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Title: SURVIVAL OF VIBRIO PARAHAEMOLYTICUS IN KOREAN-STYLE SALTED OYSTERS

Abstract approved: Dr. Margy Woodburn

This study was designed to determine the survival of Vibrio parahaemolyticus in salted oysters as prepared in Korea. Three levels of salt concentration were included: 3.0%, 6.8%, and 10.6% of the weight of the raw oysters. Two methods were used to inoculate the products, surface-inoculation and injection into the oysters, which were then held at 18°C. Multiplication of V. parahaemolyticus (Japanese strain T-3765-1) did not occur in the salted oysters, except for a slight increase in numbers after 24 hours from surface-inoculated samples with 3.0% salt and all samples of injected oysters. The samples with the higher salt concentrations showed longer survival of the organisms. Numbers of survivors in surface-inoculated oysters were markedly reduced within three days; the samples of injected oysters did not show reduced numbers in this period. There were more survivors from the injected samples over

the storage period than from the surface-inoculated samples. No viable cells were observed after seven days from the surface-inoculated samples with 3.0% salt and the injected samples with 3.0% salt, after eight days from the surface-inoculated samples with 6.8% salt, after nine days from the injected samples with 6.8% salt, and after 11 days from the injected samples with 10.6% salt. A small number of V. parahaemolyticus were recovered from the surface-inoculated samples with 10.6% salt after eight days, the last testing period for these samples. The results of pH measurement suggested that the samples with higher salt concentration may have prolonged survival of V. parahaemolyticus through the effect of the higher pH value. An analysis of variance revealed a significant difference (5% level) in recovery between a modified isolation medium (Twedt and Novelli) and thiosulfate-citrate-bile salts-sucrose agar. Salt concentrations of 3.0%, 6.8%, and 10.6% slightly reduced the total bacterial counts after three days from the two lots of oysters used for the surface-inoculated samples and further reduction was observed after seven days; reduction in numbers was not detected within 8 or 11 days from the two lots used for the injected samples. The latter two lots had much lower plate counts at the beginning of the experiment. No relationship was found between pH and total bacterial counts on the salted oysters.

Survival of *Vibrio parahaemolyticus* in Korean-Style Salted Oysters

by

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SURVIVAL OF VIBRIO PARAHAEMOLYTICUS IN KOREAN-STYLE SALTED OYSTERS

INTRODUCTION

Vibrio parahaemolyticus, an enteropathogenic organism which causes foodborne infections, was originally found by Fujino et al., 1951, in Japan (Sakazaki, 1969). It has been repeatedly isolated from sea water, sediments and shellfish in many parts of the world. The first isolation of the organism in Korea was done by Chun et al., 1967, from sea fish and shellfish caught in a southeastern coastal area. Thirty-one of 540 specimens were positive for V. parahaemolyticus. The organism has been repeatedly isolated from sea fish and sea waters in various coastal areas of Korea and outbreaks of food poisoning have been reported by several investigators (Chun, 1971). Therefore, the possibility of foodborne infection caused by eating fish in Korea must be considered. However, very few experiments have been done to determine preventive measures for this foodborne illness. Temmyo (1966) suggested that outbreaks can be prevented by washing seafish sufficiently in fresh water or in boiling water and by keeping them cold, as well as cooking them before eating. Thorough washing and drying of utensils to avoid sources of secondary contamination were also recommended.

One of the most important sources of protein in Korea is fish,

both salt and fresh-water varieties. The Korean fishery industry supplies an estimated 85% of the animal protein for the Korean people (Borgstrom, 1965). Oyster is one of the important species of fish in Korea as well as other shellfish. Salting has been widely used for preservation of fish. Over half of the oyster catch is salted and the rest is marketed fresh, sun-dried with or without salt, or canned. Many Korean wives buy fresh oysters and salt them at home. The salted oysters are then held in a cool place for a week to months. The fermented oysters are served as the raw, salted product or added im making "kimchi", which is a Korean fermented vegetable.

In this study, an experiment was designed to determine the survival of V. paraheamolyticus in salted oysters. Because this organism shows great tolerance to salt concentration, it is rather a question whether or not salting will be a safe way for preservation of raw oysters.

Based upon the preparation of salted oysters by local Koreans, three levels of salt were compared, 3.0, 6.8 and 10.6% of the total weight of the oysters. Initially, salt concentration in the liquid would be much higher than these with diffusion occurring slowly. The salted products were held at 18°C, the average autumn temperature in Korea, until the end of the storage time tested.

REVIEW OF LITERATURE

Contamination of Shellfish and Their Environment with Vibrio parahaemolyticus

V. parahaemolyticus, as it is now known, was first isolated by Fujino et al., in 1951 (Sakazaki, 1969) and is characterized by the production of a foodborne infection syndrome associated with warm summer months and the consumption of raw sea fish or shellfish. The incidence of food poisoning due to V. parahaemolyticus is high in Japan and the reported cases have represented 40 to 70% of all reported foodborne illnesses due to bacterial causes (Fishbein et al., 1969).

There is no doubt that V. parahaemolyticus is widely distributed throughout the seas and oceans of the world (Fishbein et al., 1970), and confirmed incidences of foodborne illness have been reported with increasing frequency. The first isolation of the organism in the United States was done by Baross and Liston (1968) from waters, sediments, and oysters (Crassostrea gigas) of the Puget Sound region. Numbers of vibrios ranged from 30 to 350 per ml in the water samples, from 100 to 2,000 per g in the sediment samples, and from 65 to 4,000 per g in the oyster samples. V. parahaemolyticus was isolated from oysters and clam samples taken from oyster areas in Washington (Baross and Liston, 1970). They

also found this organism in the intestines of crabs in the same area. They concluded that, in general, V. parahaemolyticus and related vibrios appear to be associated with habitats with high organic nutrient content. Krantz et al. (1969) also reported the isolation of V. parahaemolyticus from blue crabs (Callinectes sadipus) from Chesapeake Bay. They isolated strains of V. parahaemolyticus from lethargic and moribund crabs being retained in commercial tanks during the "shedding" stage. Mortality of crabs in some tanks was greater than 50%. Vanderzant et al. (1970) isolated V. parahaemolyticus from white shrimp (Penaeus setiferus) from the Gulf Coast of Mexico. They found that this organism was pathogenic for brown shrimp (Penaeus aztecus). Thomson and Trenholm (1971) isolated the organism from Canadian Atlantic shellfish. The wide distribution of V. parahaemolyticus in the coastal sea area has been reported by several workers in Japan (Horie et al., 1964; Yasunaga, 1967).

Miyamoto et al. (1962) reported a seasonal incidence of fish and shellfish food poisoning by the halophilic pathogens, mainly during the summer season, June to October. A correlation was found between season, water temperature, and the level of incidence of V. parahaemolyticus in oysters (Baross and Liston, 1970). This seasonal incidence is closely related to the organism's temperature sensitivity. Samples collected in New Hampshire during the month

of September were found to be contaminated with up to 1,000 V. parahaemolyticus per 1 of water and 500 organisms per g of oysters (Bartley and Slanetz, 1971). The numbers of this organism decreased appreciably as the temperature of the water decreased from 14 to 8°C (during October and November). No vibrios were isolated from oysters maintained in tanks receiving water with temperatures of 0 to 9°C.

V. parahaemolyticus has been recovered from market samples of shellfish. Rodriquez-Rebollo et al. (1971) found the organism in two mussels obtained from fish markets in Madrid, Spain. Shellfish from retail markets in the Canadian Atlantic coast were found to be contaminated by V. parahaemolyticus (Thomson and Trenholm, 1971). The positive samples were unfrozen oysters, clams, periwinkles and mussels, and frozen clams, periwinkles and mussels. Fifty-six strains of V. parahaemolyticus were recovered from 60 samples of processed meat of Chesapeake Bay blue crabs by Fishbein et al. in 1970. The market samples of Chesapeake Bay processed blue crab were cooked, picked, packed, and refrigerated meat. These results indicate that seafoods could be contaminated by the organisms that survive during market storage.

Survival and Growth of Vibrio parahaemolyticus in Foods under Preparation and Storage Conditions

V. parahaemolyticus is a halophilic, facultative anaerobe (Sakazaki et al., 1963). It grows in ordinary media with a salt concentration from 1 to 7% (Twedt et al., 1969). The optimum pH is 7.5-8.5 with a range of 5.0-11.0 (Fishbein et al., 1970). The optimum temperature is 37.5°C, but growth occurs between 15 and 40°C (Sakazaki, 1969). V. parahaemolyticus is an enteropathogen and is responsible for disease outbreaks that usually follow the eating of such foods as raw meat of fish and shellfish, semi-dried fish such as shirasu, and other groups of food stuffs, i.e., pickled vegetable and macaroni salad (Aiso and Matsuno, 1961).

Temperature has a marked effect on the survival of V. parahaemolyticus. Baross and Liston (1970) observed that most strains of V. parahaemolyticus appeared to be sensitive to refrigerator temperature. Matches et al. (1971) found that at 0.6°C, the reduction in numbers of viable cells of V. parahaemolyticus varied from 2.0 to 6.4 logs for storage times from 26 to 48 days. In the frozen state, at -18°C and -34°C, the reduction of viable cells was faster and, after 12 days of storage at -34°C, 99% reduction in counts was seen in fish homogenate (English sole, Paraphrys vetulus). Temmyo (1966) reported that V. parahaemolyticus with 10^6 cells

per ml in peptone solutions became extinct at -2 ± 0.5°C within five days. At 4 ± 0.5°C the Vibrio counts with 10^2 cells per ml were completely reduced after six days. He also studied the effects of salt concentrations on the survival of V. parahaemolyticus in saurel extracts at -2°C. He used 0.5, 1, 3, 5, and 7% salt and found that the organism survived after eight days storage of samples, showing that the greater the salt concentration, the longer the survival of the organism.

Heating is a common process applied to foods to kill pathogenic organisms. Temmyo (1966) found that V. parahaemolyticus was killed if heated in peptone solution at 55°C for 10 min or at 60°C for 5 min, and he concluded that heated foods are safe if they are protected from secondary contamination. Kawabata and Kozima (1963) reported that the lethality of the organisms disappeared when the 3% NaCl broth culture was heated at 100°C for 10 minutes, or when the culture was filtered through a Seitz filter.

