THE EFFECT OF TIME AND TEMPERATURE UPON SALMONELLAE IN INOCULATED BUTTER

by

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INTRODUCTION

Salmonellosis is considered currently to be the most important of the zoonotic diseases, since it affects more people than any other. As many as 2 million persons are infected each year in the United States (Steele and Galton, 1966). There is evidence the incidence is increasing in man and animals in at least the more advanced nations. Such an increase is ironic accompaning the presumed gradual improvement in sanitation for man and animals. Possibly the factors favoring increased incidence of salmonellosis, particularly sporadic cases, may include (a) changes in the feeding habits of the population with communal feeding and increased use of ready-cooked foods in the home; (b) improved laboratory facilities and better techniques for the detection and identification of Salmonella; (c) improved reporting and epidemiological investigation of food poisoning incidents; (d) centralization and extension of bulk preparation and mass distribution and consumption of human and animal food; (e) increased international commerce in bulk foods for human and animal consumption; (f) "build-up" of Salmonella in livestock and poultry as they pass from the farm to market and abattoir; (g) increased incidence of human excreters, especially among persons responsible for handling human and animal food.

Salmonellosis is a communicable disease usually transmitted from vertebrate animals to man in food or drink. The cycle of infection involves the transfer of viable Salmonella from one

host to another: directly, when one host ingests the contaminated feces of another; indirectly, when the victim ingests contaminated food.

Salmonellosis is caused by a microorganism of the genus Salmonellae. As many as 1200 + sero-types have been detected. The most commonly involved in food poisoning are S. typhimurium and S. typhimurium var copenhagen (Morbidity and Mortality Report, 14).

The most important reservoirs of human salmonellosis are livestock and poultry. Salmonella are common in swine and poultry, frequent in rodents, not uncommon in cattle, sporadic in sheep and horses, and occasional in wild animals (WHO/FAO, 1959). The organism inhabits the intestinal tract of the host.

It therefore follows that the most important vehicle of Salmonella is human or animal food, the most suspect foods being those lightly cooked and subjected to much handling.

Food may become contaminated with Salmonella in many ways, some of which are (a) intravital infection of poultry and livestock resulting in sick animals or asymptomatic carriers, (b) by failure to pasteurize milk from infected cows, (c) by infection of eggs in the oviduct or by contamination of eggs by feces, (d) by use of contaminated egg products in processed foods, (e) by food preparation in an already contaminated area, (f) by contamination with organisms from insects, (g) by contamination with feces from infected rodents or birds, and (h) by human excreters-cases, convalescents, or asymptomatic carriers.

The following general classifications of foods have been incriminated as causing salmonellosis; meat and meat products, egg and egg products (bulk broken-out), milk and milk products (unpasteurized or post-pasteurization contamination), fish, and uncooked vegetables.

Salmonella contamination of milk may be either intrinsic or extrinsic. The former more commonly occurs during the febrile stage of bovine salmonellosis, while extrinsic contamination may occur through fecal contamination.

Numerous epidemics of salmonellosis have been traced to raw or improperly pasteurized milk and milk products, however, few disease epidemics are attributable to butter. While butter is not commonly considered as a source of salmonellosis, this relative unconcern of its potential in this regards warrants further study. Due to the household practice of keeping butter at room temperature to insure its spreading, the potential may exist for Salmonella food poisoning if contaminated butter reaches the public.

Salmonellae have been isolated from butter and their presence in butter could be attributed to (a)lack of adequate pasteurization of contaminated cream, (b) contamination of pasteurized cream, (c) contamination of wash water being utilized, or (d) contamination during handling.

In view of the ways butter may become contaminated and the variations in salt content, storage time, and storage temperature, a study was devised in an attempt to observe the survival of

Salmonella typhimurium var copenhagen inoculated into butter. The variables considered were salt content and time and temperature of storage.

REVIEW OF LITERATURE

Salmonellae have been recognized as a separate genus of bacteria since 1885 when Dr. D. E. Salmon, an American bacteriologist and veterinarian, isolated Salmonella cholerae-suis, then believed to be the agent of hog cholera.

The widespread occurrence of Salmonellae is well documented (Snoeyenbos, 1967). There are in excess of 1200 sero-types of Salmonella presently recognized with more being identified each year. Salmonella are said to be ubiquitous in nature, the organism having been isolated from almost every variety of mammals, birds, amphibians, reptiles, and even from some insects (Schroeder, 1967).

Animal Reservoirs:

Livestock and poultry are most significant reservoirs of human salmonellosis (Bowmer, 1965). Surveys conducted in the United States have revealed a widespread distribution of Salmonella. From 47 animal species Edwards et al (1948) isolated 111 different types, 61 of which were also recovered from man; these 61 types were responsible for 93% of human infections.

Moran (1962) reported that from 5000 recoveries from animals between 1957 and 1961 (excluding avian species S. pullorum and S. gallinarum); there were 84 different types from 35 animal species. The most common types were S. typhimurium (28%); S.

cholerae-suis (8%); S. anatum and S. heidelberg (each 6%); S. enteritidis, S. newport and S. san diego (each 5%); S. infantis (4%); S. chester and S. saint paul (each 3%); and S. blockey, S. derby, and S. muenchen (each 2%). These 13 sero-types accounted for 78% of the animal isolations.

Pigs:

In Florida, Galton et al (1954) found that 27 (7%) rectal swabs from 374 pigs on 11 of 28 farms yielded Salmonella.

In 1961 and 1962 Shotts et al recovered Salmonella from 95 (54%) of 176 samples taken at Kentucky abattoirs. The contaminated areas included mud in the lairage, the ramp to the kill room, the dehairing machine and chute, the scraping table, the hand saw, and the edible viscera pan.

Hansen et al (1964) reported that keeping hogs in holding pens for prolonged periods prior to slaughter resulted in an increased evidence of Salmonella in the intestine at the time of slaughter. Williams and Newell (1967) concluded that stress and possibly antibiotics fed on the farm, markedly influenced the excretion of Salmonella.

Barnes and Bergeland (1968) reported an epizootic of S. typhisuis infection of swine on 5 Minnesota premises. Mortality was approximately 10-30% in affected groups. These were widely scattered occurrences and no attempt to trace the source of infection was evident.

Poultry and Poultry Processing Plants:

Galton et al (1955), in a survey of American processing plants, found Salmonella in 11% of 118 swabs from tubs containing iced birds. Wilder and MacCready (1966) reported that 11.2% of cultures from 2,057 samples from 2 poultry processing plants (1 inspected (5.5%) and the other uninspected (17%)) were positive for Salmonella. They also reported 50.2% of dressed chickens and 39% of unpasteurized frozen eggs were positive for Salmonella.

