AN ABSTRACT OF THE THESIS OF

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Title: BACTERIAL FLORA IN THE OREGON MOIST PELLET, A DIET FOR SALMONID FISH

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Abstract approved: Dr. J. L. Fryer

Enumeration and isolation of bacteria, including fish and human pathogens, in the Oregon Moist Pellet (OMP) and its ingredients were made. A comparison of the bacterial numbers in the OMP fish digest and the final OMP pellet resulting from recently remodeled fish digest pasteurization equipment was made with those produced by the previous system. The total bacterial count was determined from seven samples of OMP before the equipment change and averaged 4.8×10^7 CFU/g. After the equipment change, the bacterial count of 26 OMP samples averaged 1.9×10^6 CFU/g resulting in a 96% decrease in the bacterial load of OMP. Bacterial counts of the fish digest, which comprises 38% of OMP, averaged 5.5×10^8 CFU/g before the change in equipment; however, after the equipment modification the bacterial load decreased approximately 99% to $<1.0 \times 10^4$ to 4.3×10^6 CFU/g.

The OMP diet was examined for a total of 16 bacteria. Two isolations of Yersinia ruckeri serotype II and Salmonella pullorum were made from six samples of fish digest; however, they were not isolated from the final pellet. The following bacteria were isolated from OMP: Aeromonas hydrophila, Clostridium perfringens, Pseudomonas, Staphylococcus, Streptococcus, four Salmonella sp., and 13 different members of the Enterobacteriaceae.

Bacterial Flora in the Oregon Moist Pellet, a Diet for Salmonid Fish

by

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BACTERIAL FLORA IN THE OREGON MOIST PELLET, A DIET FOR SALMONID FISH

I. INTRODUCTION

The Oregon Moist Pellet (OMP) is a fish feed that is utilized primarily for the rearing of Pacific salmon. This diet is used throughout North America and results in the consumption of 12-13 million pounds per year. In recent years the State of Oregon has used as much as 4-5 million pounds amounting to more than one third of the OMP fed to fish in North America.

Frequently fish carcasses and viscera are included in the ingredients of fish feeds as a source of protein. In the past these products were not pasteurized resulting in the transmission of mycobacterial infections to juveniles. Raw fish products are now subjected to pasteurization to eliminate the possibility of transmitting fish or human pathogens. A pasteurized fish digest comprises 38% of OMP.

There are no FDA (Food and Drug Administration) regulations on the limitations of total bacterial counts in fish diets. As a result, fish feeds have been detected with high bacterial levels and contain both fish and human pathogens. Feeding a diet contaminated with these pathogens could result not only in the fish becoming infected, but also serving as carriers of bacterial diseases.

Because of the widespread use of OMP and the possibility of the product containing fish or human pathogens, this diet was examined for total bacterial counts, the major groups of bacteria existing in OMP, and specific fish and human pathogens. All OMP and related ingredients were taken from Bioproducts, Inc., a fish feed manufacturer in Warrenton, Oregon. At the time of this study, Bioproducts modernized its pasteurization equipment. This allowed for a comparison of bacterial counts and isolates from before and after pasteurization equipment modification.

II. LITERATURE REVIEW

Historical Background

One of the earliest documented cases of disease caused by fish food was described by Wood and Wallis in 1955. They demonstrated that feeding infected flesh and viscera of adult chinook salmon to juvenile chinook for a 41 to 52 day period resulted in complete (100%) transmission of bacterial kidney disease (BKD). Ross, et al. (1959), and Wood and Ordal (1958) continued the investigation of salmonid foodborne diseases by examining the possibility of transmitting mycobacterial infections in the same manner. Mycobacteriosis was generally associated with fish that had been maintained in hatcheries for at least one year. The inclusion of raw carcasses and viscera of adult salmon in the diet of juveniles was the suspected source of infection. When this practice was discontinued and pasteurized salmon products were used in the diet, the disease was virtually eradicated from fish reared in Pacific Northwest hatcheries.

These experiments were significant because they brought about the discontinuance of feeding raw salmon carcasses and viscera to juvenile salmon. Raw fish products when included in the diet are now subjected to pasteurization (Fryer and Sanders, 1981). The proce-

dures for this are outlined in the Oregon pellet specifications (Westgate, 1983).

Recently a third foodborne disease resulting from feeding fish products occurred in broiler chicks and trout which had been fed high levels of fish meal (Okuzumi, et al., 1984). The disease was manifested primarily as gizzard and stomach erosion. Erosion was caused by a toxic substance formed from free histidine or histamine which combined with protein in the fish meal during the heating process. The authors considered that histamine in fish meals might be produced by histamine-forming bacteria during storage of the raw materials prior to meal production.

During the 1970's, three papers were published describing the bacterial populations of fish diets. Trust (1971) initiated this work using two commercial fish diets. The following year, Trust and Money examined the bacterial populations of diets for aquarium fishes. Both papers described similar results regarding total aerobic bacterial counts which ranged from 10^3 to 10^7 bacteria/g. The diets also contained members of the Enterobacteriaceae, including species of Salmonella. In 1976, Kitao and Aoki discussed the microbial flora of artificial fish diets prepared and marketed in Japan. All reports concluded that the bacterial flora in the diets consisted mainly of gram-positive bacteria and

contained coliforms. However, the Japanese reported the total aerobic bacterial counts to be significantly less, 10^3 to 10^5 bacteria/g. This may have resulted from the fact that the Japanese fish diets tested were all dehydrated products. It should be noted that Vibrio was detected in 3 of 16 diet samples tested by Kitao and Aoki.

Trust (1971) and Bell (personal communication) have suspected the high bacterial load in fish feeds may affect fish health. The most direct method of affecting fish health would be the transmission of fish pathogens from the feed to the fish. Another example would be under hatchery conditions where fish are sometimes crowded, or during high summer temperatures, the fish are stressed thereby becoming more susceptible to infectious disease. The fish waste products from eating a contaminated diet as well as the diet itself could become the inoculum for an epizootic by increasing the bacterial level in the hatchery water and infecting the susceptible fish.

It is apparent the fish digest in OMP is a source of nitrogen resulting from the breakdown of protein to peptides and amino acids. This provides a good substrate for bacterial growth (Clausen, et al., 1985). The OMP consists of 38% pasteurized fish digest which is made from raw, frozen fish. It is this raw fish that

most likely contains a number of fish pathogens prior to pasteurization. If the fish digest manufacturing procedure was adulterated, it is evident the environment for microbial growth in the digest is ideal. The high moisture content (26-28%) of the final pellet would also contribute to the condusive environment for microbial growth. Because of this, OMP would be expected to have higher bacterial levels than a dry diet which contains only 8% moisture.

Feeding a diet, highly contaminated with fish or human pathogens, could result not only in the fish themselves being affected, but also becoming carriers of bacterial diseases. Janssen (1970), after extensive review of the literature, suggested fish may be more important vectors of human disease than has generally been recognized. In 1966, Martin found that a variety of fish caught as far as 1.5 miles below sewage treatment plants contained Salmonella. Three of the original 17 serotypes isolated, including Salmonella typhimurium, continued to be excreted into the water of holding tanks for the entire 29 day duration of Martin's study. A hatchery's effluent could become a source of contamination for not only downstream water users, including livestock, but native fish as well.

The normal flora of fish and fishery products has been the subject of many studies (Griffiths, 1937; Col-

well, 1962; Okuzumi, 1969; Shewan, 1971; Sera, 1972; Trust, 1974). One of the more recent reports was a study on the intestinal microflora of salmonids by Yoshimizu and Kimura (1976). The results from their work demonstrated the intestinal microflora of healthy salmonids living in fresh water is mainly composed of the genus Aeromonas and the family Enterobacteriaceae. These results agreed with the report of Trust (1974) which indicated that the genera Aeromonas, Enterobacter, and Acinetobacter were the main constituents of intestinal microflora in fish. In contrast, the flora of fish living in sea water is mainly composed of Vibrio of marine or halophilic type. If fish migrate between fresh and salt water during their life cycle, the flora in their intestines would undergo changes in order to help adapt the fish to their living environments. This supported Shewan and Griffiths' conclusion that the bacterial flora of the environment in which the animal lives plays a major role in determining the flora of the fish.

Feed Regulations

The Oregon Revised Statutes prohibits the distribution of adulterated commercial feeds (Anon., 1983). A commercial feed is deemed adulterated

> "...if any poisonous, deleterious or nonnutritive ingredient is therein present

> in sufficient amount to render it injurious to health when fed in accordance with directions for use shown on the label...."

The 1985 Code of Federal Regulations (CFR) on animal feeds (Anon., 1985) further states:

> "Salmonella contamination of such animal feeds having the potentiality for producing infection and disease in animals must be regarded as an adulterant...."

Definitions of animal feeds by the CFR included fish solubles, fish meal, similar animal byproducts, or blended mixtures of these.

The Oregon Department of Agriculture interpreted the FDA regulations by stating that even though the 1986 regulations indicate Salmonella contamination was an adulterant to animal feeds, two 1980 compliance policies (Anon., 1980a,b) remain in effect. These are:

> "...FDA will not routinely inspect or sample animal feed or animal feed ingredients of either domestic or import origin for salmonella contamination...The tolerance for Salmonella in rendered animal by-products intended for use in animal feed or in pet food is found in Compliance Policy Guide 7126.13. No tolerance has been set for finished animal feed or pet food."

The Compliance Policy Guide 7126.13 states the regulatory action for Salmonella contamination in animal by-products is

> "Actionable where 30% of the sub-divisions are positive for Salmonella, and there is corroborating factory evidence of continuing gross insanitation...."

In recent years FDA participated in a joint enforcement program with the United States Department of Agriculture (USDA) in an effort to reduce the incidence of Salmonella in rendered animal and marine by-products. This program was terminated in 1972 when the USDA came to the conclusion that it had higher priorities and this was not a cost effective method to control Salmonella.

Although there are more defined regulations for Fish Protein Concentrate (FPC) (Anon., 1967), there should be a distinction made between this and the fish digest in OMP. The FPC is used as a supplement for human food. Therefore, not only is the FPC used in different products, but it is also in powder form and not a wet hydrolysate.

Composition and Production of OMP

In 1948 the Oregon Fish Commission and Astoria Seafoods Laboratory, an agricultural experiment station of Oregon State University, began a cooperative study of nutritional requirements of Pacific salmon and steelhead trout (Hublou, et al., 1959). Individual components of potential fish foods were investigated with the emphasis on the utilization of marine fishery products. An economical and practical diet of combined tested materials was formulated resulting in the Oregon Moist Pellet.

The Oregon Moist Pellet is labeled a moist pellet

because it contains 26-28% moisture. A diet with a high moisture content is more palatable for salmonids when compared to a dry diet of 8% moisture. The Oregon Moist Pellet is also labeled an open formula which means any manufacturing company may make this product providing specifications are met.

The Oregon Moist Pellet consists of 60% meal mix, 38% wet mix, and 2% vitamin premix (Table 1). The meal mix is a combination of fish and plant meals, whey, grain, and minerals. The wet mix is a pasteurized fish digest and the vitamin premix is a ready-made powdered product.

The production of OMP begins with 50 pound bags of frozen, raw fish which could be, in descending order, herring, black cod, hake, bottomfish, or salmon (Figure 1). This is coarsely ground to 1/2 inch particles prior to being finely minced to 1/16 inch particles. This requires approximately 10 min before being pumped into the pasteurization tank.

It is at the point of pasteurization that a remodeling of old equipment was made in order to decrease the bacterial load in OMP. The old pasteurization tank had a 5,000 pound capacity and was a completely open container five feet in diameter and five feet deep. The inside walls of this tank were corroded from years of use making complete sanitation

Table 1. Ingredient specifications for OMP.[a]

Ingredient	% of Diet	
I. Meal Mix		
Fish meal	28.0	60%
Cottonseed meal	15.0	
Dried whey	5.0	
Wheat germ meal	remainder	
Corn distillers dried solubles	4.0	
Trace mineral premix	0.1	
II. Vitamin Premix	1.5	2%
III. Wet Mix		
Pasteurized wet fish	30.0	38%
Fish oil	6.5	
Choline chloride	0.5	
	100.0	

[a]Westgate,1983.

Figure 1. Procedure for manufacturing the Oregon Moist Pellet. Samples for bacterial analysis were obtained at points marked by a *.

