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Numerical taxonomy and changes of the bacterial flora on frozen chicken

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Numerical taxonomy and changes of the bacterial flora on frozen chicken

by

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INTRODUCTION

Nearly 90 percent of the fresh, ready-to-cook chicken produced in the United States is distributed and marketed unfrozen-refrigerated. Refrigerated chicken has a short shelf life (Ayres et al., 1950) and requires a rapid turnover. The freezing of chicken would greatly extend holding times for marketing; but the American consumer prefers refrigerated over frozen chicken even though other poultry such as turkey and cornish hens are well accepted as frozen products.

Tissue damage and drip loss are some of the factors that seem to limit the application of freezing for marketing ready-to-cook chicken. Spray freezing with liquid nitrogen seems to result in improved general appearance for many frozen food products. Li et al. (1969) and Kuschfeldt and Thiel (1970) observed some decrease in drip loss and improved general appearance on chicken frozen with liquid nitrogen as compared to air blast freezing. The freezing of chicken with liquid nitrogen for marketing and its acceptance by the consumer are possibilities to be explored.

In spite of the apparent preference for fresh refrigerated chicken, with the abundance of home freezing facilities, it is a widespread practice to select fresh, unfrozen chicken at the retail store and keep it at home under frozen conditions for undetermined periods of time. Under such circumstances, it is entirely possible that during days of slow turnover not so fresh chicken can be chosen at the supermarket to be held under frozen conditions in the domestic freezer for several weeks or months. This creates an interest in

studying the effect that freezing can have on the natural bacterial flora of chicken.

Bacterial death due to freezing causes some reduction in numbers of bacterial populations (Kitchell and Ingram, 1956; Elliott and Michener, 1960; Rey et al., 1969). Freezing may also cause non-lethal injury to bacterial cells which is detected by changes in their metabolic functions (Straka and Stokes, 1959). Arpai (1962) established that restoration of growth and normal functions of bacteria surviving freezing depend to a large extent on the suspending medium. In general, bacteria which remain viable in frozen foods grow faster when the food is defrosted than they would have done under similar conditions before the food was frozen (Mossel and Ingram, 1955; Kitchell and Ingram, 1956). Chicken, in particular, seems to provide excellent conditions for recovery of bacteria surviving freezing. According to Spencer et al. (1955) the overall effect of freezing chicken is that the number of days to spoilage after thawing broilers is the same as for their unfrozen counterparts.

The purpose of the present investigation was to compare changes in bacterial numbers and in incidence of potential pathogens on chicken frozen by mechanical refrigeration and by spraying with liquid nitrogen. Numerical taxonomy and multiple inoculation procedures were employed to detect changes in type of bacterial flora due to freezing and freezing method.

LITERATURE REVIEW

The Poultry Industry

Chicken in our diet

Strandine (1963) reviewed developments in the poultry industry which have made available improved varieties of eggs and meat-type poultry. Consumer demands for poultry meat have paralleled these improvements. Figure 1 shows that the production of chicken in the United States tripled in the last 20 years while retail prices have decreased. This indicates an industry which has grown in efficiency as well as in production. At the present time, chicken makes an important contribution to the supply of meat in the American diet. Table 1 shows per capita consumption in the United States of various foods that are considered good sources of protein. Chicken consumption steadily increased from 1960 through 1972 while other protein sources such as eggs and fluid milk decreased. From the trends in price and consumption during the last few years, a future increase in consumption of chicken can be predicted.

Development of the industry

Strandine (1963) pointed out many technological advances in the production of poultry that have reinforced the improvements on poultry breeding and have resulted in a continuous growth of the industry. Commercial hatcheries appeared. The earliest broiler growing area located in Delaware, Maryland and Virginia expanded. Even though feed supply and accessibility to market are important

Figure 1. Production and price of chicken in the United States from 1950 through 1972. Includes chicken from commercial sources plus chicken sold from and consumed on farm plus non-farm production

A. U.S. Dept. of Agric. (1973b, Table 30)
B. U.S. Dept. of Agric. (1973b, Table 45)

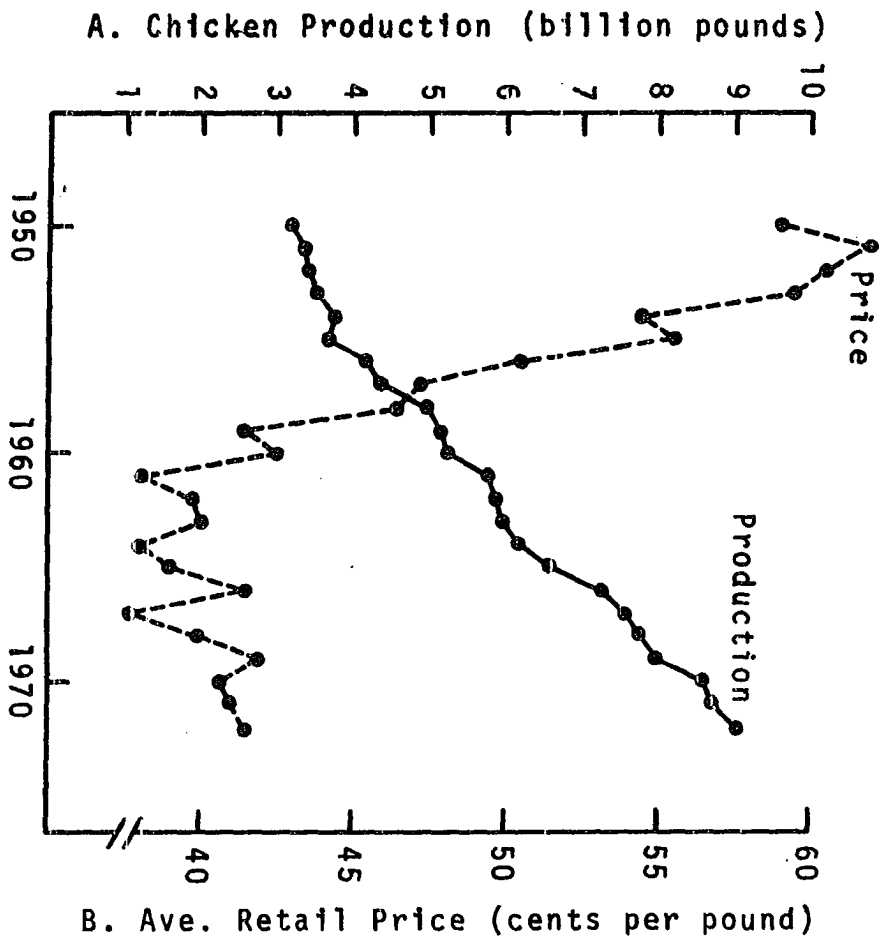

A. Chicken Production (billion pounds)
1
2
3
4
5
6
7
8
9
10
1950
1960
1970
Price
Production
40
45
50
55
60
B. Ave. Retail Price (cents per pound)

Table 1. Per capita consumption of several protein source foods in the United States from 1960 through 1972[a]

Year	Beef and veal[b]	Pork[b]	Chicken[b]	Turkey[b]	Lamb and mutton[b]	Eggs[c]	Hard cheese[d]	Fluid milk and cream (milk equiv.)[d]
	(lbs)	(lbs)	(lbs)	(lbs)	(lbs)	(numbers)	(lbs)	(lbs)
1960	91	65	28	6	5	324	8	322
1961	93	62	30	7	5	328	9	312
1962	94	64	30	7	5	326	9	308
1963	99	65	31	7	5	317	9	307
1964	105	65	31	7	4	318	9	304
1965	105	59	33	8	4	314	10	302
1966	109	58	36	8	4	312	10	297
1967	110	64	37	9	4	320	10	285
1968	113	66	37	8	4	316	11	280
1969	114	65	39	8	3	310	11	272

1970	117	66	42	8	3	311	11	264
1971	116	73	41	9	3	314	12	259
1972	118	67	43	9	3	307	13	258

[a]Total disappearance divided by July 1st population.

[b]U.S. Dept. of Agric. (1973b, Table 33).

[c]U.S. Dept. of Agric. (1973b, Table 32).

[d]U.S. Dept. of Agric. (1973a, Table 6).

factors the geographic development of broiler growing areas seems to have been influenced to a great extent by climatic conditions. Low temperatures are detrimental to broiler raising. Feed consumption and conversion are reflected in production cost and are greatly dependent on temperature. Statistics from the Department of Agriculture (U.S. Dept. of Agric., 1973b) show that almost 90 percent of the broilers produced in 1972 were raised in the Southern Atlantic and Southern Central regions. Location of processing plants specializing in supplying broilers to the consumer market is determined by the availability of poultry in the area. Thereby 90 percent of the broilers sold in the United States in 1972 and 1973 were processed in and transported from the South-eastern and South-central regions.

Processing operations

Processing plants are highly mechanized and line-operated. Until 1972 mandatory federal inspection of the processing plants was required if the product was to be sold in interstate commerce. The Poultry Products Inspection Regulations (Federal Register, 1972) have made inspection for wholesomeness mandatory for all poultry processed for commerce. Inspection for U.S. quality grade is also available on a voluntary basis.

The Poultry Products Inspection Regulations also provide for reinspection of poultry during and/or after cutting, packaging and preparation of further processed poultry products, inspections for establishment requirements, shipping requirements and disposition of condemned products.

According to Childs et al. (1970) a schematic flow of poultry processing operations is as follows:

Antemortem inspection

Unloading and hanging

Slaughter: shackle and bleeding

Defeathering: scalding

mechanical picking

singeing

pinning

washing

Automatic feet cutting

Evisceration: removal of uropial gland, cut around vent, abdominal cut and pull out viscera

postmortem inspection

removal of liver, gizzard, heart, head and alimentary canal

slitting of neck and removal of lungs

vacuum removal of lungs

cut-off neck

washing

Chilling

Juiciness, flavor and delay of bacterial spoilage of poultry greatly depend on the efficiency of the chilling operation. Onset of rigor takes place shortly after dressing and lasts from 8 to 14 hours. Freezing or cooking birds while muscles are in rigor results in tough meat; thus freezing and cooking are normally delayed until resolution of rigor and are usually preceded by chilling (Strandine, 1963).

Whole birds can be chilled in ice slush in tanks with air agitation but in most instances chillers operated on continuous flow of ice-water or ice-water with slush-ice have replaced the bulk type chiller (Childs et al., 1970).

Poultry may be sold whole or cut-up as halves, quarters, or parts, or it can be further processed into a large variety of special poultry products. In the past, most cutting up and tray-packaging was done at the retail level. Still an undetermined but probably considerable proportion of the broilers distributed as whole by the processing plants are cut-up by stores. Nineteen percent of the chicken produced in 1965 was cut up at plant level. This proportion has steadily increased for the last 8 years. Breakdown of the production of chicken for 1972 and 1973 (U.S. Dept. of Agric., 1974) shows that close to one-third of the chicken produced in the United States is presently cut up at processing plants. The actual trend is then for centralized cutting operations at the plant level.

Chilled and frozen chicken

Whole birds are usually packed in wooden crates or waxed fiber board boxes (20 to 30 head per box) and covered with ice for shipping. Ice is spread over the boxes after loading the trucks and replenished as needed while in route.

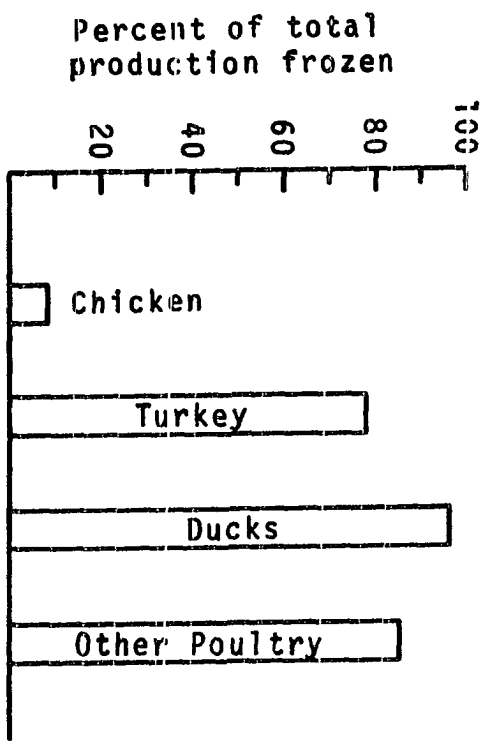
Data plotted in Figure 2 shows that the greatest proportion of broilers entering the consumer market in the United States is chilled. All chicken for exportation is frozen. Exported chicken is included as a part of the figures on frozen chicken presented in the figure as reported by the U.S. Dept. of Agric. (1974). The amount of frozen chicken sold per year since 1960 has been very steady and most of it is presumably used by food service establishments and institutions with very small amounts of frozen chicken, if any, entering the retail market.

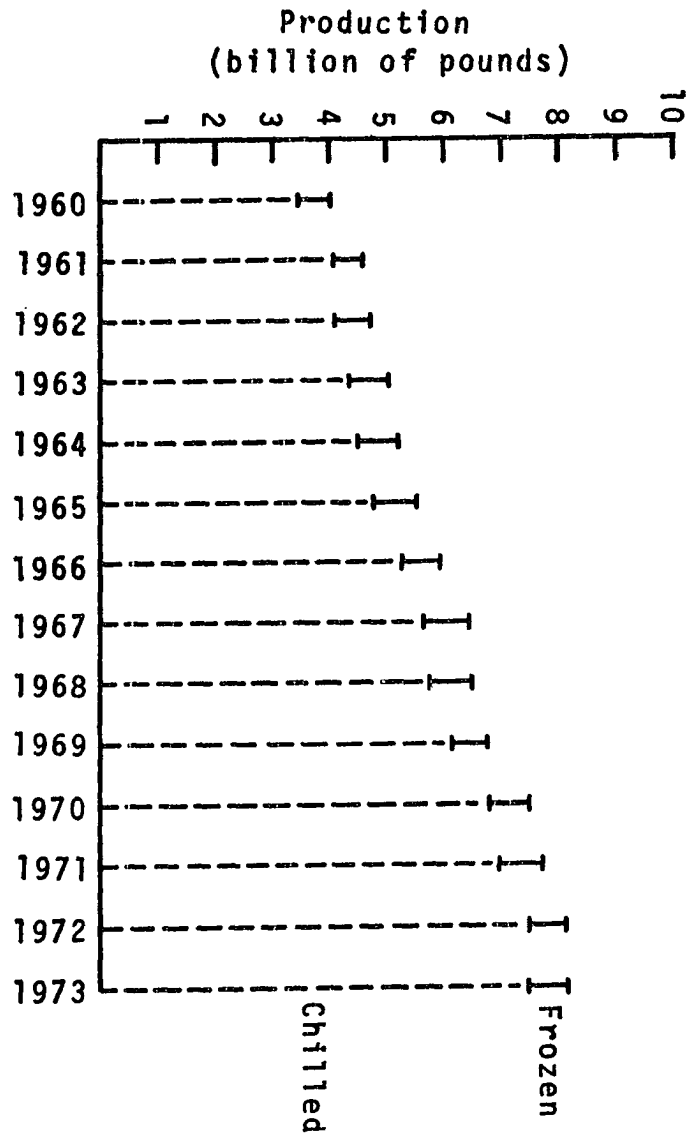
The United States has the largest per capita consumption of frozen foods around the world. Data from the Quick Frozen Foods Institute (1972) show that 76-80 lbs of frozen foods were consumed per capita in the United States in 1970. These figures are more than double the consumption in Sweden (33-36 lbs per capita) which ranked second in the world in order of frozen food consumption.

Percentages of the total production of different types of poultry that were frozen at the processing plant in 1973 are presented in Figure 3 (U.S. Dept. of Agric., 1974). It is evident that in spite of the high consumption of frozen foods and the acceptance of frozen turkeys, ducks and other poultry, the domestic

Figure 2. Chicken chilled and frozen at the plant level in the United States from 1962 through 1973 (J. E. Cochrane, Head of Poultry Section, Statistical Reporting Service, U.S. Dept. of Agric., personal communication, April 5, 1974)

Figure 3. Percent of total production of different types of poultry inspected for freezing in the United States in 1973 (U.S. Dept. of Agric., 1974)


Percent of total production frozen
20
40
60
80
100
Chicken
Turkey
Ducks
Other Poultry


Production (billion of pounds)
1
2
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4
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6
7
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9
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1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
Chilled
Frozen

market in the United States prefers chilled over frozen chicken. Dawson (1969) showed that better flavor is the leading reason given in several consumer surveys for preference of chilled over frozen fryers. Turkeys are less stable than chicken under frozen storage due to the high susceptibility of turkey fat to the development of rancidity (Mecchi et al., 1956). However, turkey is well accepted as a frozen product which indicates that factors other than flavor are involved in the preference for chilled over frozen chicken.

Bone and meat darkening is a problem related to frozen broilers. This defect does not affect aroma, flavor or texture of the cooked meat (Ellis and Woodroof, 1959). Bone discoloration seldom occurs in older chickens and has not been reported for turkeys, ducks or geese (Dawson, 1969). The defect is due to rupture of blood vessels during freezing. Upon thawing, bloody discoloration appears around the bones in the vicinity of the joints where the most tender growing tissues are present (Brant and Stewart, 1950). Cooking oxidizes the hemoglobin to methemoglobin developing the dark color. It is then understandable why broilers, being young birds of fast growing varieties which usually have not reached full maturity when slaughtered, are most susceptible to bone darkening.

The influence of bone darkening on the acceptance of frozen broilers by the consumer is questionable. United States is the major producer of household freezers in the world (Officiel Mag. Arts Menang., 1972). Bone darkening is related to rate of freezing

and is very prominent at the slow freezing rate of household freezers (Ellis and Woodroof, 1959). However, fresh broiler quantity lots sold as chilled chicken are frequently preserved in home freezers for later use.

Color can greatly affect consumer preference. Light colored poultry meat is favorably associated with degree of finishing and is preferred by the majority of the consumers (Dawson, 1969). Frozen chicken has a dark appearance. This results from changes taking place in both the skin and the surface layer of the flesh. Klose and Poole (1956) found that during defeathering part of the epidermal layer of the skin is removed increasing the transparency of the frozen skin. Baker (1954) observed a darkening of color of the flesh by freezing. Similar to bone darkening, flesh darkening is inversely related to rate of freezing. Li et al. (1969) and Kuschfeldt and Thiel (1970) reported improved general appearance and decrease in bone discoloration for poultry frozen with liquid nitrogen as compared to air blast. Clough (1969) reports that chicken portions frozen by spraying with liquid nitrogen to form a crust and them moved into regular freezers have met customer acceptance in the United Kingdom.

Review on Freezing

Freezing applied to food

Freezing is regarded as the long term preservation method that renders food the closest to its original quality. During freezing ice is formed at the expense of the water content of the food.

Solidification of the moisture present hinders biological and chemical changes in the food. Upon thawing, the inability to reestablish the original aqueous system results in dripping which is the major defect observed with frozen foods.

Most foods can be damaged if frozen too fast (Morphew, 1969); but it has been observed that increasing the rate of freezing within certain limits results in less structural damage, better taste, and less drip loss for many food products. This has been reported for meats (Gray, 1967; Bengtsson and Jakobsson, 1969), fish (Piskarev and Bomovalova, 1969), baked goods (Breyer et al., 1966), peppers, green beans, cauliflower (Monzini et al., 1969), mushrooms (Åström and Löndahl, 1969), tomatoes (Anonymous, 1964) and many other foods.

Development of fast food freezing

There are no true limits between slow, quick or ultra fast freezing (Somn, 1969). The speed at which each food product is frozen is determined by microbiological stability, hygienic considerations and consumer acceptance.

Average freezing times for poultry with conventional type refrigeration have been reported to range from as much as 30 hours for still air to 5 hours for air blast freezing (Ryan, 1966). A search for improvement of quality has led to the development of faster freezing methods. Carbon dioxide sublimizes at -78.5 C. The use of carbon dioxide as a refrigerant started to develop in the early 30's. Freezing systems with solid carbon dioxide require

mechanical action such as tumbling to increase contact between refrigerant and food. These types of freezers even when offering high freezing rates lend themselves to particular type of foods and have not been widely used for commercial food freezing.

The first attempt at freezing foods with liquids of extremely low boiling points were made in the decade of the 40's by immersion of food in nitrous oxide (Breyer et al., 1966). Technological developments in the aerospace industry, chemical industry, welding processes, steel industry and the medical field, have created a great demand for liquid oxygen. Liquid oxygen is mainly produced by electrolytic separation from air. From this process, argon and liquid nitrogen are separated as by-products. By 1960 the production of liquid nitrogen per year in the United States surpassed 10 billion standard cubic feet which allowed for consideration of this cryogenic fluid as an expendible refrigerant (Flynn and Smith, 1970).

