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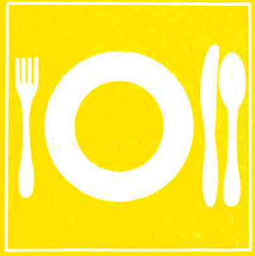

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Research Papers

General Interest Papers

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A Research Note

Occurrence of Orthophenylphenol During Manufacture of Lemon Marmalade

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(Received for publication July 17, 1978)

ABSTRACT

Orthophenylphenol (OPP) content in lemon peels was reduced to less than half of its original content with the procedure used to remove bitter substances. OPP content in peels decreased little after dipping them in water. The increment of OPP content in marmalade was proportional to the ratio of peeled pieces of lemon. OPP content of lemon marmalade prepared by an ordinary procedure from lemons containing 2.3 ppm of OPP on whole basis was 0.39 ppm, about 17% of the initial amount of OPP being present in the final product.

Orthophenylphenol (OPP) and diphenyl (DP) are effective preservatives to prevent putrefaction of citrus fruits and vegetables caused by *Oidium* species, and their use on various kinds of foods is approved in the U.S.A. and in EC countries with limitation. Contrary to this, in Japan since 1971 only diphenyl is allowed to be used as a preservative for grapefruit, lemon and oranges with a maximal residual level of 70 ppm. Use of OPP and other preservatives was strictly prohibited. In April 1975 quantities of lemon and other citrus fruits had to be destroyed because they contained detectable amount of OPP (*1*). It was quite recent (April 30, 1977) that OPP as well as its sodium salt were added to the Japanese list of acceptable chemically synthesized food additives (*2*). Its use, however, is restricted to citrus fruits with a maximal permitted residual level of 10 ppm as OPP.

It must be taken into account, however, that, besides being consumed raw, citrus fruits are often used as one of the raw materials in the manufacture of marmalade. At present, OPP is not allowed to be used in the manufacture of marmalade or jams. Accordingly, it was undertaken by us to ascertain the occurrence of OPP in marmalade when it was made from lemons containing permissible level of OPP.

MATERIALS AND METHODS

Preparation of lemon marmalade

Marmalade was prepared by an ordinary method in the following way. Lemons imported from the U.S.A. were cut into quarters and peeled. Peels were cut into pieces, in some instances boiled for 30 min in 1% NaCl solution to remove bitter substances, and finally partially dehydrated to obtain peel pieces for marmalade preparation. Pulps were crushed and divided into endocarps and edible portions; the former was mixed with twice as much, by weight, of 0.25% citric acid solution being followed by boiling for 30 min to obtain crude pectin while the latter was boiled for 3 min to obtain the jelly base. Crude pectin and jelly base were mixed in the ratio of 4:6; addition of peel pieces to pectin jelly mixture was arranged to obtain six kinds of final products.

Sample	Added ratio of peel pieces to the mixture	Note
No. 1	9.1% by weight	Removal of bitter substances in peel was omitted.
No. 2	9.1% by weight	
No. 3	13% by weight	
No. 4	17% by weight	
No. 5	23% by weight	
No. 6	13% by weight	Peel was dipped in water for one night instead of boiling for 30 min in 1% NaCl solution.

Pectin powder was added at the level of 0.2%, then the mixture was successively heated, sugared, concentrated and finished; the final product was packed in glass bottles each of ca. 300 ml.

Analysis of OPP

OPP contents in samples were determined gas chromatographically by use of the *Standard Methods for Hygienic Chemists* (*3*). A Yanaco G-80 gas chromatograph (Yanagimoto Mfg. Co. Ltd.) with flame ionization detector attached, being fitted with glass tubing of 1.5 m in length packed with 3% SE-30 on Chromosorb W 60/80, was used. Under this condition, recovery of OPP at the level of 1 ppm added to sample No. 1 was found to be 97.4%.

RESULTS AND DISCUSSION

OPP content in lemons used as raw material was determined, on a whole basis, to be 2.3 ppm. The values obtained on samples are summarized in Table 1. It was confirmed that 57% of OPP was lost during removal of bitter substances.

The ratio of OPP content of sample No. 3 to No. 6 was 0.45 which was nearly the same as that of sample No. 2 to No. 1. Since the composition of raw materials was the same among samples No. 1 and 2 and No. 3 and 6, it is suggested that OPP content in sample No. 6 was little affected by dipping the peels in water. Furthermore, a comparison of OPP contents was done by changing the

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TABLE 1. *OPP contents in prepared jams.*

Sample	Removal of bitter substances	Ratio of Pc, J and Pl[a]	OPP content (ppm)[b]	Ratio to No. 2	Ratio to No. 1	Ratio to No. 6
No. 1	Not carried out	4:6:1.0	0.90	—	1	—
No. 2	Carried out	4:6:1.0	0.39	1	0.43	—
No. 3	"	4:6:1.5	0.54	1.38	—	0.45
No. 4	"	4:6:2.0	0.82	2.10	—	—
No. 5	"	4:6:3.0	1.13	2.90	—	—
No. 6	"	4:6:1.5	1.21	—	—	1

[a]Pc stands for pectin, J for jelly base and Pl for peel pieces, respectively.
[b]Averages of three trials.

amount of peel pieces in samples No. 2-5. From the results it became clear that change in OPP content was proportional to the increase in the combined ratio of peel pieces, indicating that most of OPP contained in the raw material was in the peel fraction.

Only 17% of the initial amount of OPP remained in the finished product, lemon marmalade (cf. Sample No. 2) prepared by an ordinary procedure from lemon which contained 2.3 ppm of OPP.

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Journal of Food Protection Vol. 42, No. 4, Pages 294-296 (April, 1979)

A Research Note

Ascorbic Acid Stimulation of Diacetyl Production in Mixed-Strain Lactic Acid Cultures

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ABSTRACT

The effect of three levels of ascorbic acid on diacetyl and acid production by two mixed-strain cultures was investigated. Each culture was grown in nonfat dry milk reconstituted to 11% total solids with 0.1% added sodium citrate. Ascorbic acid concentrations in the milk were 0, 0.10, and 0.25%. Stimulation of diacetyl production was observed at both concentrations of ascorbic acid. The stimulatory effect of ascorbic acid at both concentrations was approximately the same for each culture. Diacetyl production was initiated earlier in samples containing ascorbic acid. Ascorbic acid did not stimulate acid production.

Oxidation of dairy products is a concern to consumers and product processors. Oxidized or metallic was listed as a flavor defect in 25% of the sour cream samples evaluated at the 1974 American Cultured Dairy Products Association Clinic (*13*). Since 1974 the frequency of the oxidized or metallic flavor defect varied each year but the defect is still detected.

The effect of antioxidants on bacterial growth is not specific. Shih and Harris (*19*) reported that nordihydroguaiaretic acid had greater antimicrobial activity against *Staphylococcus aureus* than did butylated hydroxyanisole or propyl gallate. At 400 ppm, propyl gallate and nordihydroguaiaretic acid were more lethal than butylated hydroxyanisole against *Escherichia coli*. Robach et al. (*18*) have shown that 400 ppm of butylated hydroxyanisole will inhibit growth of *Vibrio parahaemolyticus* 04:kll in crab meat. *Salmonella typhimurium* is more resistant to butylated hydroxyanisole than *Staphylococcus aureus* 100 and certain enteropathogenic strains of *Escherichia coli* (*4*). Ascorbic acid is known to have antioxidant properties (*8,12*) and is used for this purpose in foods (*11*). Information relating to the effect of ascorbic acid on microbial growth is limited (*5*). Prevot and Taffanel (*16*) found that ascorbic acid stimulated volatile compound production by some bacteria. Thermally injured cells of *Streptococcus lactis* have been reported to respond to ascorbic acid with increased acid production (*17*). However, ascorbic acid did not stimulate acid production from uninjured cells.

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The objective of this study was to investigate diacetyl production and growth of mixed lactic acid cultures in the presence of antioxidants. This paper reports the stimulation of diacetyl production by ascorbic acid.

MATERIALS AND METHODS

Spray-dried nonfat dry milk was reconstituted to 11% total solids with distilled water. Sodium citrate was added to achieve a final concentration of 0.1%. The milk was divided into four portions and heated at 85 C for 30 min. Filter-sterilized ascorbic acid (0.22-μ Millipore filter) was added as a 25% solution to achieve concentrations of 0.10 and 0.25%. The control contained no added ascorbic acid. Two commercially available mixed cultures were used. Each was a frozen concentrate containing *Leuconostoc cremoris* with either *Streptococcus cremoris*, *S. lactis*, or both. Milk was inoculated at the level recommended by the culture supplier and direct from the concentrate to avoid strain dominance that might occur during culture transfer (*6*). Accuracy of inoculation was increased by diluting the culture with 10 volumes of sterile distilled water. After inoculation, 20-ml portions of the control and milk containing each concentration of ascorbic acid were aseptically transferred into six sterile test tubes. Sterilized stoppers were placed in the tubes after they were filled. A 0-h sample was immediately placed in ice water and the remaining samples incubated at 22 C.

A control and one tube at each ascorbic acid concentration were removed from the incubator and immediately placed in ice water after 6, 10, 14, 18, and 24 h of incubation. Diacetyl determinations according to Pack et al. (*14*) and pH measurements were made after each incubation period.

RESULTS

Ascorbic acid caused increased diacetyl production by both cultures (Table 1). The amount of diacetyl produced by the cultures differed but the magnitude of increased diacetyl production was similar. Diacetyl production in samples containing ascorbic acid was four-fold greater than the control after 24 h for culture A and three-fold greater than the control for culture B. Additional experiments showed that the amount of diacetyl produced by cultures A and B under identical conditions was similar. Differences in diacetyl production by these cultures reported here could be caused by the reagents used in the diacetyl determinations. Intensity of the developed color in the diacetyl determination is affected by the age of the ferrous sulfate solution. The solution should be replaced each time a slight yellow color begins to appear (*14*). Ascorbic acid both increased diacetyl

production and initiated production sooner.

