# MICROFLORA OF FRESH AND FROZEN DEBONED FISH PATTIES MADE FROM WHITE SUCKER

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ABSTRACT

MICROFLORA OF FRESH AND FROZEN DEBONED FISH PATTIES MADE FROM WHITE SUCKER

By

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This study was carried out to observe the effects of frozen storage on the microbial population of deboned fish patties made from white sucker (Catostomus commersoni) from Lake Huron.

The fish patties were stored at -18°C for one to six months, and analyzed for aerobic plate counts, percentages of Bacillus, pigmented microorganisms and molds. Bacteria were isolated and identified from the patties after thawing at room temperature for two hours, and again after refrigeration at 4°C for five days.

Freezing at -18°C for six months caused 70-84 percent destruction of microorganisms present. The greatest amount of destruction occurred during freezing and during the first three months of storage. During frozen storage, the percentage of Bacillus increased, while the percentage of pigmented microorganisms decreased. After refrigeration of thawed samples at 4°C for five days, aerobic plate counts increased about one hundred-fold, and

the percentages of Bacillus and pigmented microorganisms decreased.

Fresh deboned fish and fish patties contained primarily pigmented gram-negative, oxidase-positive rods including: groups I and II Pseudomonas, Aeromonas, Vibrio, Flavobacterium, Branhamella, Acinetobacter, Neisseria, Bacillus, Pediococcus, Pediococcus-like, Neisseria-like, Photobacterium-like, Moraxella-like, and unidentified organisms. Aeromonas, Acinetobacter, Neisseria, Pediococcus, Photobacterium-like, Vibrio-like and unidentified organisms were not detected after freezing and thawing. Vibrio, Flavobacterium, Bacillus and Moraxella-like organisms were isolated from the thawed and refrigerated patties.

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# INTRODUCTION

The storage of fish by freezing is an important method of fish preservation because if properly done, freezing can preserve fish without significant changes in size, shape, texture, color, and flavor. Although freezing causes a destruction in the number of microorganisms present, studies on the microbial content of frozen fish usually show that fish contain a certain number of microorganisms. Many factors influence the number of microorganisms in the frozen fish such as cooling and freezing conditions, time and temperature during frozen storage, and the original number of microorganisms in the fresh fish which is influenced by the species and physiology of the fish (Wood, 1953; Shewan, 1961; Nickerson and Sinskey, 1972).

Currently a large amount of fish is prebreaded or precooked such as in the preparation of fish patties, fish fillets, and other fish products. These fish products are then packaged and stored in freezers. However, investigation concerning the microbial population of these fish products is limited. This study was carried out to observe the effects of frozen storage and thawing on the microbial

population of deboned fish patties prepared from white sucker (Catostomus commersoni) from Lake Huron, and to identify the bacteria isolated from fresh and frozen fish patties.

In recent years several methods have been developed for the identification of bacteria isolated from fish. The generic identification methods of Shewan et al. (1960a) and Shewan (1971) for gram-negative bacteria have been used widely for identifying bacteria commonly found in marine and fresh-water fish. However, in this investigation some modifications of these identification schemes were considered necessary in order to comply with current bacterial nomenclature as presented in the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

# LITERATURE REVIEW

## Microbiology of Fresh Fish

Although the flesh and body fluids of fresh, healthy fish are generally sterile in nature, the skin, slime, gill, and the intestine usually contain a great number of microorganisms, particularly bacteria. Many of these bacteria are responsible for the spoilage of fish. The numbers of bacteria in fish reported by some investigators ranged from $10^2$ to $10^6$ per $cm^2$ on the skin, from $10^3$ to $10^5$ per gram on the gill tissue, and from very few to $10^7$ or more per gram in the intestine (Georgala, 1958; Liston *et al.*, 1963; Hawker and Linton, 1971). Both aerobic and anaerobic bacteria were found in the intestine of fish.

Many factors influence the microbial content of fresh fish, such as species of fish, water environment, season, method of catching, and method used to determine microbial counts including sampling technique, media, and incubation time and temperature. It is believed that the fish species affects the microbial population, particularly due to the differences in slime content on the skin between one fish species and another. The microflora in the

intestine of fish is greatly influenced by the kind of food ingested and the nature of the water from which the fish are caught.

The microflora of fish is primarily composed of gram-negative bacteria, and some gram-positive bacteria. Kazanas (1966) isolated aerobic bacteria from fresh-water fish (yellow perch fillets). They consisted of gram-negative bacteria such as Achromobacter-Alcaligenes, Pseudomonas, Flavobacterium, Aeromonas, Vibrio, coliforms, and gram-positive bacteria including Brevibacterium, Micrococcus, Bacillus, Corynebacterium, Sarcina, Micro-bacterium, Lactobacillus, and Mycobacterium. About 60 percent of the microbial counts consisted of gram-negative bacteria, particularly Achromobacter-Alcaligenes, Pseudomonas and Flavobacterium. Trust and Sparrow (1974) examined the bacterial flora in the alimentary tract of fresh-water salmonid fishes and found mostly gram-negative rods with Enterobacter, Aeromonas and Acinetobacter present in the largest numbers.

As stated earlier, the water environment influences the microflora of fish. A higher percentage of mesophiles such as Bacillus, coryneforms and micrococci, and fewer psychrophiles such as Pseudomonas, Alcaligenes and Flavobacterium, should be present in fish caught from warmer waters than in fish caught from colder waters (Shewan, 1961). Even though a fish is caught from

salt-water, it is believed that fresh-water bacteria become predominant during storage since fish are usually held in fresh-water ice during fishing and transporting.

The microflora of fish is also influenced by the season. In both skate and sole, Liston (1957) found that bacteria which liquified agar were mostly present at the beginning and end of the year. Another investigator showed that Achromobacter and luminous bacteria were found more frequently in the winter, whereas the number of Pseudomonas usually increased in the summer (Georgala, 1958).

The temperature of incubation also has a significant effect on the microflora of fish. Georgala (1958) reported that Corynebacterium, Flavobacterium, Micrococcus and Vibrio were found in greater numbers in cod after incubation at 20°C than at 0°C. Reed and Spence (1929) found a high percentage of Bacillus in haddock, which probably was due to the high incubation temperatures used (15, 25 and 37°C).

Fresh fish generally are not contaminated with foodborne pathogens such as salmonellae and staphylococci unless the fish are caught in polluted water. However, the fish can be contaminated during handling and processing, and may contain mesophilic spoilage and indicator organisms such as Escherichia coli, fecal streptococci, staphylococci, and salmonellae. Cook et al. (1974) found Salmonella enteritidis in fish caught from water polluted

by poultry processing wastes. Other investigators found nonproteolytic Clostridium botulinum types B, E and F (Craig et al., 1968; ICMSF, 1974) and Vibrio parahaemolyticus (Barros and Liston, 1970; Johnson et al., 1971) in fish. However, both these organisms are usually found in low numbers and rarely cause illness.

## Microbiology of Fish Spoilage

Spoilage of fish may be caused by autolysis by fish enzymes, oxidation, or microbial action. It is known that fish flesh is more perishable than meat, particularly due to the rapid autolysis by fish enzymes. Many factors influence the rate of spoilage of fish such as the species of fish, microbial content of fresh fish, condition of fish when caught, temperature during handling and storage, and the use of preservatives (Frazier, 1967).

Bacteria are the major cause of fish spoilage. As stated earlier, bacteria are present naturally on the skin, slime, gill and intestine. After the fish dies, the fish tissues cannot prevent bacterial invasion, and bacteria can penetrate the tissues, causing spoilage of the fish. Some investigators have agreed that the main routes of invasion are from the gill and kidney into the flesh through the vascular system, or directly through the skin and peritoneal lines (Shewan, 1961; Burgess et al., 1967). However, it is not clear how fast spoilage begins. It is generally believed that spoilage of fish occurs after rigor mortis.

Therefore, the longer rigor mortis is delayed, the longer will be the shelflife of the fish. Rigor mortis can be prolonged by holding the fish at low temperatures such as in ice or at cooling temperatures. Dyer et al. (1946) reported that spoilage of fish occurred mainly on the skin, and most of the flesh remained sterile for about ten days. Another investigator found an increasing number of bacteria in the flesh, particularly along the lateral lines, on the second day of postmortem (Shewan, 1961).

Bacteria responsible for the spoilage of fish are those which exhibit proteolytic activity, and usually are those which can grow at low temperatures since fish are usually held in ice or at cool temperatures. Investigations concerning the spoilage of fish and fish products generally showed that the majority of bacteria in spoiling fish are gram-negative rods. The bacteria causing spoilage of fish consisted primarily of species of the genera Pseudomonas and Achromobacter-Alcaligenes (Shewan et al., 1960b; Shewan, 1961), and usually 20 to 50 percent of the Pseudomonas species were P. fragi (Nickerson and Sinskey, 1972). Chai et al. (1968) found that P. putrefaciens and fluorescent pseudomonads were the major species of spoilage bacteria in haddock fillets.

Some analyses can be used to determine the degree of spoilage in fish such as organoleptic and chemical analyses. Lerke et al. (1965) used production of off-odor,

volatile reducing substances (VRS), and trimethylamine (TMA) to compare the quantitative and qualitative effects of bacterial flora on the fish spoilage. They discovered that no micrococci, flavobacteria, or coryneforms caused spoilage in fish. Shaw and Shewan (1968) reported that certain subgroups of the genus Pseudomonas, particularly groups III and IV, caused spoilage in fish, whereas some members of groups I and II were inactive. Likewise, the genus Achromobacter consisted of spoilers and nonspoilers. However, members of the latter genus appeared to play a minor role in the production of off-odor. Members of group I Pseudomonas are not known to cause spoilage of fish, but recently it was reported that they have contributed to the organoleptic changes in fish (Nickerson and Sinskey, 1972). According to Lerke et al. (1965) coliforms can also cause spoilage.

Kazanas (1968) investigated the proleolytic activity of bacterial cultures isolated from fresh and spoiling nonirradiated and irradiated fresh-water fish (yellow perch fillets) during storage at 1 and 5°C. Species of Achromobacter-Alcaligenes, Aeromonas, Microbacterium and Lactobacillus did not have proteolytic activity. About 55 percent of the total proteolytic bacteria consisted of pigmented cultures, including Sarcina and Micrococcus spp. which were actively proteolytic. All Flavobacterium

spp., but only a few pigmented Brevibacterium cultures had proteolytic activity.

## Effect of Freezing on the Microorganisms of Fish

### Death and Injury of Microorganisms During Freezing

Generally, there are five operational steps in the preservation of foods by freezing. These are prefreezing or cooling, progressive freezing, frozen storage, defrosting, and postthawing treatments. All of these steps have significant effects on the microbial population in frozen foods. The important variables in freezing preservation are the freezing rate and the temperature of frozen storage. Freezing rates may be defined as follows: slow freezing (less than 1°C/50 minutes), rapid freezing (1°C/50 minutes to 100°C/minute), and ultrarapid freezing (5°C/second to 100°C/second) (Nickerson and Sinskey, 1972). In commercial practice the temperatures used for freezing food products generally vary from -15 to -40°C.

According to Kiser (1944) and Ingraham (1958), during freezing of food products which contain a number of microorganisms, an extension of the logarithmic phase of microbial growth occurs initially. When the minimum temperature for microbial growth is reached, growth ceases. However, yeasts and molds are not as sensitive as bacteria, and, according to Shewan (1961), they may grow in partially

frozen media below -7.5°C. Generally, growth of most microorganisms is inhibited at temperatures below -10°C, although McCormack (1956) found bacterial growth in frozen fish during storage at -11°C after 16 months, and growth of a pink yeast in frozen oysters at -19°C. Below the minimum temperature for growth, some of the microorganisms usually are killed. During freezing there is a gradual destruction of microorganisms present, the rate depending upon the type of the microorganism.

Many theories explain the death and injury of microorganisms at low temperatures (freezing). Some of them are: (1) increase in the concentration of intracellular solutes (Smith, 1961; Brock, 1974), (2) leakage of low molecular weight materials from the cells (Sakagami, 1959; MacLeod and Calcott, 1976), and (3) formation of ice crystals, particularly intracellular ice crystals (Mazur, 1961b). Increase in the concentration of intracellular solutes is due to the separation of water from the cells during the progressive freezing of the surrounding medium since the microbial cytoplasm does not freeze as fast as the water solution surrounding the cells (Smith, 1961; Brock, 1974). Mazur (1970) believed that supercooled water in the cells had a higher vapor pressure than ice in the surrounding medium, therefore cells began to lose water to obtain an equilibrium with the medium. According to Nei (1960), about 80 percent of water was separated from

bacterial cells during commercial freezing. Mazur (1961a) discovered that the concentration of solids in yeast cells (Saccharomyces cerevisiae) increased from 20 to 28 percent during freezing, whereas the total mass of solids decreased. Shewan (1961) suggested that desiccation was apparently responsible for the damage of bacterial cells during freezing.