Glezen et al (1966) reported an epidemic of febrile gastroenteritis occurred in 170 of 1200 persons who attended a chicken barbecue supper at a North Carolina public school in 1958. Salmonella typhimurium was recovered from 82 of 176 fecal specimens. In reviewing food handling methods utilized, it was evident there was opportunity for contamination of prepared foods by persons who were handling raw chickens. The source plant of the chickens was surveyed for Salmonella organisms in conjunction with another larger plant. The larger plant (good sanitation) had less than 1% positive Salmonella recoveries while the source plant (poor sanitation) had 21% positive Salmonella recoveries. There was no direct evidence that chickens were the source of S. typhimurium but the food handling practices were inadequate to prevent its spread. Edwards (1958) has stated that domestic fowl are probably the largest single source of Salmonella in animals.

Pets:

Salmonellosis of children was traced to pet turtles in two instances in Minnesota (1964). The same sero-type was readily isolated from the patients feces and the turtle water.

Young puppies and kittens are mentioned by Thomsett (1963) as being as much as 35% and 12% infected with Salmonella, respectively, and serve as ready reservoirs for household infections.

Pet rabbits were reported as a source of S dublin by Rokey and Erling (1960). Frogs also served as a good reservoir of Salmonella as reported by Pantaleon and Rosset in 1964. They isolated Salmonella 31 times from 164 samples. Frogs originating in South America were particularly contaminated.

Cattle:

Recent reports indicate that salmonellosis among dairy and beef cattle is becoming a major problem. In 1959 and 1960 Ellis reported 40 isolations of Salmonella from cattle with enteritis in Florida, many of which became permanent excreters.

Rokey and Erling (1959) reported an outbreak of S. dublin in calves in an Arizona dairy herd. Mortality was 33% and morbidity was 85%. Henning (1959) reported that S. dublin remained viable in dried feces for up to 3 years on the same premises. Mature cattle, after apparent recovery, have been shown to shed S. dublin for 3 years (Field, 1949).

In 1962, (Vet. Rec., 1963), a dairy cow became ill after calving and the calf died suddenly. Her surplus colostrum severly scoured other calves and the cow soon died. Salmonella

typhimurium var copenhagen was isolated from the cow, one calf, and 4 human contacts.

Moore et al (1962) incriminated S. newport as a cause of septicemic and toxic enteritis in cattle, resulting in serious losses of valuable dairy cattle in the United States.

Rude (1963) reported that S. typhimurium and S. typhimurium var copenhagen caused 46 cases of salmonellosis in cattle referred to the Central Animal Diagnostic Laboratory, Madison, and the Regional Animal Diagnostic Laboratory, Borron, Wisconsin. He reported a watery, fetid diarrhea and persistent, highly elevated body temperature as the main clinical signs in mature cattle accompanied with depression and decreased milk flow. Calves rapidly became dehydrated and had rough hair coats, yellow feces, often blood-tinged, with temperatures as high as 107 F and depression associated with pneumonia. The incidence was definitely higher in calves than mature cattle. Sources of infection were not established. Rankin and Taylor (1966) stated that the LD_{50} of a strain of S. typhimurium for calves was 100,000 viable units.

Rothenbacher (1965) reported that S. typhimurium proved to be the predominating sero-type incriminated in calf losses on 39 Michigan farms during a 20 month period. Mortality experienced was 23.6%. It was thought that adult carriers played an important role in these cases.

Salmonella typhimurium affected 80% of a 70 cow dairy herd resulting in 9 deaths in Oregon (Peterson and Coon, 1967). Milk production dropped from 2,500 to 300 lb./day. Strangely enough calves running with the infected cows did not become ill. Three

children on the dairy farm also became ill enough to be hospitalized. A definite source could not be found.

Woods et al (1968) reported S. typhimurium and infectious bovine rhinotracheitis concurrently infected 75 dairy cattle resulting in 11 deaths and 12 abortions. The combination of the 2 diseases produced decreased milk production, profuse diarrhea, abortion, and death. S. typhimurium was isolated from a dairy feed supplement, a bulk raw milk sample, and rectal samples of the affected cows. The source of Salmonella was believed to be the dairy supplement. Even though no human cases developed on the farm, the necessity of adequate pasteurization of milk is emphasized.

Animal By-Products and Feeds:

Wedman and Ellis (1963) attributed inadequate sanitation in the processing and handling of animal by-products and feeds as being responsible for contamination with Salmonella organisms.

Moyle (1966) found 10.8% of rendering plant by-products to be contaminated with Salmonella. No correlation existed between the incidence of Salmonella and plant sanitation. He did, however, attribute some salmonella-inhibition properties to the putrefactive process (possibly free fatty acids).

A survey of animal feed and organic fertilizers resulted in 88 sero-types of Salmonella isolated from 1,262 samples (Bull. Ministry of Health, 1959).

Foods:

As mentioned before, the most important vehicle of Salmonella is human or animal food.

In England and Wales between 1949 and 1960 there were in excess of 3100 general and family outbreaks where there was a food vehicle identified. Of these outbreaks, 73% were associated with meat, 8% with sweatmeats, 6% with fish, 6% with egg and egg* products, 3% with milk and milk products, 2% with vegetables, 1% with fruits, and 1% with other foods (Cockburn, 1962).

Many meats, especially processed meats, have been incriminated as a source of Salmonella. Bulk sausage was contaminated at an 11% rate in a study conducted by Wilson et al in 1961. Salmonella typhimurium was incriminated in 105 scattered cases eminating from fresh meat by Harvey et al in Wales in 1963. Eight persons were ill after eating raw or slightly fried ham slices containing 23,000 S. infantis per gram (Angelotti et al, 1961).

Beasley et al (1967) reported that people contracted salmonellosis by ingesting pet food contaminated with S. typhimurium and S. dublin.

Turkey meat (Bowmer, 1961 and 1963, McDonagh and Smith, 1958) has been incriminated in several instances as was chicken by Caraway and Bruce in 1961.

Cooked meat was incriminated as a vehicle for typhoid dissemination by Wilson et al in 1961. Meat pies have been indicated as a vehicle by Hobbs (1953) and potted meat caused S. typhimurium infection according to Evans and Suggitt (1949). Most of these cases involved inadequate cooking or contamination after cooking.

Eggs and Egg Products:

Mass production of many foods that are eaten without adequate cooking includes bulk-broken egg products. Cake mixes containing S. thompson and S. heidelberg caused salmonellosis in two Canadian outbreaks (Butler and Josephson, 1962 and Skoll and Dillenberg, 1963). Commercial egg products were shown by Thatcher and Montford (1962) to contain Salmonella (up to 54% in cake mixes and 21% in frozen egg products) in a Canadian survey. The Canadian Department of National Health and Welfare instituted a regulation prohibiting sale of egg products containing Salmonella; the contamination rate was reduced to 2% (Thatcher, 1963).

Egg-nog originating from fresh duck eggs was incriminated as the vehicle in an outbreak involving 104 of 1,850 patients in a Massachusetts mental hospital. Three duck eggs and the patients yielded S. typhimurium (Philbrook, et al., 1960).