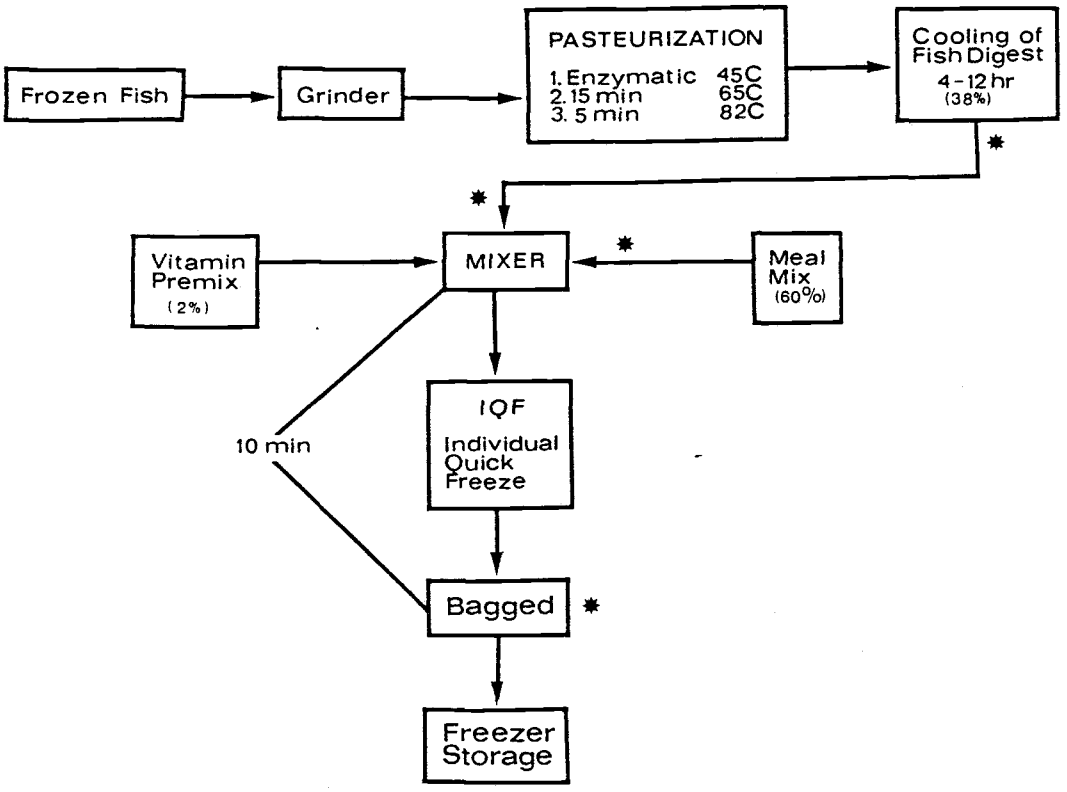

Frozen Fish
Grinder
PASTEURIZATION
1. Enzymatic 45C
2. 15 min 65C
3. 5 min 82C
Cooling of Fish Digest
4-12 hr
(38%)
Vitamin Premix
(2%)
MIXER
Meal Mix
(60%)
10 min
IQF
Individual Quick Freeze
Bagged
Freezer Storage

Figure 1

impossible. Steam was injected into the tank in order to reach pasteurization standards. The fish digest was cooled by a refrigeration jacket that surrounded the pasteurization tank. Both the steam injection and refrigeration cooling are the same procedures for the old and new pasteurization equipment. The new pasteurization equipment consists of two seamless, stainless steel tanks with a 15,000 pound capacity; however, fish digest loads are routinely 12,000 pounds. The new tanks are completely enclosed and contain a churning wheel that remains in motion during the pasteurization stages.

The fish hydrolysate is digested at 45^{o}C with a small amount of papain, and acid, for enzymatic digestion of bones. Once this is complete, the temperature is raised to 65^{o}C at which the fish digest is held for 15 min. For the final pasteurization step, the digest temperature is raised to 82^{o}C for five minutes. As the mixture is cooled by the surrounding refrigeration jacket, the churning wheel mixes air through the digest to evaporate excess moisture, cool, and to ensure the system remains aerobic. The cooling process may take as little as 4 h or as long as 12 h depending on the refrigeration load throughout the remainder of the plant.

After cooling, the fish digest is pumped to the mixer where the meal mix and vitamin premix are

combined. From the mixer the combined ingredients are taken by a conveyor belt to the Individual Quick Freeze (IQF). At this point the pellets are formed, individually frozen and bagged. It takes approximately 10 min for the material to travel from the mixer to the completed IQF. Bags are stored in a freezer prior to shipping. As a result of the vitamin deterioration following production, the OMP has a 90 day shelf life.

Bacterial Isolates Tested for in OMP

A variety of general and specific bacterial isolates were tested for in OMP (Table 2). Specific isolates included both fish and human pathogens. A review of all the bacteria tested for in OMP is not appropriate at this time. Reviews of bacterial fish pathogens are provided by Fryer and Rohovec (1984), and Austin and Allen-Austin (1985). The Manual of Clinical Microbiology (Lennette, et al., 1980) is one of several texts on human pathogenic bacteria that includes bacterial isolates chosen for this study.

Table 2. General bacterial counts and specific bacteria for which the Oregon Moist Pellet was tested.

General Bacterial Counts
1. Total aerobic plate count
2. Mesophilic aerobic spore count
3. Total gram-positive count
4. Total gram-negative count
5. MPN fecal coliform count

Specific Bacterial Isolates	
Fish Pathogens	Human Pathogens
Aeromonas	Anaerobes
A. hydrophila*	Clostridium perfringens
A. salmonicida	
	Salmonella
Mycobacteria*	
	Shigella
Pseudomonas*	
	Staphylococcus
Renibacterium salmoninarum	S. aureus
	Streptococcus
Vibrio	Groups A and B*
V. anguillarum	
	Yersinia enterocolitica
Yersinia ruckeri	

*Both fish and human pathogen

III. MATERIALS AND METHODS

Sample Collection

The pellets were collected prior to being placed in shipping bags and freezer storage (Figure 1). After collection the pellets were placed in sterile plastic twirl bags and transported in insulating material to Oregon State University (OSU), Corvallis. The meal mix, as well as its individual dry ingredients, were collected prior to mixing with the vitamin premix and fish digest and transported as previously described. The fish digest was collected in sterile plastic bottles after cooling from pasteurization. These bottles were stored at 4°C prior to being packed with insulating material and shipped. If there was a delay in transport, the fish digest remained at 4°C, the pellets were stored at -20°C, and dry ingredients were held at room temperature. All were shipped the following day. The majority of the samples were transported and processed at the OSU laboratory the same day of manufacture. Preliminary tests indicated delivering OMP and the fish digest on ice was not necessary because bacterial plate counts remained the same whether samples were shipped in insulating material or on ice.

Upon receipt of the samples, a 1:10 dilution was made by adding 10 g of the sample to 90 ml of phosphate buffered saline (PBS, pH 7.1). Aerobic bacterial plate

counts were performed by making serial dilutions of this original suspension.

Culture Media

Numerous media were used for the isolation and enumeration of bacteria from OMP (Table 3). The majority of the solid media was purchased in powdered form from commercial sources and prepared according to the manufacturers recommendations. Individual media components were combined according to references cited. Methods for media interpretation are referenced (Table 3).

Aerobic Bacterial Plate Counts

Aerobic bacterial plate counts were performed in triplicate by the standard spread plate method (Speck,1984). A 0.1 ml sample of serial dilutions of the OMP, or OMP ingredient, was spread on tryptic soy agar (TSA) plates and incubated at 25^{o}C for 72 h. Bacterial colonies were counted following incubation and reported as colony forming units per gram (CFU/g).

A comparison of plate counts was made using brain heart infusion (BHI), cytophaga (CA), plate count, and tryptic soy agar at 18, 25, and 35^{o}C. Tryptic soy agar, at 25 and 35^{o}C, and CA, at 18^{o}C, consistently gave the highest bacterial counts.

Table 3. Media used for bacterial isolation and identification.

Media	Media preparation and interpretation may be found in the following:		
	Difco Manual[a]	Compendium[b]	Other
Alkaline peptone water (APW) (B)	-	+	
Azide blood agar (ABA) (P)	+	+	
Bismuth sulfite agar (BS) (P)	+	+	
Blood agar (BA) (P)	+	+	
Cytophaga agar (CA) (P)	-	-	c
Egg-yolk free tryptose-sulfite cycloserine agar (EYF TSC) (P)	-	+	
Escherichia coli broth (EC) (T)	+	+	
Gram-negative broth (GN) (B)	+	+	
Lactose gelatin medium (LAC GEL) (T)	-	+	
Lauryl sulfate tryptose broth (LST) (T)	+	+	
Lysine decarboxylase test medium (LDC) (T)	+	+	
MacConkey agar (MAC) (P)	+	+	
Methyl red/Voges-Proskauer test medium (MR,VP) (T)	-	+	
Motility agar (MOT) (T)	+	+	
Nitrate-motility medium (NO_3-MOT) (T)	-	+	
Ogawa agar (Ogawa) (T)	-	-	d
Ornithine decarboxylase test medium (ODC) (T)	+	+	
Oxidative-fermentative test medium (O/F) (T)	+	+	

Table 3. Continued.

Media	Media preparation and interpretation may be found in the following:		
	Difco Manual[a]	Compendium[b]	Other
Phosphate buffered saline (PBS) (B)	-	+	
Pseudomonas agar (PA) (P)	+	-	
Rimler-Shotts agar (RS) (P)	-	-	e
Salmonella Shigella agar (SS) (P)	+	+	
Selective kidney disease medium (SKDM) (P)	-	-	f
Selenite F broth (SF) (T)	+	+	
Staphylococcus 110 medium (Staph 110) (P)	+	-	
Starch-ampicillin agar (SA) (P)	-	-	g
Tetrathionate broth (TTB) (T,B)	+	+	
Thiosulfate-citrate-bile salts-sucrose agar (TCBS) (P)	+	+	
Triple sugar iron agar (TSI) (T)	+	+	
Tryptic soy agar (TSA) (P)	+	+	
Tryptone glucose extract agar (TGE) (P)	+	+	
Tryptophan broth (IND) (T)	+	-	
Urea broth (URE) (T)	+	+	
Xylose lactose deoxycholate agar (XLD) (P)	+	+	
Yersinia agar (YA) (P)	+	-	

Table 3. Continued.

(B) Broth medium $\geq$ 100 ml per sample

(P) Plated agar

(T) Tubed agar or broth

[a]Difco Laboratories, 1984.

[b]Compendium of Methods, 1984.

[c]Amos, 1985.

[d]Tsukamura, 1961.

[e]Shotts and Rimler, 1973.

[f]Austin et al., 1982.

[g]Palumbo et al., 1985.

Mesophilic Aerobic Spore Counts

The mesophilic aerobic spore counts were performed in triplicate on tryptone glucose extract (TGE) agar following standard methods described in the Compendium of Methods (Speck, 1984). A 50 g sample was added to 450 ml sterile peptone water (0.1%) in a blender jar and the suspension blended at high speed for 2 min. Triplicate samples of 0.1 ml, 1 ml, and 10 ml of the suspension were placed into nine 500 ml flasks containing 100 ml TGE equilibrated to 45°C. An additional flask was used as a sterility control. All ten flasks were placed in an 80°C water bath for 30 min. The flasks were immediately cooled with cold tap water and placed in a 45°C water bath. Within 10 min of cooling, five pour plates were made from each flask.

Total Gram-Positive Counts

To determine the total gram-positive counts, serial dilutions made from the 1:10 suspension of OMP were spread on azide blood agar (ABA) in triplicate according to standard methods. These plates were then incubated in a CO_2 candle jar at 35°C for 48 h (Figure 2). Bacterial colonies were counted and the total numbers of gram-positive bacteria were determined.

Total Gram-Negative Counts

To determine the total gram-negative counts, serial

Figure 2. Procedures for the isolation and identification of general bacterial counts and specific bacterial isolates from the Oregon Moist Pellet. Refer to Table 3 for media abbreviations.[a]

[] - Selective treatment prior to plating to inhibit overgrowth of contaminating bacteria.

* - Look for brown pigmented colonies of Aeromonas salmonicida.

ds - double strength
ss - single strength

CAT, catalase test; COAG, coagulase test; GS, gram stain; OX, cytochrome oxidase test.