This is evident by the concentration of diacetyl in the samples after 10 h of incubation. However, this effect is probably caused by a lower pH in the samples containing ascorbic acid at that time since citrate permease is more active at pH values below 6.0 (*10*). Samples containing ascorbic acid achieved this pH before the control. A low initial pH value caused by addition of ascorbic acid and not the rate of acid production was responsible for reaching pH 6.0 more quickly. These results indicated that 0.1% ascorbic acid was sufficient to cause stimulation of diacetyl production. Higher concentrations did not result in greater diacetyl production. The lowest concentration of ascorbic acid that will cause stimulation was not determined during this study but is currently being investigated.

Ascorbic acid addition did not affect acid production. Initial pH values for samples containing ascorbic acid were lower than pH values for the controls. After 24 h of growth the controls and their respective ascorbic acid-containing counterparts had essentially the same pH. Since bacterial counts were not made, it is not evident if any of the bacteria in the cultures were stimulated or inhibited.

DISCUSSION

Stimulation of volatile compound production by bacteria with ascorbic acid has been reported previously. Prevot and Taffanel (*16*) reported stimulation of volatile acid production by several species of *Clostridium.* Collins (*7*) has stated that to enhance diacetyl production requires that (*a*) the strains of bacteria or yeast must be capable of producing diacetyl, (*b*) the environment must be controlled to get good growth of the organism, and (*c*) the environment must be such that appreciable quantities of pyruvic acid will be produced that are not required for synthesis of cell materials or for oxidation of $NADH_2$. Pack et al. (*15*) have been able to stimulate diacetyl production and stabilize it by treatment of growth media with hydrogen peroxide. They attributed this to the oxidative effects of nascent oxygen liberated by catalase. Stimulation of diacetyl production by ascorbic acid would be different since it acts as a reducing agent. Consequently, the stimulatory effect of lowered E_h on lactic culture growth can not be ignored (*9*). Stimulation of culture growth does not seem likely since growth as determined by pH change was not significantly different. Additionally, Anderson and Elliker (*1*) reported that ascorbic acid stimulated acid production by only six strains of 32 strains of *S. cremoris* and *S. lactis.*

Little information is available concerning the effect of ascorbic acid on metabolism of lactic acid bacteria. Ascorbic acid has a protective action on *Lactobacillus arabinosus* deficient in biotin or pantothenic acid. However, it will not stimulate growth of *Lactobacillus arabinosus* deficient in nicotinic acid nor *Lactobacillus casei* deficient in pantothenic acid (*20*). Other reports (*2,3*) present evidence that ascorbic acid and D-isoascorbic acid act as reducing agents which convert inactive thiamine disulfide to active thiamine in *Lactobacillus*. Most evidence indicates that the stimulatory effect of ascorbic acid on microorganisms is caused by the reducing properties of ascorbic acid. We are presently investigating other antioxidants for their ability to stimulate diacetyl production.

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TABLE 1. *Effect of ascorbic acid on diacetyl production and pH change during growth of mixed lactic cultures.*

		Diacetyl (ppm)			pH		
		Ascorbic Acid (%)			Ascorbic Acid (%)		
Hour	Culture	0.0	0.10	0.25	0.0	0.10	0.25
0	A	0	0	0	6.80	6.60	6.30
	B	0	0	0	6.65	6.50	6.10
6	A	0	0	0	6.40	6.20	6.00
	B	0	0	0	6.50	6.30	6.00
10	A	0.21	0.67	0.67	5.40	5.20	5.30
	B	0	0.21	0.21	5.85	5.68	5.42
14	A	0.44	0.88	0.88	4.60	4.60	4.60
	B	0.21	0.29	0.25	5.00	4.78	4.70
18	A	0.44	1.15	1.45	4.50	4.50	4.50
	B	0.25	0.40	0.48	4.60	4.55	4.50
24	A	0.48	1.55	1.45	4.50	4.50	4.50
	B	0.49	1.01	0.88	4.40	4.40	4.35

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Elution of Artificial Colors from Cardboard and Wrapping Paper Used for Food Packages

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ABSTRACT

In Japan artificial colors and pigments can be used for food packages if they are processed so that the artificial color or pigment will not be eluted to contaminate food. Ten samples of cardboard and five samples of wrapping paper were subjected to the elution test. One hundred cm^2 of cardboard or paper was immersed in water at 40 C and held for 30 min. Artificial color was eluted from all samples of whole cardboard and, when it was divided into three parts (surface, inner part and reverse side) to do the elution test separately, artificial color was eluted from all parts except with one sample when color was eluted only from inner part. By comparing R_f values on a paper chromatogram as well as its maximal absorbance with color standards, the eluted color was identified to be croceine orange. Congo red and croceine orange were eluted each from one sample of wrapping paper.

In Japan, the Notification regarding Standards and Specifications of Food, Food Additives etc. (Ministry of Health and Welfare Notification No. 370, December 28, 1959) prescribes that apparatus and package shall not contain artificial colors except those listed in Table 2 of the Enforcement Regulation of the Food Sanitation Law (Ministerial Ordinance No. 23, July 13, 1948). It is permitted, however, to use artificial colors and pigments not listed in Table 2, if the apparatus or package is processed so that the artificial color or pigment will not be eluted to contaminate food. The elution test is prescribed in Notification No. 370 and the test of plastic apparatus and packaging material should be carried out by use of water at 60 C for 30 min. In the same notification it is prescribed that the test for origami (colored paper for folding play by babies) should be carried out by use of water at 40 C for 30 min. As yet, no elution procedure is prescribed for the test of cardboard and wrapping paper.

In the Sanitary Inspection Guide (*1*), however, it is tentatively indicated that solution for checking elution from paper and converted paper is to be prepared by use of either water, 4% acetic acid or 0.1% ammonia-water. Examples for eluting conditions are as following: (*a*) leave for a definite period (10 min, 24 h) at room temperature, (*b*) use eluting solution at 40 C and leave for 30 min at 40 C, (*c*) use eluting solution at 60 C and leave for 30 min at 60 C, (*d*) use eluting solution of 80 ± 2 C and leave for 30 min at room temperature, or (*e*) use boiling eluting solution and boil for 30 min.

Conditions (*a*) and (*b*) are recommended for the elution test of paper products in general, (*c*) is to be applied for the elution test of laminated paper products (for instance, paper drinking glass and paper dish), (*d*) is applicable to test paper containers for hot coffee and other hot products, while (*e*) is to be applied to test tea-bags and paper pans.

Japan imports quantities of dried fish every year from abroad as raw material for preparation of "Tsukudani" (preserved food boiled down in soy sauce). The fish are usually imported directly packed in cardboard with bottom and sides being covered with thin wrapping paper. By request of government food sanitation inspectors residing at the Moji port, we had many chances to inspect such paper products during the first half of 1978.

MATERIALS AND METHODS

Paper samples

Ten kinds of cardboard and five kinds of wrapping paper were used as test samples. Cardboard was divided into three parts (surface, inner part and reverse side), and the elution test was done on whole cardboard and the three parts.

Preparation of test solution for dye elution and identification

One hundred cm^2 of paper sample was dipped into 200 ml of water of 40 C and was held 30 min at 40 C. Elution of dye from paper was judged by taking 50 ml of the test solution into a Nessler's tube of 50 ml (specifications : internal diameter, 20 mm; the distance from the base to the bottom face of glass stopper, 20 cm) and by observing the color from the top and side against a white background. When the test solution was judged to be colored, the extract solution was concentrated to 5 ml in vacuo and the eluted artificial color in the concentrate was extracted and purified by the wool dyeing method (*2*). Dyes were eluted from wool with dilute ammonia water and the preparations were separated by paper chromatography. Toyo filter paper No. 50, 2 × 40 cm, was used with two developing systems of 25% ethanol:1% ammonia water (1 + 1) and n-butanol:ethanol:1% ammonia water (6:2:3). The developed color was identified by comparing its R_f value with color standards as well as by measuring its maximal absorbance.

RESULTS AND DISCUSSION

From data in Table 1 it is clear that all the test solutions of whole cardboards were judged to be colored. Results of separate inspection confirmed that colors were eluted from the surface, inner part and reverse side except for sample No. 2 where color was eluted from only the inner part.

On the paper chromatogram, the eluted color had a R_f value of 0.45 with the first developer and 0.68 with the second developer, respectively. The maximal absorbance

TABLE 1. *Elution test on cardboard.*

Sample	Elution of color				Identification of eluted color
	Surface	Inner part	Reverse side	Whole	
No. 1	+[a]	+	+	+	Croceine orange
No. 2	–[b]	+	–	+	Croceine orange
No. 3	+	+	+	+	Croceine orange
No. 4	+	+	+	+	Croceine orange
No. 5	+	+	+	+	Croceine orange
No. 6	+	+	+	+	Croceine orange
No. 7	+	+	+	+	Croceine orange
No. 8	+	+	+	+	Croceine orange
No. 9	+	+	+	+	Croceine orange
No. 10	+	+	+	+	Croceine orange

[a] + : Color was eluted.
[b] – : Color was not eluted.
Detection limit: One μg of Croceine orange in one cm² of cardboard.

of the colored solution and extracted dye were known to be around a wavelength of 483 nm, being quite coincident with R_f values and maximal absorbance of croceine orange (Fig. 1).