Trifle, including cake made from egg products, was responsible for an outbreak involving over 100 school children. Salmonella typhimurium and S. thompson were recovered from the patients, trifle, stools of bakery personnel, and drain swabs (Harvey et al, 1961).

Bierer and Barnett (1965) conducted an experiment designed to evaluate wash water temperature kill of Salmonella on egg shells. They concluded that the 3 minute washing temperature should be between 140 and 150 F.

Vegetables:

Vegetables have been only rarely associated with salmonellosis. Desiccated coconut, containing S. typhi and S.

paratyphi B plus 12 other sero-types, was incriminated as causing an outbreak of typhoid fever and salmonellosis (Kovacs, 1959).

Milk and Milk Products:

As recently as 2 years ago, no one thought Salmonella posed much of a problem for the milk industry. Discovery of Salmonella in powdered milk in 1966 caused the recall of many nationally distributed brands, resulting in economic loss of millions of dollars. Apparently one plant was the source of the S. new brunswick contaminated product. Temperatures employed for pasteurization and subsequent heat treatment were insufficient to kill Salmonella (Schroeder, 1967).

Price and Carter (1967) reported an outbreak of Salmonellosis in Pennsylvania due to consumption of unpasteurized cup cheese contaminated with S indiana.

Butter was incriminated as a vehicle of typhoid bacillus in 1910 in Anoka, Minnesota (Hammer and Bell).

Experimental evidence of Salmonella and other bacterial response in different foods is available. Angelotti et al (1961) reported that growth of Salmonella, Staphylococci, and Clostridia botulinum was prevented in perishable foods (custard, chicken ala king, and ham salad) when the internal temperature was at or below 42 F. Their conclusion was that prompt and adequate refrigeration appeared to be the best safeguard against the development of conditions favoring food poisoning during storage.

Mossel (1963) wrote that one should always expect that certain dehydrated products, commodities of slightly reduced pH or increased salt content, and perishable low acid foodstuffs kept under refrigeration or frozen may contain Salmonella.

Salmonella inoculated into bouillon, bouillon with egg, vanilla sauce with egg, tomato juice, and carrot juice diminished in the course of 6 days (at temperatures of 7, 4, and 2 C) down to about 10% of the quantity introduced. Freezing at -10 C resulted in a rapid decrease of the number of Salmonella, down to 10-20% within 48 hours and down to 3-5% in 6 days (Wundt and Schittenhelm, 1965).

Todorov (1962) reported that Salmonella survived in sweet and ripened-cream butter kept at room temperature well beyond the shelf-life of the butter, and at 0 C the organisms remained viable up to 124 and 55 days respectively.

Zagaevskii (1963) studied the viability of S. typhimurium and S. dublin in milk and 6 dairy products during storage at 18 to 23 C or 0 to 4 C and indicated viability of up to 9 months in butter.

Berry (1927) reported that in butter stored at 50.9 F, S. schottmulleri survived 324 days, S. paratyphi 107 days, and a strain of S. typhosa 290 days. Washburn (1908) noted that in an ice chest S. typhosa survived 151 days in butter and 43 days in milk.

Butter, stored at -10 to -12 C for 6 months, evidenced a gradual decline of Escherichia coli with complete disappearance by the end of the storage period. In butter stored at 2 to 4 C for 3 months, E. coli decreased significantly (Avakyan, 1965).

Shigella flexneri and Shigella sonnei were reported to survive -20 C for 10 months in butter samples when inoculated at levels of 6×10^7/g (Dobberkan and Lenk, 1966).

Goepfert et al (1968) contaminated cheddar cheese with Salmonella typhimurium after pasteurization. They reported that during curing at 7.5 C and 13 C a 2 log reduction in Salmonella level was achieved during a 60 day holding period. The 13 C curing effected the largest decline. Evidence was presented indicating that the production of volatile fatty acids in the curd during curing attributed to the decline.

Wethington and Fabian (1949) reported that Staphylococci were more viable than Salmonella when inoculated in salad dressing and mayonnaise. All organisms survived longer in mayonnaise than salad dressing. Acetic acid levels were higher in the salad dressing (pH 3.2) than mayonnaise (pH 3.8) and were deemed responsible for decreased survival.

Hunziker has amply outlined butter's physical and chemical properties. He describes butter as principally composed of milk fat (77-84%), moisture (13-19%), curd (0.89%), and salt (0-4%). Butter is essentially a water in oil emulsion derived from cream, an oil in water emulsion. Butter fat is composed of a mixture of numerous fatty glycerides. The definitely known fatty acids include; butyric, lauric, caproic, myristic, palmitic,

oleic, stearic, capric, and caprylic. The moisture is attributable to two sources; the buttermilk and the wash water. Buttermilk droplets are exceedingly minute and although they contain suitable food for bacteria, they are too small for much bacterial activity and the majority of them are sterile. On the other hand, wash water droplets are large enough in water content but lack enough nutrients for bacterial growth.

Salt in butter is present in the water droplets. Therefore, the concentration of salt in the water droplets of the butter controls any inhibitory properties acting on microorganisms present. The addition of salt (due to the hydrophilic nature of salt) to butter decreases the proportion of small water droplets and greatly increases the proportion of large droplets when compared with unsalted butter.

King (1964) attributed the sole site of biochemical and microbiological reactions taking place either inside the droplets or at the interface between the water and the fat. He stated that bacterial cells are located within the moisture droplets, and butter does not seem to be a very suitable substrate for growth of microorganisms.

MATERIAL AND METHODS

Bacterial Cultures:

The culture of *Salmonella typhimurium* var *copenhagen* used in this study was procured from the Biology Department, Kansas State University. The stock culture was maintained at room temperature after an initial 24 hours at 37 C on trypticase soy agar slants*. Transfer to new slants were made at 30 day intervals.

The inoculum was prepared by washing the cells from 24 hour slants with sterile 0.1% tryptone solution* (St. Julian *et al*, 1963). The suspension was centrifuged** at 500 G's for 30 minutes and the supernatant fluid discarded. The cells were resuspended in sterile 0.1% tryptone solution and adjusted to an optical density of 0.4 at 520 mu on a spectrophotometer***. This resulted in a cell concentration of 6.5×10^8/ml (variance of 34.8) as predetermined by the least squares regression technique of Fryer. The inoculum for batches 1 and 2 consisted of 5.0 ml of this suspension. The inoculum for batch 3 consisted of 500 ml water-tryptone cell suspension (O.D. 0.4) utilized as wash water. The concentration was confirmed by plating on Brilliant Green sulfadizine (BGS) agar* using the spreader technique.

*Difco Laboratories, Detroit, Michigan 48201

**International Centrifuge Size 1, Type SB; International Equipment Co., Boston, Mass.

***Bausch & Lomb Spectronic 20; Bausch & Lomb, Rochester, New York 14602

Manufacture and Sampling of Butter:

Butter was manufactured in s sterilized Dazey* glass electric churn. Commercial grade A cream was used and each churning consisted of 4-1/2 pints. Fat content, standard plate count, and coliform count were performed on the cream as described in Standard Methods (1960). All cream was plated on BGS agar prior to inoculation with Salmonella to assure its being Salmonella free.