[a]Circled numbers represent plating order in which plate counts will be least affected.

[b]Duplicate plating.

[c]Triplicate plating.

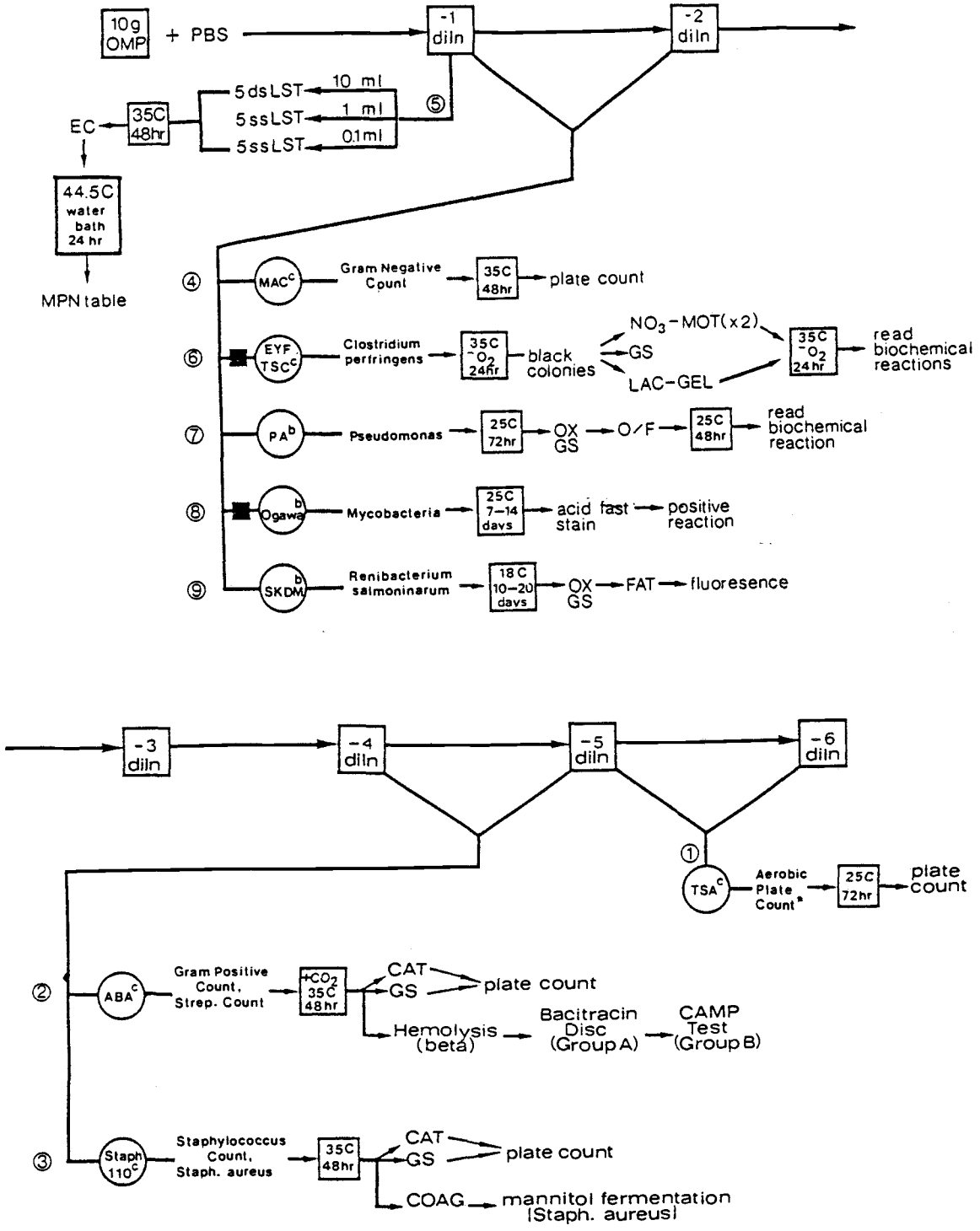

10g OMP
+ PBS
-1 diln
-2 diln
10 ml
1 ml
0.1ml
5 ds LST
5 ss LST
5 ss LST
⑤
EC
35C 48hr
44.5C water bath 24 hr
MPN table
④
MAC^c
Gram Negative Count
35C 48hr
plate count
⑥
EYF TSC^c
Clostridium perfringens
35C -O2 24hr
black colonies
NO3-MOT(x2)
GS
LAC-GEL
35C -O2 24hr
read biochemical reactions
⑦
PA^b
Pseudomonas
25C 72hr
OX GS
O/F
25C 48hr
read biochemical reaction
⑧
Ogawa^b
Mycobacteria
25C 7–14 days
acid fast stain
positive reaction
⑨
SKDM^b
Renibacterium salmoninarum
18C 10–20 days
OX GS
FAT
fluoresence
-3 diln
-4 diln
-5 diln
-6 diln
①
TSA^c
Aerobic Plate Count*
25C 72hr
plate count
②
ABA^c
Gram Positive Count, Strep. Count
+CO2 35C 48hr
CAT
GS
plate count
Hemolysis (beta)
Bacitracin Disc (Group A)
CAMP Test (Group B)
③
Staph 110^c
Staphylococcus Count, Staph. aureus
35C 48hr
CAT
GS
plate count
COAG
mannitol fermentation (Staph. aureus)

Figure 2

dilutions made from the 1:10 suspension of OMP were spread on MacConkey agar (MAC) in triplicate according to standard methods (Figure 2). After plates were incubated at 35°C for 48 h, the bacterial colonies were counted and the total numbers of gram-negative bacteria were determined.

MPN Fecal Coliform Counts

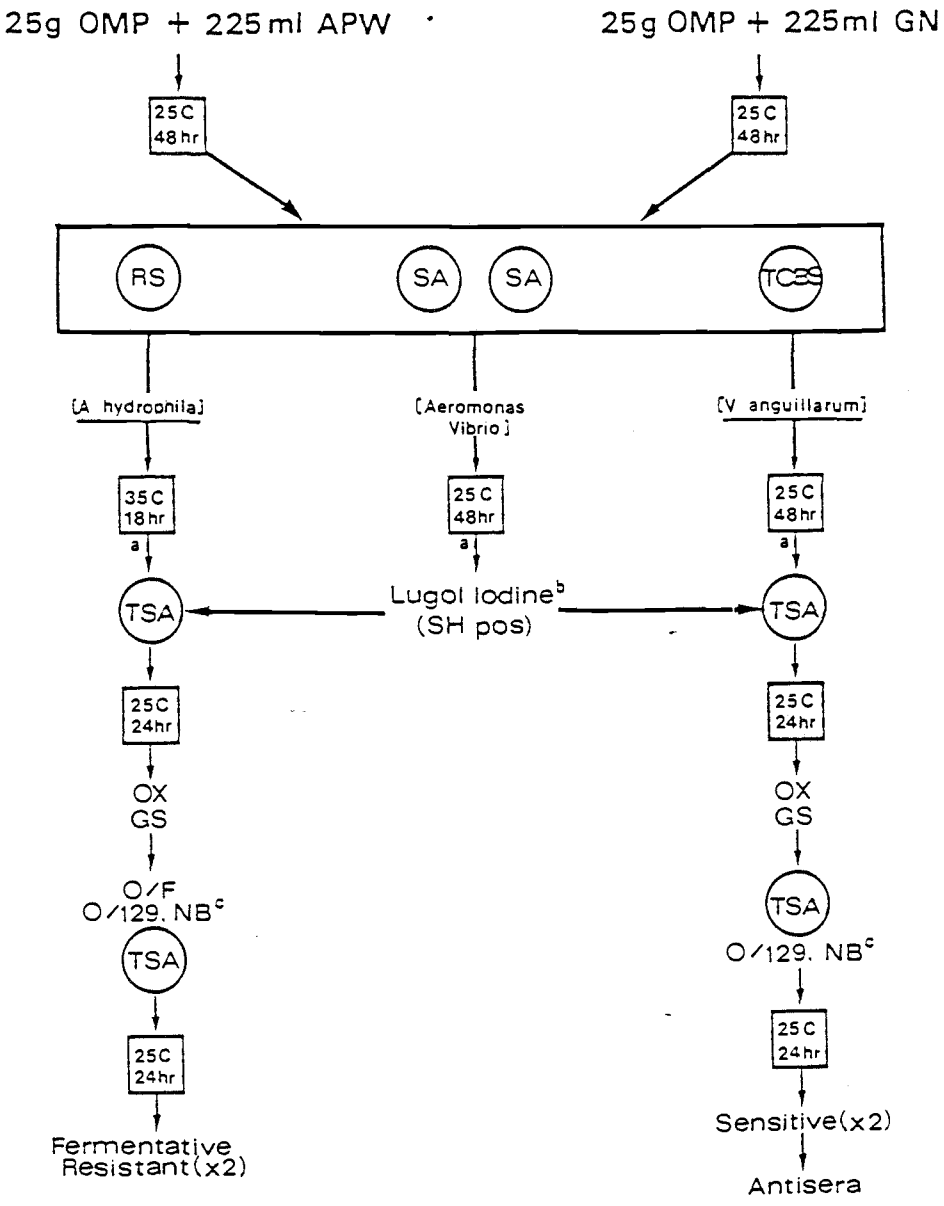
Standard methods for the most probable number (MPN) fecal coliform count are described in the Compendium of Methods (Speck, 1984). The 1:10 OMP suspension provided the inoculum for the presumptive identification in lauryl sulfate tryptose broth after incubating 48 h at 35°C (Figure 2). Bacteria from tubes showing gas production were transferred using a 3 mm loop into Escherichia coli (EC) broth tubes for the definitive test. After the 44.5°C water bath incubation, the number of EC tubes showing bacterial gas production were recorded for interpretation of MPN fecal coliforms per gram from MPN tables also listed in the Compendium of Methods.

Procedures for Isolation and Identification of Fish Pathogens from Oregon Moist Pellets

Aeromonas hydrophila

The isolation of A. hydrophila began by placing 25 g OMP into 225 ml alkaline peptone water (APW) and 25 g OMP into 225 ml gram-negative (GN) broth (Figure 3).

Figure 3. Procedures for isolation and identification of Aeromonas hydrophila, Aeromonas salmonicida, and Vibrio anguillarum. Refer to Table 3 for media abbreviations.



[a] Use positive control plates as a guide for selecting isolates.

[b] Diluted 1/20 for detection of starch hydrolysis (SH).

[c] Vibriostat (O/129) and novobiocin (NB) disks for sensitivity reactions on tryptic soy agar.

Figure 3

After an enrichment incubation of 48 h at 25°C, one Rimler-Shotts (RS) and two starch ampicillin (SA) plates were inoculated using a sterile cotton swab and then streaked for isolation. By using a swab rather than a 3 mm bacteriological loop a larger inoculum is transferred; therefore, enhancing the probability of isolating A. hydrophila. Both RS plates were incubated at 35°C for 18 h and the four SA plates were incubated at 25°C for 48 h. Similar procedures were performed on cultures of A. hydrophila for positive controls.

After incubation of the RS, yellow colonies resembling the positive control were transferred to TSA and incubated for an additional 24 h at 25°C. The colonies from TSA were tested for oxidase (Pathotec strips) and Gram reaction. Those isolates that were oxidase positive and gram-negative rods were tested for oxidation/fermentation (O/F) of carbohydrates using 1% glucose. Two O/F tubes were used per isolate with one overlayed with sterile mineral oil. These tubes, including controls, were incubated at 25°C for 48 h.

The isolates were also tested for their sensitivity to the vibriostat O/129 (Calbiochem) and novobiocin (NB) (Sigma). Sensitivity disks were placed on the TSA plates and incubated overnight at 25°C. Those gram-negative isolates that were resistant to both O/129 and

NB and fermented glucose were identified as being A. hydrophila.

To ensure A. hydrophila isolation, an additional procedure using SA plates was tested. Bacteria growing on the SA plates were tested for positive starch hydrolysis (SH) by flooding the plates with Lugol iodine solution (Palumbo et al., 1985). A 1:20 dilution of Lugol was used rather than the recommended 1:5 dilution because no bacterial colony growth occured after transfer to TSA when the higher concentration was used. Those SH positive colonies were transferred to TSA, incubated at 25°C for 24 h, then tested for oxidase, Gram stain reaction, O/F, and O/129 and NB sensitivity.