Secondary contamination may be an important factor. Food poisoning by V. parahaemolyticus following the eating of cooked shellfish has been recently reported. One was an incident in New Jersey in October, 1972. A total of four family units consisting of 15 members were affected after eating cooked shrimp and crabs from the same seafood bar on the same day. Both the crabs and shrimp were cooked at the seafood bar and then placed in the

baskets used for shipping the raw crabs. V. parahaemolyticus was recovered from the swabs of these shipping baskets (Center for Disease Control, 1972b). Another example was reported after illnesses following a flight from Bangkok, Thailand, to London (Center for Disease Control, 1972a). Crab meat prepared in the flight kitchen at the Bangkok airport was suspected as the vehicle of infection. V. parahaemolyticus was isolated from all stool specimens examined.

In the prevention of food poisoning, the use of ice during preservation, preliminary washing with fresh water, and the use of vinegar at the time of cooking are to be recommended. Kawabata and Kozima (1963) reported that washing the cells with 3% NaCl broth or physiological saline did not change the lethality for mice, but that bacteria lost its lethality completely if washed with distilled water. Temmyo (1966) found that seasonings such as vinegar, soy and Worcester sauce exerted a growth inhibitory effect on the organism. Worcester sauce was effective in 15 min, soy in 10 min.

Isolation Media and Identification of Vibrio parahaemolyticus

V. parahaemolyticus is an anaerobic, gram-negative rod exhibiting pleomorphism. It has biochemical characteristics of oxidase positive, catalase positive, Voges-Proskauer reaction

negative, methyl red positive, and urease negative. It ferments glucose, maltose, and starch without producing gas, does not ferment lactose and sucrose, and utilizes citrate. Because there is a wide variation in biochemical reactions, it may be difficult to identify the organism.

Development of a method for the isolation and identification of the pathogenic organism from foods is still an important part of the research which is needed. Sakazaki et al. (1963) used trypticase soy agar containing 3% NaCl and 0.2% Teepol as a first isolation medium. Almost all the colonies grown were moist, smooth, circular, and opaque in appearance. They found that all the cultures studied grew well on/in ordinary media containing 3% NaCl, but grew very poorly or did not grow at all on/in those containing no NaCl. However, the optimum concentration of NaCl for growth of the organism was changed by medium and temperature of incubation. When the organisms were inoculated into brain heart infusion broth, they grew well in the broth without additional salt. Bromothymol blue-Teepol agar (BTB-Teepol agar, pH 7.8) was used for isolation of the organism from raw fish, shellfish, utensils used by suchi cooks, and stools from in-patients and healthy persons by Zen-Yoji et al. (1965). They used peptone water containing 3% NaCl for propagation and selection. A selective medium, taurocholate-bromothymol blue-sucrose agar developed by

Kobayashi et al. in 1963 (Kampelmacher et al. 1970), has been used by many researchers along with BTB-Teepol agar for isolation of V. parahaemolyticus. Thomson and Trenholm (1971) reported that BTB-Teepol medium in combination with water blue-alizarin yellow (WA) medium or taurocholate-bromothymol blue-sucrose agar was sufficient for primary plating of shellfish samples. The use of these three media was based on the differential fermentation of sucrose by the Vibrio.

Baross and Liston (1968) formulated a semi-selective, solid medium on the basis of the invariant ability of the Vibrio to utilize starch. The components were: 0.3% peptone; 0.1% yeast extract; 0.5% soluble potato starch; 0.5% sodium chloride; 1.5% agar, adjusted to pH 7.5. In this work, surface-inoculated plates of the medium were incubated in an anaerobic jar at 37°C for 36-48 hr. The incorporation of 2 units per ml of penicillin in the medium was found to be effective in greatly reducing contamination. Twedt and Novelli (1971) modified the semi-selective salt-starch-agar formulation as the result of a systematic study of the effect of each constituent on the growth of V. parahaemolyticus and competitive species characteristic of the marine environment. The modified formulation included: 2.0% peptone; 0.2% yeast extract; 0.5% corn starch; 3.0% sodium chloride; 1.5% agar (pH 8.0). Penicillin at 2 to 5 units per ml of medium increased selectivity without

inhibition; inhibitory levels were 8.4 to > 20 units per ml. Partially soluble corn starch was substituted for the soluble starch in the basic isolation medium. Flooding a plate of the modified medium with Lugol's iodine solution markedly improved visibility and countability of V. parahaemolyticus.

The isolation medium presently used in the Food and Drug Administration laboratories is thiosulfate-citrate-bile salts-sucrose agar (TCBS-agar). Glucose salt Teepol broth (GSTB) is used as an enrichment medium and, after incubation of the food sample in this broth, direct plating onto TCBS-agar is utilized (Food and Drug Administration, 1972).

Differentiation between V. parahaemolyticus and V. alginolyticus has been studied by many investigators. Zen-Yoji et al. (1965) reported that V. alginolyticus (biotype 2 of V. parahaemolyticus) is not enteropathogenic. Their comparative data from raw fish, shellfish, utensils of sushi cooks, and suspected patients, food poisoning patients, and healthy persons supported this view. Sakazaki (1968 and 1969) and Sakazaki et al. (1963) studied and reported the different biochemical characteristics of V. parahaemolyticus, V. alginolyticus, and V. anguillarum. Their results are summarized in Table 1. In the Table, the biochemical characteristics of V. parahaemolyticus were based on the Food and Drug Administration (1972) and Sakazaki's

Table 1. Differentiation Among V. parahaemolyticus, V. alginolyticus, and V. anguillarum. 1/

Characteristics	V. parahaemolyticus	V. alginolyticus	V. anguillarum
Growth in peptone with:			
0% NaCl	-	-	-
3% NaCl	+	+	+
7% NaCl	+	+	-
10% NaCl	-	+	-
Voges-Proskauer reaction	-	+	-
Sucrose fermentation	-	+	-
Cellobiose fermentation (within 24 hr)	d 2/	-	+
Arabinose fermentation	d 2/	-	-

1/ Differentiating characteristics based on data of Sakazaki et al. 1963. Japanese Journal of Medical Science and Biology 16:161-188; Sakazaki, 1968, Japanese Journal of Medical Science and Biology 21:359-362; and Sakazaki, 1969, In: Food-borne infections and intoxications, ed. by H. Riemann, New York, Academic Press, 1969, 115-129; and Food and Drug Administration, 1972.

2/ Different with subgroups.

reports (1968 and 1969, and Sakazaki et al., 1963). Sakazaki reported different reactions with subgroups of V. parahaemolyticus in Voges-Proskauer and sucrose fermentation.

Biochemical characteristics of V. parahaemolyticus as identified by Sakazaki et al. (1963) and Sakazaki (1969) are presented in Table 2. The characteristics described by the Food and Drug Administration (1972) are the same except for those marked "d".

Table 2. Biochemical Characteristics of Vibrio parahaemolyticus Isolates as Reported by Sakazaki et al. 1/

Tests	V. parahaemolyticus isolates	Tests	V. parahaemolyticus isolates
Growth in broth with:		Fermentation of:	
0% NaCl	-	Glucose	+ (no gas)
3% NaCl	+	Maltose	+ "
7% NaCl	+	Trehalose	+ "
10% NaCl	-	Mannitol	+ "
Cytochrome oxidation	+	Starch	+ 2/ "
Catalase	+	Sucrose	d 2/ "
Phenylalanine deaminase	-	Cellobiose	d 2/ "
Gelatinase	d 2/	Arabinose	d 2/ "
Urease	-	Lactose	- "
Nitrate reduction	+	Rhamnose	- "
Indole production	+	Xylose	- "
Voges-Proskauer reaction	d 2/	Adonitol	- "
Methyl red reaction	+	Dulcitol	- "
Malonate utilization	-	Inositol	- "
Citrate utilization	d 2/	Salicin	- "
Haemolysis	+	Hugh-Leifson test	F 3/

1/ Sakazaki, R., S. Iwanami and H. Fukumi, 1963. Japanese Journal of Medical Science and Biology 16:161-188. Sakazaki R., 1969. In: Food-borne infections and intoxications, ed. by H. Riemann, New York, Academic Press, p. 115-129.

2/ Different with subgroups.

3/ Fermentation.

Several other investigators have reported the biochemical characteristics of V. parahaemolyticus isolates, and most of their results are similar to those presented in Table 2 (Colwell, 1970; Baross and Liston, 1970; Fishbein et al., 1970; Thomson and Trenholm, 1971; and Twedt et al., 1969). However, differences with subcultures have been seen in fermentation of sucrose, cellobiose, and arabinose, malonate utilization, urease and indole

production, Voges-Proskauer reaction, and growth in 10% NaCl broth. The characteristics used for definition of the species by the authors also differed.

MATERIALS AND METHODS

Preparation of Inoculated Salted Oysters

Salting Process

Preliminary tests were conducted to determine the proportion of sodium chloride to raw oyster in preparing salted oysters, since no published methods were available. Eight Korean wives living in Corvallis agreed to prepare salted oysters. All of these had made or helped in making salted oysters in Korea. Two hundred and thirty grams of raw oysters (Crassostrea gigas) and 737 grams of sodium chloride (table salt) were provided by the experimenter. After salted oysters were prepared by each individual in her home, the products were returned to the laboratory and the amount of sodium chloride used by each of the wives was determined by the difference between the original and remaining sodium chloride. The range in sodium chloride added was 3.3 to 8.7% of the weight of the raw oysters, with an average of 6.8%. For the experiment, three levels of added sodium chloride were chosen: (1) the lowest level of 3.0%, (2) the average level of 6.8%, and (3) above the maximum level permitting the growth of V. parahaemolyticus, 10.6%.

One thousand grams of raw oysters (Crassostrea gigas) constituted each sample in a sterile 2 l glass beaker covered with

sterile aluminum foil and cotton. To determine if the raw oysters had been contaminated by V. parahaemolyticus before salting, control samples of raw oysters were salted and tested in the same way.