Liquid nitrogen boils at -196 C. When boiling in contact with food it absorbs 86 BTU per pound (heat of vaporization). The gas produced continues to absorb heat at the rate of 0.45 BTU/pound/degree centigrade until the temperature of the food and the temperature of the gas equilibrate. From this comes the tremendous potential of liquid nitrogen as a refrigerant. Immersion techniques for freezing with liquid nitrogen have not been satisfactory (Breyer et al., 1966). The thermal shock results in cracking and peeling of many products, the cold gases are wasted with inefficient use of the refrigerant and violent boiling at the surface of the product

prevents efficient contact between the refrigerant and the food. Most liquid nitrogen freezers employ spray methods (Novak and Ramachandra Rao, 1966). They are basically conveyor-type tunnels in which the product flows counter current to the refrigerant. Liquid nitrogen is sprayed close to the outlet while the cold gas circulates toward the inlet. Pre-cooling of the product by nitrogen gas conditions it for the low temperature of the spray zone diminishing the effect of thermal shock. The aim of these processes is to supercool the outer shell which freezes while the core temperature is still warm. After spraying the products are stored under conventional refrigeration where the core and the surface temperature reach equilibrium achieving freezing throughout the product in a very short time. The temperature at equilibrium depends on the depth at which the outer shell is frozen as well as on the temperature at which the product is stored.

It is unlikely that freezing as applied to foods would require temperatures below -23.3 C (-10 F). Somm (1969) made a survey on international legislation for frozen foods. He found that a storage temperature of -18 C was generally accepted. In the United States, provisions for freezing poultry are included in Poultry Products Inspection Regulations (Federal Register, 1972). Poultry labeled "frozen fresh", "quick frozen" or "fresh frozen" shall be maintained at -17.8 C (0 F) or less. The rate of freezing shall be such as to bring the internal temperature to -17.8 C within 72 hours for ready-to-cook poultry.

Evaluation of liquid nitrogen freezing

Liquid nitrogen freezing is an expensive process. Trauberman (1966) estimated freezing cost to be 1.5 to 2.5 times more expensive with liquid nitrogen than with conventional refrigeration. Over the years these proportions might have changed but the author has not found any nonpartisan reference on the subject more recent than that of Trauberman.

The price per standard cubic foot of liquid nitrogen decreases with increasing volume for each delivery. The storage tank, which is usually owned and maintained by the industrial gas supplier is rented to the food plant on a monthly basis. The larger the capacity of the tank the lower the rent paid per cubic feet. Therefore, large scale operations and continuous production reduce the cost of the freezing process. The location of the food plant is an important factor in determining operational cost since distribution cost, which is added to the basic price of liquid nitrogen, increases proportionally to distance of transportation. Other factors such as efficiency of storage and refrigeration equipment, shape and size of the food, and use of packaging films influence operational cost (Brown, 1967). The actual cost for liquid nitrogen freezing can be determined only by analysis of all factors involved in each individual case.

Commercial freezing with liquid nitrogen has constantly expanded not only in the United States but also in Canada, Japan, South Africa, Israel, Scandinavia, France, Belgium, Holland, Russia,

Germany, Ireland and the United Kingdom (Clough, 1969). This indicates that there are advantages with liquid nitrogen freezing that can off-set the high cost of the operation. Clough points out that mechanical simplicity, minimum labor, adaptability to full automatic operations, low investment cost, reduction of floor space, rapidity of freezing, quick adjustment to different rates of production and the suitability of liquid nitrogen systems to produce individual-quick-frozen products are some of the advantages of liquid nitrogen over conventional freezing.

Application of liquid nitrogen to poultry

A weighted analysis on the economic and technical aspects seems to be necessary before ascribing a freezing method to a certain food. Freezing meat by conventional methods is generally satisfactory. But, a reduction of drip loss and improved water-holding-ability are reported for meats with increased freezing rates. A disadvantage of ultra-fast freezing of red meats with liquid nitrogen is that they develop a light color (Anonymous, 1967). The development of a light color is due to formation of very fine ice crystals at the surface which increases reflectance. A quartz infrared unit is added to tunnel systems for red meats. Short exposure to infra-red heating melts the ice crystals at the surface without impairment of the frozen state. A light color is a desirable property for poultry meat. The lightening of color, regarded as a defect for red meats, would be an advantage for poultry, especially chicken,

in meeting consumer acceptance since it would balance the red discoloration observed on frozen chicken.

Microbiological Aspects

Microbial contamination of poultry

Even with the highest standards for processing, the presence of microorganisms on poultry is unavoidable. The sanitary conditions under which poultry is processed influences the numbers (Walker and Ayres, 1956; Wilkerson et al., 1961) as well as the kinds (Nagel et al., 1960) of contaminants initially present. If these contaminants are allowed to grow, they cause spoilage and possibly health hazards.

Microbial growth and survival in relation to temperature

Microorganisms are broadly classified as thermophiles, mesophiles and psychrotrophs according to their ability to grow at high, intermediate or low temperatures. Minimum growth temperature for most mesophiles is between 10 to 15 C, while some thermophiles have minimal growth temperatures as high as 40 to 45 C (Stanier et al., 1970). Psychrotrophs have considerably lower minimal temperatures than mesophiles and are the major problem with refrigerated foods.

Microbial growth has been reported at temperatures as low as -18 C (Sulzbacher, 1950); but it is generally agreed that for practical purposes freezing at -10 C (14 F) controls bacterial growth, -12 C (10 F) controls yeasts and -18 C (0 F) controls fungi

(Institut International du Froid, 1972). Microorganisms, however, can contribute to deterioration of frozen foods even at temperatures at which growth is restricted. Some extracellular enzymes, if released prior to freezing, can remain active at temperatures as low as -18 C (Rey et al., 1969). Such enzymes would continue their catalytic action in the frozen food even though at a reduced rate (Institut International du Froid, 1972).

Studies of Mazur (1966) in bacteriological media and of Michener and Elliot (1969) in foods show that 90 to 99 percent of the bacterial populations die at, or near, the freezing point. In these studies, when the bacterial suspension or the food was stored frozen for considerable periods of time, bacterial numbers continued to decrease but at a reduced rate. In some cases, these authors observed that bacterial numbers leveled off after a few weeks of frozen storage, remaining stationary for several months.

Average numbers of total aerobic bacteria on poultry right after slaughter were reported to be 35,000 per cm^2 by Ayres et al. (1956) and 60,000 by Gunderson et al. (1954). To bring the effect of freezing into perspective, if birds are frozen immediately after slaughter and a reduction in numbers of 99 percent takes place upon freezing, still 350 to 600 aerobic bacteria per cm^2 might remain viable. Consequently there would be enough bacteria present in the frozen poultry to resume growth after thawing. In fact, it has been found that the reduction in numbers taking place during freezing and frozen storage has no practical significance. The food usually

spoils as fast after thawing as if it had never been frozen (Spencer et al., 1955; Kitchell and Ingram, 1956).

Storage life of chilled versus frozen chicken

According to the Poultry Products Inspection Regulations (Federal Register, 1972) poultry shall be chilled to an internal temperature of 4.4 C (40 F) within 6 hours after processing, if chilled in ice or ice-water, or within 24 hours if placed in a refrigerated room for air chilling. Chilled poultry is shipped and stored at retail stores under refrigeration. Under these conditions spoilage is mainly caused by development of a psychrotrophic bacterial flora in which spoilage types of Pseudomonas species predominate (Barnes and Shrimpton, 1959; Ayres, 1960; and Michener and Elliott, 1969). A musty-fermented odor and eventually slime develops on the product frequently accompanied by a greenish-fluorescent discoloration (Kraft and Ayres, 1961). These changes are defined as "decomposition" by the Poultry Products Inspection Regulations and are cause for condemnation of the product.

Ayres et al. (1950) found that an average shelf life of 1 to 2 weeks was feasible for chilled poultry when the temperature of storage was maintained close to 0 C (32 F); but for every degree centigrade increase in temperature of storage, the shelf life decreased almost exponentially. In fact, under commercial conditions, chilled chicken spoils very fast and requires a rapid turnover (Michener and Elliott, 1969).

The shelf life of chicken is greatly extended under frozen storage and will vary from 6 to 12 months or longer (Strandine, 1963). The changes in quality that limit shelf life under frozen conditions are correlated with oxidative rancidity rather than with bacterial spoilage (Dawson, 1969).

Role of chicken in food borne illness

On the basis of the implications that microorganisms can have on food-borne disease, the presence of specific types is more relevant than the total concentration of bacteria present.

Bacterial pathogens have been the most common etiological agents of food-borne illness reported in the United States from 1968 through 1970 (Table 2). The bacterial agents most frequently and consistently associated with chicken during this period were Staphylococcus, Clostridium perfringens and Salmonella. C. perfringens food poisoning involved the largest number of cases for all foods, followed in decreasing order by staphylococcal food poisoning and salmonellosis. In 1968 two outbreaks of botulism and two of shigellosis resulting from ingestion of chicken were reported. In the following years shigellosis and botulism were reported for other foods but not for chicken.

Food eaten away from home predominated in the outbreaks involving C. perfringens, Staphylococcus and Salmonella. The factors contributing to these food borne outbreaks as reported in decreasing order of frequency were: 1) improper holding temperature; 2) poor personal hygiene of food handlers; 3) contamination from equipment and 4) inadequate cooking.

Table 2. Foodborne outbreaks reported for chicken as vehicle of infection from 1968 through 1972 as compared to outbreaks and cases reported for all foods for each of the etiological agents associated with chicken[a]

Etiological agent	Vehicle	Cases or outbreaks	Year				
			1968	1969	1970	1971	1972
C. perfringens	Chicken	Outbreaks	6	4	2	3	4
	All foods	Outbreaks	56	65	54	51	9
	All foods	Cases	5,966	18,527	6,952	3,856	973
Staphylococcus	Chicken	Outbreaks	6	7	7	4	3
	All foods	Outbreaks	90	94	102	92	34
	All foods	Cases	4,419	3,481	4,699	5,115	1,948
Salmonella	Chicken	Outbreaks	2	7	5	1	3
	All foods	Outbreaks	44	49	48	30	36
	All foods	Cases	1,287	1,892	4,747	760	1,880
C. botulinum	Chicken	Outbreaks	2	0	0	0	0
	All foods	Outbreaks	9	10	7	9	4
	All foods	Cases	10	17	14	21	24
Shigella	Chicken	Outbreaks	2	0	0	0	0
	All foods	Outbreaks	7	10	8	7	3
	All foods	Cases	407	1,444	1,668	906	86

Multiple	Chicken	Outbreaks	2	0	0	0	0
	All foods	Outbreaks	22	1	0	4	0
	All foods	Cases	-[b]	30	-[b]	153	-[b]
Chemical	Chicken	Outbreaks	2	0	0	0	0
	All foods	Outbreaks	22	28	28	30	28
	All foods	Cases	113	172	295	220	146
Unknown	Chicken	Outbreaks	6	5	3	2	19
	All foods	Outbreaks	85	83	99	81	165
	All foods	Cases	2,441	2,310	3,388	1,103	-[b]

[a]U.S. Dept. Health, Education and Welfare (1973, 1972, 1971, 1970, 1969).

[b]No data available.

The bacterial pathogens associated with chicken

Botulism resulting from ingestion of chicken is quite rare in the United States. Data from the U.S. Department of Health, Education, and Welfare (1968a, and 1968b) show that from 679 outbreaks reported for all foods from 1899 through 1972 only 3 were attributed to chicken. Greenberg *et al.* (1967) reported the isolation of *C. botulinum* spores from chicken sampled right after slaughtering at the processing plant.

C. botulinum is the most dreadful of the bacterial pathogens causing food borne illness. The two outbreaks resulting from ingestion of chicken reported in 1968 involved three people and caused two deaths (U.S. Department of Health, Education, and Welfare, 1968a, 1968b). This organism being an anaerobe is likely to grow in hermetically sealed containers or in the interior of foods not hermetically packed if oxygen fails to penetrate. It forms endospores with sufficient heat resistance to survive the heat processes normally used in the preparation of precooked frozen foods (Michener and Elliott, 1969). The spores are capable of surviving prolonged frozen storage. In fact, the two outbreaks reported in 1968 for chicken as the vehicle of infection were caused by chicken stew and liver paste, both prepared from frozen chicken.

To produce toxin, which is the direct cause of the disease, the spores have to germinate and grow. Minimum growth temperature for *C. botulinum* type A and type B is 10 C (Rieman, 1969). These two types are those that have been isolated from the 3 outbreaks of

botulism resulting from ingestion of chicken reported in the United States since 1899. C. botulinum type E, which has a fairly low minimal temperature (3 C), produces spores with very low heat resistance. The outbreaks of botulism type E reported have been related to fish, uncooked or with very little heat treatment (Sakaguchi, 1969).

A history of gross temperature abuse was reported for the two outbreaks of botulism from chicken occurring in 1968. Adequate refrigeration is a good safety measure in preventing botulism.

C. perfringens is frequently found in foods. The spores of C. perfringens are widely distributed in feces, soil and dust (Breed et al., 1957). This organism was confirmed as a food poisoning agent for 36,274 cases from 1968 through 1972 (Table 2). Fortunately the disease is generally mild and of short duration. It is caused by ingestion of large numbers of cells and is characterized by abdominal cramps and diarrhea.

C. perfringens is microaerophilic and produces endospores that are more heat resistant than the vegetative form of the organism (Hobbs, 1969). Minimal temperature for growth is 6.5 C as reported by Hobbs (1969). Vegetative forms do not survive well under refrigeration but the spores can survive prolonged frozen storage (Despaul, 1964).

Cooking eliminates competition from the natural saprophytic bacteria that might be present in the food since, in general, they are more sensitive to heat than the spores of C. perfringens.

Heating also drives off gases lowering the oxygen tension in the food and heat activates the spores. The process of cooking large quantities of food in large containers results in poor heat penetration and a slow cooling rate. These conditions, which are ideal for growth of C. perfringens, are more frequently found in institutional kitchens than in home cooking. This explains to some degree the high incidence of C. perfringens from institutional foods.

Staphylococcal gastroenteritis produced the largest numbers of food-borne outbreaks in the United States from 1968 through 1972 (Table 2). It was second to C. perfringens food poisoning in number of cases.

Some strains of Staphylococcus aureus produce a toxin that when ingested results in acute self-limiting gastroenteritis. The disease is characterized by a short incubation period after which nausea, vomiting, abdominal cramps, and diarrhea appear. Fever is common, but temperature drops may occur also (Michener and Elliott, 1969).

Minimal temperature for growth and toxin production by Staphylococcus aureus is 6.7 C (Angelotti et al., 1961). The organism, however, survives well during prolonged frozen storage (Woodburn and Strong, 1960). For formation of sufficient toxin in foods to cause illness, the organism has to multiply to high numbers. Peterson et al. (1962) demonstrated that this organism is capable of multiplication to high numbers in foods even with the competition of a mixed saprophytic microflora.

Staphylococcal toxin involved in food poisoning is fairly

stable to heat; therefore, cooking does not necessarily render the food safe. The bacterial cells are easily killed by heat and the presence of living forms of the organism in cooked foods generally indicates contamination after cooking. Contamination after cooking is a frequent mode of entry of the organism in foods. Habitats for Staphylococcus aureus are primarily the mucus membranes of the nasal passages and the skin. Poor personal hygiene of food handlers was recognized as the contributing factor for 50 percent of all outbreaks of staphylococcal food poisoning occurring in the United States in 1972 (U.S. Dept. of Health, Education, and Welfare, 1973).

Any of several species of Salmonella when ingested, can multiply within the small intestine, which is their natural habitat, causing illness. Ordinarily the infection occurs only after ingestion of large numbers of Salmonella cells.

Salmonellosis ranks close to C. perfringes and staphylococcal food poisoning in numbers of outbreaks and cases of food-borne illness (Table 2). Taylor and McCoy (1969) made an extensive review of Salmonella infection. The illness is characterized by a relatively long incubation time (24 to 48 hr) and long recovery. Vomiting, diarrhea, abdominal pain, and fever are common symptoms. Fatalities are not uncommon in children, elderly people or otherwise debilitated patients; these individuals are susceptible to infection from ingestion of fewer cells than normal healthy adults. The affected individuals continue to excrete the infective organism during convalescence for a few days or weeks after recovery. In

some cases they become asymptomatic carriers.

Salmonella from animal fecal sources are frequently present in animal products. High incidence of Salmonella is reported for animal feeds, unpasteurized liquid and dried eggs, red meats and poultry meat (Taylor and McCoy, 1969).

Temperatures below 6.7 C control growth of Salmonella (Michener and Elliott, 1964). Oblinger and Kraft (1973) observed that Salmonella can grow to high numbers in competition with psychrotrophic species of Pseudomonas at 15 C. This indicates a high level of probability of salmonellosis from food bearing Salmonella, and the need for a careful control of the temperature of storage of foods.

As observed with other bacterial species, freezing reduces viable counts of Salmonella, but thereafter, the survivors die at low rate (Committee on Salmonella, 1969).

Rieman (1969) summarized data from studies on heat resistance of Salmonella. Most reports show thermal death times below 10 minutes at 60 to 65 C for most strains and serotypes. In practice, adequate cooking or pasteurization are effective methods for eliminating viable Salmonella from foods.

Significance of indicator organisms in chilled and frozen chicken

Escherichia coli does not survive freezing or frozen storage well (Kraft et al., 1963). The presence of high numbers of E. coli in frozen chicken can be considered as indication of recent fecal pollution. Coliform bacteria, in general, are able to multiply

to high numbers under refrigeration losing significance as indicators (Rey et al., 1970, 1971).

Kraft (1969) evaluated the significance of enterococci as indicators for frozen poultry. These organisms survive well during frozen storage and cannot be regarded as indicators of recent pollution in frozen foods. However, they would be more significant than E. coli in trying to establish the sanitary aspects of frozen poultry prior to freezing. Rey et al. (1971) observed that numbers of enterococci remain constant during refrigerated storage of meat. As stated for frozen poultry, they lose significance as indicators of recent pollution on refrigerated chicken but can give some indication of the sanitary history of the product.

Bacterial Classifications

Taxonomic systems

Cain (1962) has presented a detailed discussion on the evolution of classifications of bacteria. The early classifications were typological and were based on Linnaeus's principle of the existence of distinctive kinds of living things. Each kind was considered separately created without intermediates or transitorial states. Each kind represented a species. Parallel to the evolution of biological sciences, the concept of bacterial taxonomy changed. As it now stands, the most refined techniques for grouping bacteria involve systematic formation of groups or taxa based on their presumed natural relationship. Current systematic classifications are

based upon probable evolutionary development. Since bacteria do not fossilize, there is no supporting evidence from geological studies for tracing their evolution. As described by Breed (1957) the phylogeny is traced under the assumption that certain characteristics of bacteria are related to primitive undifferentiated forms while other characteristics have developed by evolution alone with the changes from the early conditions to the actual environment of the earth. The weights given to the characteristics place them in categories or ranks. The hierarchy of characters determines the hierarchy of the taxa. For example, certain characters define the genera, other characters define the species within genera and in turn varieties within species are defined by another set of characters.

With an evolutionary approach, Bisset (1962) assumes that most primitive bacteria are aquatic, polar flagellated and nutritionally unexacting. Evolution of the primitive forms have led to terrestial types and forms with nutritional specificity. He condiders Pseudomonas to be a form in the early stages of evolution while Bacillus species are recent links in the gradual transition from aquatic to terrestial forms. The anaerobic Clostridium are regarded by him as retrogressive forms evolved from Bacillus.

With additional developments in the fields of biochemistry, immunology, genetics and microscopy, new characteristics are recognized and the weight given to known characteristics upon which bacterial classifications have been based are constantly

reconsidered. This results in shifts and rearrangements of the taxa observable through the seven editions of Bergey's Manual of Determinative Bacteriology.

Opposed to the phylogenetic relationships, there is the phenologic model used in recent approaches to classification. Cain (1962) refers to this type of classification as "natural". In phenologic classifications as many characters of the organisms as possible are compared but without giving any weight to any particular character. The groups are formed by sorting the organisms with maximum characters in common. The sorting of characters when handling large numbers of features and organisms is not a simple task and requires the use of mathematical methods.

Sneath (1956) was the pioneer of applying numerical methods for classification of bacteria. He described the application of computers to the mathematical analysis of data related to the observed features of bacteria (Sneath, 1957). He defined the coefficient of similarities between a pair of organisms by

$$S = \frac{NS}{NS + ND}$$

where NS is the number of similar characters between the pair, ND the number of characters not shared by the two organisms and S is the coefficient of similarities.

Sokal and Sneath (1963) described a method for formation of clusters from the coefficient of similarities. A similarity matrix

is constructed by arranging the similarity coefficients obtained by comparison of all possible pairs of organisms. The process continues by comparing individuals to pairs already formed and grouping them on the basis of maximum common level of similarities. By repetition of the procedure, distinctive clusters of individuals can be formed. Grower (1967) evaluated some of the current methods used for cluster analysis. He recommended the agglomerative method of Sokal and Sneath for general purpose classifications.