Aeromonas salmonicida

Procedures for isolating A. salmonicida were similar to those used for isolation of A. hydrophila from SA plates (Figure 3); however, A. salmonicida had brown pigmented SH-positive colonies. Tests were followed according to the above instructions for A. hydrophila. A brown-pigmented colony demonstrating starch hydrolysis which was an oxidase positive, gram-negative, nonmotile rod, fermented glucose and was resistant to both O/129 and NB was presumed to be A. salmonicida.

Vibrio anguillarum

Methods used to enrich for aeromonads were also

used for *V. anguillarum* (Figure 3). After enrichment incubation, one thiosulfate-citrate-bile salts-sucrose (TCBS) agar and two SA plates were inoculated from each flask. The TCBS, both test and controls of known *Vibrio*, were incubated at 25°C for 48 h then observed for *Vibrio* colonies. Test isolates were then transferred to TSA before performing the oxidase test because ingredients of TCBS interfere. After incubation at 25°C for 24 h, those isolates which were oxidase positive and small gram-negative rods were transferred to an additional TSA for testing sensitivity to 0/129 and NB. Isolates sensitive to both antimicrobial agents were definitively identified with *V. anguillarum* antisera.

Mycobacteria

Ogawa agar (OA) was used for the detection of *Mycobacteria* from OMP. This medium was liquified by contaminating organisms when OMP was directly inoculated; therefore, a selective procedure was necessary. The method used was developed by Corper and Uyei and presented in Diagnostic Microbiology (Finegold and Martin, 1982). Equal volumes of the 1:10 OMP suspension and 5% (w/v) oxalic acid (Sigma) were placed in a 50 ml plastic, screw capped centrifuge tube. The sample was homogenized using a vortex mixer then allowed to stand at room temperature for 30 min. During this time the tube was shaken occasionally. Sterile PBS was added to

within one inch of the tube top, then the sample was centrifuged at 3,000 rpm for 15 min. After the supernatent was decanted, the sediment was neutralized with 4% NaOH containing 0.004% phenol red indicator until a faint pink end point was reached. Three OA tubes were inoculated with 0.33 ml each and incubated at 25°C for seven days. During the incubation time the tubes were observed for growth resembling Mycobacteria chelonei, the positive control. A Ziehl-Neelson acid-fast stain was performed on potential isolates. Those isolates demonstrating a positive acid-fast reaction were considered to be rapidly growing mycobacteria (Arakawa and Fryer, 1984).

Pseudomonas

Methods used to detect pseudomonads began by inoculating two pseudomonas agar (PA) plates with the 1:10 OMP suspension using spread plate standard methods (Figure 2). After incubation at 25°C for 72 h, colonies were tested for oxidase and Gram stain reactions. The PA medium contains Irgasan^{R} which is a potent broad spectrum antimicrobial not active against Pseudomonas. Oxidase positive, gram-negative rods were then tested for O/F by procedures described for A. hydrophila. Selected known organisms were included as controls. All oxidative isolates were considered to be Pseudomonas.

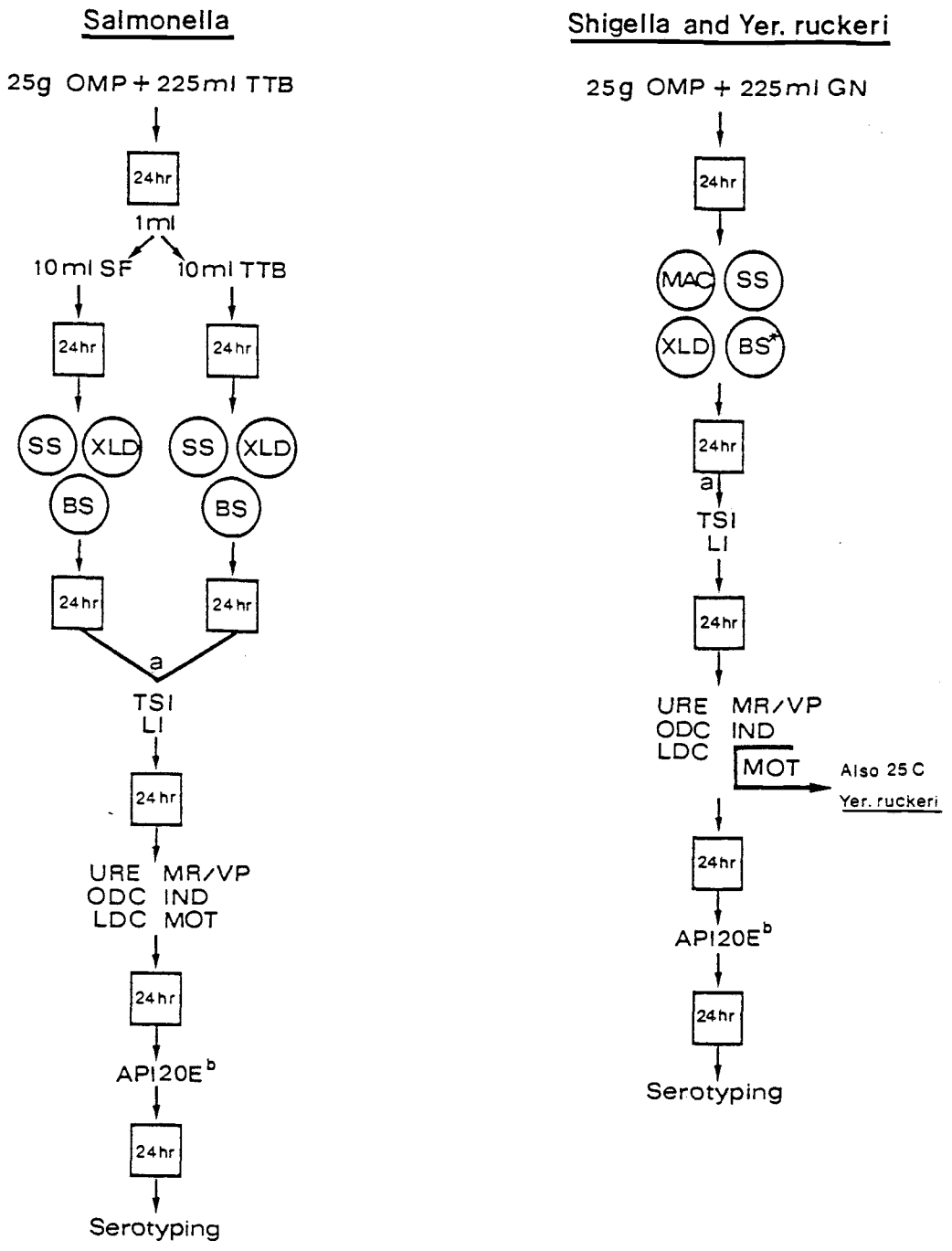
Yersinia ruckeri

The isolation of Y. ruckeri began by placing 25 g OMP in 225 ml GN broth and incubating at 35°C for 24 h (Figure 4). MacConkey (MAC), Salmonella-Shigella (SS), and xylose lactose deoxycholate (XLD) were then inoculated using a sterile cotton swab and then streaked for isolation. All plates and biochemical tests were incubated overnight at 35°C unless otherwise noted. Positive control plates were used as a guide for selecting colonies to be tested. Transfers were made to triple sugar iron (TSI) and lysine iron (LI) agar tubes. After incubation, those isolates giving alkaline over acid (K/A) results in both TSI and LI were further tested for the following biochemical reactions: urease, ornithine decarboxylase, methyl red, Voges-Proskauer, indole, and motility at 25 and 35°C (Finegold and Martin, 1982). Results were compared to biochemical reactions listed in Table 4. Isolates duplicating Y. ruckeri biochemical reactions were then identified by an API20E (Analytab) strip. Confirmative identification of Y. ruckeri was made by serotyping using Y. ruckeri antisera against serotypes I and II (O'Leary, et al., 1982).

Renibacterium salmoninarum

Selective kidney disease medium (SKDM) was used to culture for R. salmoninarum from the 1:10 OMP suspension (Figure 2). Two plates were streaked for isolation,

Figure 4. Procedures for the isolation and identification of Salmonella, Shigella, and Yersinia ruckeri from the Oregon Moist Pellet. Refer to Table 3 for media abbreviations.



*Streak BS for additional check for Salmonella.

[a]Use positive control plates as a guide for selecting isolates.

[b]Analytab product for identification of Enterobacteriaceae.

Figure 4

Table 4. General biochemical reactions of select members in the family Enterobacteriaceae.[a,b]

Genus and species	TSIA			LIA									
	Slant/ Butt	Gas	H_2S	Slant/ Butt	Gas	H_2S	IND	MR	VP	URE	ODC	LDC	MOT
Yersinia ruckeri	K/A	-	-	K/A	-	-	-	+	-	-	+	+(+)	- 35°C +/-25°
Shigella	K/A	-	-	K/A	-	-	-/+	+	-	-	d	-	-
Salmonella	K/A	+	+/-	K/K*	-	+/-	-	-	-	-	+	+	+
Citrobacter	KorA/A	+	+/-	K/A	-/+	+/-	-/+	+	-	d	+	-	+
Hafnia alvei	K/A	+	-	K/K	-/+	-	-	-/+	+/-	-	+	+	+
Proteus vulgaris	AorK/A	+	+	R/K	-	-	+	+	-	+	-	-	+
Proteus mirabilis	K/A	+	+	R/A	-	-	-	+	-/+	+	+	-	+
Enterobacter	KorA/A	+/-	-	K/d	+/-	-	-	-	+	d	+/-	d	+

K, alkaline; A, acid; R, red.

Sign: +, 90% or more are positive within one or two days; (+), delayed positive reaction occurs within three to four days; -, no reaction (90% or more); +/-, reaction of most species/reaction of some species; d, different reactions (+, +/-, or -); *, key reaction.

[a]Modified from Finegold and Martin, 1982.

[b]All reactions are incubated at 35°C except where noted.

sealed with Parafilm (American Can Company), and incubated at 18°C for 10-20 days (Evelyn, 1977). Plates were examined the fourth and seventh day for contaminating bacteria which were aseptically excised. Suspect isolates were Gram stained. Gram-positive coccobacilli were examined using a fluorescent antibody test (FAT) (Banner et al., 1982). Fluorescence identified the isolate to be R. salmoninarum.

Elliott (1986) described another FAT procedure for examination of filtered salmonid ovarian fluid for R. salmoninarum. This procedure was useful in this study because the bacterial flora in OMP could be concentrated onto a filter. Slight adaptations were made in order to filter the OMP. A 10 g sample of OMP was diluted in 20 ml PBS and, depending on pellet size, allowed to dissolve approximately 10-20 min. Large feed particles were removed by filtration through four single layers of cheesecloth. The filtrate was collected in a test tube and allowed to stand 5-10 min. This let the sediment of the filtrate to settle out. A 3 ml syringe was used to aspirate 0.5 ml supernatent which was placed in a plastic centrifuge tube and mixed with 0.5 ml PBS (pH 7.1), containing 0.5% (v/v) Triton X-100 (Sigma) (PBS-Triton), and 0.5 ml 1% (w/v) trypsin (Sigma) solution. Samples were vortexed 30 sec then heated in a 50°C water bath for 10 min. A 3 ml syringe equipped with a 22

gauge needle was used to triturate each sample to break up any remaining clumps of material. The syringe was attached to a Swinney-type filter holder (VWR) containing a 13 mm, 0.2 um Nuclepore polycarbonate filter (VWR#28157-880). Samples were filtered then rinsed with 3 ml PBS-Triton. The filter remained in the holder and 100 ul of FITC conjugated anti-R. salmoninarum antisera was added. The top of the filter holder was covered with Parafilm then incubated in the dark for one hour. Following incubation each filter was rinsed with PBS containing Eriochrome black T (1:20,000 solution filtered through Whatman No. 1) (Sigma) for the counterstain. Evans Blue dye does not work well as a counterstain for this procedure (Elliott, personal communication). Filters were removed and placed on a slide to air dry. One drop of glycerol mounting medium (pH 9) was placed in the center of the filter and mounted with a cover slip. Slides were then examined for FITC epifluorescence with a Zeiss standard microscope using an IC Fl epi-fluorescence condenser, 12 V 100 watt halogen-tungsten light source, and a LP 520 orange barrier filter. Each sample was performed in duplicate.