Inoculation

Each oyster sample was inoculated with V. parahaemolyticus (Japanese strain, T-3765-1 , from a Japanese food poisoning patient in 1969, provided by H. Zen-Yoji, Tokyo-to Laboratories for Medical Sciences, Tokyo, Japan). The inoculum was grown on a trypticase soy agar slant (TSA, Difco, Detroit, Michigan) with 3% additional sodium chloride for 24 hr at room temperature ($20 \pm 1^{\circ}C$). The culture of V. parahaemolyticus on the slant was washed off with 0.1% peptone-3% salt broth and the optical density of the suspension was adjusted to 50% at 620nm. Two methods were used in the inoculation. One method was the inoculation of the organisms onto the surface of the oysters followed by thorough mixing with a sterile spoon (surface-inoculation). The other method was the injection of the organisms into oysters internally using a sterile syringe (injected-inoculation). Two replications were done for each method. Within one hr of inoculation, salt was added to each sample and thoroughly mixed in.

The samples were then held at $18^{\circ}C \pm 0.5^{\circ}C$ during the period of the laboratory tests. To determine the survival pattern of

V. parahaemolyticus in the salted oysters, samples were removed after one day (24 ± 2 hr), three days (72 ± 2 hr), and seven days (168 ± 2 hr) of storage. Sampling was continued until no V. parahaemolyticus were recovered.

Determination of Numbers of Vibrio parahaemolyticus

Laboratory Sampling

For each sample, 50 g of the salted oysters and liquid were weighed into 450 ml of 0.1% peptone-3% salt broth in a sterile one quart jar to make a 1:10 dilution of the sample. For taking the salted oysters out of the container, an alcohol-flame sterilized spoon was used. The blade and gasket of an electric blender were sterilized in 70% ethanol for 20 min, rinsed three times in sterile distilled water and then transferred to the jar. The diluted sample was blended on low speed for 30 sec and then on high speed for 2 min. The 1:10 dilution of the sample was used for further dilutions. Dilutions of 1:100, 1:1,000, 1:10,000 and 1:100,000 were made of all the salted samples. These dilutions were used for determination of numbers of V. parahaemolyticus and also for Total Plate Counts. The outline of the procedure is presented in Figure 1.

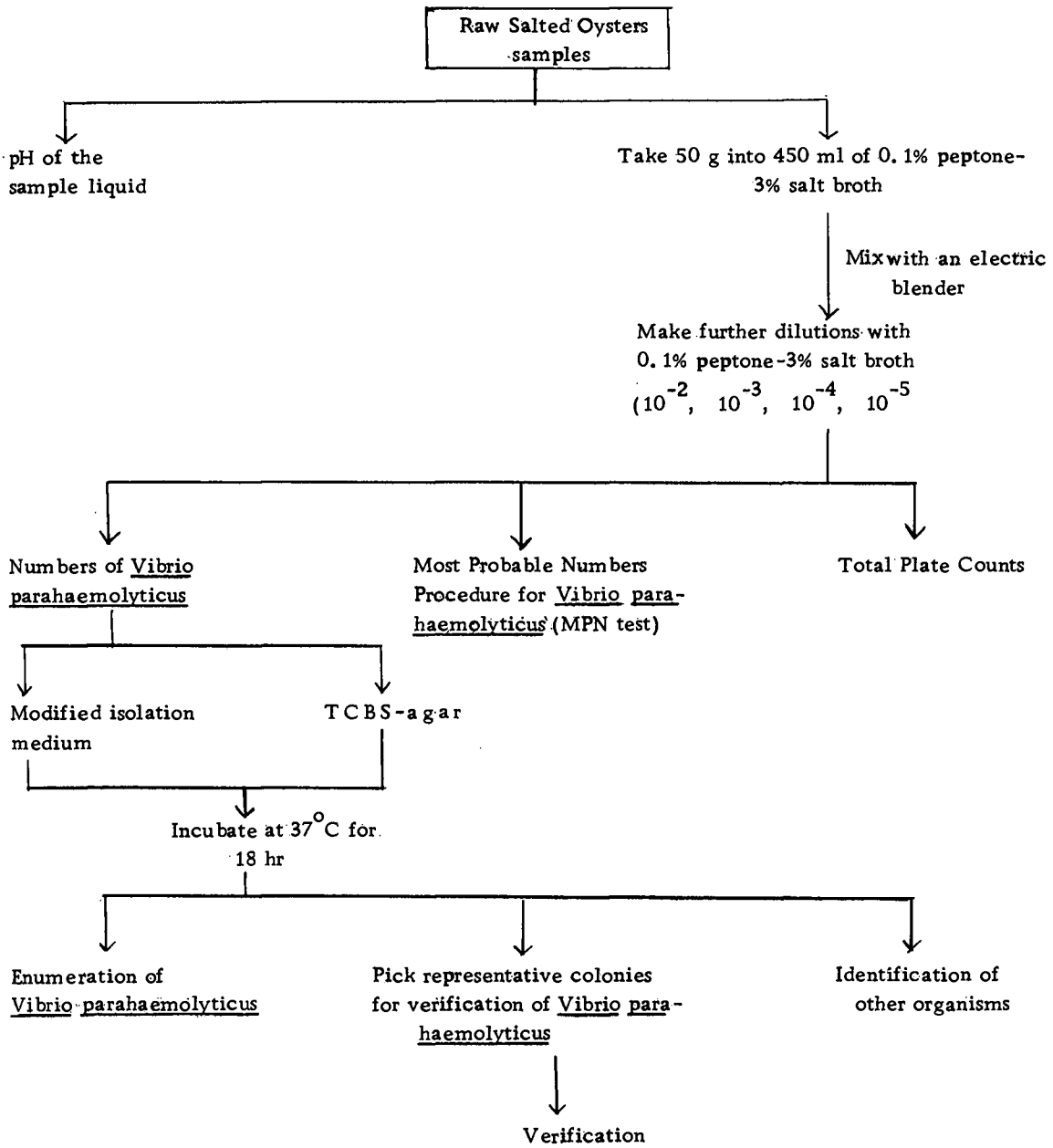

Figure 1. Outline of sampling procedure and enumeration of Vibrio parahaemolyticus in salted oyster samples.

Comparison of Two Selective Media

Two media were used for the determination of numbers of V. parahaemolyticus. One medium was a modified isolation medium which was developed by Twedt and Novelli (1971), and the other was thiosulfate-citrate-bile salts-sucrose agar (TCBS, BBL, Cockeysville, Maryland) which is used in the FDA method (Food and Drug Administration, 1972).

The modified isolation medium formulation included: peptone, 2.0%; yeast extract, 0.2%; corn starch, 0.5%; NaCl, 3.0%; and agar, 1.5% (final pH 8.0) (Twedt and Novelli, 1971). Penicillin at 8 units/ml was added to the modified medium to increase selectivity. Pour plates were dried for about 2 hr at room temperature before inoculation with the sample. From each of the 1:10, 1:100, 1:1,000, and 1:10,000 dilutions of salted oysters, 0.1 ml was pipetted onto the surface of a plate and spread by the spread plate technique. These plates were incubated aerobically at 37°C for 18 hr and the numbers of V. parahaemolyticus were counted. Colonies of V. parahaemolyticus from the modified isolation medium were characterized by large zones of starch hydrolysis within 18 hr of incubation. To improve visibility and thus the accuracy of colony count, the plate was flooded with gram's iodine solution (Difco) after a representative number of colonies had been picked for verification. Selected colonies were transferred to slants of 3%

salt-TSA (STSA) and incubated for 24 hr at room temperature (20 ± 1°C).

The use of thiosulfate-citrate-bile salts-sucrose agar (TCBS) was described in the Food and Drug Administration - Bacteriological Analysis Manual (1972) for the isolation and identification of V. parahaemolyticus. From each dilution of the salted oyster sample, 0.1 ml was pipetted onto the surface of a dried TCBS agar (BBL) plate and spread by means of the spread plate technique. The colonies of V. parahaemolyticus were counted after incubation aerobically at 37°C for 18 hr. Typical colonies of V. parahaemolyticus from the TCBS-agar plates were round, 2-3 mm in diameter, with green centers. Representative colonies were picked for verification. Each selected colony was transferred to a slant of STSA and incubated for 24 hr at room temperature (20 + 1°C).

Most Probable Numbers Procedure

In order to determine small viable numbers of V. parahaemolyticus, the Most Probable Numbers procedure (MPN) (Food and Drug Administration, 1972) was utilized. Three-10 ml portions of the 1:10 dilution of the salted oyster sample were inoculated into 10 ml of glucose-salt-Teepol broth (GSTB) of 2-fold concentration. From the dilutions of 1:10, 1:100, and 1:1,000, three-1 ml portions of each dilution were inoculated into 10 ml of

single strength GSTB. The tubes were incubated at 37°C for 18 hr. After incubation, a 3 mm loopful of the broth culture was streaked onto a TCBS agar plate. These plates were incubated at 37°C for 18 hr. MPN Tables were used to enumerate viable numbers of V. parahaemolyticus (American Public Health Association, 1970).

Verification of Vibrio parahaemolyticus

Representative colonies of V. parahaemolyticus from the modified isolation medium and TCBS agar plates were identified after incubation for 24 hr at room temperature on STSA slants. Gram staining and the following identification procedures were done: growth in halophilism shake culture medium (HSCM) (Food and Drug Administration, 1972) containing 8% and 10% sodium chloride, Voges-Proskauer reaction, sucrose fermentation, and reaction on triple sugar iron agar (TSI, Difco). These procedures were also followed on the V. parahaemolyticus culture used for inoculation of the salted oyster samples.

Identification of Other Microorganisms

Organisms other than V. parahaemolyticus that grew on the media used in this experiment were isolated and identified. The two media are very selective for V. parahaemolyticus. However,

other vibrios will grow well too although colony form will differ.

pH Measurements

To study the relationship between the survival of V. parahaemolyticus and pH of the salted oysters, the pH of the liquid of the salted sample was measured on each sample. The relationship of pH to the total plate counts was also studied. A Beckman pH meter (Model G) was used in measuring pH.

