyed during that peroid of storage. The study of V. parahaemolyticus was also indicated gradual initial decline upto seven days and in further refrigerated storage (3° 6°C) the recovery of V. parahaemolyticus was inconsistant. Longer survival upto 14 days was noted with P. indicus and S. serrata.

Cyclic defrosting and freezing reduce the viability of Salmonella and V. parahaemolyticus quicker than in frozen storage at -20°C. The study of injury and death during freezing process, showed that broths indicated higher percentages of injury and death of Salmonella strains studied than in the homogenates of fish and shellfishes. The death percentages varied from 25 to35% in homogenates. The different serotypes of Salmonella showed variation (50 to60%) of injury. But however, the injured cells recovered when plced in noninhibitory media. V. parahaemolyticus showed death (61 to 75%) in various homogenates and revealed that injury was comparatively greater than in the

Salmonella organisms. Cell recovery and repair of frozen organisms were higher in homogenated and it was that the reapairing process was almost completed within two hours. Freeze injured Salmonella and V.parahaemolyticus required for cell repair different components such as vitamins, aminoacids and nucleosides bases. Among the broths, Tripticase soy broth, Brain heart infusion broth and Nutrient broth were superior to Minimal broth.

The study indicated that the various Salmonella serotypes survived when exposed to higher temperatures of 50° to 60°C for 15 min to 30 minutes The fish and shellfish homogenates gave better protection than the liquid broths of TSB and M-9 . On comparing the liquid broths M-9 gave the least protection. At higher temperature of 55°C and 60°C S. senftenberg showed higher survival than the other serotypes. The results indicated that the survival of various serotypes of Salmonella was not proportional to the initial concentration of 1.0×10^8 per gram. The protective aspects of various amino acids and carbohydrates at 55° and 60°C were almost nil. The commercial peptones which are commonly used for the bacteriological media gave better protection when compared few pure aminoacids. Yeast extracts (1% level) gave

better protection than the other peptones. At 60°C and above, these nutrients did not protect the serotypes of Salmonella used in this study. But fish meat muscle protein gave substantial protection at a temperatures of 55° and 60°C for 15 min. and 30min. respectively. This is a very significant observation that fish muscle proteins protect the Salmonella organisms from virtual elimination. The time required to kill the various serotypes of Salmonella organisms at a level of 10^8 per gram to a detectable level in various fish and shellfishes homogenates was evaluated. The C_{60} value (minutes of exposure at 60°C required to effect 100% destruction) are as follows: S. senftenberg in fish muscle, crab (Scylla serrata) and prawn (P. indicus) muscle homogenates (50%) 80; for other strains of Salmonella the values varied from 25 to 30. The thermal death times (average D values) of various serotypes of Salmonella on different temperatures of exposure were found to be higher values for the homogenates than for liquid broths of TSB and A-9 broth. The average D values at 50°C in TSB and A-9 broths and in homogenates were found in the range of 61.6 to 86.4 and 65.8 to 93.3 respectively. Thermally injured cells normally recovered in two hours when placed on Xylose Lysine Desoxycholate agar.

Eventhough V. parahaemolyticus registered higher value of survival in homogenates than in liquid broths, the cells were killed rapidly in the heating menstrua when compared to Salmonella organisms. With high salt concetration (7.5%) in the homogenates V. parahaemolyticus was protected. Obviously this organism is mildly halophilic and requires salt for their growth. Commercial peptones, Tryptones, amino acids, vitamins and nucleosides of bases in posphate buffer did not enhance the survival as in the case of homogenates. It was found that V. parahaemolyticus organisms were more heat sensitive than Salmonella. Resistance to heat stress of microorganisms is dependant on the chemical make up od the cell as well as the physiological nature of the heating environment. The changes of fatty acid content in the cellular components have a marked influence on the heat resistance. D values of V. parahaemolyticus icreased with higher concentration of sodium chloride at 50° and 55°C.

In this investigation, the isolated strains of V. parahaemolyticus showed lesser injury when the salt concentration increased from 0.5% to 7.5% and further the injured cells recovered within two to three hours when placed in the noninhibitory media. The recovery was independent on the protein, Ribonucleic acid and decoxy ribonucleic acid synthesis. The alteration of the

metabolic mechanisms of injured cells is related to the increased time lag for growth repairtaken they are placed in nutrient broth. Thermally injured cells were found to recover well in the rich nutrient media. The fish pf processing technique where the low and high temperatures used for the preservation and manufacture of seafoods for export and internal outlets, can produce stress or injury to the organisms and affect the metabolic and reproductive function which obstruct the growth in the selective media and thereby mmmmm as escape the detection.