Sneath (1962) recommends that as many characters as possible be chosen for general purpose classifications; however, special types of classifications can be constructed by selection of fewer specific characters.

Lockhart and Liston (1970) have described the mechanics of different methods for collecting, coding, analyzing and presenting data for numerical taxonomy.

Numerical versus conventional taxonomy

In spite of the apparent differences between numerical and conventional taxonomic methods, several reports show satisfactory agreement between them (Sneath, 1957; Rhodes, 1959, 1961; Thornley, 1960, 1967).

Krieg and Lockhart (1966) did not find agreement between clusters of enterobacteria formed by numerical taxonomy and groups formed by any of the schemes currently used for their classification. These authors compared a total of 105 characters for their numerical analysis, while fewer characters are considered with any of the

common schemes for classification of enterobacteria: 1) Bergey's Manual (Breed et al., 1957); 2) Edwards and Ewing (1962) and 3) Kauffman-White scheme as listed by Edwards and Ewing (1962). Krieg and Lockhart concluded that according to the principles for natural grouping, some tribes or genera of enterobacteria formed by classical taxonomy have no justification and that some species separate distinctively from the tribe or genera to which they have been assigned. Sneath (1962) considers that the concept of natural classifications may have its roots in the structure of the genes which will determine the phenotypes. Likewise, any permanent phylogenetic transformation is presumably represented in the genome. Therefore, if the exact phyletic lineage could be determined and all possible phenotypic characters could be tested, there might not be any essential difference between the two types of classifications.

Application of numerical taxonomy to food bacteriology

Some morphological and many functional characteristics of bacteria used to differentiate systematic units such as genera and species are subjected to changes with time and environment (Ingram and Shewan, 1960).

Many authors (Thornley, 1960; Ingram and Shewan, 1960; Corlett et al., 1965; Shiflett et al., 1966; and Lee and Wolfe, 1967) have successfully applied numerical taxonomy for identification of bacteria in foods. Simple analytical procedures and computerized calculation of the results, as employed for numerical taxonomy, allow the investigator to use a more representative sample by

increasing sample size. Hartman (1968) has reviewed the micro-techniques, some of which are simple, economic and reliable tests, that allow for synchronized testing of many individuals from a bacterial population. Multiple inoculation procedures such as the one developed by Hartman and Pattee (1968) can also be of value in handling large numbers of bacteria for taxonomic classification of bacteria isolated from foods.

For the purpose of making generalizations about the bacterial flora on foods, natural or phenetic classifications are well suited. The knowledge of the behavior of the bacterial flora of foods under fixed environmental conditions can be more objective in certain cases than the recognition of distinct systematic units within the population on the basis of their phylogeny.

MATERIALS AND METHODS

Description of Procedures

Description of samples

Prepackaged chicken wings were purchased from a local retail store. The wings had been cut and packaged at the store. The packages were selected at random from the display case of the store.

At the laboratory, the wings were asceptically removed from the commercial packages and assigned at random to one of the various treatments.

Experimental design

Ten wings were sampled immediately after purchasing to serve as reference for the types and numbers of bacteria present. The remaining forty wings were divided into two lots of twenty. One of these lots was frozen in air while the other was frozen with liquid nitrogen. Ten frozen wings from each freezing method were sampled right after freezing to determine the effect of freezing on the bacterial population. The rest of the frozen wings were stored at -29 C ($\pm$ 2^{o}) being sampled after 8 weeks of frozen storage to determine effect of storage under frozen conditions on survival of the microbial population.

Packaging, freezing, and thawing procedures

The wings to be air frozen were packaged in Polyethylene bags 0.0025 in thick (Curden Martin Manufacturing Co., St. Louis, Mo.).

Air freezing was accomplished in 2-3 hr by placing the prepackaged wings in a freezer room at -29 C. Liquid nitrogen freezing was achieved in 3 min with a spray of liquid nitrogen in a tunnel-type liquid nitrogen freezer following the method described by Rey et al. (1971). The spray of liquid nitrogen was applied directly to the surface of the samples. The liquid-nitrogen-frozen samples were packaged right after freezing as explained for the air-frozen wings. The frozen wings were thawed before sampling by placing them overnight in a cold room at 2 C (± 2°).

Sampling procedure

An area of 2 cm^2 of the surface of each wing was swabbed for enumeration of various types of bacteria present. One swab from the total underside of the wings was used to determine incidence of Staphylococcus and one swab from the total upperside was used to determine incidence of Salmonella. A consistent location was maintained for each of the three types of swabs in all the samples.

Bacteriological methods

The analytical procedures employed to determine numbers of total aerobes, enterococci, fluorescent Pseudomonas and Clostridium perfringens and incidence of coagulase positive Staphylococcus and Salmonella are summarized in Table 3.

Table 3. Procedures employed for determination of bacterial densities and incidence of *Salmonella* and coagulase positive *Staphylococcus*

Quantitative determinations	Basal media	Plating technique	Incubation Temperature	Incubation Time	Confirmatory
Total aerobes	Trypticase soy agar (BBL)[a]	Pour plate	15 C	5 days	
Fluorescent *Pseudomonas*	Medium B of King *et al.*, (1954)	Surface plating	15 C	6 days	Fluorescence under U.V. light
Enterococci	KF *Streptococcus* medium (Difco)[b]	Pour plate	37 C	2 days	
Clostridium perfringens	SPS of Angelotti *et al.*, (1962)	Pouch of Bladel and Greenberg (1965)	37 C	24 hours	Motility, H_2S and dentrification

Qualitative determinations	Enrichment	Isolation	Confirmatory
Salmonella	Meat procedure of Galton *et al.*, (1968)	Galton *et al.* (1968)	Galton *et al.* (1968)
Coagulase + *Staphylococcus*	Wilson *et al.* (1959)	Herman and Morelli (1960)	Tube coagulase test

[a]Baltimore Biological Laboratories, Baltimore, Maryland.

[b]Difco Laboratories, Detroit, Michigan.

Bacterial cultures used in taxonomic studies

Plates from the total aerobic count showing 30 to 60 colonies per plate were selected from every treatment applied to the wings. Five to 10 isolated colonies were selected at random from each plate. Each bacterial colony was picked and streaked twice onto TSA agar, incubating the plates at 30 C for 3 days for purification. Gram stain preparations of each culture were examined with a light microscope to determine if they were morphologically homogeneous. The bacterial suspensions that had not been purified after two transfers were discarded. The number of bacterial cultures isolated and purified from each treatment is given in Table 4.

Thirty cultures from different genera and species (Table 5) were used as references for the taxonomic classification. Stocks of all bacterial cultures were maintained on slants of Trypticase soy agar at 5 C.

Table 4. Number of unknown cultures isolated per treatment

Treatment applied to the chicken	Number of cultures
Before freezing	68
After freezing:	
in air	85
after spraying with liquid nitrogen	81
Air frozen samples stores at -29 C for 8 weeks	89
Liquid nitrogen frozen samples stored at -29 C for 8 weeks	92

Table 5. Identified cultures used as references for taxonomic classification

Name	Designation and/or source
Streptococcus faecalis	G 13 (Frozen turkey)
S. faecalis	G 21 (Frozen turkey)
S. faecalis	50-1-10-(1) (Frozen turkey)
S. faecium	B 20 (Frozen turkey)
Micrococcus lysodeikticus	Jeffries, Dept. Bacteriol. Iowa State Univ. (ISU)
Staphylococcus aureus	No. 149 (Bergdoll, Food Res. Inst., Madison, Wisc.)
S. aureus	SR-18-1 (Dept Food Technol. ISU)
S. aureus	S"6"B (Bergdoll, Food Res. Inst., Madison, Wisc.)
Enterobacter hafniae	ST3A (Dept. Food Technol., ISU)
E. aerogenes	American Type Culture Collection (ATCC) 13048
Salmonella typhimurium	ATCC 6994 (Matches)
S. heidelburg	ATCC 8326 (Matches)
Acetobacter orleanenses	B-55 NRRL (National Regional Research Laboratories)
Arthrobacter globiformis	ATCC 4336
A. pascens	ATCC 13346
A. crystallopoietes	ATCC 15481
A. simplex	ATCC 13260
A. citreus	ATCC 11624
A. globiformis	ATCC 8010
A oxydans	ATCC 14358
A. variabilis	ATCC 15753
A. polychromogenes	ATCC 15216
A. ramosus	ATCC 13727
A. albidus	ATCC 15243
Pseudomonas sp.(Fluorescent)	F 17 (Dept. Food Technol. ISU)
Pseudomonas sp.(Fluorescent)	F 21 (Dept. Food Technol. ISU)
P. aeruginosa	Goetze (Hamon, Inst. Pasteur, Lille)
Pseudomonas sp.(Nonfluorescent)	A-6 (Dept. Food Technol. ISU)
P. fragi	(Dept. Food Technol. ISU)
P. Fluorescens	(Dept. Food Technol. ISU)

Tests used for classification

Fifty-one characters with a total of 163 states were tested for every culture. States can be defined as the various responses that can be observed with a character for example, nitrate utilization (character): 1) nitrate not utilized, 2) nitrate utilized being reduced to nitrite, or 3) nitrate utilized yielding end products past the reduction stage of nitrite. The three responses that could be detected for utilization of nitrate with the procedure employed would be the 3 states for this character. Table 6 summarizes the features used.

All the tests requiring surface inoculation of solid media were made with the replicator instrument described by Hartman and Pattee (1968). The cultures to be tested were grown in Trypticase soy broth (B.B.L.) in medicine dropper flasks (Standard ½ oz. from Arthur H. Thomas Co., P.O. Box 779, Philadelphia, Pennsylvania). The flasks were incubated at 30 C for 24-48 hr. The broth cultures were transferred with the droppers into the wells of Microtiter plates (Cook Engineering Co., Alexandria, Virginia) in a predetermined sequence to charge the capillary tubes of the inoculator. Square plastic petri plates (Lab Tek Products, Westmont, Illinois) were prepared with all the testing media. The media were allowed to solidify and dried when necessary in a 37 C incubator to avoid any exudation on the surface of the agar by the time of inoculation. Every plate was inoculated simultaneously with 68 different cultures by touching the surface of the agar with the pre-charged capillaries

Table 6. Characters used for numerical taxonomy

Description	No. of features	No. of states
Anaerobic growth at 30 C on Thioglycolate agar (Difco)[a]	1	3
Cell morphology[a], gram reaction[a]	2	7
Form, elevation, color and consistency of colony agregation on TSA[b]	4	14
Growth at 5[a], 30, 37[a] and 45[a] C on TSA[b]	4	13 (3 each)
Growth at pH 3.5, 4.5[a], 5.5[a], 8.1 and 9.6	5	15 (3 each)
Growth and reaction on VRB[c], EMB[d], and KF[e] media at 37 C	6	31
Growth, chromogenesis, lecithinase, gelatinase and mannitol fermentation on S110[f] at 37 C	5	12
Hemolysis of bovine blood at 15 and 30 C[a]	2	6 (3 each)
Hydrolysis of gelatin[a], casein[a], starch[a], cellulose, tributyrin[a] and chicken fat[a] at 15 and 30 C	12	36 (3 each)
Motility[a] and reduction[a] of nitrate at 22 C	2	5
Production of catalase[a], cytochrome oxidase[a] and lecithinase[a] at 30 C	3	6 (2 each)
Production of fluorescent pigment[a] at 15 and 30 C	2	6 (3 each)
Utilization of glucose[a], sucrose[a] and lactose[a] at 15 and 30 C	3	9 (3 each)

[a]Responses evaluated by homogeneity chi square to test for changes in the morphology and physiology of the microflora due to freezing and frozen storage.

[b]Trypticase soy agar (B.B.L.).

[c]Violet red bile (Difco).

[d]Eosin methylene blue agar (Difco).

[e]KF Streptococcus agar (Difco).

[f]Modification of Herman and Morelli (1960) of the Staphylococcus medium No. 110 (Difco).

of the inoculator.

TSA was used in general as the basal medium for replica plating. The medium was modified as follows: pH was adjusted to 8.1 and 9.6 with 6 N NaOH; 5% egg yolk, 0.2% soluble starch, 1.5% skim milk, or 0.1% cellulose were added for hydrolysis of lecithin, starch, casein, and cellulose respectively; 0.5% tributyrin and 0.5% tragacanth gum (Mathison Coleman and Bell Norwood, Cincinnati, Ohio) were added with homogenization for hydrolysis of tributyrin.

The medium of Goldman and Rayman (1952) with the modification of Rey (1968) was used for hydrolysis of chicken fat. Gelatinase activity was tested on the medium of Smith and Goodner (1958). Growth at pH 3.5 and 4.5 was tested on Potato dextrose agar (Difco) adjusting the pH with a 10% solution of tartaric acid. Growth at pH 5.5 was tested on Potato dextrose agar without adjustment. Medium B of King et al. (1954) was used for production of fluorescent pigment. Acid from glucose, sucrose and lactose was tested on Phenol red broth base (Difco) with 1.5% agar. Motility and reduction of nitrate were assayed as described by Angelotti et al. (1962). The method of Kovacs (1956) was employed for detection of cytochrome oxidase activity. Catalase was determined by flooding the surface of the bacterial colonies with hydrogen peroxide.

Incubation time for the various tests was 24 hr at 45 or 37 C, 48 hr at 30 C or room temperature, 3 days at 15 C or 2 weeks at 5 C. To test for hydrolysis of chicken fat the plates were incubated for 10 to 15 days regardless of the incubation temperature used.

Statistical procedures

Differences in numbers of different types of bacteria recovered from the samples were compared by analysis of variance after logarithmic transformation of the data.

A phenotypic approach was used for taxonomic classification. Differences between the microflora isolated from samples from the various treatments were determined by cluster analysis with the technique of Sneath and Sokal (1962). The computer program was written by Mr. Roger Maracheck (Department of Statistics, Iowa State University). Affinity between organisms was the basis for clustering. The degree of similarity was calculated for every possible pair of organisms according to the number of morphological and physiological characters shared by each pair. The formula of Sneath (1957) was used for calculation of similarities in percent:

$$S = 100 \times \frac{NS}{NS + ND}$$

where S = percent similarities, NS = number of similar characters and ND = number of characters not shared by the pair.

To calculate the percent similarities, equal weight was given to positive and negative characters. Primary and secondary characters such as ability to grow on a selective medium (primary) and type of reaction manifested in the medium (secondary) were also coded so as to contribute equal weight to the percent similarity. Similarly the responses of dichotomous characters such as motility (+ or -),

multivalued qualitative characters such as colony consistency (smooth, rough or mucoid) and semi-quantitative multivalued characters such as growth (absent, scant, moderate or abundant) were given equal scores for the calculation of affinities between organisms.

With the percent similarity, a similarity matrix was computed for the bacteria recovered from each treatment using the single linkage method of Sneath (1957). From the similarity matrix, dendograms were printed showing phenotypic relationships between organisms. For graphical presentation of the results, simplified versions of these dendograms were made.

Homogeneity chi-square was used to evaluate differences in morphology and physiology of the bacterial populations isolated from the chicken samples subjected to the various treatments. For example, the proportion of organisms utilizing glucose among those isolated before freezing was compared to the proportion of organisms utilizing the sugar among those isolated after freezing. The characters used for these evaluations are listed in Table 6. The comparisons made were: unfrozen versus frozen chicken, air freezing versus liquid nitrogen freezing and immediately after freezing versus storage for 8 weeks under frozen conditions.

RESULTS AND DISCUSSION

Density of Bacterial Populations on the Samples

Bacterial profile before freezing

The initial contamination of poultry is a variable factor and can serve as an index of the sanitary conditions observed during processing. Enterococci were detected in four of the samples before freezing; however, they were present in low numbers in these samples ranging from less than 1 to 26 per cm^2. Clostridium perfringens and Salmonella were not recovered at any time from any of the samples. The low contamination of the meat with organisms that could be traced to probable fecal contamination was taken as an indication that good processing practices had been applied to these samples.

The average total aerobic counts before freezing as shown in Figure 4, exceeded the average counts reported in the literature for freshly processed poultry (Ayres et al., 1950; Gunderson et al., 1954). This could have been indicative of improper handling during processing and marketing or of bacterial growth taking place between the time of processing and time of sampling. Low numbers of Pseudomonas are generally present on poultry immediately after slaughtering (Walker and Ayres, 1956). But it has been demonstrated that upon cold storage Pseudomonas soon outnumber other bacterial species on refrigerated meats (Kirsch et al., 1952; Wolin et al., 1957; Nagel et al., 1960; and Ayres, 1960). Among the total load of Pseudomonas developing in cold storage of meats, fluorescent

Pseudomonas usually comprise a relatively low proportion of this class of organism. Figure 4 shows that high numbers of fluorescent Pseudomonas were present on the samples before freezing. This was interpreted as evidence that the meat had been under refrigerated storage for a relatively long period of time between processing and sampling allowing bacterial growth under conditions where psychrotrophic bacteria were favored.

The density of total aerobes ranged from 65,000 to 34,000,000 per cm^2 among the samples from different packages tested before freezing. This indicated a difference in the length of time of refrigerated display storage between the packages of wings purchased at the retail store.

All the samples tested before freezing yielded coagulase positive Staphylococcus (Figure 5). These organisms are commonly present on the human skin and nasal passages. The high frequency of isolation of coagulase positive Staphylococcus from the chicken wings reflected the high degree of manipulation to which cut-up chicken is subjected.

Bacterial densities after freezing

The analysis of variance presented in Table 7 shows that the freezing effect was significant at the 5% level of probability for total aerobes and at 1% level for fluorescent Pseudomonas. The plots in Figure 4 show that upon freezing the average numbers of total aerobic bacteria and fluorescent Pseudomonas decreased. Kraft et al. (1963) also reported a reduction in bacterial numbers

Figure 4. Average number of total aerobic bacteria and fluorescent Pseudomonas present on the samples before freezing, after freezing and after storage at -29 C for 8 weeks. Data from both methods of freezing are included in these averages

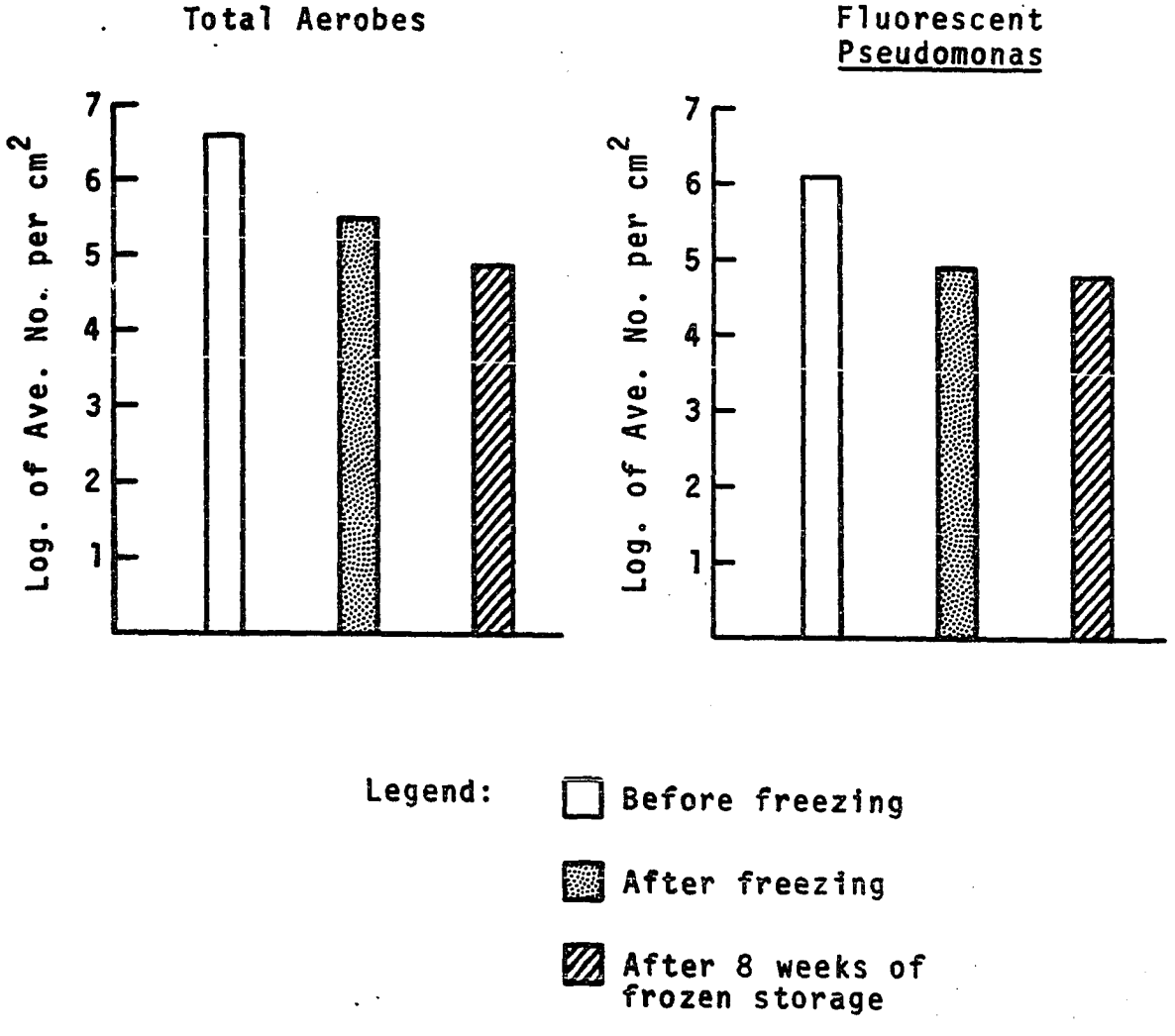

Total Aerobes
Fluorescent
Pseudomonas
Log. of Ave. No. per cm2
7
6
5
4
3
2
1
Legend:
Before freezing
After freezing
After 8 weeks of
frozen storage