Batch 1 (Unsalted butter, cream inoculated)- To 4-1/2 pints of 36.5% fat cream (45 F) was added 5.0 ml tryptone cell suspension of S. typhimurium var copenhagen (O.D. 0.4). The inoculated cream was churned approximately 45 minutes and the butter placed in a sterilized pyrex bowl after discarding the buttermilk. The butter was washed with 300 ml sterile deionized distilled water (40 F) and worked with a sterile wooden paddle to distribute the moisture evenly. The butter was placed in sterile screw-top jars of 130 ml capacity, each jar receiving 10 grams of butter. Five lots were formed (13 jars/ lot) and one lot placed at each of the following temperatures; 77 F, 40 F, 32 F, 0 F, and -10 F (25 C, 4.44 C, 0 C, -17.77 C, and -23.33 C). Samples were withdrawn and placed in the above mentioned jars for initial Salmonella count and chemical analysis of the butter. Chemical analysis was performed by the Kohman method (Goss, 1953).

Batch 2 (Salted butter, cream inoculated)- To 4-1/2 pints of 37.5% fat cream (45 F) was added 5.0 ml of tryptone cell suspension

*The Dazey Corporation, St. Louis 7, Missouri.

of S. typhimurium var copenhagen (O.D. 0.4). Working, churning, and washing were accomplished similar to that performed on batch 1, except for the addition of 20 grams of sterile NaCl to the butter. The butter was divided, stored, and sampled as batch 1.

Batch 3 (Salted, wash water inoculated)- Churning was accomplished in 45 minutes using 4-1/2 pints of 30.0% fat cream. Buttermilk was discarded and the inoculated wash water (500 ml) prepared by adding 75 ml tryptone cell suspension to 425 ml sterile deionized distilled water (40 F). The resulting cell suspension (O.D. 0.4) was used to wash the butter and act as another method of butter contamination. Nineteen grams of sterile NaCl was worked into the butter mass after washing. The butter was divided, stored and sampled as batches 1 and 2.

The following table (Table 1) will summarize the cream and butter composition and inoculum levels:

Table 1: Composition of Cream, Butter, and Inoculum Levels of the Different Batches.

Constituent	Batch 1	Batch 2	Batch 3
CREAM			
% Fat	36.5	37.5	30.0
Standard Plate Count/ml	6000	3300	3000
Coliform/ml	<10	<10	<10
Quantity (pts)	4-1/2	4-1/2	4-1/2
SALMONELLA CELL SUSPENSION			
Concentration	8.5×10^8/ml	1.0×10^9/ml	6.4×10^8/ml
Volume Added (ml)	5.0	5.0	5.0
BUTTER			
% Fat	85.8	82.0	84.1
% Moisture	13.3	15.3	14.1
% Salt	0.0	2.2	1.7
% Curd	0.9	0.5	0.1
Initial Salmonella Count	1.14×10^5/g	2.7×10^3/g	2.196×10^5/g

All lots were sampled initially and at the following intervals; 3, 6, 9, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days.

Enumeration of Salmonella:

Enumeration of Salmonella throughout this study was accomplished by use of the BGS spreader plate technique as suggested by V. D. Foltz*.

Samples were removed from their storage temperatures at intervals indicated above and immediately a 5 gram sample aseptically removed by heat sterilized spatula and placed in a 50 ml sterilized beaker. One ml of 10% Tergitol No. 7 solution** was added by sterile pipette for the purpose of emulsification (Galton, et al, 1968). The amount of Tergitol No. 7 solution used per sample was increased to 3 ml in cases where undiluted butter was spread on BGS agar plates as otherwise even spreading necessary for uniform colony distribution was hampered. The beaker containing the butter-Tergitol mixture was then placed in a 45 C (113 F) magnetically agitated water bath *** until the butter had completely melted (Methods of Analysis of milk and its Prod.). Concurrently warming in the same water bath were 9 and 99 ml sterile dilution blanks containing 0.1% tryptone solution. The blanks were warmed to prevent the melted better from solidifying when dilution was in progress. The butter

*V. D. Foltz, Department of Biology, Kansas State University

**Carbide and Carbon Chemical Co., 30 E. 42nd St., New York 17, N.Y.

***Magni Whirl Serological Water Bath, Blue Electric Co., Blue Island, Illinois

Tergitol mixture was then serially diluted and 0.1 ml from each dilution spread on BGS agar plates with sterile 3 mm glass rods (bent at right angles) immediately upon pipetting. Duplicate plates were utilized. When undiluted butter was spread on BGS agar plates the plates were prewarmed to 39 C to enhance spreading.

Plates were then incubated at 37 C for 24 hours in the inverted position. Plates were examined at the end of 24 hours and if colonies were excessively small the plates were allowed to incubate an additional 24 hours before counting. Plates were counted on a Quebec dark field colony counter*. Only plates having 30-300 colonies of typical Salmonella were counted. Typical Salmonella colonies were streaked and stabbed on triple sugar iron and lysine iron agar slants** and streaked on trypticase soy agar slants***. These slants were incubated at 37 C for 24 hours and the results recorded. Confirmation of isolates as S. typhimurium var copenhagen was accomplished by slide agglutination with group B antisera**** and by flagellar agglutination with Salmonella H Antiserum Poly****.

*American Optical Co., Bufalo, N.Y.

**Difco Laboratories, Detroit, Michigan 48201

***Baltimore Biological Laboratory, Baltimore, Maryland

****Difco Laboratories, Detroit, Michigan 48201

All used BGS agar plates were disposed of by gas incineration. A 2% Amphyl* solution was used for disinfection of Salmonella contaminated jars, glass spreader rods, dilution blanks, butter churn, and table tops. An autoclave** was utilized to sterilize the BGS, TSI, lysine, and trypticase medias as well as 9 and 99 ml dilution blanks, pyrex bowl, wooden paddle, clean glass spreader rods, 50 ml beakers, pipetts, wash water, Tergitol No. 7 solution, and miscellaneous articles.

*Lehn and Fink Products Corp., Toledo, Ohio

**Scanlan Morris Co., Madison, Wisconsin

RESULTS

Salmonella typhimurium var copenhagen was recovered from all butter samples at all time intervals and at all temperatures. At no time did the recovery level at temperatures ≤ 40 F (Tables 3, 4, 5, and 6) exceed the initial count of the same batch. All batches supported the growth of Salmonella when stored at 77 F as illustrated in Table 2 and Fig. 1.

Batch 1 (Unsalted butter, cream inoculated) was shown to have about a 1.5 to 2 log decrease (5.26 to 0.709% of original) in viable Salmonella count at temperatures ≤ 40 F at the end of the 10 week storage period as illustrated in Tables 3, 4, 5, and 6 and Fig. 2, 3, 4, and 5. The same butter stored at 77 F became rancid and was shown to have about a 3.35 log increase (368421% of original) in viable Salmonella within 3 days followed by a gradual decline of viable Salmonella to a level 1.3 logs below (3.42% of original) the initial count at the end of the 10 week storage period.