The increase in the concentration of intracellular solutes during freezing may result in physical and chemical changes in bacterial cells such as changes in pH, vapor pressure, freezing point, surface and interfacial tension, and oxidation-reduction potential (Nickerson and Sinskey, 1972). At high salt concentration in the cells, denaturation of polymers such as RNA, DNA, polypeptides and proteins may occur. According to Heen and Karsti (1965) the temperatures between 0 and -10°C have more lethal effects than lower temperatures. However, Mazur (1961b) reported that yeasts cells were destroyed primarily at temperatures between -10 and -30°C. No matter what temperature is used, some microorganisms survive for long periods of frozen storage. In general, large cells are more sensitive to freezing than are small cells (Brock, 1974).

Water which separates from the cells can bring low molecular weight materials out of the microbial cells. In yeast cells, Sakagami (1959) discovered that the lost materials included potassium ions ($K^+$), inorganic

phosphate, sugars, fatty acids, amino acids and esters, and according to Mazur (1961a), the lost materials had molecular weights below 600.

One theory stated that injury of microbial cells during freezing was due to the formation of ice crystals, particularly because the ice crystals caused physical damage to the cells (Mazur, 1961b). Damage by intracellular ice formation was influenced partly by the rate of water separation from the cells to form an equilibrium with the surrounding (frozen) medium, and by the degree of water crystallization inside the cells. Ultra-rapid freezing caused the formation of tiny ice crystals inside the microbial cells, so that the size of the cells was not changed. On the other hand, slow freezing causes the microbial cells to shrink because most of the water separated out, resulting in damage of the cells (Nei, 1960; Mazur, 1970). The lower the temperature of freezing or the more rapid the freezing, the higher the number of survivors. According to this theory, preservation by slow freezing appears to be better than rapid freezing because more microbial cells are destroyed. However, slow freezing is not recommended for preservation of food products since the longer time needed to achieve the freezing temperature in slow freezing gives the bacteria more time to grow and multiply. This may result in changes in the qualities of the frozen foods.

Kiser and Beckwith (1942) reported that freezing caused 60 to 90 percent destruction of the bacterial population present. Destruction generally occurs exponentially for the initial period of freezing, followed by more gradual decreases in the number of bacteria. However, sterility is never achieved even after prolonged storage. The viable bacteria remaining can then grow and multiply again after thawing.

Factors affecting the apparent survival of microorganisms subjected to freezing are the type and strain of microorganism, microbial population of the fresh products, nutritional condition of the microorganism, growth phase, composition of the freezing substrate, length of precooling, freezing rate, time and temperature of frozen storage, and methods used to determine the survival counts, such as sampling technique, media, and time and temperature of incubation (MacLeod and Calcott, 1976). The heavier the microbial population of the fresh products, the greater the number of survivors after freezing. Composition of the freezing substrate is important due to the suitability and availability of the substrate to support microbial growth.

## Changes in the Microflora During Freezing

Generally, the gram-negative bacteria, particularly Pseudomonas spp., are sensitive to cold storage, whereas

gram-positive bacteria such as micrococci and lactobacilli (Lund and Halvorson, 1951) and streptococci (Shewan, 1961) are more resistant. Freezing usually has no substantial effect on bacterial spores, and vegetative cells of yeasts and molds are not as sensitive to freezing as those of bacteria. Some of the cold-sensitive bacteria may be killed during freezing. Microorganisms which can survive the initial freezing usually remain viable for long periods of time during frozen storage (Brock, 1974).

Shewan (1961) reported that mesophiles generally fail to grow at about 5°C, and at lower temperatures, growth of some psychrophiles may be inhibited. According to Hartsell (1951), mesophilic bacteria required a very good substrate to grow at temperatures below 0°C. Bedford (1933) discovered that of the bacterial cultures isolated from marine fishes, only a few serratia, flavobacteria and achromobacteria did not grow at -5°C. At -7.5°C, only a few micrococci and achromobacteria continued to grow, whereas other bacteria were eliminated completely.

Stewart (1935) reported that in fish (haddock) stored at -12°C for three months, Pseudomonas was not detected, Achromobacter decreased and the percentage of Flavobacterium increased four-fold. Another study (Laycock and Regier, 1970) indicated that in iced haddock, Achromobacter was the predominant bacterium after two days, and some Pseudomonas and Flavobacterium were also present.

After five days of storage, the percentage of Achromobacter increased, Pseudomonas remained constant, whereas the percentage of Flavobacterium decreased substantially. After nine days, at which time the fish were spoiled, the percentages of groups I and II Pseudomonas and P. putrefaciens increased, whereas Achromobacter decreased.

Although microbial spoilage of fish can be inhibited by freezing, undesirable physical and chemical changes in the fish may occur. Important factors affecting the undesirable changes in frozen fish are the time and temperature of frozen storage. According to Awad et al. (1969) fish should be stored at -18°C or lower to decrease the rate of undesirable changes which can result in loss of the quality of fish. The most sensitive components to frozen damage in fish flesh are proteins and lipids. Denaturation of protein during frozen storage may cause a decrease in water binding capacity or loss of juice in thawed fish flesh, whereas deterioration of lipids, which is due to lipolytic activity or oxidation, produces rancid flavors in the fish. To increase the water binding capacity of the frozen fish, alkaline phosphates, such as disodium phosphate, hexametaphosphate, sodium tripolyphosphate and sodium pyrophosphate, are now used in mixtures with antioxidants, which inhibit the oxidative rancidity of the fish lipids.

Microbiology of Thawed Fish

It is believed that most of the microbial deterioration of frozen foods occurs during precooling and after thawing. This is particularly due to the psychrophilic bacteria which have optimum temperatures for growth between -6.7 and 3.3°C (20 and 38°F). However, food-borne pathogens, which have higher optimum temperatures, cannot grow at these temperatures (Nickerson and Sinskey, 1972).

Although freezing and frozen storage cause a substantial decrease in the number of microorganisms, the spoilage of frozen and thawed fish occurs more quickly than spoilage of unfrozen fish (Frazier, 1967). According to Chistyakov and Noskova (1955), there is microbial adaptation to low temperatures during freezing, so that the generation time of thawed microorganisms can be shortened. However, Luijpen (1958) discovered that thawed fish which had been frozen at -30°C, spoiled at +2°C more slowly than unfrozen fish.

Besides the method of thawing, all factors affecting the microbial population of frozen foods also influence the microbial population of thawed products. The longer times of thawing give the microorganisms more time to grow and multiply. Due to the survival of some microorganisms in frozen foods, bacterial standards have been recommended for various types of frozen foods. The primary purpose of the bacterial standards is to check and improve the

sanitary quality of the foods. For frozen fish the suggested bacterial standard is a maximum viable count of 100,000 aerobic cells per gram (Frazier, 1967).

## Identification of Bacteria From Fish

### Gram-Negative Bacteria

The methods of bacterial identification described by Shewan et al. (1960a) and Shewan (1971) have been used widely for the generic identification of gram-negative bacteria commonly found in fish. Although these methods had been developed primarily for the bacteria isolated from marine fish, some investigators (Kazanas, 1966, 1968; Cox and Lovell, 1973; Trust and Sparrow, 1974) have used these methods for identification of gram-negative bacteria isolated from fresh-water fish. The bacteria were identified based on gram stain, morphology, pigmentation, presence or absence of oxidase, motility, flagellation, type of carbohydrate metabolism, and sensitivity to penicillin and 2,4-diamino-6,7-diisopropyl pteridine (0/129).

Employing these methods, most of the bacteria isolated from marine fish were psychrophiles. The incubation temperature used for the identification tests was usually 20°C (Shewan et al., 1960a) to 22°C (Pelroy and Eklund, 1966), at which psychrophilic bacteria and some mesophiles can grow. Certain gram-negative bacteria, such as members of the genera Acetobacter, Azotobacter and

Cellulomonas, were excluded from the identification scheme because they normally were not isolated from fish. The only members of the Enterobacteriaceae normally found in fish were paracolons and Escherichia coli. Other enteric bacteria such as Shigella were not found.

According to Shewan et al. (1960a), there were four broad groupings of gram-negative rods based on motility, flagellation, oxidase test reaction, and pigmentation. These were: (1) the polar-flagellate, oxidase-positive group, (2) the peritrichous-flagellate, oxidase-negative group, (3) the nonmotile, nonpigmented group, and (4) the nonmotile, pigmented group. The polar-flagellate, oxidase-positive group included members of the genera Pseudomonas, Aeromonas, Vibrio, Spirillum and Xanthomonas. Paracolons, Escherichia coli and other motile enteric bacteria were in the peritrichous-flagellate, oxidase-negative group. Members of the genera Achromobacter and Alcaligenes were included in the nonmotile, nonpigmented group, whereas other nonmotile bacteria such as Flavobacterium and Cytophaga were in the nonmotile, pigmented group. However, according to the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), some modification of these groupings should be made since members of the genus Alcaligenes and some members of Flavobacterium are now known to be motile with peritrichous

flagella, while members of the genus Cytophaga are considered to be motile via a gliding mechanism.

Members of the genus Achromobacter and closely related bacteria are sometimes called the Achromobacter-Alcaligenes group. Due to the close similarity between these two genera, Hendrie et al. (1974) suggested that the generic name Achromobacter be rejected. They believed that members of the genus Achromobacter were indefinable species of Alcaligenes.

Shewan et al. (1960a) used Hugh and Leifson's medium (Hugh and Leifson, 1953) to further differentiate members of the polar-flagellate, oxidase-positive group. Depending upon the type of carbohydrate metabolism in Hugh and Leifson's medium, the polar-flagellate, oxidase-positive group can be differentiated into: (1) fermentative bacteria including Aeromonas and Vibrio, (2) oxidative bacteria such as groups I and II Pseudomonas, (3) bacteria which produce an alkaline reaction such as group III Pseudomonas, and (4) those which produce no reaction including group IV Pseudomonas and Spirillum. Group I Pseudomonas includes P. fluorescens and P. aeruginosa, which are oxidative and produce fluorescent pigments. Group II Pseudomonas, such as P. fragi, are also oxidative, but do not produce fluorescent pigments. Pseudomonas aureofaciens is in the group III Pseudomonas, while P. rubescens is in group IV (Shewan et al., 1960a). In Hugh

and Leifson's medium, some strains of Alcaligenes produce no reaction or an alkaline reaction, and some are fermentative. Alcaligenes is different from Pseudomonas primarily in motility, morphology, and sensitivity to penicillin.

Table 1 shows typical reactions used to differentiate the genus Pseudomonas from the related genera, Aeromonas, Vibrio and Photobacterium (Shewan, 1971; Buchanan and Gibbons, 1974). Members of the genera Aeromonas and Photobacterium are characterized by fermentation of glucose with the production of acid and gas in Hugh and Leifson's medium, whereas Vibrio spp. ferment glucose and produce acid, but do not produce gas. Aeromonas spp. are not sensitive to 2,4-diamino-6,7-diisopropyl pteridine (0/129), but Pseudomonas, Vibrio and Photobacterium are sensitive. Genera of the polar-flagellate, oxidase-positive group are not sensitive to penicillin (2.5 units), except Spirillum. Members of the genus Spirillum, which have no action upon glucose in the Hugh and Leifson's medium, are not sensitive to the pteridine compound (Shewan et al., 1960a; Shewan, 1971).

The genera Photobacterium, Lucibacterium and some strains of the genus Vibrio are known as luminous bacteria since these bacteria produce luminescence (Hendrie et al., 1970). All luminous bacteria are gram-negative rods which are insensitive to penicillin, and most of them are motile

Table 1.--Differentiation of the genus Pseudomonas from related genera.*

| Tests | Pseudomonas | Aeromonas | Vibrio | Photobacterium |
|---|---|---|---|---|
| Flagellation | Polar | Polar | Polar | Polar (or none) |
| Oxidase | + (occasionally -) | + | + | + or - |
| 0/129 sensitivity | + | - | + | + |
| Carbohydrate metabolism | Resp. or no reaction | Ferm. | Ferm. | Ferm. |
| Gas production | - | d | - | + |
| Diffusible pigments | Green fluorescent | - or brown | - | - |
| Luminescence | d | - | d | + |

*Shewan (1971); Buchanan and Gibbons (1974).

+ = > 90% strains positive; - = > 90% strains negative; d = > 90% strains positive or negative.

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with polar or peritrichous flagella. Growth and luminescence usually occur on media containing 0.5 to 5 percent socium chloride (NaCl). Members of the genus Lucibacterium are motile with peritrichous flagella, insensitive to pteridine (0/129), oxidase positive, and fermentative with the production of gas.

The members of the genera Moraxella, Neisseria, Branhamella and Acinetobacter have many similarities. They are gram-negative bacteria which consist of plump cells of coccal or coccobacillary shape, arranged in pairs (Henriksen, 1976). Acinetobacter is different from Moraxella, Neisseria and Branhamella, particularly in growth requirement, physiological and biochemical characteristics, and sensitivity to penicillin. Acinetobacter does not produce oxidase and has moderate or low sensitivity to penicillin, while the other three genera produce oxidase and are highly sensitive to penicillin. Branhamella and Moraxella (except M. kingae) do not attack sugars, Neisseria does not attack sugars or attacks a few mono- and disaccharides, while Acinetobacter does not attack sugars or only attacks aldoses oxidatively. Other characteristics of the four genera are presented in Table 2.