Figure 5. Incidence of coagulase positive Staphylococcus before freezing, after freezing and after 8 weeks of storage at -29 C

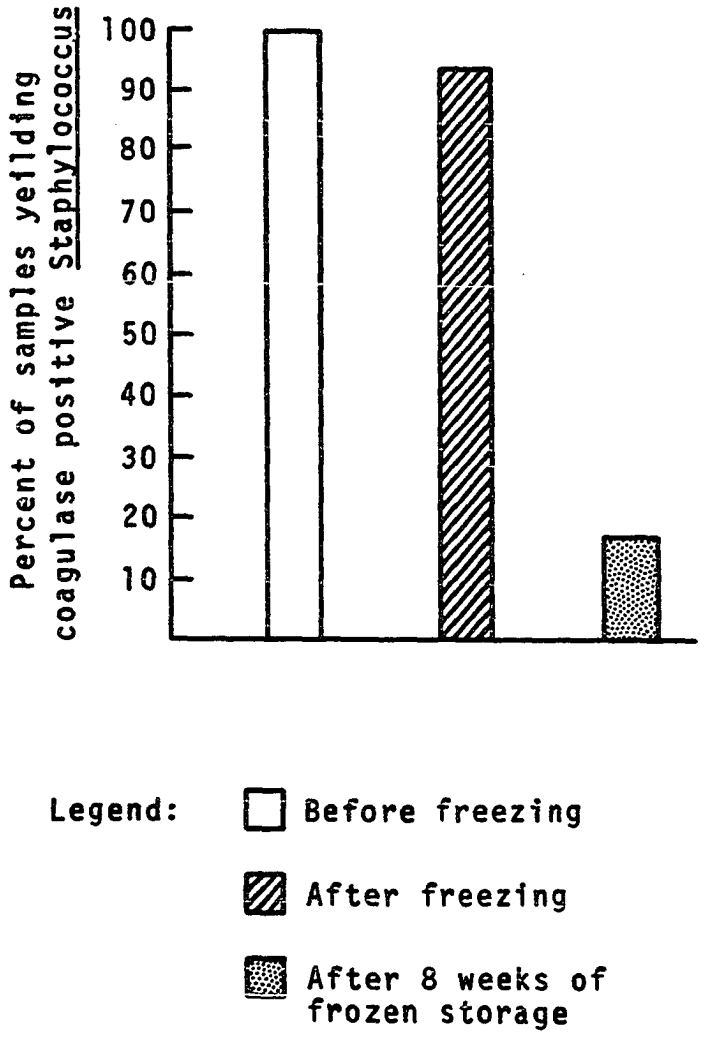

Percent of samples yielding coagulase positive Staphylococcus
100
90
80
70
60
50
40
30
20
10
Legend:
Before freezing
After freezing
After 8 weeks of frozen storage

Table 7. Analysis of variance on numbers of total aerobes and fluorescent Pseudomonas recovered from the samples

Source of variation	Degrees of freedom	Mean squares	
		Total aerobes	Fluorescent Pseudomonas
All treatments	4	2.774*	2.465**
Unfrozen vs. frozen	1	5.281*	7.801**
A - Air freezing vs liquid nitrogen freezing	1	1.047	0.064
B - After freezing vs 8 weeks in frozen storage	1	4.394*	1.726
A X B	1	0.375	0.269
Residual	45	0.848	0.643
Total	49		

*Significant at 5% probabilities.

**Significant at 1% probabilities.

on chicken subjected to several freezing methods.

Fanelli and Ayres (1959) observed decreased incidence of fecal streptococci and staphylococci as a result of air blast freezing of chicken pies. Salt and other ingredients of chicken pies might influence the response of bacteria to environmental factors. In the

present investigation numbers of enterococci did not vary greatly after freezing which indicated that this type of organism when present in chicken meat can be quite stable to the cold shock of the freezing process. Similarly, Figure 5 shows that the frequency of isolation of coagulase positive Staphylococcus varied very little after freezing. Chi-square tests indicated that the slight decrease in incidence of coagulase positive Staphylococcus after freezing had no statistical significance.

The overall effect of the freezing process can be summarized as a decrease in total aerobic bacteria but the effect is not uniform for all aerobes occurring on chicken and seems more detrimental to certain types of bacteria than to others.

Effect of frozen storage on bacterial densities

The analysis of variance (Table 7) indicated at a 5% level of probability that there was a variation in numbers of total aerobic bacteria that could be attributed to frozen storage.

Sulzbacher (1950) reported growth of bacteria in pork stored at -4 and -18 C. Enough information exists in the literature, however, to support the contention that bacterial growth does not take place in most foods during storage under adequate frozen conditions (at or below -10 C) even though large numbers of microorganisms present in the food retain viability for long periods of time (Desrosier, 1959; Michener et al., 1960; Frazier, 1967 and Stanier et al., 1970). The results of our studies are in general agreement

with this view. Plots of total aerobic counts in Figure 4 show that bacterial numbers decreased during frozen storage even though survival after 8 weeks of frozen storage was still high.

Although enterococci survived cold shock during freezing they did tend to die off during storage and they were not isolated from any of the samples that had been stored at -29 C for 8 weeks. Likewise, the frequency of isolation of coagulase positive Staphylococcus which did not decrease significantly during freezing was greatly reduced by frozen storage (Figure 5).

The opposite was observed with fluorescent Pseudomonas. Numbers of this type of organism that had diminished significantly during freezing were maintained at practically the same level under frozen storage (Figure 4). The analysis of variance (Table 7) corroborated that storage of the samples at -29 C for 8 weeks did not cause significant variation on the numbers of this type of bacteria that had survived the freezing process.

On the basis of these observations it was concluded that the stability of certain bacterial species to cold shock during freezing does not necessarily imply stability to prolonged frozen storage and, vice versa, some bacteria can be quite susceptible to cold shock during the freezing process but the cells surviving freezing might be, as observed with fluorescent Pseudomonas, quite resistant to frozen storage.

Consideration of the overall effect of frozen storage leads to the same conclusion stated for effect of freezing; namely, a

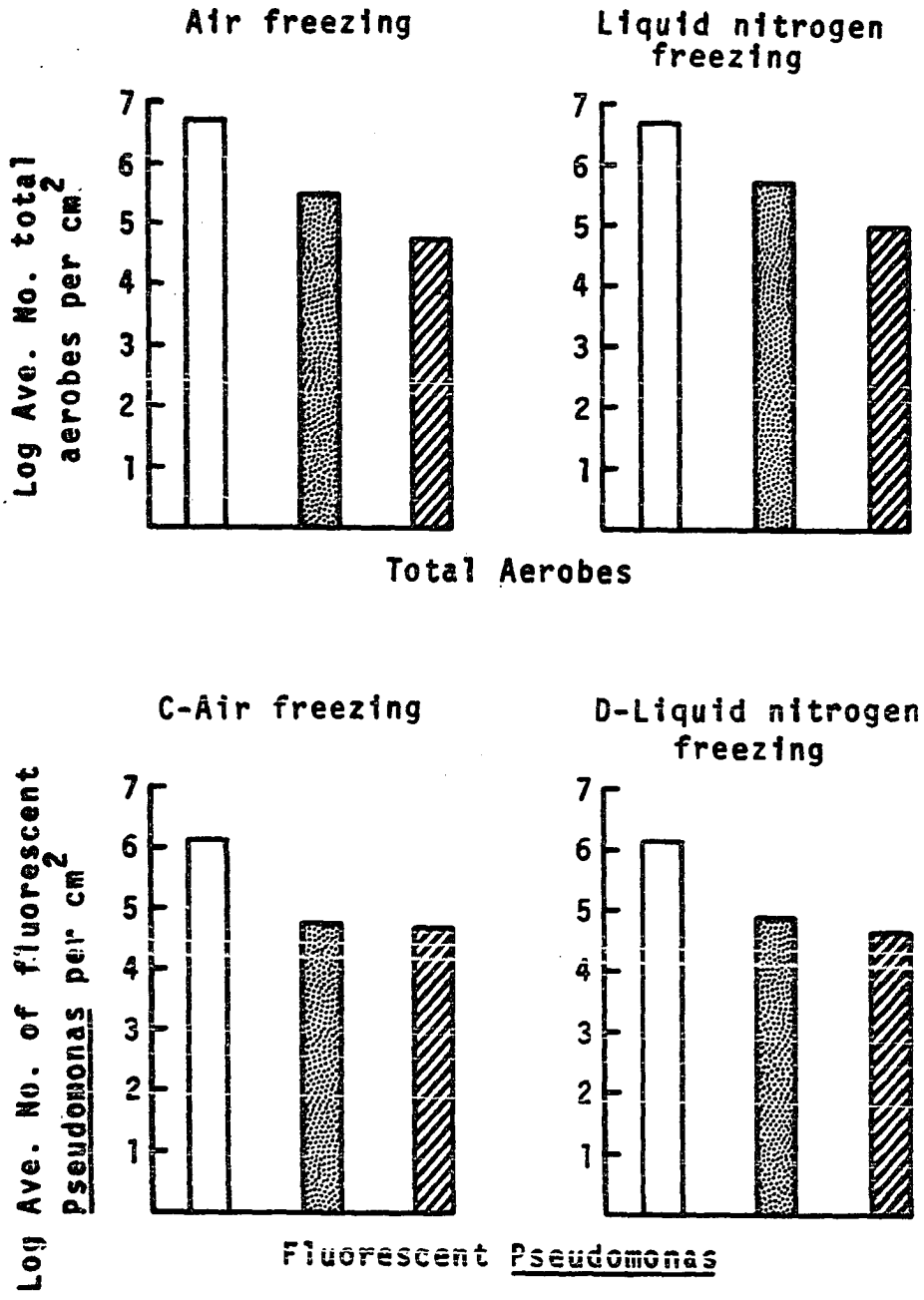
decrease in total aerobic bacteria that was not uniform for every type of microorganism comprising the microflora of chilled chicken was observed during frozen storage.

Air freezing versus liquid nitrogen freezing

There were very small differences in average numbers of total aerobes and fluorescent Pseudomonas isolated from samples frozen in air and those frozen with liquid nitrogen (Figure 6). No trend or consistency among the individual samples existed to indicate that one method of freezing yielded higher bacterial counts than the other. The analysis of variance (Table 7) corroborated that the differences in total aerobes or fluorescent Pseudomonas that could be explained by the effect of freezing method had no statistical significance. Differences on the frequency of isolation of coagulase positive Staphylococcus with each freezing method were also very small and did not show statistical significance when tested by chi-square.

An outstanding difference between the two methods of freezing was their effect in the general appearance of the frozen meat as shown in Plate 1. During preparation for freezing the wings were stretched for the purpose of conferring the most appealing form to the frozen product. With the liquid nitrogen freezing this operation was done by the time the wings were placed in the conveyor of the freezing unit. With the fast freezing operation the samples maintained the stretched form. The wings frozen in air, being placed on the shelf of the freezer room in stretched position as well, contracted during

Figure 6a. Comparison between the average numbers of total aerobic bacteria and fluorescent Pseudomonas isolated from samples frozen in air and frozen with liquid nitrogen


Air freezing
Liquid nitrogen freezing
Log Ave. No. total aerobes per cm²
7
6
5
4
3
2
1
Total Aerobes
C-Air freezing
D-Liquid nitrogen freezing
Log Ave. No. of fluorescent Pseudomonas per cm²
Fluorescent Pseudomonas
Legend:
Before freezing
After freezing
After 8 weeks of frozen storage

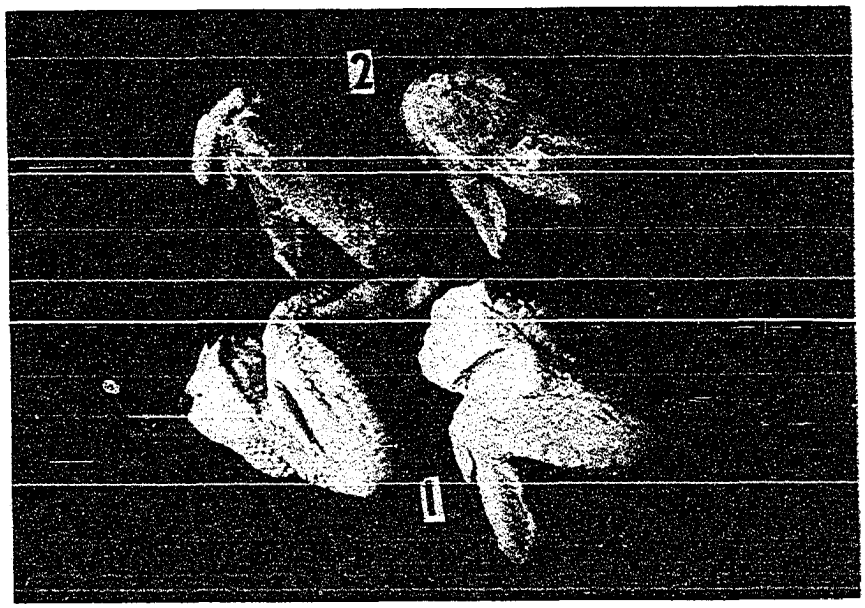


Plate 1. Chicken wings in the frozen state: 1) wings frozen with sprays of liquid N_2 and subsequently stored for 8 weeks in a freezer room at -19 C; 2) wings held in the same freezer room for identical period of time but placed in the freezer room unfrozen

the slow freezing process appearing after freezing as shown in Plate 1. Perhaps the unavoidable deformation inherent to frozen chickens when they are stacked on shelves of mechanical freezing units could be overcome with liquid nitrogen freezing.

Moreover, the air frozen wings exhibited the usual uneven reddish discoloration resembling ruptured blood vessels which is characteristic of frozen chicken. The wings frozen with liquid nitrogen not only had no trace of discoloration, which concurs with the statement of Kuschfeldt and Thiel (1970), but remarkably, they maintained their normal appearance while exposed for eight weeks to conventional frozen storage. These facts indicate that there could be great possibilities for marketing chicken with appealing appearance by freezing with liquid nitrogen. This would guarantee not only an increased storage time to processors and retailers but most important it would increase the margin of safety from the public health standpoint over that of chilled chicken.

Taxonomic Studies

Interpretation and presentation of results

Taxonomic grouping of the microorganisms isolated from the chicken was done for the purpose of detecting changes in the type of microflora during freezing and during frozen storage of the samples.

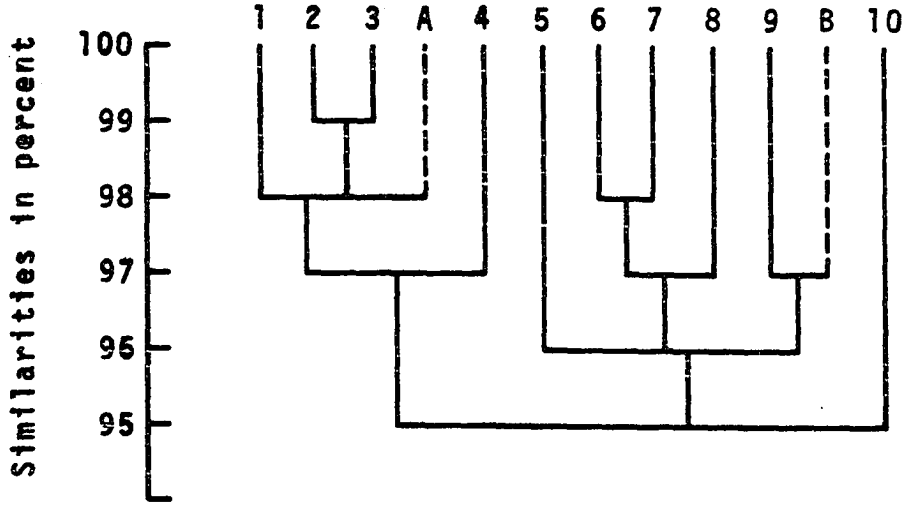
Independent analysis was made for every group of isolates according to their source of isolation: 1) chilled chicken as

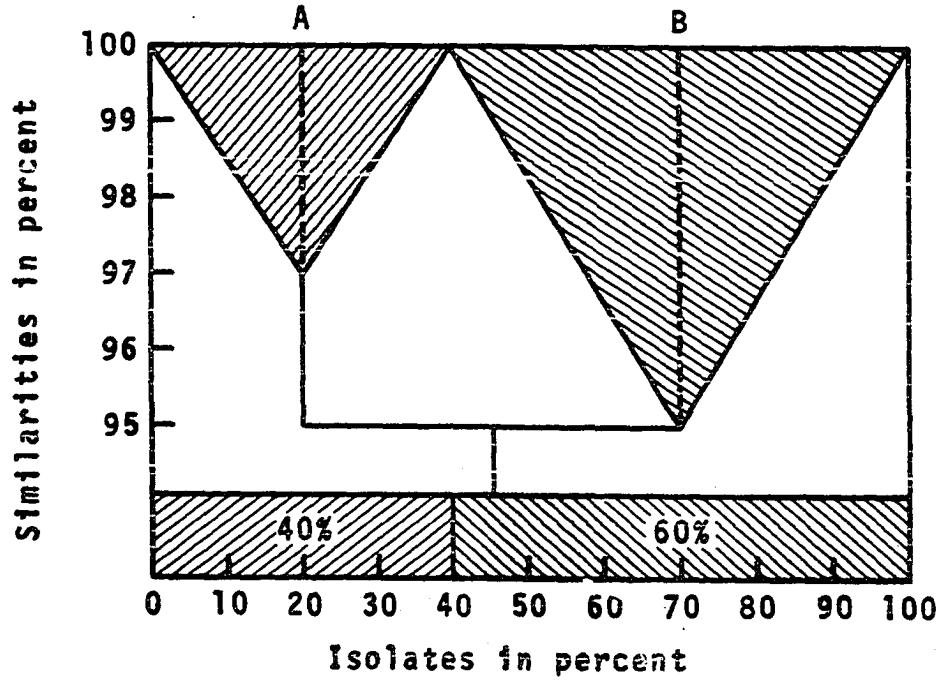
purchased from the retail store, 2) chicken frozen in air after purchasing from the retail store and tested right after freezing, 3) chicken frozen with liquid nitrogen after purchasing and tested right after freezing, 4) chicken frozen in air after purchasing and then stored at -29 C for eight weeks, and 5) chicken frozen with liquid nitrogen after purchasing and stored at -29 C for eight weeks. When calculating coefficient of similarities for each of these treatments the set of 30 known cultures was included with the cultures isolated from the chicken for comparative purposes. The known cultures also were analyzed individually.

The dendograms printed by the computer were branched tree diagrams. Figure 6b shows a hypothetical dendogram as printed by the computer. Each branch or vertical line represented a different culture. At the top of each branch a code number was printed for identification of the organism represented by each line. The ordinate had a scale in percent to measure the level of similarities. The branches were connected by horizontal lines similar to the top of a bar in a bar graph and showed the level of similarities between the organisms so connected by reading on the scale on the ordinate. For example, cultures 2 and 3 in Figure 6b would have had 99% of similar responses or shared 99% of the characters tested. These dendograms simply showed phenotypic relationships between microorganisms. The computer dendograms linked the isolates from chicken (represented in Figure 6b by numbers) among themselves as well as with the known cultures which were represented by letters in Figure 6b.

Figure 6b. Hypothetical dendogram as printed by the computer

Figure 7. Simplified version of the dendogram in Figure 6


1 2 3 A 4 5 6 7 8 9 B 10
100
99
98
97
96
95
Similarities in percent


A
B
100
99
98
97
96
95
Similarities in percent
40%
60%
0 10 20 30 40 50 60 70 80 90 100
Isolates in percent

To prepare the simplified versions from the computer dendograms the probable phylogenetic relationships between organisms was considered. The closeness between the microorganisms isolated from chicken and the known cultures was determined by the highest linkage clustering method.