Batch 2 (Salted butter, cream inoculated) Salmonella count was demonstrated to decline about 0.7 to 0.8 logs (22.52 to 13.04% of original) at temperatures ≤ 40 F at the end of the 10 week storage period. The same butter stored at 77 F became rancid and was shown to have a 3.1 log (123,444% of original) increase in viable Salmonella within 3 days followed by a fluctuating recovery level, declining to a level 0.2 logs (44.44% of original) below the initial count at 56 days, then followed by a slight

increase of about 0.6 logs (506.67% of original) at the end of the 10 week storage period.

Batch 3 (Salted butter, wash water inoculated) was shown to have about a 0.8 to 1.1 log (5.02 to 14.06% of original) decrease in viable Salmonella at temperatures $\leq$ 40 F at the end of the 10 week storage period. The same butter stored at 77 F was shown to increase by about 1.6 logs (5,661% of original) within 3 days, gradually increasing to a level 2.4 logs (42349% of original) above original counts at 35 days. The recovery level then declined to a level about 0.8 logs (837% of original) above the initial count at the end of the 10 week storage period.

All isolates from the BGS agar plates were serologically and biochemically identifiable as S. typhimurium var copenhagen.

Tables 2, 3, 4, 5, and 6 are the counts on BGS plates for Batches 1, 2, and 3 for all temperatures involved. They are complete for all sampling intervals from the initial count to day 70 which completed the study.

Table 2: Salmonella Counts/g at 77 F for Batches 1, 2, and 3.

Days	Batch 1	Batch 2	Batch 3
Initial	114,000	2,700	219,600
3	420,000,000	3,333,000	12,432,000
6	390,000,000	3,504,000	20,520,000
9	339,000,000	4,860,000	19,260,000
14	138,600,000	7,980,000	22,980,000
21	142,200,000	3,906,000	63,000,000
28	142,200,000	588,000	16,080,000
35	840,000	12,540,000	93,000,000
42	942,000	1,560,000	1,542,000
49	978,000	22,200	1,410,000
56	94,200	1,200	1,760,000
63	4,320	2,000	1,760,000
70	3,960	13,680	1,840,000

Table 3: Salmonella Counts/g at 40 F for Batches 1, 2, and 3.

Days	Batch 1	Batch 2	Batch 3
Initial	114,000	2,700	219,600
3	14,760	2,370	147,200
6	9,780	1,672	92,800
9	11,340	1,680	22,000
14	3,240	1,216	24,240
21	2,322	872	6,720
28	2,872	2,112	5,760
35	2,512	1,480	15,040
42	1,400	1,768	15,520
49	2,880	1,424	14,480
56	816	504	3,360
63	1,280	736	14,560
70	904	608	29,760

Table 4: Salmonella Counts/g at 32 F for Batches 1, 2, and 3.

Days	Batch 1	Batch 2	Batch 3
Initial	114,000	2,700	219,600
3	32,040	2,280	62,400
6	23,160	1,016	12,800
9	31,380	1,648	30,640
14	7,860	2,024	15,200
21	4,680	2,360	7,440
28	6,040	888	4,080
35	3,248	1,576	5,600
42	1,344	952	6,880
49	264	592	16,160
56	1,624	456	12,000
63	720	368	27,600
70	808	512	21,280

Table 5: Salmonella Counts/g at 0 F for Batches 1, 2, and 3.

Days	Batch 1	Batch 2	Batch 3
Initial	114,000	2,700	219,600
3	85,800	1,020	96,000
6	17,520	240	16,240
9	15,660	936	11,440
14	4,800	1,768	37,360
21	5,820	504	3,200
28	4,240	144	2,640
35	1,840	256	4,400
42	3,472	1,208	7,840
49	2,640	200	2,160
56	2,240	336	14,320
63	2,400	192	12,320
70	1,000	352	11,040

Table 6: Salmonella Counts/g at -10 F for Batches 1, 2, and 3.

Days	Batch 1	Batch 2	Batch 3
Initial	114,000	2,700	219,600
3	36,000	558	18,400
6	8,880	280	4,960
9	15,180	432	8,560
14	9,240	152	9,200
21	4,320	80	3,600
28	3,608	80	5,840
35	2,272	336	6,400
42	2,144	392	29,770
49	1,688	376	16,240
56	6,080	864	14,240
63	1,600	344	21,680
70	6,000	560	30,880

DISCUSSION

Due to the methods employed in manufacture of butter the author recognizes some difference between each batch and that differences exist between the experimental butter and commercial butter. There were, theoretically, differences in moisture droplet size and dispersion as well as salt distribution.

Contamination levels were necessarily higher than is probable under commercial conditions to enable observation of trends.

Count data as recorded in Tables 2, 3, 4, 5, and 6 were converted to percentages of original count for all batches stored at $\leq$ 40 F. All counts from samples of butter stored at 77 F were converted to log deviations from initial count. These transformations were necessary to enable comparison to be derived from different original population levels. Figures 1, 2, 3, 4, and 5 graphically represent these transformed populations for the time, temperatures, and batches involved.

A three way analysis of the variance was computed* on all counts derived from butter stored at $\leq$ 40 F. The data from Tables 3, 4, 5, and 6 was converted to a square root of the log of the percentage for programing**. Table 7 illustrates the analysis derived.

It was evidnet from the computer analysis results in Table 7 that all variables had a significant effect upon the population means. Time of storage had a significant effect in that the longer a sample was held at temperatures $\leq$ 40 F the lower became the level of surviving Salmonella.

*IBM 360 Model 50
**Department of Statistics and Computer Science, Kansas State University