Total Plate Counts

Total plate counts at 20 $\pm$ 1°C were done to determine the total number of mesophilic organisms present originally and the number of organisms in the salted oyster samples during storage at 18°C. From the dilutions of $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$, 1 ml portions were transferred into plates and poured with trypticase soy agar (Difco). The plates were incubated at room temperature (20° $\pm$ 1°C) for 72 $\pm$ 2 hr and then all colonies were counted.

Statistical Analysis

An analysis of variance for the three-factorial experiment was made in order to measure the effectiveness of salt levels, storage times, and media on the survival of V. parahaemolyticus in the salted oysters at 18°C. Each method of inoculation was planned and

analyzed as a separate experiment. To determine the relationship between total plate counts and pH of the salted oysters, the correlation coefficient, r, was calculated for each concentration of salt.

RESULTS AND DISCUSSION

Survival of Vibrio parahaemolyticus with Different Levels of Salt in Salted Oysters

Survival in Surface-Inoculated and Injected Samples

V. parahaemolyticus (Japanese strain, T-3765-1) was unable to multiply to an important degree in salted oysters during the storage period at 18°C. The organism was inoculated onto the surface of the oysters to see the survival pattern in salted oysters contaminated during processing or storage. In the surface-inoculated oysters, samples with 3.0% salt showed a slight increase in numbers of V. parahaemolyticus after one day storage (Table 3). After three days, the numbers of the organism were reduced to 1.0×10^3 from the initial numbers of 7.2×10^4 per g of the sample (on the modified isolation medium) and to 1.0×10^2 from 1.0×10^5 per g (on TCBS-agar). No viable organisms were recovered after seven days from the samples. Samples with 6.8% salt showed a slight decrease in numbers of V. parahaemolyticus after one day. The numbers were markedly reduced after three days to 2.6×10^3 from the initial numbers of 8.3×10^4 per g of the sample (on the modified isolation medium) and to 2.9×10^3 from 1.0×10^5 per g (on TCBS-agar). After seven days the numbers decreased to $< 6.0 \times 10$ per g of the sample (on the modified isolation medium) and to $< 2.0 \times 10$ per g (on TCBS-agar);

viable cells were not detected after eight days. A decrease in numbers of V. parahaemolyticus after one day storage was also seen in samples with 10.6% salt. After three days the numbers of the organism were 4.4×10^2 as compared to the initial numbers of 4.4×10^4 per g of the sample (on the modified isolation medium) and 1.5×10^3 from 5.7×10^4 per g (on TCBS-agar). The numbers were further decreased after seven days to $< 1.0 \times 10^2$ per g (on the modified isolation medium) and $< 1.1 \times 10^2$ per g (on TCBS-agar), but surviving vibrios were still present after eight days in the samples in one replication.

Most probable numbers of V. parahaemolyticus showed marked reduction to less than 3.0 x 10 after seven days storage of oysters with 3.0% salt, but survival was also seen until eight days storage in samples with 6.8% and 10.6% salt (Table 3).

From an analysis of variance (Table 4), the F values for the effects of salt levels and of storage times were significant at the 1% level. However, a significant interaction at the 1% level between salt levels and storage times occurred. Figures 2 and 3 illustrate that the effect of salt level on survival did not show consistency over the period tested.

To simulate the survival pattern in the digestive tract of oyster, in which the organism may be originally found, V. parahaemolyticus was injected into oysters before salting. In the injected samples

Table 3. Numbers of Viable Vibrio parahaemolyticus Following Incubation at 18°C of Surface-Inoculated Salted Oysters.

NaCl %	Storage time (days)	Recovery medium: Modified isolation medium [1] Replication 1 Cells/g	Modified isolation medium [1] Replication 2 Cells/g	Modified isolation medium [1] Average Cells/g	TCBS-agar [2] Replication 1 Cells/g	TCBS-agar [2] Replication 2 Cells/g	TCBS-agar [2] Average Cells/g	Most probable numbers [3] Replication 1 Cells/100g	Most probable numbers [3] Replication 2 Cells/100g
3.0	0	7.8×10^4	6.5×10^4	7.2×10^4	1.2×10^5	8.7×10^4	1.0×10^5	$>1.1\times10^5$	$>1.1\times10^5$
	1	1.1×10^5	1.2×10^5	1.2×10^5	3.2×10^5	1.2×10^5	2.2×10^5	2.4×10^4	$>1.1\times10^5$
	3	1.7×10^3	3.0×10^2	1.0×10^3	M.D. [4]	1.0×10^2	1.0×10^2	2.3×10^3	4.3×10^2
	7	<10	<10	<10	<10	<10	<10	$<3.0\times10$	$<3.0\times10$
	8	<10	<10	<10	<10	<10	<10	$<3.0\times10$	$<3.0\times10$
6.8	0	9.5×10^4	7.1×10^4	8.3×10^4	1.2×10^5	7.9×10^4	1.0×10^5	$>1.1\times10^5$	$>1.1\times10^5$
	1	1.8×10^3	6.9×10^3	3.5×10^3	1.6×10^4	3.4×10^3	2.5×10^4	2.4×10^4	1.1×10^5
	3	1.5×10^3	3.7×10^3	2.6×10^3	4.2×10^3	1.5×10^3	2.9×10^3	9.3×10^2	2.4×10^4
	7	<10	1.1×10^2	$<6.0\times10$	<10	3.0×10	$<2.0\times10$	6.2×10	2.9×10^3
	8	<10	<10	<10	<10	<10	<10	3.6×10	9.1×10
10.6	0	4.7×10^4	4.0×10^4	4.4×10^4	8.7×10^4	2.6×10^4	5.7×10^4	$>1.1\times10^5$	$>1.1\times10^5$
	1	1.2×10^3	5.8×10^3	3.5×10^3	1.5×10^3	2.2×10^3	1.8×10^3	9.3×10^3	1.1×10^5
	3	6.0×10^2	2.8×10^2	4.4×10^2	2.8×10^3	2.0×10^2	1.5×10^3	4.3×10^3	1.1×10^5
	7	<10	1.9×10^2	$<1.0\times10^2$	<10	2.0×10^2	$<1.1\times10^2$	2.0×10^2	$>1.1\times10^5$
	8	<10	1.0×10^2	$<5.5\times10$	<10	1.0×10^2	$<5.5\times10$	3.6×10	2.3×10^3

[1] Modified selective and differential isolation medium for V. parahaemolyticus (Twedt and Novelli, 1971).

[2] Thiosulfate-citrate-bile salts-sucrose agar (BBL) (Food and Drug Administration, 1972).

[3] MPN Tables from Recommended Procedures for the Examination of Sea Water and Shellfish, 4th ed. 1970, p. 101. The American Public Health Association, Inc., New York.

[4] Missing data.

Table 4. Analysis of Variance for Effects of Media Used, Salt Levels, and Storage Times on the Survival of Vibro parahaemolyticus in Surface-Inoculated Oysters at 18°C.

Source of variation	Degrees of freedom	Mean square	F value 1/
Media	1	$7,998 \times 10^6$	5.91*
Time	3	$14,760 \times 10^6$	10.90**
Salt	2	$7,711 \times 10^6$	5.69**
Salt x Media	2	$2,632 \times 10^6$	1.94
Salt x Time	6	$8,281 \times 10^6$	6.11**
Time x Media	3	$3,336 \times 10^6$	2.46
Salt x Time x Media	6	$3,249 \times 10^6$	2.40
Error	24	$1,354 \times 10^6$	

1/ * indicates significance at the 5% level; **, 1% level.

with the three levels of salt, the numbers of the organism were slightly increased after one day storage at 18°C (Table 5). Samples with 3.0% salt showed a slight decrease in numbers after three days on the modified isolation medium (6.6×10^4 from the initial numbers of 1.1×10^5 per g of the sample), but on TCBS-agar, the numbers were constant (1.8×10^5 from 9.4×10^4). However, no viable organisms were counted after seven days. Samples with 6.8% salt