For the construction of taxonomic groups Sneath (1962) recommended as the criterion for admission to a group either the highest similarity value between an entrant and the existing members of the group or the use of the mean similarity values. In calculating mean similarity values each strain of the group has equal weight in determining similarity level for the group. A nucleus of a group is formed by the pair of strains having the highest similarity. Strains which have the highest mean similarity with the members of the nucleus are added sequentially. After incorporation of each new strain to the nucleus, the mean similarity for the group is recalculated. The mean similarity of the group is taken to represent the similarity level at which the cluster forms. Two groups fuse at a similarity level which is equal to the mean of the intergroup similarity value. This method gives very defined clusters. Thornley (1967) used mean similarity on a taxonomic study of Achinetobacter. To make simplified versions of the computer dendograms, the phenetic groups formed by Thornley were prefixed by choosing 72.5, 82.5, and 92.5% similarity as limits or boundaries to form 4 phenons. Therefore, defined groups formed above 92.5% similarity were considered more closely related than groups formed between 82.5 and 92.5% even though some groups

formed close to the 92.5% level, either above or below this boundary. Such groups may be closely related but separated from each other arbitrarily because of the chosen limits.

Another approach is to select differential characters to form groups (Colwell, 1964). Characteristics are chosen according to their significance as a diagnostic key. This method assumes the existence of a hierarchy of characters and the groups are formed on the same basis used for phylogenetic taxa. In the present investigation, where a known set of cultures was used, the highest linkage method of Sneath (1962) was considered the most adequate. However, in preparing the simplified dendograms the highest linkage of the isolates from chicken with the known cultures was the criteron for group formation. The groups were formed at the highest common level of similarities between the unknown and the known cultures under the assumptions that the known cultures used were typical representatives of their taxa and that differences as small as a 1% similarity for intergroup linkage were acceptable variability. These two assumptions were made with the recognition that aberrant forms of a class might have been misplaced.

In the hypothetical dendogram in Figure 6b, cultures 1, 2, 3, and 4 (unknown) would have been considered to form a phylogenetic cluster with A (a known culture) rather than with B since they linked at highest level of similarities with A (97%) than with B (95%). The same consideration would have led to cluster cultures 5, 6, 7, 8 and 9 with culture B at 96% similarities rather than

with A at 95%. In cases similar to that of culture 10 in Figure 6b, which linked at 95% with either A or B the highest linkage could not be used in determining its placement into either cluster and other judgments had to be made. To be able to include culture 10 within the cluster formed by 1, 2, 3, A and 4, the highest common level of similarities of the cluster would have had to be lowered from 97 to 95% or 2 units on the similarity scale. By including culture 10 within the cluster formed by 5, 6, 7, 8, 9 and B the level of similarities of the cluster would have to be lowered only one unit on the similarity scale (from 96 to 95%). With these considerations culture 10 would have been placed in the cluster with B with the least alteration of the phenotypic level of similarities for the clusters printed by the computer.

The tree diagram in Figure 6b could be represented as shown in Figure 7. The triangles represent clusters of microorganisms that grouped at equivalent levels of similarities: cluster A at 97% and cluster B at 95%. The maximum common level of similarities among the microorganisms forming each cluster is indicated by the vertices of the triangles by reading on the similarity scale on the ordinate. The computer dendograms ranged from 98 cultures for classification of microorganisms isolated before freezing (68 unknown plus 30 known) to 122 for liquid nitrogen frozen samples stored for 8 weeks at -29 C (92 unknown plus 30 known) (Table 4).

To prepare graphs for presentation of results the tree diagrams had to be simplified and adapted to fit into conventional size graphs.

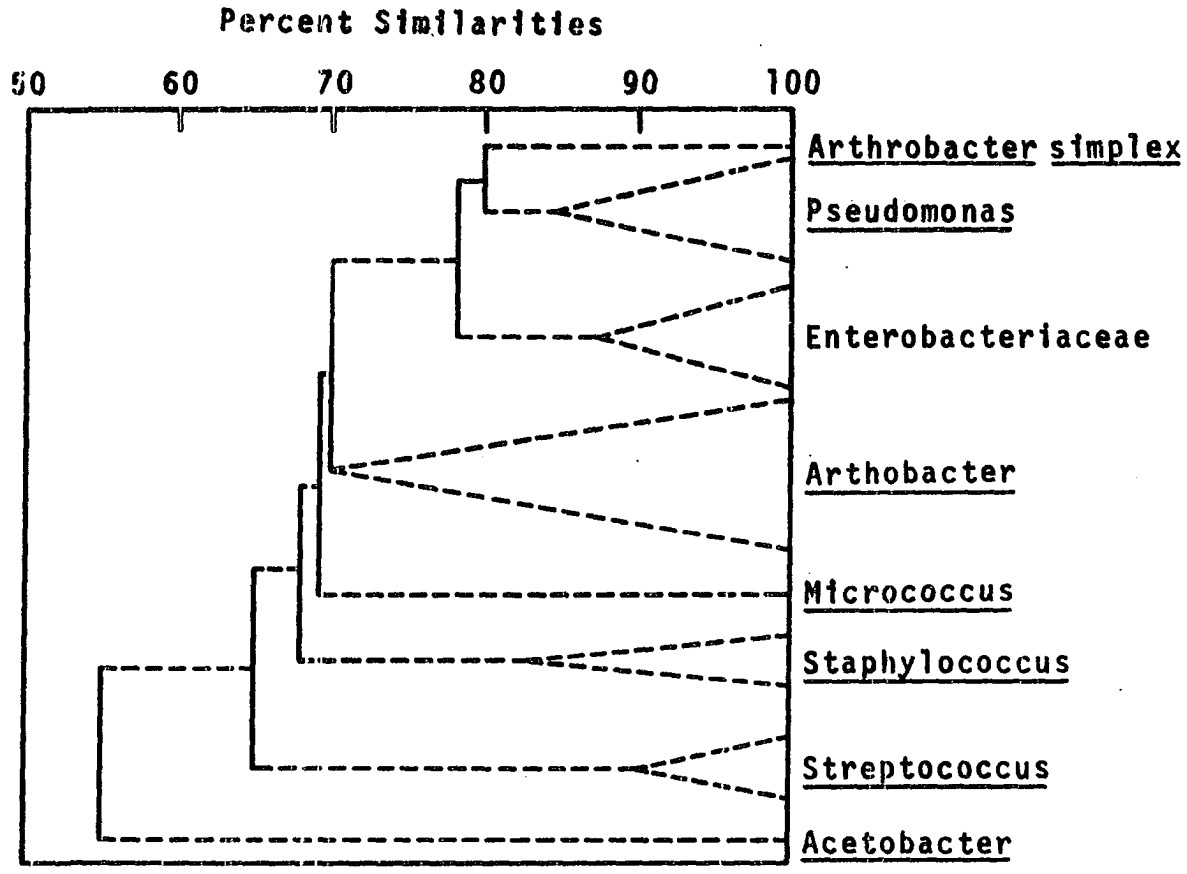
To keep the size of the clusters into perspective, the base of the triangles were drawn proportional to the numbers of isolates or unknown cultures included into each cluster as a percent of the total number of microorganisms isolated from the particular treatment. For example, cluster A in Figure 6b which was formed by 4 of the 10 unknown cultures (1, 2, 3, and 4) was to be represented by triangle A in Figure 7. The size of the base of this triangle is 40 units on the scale in percent on the abscissa indicating that the cluster includes 40% or 4 of the 10 isolates in the graph. The known cultures A and B were represented by discontinuous lines in Figure 7. On the simplified dendograms to follow (Figures 9 through 11) the reference or known cultures were also identified by names of species, genus or the taxonomic family.

Similarities between the known cultures

Figure 8 shows a simplified version of the dendograms printed for the set of known cultures. According to the diagnostic characters tested, similarities among different genera ranged from 80% for the linkage of Pseudomonas species with Arthrobacter simplex to almost 55% at which level the strain of Acetobacter orleanense separated from all the other known cultures.

The most homogeneous cluster was formed by the strains of Streptococcus that linked at a level of 90% similarity. The most heterogeneous group was formed by the species of Arthrobacter which linked at 69% similarity with A. simplex linking at a higher level

Figure 8. Phenotypic relationships between the known cultures used as reference

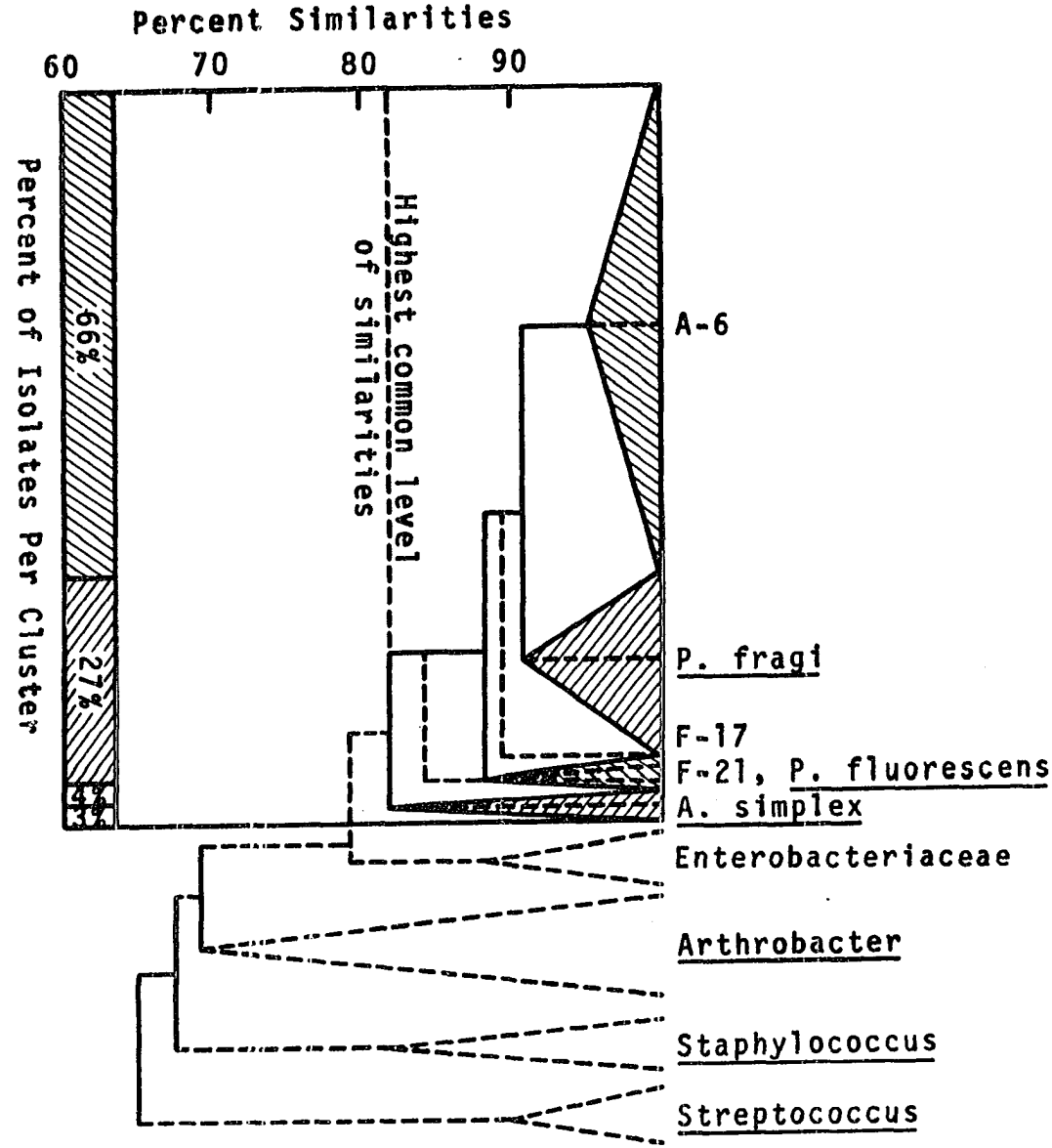

Percent Similarities
50
60
70
80
90
100
Arthrobacter simplex
Pseudomonas
Enterobacteriaceae
Arthobacter
Micrococcus
Staphylococcus
Streptococcus
Acetobacter

of similarity with Pseudomonas sp. and the strains from the Enterobacteriaceae family than with the other strains of Arthrobacter tested.

Microflora present in the chicken before freezing

The microorganisms isolated from chicken before freezing were very homogeneous in their responses (Figure 9). Ninety-seven percent of these organisms grouped into three clusters (66, 27 and 4%). The largest cluster (66% of the isolates) grouped at 96% similarity and associated with Pseudomonas A-6, a nonfluorescent strain isolated from refrigerated chicken. The five known strains of Pseudomonas linked at 84% similarity (Figure 8) while the three clusters formed by the organisms isolated from commercially refrigerated chicken, which associated with the known Pseudomonas, linked at 88%. This gives an estimate of the high level of homogeneity of the microflora developing on chilled chicken. The microorganisms forming the first three clusters, representing 97% of the isolates, were considered Pseudomonas-like due to the high level of similarities with known strains of this genus. Most of the Pseudomonas-like organisms associated closely with the nonfluorescent Pseudomonas (A-6 and P. fragi). They comprised 93% of all the isolates from chicken. The remaining Pseudomonas-like organisms (4% of all the isolates) linked with the fluorescent strains F-17, F-21 and P. fluorescens. These results are in general agreement with the previous conclusion under "Bacterial profile before freezing" stating that Pseudomonas,

Figure 9. Taxonomic relationships between the micro-organisms isolated from chilled chicken right after purchasing (A-6 - nonfluorescent *Pseudomonas*; F-21 and F-17 - fluorescent *Pseudomonas*)


Percent Similarities
60
70
80
90
Percent of Isolates Per Cluster
66%
27%
4%
3%
Highest common level of similarities
A-6
P. fragi
F-17
F-21, P. fluorescens
A. simplex
Enterobacteriaceae
Arthrobacter
Staphylococcus
Streptococcus

in general, and non-fluorescent species of the genus, in particular, are the predominant saprophytic flora encountered on chilled chicken.

Only a very small proportion of the organisms isolated before freezing did not associate closely to the known Pseudomonas. They formed a cluster containing 3% of the isolates and separated from the Pseudomonas-like group at 81% similarity. This cluster associated with A. simplex at 85% similarity. The microorganisms in this cluster were Gram variable club shaped cells. Even though they shared many characteristics with A. simplex they varied in that they did not reduce nitrate, they were psychrotrophs growing well at 5 C and did not grow at 37 C. Organisms in this cluster represented 3% of a population of 10^6 total aerobes per cm^2. Evidently their psychrotropic tendencies allowed them to multiply to high densities on the surface of the meat during refrigerated storage. There are reports in the literature associating coryneforms with refrigerated poultry products. Board et al. (1964) reported Arthrobacter comprising a substantial percent of organisms from eggs. In a survey on liquid eggs from different egg breaking plants in Iowa, Kraft et al. (1966) found Pseudomonas and Arthrobacter to be the most prevalent organism isolated from the product. Salzer et al. (1967) isolated coryneforms from turkey giblets that were able to grow at 5 C. Soil is the habitat for all Arthrobacter species listed in Bergey's Manual (1957). Kraft (ca. 1975) states that contamination of the live bird with psychrotrophic coryneforms from soil is apparently the source of these organisms in chicken.

Comparison between the two freezing methods

The microorganisms isolated from either freezing method formed similar taxonomic groups. Table 8 shows that an equal number of clusters was observed for the organisms isolated from either air frozen or liquid nitrogen frozen samples. The percentage of isolates associating with known cultures were approximately the same for either freezing method. The levels of similarity of the clusters that associated with each of the known cultures were also practically the same between freezing methods. No significant difference in microflora could be attributed to freezing method. Thus, the subsequent discussion is based on the results of the samples frozen with liquid nitrogen and is pertinent as well to the air frozen samples.

Microorganisms present right after freezing

The organisms isolated after freezing formed 8 distinctive clusters (Figure 10) indicating that the microflora isolated after freezing was more heterogeneous in character than the microflora isolated before freezing. The percent similarity at which the first cluster separated (highest common level of similarity) dropped from 82% before freezing (Figure 9) to 62% after freezing (Figure 10). Still the largest cluster (including 36% of the isolates) were closely associated with the non-fluorescent Pseudomonas (A-6 and P. fragi). However, the proportion of isolates linking with the known strains of Pseudomonas dropped from 97% before freezing to 57% after freezing. A cluster associating with Arthrobacter

Table 8. Taxonomic relationship between microorganisms isolated from air frozen and liquid nitrogen frozen chicken

	Proportion of isolates forming distinctive clusters	Known cultures associated with each cluster	Level of similarity at which the cluster was formed
After freezing in air:			
	41%	P. fragi and A-6	89%
	10%	F-17 and F-21	86%
	8%	P. fluorescens	84%
	12%	Enterobacteriaceae	81%
	9%	A. Simplex	80%
	8%	Staphylococcus	79%
	9%	Streptococcus	77%
	3%	(-[a])	65%
After freezing with liquid nitrogen:			
	36%	P. fragi and A-6	91%
	12%	F-21	88%
	9%	F-17 and P. fluorescens	85%
	16%	A. simplex	82%
	11%	Enterobacteriaceae	81%
	8%	Staphylococcus	78%
	7%	Streptococcus	76%
	1%	(-[a])	62%

Samples frozen in air and stored for 8 weeks at -29 C:

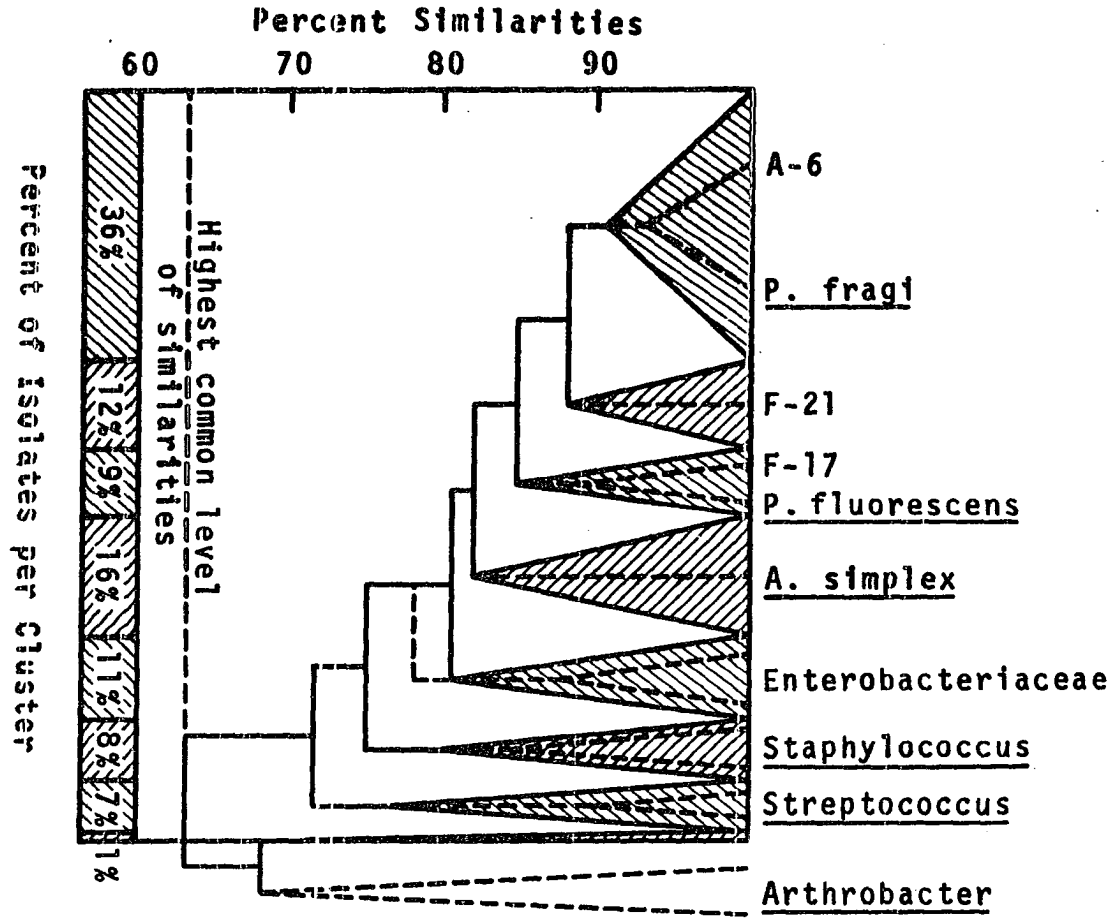
58%	A-6	93%
11%	P. fragi	90%
20%	F-21 and P. fluorescens	88%
4%	F-17	86%
6%	Enterobacteriaceae	82%
1%	Arthrobacter	75%

Samples frozen with liquid nitrogen and stored for 8 weeks at -29 C:

51%	A-6	94%
19%	P. fragi and F-17	90%
24%	F-21 and P. fluorescens	85%
4%	Enterobacteriaceae	81%
2%	Arthrobacter	75%

[a]Identified as yeasts by cell morphology.