All of the members of the genus Flavobacterium and many of the Cytophaga are characterized by the formation of yellow to orange colonies on agar. According to Shewan et al. (1960a) pigmentation is more readily detected on agar

Table 2.--Characteristics of the genera *Branhamella*, *Moraxella*, *Neisseria* and *Acinetobacter*.*

| Characteristics | *Branhamella* | *Moraxella* | *Neisseria* | *Acinetobacter* |
|---|---|---|---|---|
| Cell shape | Cocci | Short rods or cocci | Cocci | Cocci or short to medium rods |
| Arrangement | Pairs | Pairs | Pairs | Pairs |
| Division | 2 planes | 2 planes | 1 plane | 1 plane |
| Oxidase | + | + | + | - |
| Penicillin sensitivity | High | High (moderate in *M. osloensis*) | High | Moderate or low |
| Sugar metabolism | None | None (except) *M. kingae*) | Few mono- and disaccharides or none | Oxidation of aldoses or none |
| Reduction of nitrate | - or d | + | d | - |

*Buchanan and Gibbons (1974); Henriksen (1976).

+ = > 90% strains positive; - = > 90% strains negative; d = > 90% strains positive or negative.

containing 30 percent skim milk. Although Alcaligenes, Flavobacterium and Cytophaga were considered as being non-motile by Shewan et al. (1960a), the genera Alcaligenes and Flavobacterium now contain strains which are motile by peritrichous flagella, whereas Cytophaga is motile by gliding (Buchanan and Gibbons, 1974).

Gram-Positive Bacteria

Although there is no specific scheme to identify the gram-positive bacteria isolated from fish, Kazanas (1966) differentiated gram-positive bacteria isolated from fresh-water fish (yellow perch fillets) into: (1) those which did not produce catalase such as Lactobacillus (rods) and Pediococcus (cocci), and (2) those which produced catalase. Most of the catalase-positive found in fresh-water fish were Micrococcus (cocci), Sarcina (cocci in tetrads or clumps), Microbacterium (tiny rods or cocco-bacilli, pigmented or nonpigmented), Bacillus (endospore-forming rods), Mycobacterium (nonmotile, pigmented rods), and Corynebacterium (coryneforms).

According to the eighth edition of Bergey's Manual (Buchanan and Gibbons, 1974), the Family Micrococcaceae consisted of three genera, Micrococcus, Staphylococcus and Planococcus. Aerococcus, which is in the Family Streptococcaceae, is considered as an intermediate genus between the Family Micrococcaceae and the Family Streptococcaceae, but it is more closely related to

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Streptococcus than to Micrococcus, Staphylococcus or Planococcus. Table 3 is presented to differentiate the genus Aerococcus from the Family Micrococcaceae (Buchanan and Gibbons, 1974).

For a routine and rapid test to differentiate the genus Staphylococcus from Micrococcus, Davis and Hoyling (1974) used Hugh and Leifson's medium (Hugh and Leifson, 1953) to observe the metabolism of glucose under anaerobic conditions. Staphylococci ferment glucose (produce acid anaerobically from glucose), while micrococci do not.

Table 3.--Differentiation of the genus Aerococcus (Streptococcaceae) from genera of the Family Micrococcaceae.*

| Characteristics | Aero-coccus | Micro-coccus | Staphy-lococcus | Plano-coccus |
|---|---|---|---|---|
| Spherical cells | + | + | + | + |
| Arrangement: | | | | |
| Irregular clusters | - | + | + | - |
| Tetrads | + | v | - | + |
| Motility | - | - | - | + |
| Catalase: Heme | - | + | + | + |
| Non-heme | v | - | - | - |
| $H_2O_2$ formation | + | - | - | - |
| Glucose fermentation | d | - | + | - |
| Yellow brown pigment | - | - | - | + |

*Buchanan and Gibbons (1974).

+ = > 90% strains positive; - = > 90% strains negative; d = > 90% strains positive or negative; v = in one strain sometimes positive or negative.

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# MATERIALS AND METHODS

## Preparation of Samples and Frozen Storage

The samples of fish used in this study were white sucker (Catostomus commersoni) which were caught using trap nets from Saginaw Bay in Lake Huron. The samples were packed in ice in stainless steel lugs covered with plastic, cardboard and canvas, and transported to M.S.U. by truck. The following morning the fish were headed and gutted with the scales on, washed and cut into halves. The fish were deboned using a Bibun meat-bone separator with 5-mm holes, and stored in lugs at 4.4°C (40°F).

The samples of deboned fish were mixed with commercial antioxidant (Freez-Gard® powder, FP 88E) which consisted of 50 percent sodium hexametaphosphate, 45 percent sodium chloride, and 5 percent sodium erythorbate. Up to 830 ml of solution containing 24 percent Freez-Gard® were added to 18.1 kg (40 lbs.) of the fish flesh and mixed for three minutes in a mixer. The samples with and without antioxidant were stuffed into fibrous oxygen-moisture permeable casings, 88 mm in diameter, using a hydraulic sausage stuffer. The samples were frozen at -18°C and cut the following day into 57-gram (2-oz.)

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patties using a Hobart meat saw. The patties were stored in cryovac bags overnight and dipped into batter which consisted of 4.5 cups Drake's batter mix, 945 ml water, 60 grams salt and 80 grams Old Bay spice (The Baltimore Spice Co.). The patties were allowed to drain on racks, and placed in the freezer. After the patties were frozen, each patty was sealed in a cryovac bag and stored at -18°C for six months.

Samples were taken for microbial analysis on the fresh deboned fish and on fish patties, with and without antioxidant, after one, three and six months of frozen storage. The frozen samples were analyzed after thawing at room temperature for two hours and again after refrigerated storage at 4°C for five days. Bacteria were isolated and identified from samples of the fresh fish, thawed fish (three month storage), and also on the thawed fish which had been refrigerated at 4°C for five days.

## Standard Plate Counts

Fifty grams of fish were blended aseptically with 450 ml of sterile 0.1 percent peptone solution to make a 1:10 dilution of the sample. From this dilution two dilutions of 1:1,000 and 1:100,000 in 0.1 percent peptone solution were prepared. Pour plates, in duplicate, containing 1.0 and 0.1 ml of each dilution were made with plate count agar (PCA, Difco). The plates were incubated at 21°C for five days.

After incubation the pair of plates having 30-300 colonies were selected and the aerobic plate counts were calculated and recorded as microorganisms per gram of fish sample. Surface colonies were counted on selected plates, and the numbers of endosporeforming bacteria (Bacillus), pigmented microorganisms and molds which grew on the surface of the agar were counted and calculated as the percentages of total surface counts. Typical colonies of Bacillus usually have rough surfaces, are flat, and spread widely on the surface of the agar after five days of incubation.

## Isolation and Identification of Bacteria

### Isolation of Bacteria

Bacterial isolates were obtained by randomly picking colonies from surfaces of the selected plates. The cultures were purified by streaking onto PCA agar plates two times. The pure cultures were transferred onto PCA slants and identified using a modification of the identification methods of Shewan et al. (1960a) and Shewan (1971) for gram-negative bacteria. Generic identification of gram-negative and gram-positive bacteria was confirmed using the descriptions of genera in the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The bacterial isolates were identified on the basis of gram stain, morphology, growth on MacConkey

agar, spore formation, oxidase test, catalase test, motility, type of flagellation, pigmentation, hydrogen sulfide production, carbohydrate utilization, nitrate reduction, sensitivity to penicillin, sensitivity to 2,4-diamino-6,7-diisopropyl pteridine (0/129), proteolytic activity, methyl red test, Voges-Proskauer test or production of acetyl methyl carbinol (acetoin), and starch hydrolysis. The production of fluorescent pigments was also determined in order to differentiate the subgroups of Pseudomonas. A summary of tests used in the identification of bacteria is presented in the Appendix (Table A1).

Gram Stain and Morphology

A dried, fixed film of a 24-hour old culture from nutrient broth was covered for one minute with crystal violet stain, rinsed with tap water, drained, and covered with Gram's iodine solution for one minute. The film was rinsed with tap water, blotted dry, decolorized with 95 percent ethanol, rinsed, and counterstained with safranin solution for 10 seconds. Then the film was rinsed in water, dried, and examined with the oil-immersion objective of the microscope to determine (1) the gram reaction of the organism, (2) morphology, and (3) cell arrangement (i.e., singly, in pairs, in chains, in clumps). Bacteria which retained the original stain (violet) were gram-positive, while those which were decolorized by the alcohol and took the counterstain (red) were gram-negative.

Observation of morphology and arrangement were also done on wet mounts of 24-hour and 5-day old cultures from nutrient broth using a phase microscope.

Growth on MacConkey Agar

The gram stains of gram-positive bacteria such as Micrococcus, Corynebacterium and Brevibacterium may be readily decolorized, so that difficulties might be obtained in establishing their gram reaction. Therefore, all bacterial isolates were streaked on MacConkey agar (Difco) plates and incubated at 21°C for five days. Growth of the gram-positive bacteria should be inhibited by the dyes present in this agar medium.

Spore Stain

A dried, fixed film of a 7-day old culture from a PCA slant was covered for 15 minutes with malachite green. The film was washed with tap water, drained, counterstained with safranin solution for 15 seconds, rinsed in tap water, and dried. The stained film was examined with the oil-immersion objective of a microscope to determine the presence of spores and the location of spores in sporangia. The spores were stained green, and the bacterial cells and sporangia were counterstained red.

Oxidase Test

The presence of oxidase was determined by Kovacs' test (Kovacs, 1956). A piece of Whatman's No. 1 filter

paper was laid in a petri dish, and two to three drops of 1 percent tetramethyl-para-phenylenediamine dihydrochloride (Eastman Organic Chemicals Co.) solution were placed on the paper. A loopful of solid growth taken from a PCA culture was smeared thoroughly on the reagent-impregnated filter paper. If the reaction was positive, the smear turned dark purple in 5-10 seconds.

Catalase Test

The presence of catalase was determined by transferring a loopful of solid growth taken from a PCA culture to a glass slide. A drop of 3 percent hydrogen peroxide was then added. The production of gas bubbles indicated the presence of catalase.

Motility

Motility was determined by stabbing the center of a tube of nitrate-motility medium and withdrawing the needle along the inoculated stab. Diffuse growth along the line of inoculation after incubating at 21°C for five days indicated motility. Observation of motility was also done on the wet mount of an 18- to 24-hour old culture using phase microscopy.

Nitrate Reduction

For the detection of reduction of nitrate to nitrite a few drops of sulphanilic acid solution (8 g sulphanilic acid in 1000 ml of 5 N acetic acid) were added

into the growth in the nitrate-motility tube, followed by an equal amount of alpha-naphthylamine solution (5 g alpha-naphthylamine in 1000 ml of 5 N acetic acid). The development of a pink color indicated the presence of nitrite. If no color was developed, zinc dust was added, and a pink color indicated the presence of nitrate which indicated that the organism did not have the ability to reduce nitrate. A lack of color after adding zinc dust indicated that the organism had the ability to reduce nitrate to nitrite, and the nitrite was further reduced to nitrogen or ammonia.

Flagella Stain

For motile cultures, the type of flagellation was determined with the aid of Gray's flagella stain (Gray, 1926; Bailey and Scott, 1974). A suspension of a young culture was made by transferring the growth of a 24-hour old culture on a PCA slant to a small tube containing 2 ml of sterile distilled water. The suspension was incubated at room temperature for 30 minutes. Using a grease-free slide that had been flamed and cooled, a drop of distilled water was spread on the slide to cover an area of approximately 2 $cm^2$. A loopful of culture suspension was touched gently into the drop of water at several places on the slide, the slide was gently rotated, and the film was air dried. The mordant, containing 5 ml saturated aqueous aluminum potassium sulfate solution, 2 ml saturated aqueous mercuric chloride solution, and 0.4 ml saturated alcoholic

solution of basic fuchsin, was added to the slide, allowed to act for 10 minutes, and rinsed gently with tap water. Then Ziehl's carbol fuchsin solution was added and left on the slide for five minutes. The slide was rinsed with tap water, air dried, and examined under the oil-immersion objective of a microscope to determine the number and arrangement of the flagella.

## Pigmentation and Proteolytic Activity

All cultures were tested for the production of pigments on plates containing nutrient agar and 15 percent skim milk. All plates were incubated at 21°C for five days. This medium was also used for the detection of proteolytic activity because proteolytic cultures hydrolyzed casein and caused the medium to lose its opacity.

The production of fluorescent pigments by *Pseudomonas* spp. was determined using Pseudomonas agar F (Frazier *et al.*, 1968). One loopful of each culture which was classified in the genus *Pseudomonas* was streaked on a plate of solid Pseudomonas agar F. After incubation at 21°F for five days, the presence or absence of green fluorescent pigments on the plate was determined by observation under ultraviolet light.