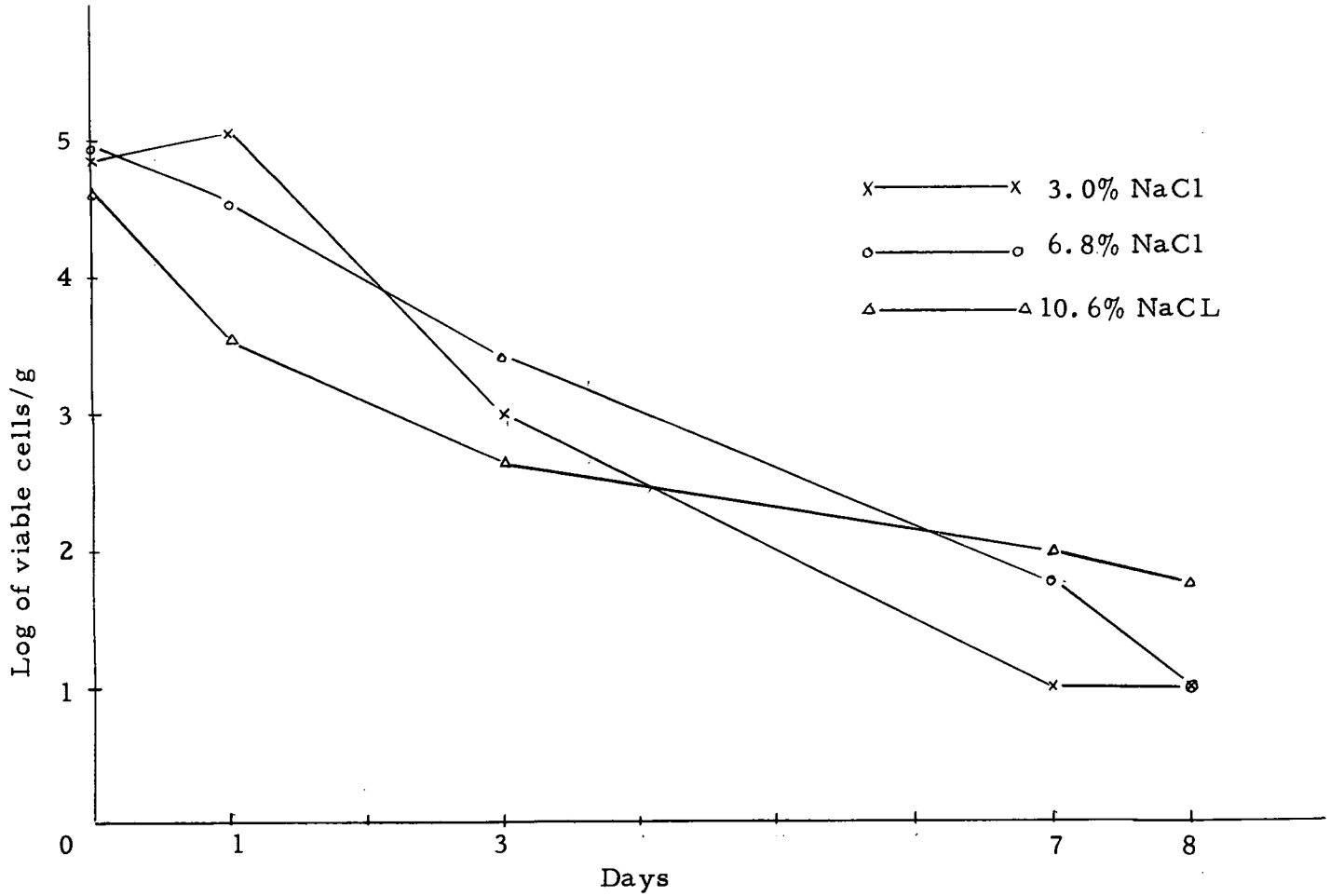

Figure 2. Survival of Vibro parahaemolyticus at 18°C in surface-inoculated salted oysters (recovered on modified isolation medium).

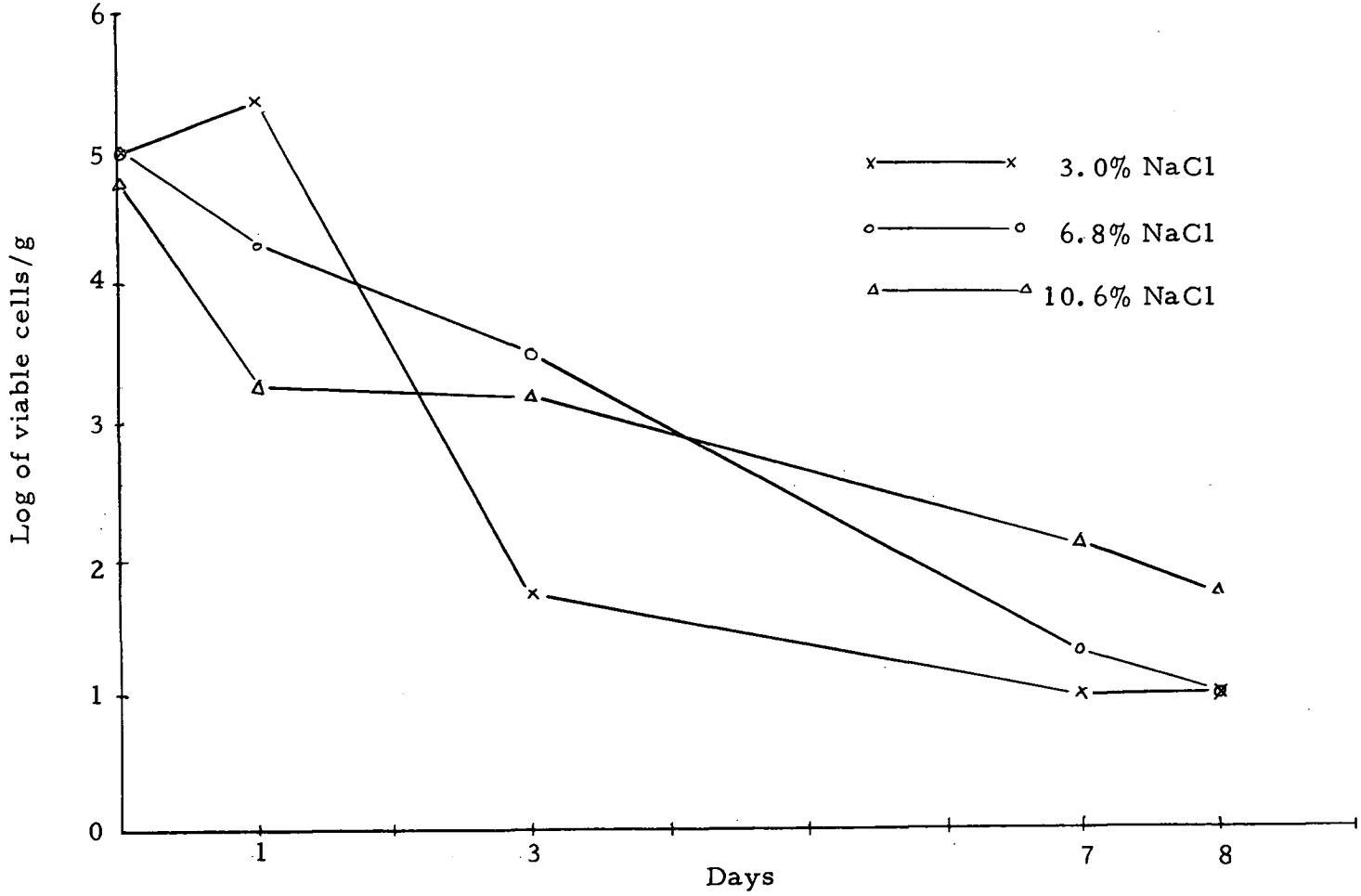

Figure 3. Survival of Vibrio parahaemolyticus at 18°C in surface-inoculated salted oyster (recovered on TCBS-agar).

showed a slight decrease in numbers of V. parahaemolyticus on the modified isolation medium after three days (4.3×10^4 from the initial number of 1.0×10^5 per g of the sample) and a slight increase on TCBS-agar (4.7×10^5 from 8.9×10^4 per g). The numbers decreased by seven days; survival still occured on the modified isolation medium after eight days but no viable cells were found after nine days storage. Samples with 10.6% salt showed the same pattern of survival until seven days as the samples with 6.8% salt. The numbers of the organism after three days were 2.6×10^4 from the initial number of 1.3×10^5 per g of the sample (on the modified isolation medium) and 4.2×10^4 from 1.4×10^4 per g (on TCBS-agar). A marked reduction in numbers was found after seven days; survival of the organism was still detected after nine days. The reduction in numbers of V. parahaemolyticus after nine days was to 8.5×10^2 per g of the sample on the modified isolation medium and 1.0×10^2 per g on TCBS-agar. No viable organisms were counted after 11 days storage.

The most probable numbers of V. parahaemolyticus showed agreement in that no surviving cells were found from oysters with 3.0% salt after seven days storage, after nine days in oysters with 6.8% salt, and after 11 days in samples with 10.6% salt (Table 5).

The analysis of variance for the effects of salt concentrations

and storage times and including media used on the survival of the injected organisms is presented in Table 6. A significant interaction at the 1% level between salt concentrations and storage times was seen. The effect of salt level varied with time (Figures 4 and 5).

Sakazaki et al. (1963) reported that growth of V. parahaemolyticus occurs between 15 and 40°C and in media with a salt concentration of 0.5 to 9.0%, sometimes to 11.0%, with the most abundant growth in 2 to 4% salt. Temmyo (1966) studied the effects of 0.5, 1, 3, 5, and 7% salt concentrations on the survival of V. parahaemolyticus in saurel extracts at -2°C. He found that after eight days storage of samples with 10^5 cells per ml originally, survival of the organism was still positive for all salt concentrations. From the results of his experiment he noted that salt concentration could protect V. parahaemolyticus during low temperature storage, indicated the greater the salt concentration the longer the survival of the organism. Protection of the cells of V. parahaemolyticus by addition of salt was also found by Covert (1972). She studied survival of V. parahaemolyticus in fish homogenate with 0, 3, 6, 9, and 12% sodium chloride at various temperatures. At low temperatures, -5 ± 1°C and -18 ± 1°C, effects of sodium chloride were strain dependent, and 12% sodium chloride was best for survival of strain T-3765-1. She concluded that such low temperatures reduce the numbers of V. parahaemolyticus in fish homogenates and that the presence of

Table.5. Numbers of Viable Vibrio parahaemolyticus Following Incubation at 18°C of Injected Salted Oysters.