Figure 10. Taxonomic relationships between the microflora isolated from chicken right after freezing (A-6 - nonfluorescent Pseudomonas; F-21 and F-17 - nonfluorescent Pseudomonas)


Percent Similarities
60
70
80
90
Percent of Isolates per Cluster
36%
12%
9%
16%
11%
8%
7%
1%
Highest common level of similarities
A-6
P. fragi
F-21
F-17
P. fluorescens
A. simplex
Enterobacteriaceae
Staphylococcus
Streptococcus
Arthrobacter

simplex was also detected after freezing. But the proportion of isolates forming this type of cluster increased from 3% before freezing to 16% of the organisms isolated after freezing.

The organisms isolated after freezing formed several clusters that associated with reference cultures other than Pseudomonas and A. simplex. A cluster formed by 11% of the isolates associated with strains of enterobacteria. Gram positive cocci were isolated for the first time. These cocci formed two clusters, one included 11% of the isolates and linked with Staphylococcus, the other cluster with 8% of the isolates linked with Streptococcus species. One of the cultures identified by its cell morphology as a yeast, separated from the other isolates at 62% level of similarity. This culture is represented in Figure 10 by the 1% cluster.

Two aspects of the bacteriology of chicken are to be considered at this point: the bacterial density and type of microflora. A reduction in bacterial densities due to freezing was previously discussed under "Bacterial densities after freezing." A comparison of the dendograms on Figures 9 and 10 shows that a change in the type of microflora took place as well. The reduction in numbers observed after freezing must have been primarily a consequence of a decrease in the numbers of Pseudomonas-like organisms while strains related to other genera must have been less susceptible to freezing. Therefore, changes in the proportions of the various types of microorganisms with freezing allowed for isolation of bacteria that had been outnumbered by Pseudomonas-like organisms during refrigerated

storage.

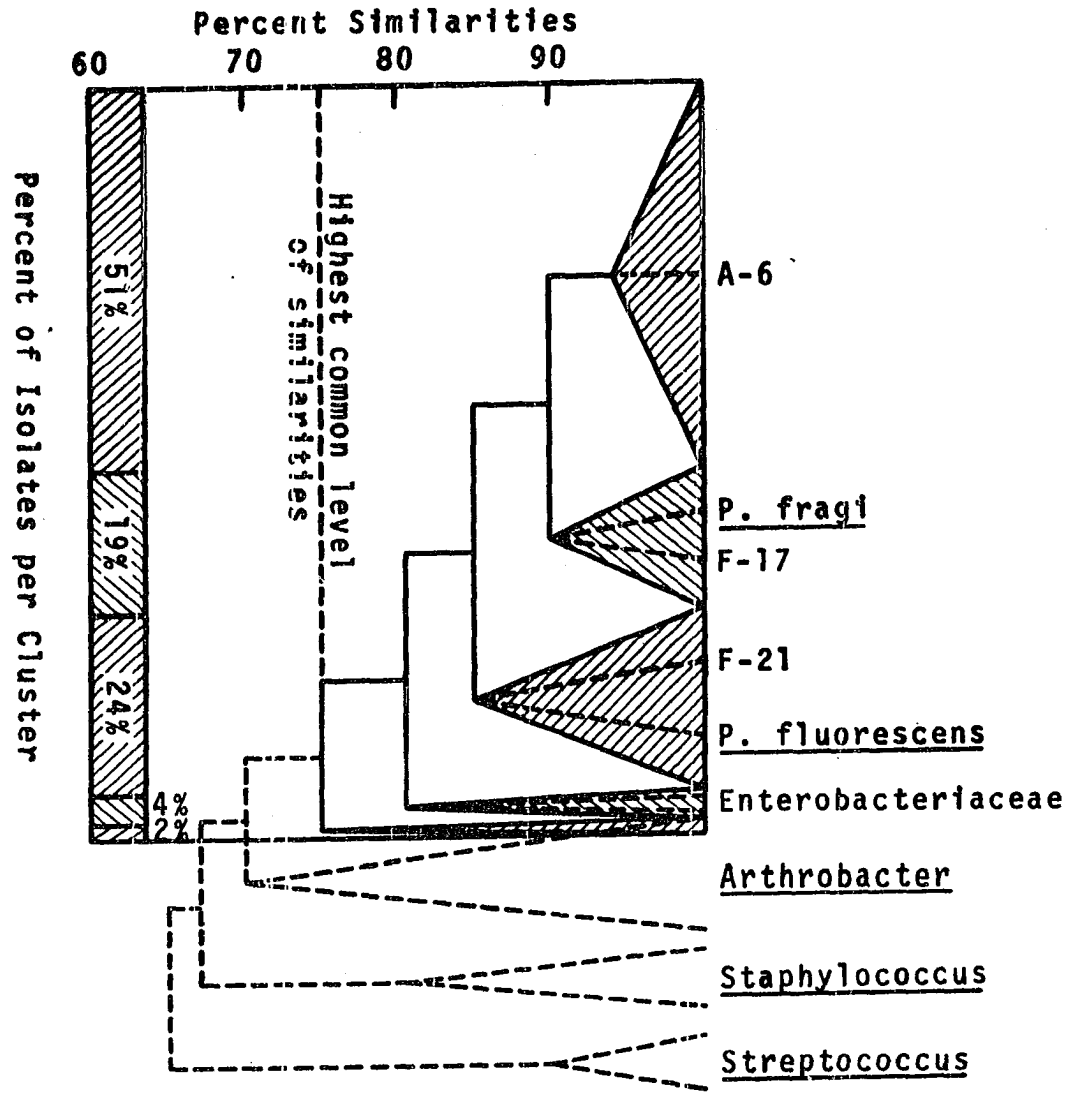
To give additional support to this assumption, a complementary test was done with the strains of bacteria used as references. The cultures were grown in Brain heart infusion broth (Difco) at 15 or 30 C, according to their psychrotrophic or mesophilic tendencies. These broth cultures were frozen and stored under identical conditions as described for the chicken samples. Cell counts of the broth cultures before and after freezing showed a more pronounced drop in cell numbers for Pseudomonas species than for strains of any other genera tested. With the limitations imposed by two different suspending media (chicken flesh versus Brain heart infusion broth), these results reinforced our hypothesis.

To summarize, freezing promoted a change in the proportions of different types of microorganisms present in the chicken apparently due to differences in susceptibility among bacterial species to cold shock. The overall effect was observed as an apparent increase in heterogeneity of the microflora.

Microflora after 8 weeks of frozen storage

The dendogram in Figure 11 shows the clusters formed by the organisms isolated from the samples after 8 weeks of storage at -29 C. A decrease in numbers of total aerobes was discussed under "Effect of frozen storage on bacterial densities". The reduction in numbers must have been greatly influenced by death of microorganisms of the types associating with known cultures other than

Figure 11. Taxonomic relationships between the microorganisms isolated from chicken stored for 8 weeks at -29 C (A-6 - nonfluorescent Pseudomonas; F-21 and F-17 - fluorescent Pseudomonas)


Percent Similarities
60
70
80
90
Percent of Isolates per Cluster
51%
19%
24%
4%
2%
Highest common level of similarities
A-6
P. fragi
F-17
F-21
P. fluorescens
Enterobacteriaceae
Arthrobacter
Staphylococcus
Streptococcus

the Pseudomonas since only two small clusters (4 and 2% of the isolates) of this type were isolated after frozen storage. These clusters are those linking with strains of the Enterobacteriaceae family and Arthrobacter species.

Frozen storage reversed the effect of freezing as discussed previously since the microflora isolated after 8 weeks at -29 C was almost as homogeneous in character as that isolated before freezing. The highest common level of similarities among all isolates within each treatment diminished from 82% before freezing (Figure 9) to 62% after freezing (Figure 10), and then rising to 75% similarity after frozen storage.

As observed in the treatments previously discussed, the majority of the organisms isolated after frozen storage (51, 19 and 24%) associated closely with the known strains of Pseudomonas. These groups represented a total of 94% of the microorganisms isolated from this treatment.

Once again, the most homogeneous and largest group clustered around the nonfluorescent Pseudomonas A-6. Also a relatively large group of isolates (24%) similar to the fluorescent strains F-21 and P. fluorescens was observed. It was pointed out previously that frozen storage caused a significant decrease in the numbers of total aerobes. However, the fluorescent Pseudomonas that survived the freezing process must have remained at constant densities during 8 weeks under frozen storage. By comparison of the clusters of organisms related to P. fluorescens and Pseudomonas F-21 in Figures

9, 10, and 11 it is evident that the proportions of organisms similar to the fluorescent strains increased from 3% on refrigerated chicken to 9% right after freezing and continued to increase to 24% during frozen storage. Thus, among the Pseudomonas-like organisms the nonfluorescent types apparently were more susceptible to the freezing process than those producing fluorescent pigment. A similar effect of freezing was reported by Rey and Kraft (1971). At equal levels of total aerobic bacteria, the proportions as well as the actual numbers of fluorescent bacteria developing on samples stored at 5 C were higher for frozen defrosted chicken than for unfrozen samples.

In the previous discussion it was noted that the nonfluorescent Pseudomonas-like groups that comprised 66 and 27% of the microflora isolated before freezing (Figure 9) decreased during the freezing process and comprised only 36% of the microflora after freezing (Figure 10). However, nonfluorescent Pseudomonas-like organisms that associated with P. fragi and A-6 among the organisms isolated after frozen storage (Figure 11) represented nearly 60% of the microflora. Evidently the nonfluorescent Pseudomonas-like bacteria, which were able to survive the freezing process seemingly survived well the 8 week period of frozen storage.

A decrease in numbers of total aerobes during frozen storage was discussed under "Effect of frozen storage on bacterial densities". The taxonomic evaluation of the microflora indicated that only a very minor proportion of the organisms isolated after frozen

storage did not associate closely to the Pseudomonas strains used as reference. These organisms formed two small clusters (2 and 4%) that linked with strains of enterobacteria and Arthrobacter (Figure 11). Therefore, the reduction in numbers of total aerobes observed during frozen storage is a consequence of the death of microorganisms of the types associating with cultures of genera other than Pseudomonas which were detected right after freezing.

Significance of the properties used for classification

Many of the characters used to determine similarities between organisms were enzymatic functions. Environmental factors such as freezing can block many normal pathways (Koepsell, 1950; Alford, 1960) or inducible enzyme systems of psychrotrophic bacteria (Kirkland, 1965; Hegeman, 1965). The experimental procedures used for these investigations involved three successive transfers onto trypticase soy agar for isolation and purification. Trypticase, an enzymatic digest of casein, is present in this medium and promotes quick recovery of bacterial cells metabolically injured by freezing (Straka and Stokes, 1959). It was assumed that the properties of the microflora used for classification were normal responses rather than erratic behavior of cells injured by freezing.

Stanier et al. (1966) have indicated that identical responses to some tests by different bacteria is not necessarily indicative of a high degree of relationship between two species since some substrates might be degraded to the same end product by biochemically

different pathways. For classification of food saprophytes, differences of this kind would not have the same weight as for phylogenetic taxonomy since the contribution to food spoilage by degradation of food components to identical end products would be equivalent, regardless of the metabolic pathway involved. Therefore, even when some of the characters used in this classification might not be valid for a typological approach, they were considered valid for the association of microorganisms contributing to food spoilage. These diagnostic characteristics indicate the behavior of the isolates from chicken under defined conditions. Chronological and environmental variability were minimized since the responses were obtained at the same age, and in the same environment for all isolates within each group. With the simplicity of the methodology it was possible to compare many characters for large groups of microorganisms. The drawing of large samples from the populations made them more representative. This, in turn, allowed for application of the law of probabilities to some extent.

Changes in properties of the microflora after each treatment

Very few of the characters tested were absolutely present or absent on the microflora of chicken. Ability to grow at initial pH of 8.1 or 9.6 were common characters, while none of the isolates was capable of growth at 3.5 ot 4.5 pH nor to hydrolyze cellulose. All other responses varied among isolates and changed to some degree with the various treatments.

Results of chi-square tests for significance of the changes in characters of the microflora with treatments are presented in Table 9. For most of the diagnostic characters the proportions of organisms possessing a given character changed significantly with freezing and with frozen storage but not with freezing method.

The change in proportions of isolates with treatment for the various characters presented in Figure 12 shows the psychrotrophic nature of the microflora isolated from the samples. All the organisms isolated before freezing and after 8 weeks of frozen storage and more than 95% of those isolated after freezing, were able to grow abundantly at 5 C. Five percent of the psychrotrophic organisms isolated after freezing and 28% of those isolated after frozen storage were able to grow well at 37 C also. The psychrotrophic behavior of the microflora isolated from the samples is also evident from the fact that many of the isolates that were capable of hydrolyzing chicken fat, tributyrin, gelatin and casein and production of fluorescent pigment when incubated at 15 C, were restricted in these functions at 30 C.

The percentage of isolates capable of hydrolyzing chicken fat ranged from 54% after freezing to 90% after frozen storage. Proteolytic activity was frequently present among the isolates from chicken as indicated by the large proportion of organisms hydrolyzing gelatin. Hydrolysis of chicken fat and proteolysis at 15 C were almost universal among the Pseudomonas used as reference (strain F-17 was negative). Cultures from other taxonomic groups showed a total lack of these

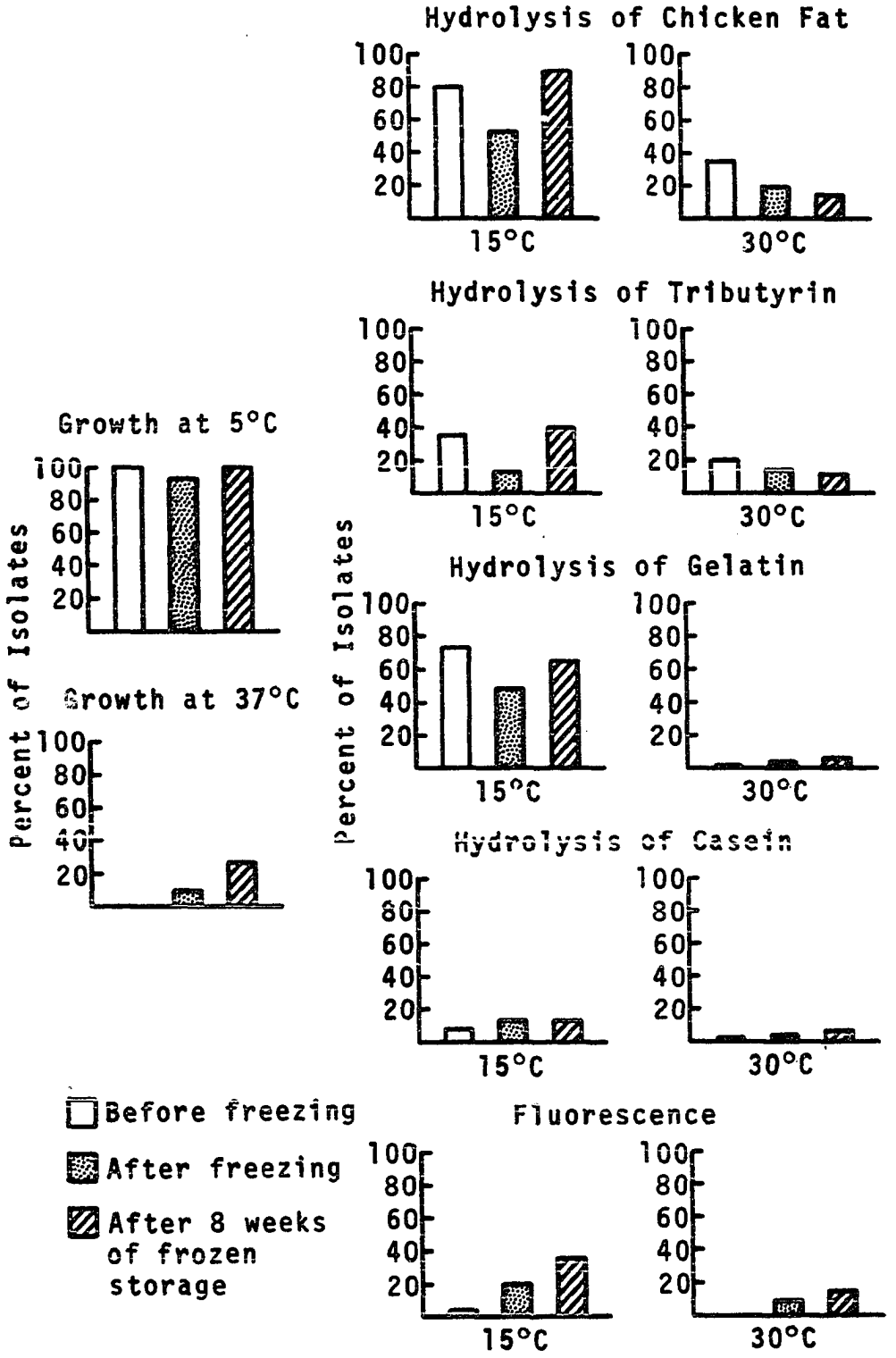
Table 9. Chi-square tests for significance of the changes in proportions of isolates with treatments for different diagnostic characters of the microflora of chicken

Diagnostic characters	Comparisons		
	Before vs. after freezing	After freezing vs. frozen storage	Frozen in air vs. liquid N_2
Growth at 5C	*[a]	*	N.S.[b]
Cell morphology	*	*	N.S.
Cytochrome oxydase	*	*	N.S.
Acid from glucose	*	*	N.S.
Hydrolysis of chicken fat at 15 C	*	*	N.S.
Hydrolysis of gelatin at 15 C	*	*	N.S.
Hydrolysis of tributyrin at 15 C	*	*	N.S.
Growth at 37 C	*	*	N.S.
Fluorescence at 15 C	*	*	N.S.
Growth at 45 C	*	*	N.S.
Anaerobic growth	*	*	N.S.
Growth at pH 5.5	*	*	N.S.
Gram reaction	*	*	N.S.
Hydrolysis of starch at 15 C	*	*	N.S.
Hydrolysis of starch at 30 C	*	*	N.S.
Hydrolysis of casein	*	N.S.	N.S.
Lecithinase activity	N.S.	*	N.S.
Acid from sucrose	*	*	*
Reduction of nitrate	*	*	*
Hemolysis at 30 C	*	*	*
Hydrolysis of gelatin at 30 C	*	*	*
Fluorescence at 30 C	*	*	*
Motility	*	*	*
Colony consistency	*	*	*
Acid from lactose	N.S.	N.S.	N.S.
Hemolysis at 15 C	N.S.	N.S.	N.S.
Catalase activity	N.S.	N.S.	N.S.
Hydrolysis of chicken fat at 30 C	N.S.	N.S.	N.S.
Hydrolysis of tributyrin at 30 C	N.S.	N.S.	N.S.

[a] * = Significant at 5% level of probabilities.

[b] N.S. = Not significant.