## Production of Luminescence

To determine the production of luminescence by *Photobacterium* spp. or *Lucibacterium* spp., yeast peptone

broth and glycerol calcium carbonate agar plates containing 1.5 and 3.0 percent NaCl (Hendrie et al., 1970) were inoculated with 2-day cultures, and examined for the production of luminescence daily for four days, and again after seven days of incubation at 21°C.

Production of Hydrogen Sulfide

The medium used as an indicator of hydrogen sulfide production by bacteria was peptone iron agar (Difco). The medium contains ferric citrate which acts as a sensitive indicator of hydrogen sulfide production. The medium in tubes was inoculated by the stab method. Blackening of the medium after incubation at 21°C for five days indicated the production of hydrogen sulfide.

Carbohydrate Utilization

Hugh and Leifson's medium was used to detect oxidation of carbohydrates and distinguish it from fermentation. The medium had the following composition (Hugh and Leifson, 1953): 0.2 percent peptone, 0.5 percent NaCl, 0.03 percent $K_2HPO_4$, 0.3 percent agar, 0.003 percent bromthymol blue, and 0.1 percent carbohydrate (glucose, sucrose or lactose). The medium was tubed to a depth of about 4 cm, and duplicate tubes of the solidified medium were inoculated by stabbing. After inoculation one of each of the pairs of tubes was covered with a layer of sterile,

melted petrolatum to a depth of 0.5 to 1 cm. All tubes were incubated at 21°C for five to ten days.

Several types of reaction were observed in the medium after incubation. Fermentative organisms produced an acid reaction in both tubes, which was indicated by a change in color from blue to yellow. Oxidative organisms produced an acid reaction in the open tube only, and the color of the covered tube remained unchanged. Nonfermentative and nonoxidative organisms produced no change in the covered tube, and no change or an alkaline reaction (dark blue color) only in the open tube.

Penicillin Sensitivity Test

For the penicillin sensitivity test 2 ml of a sterile aqueous solution containing 625 international units (IU) penicillin per ml was added to 500 ml sterile, melted PCA. Then agar plates, which contained 2.5 IU penicillin per ml, were made from this agar. A loopful of solid growth from a 24-hour old culture was smeared on the plates, and the plates were incubated at 21°C for five days. The absence of growth of the organism after incubation indicated that the organism was sensitive to penicillin (2.5 IU).

Pteridine (0/129) Sensitivity Test

The pteridine (0/129) sensitivity test (Shewan and Hodgkiss, 1954) was done using sterile disks impregnated

with a sterile saturated aqueous solution of 2,4-diamino-6,7-diisopropyl pteridine. The disk was placed on a PCA plate inoculated with a suspension of a 24-hour old culture, and then incubated at 21°C for five days. The absence of growth of the organism around the disk after incubation indicated that the organism was sensitive to pteridine.

Methyl-Red and Voges-Proskauer Tests

Tubes of proteose broth containing 0.5 percent glucose were inoculated with the bacterial isolates, and incubated at 21°C for two and seven days. In separate tubes, 0.6 ml of 5 percent alpha-naphthol and 0.2 ml of 40 percent KOH were added to 1 ml of the 2-day old cultures. The formation of a pink-red color indicated a positive Voges-Proskauer test, i.e., production of acetyl methyl carbinol (acetoin). For the methyl-red test, a few drops of methyl-red solution were added to 5 ml of the 7-day old cultures. The development of a red color indicated a positive test, whereas a yellow color indicated a negative test.

Starch Hydrolysis

For the starch hydrolysis test, nutrient agar plates containing 1 percent soluble starch (Bovre et al., 1974) were streaked with the bacterial isolates, and incubated at 21°C. After five days, the growth on the agar was flooded with iodine solution. The absence of blue

color indicated a positive test, i.e., starch was hydrolyzed.

# RESULTS AND DISCUSSION

## Aerobic Plate Counts

In this study the incubation temperature for aerobic plate counts was 21 ± 0.5°C. This incubation temperature was used since most microorganisms capable of growth on fish have an optimum temperature between 20°C (Shewan et al., 1960a) and 22°C (Pelroy and Eklund, 1966). Most psychrophiles, which were the majority of bacteria on fish, could grow at 21°C since, according to Brock (1974), obligate psychrophiles are organisms which have an optimum temperature of 15°C or below and a maximum temperature of about 20°C or slightly higher, whereas facultative psychrophiles, have an optimum temperature between 25° and 30°C, but can grow at 0°C. In addition, most mesophiles also could grow at 21°C.

The change in the aerobic plate counts of deboned fish patties during frozen storage at -18°C, and after thawing and refrigerated storage at 4°C for five days, are shown in Figures 1 and 2 for samples without and with antioxidant, respectively. The addition of commercial antioxidant (Freez-Gard®) had no substantive effect on the aerobic plate counts (Figures 1 and 2). The operational

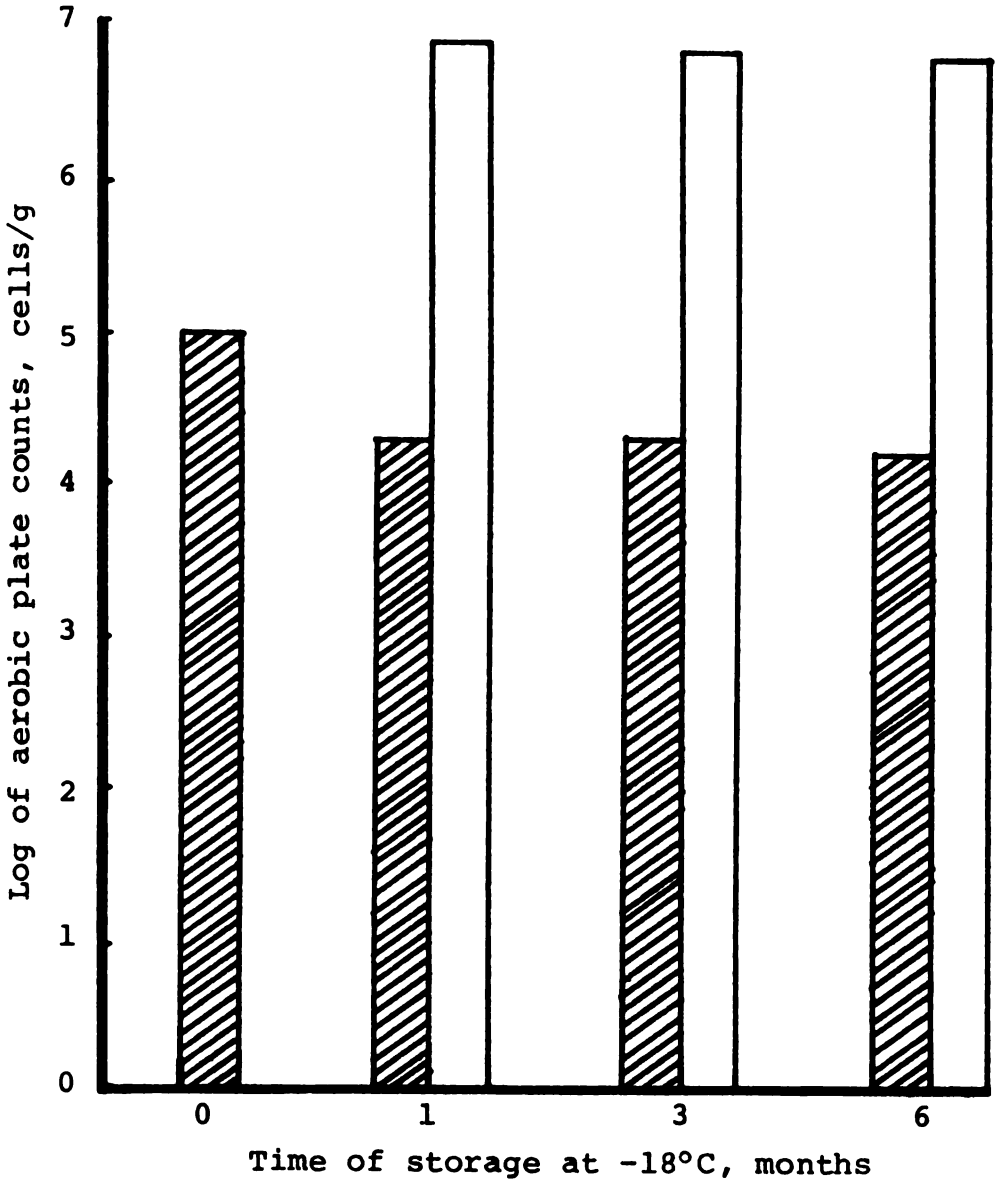


Fig. 1.--Aerobic plate counts of frozen deboned fish patties after thawing at room temperature for two hours (▨), and after thawing and refrigeration at 4°C for five days (□).

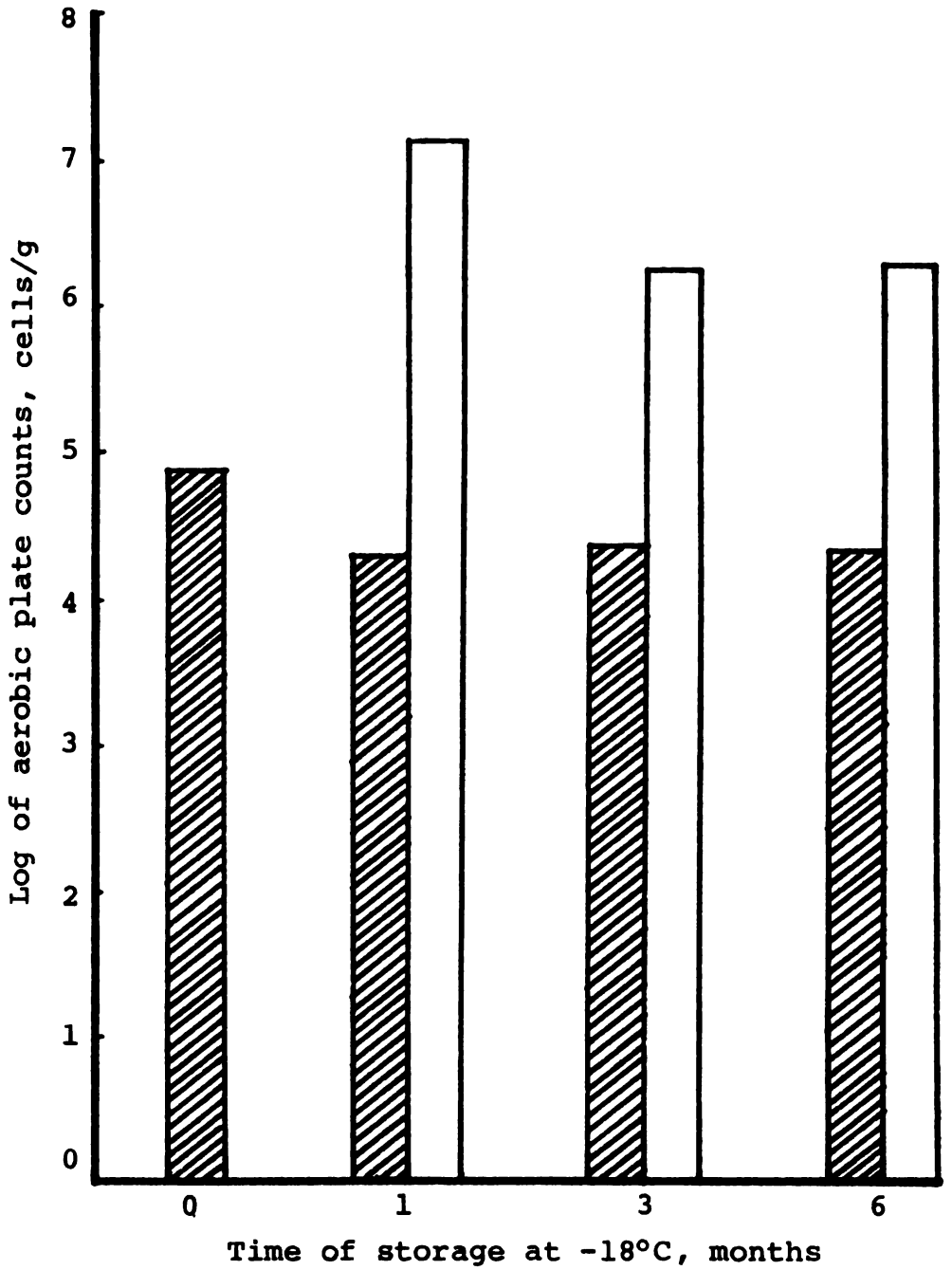


Fig. 2.--Aerobic plate counts of frozen deboned fish patties with antioxidant after thawing at room temperature for two hours (▨), and after thawing and refrigeration at 4°C for five days (□).

steps in the preparation of fish patties also did not influence the aerobic plate counts since the aerobic plate count of fresh deboned fish ($9.3 \times 10^4$ cells/g) was not substantially different from the aerobic plate count of fresh patties ($9.1 \times 10^4$ cells/g).