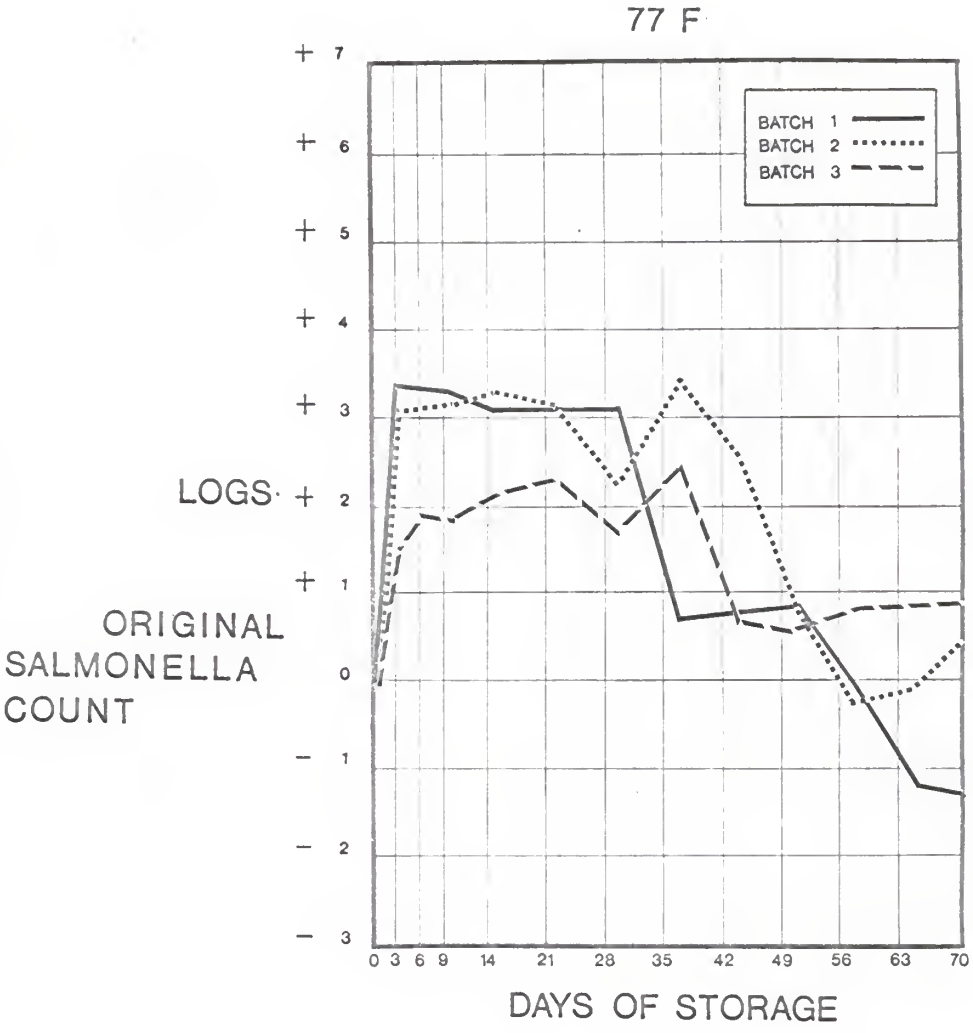

Fig. 1 - Comparison of butter batches 1, 2, and 3 regarding population deviations in logs from original count at 77 F for the 10 week period.

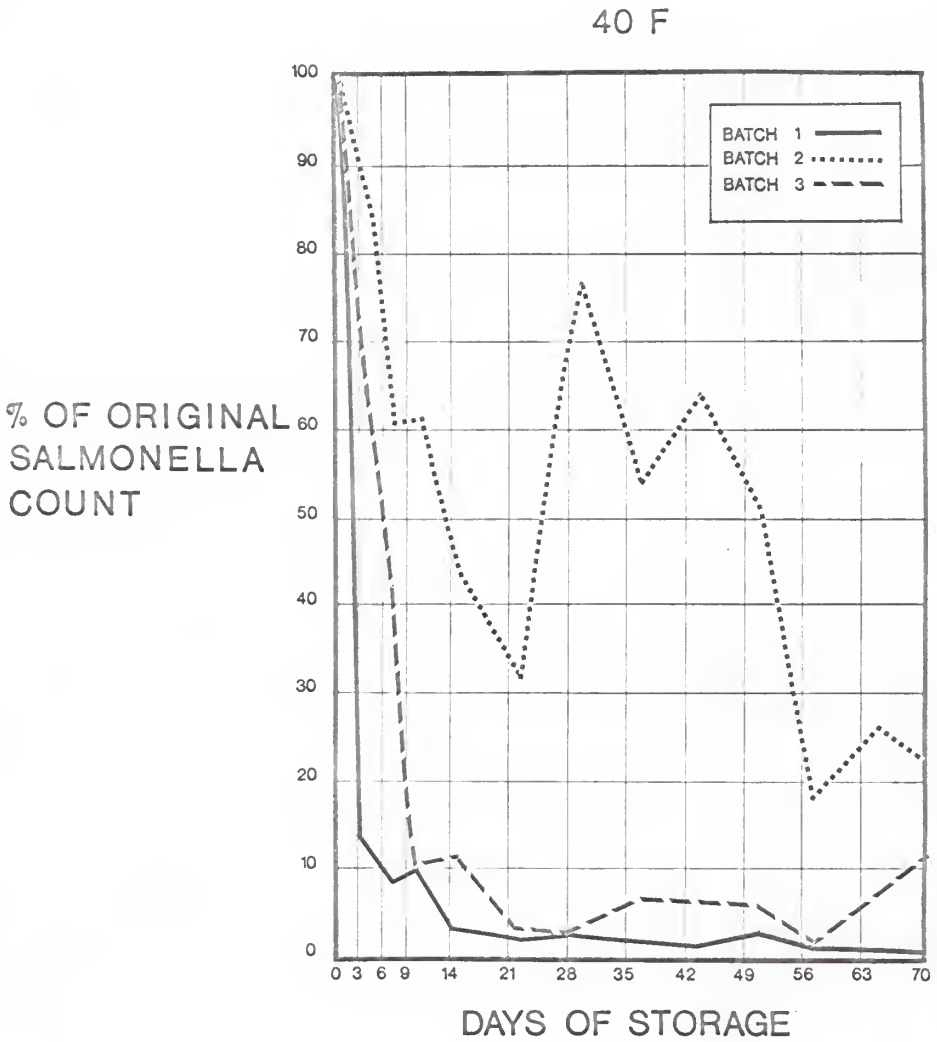

Fig. 2 - Comparison of butter batches 1, 2, and 3 at 40 F showing percentage survival during the 10 week storage period.