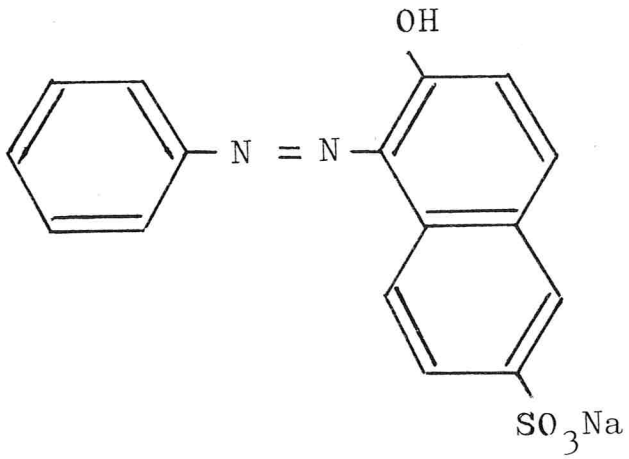

Figure 1. *Chemical structure of Croceine orange (CI Acid orange 12), $C_{16}H_{11}N_2O_4S_3Na$.*

Croceine orange (CI Acid Orange 12, CI 15970) is a kind of acid dye that is not designated as a food additive in Japan. Nine cardboards, except No. 2, were disqualified because of elution of croceine orange. Since the dye was eluted only from the inner part in sample No. 2, it may not migrate into foods. It must be noted that, besides croceine orange, unknown natural colored constituents which might have been derived from pulpwood were detected in each test solution. Such substances would not develop on paper chromatogram and remained at the starting point. As for wrapping paper, samples No. 1 and 2 were rejected because congo red (direct cotton dye) and croceine orange were detected from the test solution of No. 1 and 2, respectively.

TABLE 2. *Elution test on wrapping paper.*

Sample	Elution of color (whole)	Identification of eluted color
No. 1	+[a]	Congo red
No. 2	+	Croceine orange
No. 3	–[b]	—
No. 4	–	—
No. 5	–	—

[a] + : Color was eluted.
[b] – : Color was not eluted.
Detection limit: One μg of Croceine orange or Congo red in one cm² of wrapping paper.

In the 5th paragraph of Article 2 in Food Sanitation Law it is defined that "container-package" means the articles in which foods or additives are contained or packaged and are to be offered therein when the foods or additives are delivered. Accordingly, it will be unnecessary to carry out the elution test on cardboard and wrapping paper if foods are packed in them after having been wrapped in polyethylene or polyvinyl chloride films, for example.

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A Milk-Like Beverage from Neutralized Direct-Acid-Set Cottage Cheese Whey[1]

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ABSTRACT

An imitation milk was formulated by combining 4 parts of neutralized, direct-acid-set, cottage cheese whey with 6 parts of whole milk, and fortifying with .5% nonfat dry milk solids (NFDM). Whey collected from a commercial plant was neutralized, clarified, blended with milk, fortified with NFDM, pasteurized, homogenized, and packaged in ½-gal. paper cartons in the Kansas State University (KSU) dairy processing plant. Calculated raw material costs were compared for 2.0% low fat milk, 3.25% milk, and for imitation milks with those same milkfat concentrations. The 2.0 and 3.25% imitation milk could be formulated with savings of 35 and 25 cents per gallon, respectively. Coded samples of the product were compared with regular KSU whole milk by 112 persons. Of these, 45% identified the KSU milk samples, 35% thought the experimental product was the KSU milk, and 20% could tell no difference. The milk sample was preferred for flavor by 42% of the consumers, 32% had no preference, and 26% preferred the imitation milk. The imitation milk, containing 11.5% total solids and 2.4% protein, was .5% lower in total solids and 1.0% lower in protein than KSU whole milk.

An excellent review on whey beverages by Holsinger et al. in 1974 (*5*) covers uses in beverages of whey from cheeses made with bacterial cultures. However, they did not mention direct-acid-set (DAS) whey, a relatively new product. The clean flavor of DAS whey makes it a promising substance for further study.

Neutralized DAS cottage cheese whey is bland and does not have the characteristic flavor of cultured wheys. Its blandness should make it adaptable for use in food products. Since the DAS method for making cottage cheese is increasing substantially in the United States (*3*), the need to find uses for DAS whey is important.

With more stringent pollution regulations, milk processing plants that manufacture cottage cheese will have to consider alternatives to dumping large amounts of whey into city sewers. Unfiltered cottage cheese whey adds about 54,000 ppm to the biochemical oxygen demand (BOD) (*4*). Thus small processing plants, which often dump whey into municipal sewage systems, are facing whey disposal problems (*6*).

This paper describes development and formulation of an imitation milk fabricated from neutralized DAS whey and milk.

[1]*Contribution No. 78-416-j, Kansas Agricultural Experiment Station.*

MATERIALS AND METHODS

Preparing direct-acid-set (DAS) cottage cheese whey

Laboratory scale batches of DAS cottage cheese were made in 8-liter stainless steel tanks with Corbin's patented method (*2,9*). After the cheese was cooked, the whey was strained through a colander and the curd discarded. The collected whey was cooled to 4 C and stored in stoppered Erlenmeyer flasks.

Immediately before formulating experimental products, the whey was neutralized with 50% aqueous KOH (ca. 8 g/l of whey), vigorously mixed, then clarified by centrifuging in 100-ml plastic tubes in a Precision Universal centrifuge at 1200 RPM for 5 min to produce a clear, sweet whey.

Formulation of an acceptable whey-milk (imitation milk)

Preliminary studies were designed to establish the optimum: (*a*) type of neutralizer, (*b*) amount of milkfat, (*c*) blend of neutralized whey, and (*d*) concentration of lactose to make a milk-like beverage with acceptable flavor.

Triangle taste tests were employed to compare these milks and results analyzed according to methods described by Larmond (*7*). Results from a consumer panel were analyzed statistically to determine if customers could distinguish milk from whey-milk and if they had a preference of one over the other.

Fat, protein and ash of both KSU milk and whey-milk were analyzed by A.O.A.C. methods (*1*). Fat was determined by the Babcock method and protein by dye binding. Total solids were measured by the Mojonnier procedure and lactose was determined colorimetrically by the phenol-sulfuric acid method of Marier and Boulet (*8*).

RESULTS AND DISCUSSION

Sensory evaluation of the whey-milk formulations

A mixture of 50% neutralized-pasteurized whey and 50% commercial pasteurized homogenized milk was evaluated on a nine-point hedonic scale by five experienced judges. Four whey-milk samples were standardized, two to 2.0% milkfat and two to approximately 3.25% milkfat and neutralized with either $Ca(OH)_2$ or KOH and compared with 3.25% milk (commercial pasteurized-homogenized milk obtained from the KSU dairy plant was used throughout the study). As indicated in Table 1, results showed no difference ($P < .01$) in flavors of the whey-milks as a result of neutralizer or concentration of fat. Furthermore, the whey-milk flavors were judged not different from those of the KSU milk.

A panel consisting of both experienced and inexperienced judges, using a triangle taste test, judged threshold levels for added whey and lactose. In the first trial, whey

was added to milk at concentrations of 30, 40, and 50% and evaluated by an 11-member taste panel. At the 50% whey level, 8 of 11 correctly identified the difference. At 30 and 40% whey concentrations, the panelists could not distinguish the whey-milk from regular milk ($P < .05$; see Table 2). In a second trial with 12 taste panelists, the results were almost identical.

As a result of the first two triangle taste tests, whey-milk (40% whey) was considered to be similar in flavor to that of milk. Therefore, in a final series of triangle taste tests a 40:60 blend of whey with milk was evaluated with, 0, .5, 1.0, and 1.5% added lactose. Results in Table 3 show that the panelists distinguished samples when the lactose concentration was 1.5% or more ($P < .01$).

Consumer evaluation of whey-milk

Based on results of the triangle taste tests, a paired comparison consumer taste test was designed to compare the 40:60 whey-milk containing .5% added NFDM with milk. The .5% solids were added to approximate the solids in milk. Whey for this milk was collected from a commercial dairy plant that uses the DAS method for making cottage cheese. After being neutralized, the whey was clarified at the KSU processing plant in a motor-driven farm separator. The blended mixture of raw milk, neutralized whey, cream (to standardize milkfat to 3.25%), and NFDM (.5%) was pasteurized, homogenized, and packaged into ½-gal. cartons. These cartons were paired with cartons of KSU whole milk, coded, and sold to consumers willing to evaluate the milks. (As an incentive, the pairs were sold for the normal price of a single half-gallon carton.) Customers were asked to identify which of the two cartons contained regular milk and which they preferred.

Eighty-nine of 112 customers distinguished between the two samples. However, only 56% (50 of 89) correctly identified the milk sample. Therefore we accept the hypothesis that the customers could not distinguish between the samples (*10*).

Of the 112 consumers, 45% correctly selected the milk, 35% thought the whey-milk was regular milk, and 20% could not tell the difference between the two. The flavor of the milk sample was preferred by 42% of the consumers; 26% preferred the whey-milk, and 32% had no preference.

Composition of the whey-milk and KSU milk are presented in Table 4. The whey-milk formulated with

TABLE 2. *Triangle test results comparing regular KSU whole milk with 30%, 40%, and 50% whey-milk blends* (7).

	Sample group[1,2]		
Judgment	A	B	C
Correct	5	5	8*
Wrong	6	6	3*
Degree of difference[3]			
Slight	4	4	6
Moderate	1	1	1
Much	0	0	1
Extreme	0	0	0
Preference			
KSU milk	4	2	5
Whey-milk	1	3	3

[1]All samples contained 3.25% fat.
[2]A = KSU milk vs whey-milk containing 30% whey.
B = KSU milk vs whey-milk containing 40% whey.
C = KSU milk vs. whey-milk containing 50% whey.
[3]Degree of difference and acceptability were recorded only for those making correct judgements.
*Significantly different ($P < .05$).