Salmonella organisms rapidly proliferate well between pH 6.5 to 7.5 and grow slowly in acidic and alkaline environments. The minimum pH at which the Salmonella and V. parahaemolyticus organisms would initiate growth was found to be around pH 5.0 and some strain like S. weltevreden, S. harielly, S. newport, S. chingola, S. bredney, S. anatum and S. typhimurium were showed growth initiation at pH 4.5. Below pH 4.5 and above pH 10 these Salmonella and V. parahaemolyticus organisms were almost eliminated completely within 120 minutes of exposure. The influence of higher temperatures on the varied concentration (lows and high) of cells in the range of 10^2 and 10^6 per gram of homogenates at different pHs did not show any survival.

Salmonella serotypes isolated from different marine fishes were studied for their resistance or sensitivity to various chemical agents. S. typhimurium exhibited resistance to various antibiotics, Chlorotetracycline Chloramphenicol, Neomycin, Kanamycin, Oxytetracycline and penicillin. The other serotypes that showed resistance to three or more drugs were S. anatum, S. cubana and S. saintpaul. Out of 210 idolates of Salmonella 11.9% were resistant to CTC, 17.14% were resistant to neomycin, 28.0% to penicillin, 26.7% to streptomycin and almost all the isolated showed resistance to sulphadiazine. All the Salmonella organisms were sensitive to ampicillin Nalidixic acid, and polymyxin B. V.parahaemolyticus cultures also showed variable sensitivity to different antibiotics. 88% were sensitive to chloramphenicol, 57.9% to neomycin, 61.7% to Nalidixic acid , 82.8% to streptomycin and 50.6% to polymyxin B. 63.3% of V.parahaemolyticus indicated resistance toCTC, 79.2% to penicillin and and 94.4% to sulphadiazine.

The food preservatives Sodium benzoate (0.1%) and Potassium sorbate (0.1%) showed no conclusive evidences on the supression of Salmonella isolates and V. parahaemolyticus organisms even in the samples where the native micro flora was completely eliminated. Exposure to active chlorine concentration

of 0.5 ppm and 1.0 ppm for one to five minutes caused more injury than death for the Salmonella. But with increased chlorine concentration of 10 to 20 ppm. Salmonella and V. parahaemolyticus last their viability in the homogenates with ten minutes. This study indicated that the optimum concentration of Chlorine to kill the Salmonella and V. parahaemolyticus was 10 to 20 ppm of chlorine in active form.

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SALMONELLA IN SEAFOODS

M. Arul James, T.S.G. Iyer, and C.C. Panduranga Rao.

ABSTRACT

A brief review of investigations carried out in this laboratory on the methodology of isolation, incidence and distribution, and the rare serotypes of Salmonella occured in fish and fishery products and their sensitivity to low temperatures and antibiotics were reported.

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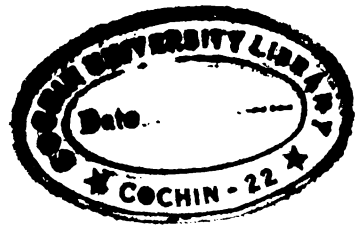

SURVIVAL OF SALMONELLA IN SHRIMP AND FROGLEGS.

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ABSTRACT

Ten serotypes of Salmonella commonly isolated from seafoods were taken for study of survival in refrigerated and frozen temperature storage and in different pH values. Salmonella serotypes were inoculated artificially in two concentrations (low $2x10^2$/g and higher $2x10^5$/g populations) in shrimps, shrimp homogenates, frog and froglegs homogenates. All the serotypes survived during refrigerated storage (7° to 5°C). Duration of survival was proportional to the initial concentrarion of inoculation. Heavily inoculated samples showed the presence of Salmonella up to five months in the muscle portion of prawns and froglegs, while survial in respective homogenates were upto three months. Lowere population of Salmonlla serotypes were distroyed at 60°C, by one minute heating of the homogenates. With the larger population ($2x10^5$/g), S. anatum, S. cubana, S. enteritidis, and Sa senftenberg survivaed at 60°C by one minute heating. Above 80°C, nome of the serotypes tested were survived by five minutes heating. All the serotypes tested were sensitive to pH values below 5.0 and above 8.0.

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