NaCl %	Storage time (days)	Recovery medium: Modified isolation medium [1] Replication 1 Cells/g	Modified isolation medium [1] Replication 2 Cells/g	Modified isolation medium [1] Average Cells/g	TCBS-agar [2] Replication 1 Cells/g	TCBS-agar [2] Replication 2 Cells/g	TCBS-agar [2] Average Cells/g	Most probable numbers [3] Replication 1 Cells/100g	Most probable numbers [3] Replication 2 Cells/100g
3.0	0	1.2×10^5	1.0×10^5	1.1×10^5	9.8×10^4	9.0×10^4	9.4×10^4	$> 1.1\times10^5$	$> 1.1\times10^5$
	1	1.3×10^6	1.2×10^6	1.3×10^6	1.6×10^5	2.8×10^5	2.2×10^5	$> 1.1\times10^5$	$> 1.1\times10^5$
	3	1.1×10^4	1.2×10^5	6.6×10^4	1.9×10^5	1.6×10^5	1.8×10^5	2.9×10^3	2.4×10^4
	7	< 10	< 10	< 10	< 10	< 10	< 10	$< 3.0\times10$	$< 3.0\times10$
	8	< 10	< 10	< 10	< 10	< 10	< 10	$< 3.0\times10$	$< 3.0\times10$
6.8	0	1.1×10^5	9.0×10^4	1.0×10^5	8.5×10^4	9.3×10^4	8.9×10^4	$> 1.1\times10^5$	$> 1.1\times10^5$
	1	6.0×10^5	2.5×10^5	4.3×10^5	1.3×10^5	1.3×10^5	1.3×10^5	$> 1.1\times10^5$	1.1×10^5
	3	4.0×10^4	4.5×10^4	4.3×10^4	9.0×10^4	8.5×10^5	4.7×10^5	$> 1.1\times10^5$	$> 1.1\times10^5$
	7	3.7×10^3	8.5×10^3	6.1×10^3	9.0×10^3	8.9×10^2	4.8×10^2	1.1×10^5	9.3×10^3
	8	1.0×10^2	2.0×10^2	1.5×10^2	< 10	< 10	< 10	$< 3.0\times10$	2.1×10^2
	9	< 10	< 10	< 10	< 10	< 10	< 10	$< 3.0\times10$	$< 3.0\times10$
10.6	0	1.3×10^5	1.2×10^5	1.3×10^5	1.6×10^4	1.2×10^4	1.4×10^4	$> 1.1\times10^5$	$> 1.1\times10^5$
	2	8.1×10^4	2.6×10^4	5.4×10^4	5.9×10^4	2.4×10^4	4.2×10^4	$> 1.1\times10^5$	$> 1.1\times10^5$
	3	3.2×10^4	2.3×10^4	2.6×10^4	5.8×10^4	2.6×10^4	4.2×10^4	$> 1.1\times10^5$	$> 1.1\times10^5$
	7	9.5×10^3	9.7×10^3	9.6×10^3	7.0×10^2	1.1×10^3	9.0×10^2	1.1×10^5	4.6×10^4
	8	1.1×10^3	3.3×10^3	2.2×10^3	2.9×10^3	2.2×10^2	1.6×10^3	$> 1.1\times10^5$	4.6×10^4
	9	1.0×10^2	1.6×10^3	8.5×10^2	1.0×10^2	1.0×10^2	1.0×10^2	2.3×10^3	2.3×10^3
	11	< 10	< 10	< 10	< 10	< 10	< 10	$< 3.0\times10$	$< 3.0\times10$

1/ Modified selective and differential isolation medium for V. parahaemolyticus (Twedt and Novelli, 1971).

2/ Thiosulfate-citrate-bile salts-sucrose agar (BBL) (Food and Drug Administration, 1972).

3/ MPN Tables from Recommended Procedures for the Examination of Sea Water and Shellfish, 4th ed. 1970, p. 101. The American Public Health Association, Inc., New York.

Table 6. Analysis of Variance for Effects of Media Used, Salt Levels, and Storage Times on the Survival of Vibrio parahaemolyticus in Injected Oysters at 18°C.

Source of variation	Degrees of freedom	Mean square	F value 1/
Media	1	$13,700 \times 10^7$	6.31*
Time	3	$49,750 \times 10^7$	22.91**
Salt	2	$6,997 \times 10^7$	3.22
Salt x Media	2	$6,924 \times 10^7$	3.19
Salt x Time	6	$8,421 \times 10^7$	3.88**
Time x Media	3	$35,560 \times 10^7$	16.48**
Salt x Time x Media	6	$5,615 \times 10^7$	2.59*
Error	24	$2,171 \times 10^7$	

1/ * indicates significance at the 5% leve; **, 1% level.

sodium chloride enhances its survival in short term storage.

Overall results from this experiment showed that multiplication of V. parahaemolyticus did not occur to an important extent in salted oysters during storage at 18°C. In the samples with 3.0% salt, the numbers of the survivors were slightly increased after one day storage from the original. Within one to three days numbers decreased rapidly and no viable organisms were detected after seven days. The surface-inoculated samples with 6.8% salt had a

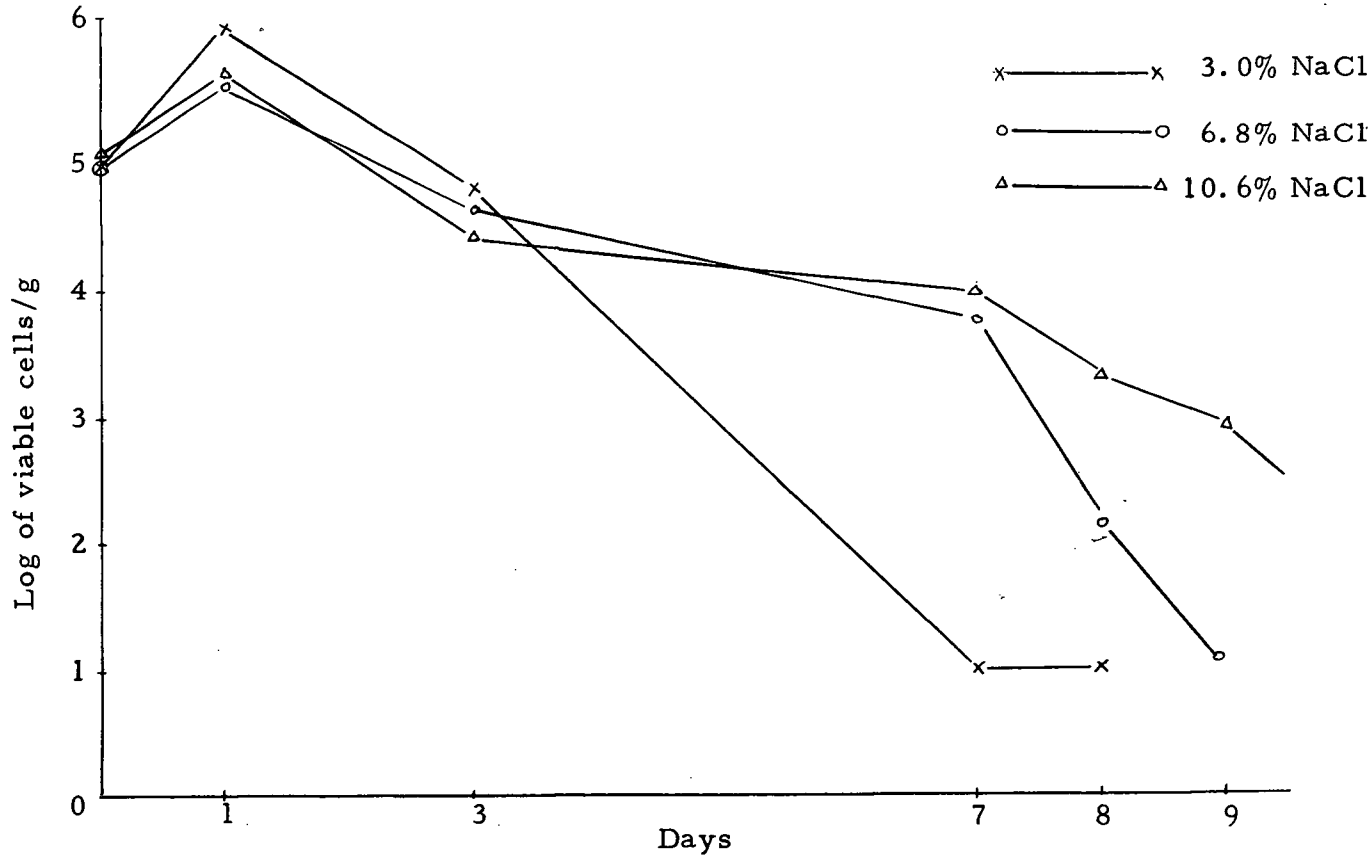

Figure 4. Survival of Vibrio parahaemolyticus at 18°C in injected salted oysters (recovered on modified isolation medium).

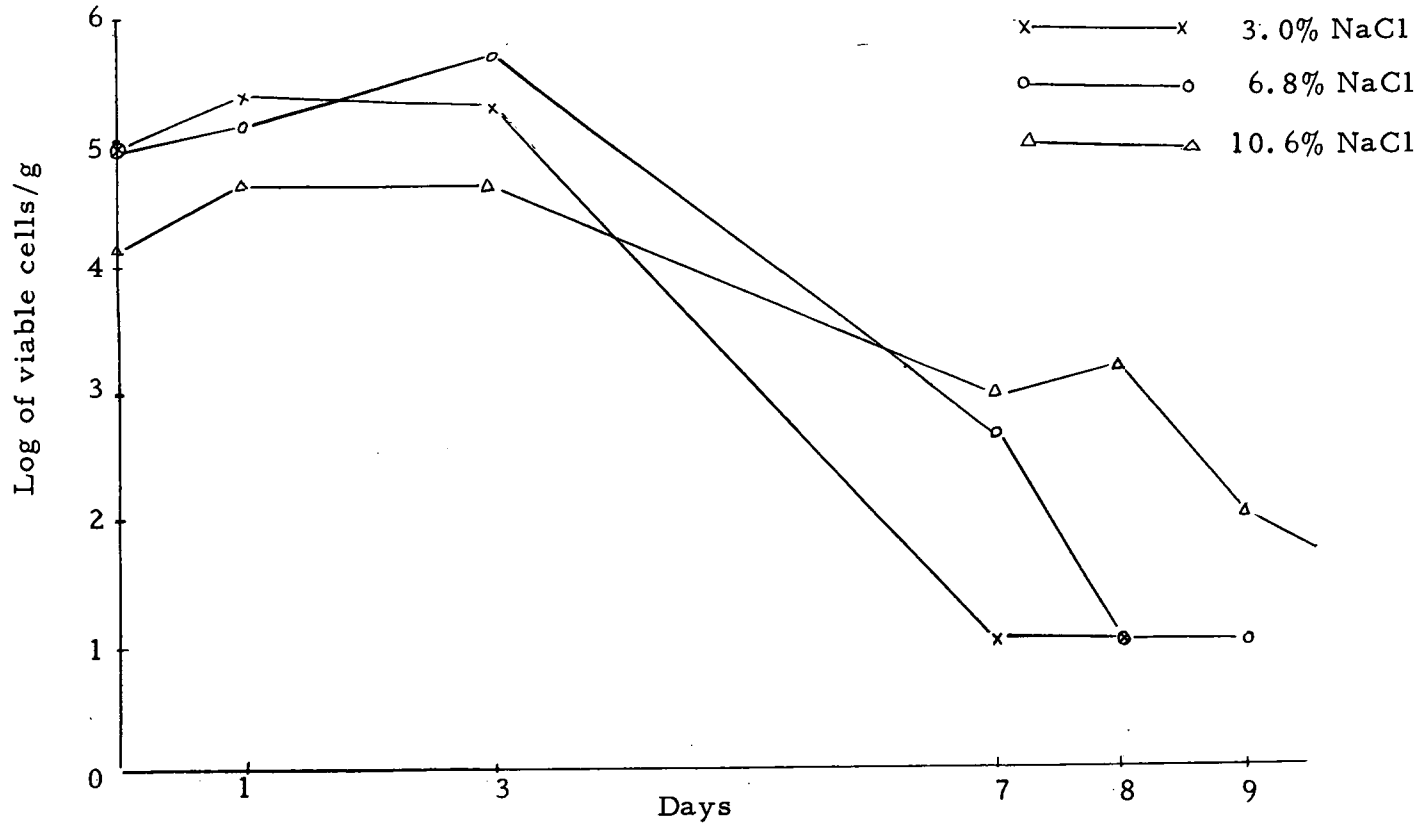

Figure 5. Suvival of Vibrio parahaemolyticus at 18°C in injected salted oysters (recovered on TCBS-agar).