Figure 12. Changes in the proportions of organisms with treatment for various of the diagnostic characters tested

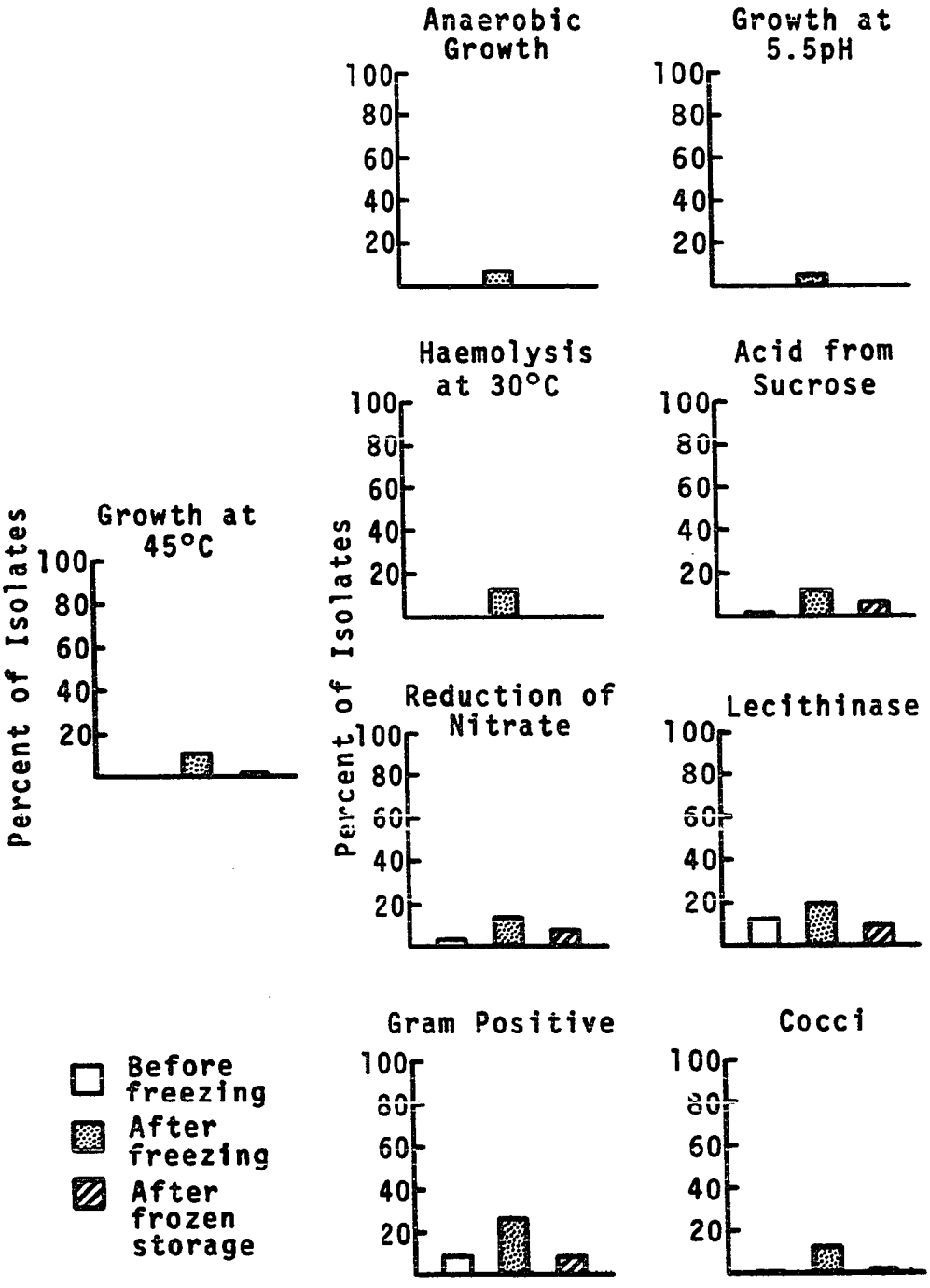

Hydrolysis of Chicken Fat
Hydrolysis of Tributyrin
Hydrolysis of Gelatin
Hydrolysis of Casein
Fluorescence
Growth at 5°C
Growth at 37°C
Percent of Isolates
100
80
60
40
20
15°C
30°C
Before freezing
After freezing
After 8 weeks of frozen storage

functions at 15 C even though some strains were positive at 30 C. The ability to hydrolyze chicken fat and to cause proteolysis at relatively low temperatures and the additional ability to grow at psychrotrophic or psychrophilic temperatures would favor the predominance of Pesudomonas-like organisms among the saprophytic flora developing on chicken stored at low temperatures.

Results presented in Figure 13 show several properties associated with the heterogeneous microflora isolated right after freezing. A group of organisms capable of abundant growth at 45 C was isolated after freezing. This supports the assumption that freezing was more detrimental to the psychrotrophic Pseudomonas-like organisms which predominated before freezing than to the other types of bacteria present. With the appearance of this group capable of growth at high temperature there was also an increase in the proportions of Gram positive isolates and in the percentage of cocci. Reduction of nitrate, acid formation from sucrose and lecithinase activity were also found more frequently among the microflora surviving freezing. Growth under anaerobic conditions and at initial acid pH, as well as hemolytic activity were properties found only among these organisms. The isolates possessing these characters were classified into clusters that associated with known Staphylococcus or Streptococcus. They contributed to a great extent to the increased heterogeneity of the microflora observed after freezing.

Changes in the proportions of organisms with characters such as catalase and oxidase activities, glucose utilization, gelatin,

Figure 13. Characteristics associated with the groups of organisms isolated for the first time right after freezing


Anaerobic Growth
Growth at 5.5pH
Haemolysis at 30°C
Acid from Sucrose
Growth at 45°C
Reduction of Nitrate
Lecithinase
Gram Positive
Cocci
Percent of Isolates
100
80
60
40
20
Before freezing
After freezing
After frozen storage

chicken fat and tributyrin hydrolysis and rod morphology were associated with the psychrotrophic flora growing abundantly at 5C (Figure 14). Organisms possessing these properties were most abundant in the homogeneous flora isolated before freezing. During the freezing process their proportions decreased, along with a drop in the level of homogeneity as shown in Figure 10 with a concomitant decrease in numbers of total aerobic bacteria as observed in Figure 4. During 8 weeks of frozen storage organisms with these characters recurred at about the same high proportion originally observed before freezing. Along with the increase in proportions of organisms with these characters, bacterial numbers continued to decrease during frozen storage as shown in Figure 4; on the other hand, the homogeneity of the microflora regressed to a level intermediate between that observed before and after freezing. These observations reinforce our assumption that the psychrotrophic groups of bacteria were more susceptible to the cold shock of the freezing process, but more stable to prolonged frozen storage than the nonpsychrotrophic organisms originally present in the samples.

A significant reduction in the proportion of organisms with Gram negative staining properties was promoted by freezing and incremented by frozen storage mainly at the expense of an increase in Gram variability (Figure 15). Similarly, motility decreased significantly after freezing and continued to decrease during frozen storage. The trend of these changes indicated that they were not particularly associated with either the psychrotrophic bacteria that

Figure 14. Changes in the proportions of microorganisms growing abundantly at 5 C and characteristics of the microflora associated with these changes

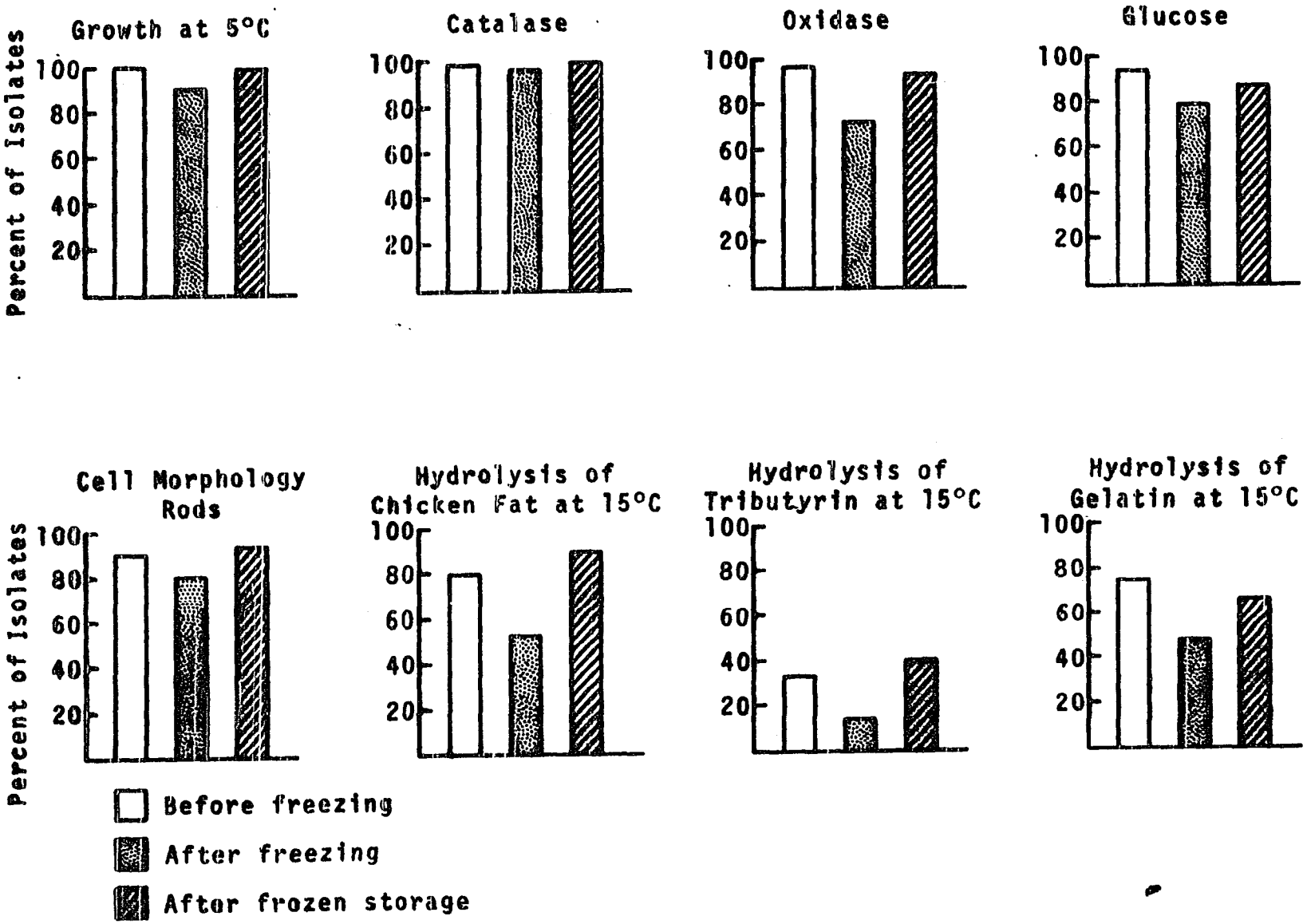
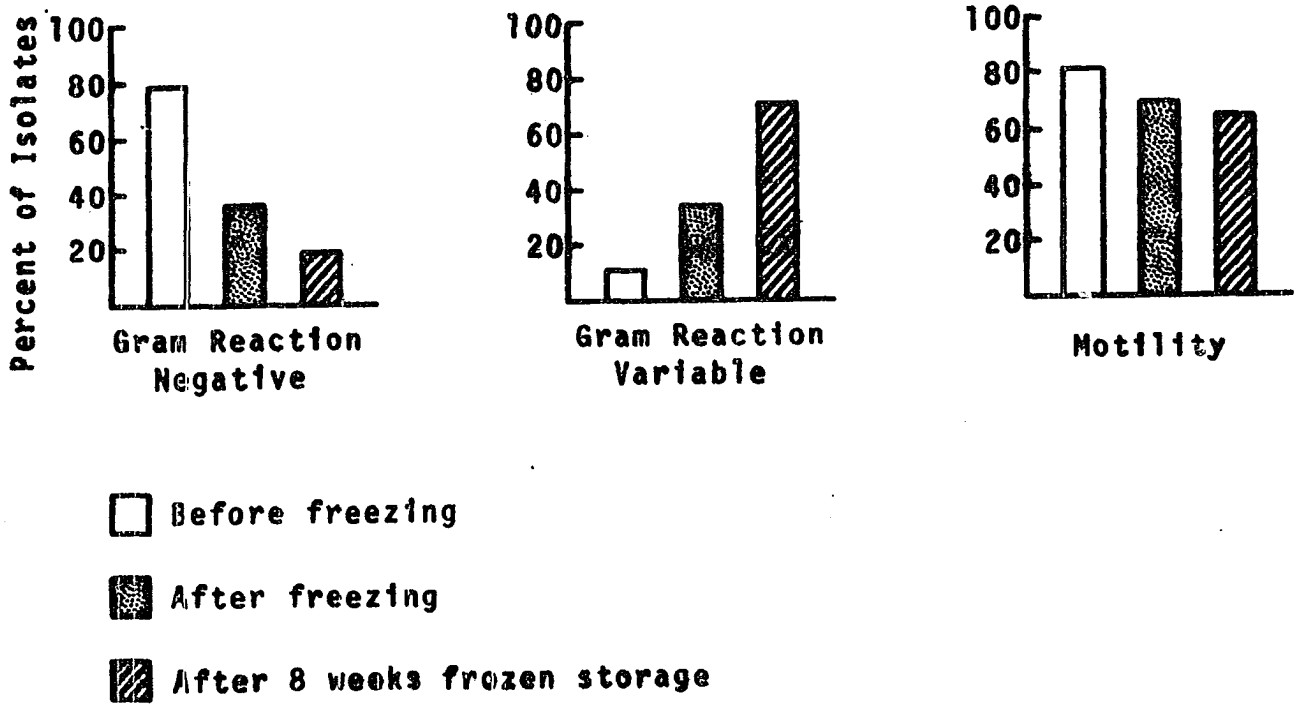

Growth at 5°C
Catalase
Oxidase
Glucose
Cell Morphology
Rods
Hydrolysis of
Chicken Fat at 15°C
Hydrolysis of
Tributyrin at 15°C
Hydrolysis of
Gelatin at 15°C
Percent of Isolates
100
80
60
40
20
Before freezing
After freezing
After frozen storage

Figure 15. Changes in motility and Gram stain reaction of the microflora of chicken with freezing and frozen storage


Percent of Isolates
100
80
60
40
20
Gram Reaction Negative
Gram Reaction Variable
Motility
Before freezing
After freezing
After 8 weeks frozen storage

decreased during freezing but were stable to frozen storage or with the non-psychrotrophic organisms that were stable during freezing but decreased during frozen storage. Therefore, impairment of bacterial motility and changes in staining reaction seem to be a general effect of freezing and frozen storage on the microflora of chicken.

Kitchell and Ingram (1956) reported that frozen defrosted meat spoils under refrigeration at the same rate as unfrozen controls. Rey and Kraft (1971) observed a selective effect of frozen storage on the natural bacterial flora of chicken which resulted in increased proportions of metabolically active psychrophiles. In the present studies, organisms with properties related to spoilage such as lipolysis of chicken fat and protein breakdown, also increased in proportions during frozen storage. An increase in relative numbers of active spoilage psychrotrophs during frozen storage could explain why in spite of lower initial numbers, frozen defrosted chicken spoils under refrigeration at the same rate as unfrozen controls.

From the foregoing discussion it is evident that most bacterial species remain viable for long periods of time under freezing temperatures. But freezing would selectively modify the bacterial flora that develops on chicken chilled under commercial conditions since the rate of death upon freezing and frozen storage is not equal for all the members of the bacterial population. The bacterial flora present in frozen defrosted chicken will be determined then by the type of bacteria present before freezing. The bacteria surviving

freezing, being those cells with the greatest resistance to injury at freezing temperatures, will be able to resume active growth upon thawing, if conditions are favorable. Therefore, it is important to minimize contamination of the fresh product especially with potential pathogens.

May (1962) reported that the cutting-up of chicken at processing plants results in lower numbers of bacteria than the cutting operation in retail stores. With the actual trend toward increased mechanization and the enforcement of inspection regulations in processing plants the differences in level of contamination of cut-up chicken prepared at the processing plant and prepared in retail stores can be even larger at the present time than when May surveyed them. Centralized cutting and packaging reduces the risks of contamination of the product during transportation from the processing plant to the consumer market. The type of commercial refrigeration used for ready-to-cook chicken allows rapid multiplication of bacteria of the psychrotrophic type that shortens the shelf life of the product. Freezing would not render the chicken safer than it was before freezing but it would prevent bacterial growth. This is equivalent to saying that if fresh chicken is to be stored at any time under frozen conditions it shall be frozen early after processing, ideally right at the processing plant.

The author recommends: 1) a rotational system for chilled chicken on display at the retail market, 2) coding of chicken packages to allow the consumer to recognize the length of time of

display storage on each package, 3) cutting and packaging of chicken parts at the processing plants, and 4) studying the economics and consumer acceptance of chicken frozen with liquid nitrogen in order to establish for the distribution and marketing of chicken frozen right after processing.

SUMMARY

The effect of two freezing methods on the natural bacterial flora that develops on chilled chicken during commercial refrigerated storage was investigated. Chicken wings that had been cut-up, packaged and placed on refrigerated display storage at a local retail store were frozen at the laboratory in 2 to 3 hr in a freezer room equipped with mechanical refrigeration or in 3 min by spraying with liquid nitrogen in a tunnel freezer. Wings frozen by either method were kept under frozen storage at -29 C for 8 weeks. Samples were examined before and after freezing and after 8 weeks of frozen storage for total aerobes, fluorescent Pseudomonas, enterococci, Salmonella, coagulase positive Staphylococcus and Clostridium perfringens. Changes in bacterial flora with treatments were also studied. Fifty-one characteristics of the isolates were investigated in order to classify the bacteria into phenotypes. Replica plating and computerized cluster analysis were tools used to compare the isolates. A set of known cultures representing various genera and species was used as reference to evaluate possible phylogenetic relationships between the bacteria isolated from the chicken and the known cultures. From the evaluation of the clusters formed by the isolates from chicken and the association of the clusters with known cultures similarities and differences between bacterial population on samples from the various treatments were observed.

Counts of total aerobes and fluorescent Pseudomonas for samples

from different packages indicated that even when all the packages were selected simultaneously at the retail store, there were probable differences in the length of time under refrigerated display storage between packages of wings. Salmonella and C. perfringens were not detected in any of the samples which would indicate good sanitary procedures for processing. All the unfrozen wings yielded coagulase positive Staphylococcus which might be related to handling during the cutting operation.

Decrease in bacterial counts was greater during the freezing process than during subsequent frozen storage. Incidence of coagulase positive Staphylococcus did not decrease during freezing but was greatly reduced by frozen storage. However, numbers of viable bacteria were still high after 8 weeks of frozen storage.

Taxonomic evaluation of the microflora showed that freezing and frozen storage had a selective effect, being more detrimental to some types of bacteria than to others. Clusters of bacteria associating with known mesophilic species of potential pathogens were detected after freezing. Freezing caused a great drop in the numbers of viable psychrotrophic bacteria. This allowed for isolation of mesophilic organisms that had been outnumbered by psychrotrophs before freezing and could not be detected with the isolation procedures employed. Because of changes in relative proportions of the various types of bacteria present, the microflora isolated after freezing appeared more heterogeneous than those isolated before freezing or after 8 weeks of frozen storage. Certain characteristics

of the bacterial flora that can be related to spoilage of chicken such as lipolysis of chicken fat and proteolysis were influenced by freezing and frozen storage. Less proteolytic and lipolytic bacteria with psychrotrophic tendencies were present among the heterogeneous microflora isolated after freezing than among the more homogeneous populations isolated either before freezing or after 8 weeks of frozen storage.

There were no detectable differences in the bacteriological profile of the samples that could be attributed to method of freezing. However, the color and general appearance of liquid nitrogen frozen samples were quite superior to the air frozen samples. The good appearance of the liquid nitrogen samples was not impaired during storage for 8 weeks under conventional frozen conditions.

CONCLUSIONS

1. Bacterial counts can vary greatly among packages of cut-up chicken during retail storage.

2. Psychrotrophic Pseudomonas-like bacteria predominate in the microflora developing during distribution and commercial display storage of chicken. Bacteria hydrolyzing chicken fat and producing proteolysis comprise a high proportion of this psychrotrophic flora. Mesophilic types of bacteria may be present also in high concentration on chilled chicken but their isolation from a nonselective plate count is difficult due to being outnumbered by psychrotrophic Pseudomonas-like organisms.

3. The microflora of commercially chilled chicken appears more heterogeneous after freezing because the freezing process causes a sharp decrease in the concentration of psychrotrophic bacteria with an increase of relative numbers of mesophiles.

4. The psychrotrophic bacteria that survive freezing are more stable to prolonged frozen storage than the surviving mesophiles.

5. Fluorescent Pseudomonas-like organisms comprise a greater proportion of the population after freezing and frozen storage than before freezing chicken from commercial display storage because these organisms seem to survive the freezing process and the exposure to frozen storage better than the nonfluorescent Pseudomonas-like bacteria.

6. Coagulase positive staphylococci can be found frequently on

commercially cut-up chicken. The process of freezing has little effect on the frequency of isolation of this type of bacteria from chicken, but prolonged frozen storage produces a decrease in incidence of coagulase positive Staphylococcus on chicken.

7. The bacteriological profile of liquid nitrogen frozen chicken was essentially the same as that of the air frozen product.

8. Numerical taxonomy as used in this investigation, had the great advantage of allowing for evaluation of a large representative sample from each of the populations of bacteria investigated; the interpretation of the results however, relays almost entirely on the researcher's reasoning because of unavailability of computational procedures that would help in recognizing sources of variation and estimating their significance.

9. Freezing by spraying with liquid nitrogen yields color and general appearance closer to fresh chicken than air freezing does. The good appearance of the frozen product is not impaired by subsequent storage for 8 weeks at -29 C.

LITERATURE CITED

Alford, J. A. 1960. Effect of incubation temperature on biochemical tests in the genera Pseudomonas and Achromobacter. J. Bacteriol. 23:591-593.

Angelotti, R., M. J. Foter, and K. H. Lewis. 1961. Time-temperature effects on Salmonella and staphylocci in foods. I. Behavior in refrigerated foods. Am. J. Public Health 51:76-83.