Procedures for Isolation and Identification of Human Pathogens from Oregon Moist Pellets

Clostridium perfringens

Two methods were used for determining the numbers

of C. perfringens. One was the direct method which utilized egg-yolk free tryptose sulfite cycloserine agar (EYF TSC) and the other was a selective alcohol treatment described by Craven and Blankenship (1985). The direct method was a procedure described in the Compendium of Methods (Speck, 1984). Serial dilutions were made from the 1:10 OMP suspension (Figure 2). A 1 ml sample was aseptically transferred to three glass petri plates for each of two dilutions made. Pour plates were made using EYF TSC, allowed to solidify, and then overlayed with 3-5 ml EYF TSC. Plates were incubated anaerobically at 35°C for 24 h using the GasPak system (BBL Microbiology Systems).

The selective treatment for C. perfringens consisted of diluting 1 ml of the original 1:10 OMP dilution with 9 ml 70% ethanol which was allowed to incubate at room temperature for 15 min. Three pour plates were immediately made according to instructions previously described. These plates were incubated in an anaerobic jar with the nonselectively treated plates.

After incubation, all EYF TSC plates were observed for nonspreading, black, sulfite-reducing colonies. The black colonies representing presumptive identification of C. perfringens were counted. Further biochemical tests were required for complete identification. Five isolates from each plate were Gram stained

and selected for transfer to one gelatin and two nitrate motility (NO_3-MOT) tubes (Speck, 1984). All tubes, including controls, were incubated anaerobically at 35°C for 24 h. Following incubation, the gelatin tubes were placed at 4°C for 30 min then read for gelatinase activity. One NO_3-MOT tube was tested for nitrate reduction by adding 5 drops sulfanilic acid then 5 drops alpha-naphthylamine (Finegold and Martin, 1982). A pink color designated a positive reaction whereas no color change indicated either a negative or a slow positive reaction. The second NO_3-MOT tube was reincubated for an additional 24 h then tested. If the second tube remained colorless, a true negative reaction was recorded. Those isolates that were nonmotile, gram-positive bacilli with blunt ends, reduced nitrate, fermented lactose, and contained the enzyme gelatinase were identified as C. perfringens.

The percent of the positive C. perfringens isolated from the five colonies tested per plate was determined. This percent of positive isolates of the original total number of nonspreading, black colonies was used to calculate the number of C. perfringens in the sample.

Salmonella

The isolation procedures for Salmonella from OMP were similar to those found in the Compendium of Methods (Speck, 1984). Two treatments were used to select for

Salmonella prior to plating samples on solid media. The first was to suspend of 25 g OMP in 225 ml tetrathionate broth (TTB) which was incubated at 35°C for 24 h (Figure 4). The second treatment was to transfer 1 ml of the first treatment to 10 ml each of selenite F (SF) broth and TTB. Both tubes were mixed and incubated. Following the second treatment, SS, XLD, and bismuth sulfite (BS) agar plates were inoculated using a sterile cotton swab and streaked for isolation. All six were incubated with control plates of known bacteria. The remainder of the Salmonella isolation and identification procedures were similar to those described for Y. ruckeri; however, only 35°C was used to incubate the MOT tube rather than both 25 and 35°C. Those isolates identified as Salmonella species by the API20E system were sent to the Oregon Department of Agriculture for serotyping.

Shigella

The isolation procedures for Shigella from OMP were similar to those found in the Compendium of Methods (Speck, 1984). An initial enrichment step of dissolving 25 g OMP in 225 ml GN broth was incubated at 35°C for 24 h (Figure 4). Then SS, XLD, and BS plates were inoculated using a sterile cotton swab and streaked for isolation. Shigella will not grow on BS; however, this plate was inoculated as an additional opportunity to

isolate Salmonella. The remainder of the Shigella isolation and identification procedures were similar to those described for Y. ruckeri; however, only 35°C was used to incubate the MOT tube rather than both 25 and 35°C. Those isolates identified as Shigella species by the API20E system were to be sent to the Oregon Department of Agriculture for serotyping.

Staphylococcus Count and Staphylococcus aureus

The Staphylococcus counts and the S. aureus isolation and identification procedures were performed in triplicate on Staphylococcus 110 (Staph 110) plates. A 0.1 ml sample of the 1:10 OMP serial dilutions was spread onto each of three Staph 110 plates following standard methods described in the Compendium of Methods (Speck, 1984) (Figure 2). After incubation at 35°C for 48 h, a minimum of ten representative colonies from each plate were Gram stained and tested for the presence of the enzyme catalase. Colonies morphologically identical to the test isolates that were catalase positive and gram-positive cocci not in tetrads, were estimated to be staphylococci.

The Staph 110 plates from the Staphylococcus count were used for the following three S. aureus tests: coagulase, gelatinase, and mannitol fermentation. All yellow or orange pigmented colonies were first tested for coagulase using the glass slide method described in Fine-

gold and Martin (1982). There were no coagulase positive colonies isolated; therefore, the gelatinase and mannitol fermentation tests were not used. These procedures are described in detail in the Difco Manual (1984).

Streptococcus Count and Streptococcal Groups A and B

A total Streptococcus count was obtained using the ABA plates described in the total gram-positive count. Colonies <1 mm in diameter were tested for catalase reaction and then examined by Gram stain (Figure 2). Those bacterial colonies that were catalase negative and small gram-positive cocci were considered streptococci. Selected known organisms were included as controls.

Streptococcal groups A and B were differentiated from the total streptoccal population by the presence of beta hemolysis (Figure 2). Separation of Group A from other beta hemolytic streptococci was accomplished using bacitracin disks (Sigma) (Levinson and Frank, 1955).

If the bacitracin test was negative, a positive CAMP factor identified group B streptococci. The test was performed following methods described in Finegold and Martin (1982). A streak of known beta hemolytic Staphylococcus was placed in the center of a BA plate. The test isolate was then streaked perpendicularly to the staphylococcal inoculum without touching it. Groups A

and B controls were included. After overnight incubation at 35°C, the plate was observed for an arrowhead pattern of hemolysis adjacent to the staphylococcal streak which indicated a positive reaction.

Yersinia enterocolitica

The Y. enterocolitica isolation and identification procedures were performed in duplicate on yersinia agar (YA). A 0.1 ml sample of the 1:10 OMP suspension was spread onto both YA plates following standard methods described in the Compendium of Methods (Speck, 1984). After incubation at 35°C for 48 h, plates were observed for colony growth similar to the positive control plate. No potential isolates were detected on YA; therefore, further identification by the API20E system was not necessary.

Determination of Bacterial Counts in OMP and Its Components

Three major components of OMP were sampled for the total bacterial count (Figure 1): 1) fish digest, 2) meal mix, and 3) OMP. The fish digest was collected after cooling from the pasteurization process. Bacterial counts from the fish digest represented the bacterial load after pasteurization and the cleanest point prior to traveling through a pipe to the mixer. Because of the modernization of the pasteurization equipment, bacterial counts were made before and after the

new equipment was installed. A smaller number of fish digest samples were taken just before being mixed with the vitamin and meal mix. The cooled fish digest had traveled through a connecting pipe to the mixer. A comparison of bacterial counts from the beginning and end of the pipe indicated bacterial contamination resulting from a lack of plant sanitation procedures.

The meal mix was collected before mixing with the fish digest and vitamin mix. Bacterial counts of the meal mix can be correlated with the bacterial levels included in the final pellet produced. Individual ingredients of the meal mix were taken directly from the packaged product or storage bin. The bacterial counts of each ingredient demonstrated which had the highest bacterial load.

The final product was collected after the IQF. The bacterial counts of the pellets can be correlated with bacterial levels in OMP that was used in one of the Pacific Northwest hatcheries. Samples were taken before and after the modernization of the pasteurization equipment. Bacterial counts of OMP before the equipment change can be correlated with bacterial levels present in pellets produced in recent years. Bacterial counts of OMP after the equipment change can be correlated with bacterial levels present in pellets currently produced.

The most popular dry diet used in Oregon hatcheries

was examined for comparison with OMP. The dry diet contains 8% moisture whereas OMP contains 26-28% moisture. Dry diet samples were mailed to OSU within a week of manufacture and stored at room temperature. The dry diet was processed in the same manner as that used for OMP evaluations.

Determination of Bacterial Growth During the 90 Day Shelf Life of OMP

The purpose of determining the bacterial counts of OMP during its 90 day shelf life was to detect a pattern of bacterial growth or reduction. As a result of the vitamin deterioration following production, the OMP has a 90 day shelf life. Aerobic bacterial plate counts were incubated at three temperatures to identify growth patterns of mesophilic bacteria contained in OMP. The OMP examined was stored at -20°C during the shelf life. At selected intervals aerobic bacteria were enumerated using the spread plate method described in the Compendium of Methods (Speck, 1984). Tryptic soy agar at both 25°C for 72 h and 35°C for 48 h, and CA at 18°C for seven days were used to determine the aerobic bacterial plate counts.

IV. RESULTS

Determination of Total Bacterial Counts in OMP and Its Ingredients

Ninety Day Shelf Life

Aerobic bacterial plate counts were determined from OMP during its 90 day shelf life (Table 5). As a result of the vitamin deterioration following production, the OMP shelf life was determined to be 90 days. Of the three temperatures tested, plate counts performed at 16°C showed the most sporatic bacterial growth pattern. Those plates incubated at 25 and 35°C demonstrated growth patterns closely paralleling each other and averaged to give the highest bacterial counts. During the first seven days of shelf life there was a decrease in bacterial growth. After this time the counts increased, most often surpassing the original plate count, till day 30 or 45 (Figure 5). It was at this point the OMP bacterial flora appeared to be at the peak of growth since its production date. For the remainder of the shelf life the bacterial numbers decreased.

Aerobic bacterial plate counts performed at 25°C were plotted (Figure 5). In general, the total bacterial counts in OMP did not appear to fluctuate much. Of the seven OMP samples tested, five decreased in bacterial counts from the day of manufacture to the

Table 5. Aerobic plate counts, determined as bacterial colony forming units x 10^5/g, from temperature studies on the Oregon Moist Pellet during its 90 day shelf life. A through H are Oregon Moist Pellet samples taken prior to pasteurization equipment modification.[a]

	CFU X 10^5 / g											
	Day 0			Day 7			Day 15			Day 30		
°C / OMP	35	25	16	35	25	16	35	25	16	35	25	16
A	540	430	440	540	390	410	470	390	350	510	450	470
B	1200	1300	1100	1200	1200	1500	1100	1200	1000	1100	1200	1000
C	490	570	690	440	480	330	490	510	380	550	630	580
D	400	570	520	530	550	480	590	540	580	460	440	410
E	310	310	270	310	400	390	360	330	460	340	400	260
F	6.6	11	12	5.4	12	11	130	140	63	140	130	96
G	TNTC	TNTC	TNTC	4.4	8.5	8	5.3	9.4	8.3	6.1	8.7	7
H	140	170	150	150	150	120	120	100	95	97	100	110
AVG	441	480	455	397	399	406	408	402	367	400	420	367

TNTC - Too Numerous To Count

[a]Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 35°C for 48 h, tryptic soy agar incubated at 25°C for 72 h, and cytophaga agar incubated at 16°C for 7 days.

Table 5. Continued.

	C F U X 1 0 ^{5} / g								
	Day 45			Day 60			Day 90		
OMP / ^oC	35	25	16	35	25	16	35	25	16
A	390	320	320	420	420	460	370	280	250
B	1400	1400	1000	1300	1200	130	1100	880	820
C	660	630	580	570	600	590	440	440	330
D	530	470	440	140	140	150	270	390	360
E	290	340	320	230	190	240	210	330	350
F	7	13	16	9.3	11	20	13	26	21
G	5.3	6.7	5.5	4	5	3.3	NSA	NSA	NSA
H	78	87	64	120	93	100	NSA	43	NSA
AVG	420	408	343	349	332	212	401	341	355

NSA - No Sample Available

Figure 5. Aerobic plate counts, determined as bacterial colony forming units/g, of Oregon Moist Pellet during its 90 day shelf life. Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 25°C for 72 h. A through F are Oregon Moist Pellet samples taken prior to pasteurization equipment change.