consistent decrease in the numbers of survivors with none observed after eight days. With 10.6% salt the organisms rapidly decreased in numbers within three days but after seven days showed the highest surviving numbers. Viable cells were still present after eight days. The greater the salt concentration, the longer the survival of the organism in both surface-inoculated and injected oysters. Higher numbers of surviving organisms were counted in injected oysters over the storage period tested than from surface-inoculated samples. However, no viable cells were detected after seven days storage of the samples with 3.0% salt, after nine days of the samples with 6.8% salt, and after 11 days of the samples with 10.6% salt. Therefore, the potential for the infection of consumers was low in the production of salted oysters when the products were stored at 18°C.

Verification of Vibrio parahaemolyticus

Representative colonies of V. parahaemolyticus recovered on the modified isolation medium and TCBS-agar plates were gram-negative, slighly curved rods with growth in HSCM containing 8% sodium chloride but not 10% sodium chloride, negative Voges-Proskauer reaction, negative sucrose fermentation, and acid butt with no gas in TSI slant. Both the inoculum and recovered culture grew in STB containing 10% sodium chloride without shaking. All of these characteristics were the same as that of the V.

parahaemolyticus (Japanese strain, T-3765-1) used for inoculation of the oyster samples. There appeared to be no difficulties in verifying V. parahaemolyticus in this experiment.

Comparison of the Recovery Media Used

Twedt and Novelli (1971) modified the semi-selective salt-starch agar formulation of Baross and Liston (1970). This medium was compared in this study to TCBS-agar which is recommended by the Food and Drug Administration (1972). At the estimated inoculation level (3.0×10^4 to 1.0×10^5 cells per g), the numbers recovered initially from the oyster samples ranged from 5.9×10^4 to 1.2×10^5 per g with the two media from the three levels of salt concentration. From the analysis of variance, the F values for the effects of media were significant at the 5% level, and an interaction between media and storage times was observed in the injected samples (Tables 4 and 6). Smith (1971) recommended TCBS-agar as selective for the isolation of V. parahaemolyticus from stool, food, and water samples. The medium also seemed to be more selective in this test, showing no growth of other organisms. Because of the cloudiness from hydrolysis of starch by V. parahaemolyticus, there was difficulty in counting the colonies on the modified isolation medium. Although flooding the plates of this medium with gram's iodine solution improved visibility and countability of

V. parahaemolyticus, it was not satisfactory.

With these media, V. alginolyticus was isolated from samples with 6.8% and 10.6% salt after seven days of storage. Yellowish green colonies from TCBS-agar and somewhat dry and rough colonies from the modified isolation medium were detected in small numbers in the least diluted samples. These cells were isolated and found to be V. alginolyticus. One sample of surface-inoculated oysters with 10.6% salt, two samples of injected oysters with 6.8% salt, and two of the same lot of oysters with 10.6% salt were positive for this organism. The organism showed characteristics of gram-negative, slightly curved rods, positive growth in STB containing 8% and 10% sodium chloride, no growth at 42°C, Voges-Proskauer reaction positive, sucrose positive, and acid slant and acid butt with no gas in TSI slant. Several of the isolates showed characteristics intermediate between V. parahaemolyticus and V. alginolyticus.

Another typical organism found in the salted oysters was S. aureus from 6.8% and 10.6% salted samples. It showed the typical morphology and yellow colonies on TSA slant. Tests for staphylococcal enterotoxins A, B, and C were negative, and the coagulase test was negative. This organism was also isolated after no viable V. parahaemolyticus were detected.

pH

The pH of all the salted samples decreased during the storage period. After eight days storage, the pH of oysters with 10.6% salt showed higher value (pH 5.2-5.7) than that of oysters with 3.0% (pH 4.4-4.7) and 6.8% salt (pH 4.5-4.9) (Tables 7, 8, 9, and 10). It was evident that samples with 10.6% salt may have prolonged survival of V. parahaemolyticus through the effect of the higher pH value during storage. It has been reported that the optimum pH for V. parahaemolyticus is from 7.5 to 8.5 and that growth occurs at pH 5.0 to 11.0 (Twedt et al., 1969). They found that 20 of 79 strains of V. parahaemolyticus from gastroenteric patients and 1 of 17 strains from wound infections showed growth at pH 4.0. Colwell (1970) reported no growth at pH 4.5 and growth at 5.0 with 63% of 32 strains. No data have been reported concerning the minimum pH for the survival of V. parahaemolyticus.

Total Plate Counts

Two lots of oysters used for the surface-inoculated series were obtained in October 1972 and two for the injected series in December 1972. Total plate counts showed high numbers at the initial day for samples used for the two replications for the surface-inoculated series (2.1×10^6 to 1.3×10^8 cells per g of salted

oysters) (Tables 7 and 8). Reduction in numbers was found after three days storage and further reduction was detected after seven days. Samples with 10.6% salt showed higher counts than other samples after seven days. The average counts were 1.7×10^4 to 9.5×10^4 per g for samples with 10.6% salt, 2.3×10^3 to 3.4×10^3 per g for samples with 3.0% salt, and 3.6×10^3 to 4.1×10^3 per g for samples with 6.8% salt.

Samples of oysters from the two lots used for the injected series showed lower initial bacterial counts (5.0×10^3 to 8.0×10^5 per g) (Tables 9 and 10). However, reduction in numbers was not seen during the storage period. In contrast to the surface-inoculated samples, the lowest salt level showed the highest bacterial counts after seven days. The average counts after seven days were 3.3×10^5 from 1.3×10^5 per g in samples with 3.0% salt, 1.2×10^5 from 2.2×10^5 per g in samples with 6.8% salt, and 4.8×10^4 from 3.8×10^4 per g in samples with 10.6% salt.

The overall result of total plate counts indicated that the effect of salt levels of 3.0%, 6.8%, and 10.6% on the numbers of viable bacteria within eight days of storage was not great.

The correlation coefficients, r, for the relationship between total bacterial numbers and pH of the salted oysters are listed in Table 11. The pH of all the samples decreased during storage. The correlation coefficients were higher in surface-inoculated samples

Table 7. Total Plate Counts and pH of Salted Oysters (18°C) Surface-Inoculated with Vibrio parahaemolyticus.

NaCl %	Storage time (days)	Total plate counts [1] Replication 1 Cells/g	Replication 2 Cells/g	Average Cells/g	pH [2] Replication 1	Replication 2	Average
3.0	0	3.2×10^{7}	1.2×10^{7}	2.2×10^{7}	6.1	6.0	6.1
	1	9.7×10^{6}	7.1×10^{6}	8.4×10^{6}	5.8	5.8	5.8
	3	4.9×10^{6}	7.0×10^{4}	2.5×10^{6}	4.9	4.8	4.9
	7	4.3×10^{3}	2.4×10^{2}	2.3×10^{3}	4.8	4.6	4.7
	8	6.9×10^{2}	6.0×10^{1}	3.8×10^{2}	4.7	4.4	4.6
6.8	0	1.3×10^{8}	6.5×10^{6}	6.8×10^{7}	6.1	6.0	6.1
	1	1.5×10^{7}	5.0×10^{6}	1.0×10^{7}	5.8	5.9	5.9
	3	7.2×10^{6}	2.0×10^{5}	3.7×10^{6}	5.7	5.7	5.7
	7	1.6×10^{3}	6.5×10^{3}	4.1×10^{3}	5.0	4.9	5.0
	8	2.4×10^{3}	3.2×10^{2}	1.4×10^{3}	4.8	4.5	4.7
10.6	0	2.1×10^{6}	2.5×10^{6}	2.3×10^{6}	6.1	6.0	6.1
	1	1.2×10^{6}	9.0×10^{5}	1.1×10^{6}	5.8	5.8	5.8
	3	9.6×10^{4}	1.1×10^{5}	1.0×10^{5}	5.7	5.8	5.8
	7	1.6×10^{5}	3.9×10^{4}	9.5×10^{4}	5.7	5.7	5.7
	8	NC [3]	4.9×10^{4}	NC	5.7	5.5	5.6

[1] On trypticase soy agar (TSA) (Difco, Detroit, Michigan).
[2] pH measured by a Beckman pH meter (Model G).
[3] Plates not countable.