TABLE 3. *Triangle test results comparing judgements on regular KSU whole milk with whey-milk containing 40% whey and .0, .5, 1.0, and 1.5% additional lactose* (7).

	Sample group[1]			
Judgment	A[2]	B[2]	C[2]	D[2]
Correct	7	8	9	11**
Wrong	6	5	4	2**
Degree of difference[3]				
Slight	3	7	5	7
Moderate	1	1	2	3
Much	3	0	2	1
Extreme	0	0	0	0
Preference				
KSU milk	4	4	3	7
Whey-milk	2	3	6	4

[1]All samples contained 3.25% fat.
[2]A = KSU milk vs whey-milk without additional lactose.
B = KSU milk vs whey-milk with 0.5% additional lactose.
C = KSU milk vs whey-milk with 1.0% additional lactose.
D = KSU milk vs whey-milk with 1.5% additional lactose.
[3]Degrees of difference and acceptability were recorded only for those making correct judgments.
**Significant at ($P < .01$).

TABLE 1. *Hedonic scores*[1] *of KSU milk compared with whey-milks made from cottage cheese whey.*

	Samples[2,3]					
Panelist	A	B	C	D	E	Total
1	8	6	7	8	6	35
2	6	6	6	5	6	29
3	7	7	8	7	3	32
4	4	4	4	7	7	26
5	4	5	5	6	7	27
Total	29	28	30	33	29	149
Avg score	5.8	5.6	6.0	6.6	5.8	

[1]Scoring range: 9 for "like extremely" — 1 for "dislike extremely".
[2]All whey-milks consisted of a 50:50 blend of whey and KSU milk.
[3]A = Whey-milk of 3.25 fat, whey neutralized with KOH + Ca $(OH)_2$.
B = Whey-milk of 3.25% fat, whey neutralized with KOH.
C = Whey-milk of 2.00% fat, whey neutralized with KOH + Ca $(OH)_2$.
D = Whey-milk of 2.00% fat, whey neutralized with KOH.
E = KSU milk of 3.25% fat.

TABLE 4. *Composition of regular KSU whole milk and plant-processed 40% whey-milk.*

Sample	Percent				
	T.S.	Fat	Protein	Lactose	Ash
KSU whole milk	12.42	3.30	3.45	4.90	.77
40:60 whey-milk	11.50	3.15	2.43	5.01	.91

.5% NFDM solids was still .5% lower in total solids than regular milk. An additional .5% NFDM could be added to adjust the solids to that of regular milk. A 2.0% (low-fat) whey-milk also could be made economically with acceptable flavor properties (see Table 1).

The calculated savings in raw material of the 40:60 whey-milk, based on Grade A raw milk at $10.83/cwt, NFDM at $.71/lb, and milkfat at $1.00/lb, were 25 cents/gal. with .5% NFDM and 3.25% milkfat, and 35 cents/gal. with .5% NFDM and 1.95% milkfat.

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Effect of Potassium Sorbate on Growth of *Staphylococcus aureus* in Bacon

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ABSTRACT

The influence of potassium sorbate on growth of *Staphylococcus aureus* in bacon was determined. Bacon was manufactured to contain 0 and 40 ppm sodium nitrite with 0.13 and 0.26% potassium sorbate, 40 and 120 ppm sodium nitrite, or neither nitrite nor sorbate. The bacon was inoculated with approximately 1,000 cells of a mixed suspension of five strains of *Staphylococcus aureus* per gram, vacuum packaged, and stored at 27 and 13 C. Bacon with 0.13 and 0.26% potassium sorbate and no nitrite was most effective in suppressing growth of *S. aureus* through 14 days of storage at 27 C. When stored at 13 C, bacon containing nitrite and potassium sorbate exhibited lower numbers of staphylococci after 7 days than did the bacon containing potassium sorbate alone.

Use of sodium nitrite in cured meats as an anti-clostridial agent has been well established (*4,9,10, 15,19*). Inhibition of several other food-poisoning bacteria, such as staphylococci, by nitrite, has also been reported (*12,17*). The other important function of this chemical additive is its role in formation of the typical color and flavor in cured meats (*13*).

In a recent article (*8*), the potential formation of carcinogenic compounds from nitrite in cured meats, particularly bacon, was reviewed. An increasing awareness of this possible hazard has prompted interest in the partial to complete removal of nitrites from meat products where nitrosamines have been detected. With removal of nitrite, the traditional cured meat products as known by the American consumer would no longer be available. However, recent studies (*11,16*) have demonstrated that with use of potassium sorbate, bacon may be produced with a level of nitrite (40 ppm) below that which is usually necessary to significantly delay growth of *Clostridium botulinum* (120 ppm), while at the same time maintaining the typical cured flavor and color in the product. Potassium sorbate is a Generally Recognized As Safe (GRAS) food ingredient. It inhibits molds, yeasts, and certain bacteria, including *C. botulinum* (*18,20*).

An additional hazard that should not be overlooked concerning cured meats, particularly bacon, is the potential of staphylococcal food poisoning. It has been reported that *Staphylococcus aureus* can occur and survive in commercial vacuum-packaged bacon (*5,7*). To date, there have been no reports concerning the survival of *S. aureus* in the low-nitrite bacon produced with potassium sorbate.

The purpose of this study was to obtain data on the ability of potassium sorbate to suppress growth of *S. aureus* in vacuum-packaged bacon containing various amounts of sodium nitrite and potassium sorbate.

MATERIALS AND METHODS

Test strains

Five strains of *S. aureus* (S-6 and 243 from the FDA and 2-10, 2-24, and 3-1 from our own collection) were grown in Trypticase Soy Broth (Difco) at 37 C. After 18 h of incubation, the five strains were pooled in 0.85% saline solution for inoculation of the bacon. All five of the strains are coagulase positive while three of the strains produce one or more enterotoxins.

Bacon

Approximately 35-45 lb. of bacon for each of seven test variables was manufactured in a commercial meat processing plant. Three pork bellies in the 12-14 lb. weight range were used for each variable. The pork bellies were pumped with pickle solutions and allowed to drain before smoking. An average of an 8% weight gain was obtained. All pickle solutions contained sodium hexametaphosphate-sodium tripolyphosphate (4%, Curafos 11-2), sodium chloride (13.3%), and sucrose (1.1%) in water. Sodium nitrite, potassium sorbate, and sodium erythorbate were incorporated into the pickle solutions so that seven variables were obtained. The desired concentration for each of these three ingredients in the bacon is shown in Table 1.

After pumping, the bellies were smoked for 7 h to an internal temperature of 53 C. The bellies were then cooled and sliced (16 slices/lb).

Inoculation of bacon

Slices of bacon for each test variable were randomly selected from each of the sliced, processed bellies. The slices were uniformly inoculated (.25 ml/50 g of bacon) with the *S. aureus* suspension to obtain a target inoculum level of approximately 1,000 cells/g. The inoculated slices for each package were layered, folded and vacuum packaged in Cryovac pouches (Cryovac, Simpsonville, S.C.). Forty-two packages (approximately 50 g of bacon/package) of inoculated bacon were prepared for each test variable.

TABLE 1. *Sodium nitrite, potassium sorbate and sodium erythorbate variables.*

Variable	$NaNO_2$ (ppm)	Potassium sorbate (%)	Sodium erythorbate (ppm)
I	0	0	0
II	0	0.13	0
III	0	0.26	0
IV	40	0	550
V	40	0.13	550
VI	40	0.26	550
VII	120	0	550

Storage and sampling of product

Thirty-two inoculated packages for each test variable were stored at 27 C. At 0,2,4,7 and 14 days, four packages from each lot were analyzed for *S. aureus*. Additional inoculated packages from each variable were incubated at 13 C and examined for *S. aureus* at 0, 7 and 14 days of storage. Four uninoculated samples for each test variable were also analyzed for total aerobic count and staphylococci.

Each 50-g sample of the product was removed from the vacuum package and blended in a Waring blender with 450 ml of sterile 0.85% saline solution for 2 min. Samples were surface-plated on Vogel and Johnson agar (V-J, BBL). The inoculated plates were examined for typical black colonies after 48 h of incubation at 37 C. Total aerobic counts of the uninoculated product were estimated using pour plates of Standard Methods agar (Difco) and incubating the inoculated plates at 30 C for 48 h.

Chemical analyses of product

Slices of bacon (1 lb. each variable) were randomly selected for each test variable, ground according to procedures of the Association of Official Analytical Chemists (AOAC 24.001;*1*) and frozen. Samples were analyzed for pH by blending bacon 1:2 with water and determining pH of the slurry. Salt, moisture, protein, and fat were determined as described in AOAC 24.007, 24.003, 24.024, and 24.005 (*1*). Sodium nitrite and phosphorus were determined according to AOAC 24.038 (modified by adding sulfanilic acid and alpha naphylamine separately) and 24.013 (*1*). A modification of AOAC 20.099 (*1*) was used to determine the sorbic acid content of the bacon samples.

RESULTS AND DISCUSSION

The chemical analyses of bacon for each of the seven variables are given in Table 2. The pH ranged from 6.17 to 6.38 and the salt content ranged from 1.06 to 1.31%. Moisture, fat, protein, phosphorus, and sorbic acid levels were as expected. Variables I, II, and III contained less than 1 ppm sodium nitrite. The bacon in variables IV, V, and VI contained 5, 9 and 7 ppm residual sodium nitrite while variable VII contained 25 ppm sodium nitrite.