As shown in Figures 1 and 2, aerobic plate counts decreased slightly during frozen storage at -18°C, however, refrigeration of the thawed fish at 4°C for five days caused a large increase in the aerobic plate counts. The rate of decrease in the aerobic plate counts of frozen fish was higher in the first month than in the following months. According to Shewan (1961) destruction of microorganisms during freezing occurred exponentially only during the initial period of freezing, and then a gradual destruction of microorganisms occurred. The higher rate of decrease in the aerobic plate counts of fish patties during the first month of frozen storage probably was due to the failure of some mesophiles to grow and the destruction of some mesophiles and psychrophiles, although a few psychrotrophs could survive temperatures below 0°C. Some of the psychrophiles are killed at -10°C (Pelczar and Reid, 1972). The slower rate of decrease in the aerobic plate counts in the following months was due to the injury and death of some of the remaining microorganisms.

After six months of frozen storage, the aerobic plate counts decreased to about 16 to 30 percent of the

original values, or there was 70 to 84 percent destruction of the microorganisms present in the fresh samples. Freezing usually causes a destruction of about 60 to 90 percent of the bacterial population present (Kiser and Beckwith, 1942). There was no substantial decrease in the aerobic plate counts after three months of storage. These results indicated that most of the cells which survived the initial freezing could survive for a long period of frozen storage.

After the thawed fish had been refrigerated at 4°C for five days, the aerobic plate counts increased about one hundred-fold compared to the thawed samples. The microorganisms which survived freezing and thawing grew rapidly at the refrigeration temperature.

## Percentages of Bacillus, Pigmented Microorganisms and Molds

The percentages of Bacillus, pigmented microorganisms and molds are presented in Tables 4 and 5 for samples without and with antioxidant, respectively. The actual surface counts and surface counts of Bacillus, pigmented microorganisms and molds are presented in the Appendix (Tables A2 and A3). As shown in Tables 4 and 5, the addition of commercial antioxidant did not have a substantial effect on the percentages of Bacillus, pigmented microorganisms and molds. The proportion of Bacillus increased during frozen storage due to destruction of some nonspore-forming organisms. However, after refrigeration,

Table 4.--Percentages of Bacillus, pigmented microorganisms and molds of deboned fish and fish patties stored at -18°C.*

| Time of storage at -18°C† (months) | Bacillus (%) | Pigmented microorganisms (%) | Molds (%) |
|---|---|---|---|
| 0 : Fresh (deboned fish) | 5.1 | 85.9 | 3.0 |
| 1 : After thawing | 32.6 | 31.6 | 4.6 |
| After thawing and refrigeration | 20.3 | 2.3 | - |
| 3 : After thawing | 60.0 | 19.3 | 1.8 |
| After thawing and refrigeration | 25.3 | 3.6 | - |
| 6 : After thawing | 67.0 | 10.5 | 3.6 |
| After thawing and refrigeration | 34.4 | 3.2 | - |

*Percentages of total surface counts.

†Samples were plated after thawing at room temperature for two hours, and after thawing and refrigeration at 4°C for five days.

Table 5.--Percentages of Bacillus, pigmented microorganisms and molds of deboned fish patties with antioxidant stored at -18°C.

| Time of storage at -18°C† (months) | Bacillus (%) | Pigmented microorganisms (%) | Molds (%) |
|---|---|---|---|
| 0 : Fresh patties | 6.2 | 82.0 | 2.7 |
| 1 : After thawing | 36.8 | 28.6 | 4.5 |
| After thawing and refrigeration | 27.4 | 2.9 | - |
| 3 : After thawing | 58.5 | 22.2 | 3.5 |
| After thawing and refrigeration | 13.6 | 2.6 | - |
| 6 : After thawing | 67.2 | 13.3 | 2.0 |
| After thawing and refrigeration | 40.1 | 4.0 | - |

*Percentages of total surface counts.

†Samples were plated after thawing at room temperature for two hours, and after thawing and refrigeration at 4°C for five days.

the proportion of Bacillus decreased due to the rapid growth of other organisms, although the level was higher than that of the fresh samples. On the other hand, the proportion of pigmented microorganisms decreased during frozen storage, and a further decrease occurred after refrigeration.

The increases in the percentages of Bacillus during freezing were due to the destruction of most of the pigmented microorganisms. The injury and death of pigmented microorganisms occurred during freezing. Spores of Bacillus, on the other hand, were more resistant to freezing and became the predominant bacteria after frozen storage. One of the lethal effects of freezing is caused by denaturation of proteins which is due to desiccation or loss of water from the cells to the surrounding frozen medium (Smith, 1961; Brock, 1974). The main reasons that bacterial spores are resistant to desiccation, according to Brock (1974), are their low available water content and the presence of calcium and dipicolinic acid in the spore.

After refrigeration at 4°C for five days there were great decreases in the percentages of both Bacillus and pigmented microorganisms. These decreases primarily were due to the growth of the nonspore-forming, nonpigmented bacteria which survived the frozen storage and grew rapidly at 4°C, so that they became the predominant bacteria during refrigeration. The vegetative cells of

most Bacillus species have optimum temperatures between 10 and 65°C (Buchanan and Gibbons, 1974), although some bacilli grow slowly at 4°C. The nonspore-forming, nonpigmented bacteria which grew during refrigeration should be psychrotrophic bacteria, whereas the pigmented microorganisms which were destroyed during freezing consisted of mesophiles and/or psychrotrophic bacteria. According to Brock (1974), organisms capable of growing at low temperatures contained more unsaturated fatty acids in their cell membranes than did other organisms, so that the cell membranes did not solidify and remained semifluid at low temperatures.

Freezing had no substantial effect on the growth of molds (Tables 4 and 5). According to Lund and Halvorson (1951), vegetative cells of molds and yeasts were more resistant to freezing than those of bacteria. However, after the thawed fish had been refrigerated at 4°C for five days there was no apparent growth of molds. Probably, after refrigeration the proportion of molds became too small to be observed and counted compared to the nonspore-forming, nonpigmented bacteria which grew rapidly and comprised about 56 to 84 percent of the total population.

## Isolation, Identification, and Broad Groupings of Bacterial Cultures

Isolation of bacteria was done by picking colonies from the plates prepared from the fresh samples and samples

frozen for three months. Cultures were purified by streaking on PCA plates. Identification was carried out using tests as shown in the Appendix (Table A1). As many as 153 colonies were isolated from the samples (Appendix, Table A4). In order to simplify the method for identification, a broad grouping of bacteria isolated from the samples was made as shown in Figures 3 and 4 for gram-negative and gram-positive bacteria, respectively.

The grouping was based on the gram stain, morphology, motility, and flagellation. Gram-negative bacteria were grouped into: (1) rods which consisted of polar-flagellate, peritrichous-flagellate, and nonmotile bacteria, and (2) cocci and coccobacilli (Figure 3). Gram-positive bacteria were grouped into: (1) rods, and (2) cocci (Figure 4).

## Descriptions of the Genera

### Gram-Negative Rods

Pseudomonas.--Characteristics of the bacteria classified in the genus Pseudomonas are presented in the Appendix (Table A5). These bacteria were part of the group of polar-flagellate, gram-negative rods (Figure 3). They consisted of single, straight asporogenous rods which produced oxidase and catalase, and did not produce $H_2S$. Some cultures produced yellow pigment(s), and cultures which produced yellow-green fluorescent pigments were

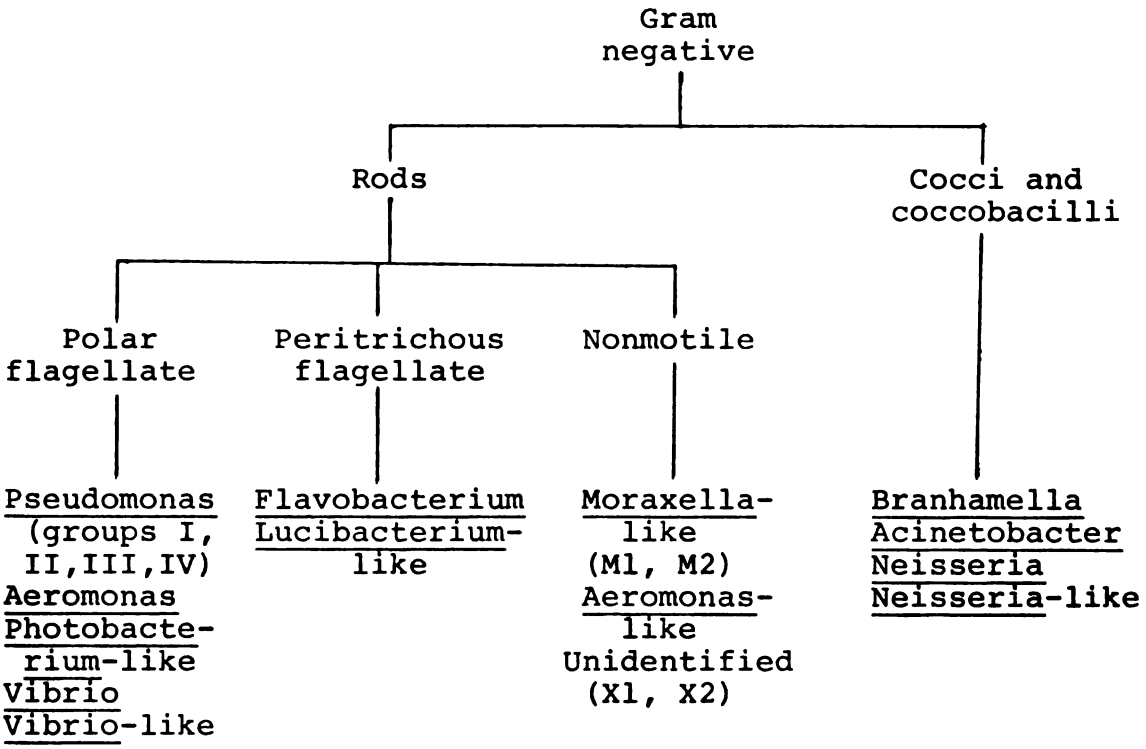


Fig. 3.--A broad grouping of gram-negative bacteria isolated from deboned fish and fish patties.

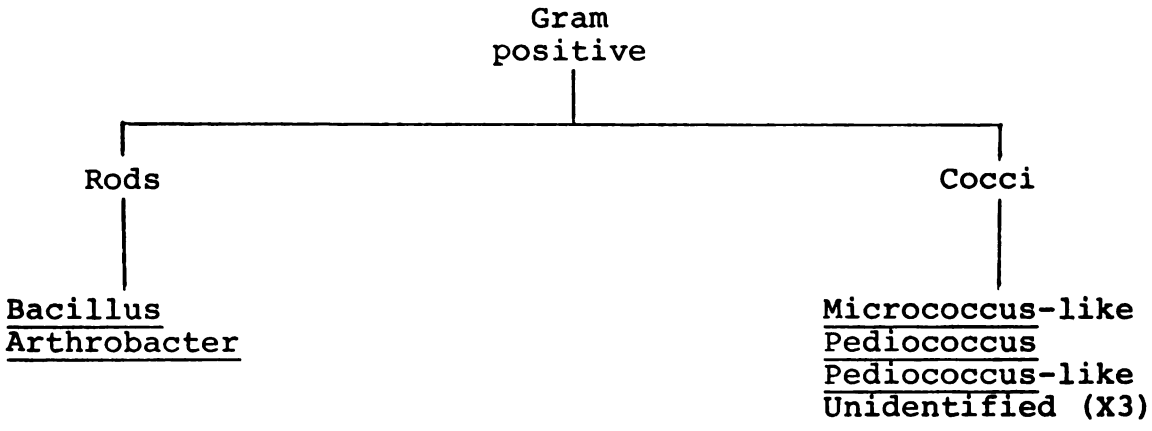


Fig. 4.--A broad grouping of gram-positive bacteria isolated from deboned fish and fish patties.

placed in group I Pseudomonas. The genus Pseudomonas was not sensitive to 2.5 units of penicillin (Shewan et al., 1960a) or 2,4-diamino-6,7-diisopropyl pteridine (Shewan et al., 1960a; Buchanan and Gibbons, 1974). As stated by Shewan et al. (1960a), in the Hugh and Leifson's medium, groups I and II Pseudomonas oxidized glucose and produced acid without the formation of gas, while group III produced an alkaline reaction, and group IV produced no reaction. Of the 16 cultures of Pseudomonas isolated from the samples, five cultures were classified in group I Pseudomonas, nine cultures in group II Pseudomonas, and one culture each in groups III and IV Pseudomonas. The nitrate reduction and proteolytic activity of members of the genus Pseudomonas were variable. According to Lerke et al. (1965), groups III, IV, and a few of group I Pseudomonas were "spoilers" which meant they exhibited proteolytic activity, while according to Shaw and Shewan (1968), groups II, III and IV were active "spoilers."