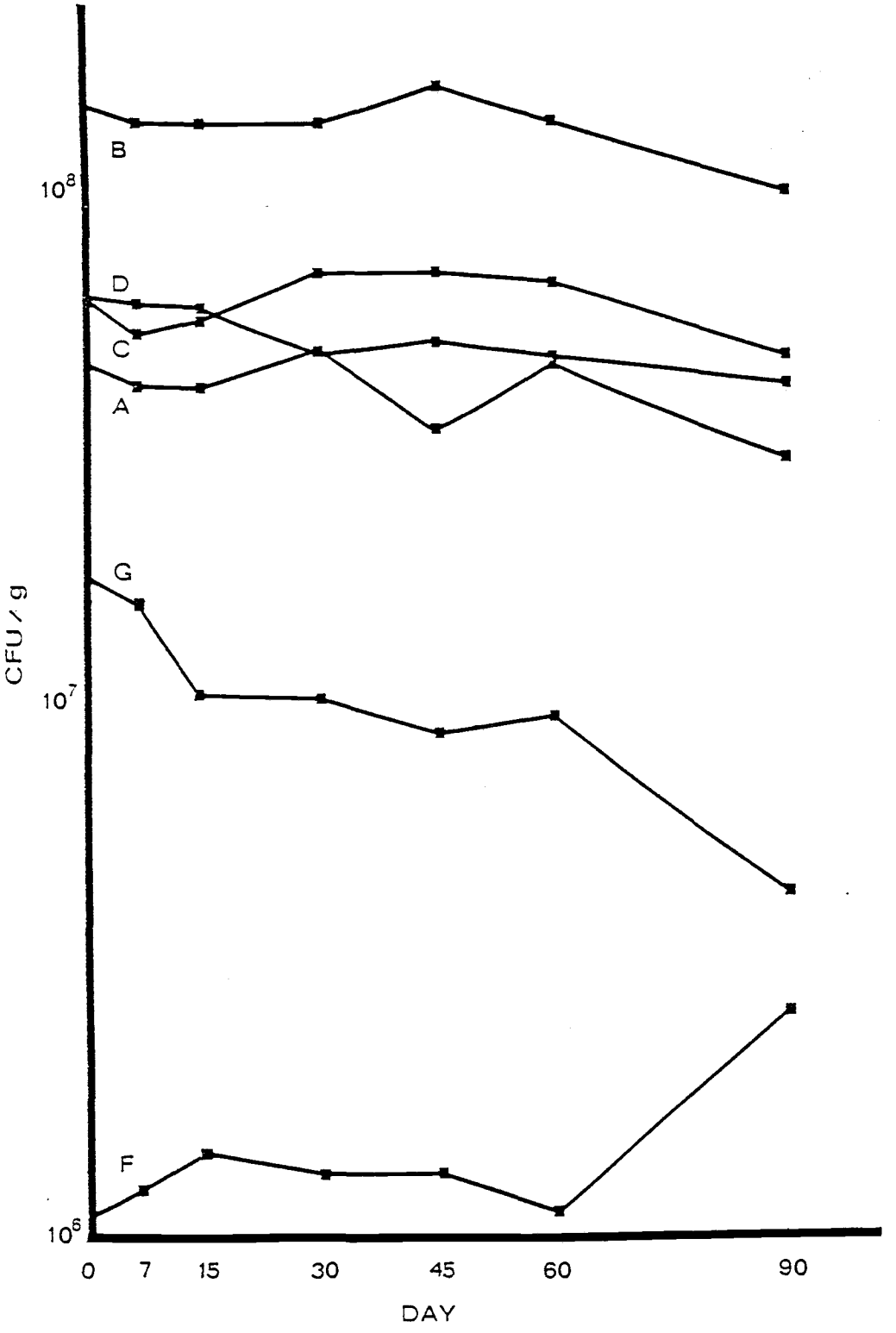


Figure 5

end of the shelf life; however, two of the samples showed an increase (Table 6).

Meal Mix

Aerobic bacterial plate counts of the OMP meal mix were determined and averaged 7.1×10^5 CFU/g (Table 7). There was no change in the manufacturing equipment or procedures for this ingredient; therefore, the total bacterial counts serve for both before and after the pasteurization equipment changed.

Individual meal mix ingredients were also tested to determine the succession of ingredients with the highest bacterial load (Table 8). Cotton seed meal demonstrated the highest bacterial count with 1.4×10^6 CFU/g. The remainder of the ingredients, in decreasing order of bacterial counts, were as follows: wheat germ, herring meal, anchovy meal, Solulac, and the vitamin premix. The ingredient with the lowest bacterial count was whey with 2.0×10^2 CFU/g.

Fish Digest

Aerobic bacterial plate counts of the fish digest, both before and after the change in pasteurization equipment, were determined (Table 9). All of the fish digest samples were taken after cooling from the pasteurization process. Before the equipment changed, bacterial counts ranged from 1.1×10^9 to 2.2×10^8

Table 6. Aerobic plate counts, determined as bacterial colony forming units/g, of Oregon Moist Pellet at the beginning and end of its 90 day shelf life. A through G are Oregon Moist Pellet samples taken prior to pasteurization equipment modification.[a]

Sample	0 Day	90 Day	% Change
A	4.3×10^7	2.8×10^7	-35
B	1.3×10^8	8.8×10^7	-32
C	5.7×10^7	4.4×10^7	-23
D	5.7×10^7	3.9×10^7	-32
E	3.1×10^7	3.3×10^7	+ 6
F	1.1×10^6	2.6×10^6	+58
G	1.7×10^7	4.3×10^6	-75
		AVERAGE	-19

[a] Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 25°C for 72 h.

Table 7. Aerobic plate counts, listed as bacterial colony forming units/g, of the meal mix, fish digest, and Oregon Moist Pellet.[a]

Before equipment modification		Meal mix used in both OMP formulations	After equipment modification	
Fish Digest	OMP		Fish Digest	OMP
2.2×10^8	4.3×10^7	5.5×10^5	6.0×10^4	8.8×10^5
NSA	1.3×10^8	9.9×10^5	1.5×10^7	4.4×10^5
5.9×10^8	5.7×10^7	8.3×10^5	$<1.0 \times 10^4$	1.6×10^6
1.1×10^9	5.7×10^7	1.1×10^6	5.0×10^4	1.4×10^6
5.7×10^8	3.1×10^7	1.3×10^6	8.0×10^4	4.1×10^5
NSA	1.1×10^6	2.7×10^5	$<1.0 \times 10^4$	1.2×10^6
4.7×10^8	1.7×10^7	4.0×10^5	1.4×10^5	5.5×10^5
		7.2×10^5	$<1.0 \times 10^4$	5.0×10^5
		1.9×10^5	NSA	1.2×10^6
			NSA	5.0×10^5
			NSA	1.2×10^6
			NSA	6.3×10^6
			NSA	1.6×10^6
			NSA	6.0×10^5
			NSA	1.8×10^6
			NSA	3.5×10^5
			NSA	9.3×10^6
			6.2×10^6	5.4×10^6
			2.0×10^2	9.5×10^5
			$<1.0 \times 10^4$	5.9×10^6
			$<1.0 \times 10^4$	2.8×10^6
			$<1.0 \times 10^4$	1.1×10^6
			1.7×10^5	1.2×10^6
			1.5×10^7	1.8×10^6
			6.0×10^4	8.8×10^5
			1.5×10^7	4.4×10^5
			$<1.0 \times 10^4$	1.6×10^6
			5.0×10^4	1.4×10^6
AVERAGE				
5.5×10^8	4.8×10^7	7.1×10^5	$<1.0 \times 10^4$ to 4.3×10^6	1.9×10^6

NSA - No Sample Available

[a] Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 25°C for 72 h. Horizontal reading of the plate counts lists the fish digest of resulting Oregon Moist Pellet.

Table 8. Aerobic plate counts, determined as bacterial colony forming units/g, of the meal mix ingredients found in the Oregon Moist Pellet.[a]

Ingredient	CFU/g
Cotton seed meal	1.4×10^6
Wheat germ	1.1×10^5
Herring meal	8.1×10^4
Anchovy meal	4.2×10^4
Solulac (Corn distillers grain)	1.4×10^3
Vitamin premix	1.2×10^3
Whey	2.0×10^2
Combined meal mix	1.9×10^5
MPN fecal coliform of combined meal mix	350[b]

[a] Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 25°C for 72 h. Dilutions were made from a 10 g sample of each ingredient for the aerobic plate count.

[b] MPN fecal coliforms/g

Table 9. Comparison of aerobic plate counts, determined as bacterial colony forming units/g, of a dry diet and Oregon Moist Pellet and its ingredients produced before and after pasteurization equipment modification. The meal mix ingredients and procedure remained the same during the pasteurization equipment change.[a]

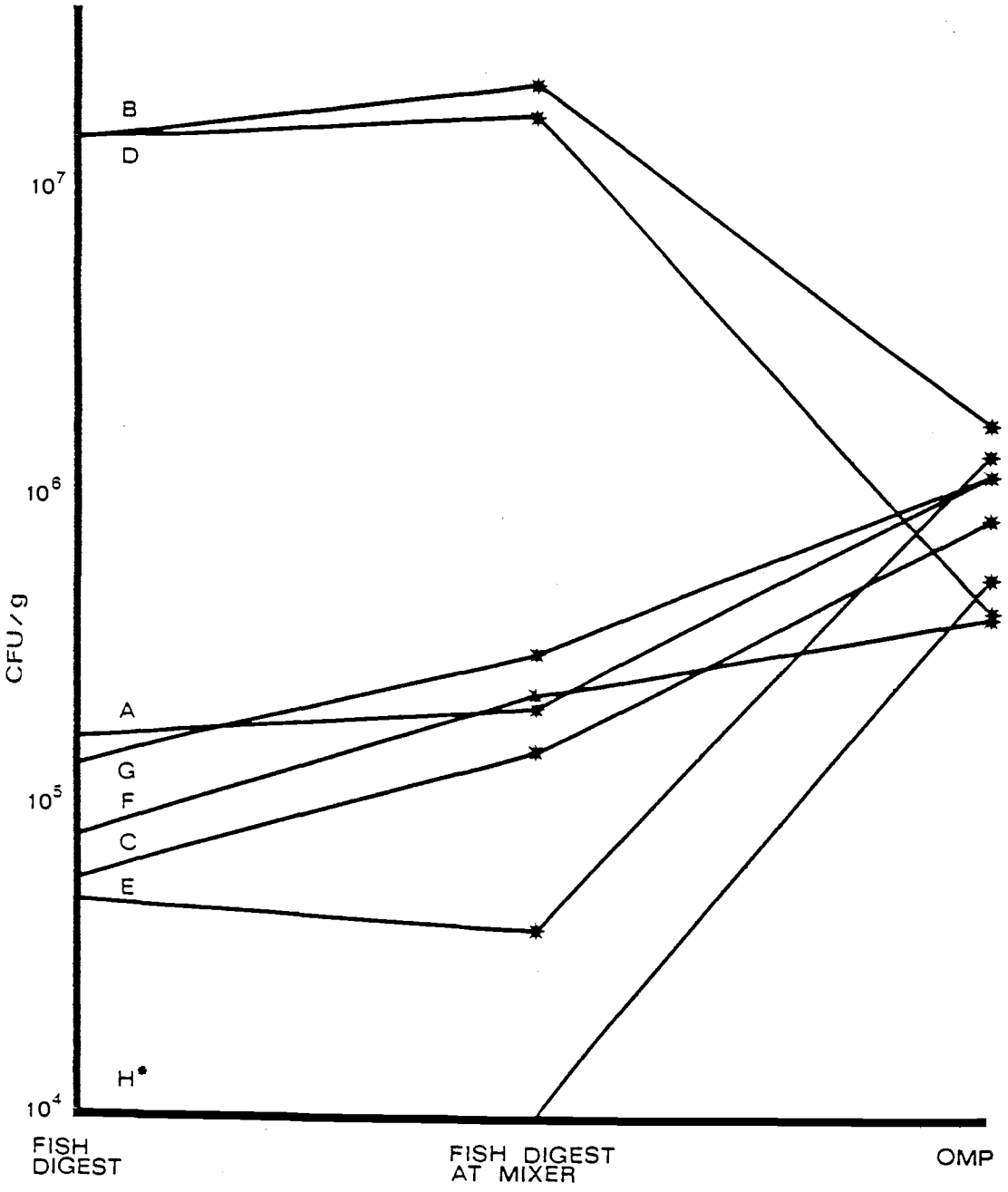
Sample (No. tested)	High	Low	Average	% Decrease
Meal Mix (9)	1.3×10^6	1.9×10^5	7.1×10^5	-
Before: Fish Digest (5)	1.1×10^9	2.2×10^8	5.5×10^8	minimum of 99.2
After: Fish Digest (19)	1.5×10^7	2.0×10^2	$<1.0 \times 10^4$ to 4.3×10^6	
Before: OMP (7)	1.3×10^8	1.1×10^6	4.8×10^7	96.0
After: OMP (28)	9.3×10^6	3.5×10^5	1.9×10^6	
Dry Diet (3)	4.7×10^5	1.5×10^5	2.6×10^5	-

[a]Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 25°C for 72 h.