The total aerobic count for each variable ranged from 10,000 to 130,000/g with a geometric mean of 32,000/g as might be expected (*5*). Counts on V-J agar for uninoculated bacon ranged from 80 to 1,300/g with a geometric mean of 250/g. Similar observations have been reported for both the cured meat product and curing brines (*7*).

The effect of the potassium sorbate and sodium nitrite on growth of *S. aureus* in vacuum-packaged bacon stored at 27 C is given in Table 3. Variable I (no nitrite or sorbate) was found to be the least inhibitory toward the staphylococci as was expected. In variables IV and VII, greater inhibition was observed as the level of sodium nitrite was increased from 40 to 120 ppm; however, counts of 10^7 cells/g or greater were obtained for both test lots of bacon. The inability of nitrite to inhibit the growth of staphylococci at the levels employed is consistent with earlier reports (*2,12,14*). For variables V and VI, where 0.13 and 0.26% potassium sorbate, respectively, were combined with 40 ppm of sodium nitrite, a slightly increased delay in growth and lower maximum numbers of staphylococci was observed when compared to variables IV and VII. Statistical analyses of the data showed no significant differences (at $P \leqslant 0.5$) between variables I, IV, V, VI and VII for each sampling time except variable VI, day 2.

The greatest inhibition of staphylococcal growth throughout the 14-day incubation at 27 C was found in test variables II and III which contained 0.13 and 0.26% potassium sorbate respectively in the product. Variable III had counts which were significantly lower than those of most of the variables especially at days 7 and 14. The phenomenon of the potassium sorbate alone being more effective in preventing staphylococcal growth than the sorbate-nitrite combination cannot be explained at this

TABLE 3. *Bacterial counts on Vogel-Johnson agar for vacuum-packaged bacon inoculated with* Staphylococcus aureus *and stored at 27 C.*

Variable	Log_{10} of viable cells/g[1,2] Day 0	Day 2	Day 4	Day 7	Day 14
I	3.27^{a}	7.70^{a}	7.89^{a}	7.27^{ab}	6.31^{a}
II	3.07^{a}	6.80^{abc}	7.13^{ab}	6.61^{bc}	5.68^{a}
III	3.09^{a}	6.51^{bc}	6.22^{b}	5.75^{c}	4.41^{b}
IV	3.14^{a}	7.83^{a}	7.50^{a}	7.79^{a}	5.79^{a}
V	3.00^{a}	6.76^{abc}	7.55^{a}	7.10^{ab}	5.56^{a}
VI	3.09^{a}	5.98^{c}	6.83^{ab}	7.23^{ab}	5.49^{a}
VII	2.99^{a}	7.03^{ab}	6.93^{ab}	7.47^{ab}	5.98^{a}

[1] Geometric mean of counts from 4 packages for each variable and time.

[2] Counts in each column that have different letters are significantly different at the level $P \leqslant .05$ according to Duncan's multiple range test (6).

TABLE 2. *Chemical analyses of bacon.*

Variable	pH	Salt (%)	Moisture (%)	Fat (%)	Protein (%)	Sodium nitrite (ppm)	Sorbic acid (ppm)	Phosphorus (%)
I	6.38	1.06	27.4	62.5	7.9	<1	18	0.12
II	6.23	1.10	30.7	58.2	8.6	<1	756	0.13
III	6.17	1.31	32.5	56.8	8.3	<1	1649	0.15
IV	6.29	1.14	27.0	63.8	8.2	5	29	0.12
V	6.28	1.14	28.0	62.8	8.8	9	713	0.12
VI	6.16	1.12	31.1	58.0	9.0	7	1375	0.13
VII	6.22	1.21	32.0	57.7	8.4	25	27	0.13

time. The only difference between these test variables is the 550 ppm of sodium erythorbate that was added to the bacon containing sodium nitrite.

Results of storage studies at 13 C for the inoculated bacon are in Table 4. Growth of the staphylococci was considerably diminished at this temperature for all test variables over the 14-day incubation period. Again, as in the 27-C study, variable I (no sorbate and nitrite) allowed the greatest growth to occur. In comparing variables II, and III, less staphylococcal growth and survival were observed as the sorbate was increased from 0.13 to 0.26%. For the remaining test variables that contained nitrite alone (IV and VII) and those with nitrite and sorbate in combination (V and VI), counts on the V-J medium decreased from the initial inoculum level after 7 and 14 days of storage of the bacon at 13 C. Similar results have been reported for survival of staphylococci in curing brines held at low temperatures (*12*). Also, it has been reported that vacuum-packaging contributed to a diminished growth by *S. aureus* on cured meats (*3*).

TABLE 4. *Bacterial counts on Vogel-Johnson agar for vacuum-packaged bacon inoculated with* Staphylococcus aureus *and stored at 13 C.*

Variable	Log_{10} of viable cells/g[1]		
	Day 0	Day 7	Day 14
I	3.27	4.87	4.04
II	3.07	4.89	3.26
III	3.09	3.49	<3.0
IV	3.14	<3.0	<3.0
V	3.00	<3.0	<3.0
VI	3.09	<3.0	<3.0
VII	2.99	<3.0	<3.0

[1] Geometric mean of counts from 4 packages for each variable and time.

The data indicate that use of 40 ppm sodium nitrite with or without sorbate or 120 ppm sodium nitrite in bacon should provide adequate protection against growth of *S. aureus* for at least 14 days when the product is properly refrigerated. There is need for additional research on the interaction of cure ingredients in relationship to *S. aureus* growth and enterotoxin production when bacon is stored at abuse temperatures.

ACKNOWLEDGMENTS

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Scanning Electron Microscopic Investigations into Attachment of Bacteria to Teats of Cows

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ABSTRACT

Scanning electron microscopy was used to examine microorganisms on the skin of cows' teats which were artificially contaminated and subsequently stored for different times. From the results it seems that bacteria were not spread uniformly on the surface of the teats. No extra-cellular polymers were observed before storage of the teats, but during storage polymers, in the form of thin fibers, were produced. These fibers became thicker and finally resulted in slime. Multiplication of bacteria during storage involved formation of microcolonies of bacteria in which bacteria were not attached directly to the skin but to other bacteria. This explains the decrease in the differences between bacterial counts obtained by the blending and rinsing methods after long periods of storage.

Bacteria attach readily to the surface of teats of cows as demonstrated by Notermans et al. (*8*). The attachment rate depends, among other factors, on bacterial strain, composition of the medium in which attachment occurs (*7*) and on the attachment surface (*3,10*).

Fletcher and Floodgate (*2*) and Marshall et al. (*6*) stated that the mechanism of attachment involves two consecutive steps, namely a primary attachment followed by a time-dependent secondary attachment. The primary attachment can be caused by different physical forces like London-van der Waals attraction, electrostatic attraction of the two surfaces or by a gain in entropy. Besides these attractive forces, bacteria which attach to the skin of teats may become locked in small holes or in the skin tissue. During secondary attachment the strength of attachment increased, as shown by Notermans et al. (*8*) and by Marshall et al. (*6*). This increment is presumably caused by metabolic activity of the bacteria once attached. Fletcher and Floodgate (*2*) demonstrated that extracellular substances, often composed of secondary acidic polyaccharides are produced by primary attached bacteria. By this process bacteria encapsulated themselves on the skin surface. It may also be possible that bacteria become located or embedded inside the skin tissue.

On the basis of the results of Notermans et al. (*8*) it becomes clear that the S-value (measured as the difference between bacterial counts obtained by the blending and rinsing methods) which increased during the secondary attachment has an optimum. This optimum was reached after 2 - 3 h. After longer storage times colonies of bacteria may be formed which cause a decrease in the S-value.

To learn more about the mechanism of attachment of different bacterial strains to the surface of meat, scanning electron microscopic (SEM) investigations were carried out using teats of cows as surface.

MATERIALS AND METHODS

Bacterial strains and their counting media

In these experiments the following bacterial strains were used: *Pseudomonas* EBT/2/143, *Staphylococcus aureus, Klebsiella* sp. and *Salmonella typhimurium*. These bacteria, their growth media and counting media, were the same as described in the previous study of Notermans et al. (*8*) regarding attachment of bacteria to teats of cows.

Teats

Teats of cows were obtained from a local slaughter-house. They were cleaned and deep-frozen until experimentation. The teats were thawed by holding them at 4 C for 15 h before experimentation.

Contamination of the teats

Teats were dipped in a bath containing 500 ml of attachment suspension. This suspension contained 0.87% NaCl and 0.01 M phosphate buffer with a pH of 7.2. To this suspension a bacterial culture was added so that about 5×10^8 bacteria were present per ml. The suspension was mixed by forced aeration and maintained at 20 C. After a residence time of 20 min the teats were removed from the bath and washed by dipping and gently moving them in sterile physiological saline solution. This procedure was repeated three times using new sterile solution. After contamination the teats were stored in closed sterile glass jars at 20 C.

Scanning Electron Microscopy (SEM)

For fixation of the samples the procedure as described by Karnovsky (*4*) was used. This procedure includes fixation in 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.5 for 24 h. Specimens were rinsed in 0.1 M phosphate buffer (4 C) for 12 h.

Samples were dehydrated through graded ethanol solutions followed by dehydration in graded amyl acetate in ethanol solutions. After dehydration, specimens were dried to critical point with CO_2 in a BALZERS critical point drying apparatus. Samples were attached to specimen studs with silver paint and subsequently coated with gold in a BALZERS sputter apparatus. The specimens were viewed with a JEOL-JSM-U_3 scanning electron microscope operating at 10-15 kV. One sample was viewed also with a Cambridge 150-S SEM operating under the same conditions.