Aeromonas and Photobacterium-like organisms.--The Aeromonas and Photobacterium-like organisms were also grouped into the polar-flagellate, gram-negative rods (Appendix, Table A5). These bacteria consisted of straight rod-shaped, asporogenous bacteria which produced oxidase and catalase, fermented glucose, sucrose and lactose with the production of acid and gas, reduced nitrate to nitrite, did not produce $H_2S$, and were not sensitive to penicillin.

The differences between Aeromonas and Photobacterium-like organisms were that Aeromonas was insensitive to 2,4-diamino-6,7-diisopropyl pteridine (0/129), hydrolyzed starch, and produced brown or no pigment, whereas Photobacterium-like organisms were sensitive to 0/129, and did not hydrolyze starch. The Photobacterium-like organisms could not be placed in the genus Photobacterium since they did not produce luminescence in yeast peptone medium or in glycerol calcium agar containing 1.5 and 3.0 percent NaCl, while Photobacterium, according to Hendrie et al. (1970) and Bergey's Manual (Buchanan and Gibbons, 1974), produces luminescence. In proteose broth containing 0.5 percent glucose, all cultures of the Photobacterium-like organisms were methyl-red positive and produced acetyl methyl carbinol (Voges-Prokauer positive). All cultures of the genus Aeromonas and some of the Photobacterium-like organisms showed proteolytic activity.

Aeromonas-like organisms.--These organisms resembled Aeromonas since they consisted of short rods in short chains, did not produce spores, pigment or $H_2S$, produced oxidase and catalase, hydrolyzed starch, and reduced nitrate to nitrite (Appendix, Table A7). They were also insensitive to penicillin and 2,4-diamino-6,7-diisopropyl pteridine, nonmotile, and fermented glucose and sucrose with the production of acid but no gas. They could not be placed in the genus Aeromonas since they did not hydrolyze

casein. According to Bergey's Manual (Buchanan and Gibbons, 1974), Aeromonas consisted of gram-negative rods, with rounded ends, to coccoid cells, which were nonmotile or motile with polar flagella, hydrolyzed casein, and fermented carbohydrates with or without the formation of gas ($CO_2$ and $H_2$).

Vibrio and Vibrio-like organisms.--Characteristics of the genera Vibrio and Vibrio-like organisms, which were in the group of polar-flagellate, gram-negative rods, are presented in the Appendix (Table A5). They consisted of short straight or curved asporogenous rods which produced oxidase and catalase, and did not produce $H_2S$. Some cultures produced yellow or orange pigments, and the others were nonpigmented. They did not reduce nitrate to nitrite, fermented glucose, sucrose and lactose with the production of acid but not gas, and were sensitive to 2,4-diamino-6,7-diisopropyl pteridine. According to Shewan et al. (1960a), Vibrio was not sensitive to penicillin (2.5 units), therefore, five of the ten cultures isolated from the samples could not be placed in the genus Vibrio and hence are called Vibrio-like organisms since they were sensitive to penicillin (2.5 units). The methyl-red and Voges-Proskauer tests on these bacteria were variable. Some cultures were methyl-red and Voges-Prokauer positive, while the others were negative.

Flavobacterium.--Members of the genus Flavobacterium were primarily grouped into the peritrichous-flagellate, gram-negative rods, although some cultures were nonmotile as shown in the Appendix (Table A6). These bacteria consisted of short and slender rods which produced yellow, orange or red pigments, and did not produce spores. They were oxidase and catalase positive, did not produce $H_2S$, and some oxidized glucose, sucrose and lactose with the formation of acid without gas, while the others produced neither acid nor gas from glucose. Some cultures reduced nitrate to nitrite, and some showed proteolytic activity. The tests for penicillin and 2,4-diamino-6,7-diisopropyl pteridine sensitivities were not done on these cultures since both tests were not specific for the genus Flavobacterium (Buchanan and Gibbons, 1974). In general, the cultures belonging to the genus Flavobacterium, particularly the peritrichous-flagellate bacteria, resembled Alcaligenes except that all of these cultures were pigmented, whereas most members of the genus Alcaligenes were nonpigmented (Buchanan and Gibbons, 1974; Hendrie et al., 1974).

Lucibacterium-like organisms.--These bacteria were called Lucibacterium-like organisms since most of the test results from these bacteria showed similarities to the genus Lucibacterium (Appendix, Table A6). These organisms were grouped into the peritrichous-flagellate,

gram-negative rods. They contained straight, asporogenous rods which produced oxidase and catalase. They were insensitive to 2,4-diamino-6,7-diisopropyl pteridine, methyl-red positive, did not produce acetyl methyl carbinol or $H_2S$, showed proteolytic activity, hydrolyzed starch, and fermented glucose and sucrose but not lactose and produced acid without gas. The differences between these bacteria and the genus Lucibacterium were that these bacteria were sensitive to penicillin, and did not reduce nitrate to nitrite, whereas according to Bergey's Manual (Buchanan and Gibbons, 1974), Lucibacterium was insensitive to penicillin and produced nitrite from nitrate.

Moraxella-like organisms.--These organisms (M1 and M2) were grouped into the nonmotile, gram-negative rods with characteristics as shown in the Appendix (Table A7). They consisted of short rods which occurred singly, in pairs or short chains (M1), or tiny rods which occurred in short chains (M2). They did not produce spores, pigment or $H_2S$, produced oxidase and catalase, did not produce acid or gas from carbohydrates (glucose, sucrose or lactose), and did not have proteolytic activity. They could not be placed in the genus Moraxella since they were insensitive to penicillin (2.5 units), whereas Moraxella should be highly sensitive to penicillin (Buchanan and Gibbons, 1974; Henriksen, 1976). There were two types of organisms belonging in the Moraxella-like organisms,

culture M1 which grew on MacConkey agar and reduced nitrate to nitrite, and culture M2 which did not grow on MacConkey agar and did not produce nitrite from nitrate. The test for sensitivity to 2,4-diamino-6,7-diisopropyl pteridine, which is not specific for the genus Moraxella, was not done on these bacteria.

Unidentified organisms.--Two cultures (X1 and X2) consisted of nonmotile, gram-negative rods which occurred singly or in short chains, produced oxidase and catalase, did not produce spores or $H_2S$, and did not reduce nitrate to nitrite (Appendix, Table A7). Culture X1 did not grow on MacConkey agar, produced yellow-orange pigments, fermented glucose, sucrose and lactose with the production of acid without gas, had proteolytic activity, and was sensitive to 2,4-diamino-6,7-diisopropyl pteridine, but insensitive to penicillin. This culture resembled Flavobacterium, Vibrio or Photobacterium, however, these bacteria could not be placed in the genus Flavobacterium since Flavobacterium does not ferment carbohydrates but metabolizes carbohydrates oxidatively (Kazanas, 1966; Buchanan and Gibbons, 1974). They also could not be placed in the genus Vibrio since they were nonmotile and did not reduce nitrate to nitrite, or in the genus Photobacterium since they did not reduce nitrate to nitrite, did not produce luminescence, and fermented carbohydrates without gas formation. According to Shewan (1971) and Bergey's Manual (Buchanan

and Gibbons, 1974), Vibrio consisted of polar-flagellate, gram-negative rods which reduced nitrate to nitrite, while Photobacterium also reduced nitrate to nitrite, produced luminescence, and fermented carbohydrates with the formation of gas.

The bacteria belonging to the culture X2 grew on MacConkey agar, did not produce pigment, oxidized glucose and lactose but not sucrose, did not have proteolytic activity, and were sensitive to penicillin (2.5 units), but insensitive to 2,4-diamino-6,7-diisopropyl pteridine. These bacteria resembled Moraxella, Neisseria or Alcaligenes, but they could not be placed in the genus Moraxella since Moraxella does not produce acid from carbohydrates. These organisms were different from Neisseria only in morphology (Neisseria are cocci), and from Alcaligenes in motility since Alcaligenes usually are motile with peritrichous flagella, whereas these organisms were nonmotile.

## Gram-Negative Cocci and Coccobacilli

Branhamella.--These bacteria consisted of gram-negative cocci which were nonmotile, and arranged in pairs or in very short chains (Appendix, Table A8). They did not produce spores, pigment or $H_2S$, produced oxidase and catalase, and reduced nitrate to nitrite. Neither acid nor gas was produced by these bacteria from carbohydrates (glucose, sucrose or lactose). They did not have

proteolytic activity, and were sensitive to penicillin. The 2,4-diamino-6,7-diisopropyl pteridine sensitivity test was not specific for this genus. According to Bergey's Manual (Buchanan and Gibbons, 1974), only one species, B. catarrhalis, has been described in the genus Branhamella.

Acinetobacter.--These bacteria consisted of gram-negative coccobacilli which occurred in pairs (Appendix, Table A8). They did not produce spores, oxidase, acetyl methyl carbinol or $H_2S$, but produced catalase. Neither acid nor gas was produced from carbohydrates (glucose, sucrose or lactose). They were nonmotile, insensitive to penicillin, methyl-red negative, did not reduce nitrate to nitrite, and did not have proteolytic activity. Only one species, A. calcoaceticus, belongs to the genus Acinetobacter (Buchanan and Gibbons, 1974). According to Bovre et al. (1974) and Bergey's Manual (Buchanan and Gibbons, 1974), the rod-shaped cells of Acinetobacter resembled Moraxella, while the coccoid cells resembled Branhamella. However, Acinetobacter were oxidase negative, whereas Moraxella and Branhamella were oxidase positive.

Neisseria and Neisseria-like organisms.--These bacteria consisted of gram-negative cocci which occurred singly or in pairs, produced oxidase and catalase, and did not produce spores or $H_2S$ (Appendix, Table A8). They were nonmotile, nonpigmented or yellow-colored bacteria which

oxidized carbohydrates, or did not produce acid from carbohydrates (glucose, sucrose or lactose). Some cultures isolated from the samples reduced nitrate to nitrite, and one culture showed proteolytic activity. Three of the ten cultures were sensitive to penicillin, which is a characteristic of the genus Neisseria (Buchanan and Gibbons, 1974; Henriksen, 1976), but seven cultures could not be placed in the genus Neisseria and are called Neisseria-like organisms since they were not sensitive to penicillin. The sensitivity test for 2,4-diamino-6,7-diisopropyl pteridine was not done on these bacteria since the test is not specific for the genus Neisseria.

Gram-Positive Rods

Bacillus.--Most of the Bacillus cultures isolated from the samples were motile with peritrichous flagella, and only a few of them were nonmotile (Appendix, Table A9). These bacteria consisted of rod-shaped cells which produced endospores, oxidase and catalase, produced cream-yellow pigment or no pigment, did not produce $H_2S$, produced acid but no gas from glucose, sucrose and lactose under anaerobic conditions, and had proteolytic activity. Some of the cultures did not reduce nitrate to nitrite, while the others did. Penicillin and 2,4-diamino-6,7-diisopropyl pteridine sensitivity tests were not done on these bacteria or the other gram-positive bacteria.

Arthrobacter.--Young cultures (24-hour old cultures) of the bacteria belonging to the genus Arthrobacter were composed of nonmotile, gram-positive rods. However, in older cultures (5-day old cultures) the cells became coccoid (Appendix, Table A9). These bacteria did not produce spores, pigment or $H_2S$, produced oxidase and catalase, did not reduce nitrate to nitrite, did not have proteolytic activity, and oxidized glucose and sucrose, but not lactose.

Gram-Positive Cocci

Micrococcus-like organisms.--These bacteria consisted of gram-positive cocci which occurred in irregular clusters. They did not produce spores or pigments, and produced oxidase and catalase (Appendix, Table A10). They resembled Micrococcus colpogenes (species incertae sedis) since they did not produce $H_2S$, reduced nitrate to nitrite, hydrolyzed casein, and did not produce acid or gas from glucose, sucrose or lactose (Buchanan and Gibbons, 1974). However, they could not be placed in the genus Micrococcus since according to Kazanas (1966) members of Micrococcus were oxidase negative.

Pediococcus and Pediococcus-like organisms.--These bacteria consisted of gram-positive cocci which occurred in pairs or tetrads, did not produce spores or $H_2S$, produced catalase (weak), fermented glucose and sucrose

without gas formation, did not produce acid or gas from lactose, did not reduce nitrate to nitrite, did not hydrolyze starch, and had proteolytic activity (Appendix, Table A10). According to Bergey's Manual (Buchanan and Gibbons, 1974), Pediococcus usually was catalase negative, but some species may be weakly positive. They were all nonmotile and produced yellow-orange pigments. One of the four cultures isolated from the samples was oxidase negative, which is a characteristic of the genus Pediococcus (Kazanas, 1966), whereas the other cultures were called Pediococcus-like organisms since they were oxidase positive.