CFU/g. The average of the five fish digest samples tested were determined to be 5.5×10^8 CFU/g. After the pasteurization equipment modification, the fish digest had reduced bacterial levels. The lowest bacterial count in the fish digest after the equipment change was 2.0×10^2 CFU/g. The average bacterial count of the fish digest after the pasteurization equipment modification ranged from $<1.0 \times 10^4$ to 4.3×10^6 CFU/g. Seven of the 19 fish digest samples taken had bacterial counts less than the lowest dilution tested. Comparison of the average of the two fish digest bacterial counts reflected a minimum decrease of 99.2% in the bacterial load resulting from the change in pasteurization equipment.

To determine whether bacterial recontamination occurred due to lack of plant sanitation procedures, fish digest samples were taken at the beginning and end of a pipe connecting the pasteurization tank and the mixer (Figure 1). These fish digest samples were taken after the pasteurization equipment changed. Aerobic bacterial plate counts were plotted (Figure 6). Of the eight fish digest samples tested, six demonstrated an increase in bacterial counts. This resulted from recontamination by residual fish digest within the connecting pipe. It should be noted that regardless of the bacterial level of the fish digest, after the meal mix

Figure 6. Aerobic plate counts, determined as bacterial colony forming units/g, of fish digest samples taken at three stages in the production of Oregon Moist Pellet: (1) after cooling the pasteurized fish digest, (2) after the same fish digest has traveled from the cooling tank to the mixer, and (3) the resulting Oregon Moist Pellet. Each determination is an average of triplicate plate counts from tryptic soy agar incubated at 25°C for 72 h. A through H are fish digest samples taken after pasteurization equipment changed.



*Fish digest value is $< 1.0 \times 10^4$

Figure 6

was added, the bacterial level in the final pellet resulted in counts close to, or one log higher, that of the meal mix. Because the meal mix comprises 60% of OMP, it generally dictates the bacterial level of the final product.

OMP

Aerobic bacterial plate counts were determined from seven OMP samples taken before, and 28 OMP samples taken after the equipment changed (Table 9). The bacterial counts in OMP before the equipment change averaged 4.8×10^7 CFU/g. After the equipment change the bacterial count decreased to 1.9×10^6 CFU/g. This resulted in a 96% decrease in the bacterial load. A dry diet was also tested for comparison of bacterial levels between a moist diet, OMP, and a dry diet (Table 9). The dry diet contained bacterial levels approximately one log less than that found in OMP produced after the pasteurization equipment modification.

Determination of Bacterial Groups in OMP

A comparison of five general bacterial counts from OMP samples taken before and after the pasteurization equipment changed was made (Table 10). These included a total aerobic plate count, mesophilic aerobic spore count, total gram-positive count, total gram-negative count, and an MPN fecal coliform count. A dry diet was

Table 10. Comparison of the bacterial counts of a dry diet and Oregon Moist Pellet produced before and after pasteurization equipment modification.

Procedure	No. bacteria x 10^2/g of diet (No. tested)					
	O M P				Dry Diet	
	Before		After		%Δ	
Total aerobic plate count[a]	480,000	(7)	19,000	(19)	-96	2,600 (3)
Mesophilic aerobic spore count[b]	39	(4)	24	(3)	-38	200 (2)
Total gram-positive count[c]	210,000	(7)	23,000	(10)	-89	620 (3)
Total gram-negative count[d]	14	(7)	340	(10)	+96	360 (3)
MPN fecal coliform count[b]	0.5	(5)	2.1	(11)	+76	0.1 (3)

[a]Triplicate plating on tryptic soy agar incubated at 25°C for 48 h.

[b]Compendium of Methods, 1984.

[c]Triplicate plating on azide blood agar incubated at 35°C for 48 h.

[d]Triplicate plating on MacConkey agar incubated at 35°C for 48 h.

included for comparison with the moist pellet. The first general bacterial level to be determined was the aerobic bacterial plate count. As previously described, the aerobic bacterial plate count decreased 96% after the pasteurization equipment changed.

Mesophilic Aerobic Spore Counts

A mesophilic aerobic spore count was determined from four OMP samples taken before, and three OMP samples taken after the pasteurization equipment modification (Table 10). All 35 OMP samples examined in the total aerobic plate counts could not be tested because of insufficient samples. There was a relatively low spore count both before and after the equipment changed. The new pasteurization equipment did decrease the spore count 38% from previously detected levels. The dry diet contained a spore count over five times that detected in OMP before the change in equipment, and over eight times that detected in OMP after the change in equipment.

Total Gram-Positive Counts

A total gram-positive count was determined from seven OMP samples taken before, and 10 OMP samples taken after the pasteurization equipment modification (Table 10). The gram-positive bacteria comprised the largest population of the bacterial flora detected in OMP. The 2.1×10^7 gram-positive bacteria/g recorded in samples

prior to equipment modification were reduced 89% after the new pasteurization equipment was installed.

The bacterial flora in the dry diet, like OMP, was largely composed of gram-positive bacteria. However, the dry diet's bacterial population was composed of fewer gram-positive bacteria per gram than detected in OMP. The dry diet contained 6.2×10^4 gram-positive bacteria/g whereas the OMP produced after the change in pasteurization equipment contained 2.3×10^6 gram-positive bacteria/g.

Total Gram-Negative Counts

A total gram-negative count was determined from seven OMP samples taken before, and 10 OMP samples taken after the pasteurization equipment modification (Table 10). The gram-negative population was a small fraction in the OMP bacterial population; however, this count increased 96% after the new pasteurization equipment was installed. The range before the equipment changed was 1.0×10^2 to 2.8×10^3 gram-negative bacteria/g. The range after the equipment changed was 3.2×10^3 to 1.8×10^5 gram-negative bacteria/g. The pastuerization equipment modification and/or plant sanitation procedures had an effect on the gram-negative bacterial population.

The dry diet demonstrated an even higher gram-negative bacterial count than that detected in OMP. Because the dry diet contains a total bacterial count

one log less than that in OMP, it is evident an increased gram-negative count in the dry diet would comprise a larger percentage of the bacterial population when compared to OMP.

MPN Fecal Coliform Counts

An MPN fecal coliform count was determined from five OMP samples taken before, and 11 OMP samples taken after the pasteurization equipment modification (Table 10). Of the five general bacterial counts tested, the fecal coliforms contributed the least to the OMP bacterial flora. However, like the gram-negative counts, the coliform levels increased after the change in pasteurization equipment. When both the before and after pasteurization equipment change averages of OMP were compared, a 76% increase in fecal coliforms was recorded. The dry diet had lower fecal coliform counts than both OMP samples taken before and after pasteurization equipment modification.

Isolation of Fish Pathogens From OMP

The isolation of eight fish pathogens from OMP, both before and after the pasteurization equipment modification, and a dry diet was tested (Table 2). Several pathogens, including A. salmonicida, Mycobacteria, R. salmoninarum, Streptococcus group B, and V. anguillarum, were not isolated. However, Pseudomonas

was isolated from all three types of diets tested. Pseudomonas was recorded to be <100 pseudomonads/g in OMP prior to the change in equipment, <100 to 400 pseudomonads/g in OMP after the equipment change, and <100 to 300 pseudomonads/g in the dry diet. One A. hydrophila was detected from 10 OMP samples tested after the pasteurization equipment modification. Yersinia ruckeri serotype II was isolated from two of six fish digest samples tested before the change in pasteurization equipment; however, it was not detected in the final pellet or dry diet.

Although R. salmoninarum was not isolated, an FAT was performed on seven OMP and two dry diet samples. The OMP samples were produced both before and after the pasteurization equipment changed. All of the samples contained bacteria that fluoresced when tested with the FITC conjugated anti-R. salmoninarum antisera. This made it impossible to have a negative control. However, after careful examination of the specimen slides, there were fluorescing bacteria morphologically identical to those on the positive control slides.

Isolation of Human Pathogens From OMP

Methodology which would detect 12 human pathogens was used to test the three types of diet. (Table 2). Several pathogens were not isolated and included Mycobacteria, Shigella, S. aureus, Streptococcus groups

A and B, and Y. enterocolitica. The isolation of Pseudomonas and A. hydrophila were previously described under the isolation of fish pathogens. A variety of other gram-negative bacteria were isolated from OMP when testing for human pathogens (Table 11).

Clostridium perfringens was isolated from eight OMP samples produced before, and 10 OMP samples produced after the change in pasteurization equipment (Table 12). The new pasteurization equipment appeared to have little effect on this spore-forming bacterium as the levels before and after were approximately the same. Clostridium perfringens was not isolated from the dry diet.

The total Staphylococcus and Streptococcus counts decreased after the pasteurization equipment modification (Table 11). This is in compliance with the previously recorded gram-positive count decreasing 89% after the change in pasteurization equipment (Table 10). The dry diet contained variable levels of Streptococcus and Staphylococcus; however, all numbers remained less than those detected in OMP.

Salmonella was isolated from the OMP fish digest and final pellet but was not detected in the dry diet (Table 11). Salmonella pullorum was isolated from two of six fish digest samples taken prior to the pasteurization equipment modification. Salmonella

Table 11. Gram-negative bacteria isolated from the Oregon Moist Pellet.

CDC enteric group 1[a]	*Enterobacter agglomerans*
CDC enteric group 2	*Enterobacter cloacae*
Cedecea	*Enterobacter sakazakii*
Citrobacter amalonaticus	*Escherichia coli*
Citrobacter diversus-levinea	*Hafnia alvei*
Citrobacter freundii	*Proteus mirabilis*
	Proteus vulgaris

[a]From Centers for Disease Control, Atlanta, GA.

Table 12. Comparison of estimated bacterial counts of a dry diet and Oregon Moist Pellet produced before and after pasteurization equipment modification.

Isolate[a]	No. bacteria x 10^2/g of diet (No. tested)		
	OMP		
	Before (8)	After (10)	Dry Diet (3)
Clostridium perfringens	<1 to 6	<1 to 4	NI
Total *Streptococcus*	<100 to 440,000	9,800	<1 to 400
Groups A and B	NI	NI	NI
Total *Staphylococcus*	18,000	4,000	<10 to 220
Staph. aureus	NI	NI	NI
	Positive samples/total samples tested		
Salmonella[b]	2/8[c]	2/10[d]	NI

NI - None Isolated

[a]No *Shigella* or *Yersinia enterocolitica* were isolated from any OMP or dry diet samples.

[b]*Salmonella pullorum* was isolated from 2 of 6 fish digest samples taken prior to the pasteurization equipment modification.

[c]*Salmonella havana*, *Salmonella meleagridis*

[d]*Salmonella cubana*

havana and *Salmonella meleagridis* were the isolates detected in two of eight pellet samples produced before the change in pasteurization equipment. Of the 10 OMP samples tested after the equipment change, two were positive for *S. cubana*.

V. DISCUSSION

Relatively little attention has been given to the bacterial flora in fish diets since the advent of pasteurization of fish carcasses and viscera included in the feed. During this time there have been no federal regulations on the limitation of bacterial levels in fish feeds. As a result, fish feeds have been detected with high levels of bacteria and containing both fish and human pathogens as reported by Trust in 1971. The Oregon Moist Pellet was not an exception to this report. The bacterial numbers in diet samples recorded by Trust actually averaged slightly lower levels than those detected in OMP.