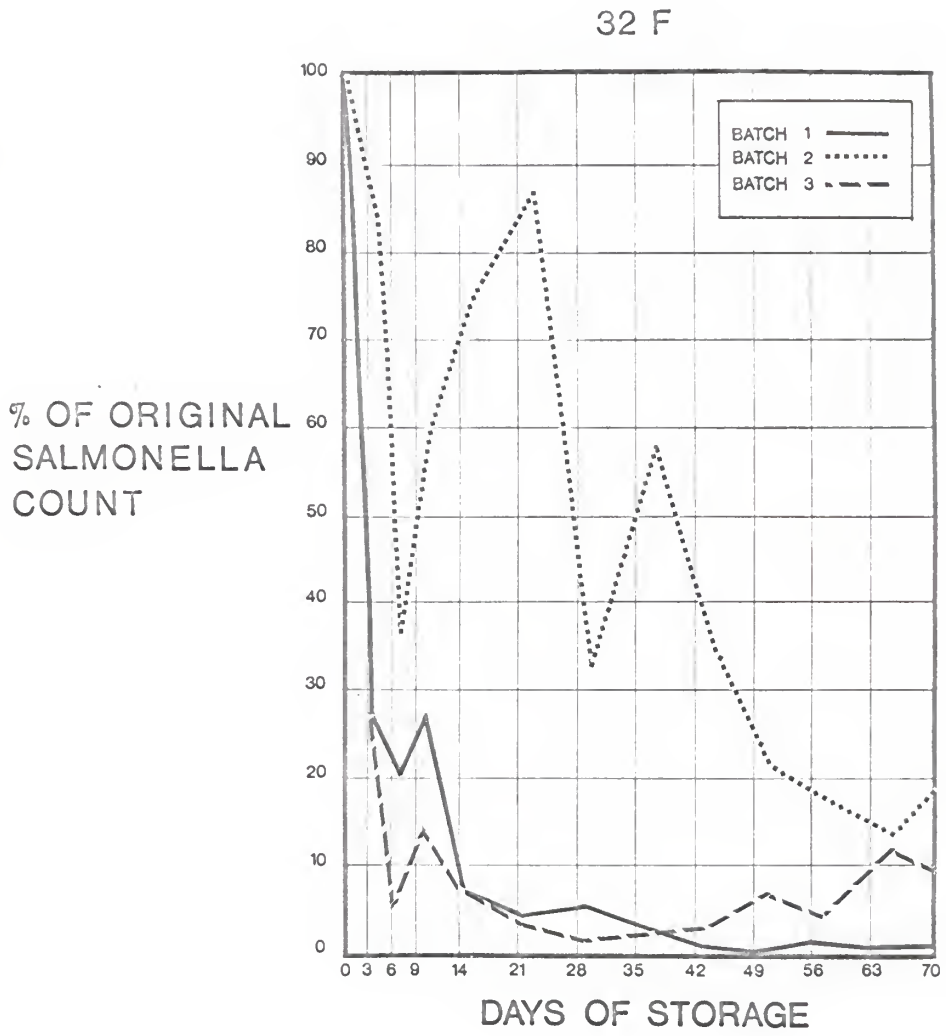

Fig. 3 - Comparison of butter batches 1, 2, and 3 at 32 F showing percentage survival during the 10 week storage period.

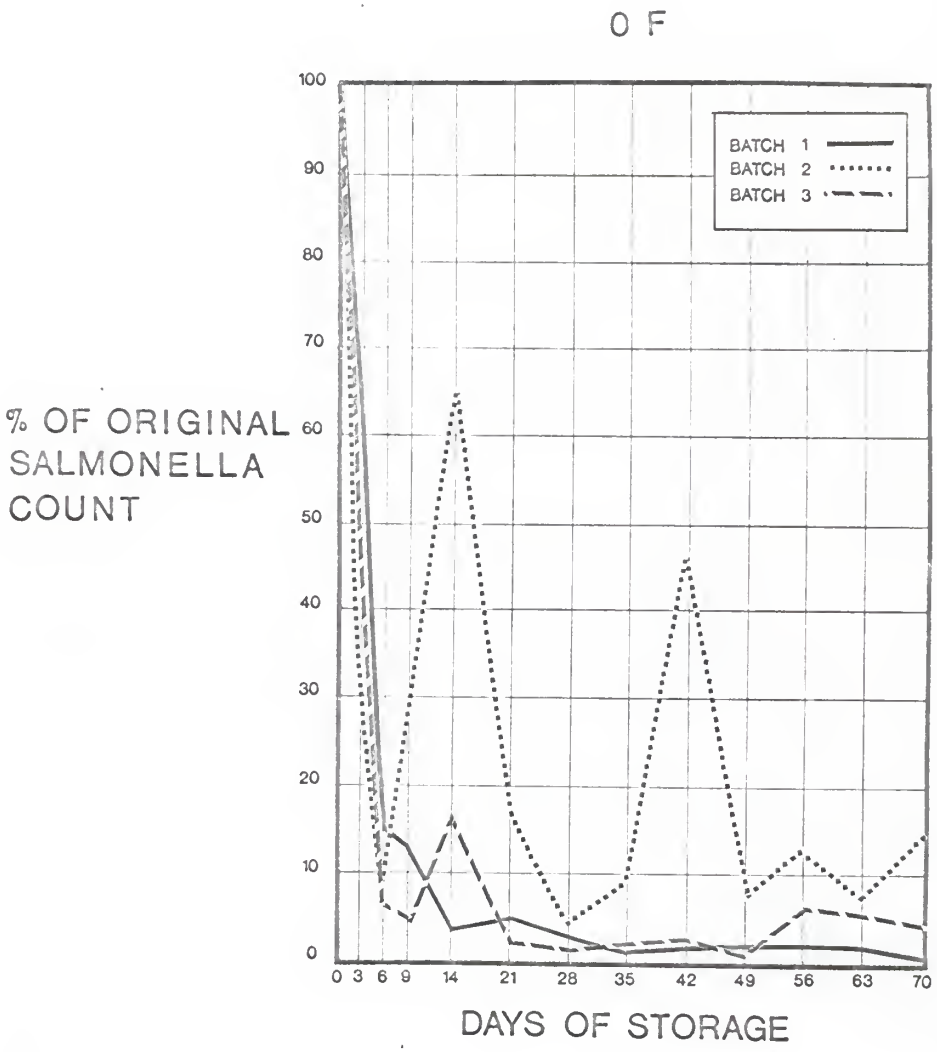

Fig. 4 - Comparison of butter batches 1, 2, and 3 at 0 F showing percentage survival during the 10 week storage period.