Exposures taken by SEM

In the first experiments exposures were made of the surface of the

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teat without artificial contamination. Next, exposures were made of the surface after artificial contamination. For these experiments teats were contaminated as described above with, respectively, *Ps.EBT*/2/143, *S. aureus, Klebsiella* sp. and *S. typhimurium*. Using *Ps. EBT*/2/143 and *S. aureus*, exposures of the samples were made immediately after attachment, after a storage time of 3 h and after a storage time of 12 h. Using *Klebsiella* and *S. typhimurium*, exposures were only made after a storage time of 3 h.

RESULTS

In Fig. 1, an exposure of the surface of the teat is shown with the natural flora generally present. The natural flora of the teat was spread very non-uniformly over the skin. Some of the bacteria were present in small niches of the skin. On the basis of the other exposures it was clear that only a very small part of the surface was covered with bacteria and that the exposure in Fig. 1 shows an extremely contaminated area.

To study the mechanism of attachment, artificially contaminated teats were used. Different exposures were made of every sample. The most representative are presented in this paper. In Fig. 2 (a,b,c,d), surfaces with *Ps. EBT*/2/143, after different storage times, are shown.

Figure 2a shows the bacteria on the teat surface immediately after contamination. The bacteria were non-uniformly spread over the teat. After storage of 3 h at 20 C (Fig. 2b) production of extracellular polymers in the form of thin fibers could be observed. The amount of those fibers and their thickness increased during storage. Slime was also noted (Fig. 2c). After 12 h of storage microcolonies were observed (Fig. 2d). In the microcolonies it could be seen that more and more bacteria were attached to each other and not to the skin of the teat. Some of the colonies were covered with slime while others contained only a very small amount of slime.

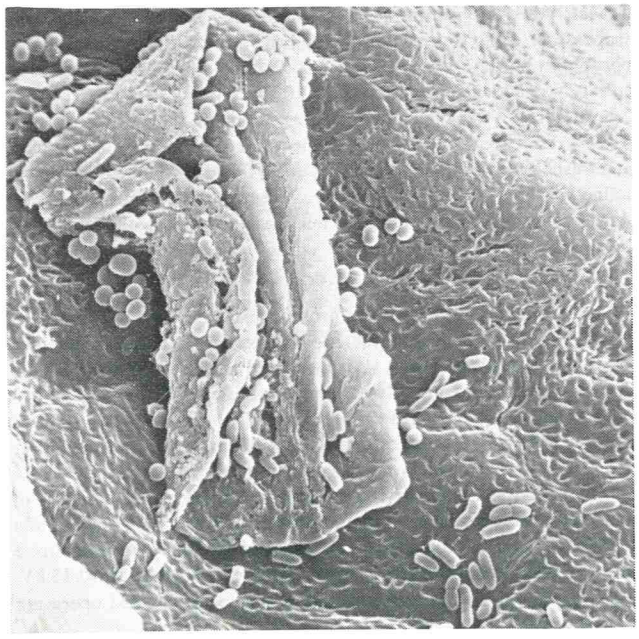

Figure 1. *The natural flora generally present on the skin of teats (× 3000).*

Figure 3 (a, b, c, d) shows teats artificially contaminated with *S. aureus*. The behavior of *S. aureus* was similar to that observed for *Ps.EBT*/2/143, i.e. immediately after inoculation the bacteria were spread non-uniformly and no extracellular material could be observed. During storage extracellular materials produced by *S. aureus* looked different from those produced by *Ps.EBT*/2/143 (less fibrous).

Inoculation of teats with *Klebsiella* sp. for 3 h brought similar results, i.e. production of extracellular fibers followed by a small amount of slime.

A picture of *S. typhimurium* attached to teat skin after 3 h of storage is presented in Fig. 4 (a, b). This bacterium also produced extracellular fibers (4b), which are absent on teat skin without bacteria. The cells of this bacterium were much longer than those of other bacteria examined and they tended to grow in short chains of 2-3 cells. This seemed to interfere with attachment of this bacterium to the skin (Fig. 4a).

DISCUSSION

Teats of cows were found to be extremely suitable for examining the attachment of microorganisms with a SEM. The surface of the cleaned teat seen in the electron microscope was smooth and did not contain fibers. SEM experiments made with chicken skin (5) showed that those surfaces were covered with fibers. This made it difficult to distinguish between those fibers and the fibers produced by the bacteria during storage.

The process of attachment of bacteria to the teats may also be divided into two sequential processes, as stated by Fletcher and Floodgate (2) and Marshall et al. (6) who used other surfaces. After primary attachment a time-dependent secondary attachment appeared during storage of the samples. No production of extracellular materials could be seen after the primary attachment. However, the non-uniform spread of the natural flora, as well as of all other bacteria with which the teats were contaminated, could indicate an interaction between bacteria and certain microecosystems on the surface. The shape of bacteria could also play a role in the primary attachment (Fig. 4a). The mechanism of primary attachment could not be explained by these experiments.

During secondary attachment, production of extracellular polymers was observed for all bacteria examined. Similar results, i.e. production of extracellular polymers by attached bacteria, was also found by Marshall et al. (6) and by Fletcher and Floodgate (2), using marine bacteria. At the beginning of secondary attachment, production of thin fibers was observed. These fibers became thicker during storage. In this stage the bacteria tended to produce slime. The fibers, as well as the slime produced by different bacteria, looked different and may have been composed of different polymers. The shape of bacteria and their fit to the surface seems also to increase the strength of attachment.

Some of the microcolonies formed were covered with slime (Fig. 2c and 3c), while others contained only small

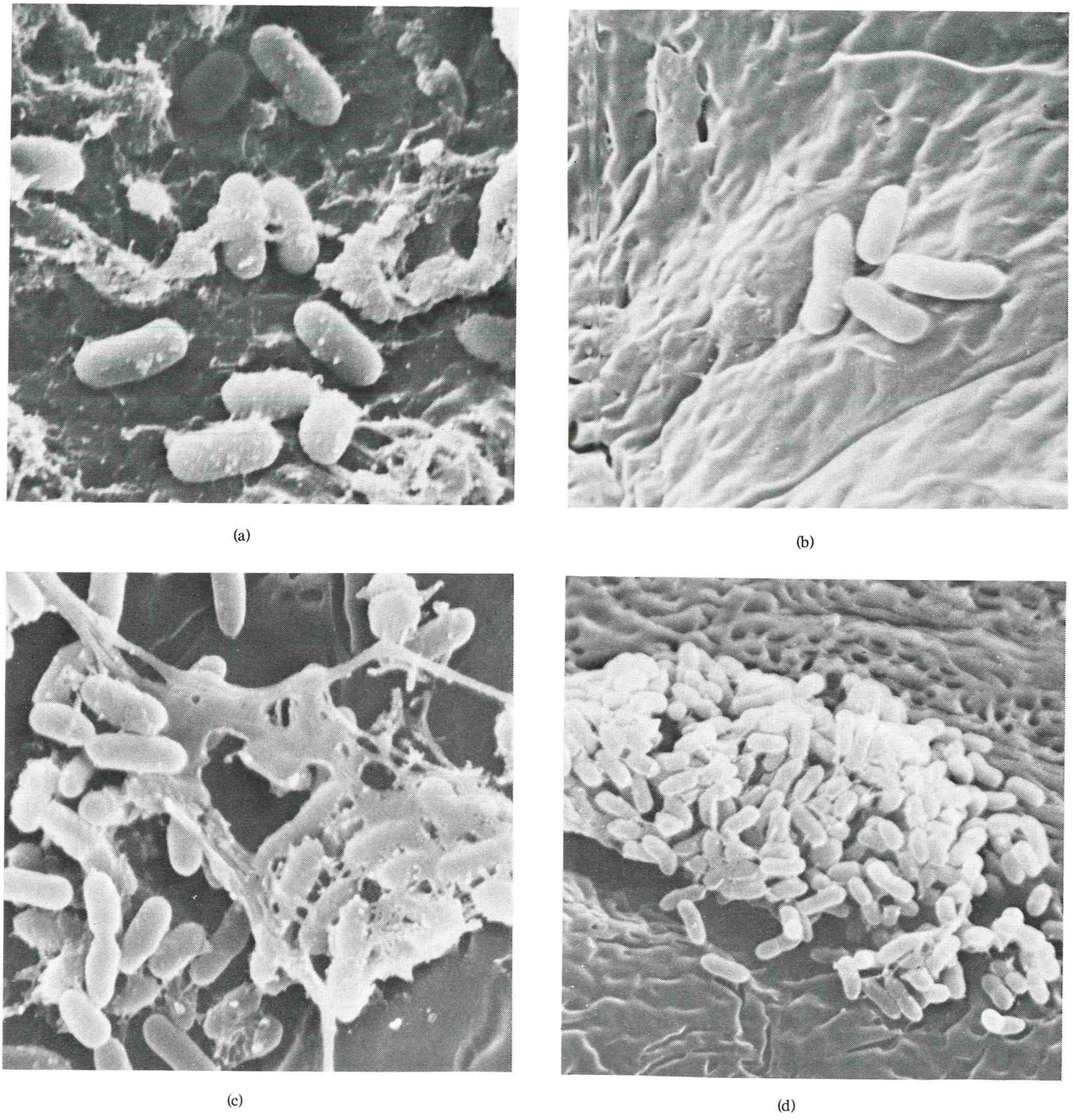

Figure 2. *Teats artificially contaminated with* Ps.EBT*/2/143:* (a) *immediately after contamination* (× *10,000*), (b) *after 3 h of storage* (× *10,000*), (c) *slime production after 12 h of storage* (× *10,000*), *and* (d) *micro-colonies formed after 12 h of storage* (× *6,000*).

amounts of slime (Fig. 2d and 3d), probably due to differences in available nutrients. Zobell (*9*) stated that slime production by attached bacteria appeared to be influenced mainly by the micro-ecosystem. He found that more slime was produced in ecosystems in which it was difficult for bacteria to survive. Costerton et al. (*1*) stated that the polymers (glicocalyx) produced by bacteria may not only position the bacteria but also conserve and concentrate the digestive enzymes and serve as a food reservoir for the bacteria.