Although the total bacterial counts in OMP appear elevated, one might be surprised at the comparison of bacterial levels of OMP, a frozen product, to a few frozen fish products for human consumption. Ingram, et al. (1974) reported 62% of 399 frozen fish cakes contained 10^5 to 10^6 bacteria/g and 7% contained $>10^6$ bacteria/g. It is understood that OMP is not for human consumption; however, it is interesting to note the bacterial levels of OMP made after the pasteurization equipment modification are similar to levels detected in some human foods.

The pasteurization specifications for the OMP fish digest are sufficient for the destruction of pathogenic

organisms. These specifications are set at even higher standards than what is used in the dairy industry due to slightly higher amounts of solid materials. The most comparable dairy product to the fish digest in relation to solid materials is ice cream mix. Both products are pasteurized by batch methods using low temperature long hold (LTLH) pasteurization techniques (Campbell and Marshall, 1975). The ice cream mix is held at 68.3°C for 30 min whereas the fish digest is held at 65°C for 15 min and 82°C for 5 min. Milk pasteurization is a high temperature short time (HTST) procedure being held at 76.7°C for 15 sec (OAMFES, 1985).

The pasteurization equipment modification had a direct effect upon the bacterial levels and flora in OMP and its fish digest. After the new equipment was installed, OMP bacterial numbers decreased 96%. When the total bacterial levels of OMP produced after the change in equipment were compared to levels in the dry diet, OMP was one log higher. Before the change in equipment, the bacterial numbers in OMP were over two logs higher than the dry diet. It would be expected the diet with the higher moisture content, being more favorable for bacterial growth, would have the higher bacterial load.

The change in pasteurization equipment had an even greater impact upon the fish digest which resulted in a

decrease of 99% in the bacterial numbers. Bacterial plate counts were made from two samples of ground, raw fish slurry prior to pasteurization and recorded to be an average of 7.7×10^5 CFU/g. When this value is compared to the average bacterial level in the fish digest after pasteurization and prior to the change in equipment, the counts increased dramatically. These high bacterial levels, 10^8 to 10^9 CFU/g, in the pasteurized fish digest could hardly represent a pasteurized product when the raw material has lower bacterial numbers. After the pasteurization equipment modification, bacterial levels in the fish digest reflected an efficient pasteurization process with counts as low as 2.0×10^2 CFU/g; however, there were a few fish digest samples that contained $>10^5$ CFU/g. In addition, the high bacterial counts in the pasteurized fish digest produced prior to the equipment change would be more likely to contain fish pathogens, as a result of such high bacterial numbers and inadequate pasteurization, than fish digest produced after the change in equipment. Therefore, because the fish digest comprises 38% of OMP, that OMP produced in recent years, as represented by OMP produced prior to the change in pasteurization equipment, would be more likely to affect fish health than OMP currently produced.

The new pasteurization equipment has proven the

ability to decrease bacterial levels in the fish digest; however, lengthy cooling times and bacterial recontamination resulting from insufficient cleaning procedures most likely lead to increased numbers of bacteria in both the fish digest and final pellet. The arbitrary cooling times of 4 to 12 h depends on the refrigeration load throughout the remainder of the plant. This long cooling step allows for growth of bacteria surviving pasteurization, bacteria in fish digest splatters not reaching pasteurization temperatures, injured cells, and heat-shocked spores into vegetative cells. It is suggested that one of the next improvements in the production of OMP should be to lower the fish digest cooling times in order to get the maximum benefit of the pasteurization process.

The second improvement suggested is adequate cleaning of equipment surfaces prior to the sanitation step. It appears there is little cleaning or line flushing before the sanitizing agent is applied. This is indicated by the increase in bacterial levels simply by traveling approximately 30 feet from the pasteurization tank to the mixer. A thorough cleaning of equipment is vital for the sanitizing agent's effectiveness.

With the installation of new pasteurization equipment, consistent sanitation measures were

initiated. Bioproducts is currently using Roccal, a quaternary ammonium compound, for its sanitizing step. Quaternary ammonium compounds, also known as quats, are excellent bactericides but poor detergents. Quats are also known to work very well against gram-positive bacteria but not gram-negatives (Troller, 1983). The combination of this and the lack of adequate cleaning procedures could explain the great decrease in OMP gram-positive bacterial levels and increase in gram-negatives. As the fish digest travels towards the mixer, the lines contain residual fish digest that has been treated with Roccal. This allows for the destruction of the gram-positive bacteria, which comprise the largest population in the OMP bacterial flora, resulting in a lack of competition for growth and a sudden increase in available nutrients for the remaining bacteria.

Therefore, to further reduce bacterial levels in OMP, there should be improved cleaning techniques, including an alkaline detergent to remove organic material, followed by an iodophor rather than a quaternary ammonium compound for the sanitation measures. If iodine residual is of concern, although it is commonly used in the dairy industry, the equipment surfaces may be rinsed with water before production. Rinsing after iodophor application is not a common

practice for refrigerated products; however, OMP is frozen which rescinds this standard.

The bacterial load in the meal mix was much higher than expected. Because the meal mix comprises 60% of OMP, it is not surprising to find it dictates the bacterial levels of the final product. If the one fecal coliform test result of 350 MPN fecal coliforms/g is any indication of the gram-negatives present within the meal mix, this could be another explanation for the increase in gram-negative levels in OMP after the change in pasteurization equipment. Because of a reduced bacterial load in the fish digest after the change in equipment, the meal mix bacterial flora would be the predominant species in the final product.

Both OMP and the dry diet contain various grains which contain gram-negative bacteria commonly associated with plants and soil. This could be misleading when evaluating the total gram-negative counts; therefore, it was important to differentiate the true fecal coliform levels from the total gram-negative counts. An excellent example of misleading, elevated gram-negative levels was provided by the dry diet. Of the three diets tested, the dry diet had the highest gram-negative count yet the lowest MPN fecal coliform count. This is most likely a result of the higher grain and lower moisture content in the dry diet than in OMP.

Several critiques of methods and media were made during the development and testing of new procedures for the isolation and identification of fish and human pathogens from OMP and its ingredients. Unless mentioned otherwise, isolation and identification procedures used in this study were satisfactory. The first medium that was not appropriate for use with OMP was peptone beef extract glycogen agar (PBEG) described by McCoy and Pilcher (1974). An intense effort was made to use this pour plate medium for the isolation of A. hydrophila and A. salmonicida for which the medium was developed. Positive control plates worked well; however, the high bacterial numbers in OMP at the low dilutions in which aeromonads would be present overgrew any yellow aeromonas colonies that might be present.

An additional procedure to the enrichment methods previously described for the detection of Y. ruckeri was used. This selective treatment prior to plating was developed by Doyle and Hugdahl (1982) for the enhanced recovery of Y. enterocolitica from meats. The procedure worked well for the control plates; however, as a result of so few Y. ruckeri isolations, it is difficult to determine if the procedure was not appropriate for OMP or if there were simply no isolates to be detected. Another important note concerning Y. ruckeri is that it will not grow on YA.

The SA medium (Palumbo, et al., 1985) used in the detection of Aeromonas and Vibrio was of great success. This medium is highly recommended for future isolation of both genera. The use of two broths, GN and APW, for the enrichment of these genera could be reduced to one. The GN produced more starch hydrolysis positive colonies than the APW broth suggested by the Compendium of Methods (Speck, 1984).

The ethanol treatment for the detection of C. perfringens (Craven and Blankenship, 1985) produced fewer positive isolates than the nontreated plates of the Compendium methodology (Speck, 1984). It is suggested the latter method be used for future studies of this bacterium. An important note on reading the EYF TSC plates used in C. perfringens isolation is that the plates should be read no later than 20 h. This prevents overgrowth of spreading, black colonies.

The isolation of R. salmoninarum by direct plating and streaking for isolation was not successful. The spread plate method could not be used, as noted by Evelyn (1977), due to the presence of fast growing contaminants in OMP. It was later discovered a pH adjustment needed to be made after the addition of the filter sterilized solutions in the SKDM; otherwise, the pH would be too low if prepared by the methods listed by Austin, et al. (1982). Another medium, KDM-2 (Evelyn,

1977), was used in addition to the SKDM; however, no R. salmoninarum isolates were detected.

The FAT worked well for the detection of FITC epifluorescent bacteria in OMP and the dry diet when anti-R. salmoninarum antisera was used. If this procedure is to be used for quanitative measures, it is suggested that <3 ml be used to filter and/or a greater dilution than 1:3 be used in order to prevent the filter from breaking. It is important to note the FAT has been criticized in the identification of R. salmoninarum for cross-reacting with other gram-positive bacteria (Austin, et al., 1985). In addition, it does not detect viable cells. Therefore, it is a possibility the fluorescence detected in both OMP and the dry diet samples could be gram-positive bacteria other than R. salmoninarum. It is proposed that the detection of R. salmoninarum from OMP and the dry diet cannot be dismissed based upon the fluorescing bacteria morphologically identical to those on the positive control slide. For future studies, counterimmunoelectrophoresis is suggested in addition to, or in place of, the FAT (Cipriano, 1985).

The procedures used for the determination of Salmonella from OMP and the dry diet were successful. In addition to the media used, a modified xylose lysine brilliant green (XLBG) medium was tested, but later

discontinued (Hussong, et al., 1984). The modified XLBG did not produce as many H_2S-positive salmonellae as detected on SS and XLD. The BS plates were difficult to read as Salmonella was not easily distinguishable from other members of the Enterobacteriaceae. It is important to note the key reactions of the TSI and LI tubes for the isolation of Salmonella. This prevented the unnecessary use of API20E strips for bacteria other than Salmonella.

The levels of Salmonella detected in OMP did not comprise 30% of the samples tested. Therefore, Bioproducts would not be in violation of federal regulations (Anon., 1980b). This may only be stated for this particular plant. Because OMP is an open formula, other plants producing this diet may have bacterial levels very different from what was detected in this study. This was found to be true after observing unpublished data on another OMP production plant (Bell, personal communication).

The isolation of fish pathogens from OMP was difficult resulting from the very high levels of contaminating bacteria. This was compounded by the lack of selective procedures and media specifically for fish pathogens. However, the presence of fish pathogens was detected which could be the basis of future in vivo studies.

Consideration should be given to the relatively high levels of fecal coliforms and to the fact that several human pathogens, including Salmonella, were isolated from OMP. It is suggested the Oregon Moist Pellet does not pose an immediate threat to the health of hatchery personnel or downstream water users; however, the potential is everpresent. As a result of not having federal regulations on the limitation of bacterial levels in fish diets, the high bacterial numbers and pathogenic bacteria will be restrained only by meeting pasteurization standards and proper cleaning and sanitizing of the production plant.

VI. SUMMARY AND CONCLUSIONS

1. The total aerobic bacterial count of OMP was determined to be 4.8×10^7 CFU/g before, and 1.9×10^6 CFU/g after the pasteurization equipment modification. The change in equipment resulted in a 96% decrease in the bacterial count.

2. The total aerobic bacterial count of the fish digest in OMP was determined to be 5.5×10^8 CFU/g before, and $<1.0 \times 10^4$ to 4.3×10^6 CFU/g after the pasteurization equipment modification. The change in equipment resulted in a 99% decrease in the bacterial count.

3. The total aerobic bacterial count of the meal mix in OMP was 7.1×10^5 CFU/g. Bacterial levels in the OMP meal mix generally determined the total bacterial count in the final pellet.

4. There was an average decrease of 19% in bacterial numbers in OMP from the day of manufacture to the end of the 90 day shelf life.

5. Gram-positive bacteria were the predominant organisms in OMP and the dry diet.

6. There was an 89% decrease in the gram-positive bacterial numbers after the change in pasteurization

equipment. Conversely, there was a 96% increase in the gram-negative numbers after the change in pasteurization equipment.

7. A variety of isolation and identification procedures for detecting fish and human pathogens from OMP and a dry diet were tested.

8. The OMP had a greater total bacterial count, gram-negative count, and MPN fecal coliform count per gram than the dry diet. However, the dry diet contained a higher mesophilic spore count and gram-negative count per gram than OMP.

9. Of the 12 human pathogens examined in OMP, A. hydrophila, Pseudomonas, C. perfringens, Salmonella, Staphylococcus, and Streptococcus were isolated. These procedures allowed the detection of 13 different members of the Enterobacteriaceae.

10. Of the eight fish pathogens examined in OMP, A. hydrophila and Pseudomonas were isolated. Yersinia ruckeri serotype II was isolated from the OMP fish digest; however, it was not detected in the final pellet.

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