Unidentified organisms.--The X3 culture consisted of gram-positive cocci which occurred singly, in pairs or clumps, did not produce spores or $H_2S$, produced oxidase and catalase, produced yellow-orange pigments, reduced nitrate to nitrite, and did not have proteolytic activity (Appendix, Table A10). They fermented glucose, but not sucrose or lactose, without gas formation. They resembled Aerococcus but could not be placed in this genus since Aerococcus spp. do not produce pigment and do not reduce nitrate to nitrite (Buchanan and Gibbons, 1974). They also resembled Pediococcus and Leuconostoc, but they could not be placed in these genera since Pediococcus and Leuconostoc do not reduce nitrate to nitrite (Buchanan and Gibbons, 1974), and also members of Pediococcus are

oxidase negative (Kazanas, 1966), while Leuconostoc spp. are catalase negative (Buchanan and Gibbons, 1974).

## Effects of Freezing and Refrigeration on the Microflora of Fish

### Fresh Deboned Fish and Fish Patties

Table 6, which presents the microflora of fresh, frozen and refrigerated samples, shows that the microflora of fresh deboned fish and fish patties primarily were pigmented gram-negative, oxidase-positive rods, and a few gram-negative cocci and gram-positive rods and cocci. It has been known that the predominant bacteria on fish are gram-negative bacteria (Shewan, 1960a, 1961; Kazanas, 1966; Trust and Sparrow, 1974). As shown in Table 6, the fresh deboned fish and fish patties contained gram-negative bacteria such as groups I and II Pseudomonas, Aeromonas, Vibrio, Flavobacterium, Branhamella, Acinetobacter, Neisseria, Neisseria-like, Photobacterium-like, Moraxella-like (M2), and an unidentified (X1) organism. The fresh samples also contained gram-positive bacteria such as Bacillus, Pediococcus, Pediococcus-like organisms, and an unidentified (X3) organism.

The presence of other bacteria on the fresh patties besides those found in the fresh deboned fish, such as groups III and IV Pseudomonas, Vibrio-like, Lucibacterium-like, Moraxella-like (M1), Micrococcus-like, and an

Table 6.--Microflora of deboned fish and fish patties.

| Genera | Without antioxidant | | | With antioxidant | | |
|---|---|---|---|---|---|---|
| | | Frozen patties* | | | Frozen patties* | |
| | Fresh deboned fish | After thawing | After thawing and refrig. | Fresh patties | After thawing | After thawing and refrig. |
| Gram-negative rods: | | | | | | |
| Pseudomonas, I | + | - | + | + | - | + |
| II | + | - | + | + | - | + |
| III | - | - | - | + | - | - |
| IV | - | - | - | + | - | - |
| Aeromonas | + | - | - | + | - | - |
| Aeromonas-like | - | - | + | - | - | - |
| Photobacterium-like | + | - | - | + | - | - |
| Vibrio | + | + | + | + | - | - |
| Vibrio-like | - | - | - | + | - | - |
| Flavobacterium | + | + | + | + | + | - |
| Lucibacterium-like | - | - | - | + | - | - |
| Moraxella-like, M1 | - | - | + | + | - | - |
| M2 | + | + | + | + | - | - |
| Unidentified, X1 | + | - | - | + | - | - |
| X2 | - | - | - | + | - | - |

Table 6.--Continued.

| Genera | Without antioxidant | | | With antioxidant | | |
|---|---|---|---|---|---|---|
| | | Frozen patties* | | | Frozen patties* | |
| | Fresh deboned fish | After thawing | After thawing and refrig. | Fresh patties | After thawing | After thawing and refrig. |
| Gram-negative cocci and coccobacilli: | | | | | | |
| Branhamella | + | - | + | + | - | - |
| Acinetobacter | + | - | - | - | - | - |
| Neisseria | + | - | - | + | - | - |
| Neisseria-like | + | - | + | + | - | + |
| Gram-positive rods: | | | | | | |
| Bacillus | + | + | + | + | + | + |
| Arthrobacter | - | + | - | - | - | - |
| Gram-positive cocci: | | | | | | |
| Micrococcus-like | - | - | - | + | - | - |
| Pediococcus | + | - | - | - | - | - |
| Pediococcus-like | + | - | - | + | - | + |
| Unidentified, X3 | + | + | - | - | - | - |

*Samples were frozen at -18°C for three months, and bacteria were isolated and identified from the samples after thawing at room temperature for two hours, and after thawing and refrigeration at 4°C for five days.

unidentified (X2) organism, probably was due to the variation of the samples plated, or additional contamination which might occur during the preparation of the fish patties, particularly during stuffing and breading. However, the total plate counts per gram of fresh deboned fish and fish patties were not significantly different.

Pseudomonas, Bacillus and Micrococcus are bacteria typically found in natural waters. Pseudomonas, Vibrio, Flavobacterium and Bacillus usually are found in the slime and intestines of both marine and fresh-water fish (Frazier, 1967). Pseudomonas, Aeromonas, Vibrio, Flavobacterium, Bacillus and Micrococcus were also found by Kazanas (1966) in fresh-water fish (yellow perch fillets).

Thawed Fish Patties

Of the bacteria present in the fresh samples, only a few bacteria such as Vibrio, Flavobacterium, Bacillus, Moraxella-like (M2) and an unidentified (X3) organism were found after freezing and frozen storage at -18°C for three months, whereas other bacteria, particularly the pigmented bacteria, were not detected (Table 6).

Some of the gram-negative bacteria such as groups III and IV Pseudomonas, Aeromonas, Acinetobacter, Neisseria, Photobacterium-like, Vibrio-like, Lucibacterium-like, and unidentified (X1 and X2) organisms, and a few of gram-positive bacteria such as Pediococcus and Micrococcus-like organisms, were not detected after freezing or after

thawing and refrigeration at 4°C for five days. According to Lund and Halvorson (1951) and Shewan (1961), the gram-negative bacteria, particularly Pseudomonas, were sensitive to cold storage, whereas gram-positive bacteria were more resistant. The higher resistance of gram-positive bacteria compared to gram-negative bacteria is due to the thicker layer of peptidoglycan present in the cell walls of gram-positive bacteria (Brock, 1974). Cell walls of gram-negative bacteria are composed of a multilayered structure which is more complex, but usually is much thinner, than that of gram-positive bacteria. Stewart (1935) discovered that Pseudomonas was not detected in fish (haddock) stored at -12°C for three months, while the proportion of Flavobacterium increased.

### Thawed and Refrigerated Fish Patties

As shown in Table 6, bacteria which survived freezing, such as Vibrio, Flavobacterium, Bacillus, and Moraxella-like (M2) organisms, grew in the thawed samples which had been refrigerated at 4°C for five days. Some bacteria which could not be detected after frozen storage, such as groups I and II Pseudomonas, Branhamella, Aeromonas-like, Neisseria-like, and Pediococcus-like organisms, could grow well at 4°C. These bacteria should be in the group of psychrotrophic bacteria which survived freezing, but their numbers in the frozen samples were so

small that they were not isolated directly after the patties were thawed at room temperature for two hours. All these bacteria, except Pediococcus-like organisms, were gram-negative bacteria, and most of them were nonpigmented. According to Frazier (1967), most of the psychrophilic bacteria found in fish which survived freezing, particularly most species of Pseudomonas and Flavobacterium, were able to initiate growth during the thawing process.

Arthrobacter, which was found in the thawed samples, could not be isolated from the fresh or refrigerated samples. Also, an unidentified (X3) organism, which was found in the fresh and thawed samples, was not isolated from the refrigerated samples. Probably, the numbers of Arthrobacter in the fresh and refrigerated samples, and the numbers of the unidentified (X3) organism in the refrigerated samples were too small to be isolated from the selected plates.

From data in Table 6, the rod-shaped bacteria appeared to be more resistant to freezing and they survived frozen storage better than cocci. However, according to Brock (1974), generally cocci are more resistant to severe conditions, including desiccation during freezing, due to their round shape. Rod-shaped bacteria, on the other hand, although they could take up nutrients more easily due to the greater surface per unit volume than cocci, were more altered due to severe conditions. Brock

(1974) also stated that small cells usually were more resistant than large cells.

# CONCLUSIONS

The results indicated that the microflora of fresh deboned fish and fish patties made from white sucker caught in Lake Huron consisted primarily of pigmented gram-negative, oxidase-positive rods, and only a few gram-negative cocci and gram-positive rods and cocci. Freezing at -18°C for six months caused a destruction of 70-84 percent of microorganisms present. The greatest amount of destruction occurred during freezing and during the first three months of storage. Refrigeration at 4°C for five days resulted in a one hundred-fold increase in the aerobic plate counts.

During frozen storage, there was destruction of pigmented bacteria present, and as a result, the percentage of Bacillus increased. Storage at refrigeration temperatures such as at 4°C is not recommended for thawed fish patties because some of the gram-negative bacteria, particularly the nonpigmented bacteria, could grow rapidly causing spoilage of the fish.

The bacteria isolated from fresh deboned fish and fish patties were groups I and II Pseudomonas, Aeromonas, Vibrio, Flavobacterium, Branhamella, Acinetobacter,

Neisseria, Bacillus, Pediococcus, Pediococcus-like, Neisseria-like, Photobacterium-like, Moraxella-like, and unidentified organisms. The only bacteria which were detected after freezing and could be isolated from thawed and refrigerated samples were Vibrio, Flavobacterium, Bacillus and Moraxella-like organisms. Aeromonas, Acinetobacter, Neisseria, Pediococcus, Photobacterium-like, Vibrio-like and unidentified organisms were not detected after frozen storage. Other bacteria, although not isolated from the frozen samples, grew at 4°C and were isolated from the refrigerated samples.

Some of the bacterial cultures isolated from the samples have not been identified completely in this study. These organisms could not be classified according to the descriptions of genera given in Bergey's Manual (Buchanan and Gibbons, 1974). Since many bacteriological tests, such as the oxidase test, are highly influenced by the methods and techniques used, certain variations of procedures may result in a weak positive reaction with some organisms which otherwise give a negative reaction.

Further research should be directed towards the study of the quantitative effects of freezing or refrigeration on each bacterial type found in the fish patties.

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## LITERATURE CITED


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# APPENDIX

# APPENDIX

Table A1.--A summary of tests used in the identification of bacteria.

| Tests | Culture Media | Stains, Indicators, Reagents |
|---|---|---|
| Gram stain | Nutrient broth (24 hours) | Crystal violet stain<br>Gram's iodine solution<br>Safranin solution<br>95% ethanol |
| Morphology | Nutrient broth (24 hours and 5 days) | |
| Growth on MacConkey agar | MacConkey agar | |
| Spore stain | Plate count agar (7 days) | Malachite green solution<br>Safranin solution |
| Oxidase test (Kovacs' method) | Plate count agar | Tetramethyl-para-phenylenediamine dihydrochloride solution (1%) |
| Catalase test | Plate count agar | Hydrogen peroxide solution (3%) |
| Motility: | | |
| Nitrate motility | Nitrate motility medium | |
| Phase microscope (wet mounts) | Nutrient broth | |

Table A1.--Continued.

| Tests | Culture Media | Stains, Indicators, Reagents |
|---|---|---|
| Flagella stain (Gray's method) | Plate count agar (24 hours) | Aluminum potassium solution<br>Tannic acid solution<br>Mercuric chloride solution<br>Saturated basic fuchsin solution<br>Ziehl's carbol fuchsin |
| Pigmentation and proteolytic activity | Nutrient agar + 15% skim milk<br>Pseudomonas agar F | |
| Luminescence production | Yeast peptone broth + 1.5% and 3% NaCl<br>Glycerol calcium carbonate agar + 1.5% and 3% NaCl | |
| $H_2S$ production | Peptone iron agar | |
| Carbohydrate utilization | Hugh and Leifson's medium | Glucose solution (10%)<br>Sucrose solution (10%)<br>Lactose solution (10%) |
| Penicillin sensitivity | Plate count agar containing 2.5 units penicillin per ml | |
| Pteridine sensitivity | Plate count agar | Saturated solution of 2,4-diamino-6,7-diisopropyl pteridine (0/129) |
| Nitrate reduction | Nitrate motility medium | Sulfanilic acid solution<br>Alpha-naphthylamine solution |

Table A1.--Continued.

| Tests | Culture Media | Stains, Indicators, Reagents |
|---|---|---|
| Methyl red test | Proteose broth containing 0.5% glucose (5 days) | Methyl red solution |
| Voges-Proskauer test | Proteose broth containing 0.5% glucose (2 days) | 5% alpha-naphthol in alcohol<br>40% aqueous KOH solution |
| Starch hydrolysis | Nutrient agar containing 1% soluble starch | |

Table A2.--Surface counts and percentages of Bacillus, pigmented microorganisms and molds of deboned fish and fish patties stored at -18°C.

| Time of storage at -18°C* (months) | Dilution factor | Total surface counts (cells/ plate) | Bacillus | | Pigmented microorganisms | | Molds | |
|---|---|---|---|---|---|---|---|---|
| | | | Surface counts (cells/ plate) | % | Surface counts (cells/ plate) | % | Surface counts (cells/ plate) | % |
| 0 : Fresh (deboned fish) | $10^{-2}$ | 85 | 4 | 4.7 | 70 | 82.4 | 2 | 2.4 |
| | | 56 | 3 | 5.4 | 50 | 89.3 | 2 | 3.6 |
| 1 : After thawing | $10^{-2}$ | 55 | 13 | 23.6 | 16 | 29.1 | 1 | 1.8 |
| | | 41 | 17 | 41.5 | 14 | 34.1 | 3 | 7.3 |
| After thawing & refrigeration | $10^{-4}$ | 68 | 15 | 22.1 | 1 | 1.5 | - | - |
| | | 65 | 12 | 18.5 | 2 | 3.1 | - | - |
| 3 : After thawing | $10^{-2}$ | 80 | 49 | 61.3 | 16 | 20.0 | 1 | 1.3 |
| | | 92 | 54 | 58.7 | 17 | 18.5 | 2 | 2.2 |
| After thawing & refrigeration | $10^{-4}$ | 145 | 40 | 27.6 | 4 | 2.8 | - | - |
| | | 140 | 32 | 22.9 | 6 | 4.3 | - | - |
| 6 : After thawing | $10^{-2}$ | 82 | 60 | 73.2 | 8 | 9.8 | 3 | 3.7 |
| | | 89 | 54 | 60.7 | 10 | 11.2 | 3 | 3.4 |
| After thawing & refrigeration | $10^{-4}$ | 63 | 27 | 42.9 | 1 | 1.6 | - | - |
| | | 62 | 16 | 25.8 | 3 | 4.8 | - | - |

*Samples were plated after thawing at room temperature for two hours, and after thawing and refrigeration at 4°C for five days.