Results obtained in the work with the SEM support the experimental data obtained previously (*8*). The increase in S-value during the first 2-3 h of storage could be explained by the formation of the extracellular fibers. The decrease in strength of attachment found after longer storage was probably due to formation of microcolonies in which more of the bacteria were attached to each other and not directly to the skin, and so could be removed easily.

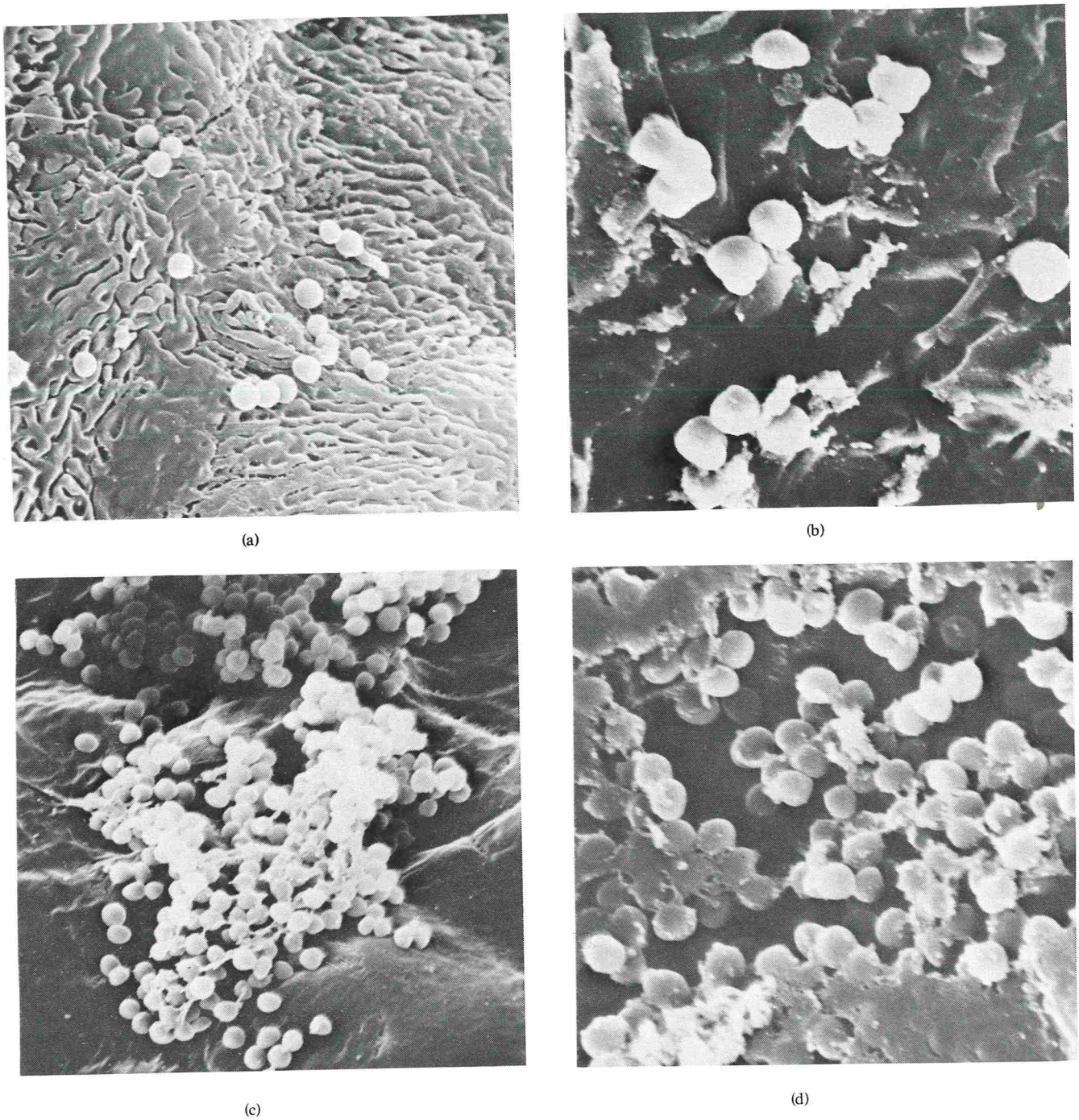

Figure 3. *Teats artificially contaminated with* S. aureus: (a) *immediately after contamination* (× *6,000*), (b) *after 3 h of storage* (× *10,000*), (c) *slime production after 12 h of storage* (× *10,000*), *and* (c) *micro-colonies formed after 12 h of storage* (× *6,000*).

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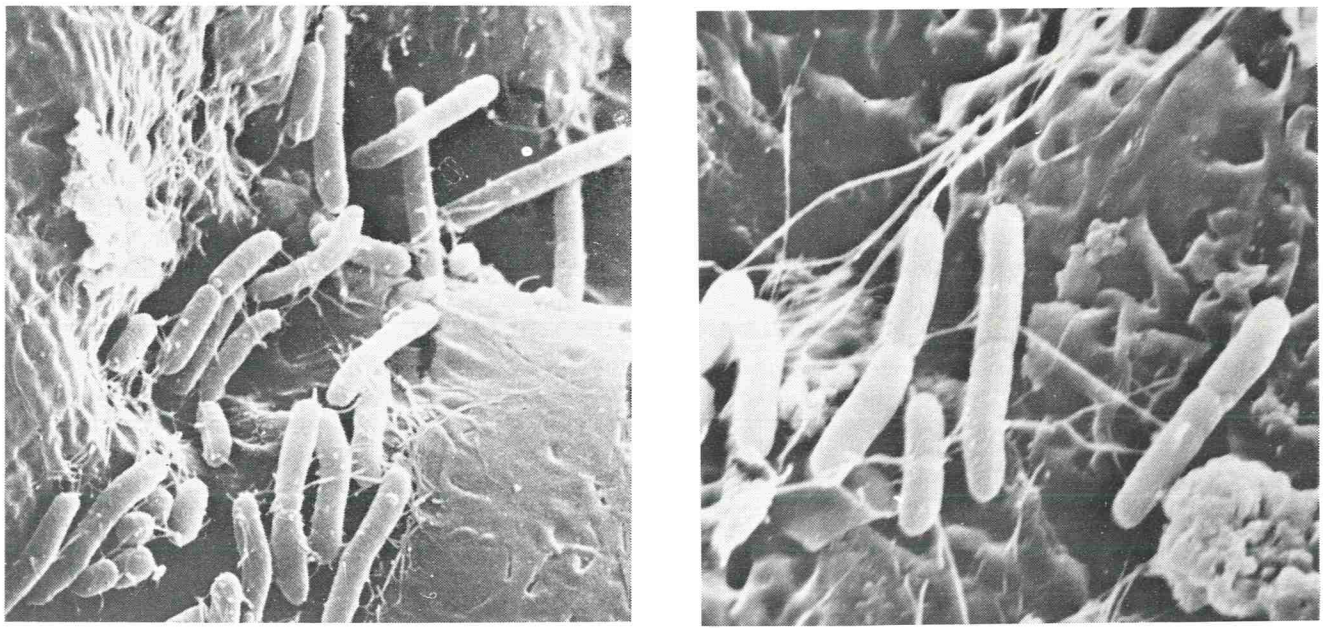

(a) (b)

Figure 4. *Teat artificially contaminated with* S. typhimurium *after 3 h of strage:* (a) *arrangement of* S. typhimurium *on the skin* (× *4,000*), *and* (b) *fibers produced by* S. typhimurium (× *10,000*).

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A Research Note

Recovery of Viable *Staphylococcus aureus* in Corn and Green Beans Stored at Ambient Temperature

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ABSTRACT

Staphylococcus aureus was recovered from post-process inoculated commercially canned green beans after periods of up to 6 weeks of storage and from inoculated commercially canned corn after up to 26 weeks of storage under various conditions. Variance in duration of viability of the culture was apparently due to intrinsic factors of the food and presence of inoculated competing organisms. Drying of the inoculated corn had no effect on viability of the test culture, but drying shortened its length of viability in green beans.

This study was initiated to determine the validity of laboratory findings in the investigation of food poisoning incidents following a time lapse of possibly several weeks between the incident and the microbiological examination of the suspect food. Viability of *Staphylococcus aureus* in whole kernel corn and whole cut green beans, during storage and under various conditions, was studied.

MATERIALS AND METHODS

Corn liquor from normal appearing, commercially processed cans, was aseptically distributed into each of 40 sterile, screw cap 20 × 130-mm test tubes in 2-ml aliquots. Thirty-two of these samples were inoculated with 1.9×10^7 *S. aureus* (Food Research Institute strain 100, a type A enterotoxin producer). One half of these same tubes were also inoculated with 2.8×10^6 *Escherichia coli* (ATCC 4157). Forty samples of the liquor from commercially processed cans of green beans were prepared and inoculated in the same manner. Eight samples of each product were retained as uninoculated controls. To facilitate drying of the inoculated products, sterile metal strips were used. Strips of tinplate, measuring approximately 10 × 100 mm, were cut from commercial food cans and sterilized. A strip was then immersed into one of the products — *S. aureus* - *E. coli* samples, aseptically transferred to a sterile 25 × 200-mm test tube, and allowed to dry at room temperature. This procedure was repeated with each of the remaining product — *S. aureus* — *E. coli* samples. All 112 tubes were incubated at ambient temperature and periodically samples were tested for the presence of viable *S. aureus*. The incubation intervals were 1, 3, 6, 9, 12, 26, 39 and 52 weeks.