Table 8. Total Plate Counts and pH of Salted Oysters (18°C) As Received (Not Inoculated). 1/

NaCl %	Storage time (days)	Total plate counts 2/ Replication 1 Cells/g	Replication 2 Cells/g	Average Cells/g	pH 3/ Replication 1	Replication 2	Average
3.0	0	3.0×10^7	1.1×10^7	2.1×10^7	6.1	6.0	6.1
	1	3.0×10^7	9.2×10^6	2.0×10^7	5.7	5.8	5.8
	3	1.3×10^5	2.2×10^4	7.6×10^4	5.1	4.8	5.0
	7	5.6×10^3	1.2×10^3	3.4×10^3	4.7	4.5	4.6
	8	1.8×10^3	3.1×10^2	1.1×10^3	4.6	4.5	4.6
6.8	0	7.2×10^7	1.1×10^7	4.2×10^7	6.1	6.0	6.1
	1	3.1×10^7	5.2×10^6	1.8×10^7	5.9	5.9	5.9
	3	1.9×10^6	2.2×10^4	9.6×10^5	5.7	5.8	5.8
	7	2.7×10^3	4.5×10^3	3.6×10^3	4.9	4.8	4.9
	8	5.8×10^2	2.9×10^2	4.4×10^2	4.8	4.6	4.7
10.6	0	5.4×10^6	2.1×10^6	3.8×10^6	6.1	6.0	6.1
	1	3.1×10^6	6.7×10^5	1.9×10^6	5.9	5.9	5.9
	3	9.8×10^4	6.3×10^4	8.1×10^4	5.7	5.8	5.8
	7	2.0×10^4	1.3×10^4	1.7×10^4	5.7	5.7	5.7
	8	NC 4/	5.1×10^3	NC 4/	5.4	5.4	5.4

1/ Controls used in Surface-Inoculated series.
2/ On trypticase soy agar (TSA) (Difco, Detroit, Michigan).
3/ pH measured by a Beckman pH meter (Model G).
4/ Plates not countable.

Table 9. Total Plate Counts and pH of Salted Oysters (18°C) Injected with Vibrio parahaemolyticus.

NaCl %	Storage time (days)	Total plate counts [1] Replication 1 Cells/g	Replication 2 Cells/g	Average Cells/g	pH [2] Replication 1	Replication 2	Average
3.0	0	4.0×10^{5}	5.0×10^{3}	2.0×10^{5}	6.2	6.2	6.2
	1	7.6×10^{5}	1.0×10^{4}	3.9×10^{5}	5.6	5.8	5.7
	3	2.1×10^{6}	1.1×10^{6}	1.6×10^{6}	5.4	5.5	5.5
	7	2.2×10^{4}	6.4×10^{5}	3.3×10^{5}	4.5	4.5	4.5
	8	3.6×10^{4}	6.6×10^{5}	3.5×10^{5}	4.4	4.4	4.4
	9	-	-	-	4.4	4.3	4.4
6.8	0	8.0×10^{5}	9.0×10^{3}	4.1×10^{5}	6.2	6.2	6.2
	1	4.0×10^{5}	8.0×10^{3}	2.0×10^{5}	5.8	5.8	5.8
	3	1.6×10^{4}	7.8×10^{5}	4.0×10^{5}	5.6	5.7	5.7
	7	6.1×10^{4}	6.9×10^{4}	6.5×10^{4}	4.8	4.9	4.9
	8	4.7×10^{4}	4.0×10^{5}	2.2×10^{5}	4.6	4.9	4.8
	9	7.0×10^{4}	1.8×10^{5}	1.3×10^{5}	4.6	4.8	4.7
10.6	0	1.1×10^{5}	6.0×10^{3}	5.8×10^{4}	6.2	6.2	6.2
	1	3.7×10^{5}	1.0×10^{4}	1.9×10^{5}	5.9	5.8	5.9
	3	3.3×10^{4}	1.1×10^{5}	7.2×10^{4}	5.7	5.7	5.7
	7	9.0×10^{4}	5.5×10^{4}	7.3×10^{4}	5.4	5.4	5.4
	8	1.3×10^{4}	2.6×10^{4}	7.8×10^{4}	5.3	5.2	5.3
	9	5.5×10^{3}	4.0×10^{4}	4.8×10^{4}	5.1	5.0	5.1
	11	8.2×10^{3}	1.3×10^{4}	1.1×10^{4}	-	5.0	5.0

[1] On trypticase soy agar (TSA) (Difco, Detroit, Michigan).

[2] pH measured by a Beckman pH meter (Model G).

Table 10. Total Plate Counts and pH of Salted Oysters (18°C) As Received (Not Inoculated). 1/

NaCl %	Storage time (days)	Total plate counts 2/ Replication 1 Cells/g	Replication 2 Cells/g	Average Cells/g	pH 3/ Replication 1	Replication 2	Average
3.0	0	1.0×10^{5}	1.4×10^{4}	5.7×10^{4}	6.2	6.2	6.2
	1	5.0×10^{5}	5.0×10^{3}	2.5×10^{5}	5.8	5.8	5.8
	3	3.0×10^{6}	4.5×10^{6}	3.8×10^{6}	5.5	5.5	5.5
	7	2.6×10^{5}	4.0×10^{5}	3.3×10^{5}	4.5	4.5	4.5
	8	5.8×10^{3}	4.6×10^{5}	2.3×10^{5}	4.5	4.5	4.5
	9	–	–	–	4.4	4.5	4.5
6.8	0	3.0×10^{4}	3.2×10^{4}	3.1×10^{4}	6.2	6.2	6.2
	1	4.1×10^{5}	1.2×10^{4}	2.1×10^{5}	5.8	5.8	5.8
	3	1.9×10^{5}	7.2×10^{4}	1.3×10^{5}	5.6	5.6	5.6
	7	8.0×10^{4}	2.5×10^{5}	1.7×10^{5}	5.2	5.0	5.1
	8	2.0×10^{4}	1.0×10^{5}	6.0×10^{4}	4.9	4.9	4.9
	9				4.8	4.8	4.8
10.6	0	2.0×10^{4}	1.4×10^{4}	1.7×10^{4}	6.2	6.2	6.2
	1	2.2×10^{5}	7.0×10^{3}	1.1×10^{5}	5.9	5.8	5.9
	3	9.3×10^{4}	9.1×10^{4}	9.2×10^{4}	5.7	5.6	5.7
	7	2.8×10^{4}	1.6×10^{4}	2.2×10^{4}	5.4	5.4	5.4
	8	1.3×10^{3}	2.3×10^{4}	1.2×10^{4}	5.2	5.3	5.3
	9	5.1×10^{3}	9.1×10^{3}	7.1×10^{3}	5.2	5.2	5.2

1/ Controls used in injected series.
2/ On trypticase soy agar (TSA) (Difco, Detroit, Michigan).
3/ pH measured by a Beckman pH meter (Model G).

Table 11. Relationship Between Total Bacterial Numbers and pH of Salted Oysters.

Sample	Correlation coefficient (r)	
	Surface-inoculated oysters [1]	Injected oysters
3.0% salt	0.92**	0.14
6.8% salt	0.92**	0.0008
10.6% salt	0.77**	0.38

[1] ** indicates significance at the 1% level.

in which the bacterial counts were slightly decreased over the storage period. The r values for the injected samples were very low. These results indicated that pH was not a good index for the bacterial count of salted oysters.

SUMMARY

The survival of V. parahaemolyticus in salted oysters as prepared in Korea was studied. A preliminary test was conducted with eight Korean wives living in Corvallis making salted oysters. On the basis of these data, three levels of salt (NaCl) concentration, 3.0%, 6.8%, and 10.6% of the weight of the oysters, were chosen for the experiment. Two methods were used for inoculation of V. parahaemolyticus (Japanese strain, T-3765-1) into oysters, surface-inoculation and injection into oysters. Inoculated and control samples of salted oysters were stored at 18°C ± 0.5°C, until no viable cells of V. parahaemolyticus were recovered.

The inoculated organism was recovered with two media, a modified isolation medium (Twedt and Novelli, 1971) and thiosulfate-citrate-bile salts-sucrose agar (TCBS-agar) (Food and Drug Administration, 1972), and the effectiveness of these two media was compared. Numbers of total aerobic bacteria were also determined. The pH of the salted oysters was measured to study the relationship between bacterial survival and pH.

Multiplication of V. parahaemolyticus was not observed in the salted samples at 18°C, except for a slight increase in numbers after one day from samples of surface-inoculated oysters with 3.0% salt and all samples of injected oysters. Numbers of the organism

surviving in surface-inoculated oysters were markedly reduced within three days. After three days, the numbers in oysters with 3.0% salt were reduced to 1.0×10^2 from the original of 1.0×10^5 per g of the sample (on TCBS-agar); the numbers in oysters with 6.8% salt were to 2.9×10^3 from 1.0×10^5 per g; and in oysters with 10.6% salt were to 1.5×10^3 from 5.7×10^4 per g. No viable cells were detected after seven days from oysters with 3.0% salt. The samples with 6.8% salt had larger numbers of survivors after three days and samples with 10.6% salt revealed the highest numbers of survivors after seven days. There were still viable cells in those samples after eight days. From samples of injected oysters, higher numbers of viable V. parahaemolyticus were present over the storage period tested than from the surface-inoculated samples. Within three days of storage, in contrast to the surface-inoculated samples, marked reduction in numbers was not observed in any injected samples. After seven days, no viable organisms were detected in samples with 3.0% salt; however, survival still occurred in samples with 6.8% and 10.6% salt. No viable cells were counted after nine days from samples with 6.8% salt and after 11 days from those with 10.6% salt. The results of most probable numbers determinations were generally in agreement with direct counts. Greater salt concentrations of the samples showed longer survival of V. parahaemolyticus in this experiment. From

the pH measurements, it was suggested that samples with greater salt concentration may have prolonged survival of V. parahaemolyticus during storage through the effect of higher pH. The pH of samples with 3.0% salt after eight days was 4.4-4.7, samples with 6.8% salt, 4.5-4.9, and samples with 10.6% salt, 5.2-5.7.

The effectiveness of the recovery media varied with different samples. TCBS-agar recovered higher numbers in surface-inoculated samples, which had greater total bacterial numbers. The modified isolation medium gave higher recovery in the injected series, which had lower total bacterial counts. The F values for the effects of the media were significant at the 5% level, and an interaction between media and storage times was observed in the injected samples.

The overall results from this experiment indicate that the potential for infection by V. parahaemolyticus of consumers of Korean-style salted oysters is low when they are stored at 18°C for 7 to 11 days in the production of the product.

Total plate counts on the initial day varied with lots, ranging from 2.1×10^6 to 1.3×10^8 per g of oysters used for the surface-inoculated experiment and 5.0×10^3 to 8.0×10^5 per g for the injected series. In samples used for the surface-inoculated series, reduction in numbers was found after three days and further reduction was observed after seven days. In samples used for the injected

series, reduction in numbers was not seen during storage. Correlation coefficients revealed no useful relationship between total bacterial counts and pH of the salted samples.

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