Table A3.--Surface counts and percentages of Bacillus, pigmented microorganisms and molds of deboned fish patties with antioxidant stored at -18°C.

| Time of storage at -18°C* (months) | Dilution factor | Total surface counts (cells/ plate) | Bacillus | | Pigmented microorganisms | | Molds | |
|---|---|---|---|---|---|---|---|---|
| | | | Surface counts (cells/ plate) | % | Surface counts (cells/ plate) | % | Surface counts (cells/ plate) | % |
| 0 : Fresh | $10^{-2}$ | 68 | 3 | 5.9 | 55 | 80.9 | 1 | 1.5 |
| (fish patties) | | 77 | 5 | 6.5 | 64 | 83.1 | 3 | 3.9 |
| 1 : After thawing | $10^{-2}$ | 69 | 26 | 37.7 | 20 | 29.0 | 4 | 5.8 |
| | | 64 | 23 | 35.9 | 18 | 28.1 | 2 | 3.1 |
| After thawing | $10^{-4}$ | 71 | 22 | 31.0 | 2 | 2.8 | - | - |
| & refrigeration | | 76 | 18 | 23.7 | 3 | 3.9 | - | - |
| 3 : After thawing | $10^{-2}$ | 45 | 29 | 64.4 | 7 | 15.5 | 2 | 4.4 |
| | | 38 | 20 | 52.6 | 11 | 28.9 | 1 | 2.6 |
| After thawing | $10^{-4}$ | 120 | 15 | 12.5 | 3 | 2.5 | - | - |
| & refrigeration | | 150 | 22 | 14.7 | 4 | 2.7 | - | - |
| 6 : After thawing | $10^{-2}$ | 50 | 32 | 64.0 | 4 | 8.0 | 1 | 2.0 |
| | | 54 | 38 | 70.4 | 10 | 18.5 | 1 | 1.9 |
| After thawing | $10^{-4}$ | 51 | 19 | 37.3 | 2 | 3.9 | - | - |
| & refrigeration | | 49 | 21 | 42.9 | 2 | 4.1 | - | - |

*Samples were plated after thawing at room temperature for two hours, and after thawing and refrigeration at 4°C for five days.

Table A4.--Numbers of bacterial cultures isolated from deboned fish and fish patties.

| Genera | No. of cultures |
|---|---|
| Gram-negative rods: | |
| *Pseudomonas*: Group I | 5 |
| Group II | 9 |
| Group III | 1 |
| Group IV | 1 |
| *Aeromonas* | 4 |
| *Aeromonas*-like | 1 |
| *Photobacterium*-like | 5 |
| *Vibrio* | 5 |
| *Vibrio*-like | 5 |
| *Flavobacterium* | 17 |
| *Lucibacterium*-like | 1 |
| *Moraxella*-like: M1 | 6 |
| M2 | 6 |
| Unidentified: X1 | 3 |
| X2 | 1 |
| Gram-negative cocci and coccobacilli: | |
| *Branhamella* | 7 |
| *Acinetobacter* | 1 |
| *Neisseria* | 3 |
| *Neisseria*-like | 7 |
| Gram-positive rods: | |
| *Bacillus* | 56 |
| *Arthrobacter* | 2 |
| Gram-positive cocci: | |
| *Micrococcus*-like | 1 |
| *Pediococcus* | 1 |
| *Pediococcus*-like | 3 |
| Unidentified: X3 | 2 |
| Total | 153 |

Table A5.--Characteristics of polar-flagellate, gram-negative rods isolated from deboned fish and fish patties.

| Tests | *Pseudomonas* (I, II, III and IV) | *Aeromonas* and *Photobacterium*-like organisms | *Vibrio* and *Vibrio*-like organisms |
|---|---|---|---|
| Morphology: 24 hours and 5 days | Rods (straight) | Rods | Short rods (straight, curve) |
| Growth on MacConkey agar | + | + | + |
| Spore | - | - | - |
| Oxidase | + | + | + |
| Catalase | + | + | + |
| Motility | + | + | + |
| Flagella | Polar (monotrichous) | Polar (mono., *Aero.* and *Photo.*) (lopho., *Photo.*) | Polar (monotrichous) |
| Pigments | Yellow or - | Brown (*Aero.*) - (*Aero.*, *Photo.*) | Yellow, orange or - |
| Fluorescent pigments | + (I) | - | - |
| $H_2S$ production | - | - | - |
| Carbohydrate metabolism: | | | |
| Glucose | Oxid. (I, II) Alkaline reaction (III) No reaction (IV) | Fermented (+ gas) | Fermented |
| Sucrose | Same or - | Fermented (+ gas) | Fermented |
| Lactose | Same or - | Fermented (+ gas) or - | Fermented or - |
| Penicillin sensitivity | - | - | - (*Vibrio*) + (*Vibrio*-like) |
| Pteridine sensitivity | - | - (*Aero.*) + (*Photo.*) | + |
| Nitrate reduction | + or - | + | + |
| Proteolytic activity | + or - | + (*Aero.*, *Photo.*) - (*Photo.*) | + or - |

Table A5.--Continued.

| Tests | *Pseudomonas* (I, II, III and IV) | *Aeromonas* and *Photobacterium*-like organisms | *Vibrio* and *Vibrio*-like organisms |
|---|---|---|---|
| Voges-Proskauer test | N.D.* | + (*Photo.*) | + or - |
| Methyl-red | N.D. | + (*Photo.*) | + or - |
| Starch hydrolysis | N.D. | + (*Aero.*)<br>- (*Photo.*) | N.D. |

*N.D. = Not done.

Table A6.--Characteristics of peritrichous-flagellate, gram-negative rods isolated from deboned fish and fish patties.

| Tests | Flavobacterium | Lucibacterium-like organisms |
|---|---|---|
| Morphology: 24 hours and 5 days | Rods (small, slender) | Rods |
| Growth on MacConkey agar | + or - | - |
| Spore | - | - |
| Oxidase | + | + |
| Catalase | + | + |
| Motility | + or - | + |
| Flagella | Peritrichous | Peritrichous |
| Pigments | Yellow, orange, or red | Orange |
| $H_2S$ production | - | - |
| Carbohydrate metabolism: | | |
| Glucose | Oxidized or - | Fermented |
| Sucrose | Oxidized or - | Fermented |
| Lactose | Oxidized or - | - |
| Penicillin sensitivity | N.D. | + |
| Pteridine sensitivity | N.D. | - |
| Nitrate reduction | + or - | - |
| Proteolytic activity | + or - | + |
| Voges-Proskauer test | N.D. | - |
| Methyl-red | N.D. | + |
| Starch hydrolysis | N.D. | + |

Table A7.--Characteristics of nonmotile, gram-negative rods isolated from deboned fish and fish patties.

| Tests | Moraxella-like org. (M1, M2) | Aeromonas-like organisms | Unidentified (X1, X2) |
|---|---|---|---|
| Morphology: 24 hours and 5 days | Short rods (singly, in pairs, short chains) | Short rods (short chains) | Rods (singly, short chains) |
| Growth on MacConkey agar | + (M1)<br>- (M2) | + | - (X1)<br>+ (X2) |
| Spore | - | - | - |
| Oxidase | + | + | + |
| Catalase | + | + | + |
| Motility | - | - | - |
| Pigments | - | - | Yellow-orange (X1)<br>- (X2) |
| $H_2S$ production | - | - | - |
| Carbohydrate Metabolism: | | | |
| Glucose | - | Fermented | Fermented (X1)<br>Oxidized (X2) |
| Sucrose | - | Fermented | Ferm. or - (X1)<br>- (X2) |
| Lactose | - | - | Ferm. or - (X1)<br>Oxidized (X2) |
| Penicillin sensitivity | - | - | - (X1)<br>+ (X2) |
| Pteridine sensitivity | N.D. | - | + (X1)<br>- (X2) |
| Nitrate reduction | + (M1)<br>- (M2) | + | - |
| Proteolytic activity | - | - | + (X1)<br>- (X2) |
| Starch hydrolysis | N.D. | + | N.D. |

Table A8.--Characteristics of gram-negative cocci and coccobacilli isolated from deboned fish and fish patties.

| Tests | *Branhamella* | *Acinetobacter* | *Neisseria* and *Neisseria*-like organisms |
|---|---|---|---|
| Morphology: 24 hours and 5 days | Cocci (in pairs, short chains) | Coccobacilli (in pairs) | Cocci (singly, in pairs) |
| Growth on MacConkey agar | + | + | + |
| Spore | - | - | - |
| Oxidase | + | - | + |
| Catalase | + | + | + |
| Motility | - | - | - |
| Pigments | - | - | Yellow or - |
| $H_2S$ production | - | - | - |
| Carbohydrate metabolism: | | | |
| Glucose | - | - | Oxidized or - |
| Sucrose | - | - | Oxidized or - |
| Lactose | - | - | Oxidized or - |
| Penicillin sensitivity | + | - | + (*Neisseria*)<br>- (*Neisseria*-like) |
| Pteridine sensitivity | N.D. | N.D. | N.D. |
| Nitrate reduction | + | - | + or - |
| Proteolytic activity | - | - | + or - |
| Voges-Proskauer test | N.D. | - | N.D. |
| Methyl-red | N.D. | - | N.D. |

Table A9.--Characteristics of gram-positive rods isolated from deboned fish and fish patties.

| Tests | Bacillus | Arthrobacter |
|---|---|---|
| Morphology: | | |
| 24 hours | Rods (singly or short chains) | Rods (singly or short chains) |
| 5 days | Rods | Cocci |
| Growth on MacConkey agar | - | - |
| Spore | + (endospores) | - |
| Oxidase | + | + |
| Catalase | + | + |
| Motility | + or - | - |
| Flagella | Peritrichous | - |
| Pigments | Cream-yellow or - | - |
| $H_2S$ production | - | - |
| Carbohydrate metabolism: | | |
| Glucose | Fermented | Oxidized |
| Sucrose | Fermented | Oxidized |
| Lactose | Fermented | - |
| Penicillin sensitivity | N.D. | N.D. |
| Pteridine sensitivity | N.D. | N.D. |
| Nitrate reduction | + or - | - |
| Proteolytic activity | + | - |

Table A10.--Characteristics of gram-positive cocci isolated from deboned fish and fish patties.

| Tests | *Micrococcus*-like organisms | *Pediococcus* and *Pediococcus*-like org. | Unidentified (X3) |
|---|---|---|---|
| Morphology: 24 hours and 5 days | Cocci (irregular clumps) | Cocci (in pairs or tetrads) | Cocci (singly, in pairs or clumps) |
| Growth on MacConkey agar | - | - | - |
| Spore | - | - | - |
| Oxidase | + | - (*Pedio.*)<br>+ (*Pedio.*-like) | + |
| Catalase | + | + (weak) | + |
| Motility | - | - | - |
| Pigments | - | Yellow-orange | Yellow-orange |
| $H_2S$ production | - | - | - |
| Carbohydrate metabolism: | | | |
| Glucose | - | Fermented | Fermented |
| Sucrose | - | - (*Pedio.*)<br>Ferm. (*Pedio.*-like) | - |
| Lactose | - | - | - |
| Penicillin sensitivity | N.D. | N.D. | N.D. |
| Pteridine sensitivity | N.D. | N.D. | N.D. |
| Nitrate reduction | + | - | + |
| Proteolytic activity | + | + | - |
| Starch hydrolysis | - | - | N.D. |