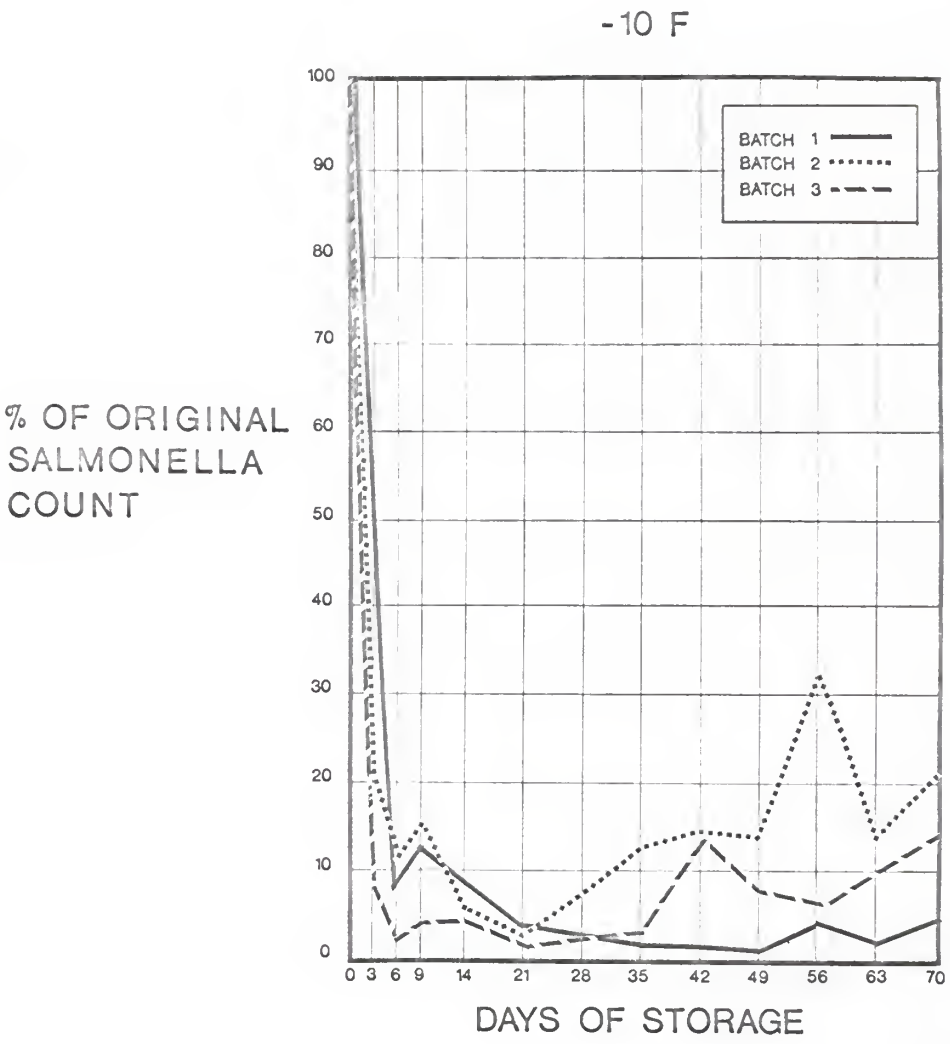

Fig. 5 - Comparison of butter batches 1, 2, and 3 at -10 F showing percentage survival during the 10 week storage period.

Table 7: Analysis of Variance Table for Butter Stored ≤ 40 F.

Source of Variance	Degrees of Freedom	Mean Square of Variables
Time	12	0.52477 *
Batch	2	1.21540 *
Temp.	3	0.11278 *
Time X Batch	24	0.02654 *
Time X Temp.	36	0.01823 *
Batch X Temp.	6	0.10608 *
Error	72	0.01104

*Mean Square is significant at .05 level of probability.

Batch composition was significant in that Salmonellae in batch 2 declined less in numbers than they did in batch 3 which also differed significantly from batch 1. The following indicates Salmonella survival means from highest to lowest:

BATCH	MEAN
2	0.56938
3	0.32817
1	0.28604

IN A THREE-FACTOR EXPERIMENT

LSD = 0.04098 (Batches are statistically unequal if the difference in means exceeds 0.04098).

Batch 2 had less of a decline than the other batches. Reference to Table 1 reveals Batch 2 had slightly more moisture (15.3%) and salt as well as a lower initial count. Theoretically, salt, being hydrophilic may have contributed to larger moisture droplets which enhanced Salmonella survival. Further the salt

(2.2% overall and approximately 11.5% in brine) may have inhibited the natural flora and presumably had little or no effect on the Salmonella. Batch 2 had more curd available for nutrients than batch 3 but less than batch 1. Batch 3 was significantly different than the other two batches and supported survival at a rate midway between batches 2 and 1. Batch 3 was higher in salt (1.7%) and moisture (14.1%) than was batch 1 but differed only slightly in original count. Apparently the low curd (0.1%) was not an important factor as batch 1 had 0.9% curd. Batch 1 was significantly less able to support Salmonella survival than the other batches. Apparently the relatively low moisture (13.3%) content was insufficient for prolonged high survival and the lack of salt may have lead to more natural flora survival.

Temperature as a variable was significant in that the lower storage temperatures (0. F and -10 F) had lower survival means than did 40 F and 32 F. The following consideration of temperature as it affected Salmonella survival means is presented:

NEW ORDER	OLD ORDER	MEANS
40 F	40 F	0.44782
32 F	32 F	0.43020
0 F	0 F	0.36742
-10 F	-10 F	0.33264

IN A THREE-FACTOR EXPERIMENT

LSD = 0.04732 (Mean differences exceeding this are significant).

The difference in survival means between 40 F and 32 F was not significant nor was there a significant difference between 0 F and -10 F.

Combinations of variables were all shown to be significant and was expected in so far as time, temperature, and batch were in themselves significant.

The literature was in wide disagreement as to what constituted an infective dose of Salmonella organisms. Much depended upon the victim's general health. The U.S. Public Health Service have a 0 tolerance for Salmonella in food for human consumption. The butter stored at 77 F supported growth of Salmonella and served to demonstrate that contaminated butter, if improperly stored presented a health hazard. No butter sample during the 10 week storage period could have been considered safe for human consumption.

CONCLUSIONS

Salt content (1-4%), as usually employed in the butter industry, did not significantly reduce Salmonella levels in butter and may have aided Salmonella survival. Salmonella readily multiplied in butter at 77 F. Salmonella contaminated butter stored at $\leq$ 40 F was shown to decline in survival levels at 40 F, 32 F, 0 F and -10 F but the survival rate was lower at 0 F and -10 F. Salmonella was recovered at all times and temperatures during the 10 week storage period and, therefore,

butter cannot be safely ignored as a potential source of Salmonella food poisoning and should be stored and handled accordingly.

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THE EFFECT OF TIME AND TEMPERATURE UPON SALMONELLAE IN INOCULATED BUTTER

by

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1969

This study was devised to determine the effect of salt content, moisture content, storage time, and storage temperature upon Salmonella typhimurium var copenhagen inoculated into butter.

Salmonella typhimurium var copenhagen was utilized to contaminate butter stored at 77 F, 40 F, 32 F, 0 F, and -10 F (25 C, 4.44 C, 0 C, -17.77 C, and -23.33 C) for 10 weeks. Butter was manufactured using 4 1/2 pints of grade A cream for each batch and varying the salt level from 0 to 2.2%. Cream was inoculated for two batches and wash water was inoculated to contaminate the third batch.

Counting of surviving Salmonella organisms was accomplished by means of brilliant green sulfadiazine agar spreader plate incubated for 24-48 hours at 37 C. Sampling intervals were 3, 6, 9, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days. All isolates were confirmed by biochemical and serological methods.

Salmonellae were readily grown in all butter stored at 77 F. No increase in Salmonella numbers above original numbers was demonstrated at temperatures $\leq$ 40 F. Temperatures of 0 F and -10 F caused a significantly greater decrease of viable Salmonella than did 40 F and 32 F. All batches at all sampling intervals and all temperatures were demonstrated to contain viable Salmonella.

Generally, at temperatures $\leq$ 40 F, an increase in storage time resulted in a decreased Salmonella survival rate.

Chemical content of butter was shown to significantly affect Salmonella survival. Butter with the greatest salt

content was shown to have the highest survival rate. Butter containing the greatest moisture content was shown to have the highest Salmonella survival rate, while the butter with the least moisture had the lowest survival rate.

Original contamination levels, when substantially lower, led to a significantly higher survival rate.