Angelotti, R., H. E. Hall, M. J. Foter and K. H. Lewis. 1962. Quantitation of Clostridium perfringens in foods. Appl. Microbiol. 10:193-199.

Anonymous. 1964. Exciting breakthrough: Freezing slices tomatoes. Food Eng. 36:56-57.

Anonymous. 1967. Ground beef to packaged frozen patties in less than three minutes. The National Provisioner 1967:48. August 5.

Arpai, J. 1962. Nonlethal freezing injury to metabolism and motility of Pseudomonas fluorescens and Escherichia coli. Appl. Microbiol. 10:297-301.

Åström, S. and G. Löndahl. 1969. Air blast in-line freezing versus ultra rapid freezing. A comparison of freezing results with some various vegetables and prepared foods. Pages 121-127 in Frozen foods. Commissions IV & V, Int. Inst. of Refrigeration, Budapest, Hungary.

Ayres, J. C. 1960. The relationship of organisms of the genus Pseudomonas to the spoilage of meat, poultry and eggs. J. Appl. Bacteriol. 23:471-486.

Ayres, J. C., W. S. Ogilvy, and G. F. Stewart. 1950. Post-mortem changes in stored meats. I. Microorganisms associated with the development of slime on eviscerated cut-up poultry. Food Technol. 4:199-205.

Ayres, J. C., H. W. Walker, M. J. Fanelli, A. W. King, and F. Thomas. 1956. Use of antibiotics in prolonging storage life of dressed chicken. Food Technol. 10:563-568.

Baker, R. C. 1954. Effect of different methods of scalding, cooling and freezing on the appearance of ready-to-cook poultry carcasses. Poultry Sci. 33:1040.

Barnes, E. M. and D. H. Shrimpton. 1959. The effect of tetracycline compounds on the storage life and microbiology of chilled eviscerated poultry. J. Appl. Bacteriol. 21:313-329.

Bisset, K. A. 1962. The phylogenetic concept in bacterial taxonomy. Pages 361-373 in 12th Symp. Soc. Gen. Microbiol. Microbial classification. The University Press, Cambridge.

Bengtsson, N. E. and B. Jakobsson. 1969. The influence of high freezing rates on the quality of frozen ground beef and small cuts of beef. Pages 165-169 in Frozen foods. Commissions VI & V, Int. Inst. of Refrigeration, Budapest, Hungary.

Bladel, B. O. and R. A. Greenberg. 1965. Pouch method for the isolation and enumeration of clostridia. Appl. Microbiol. 13: 281-285.

Board, R. G., J. C. Ayres, A. A. Kraft, and R. H. Forsythe. 1964. The microbiological contamination of egg shells and egg packing materials. Poultry Sci. 43:584-595.

Brant, A. W. and G. J. Stewart. 1950. Bone darkening in frozen poultry. Food Technol. 4:168-174.

Breed, R. S. 1957. Considerations influencing the classification used in this edition of the manual. Pages 4-14 in R. S. Breed, E. G. D. Murray, and N. R. Smith. Bergey's manual of determinative bacteriology. The Williams and Wilkins Co., Baltimore, Md.

Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed. The Williams and Wilkins Co., Baltimore, Md.

Breyer, F., R. C. Wagner, and J. P. Ryan. 1966. Application of cryogenics in the baking industry. Chem. Eng. Progress Symp. Series 62:93-104.

Brown, D. C. 1967. The application of cryogenic fluids to the freezing of foods. Ad. Cryogenic Eng. 12:11-22.

Cain, A. J. 1962. The evolution of taxonomic principles. Pages 1-13 in 12th Symp. Soc. Gen. Microbiol. Microbial classification. The University Press, Cambridge.

Childs, R. E., M. J. Reed, and J. A. Hamann. 1970. Guidelines for poultry-processing plant layouts. U.S. Dept. Agric., Marketing Research Report No. 878.

Clough, C. J. 1969. Technical aspects of liquid nitrogen freezing. Pages 137-140 in Frozen foods. Commissions IV & V, Int. Inst. of Refrigeration, Budapest, Hungary.

Colwell, R. R. 1964. A study of features used in the diagnosis of Pseudomonas aeruginosa. J. Gen. Microbiol. 37:181-194.

Committee on Salmonella. 1969. An evaluation of the Salmonella problem. National Academy of Sciences, Division of Biology and Agriculture, National Research Council, Washington, D.C.

Corlett Jr., D. A., J. S. Lee and R. O. Sinnhuber. 1965. Application of replica plating and computer analysis for rapid identification of bacteria in some foods. I. Identification scheme. Appl. Microbiol. 13:808-817.

Dawson, L. E. 1969. Stability of frozen poultry meat and eggs. Pages 143-167 in W. B. VanArsdel, M. J. Copley, and R. L. Olson. Quality and stability of frozen foods. Wiley-Interscience, New York, N.Y.

Despaul, J. E. 1964. A study of characteristics and methods of detection of food poisoning microorganisms with particular emphasis on Clostridium perfringens. Laboratory Division Directorate of Technical Operations Defense Subsistence Supply Center, Chicago, Ill.

Desrosier, N. W. 1959. The technology of food preservation. AVI Publishing Co., Westport, Conn.

Edwards, P. R. and W. H. Ewing. 1962. Identification of Enterobacteriaceae. 2nd ed. Burgess Publishing Co., Minneapolis, Minn.

Elliott, R. P. and H. D. Michener. 1960. Review of the microbiology of frozen foods. U.S. Dept. of Agric., Western Utilization Res. and Dev. Div., Albany, Calif.

Ellis, C. and J. G. Woodroof. 1959. Prevention of darkening in frozen broilers. Food Technol. 13:533-538.

Fanelli, M. J. and J. C. Ayres. 1959. Methods of detection and effect of freezing on the microflora of chicken pies. Food Technol. 13:294-300.

Federal Register. 1972. Poultry products inspection regulations. Federal Register 37, No. 95, Tuesday, May 16, 1972.

Flynn, T. M. and C. N. Smith. 1970. Trends in cryogenic fluid production in the United States. Pages 241-247 in Cryophysics and Cryoengineering. Commission I, Int. Inst. of Refrigeration, Tokyo, Japan.

Frazier, W. C. 1967. Food microbiology. 2nd ed. McGraw-Hill Book Co., New York, N.Y.

Galton, M. M., J. R. Boring, and W. T. Martin. 1968. Salmonellae in foods. U.S. Dept. of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Georgia.

Goldman, M. L. and M. M. Rayman. 1952. Hydrolysis of fats by bacteria of the Pseudomonas genus. Food Res. 17:326-337.

Gray, R. 1967. Red meat commercially frozen with liquid nitrogen system. Quick Frozen Foods, February:129-130.

Greenberg, R. A., R. B. Tompkin, B. O. Bladel, R. S. Kittaka, and A. Anellis. 1966. Incidence of mesophilic Clostridium spores in raw pork, beef and chicken in processing plants in the U.S. and Canada. Appl. Microbiol. 14:789-793.

Grower, J. C. 1967. A comparison of some methods of cluster analysis. Biometrics 23:623-637.

Gunderson, M. F., H. W. McFadden, T. S. Kyle. 1954. The bacteriology of commercial poultry processing. Burgess Publishing Co., Minneapolis, Minn.

Hartman, P. A. 1968. Miniaturized microbiological methods. Advances in Appl. Microbiol. Supplement 1. Academic Press, New York, N.Y.

Hartman, P. A. and P. A. Pattee. 1968. Improved capillary action replicating apparatus. Appl. Microbiol. 16:151-153.

Hegeman, G. D. 1965. Studies on induced enzyme synthesis in Pseudomonas fluorescens. Diss. Abstr. 26:627.

Herman, L. G. and F. A. Morelli. 1960. The growth and isolation of coagulase-positive staphylococci on medium No. 110 fortified with egg yolk. Bacteriol. Proc. 102.

Hobbs, B. C. 1969. Clostridium perfringens and Bacillus cereus infections. Pages 131-173 in H. Rieman, Food-borne infections and intoxications. Academic Press, New York, N.Y.

Ingram, M. and J. M. Shewan. 1960. Introductory reflections on the Pseudomonas-Achromobacter group. J. Appl. Bacteriol. 23: 373-378.

Institut International du Froid. 1972. Recommendations for the processing and handling of frozen foods (Translated title). Annexe au Bulletin de l'Institut International du Froid (Paris, France) 1972:52-54.

King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. (St. Louis, Mo.) 44:301-307.

Kirkland, J. J. 1965. Influence of exogenous carbon sources on inducible enzyme formation in Pseudomonas fluorescens. Diss. Abstr. 26:1289-1290.

Kirsch, R. H., F. E. Berry, G. L. Baldwin, and E. M. Foter. 1952. The bacteriology of refrigerated ground beef. Food Res. 17: 495-503.

Kitchell, A. G. and M. Ingram. 1956. A comparison of bacterial growth on fresh meat and on frozen meat after thawing. Ann. Inst. Pasteur, Lille 8:121-131.

Klose, A. A. and M. F. Pool. 1956. Effect of freezing conditions on appearance of frozen turkeys. Food Technol. 10:34-38.

Koepsell, H. J. 1950. Gluconate oxidation by Pseudomonas fluorescens. J. Biol. Chem. 186:734-751.

Kovacs, N. 1956. Identification of Pseudomonas pyocyanea by the oxydase reaction. Nature 174:703.

Kraft, A. A. 1969. Significance of various bacteria considered for microbial standards for poultry and poultry products. Symposium on "Microbiological Standards for Poultry Products", 58th Ann. Meeting of Poultry Sci. Assn. Colorado State University, Ft. Collins, Col. Institute of American Poultry Industries (Poultry and Egg Inst. of America), Chicago, Ill.

Kraft, A. A. ca. 1975. Gram positive non-sporeforming rods. Coryneforms and their significance in foods. Unpublished manuscript. Iowa State University, Ames, Ia.

Kraft, A. A. and J. C. Ayres. 1961. Production of fluorescence on packaged chicken. Appl. Microbiol. 9:549-553.

Kraft, A. A., J. C. Ayres, K. F. Weiss, W. W. Marion, S. L. Balloun, and R. H. Forsythe. 1963. Effect of method of freezing on survival of microorganisms on turkey. Poultry Sci. 42:128-137.

Kraft, A. A., J. C. Ayres, G. S. Torrey, H. R. Salzer, and G. A. N. daSilva. 1966. Coryneform bacteria in poultry, eggs and meat. J. Appl. Bacteriol. 29:161-166.

Krieg, R. E. and W. R. Lockhart. 1966. Classification of enterobacteria based on overall similarity. J. Bacteriol. 92:1275-1280.

Kuschfeldt, D. and W. Thiel. 1970. Chilling and freezing of poultry with liquid nitrogen (Translated title). Bull. Int. Inst. of Refrigeration L2:390. (Abstract).

Lee, J. S. and G. C. Wolfe. 1967. Rapid identification of bacteria in foods: Replica plating and computer method. Food Technol. 21:35-39.

Li, K. C., E. K. Heaton, and J. E. Marion. 1969. Freezing chicken thighs by liquid nitrogen and sharp freezing process. Food Technol. 23:107-109.

Lockhart, W. R. and J. Liston. 1970. Methods for numerical taxonomy. Am. Soc. Microbiol., Bethesda, Maryland.

May, K. N. 1962. Bacterial contamination during cutting and packaging chicken in processing plants and retail stores. Food Technol. 16:89-91.

Mazur, P. 1966. Physical and chemical basis of injury in single-celled microorganisms subjected to freezing and thawing. Pages 213-315 in Cryobiology. Academic Press, New York, N.Y.

Mecchi, E. P., M. F. Pool, G. A. Belmain, M. Hamachi, and A. A. Klose. 1956. The role of tocopherol content in the comparative stability of chicken and turkey fat. Poultry Sci. 35:1238-1246.

Michener, H. D. and R. P. Elliott. 1964. Minimum growth temperature for food-poisoning, fecal indicator and psychrophilic microorganisms. Adv. Food Res. 13:349-396.

Michener, H. D. and R. P. Elliott. 1969. Microbiological conditions affecting frozen food quality. Pages 43-84 in W. B. Van Arsdel, M. J. Copley, and R. L. Olson, Quality and stability of frozen foods. Wiley-Interscience, New York, N.Y.

Michener, H. D., P. A. Thompson, W. C. Dietrich. 1960. Time-temperature tolerance of frozen foods. XXII. Relationship of bacterial population to temperature. Food Technol. 14:290-294.

Monzini, A., M. Bassi, and G. Cricelli. 1969. Freezing rates and ultrastructural modifications in some vegetables. Pages 47-51 in Frozen foods. Commissions IV & V Int. Inst. of Refrigeration, Budapest, Hungary.

Morphew, A. E. 1969. General discussion on cryogenic freezing. Pages 171-174 in Frozen foods. Commissions IV & V, Int. Inst. of Refrigeration, Budapest, Hungary.

Mossel, D. A. A. and M. Ingram. 1955. The physiology of the microbial spoilage of foods. J. Appl. Bacteriol. 18:232-268.

Nagel, C. W., K. L. Simpson, R. H. Vaughn, and G. F. Stewart. 1960. Microorganisms associated with spoilage of refrigerated poultry. Food Technol. Champaign 14:21-23.

Novak, A. F. and M. R. Ramachandra Rao. 1966. Freezing more efficient with liquid nitrogen. Food Eng., 38:53-55.

Oblinger, J. L. and A. A. Kraft. 1973. Oxidation-reduction potential and growth of Salmonella and Pseudomonas fluorescens. J. Food Sci. 38:1108-1112.

Officiel Mag. Arts Menang. Fr. 1972. Comparative statistics for the manufacture of household equipment in the world. Bull. Int. Inst. of Refrigeration L2:1264.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1962. Staphylococci in competition. Appl. Microbiol. 10:16-22.

Piskarev, A. I. and A. P. Bomovalova. 1969. Investigations on quality changes during cold storage of fish frozen in liquid nitrogen. Pages 151-154 in Frozen foods. Commissions IV & V, Int. Inst. of Refrigeration, Budapest, Hungary.

Quick Frozen Foods Inst., U.S.A. 1972. Per capita consumption of frozen foods around the world, 1970. Bull. Int. Inst. of Refrigeration L2:93.

Rey, C. R. 1968. Survival, growth and biochemical activity of Pseudomonas associated with spoilage of poultry. Unpublished M.S. thesis. Library, Iowa State University of Science and Technology, Ames, Ia.

Rey, C. R. and A. A. Kraft. 1971. Effect of freezing and packaging methods on survival and biochemical activity of spoilage organisms on chicken. J. Food Sci. 36:454-458.

Rey, C. R., A. A. Kraft, R. G. Seals, and E. W. Bird. 1969. Influence of temperature on some biochemical characteristics of Pseudomonas associated with spoilage of chicken. J. Food Sci. 34:279-283.

Rey, C. R., A. A. Kraft, H. W. Walker, and F. C. Parrish, Jr. 1970. Microbial changes in meat during aging at elevated temperature and later refrigerated storage. Food Technol. 24:67-71.

Rey, C. R., A. A. Kraft, and R. E. Rust. 1971. Microbiology of beef shell frozen with liquid nitrogen. J. Food Sci. 36: 955-958.

Rhodes, M. E. 1959. The characterization of Pseudomonas fluorescens. J. Gen. Microbiol. 21:221-263.

Rhodes, M. E. 1961. The characterization of Pseudomonas fluorescens with the aid of an electronic computer. J. Gen. Microbiol. 25:331-345.

Rieman, H. 1969. Food-borne infections and intoxications. Academic Press, New York, N.Y.

Ryan, J. F. 1966. Liquid nitrogen freezing of poultry products. Quick Frozen Foods Magazine, July-August.

Sakaguchi, G. 1969. Botulism type E. Pages 329-358 in H. Rieman, Food-borne infections and intoxications. Academic Press, New York, N.Y.

Salzer, R. H., A. A. Kraft, and J. C. Ayres. 1967. Microorganisms isolated from turkey giblets. Poultry Sci. 46:611-615.

Shiflett, M. A., J. S. Lee, and R. O. Sinnhuber. 1966. The microbial flora of irradiated Dungeness crabmeat and Pacific oysters. Appl. Microbiol. 14:411-415.

Sneath, P. H. A. 1956. Cultural and biochemical characteristics of the genus Chromobacterium. J. Gen. Microbiol. 15:70-98.

Sneath, P. H. A. 1957. The application of computers to taxonomy. J. Gen Microbiol. 17:201-226.

Sneath, P. H. A. 1962. The construction of taxonomic groups. Pages 289-332 in 12th Symp. Soc. Gen. Microbiol. Microbial classification. The University Press, Cambridge.

Sneath, P. H. A. and R. R. Sokal. 1962. Numerical taxonomy. Nature 193:855-860.

Smith, H. L. and K. Goodner. 1958. Detection of bacterial gelatinases by gelatin-agar plate method. J. Bacteriol. 76: 662-665.

Sokal, R. R. and P. H. A. Sneath. 1963. Principles of numerical taxonomy. W. H. Freeman and Co., San Francisco, Calif.

Somn, H. 1969. A brief survey of some trends in modern legislation regarding frozen foods. Pages 311-317 in Frozen foods. Commissions IV & V, Int. Inst. of Refrigeration, Budapest, Hungary.

Spencer, J. V., E. A. Santer, and W. J. Stadelman. 1955. Shelf life of frozen meat after thawing. Poultry Sci. 34:1222-1223.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic Pseudomonads: A taxonomic study. J. Gen. Microgiol. 43:159-271.

Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1970. The microbial world. 3rd ed. Prentice-Hall Inc., Englewood Cliffs, N.J.

Straka, R. P. and J. L. Stokes. 1959. Metabolic injury to bacteria at low temperatures. J. Bacteriol. 78:181-185.

Strandine, E. 1963. Poultry production and processing. Pages 63-86 in J. L. Heid and M. A. Joslyn, Food processing operations. Vol. II. The AVI Publishing Co., Westport, Conn.

Sulzbacher, W. L. 1950. Survival of microorganisms in frozen meat. Food Technol. 4:386-390.

Taylor, J. and J. H. McCoy. 1969. Salmonella and Arizona infections. Pages 3-72 in H. Rieman, Food-borne infections and intoxications. Academic Press, New York, N.Y.

Thornley, M. J. 1960. Computation of similarities between strains of Pseudomonas and Achromobacter isolated from chicken meat. J. Appl. Bacteriol. 23:395-397.

Thornley, M. J. 1967. A taxonomic study of Acinetobacter and related genera. J. Gen. Microbiol. 49:211-257.

Trauberman, L. 1966. Cryogenics: for which products? Food Eng. 38:86-89.

U.S. Dept. of Health, Education, and Welfare, Public Health Service, National Communicable Disease Center. 1968a. Clostridium botulinum type A due to home-cooked chicken. Morbidity and Mortality Weekly Report 17:348.

U.S. Dept. of Health, Education, and Welfare, Public Health Service, National Communicable Disease Center. 1968b. Epidemiological notes and reports: Clostridium botulinum type A. Morbidity and Mortality Weekly Report 17:446.

U.S. Dept. of Health, Education, and Welfare, Public Health Service, National Communicable Disease Center. 1969, 1970, 1971, 1972, 1973. Center for Disease Control: Foodborne outbreaks annual summary Nos. 70-74.

U.S. Dept. of Agric., Economic Research Service. 1973a. Dairy Situation. U.S. Dept. of Agric., Economic Research Service, Bull. DS-347.

U.S. Dept. of Agric., Economic Research Service. 1973b. Poultry and egg statistics through 1972. U.S. Dept. of Agric., Economic Research Service, Statistical Bull. No. 525.

U.S. Dept. of Agric., Statistical Reporting Service, Crop Reporting Board. 1974. Poultry slaughter. U.S. Dept. of Agric., Statistical Reporting Service Bull. Pou. 2-1(2-74).

Walker, H. W. and J. C. Ayres. 1956. Incidence and kinds of microorganisms associated with commercially dressed poultry. Appl. Microbiol. 4:345-349.

Wilkerson, W. B., J. C. Ayres, and A. A. Kraft. 1961. Occurrence of enterococci and coliform organisms on fresh and stored poultry. Food Technol. 15:286-292.

Wilson, E., M. J. Foter, and K. H. Lewis. 1959. A rapid test for detecting Staphylococcus aureus in food. Appl. Microbiol. 7: 22-26.

Woodburn, M. J. and D. H. Strong. 1960. Survival of Salmonella typhimurium, Staphylococcus aureus and Streptococcus faecalis frozen in simplified food substrates. Appl. Microbiol. 8: 109-113.

Wolin, E. F., J. B. Evans, and C. F. Niven, Jr. 1957. The microbiology of fresh and irradiated beef. Food Res. 22:682-686.

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