At each specific incubation interval duplicate tubes of each product with *S. aureus* alone, each product with *S. aureus* and *E. coli*, and each product in a dried film were assayed. One ml of liquid was removed from each liquid sample and placed in 10 ml of Brain Heart Infusion (Difco) as enrichment and incubated at 35 C for 48 h. Ten milliliters of BHI were added to those tubes containing the metal strips coated with dried films of organisms. These were also incubated for 48 h at 35 C. A 3-mm loopful of growth from positive tubes was streaked for isolation on Staphylococcus Medium 110 (Difco) and incubated an additional 48 h at 35 C. The staphylococci were considered viable if there was growth of gram positive cocci on Staphylococcus 110 medium.

RESULTS AND DISCUSSION

The data indicate that the duration of viability of a culture of *S. aureus* will vary, depending upon food type and conditions of storage (Tables 1 and 2). Two products with a high level of consumption, corn and green beans, were chosen for study. These products represent extremes in the range of pH for low-acid vegetables (average pH for brine packed corn equals 6.1 and for green beans equals 5.3) (*2*). Canned product was used in this survey because of its ease in handling and its sterile nature. Incubation at room temperature for periods up to 1 year was felt to represent adverse conditions under which suspect product could be held.

The test organism was recovered through the 26-week interval in corn and in a dried film of corn. The culture

TABLE 1. *Recovery of viable* Staphylococcus aureus *from corn stored at ambient temperatures.*

Storage time (weeks)	Corn w/ *S. aureus*	Corn w/ *S. aureus* & *E. coli*	*S. aureus* & *E. coli* in dried product	Uninoculated control
1	+[a]+	+ +	+ +	–
3	+ +	+ +	+ +	–
6	+ +	+ +	+ +	–
9	+ +	+ +	+ +	–
12	+ +	+ +	+ +	–
26	+ +	– –	+ +	–
39	–[b]–	– –	– –	–
52	– –	– –	– –	–

[a] + Recovery of staphylococci in growth medium.
[b] – No recovery of staphylococci in growth medium.

TABLE 2. *Recovery of viable* Staphylococcus aureus *from green beans stored at ambient temperatures.*

Storage time (weeks)	Green beans w/ *S. aureus*	Green beans w/ *S. aureus* and *E. coli*	*S. aureus* and *E. coli* in dried product	Uninoculated control
1	+[a]+	+ +	+ +	–
3	+ x[b]	+ +	+ +	–
6	+ +	+ +	– –	–
9	–[c]–	– –	– –	–
12	– –	– –	– –	–
26	– –	– –	– –	–
39	– –	– –	– –	–
52	– –	– –	– –	–

[a] + Recovery of staphylococci in growth medium.
[b] x Mold contamination.
[c] – No recovery of staphylococci in growth medium.

lost it's viability some time between the 12-week and 26-week intervals when competing with *E. coli* in the corn sample. It is known that growth of enterotoxigenic *S. aureus* is adversely affected by competitive growth of other microorganisms (*1*). Troller and Frazier (*3,4*) found that *E. coli* inhibited *S. aureus* by means of an antibiotic substance which is somewhat heat stable and to which *S. aureus* does not develop resistance after consecutive transfers.

S. aureus was recovered up to 6 weeks after inoculation from the green bean samples with *S. aureus* alone, and from green beans with both organisms. The test culture did not survive as long in the dried green beans, the last positive interval being 3 weeks.

The variation between the two foods is likely due to some nutritional differences and/or the slightly lower pH of green beans (5.3 vs 6.1).

The effect of drying the organisms in thin films of the respective products on the metal strips was not as expected. Viability apparently was not altered significantly by drying in the product. The culture remained viable in a dried corn film through the 26-week interval. But loss of viability occurred one test interval earlier in the green beans as a result of drying compared to the liquid samples. This loss of viability may have occurred at any time between the 3-week and 6-week intervals and may not be significant.

Storage conditions, length of storage and intrinsic factors of a food have an effect on the viability of a culture of *S. aureus* when stored in food products. Therefore the validity of the laboratory investigation into the epidemiological source of a foodborne illness associated with *S. aureus* will vary with the food product and the length and conditions of storage.

ACKNOWLEDGMENT

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A Research Note

Influence of Potassium Sorbate on Growth of *Pseudomonas putrefaciens*

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(Received for publication August 14, 1978)

ABSTRACT

The effect of potassium sorbate on growth of two strains of *Pseudomonas putrefaciens* (*Alteromonas*)[1] was studied. Addition of 0.2% sorbate to trypticase soy broth (pH 6.0) inactivated strain P19X and resulted in a 3-log cycle reduction in number of viable cells of strain P5LIN through 6 days of incubation at 24 C.

Pseudomonas putrefaciens produces hydrogen sulfide and trimethylamine from refrigerated fish and poultry (*2,3,6*). *P. putrefaciens* can grow in the range of -2 C up to 30 C (*3,5*). Although *P. putrefaciens* makes up only 10-14% of the spoilage flora of fresh poultry (*1,3,7*), it must be considered a vital fraction of the flora due to its low-temperature growth and ability to produce strong off-odors (*4*).

Sorbic acid and potassium sorbate are both generally recognized as safe (GRAS) and have been widely used as food preservatives for over 30 years. Robach (*7*) reported that the spoilage flora of fresh broilers dipped in a 5% potassium sorbate solution did not contain any *P. putrefaciens* in the 1-cm² sample after 20 days of storage at 3 C. The same author observed that *P. putrefaciens* comprised 14% of the spoilage flora of the control broilers stored at the same temperature.

This investigation was undertaken to determine the effect of potassium sorbate on growth of two strains of *P. putrefaciens* in broth at a pH near that of fresh poultry.

MATERIALS AND METHODS

Test organisms

Two strains of *P. putrefaciens* were obtained from Dr. R. E. Levin, Department of Food Science and Technology, University of Massachusetts, Amherst. Strain P19X was isolated from haddock and strain P5LIN from hamburger. The cultures were prepared by inoculating a 250-ml shake flask containing 50 ml of sterile TSB with a loop of a slant culture of the appropriate organism. The flask was incubated in a shaker water bath (American Optical, Buffalo, N.Y.) for 24 h at 24 C.

Growth studies

The growth medium was trypticase soy broth (TSB;BBL) adjusted to pH 6.0 with 5 N HCl. Growth studies were done using 250-ml shake flasks containing 50 ml of sterile TSB. Filter-sterilized potassium sorbate was added aseptically to the cooled, sterile broth.

The TSB was then inoculated with sufficient of the 24-h-old culture of the appropriate test organism to contain approximately 10^4 cells/ml. Flasks were incubated at 24 C in the shaker water bath at 175 cycles per minute. Samples were withdrawn at appropriate intervals, and serial dilutions were made in sterile 0.1 M potassium phosphate buffer (pH 7.2). Organisms were enumerated by pour plating with trypticase soy agar (TSA; BBL). Plates were incubated for 48 h at 24 C before counting.

RESULTS AND DISCUSSION

Addition of 0.10% potassium sorbate to the TSB resulted in growth of strain P19X being slowed, and total cell numbers reaching a maximum one log cycle lower than the control (Fig. 1). When 0.20% sorbate was added to the TSB, an initial decrease in number of viable cells

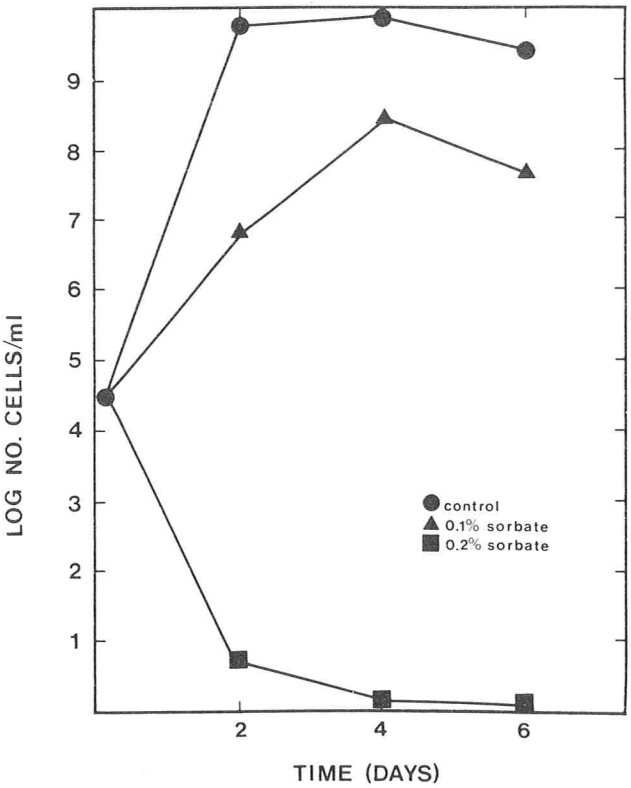

Figure 1. *Effect of potassium sorbate on the growth of* Pseudomonas putrefaciens *P19X in trypticase soy broth (pH 6.0) at 24 C.*

[1]*British nomenclature.*

of strain P19X resulted, and after 6 days of incubation no viable cells could be recovered upon subculturing (Fig. 1).

The same trend was observed for strain P5LIN. When 0.10% sorbate was incorporated into the TSB a slower growth rate and a lower maximum cell population in comparison to the control were observed (Fig. 2). Addition of 0.20% sorbate to the TSB resulted in a steady decline of viable cells of strain P5LIN through 4 days, after which a slight increase in cell population was observed (Fig. 2).

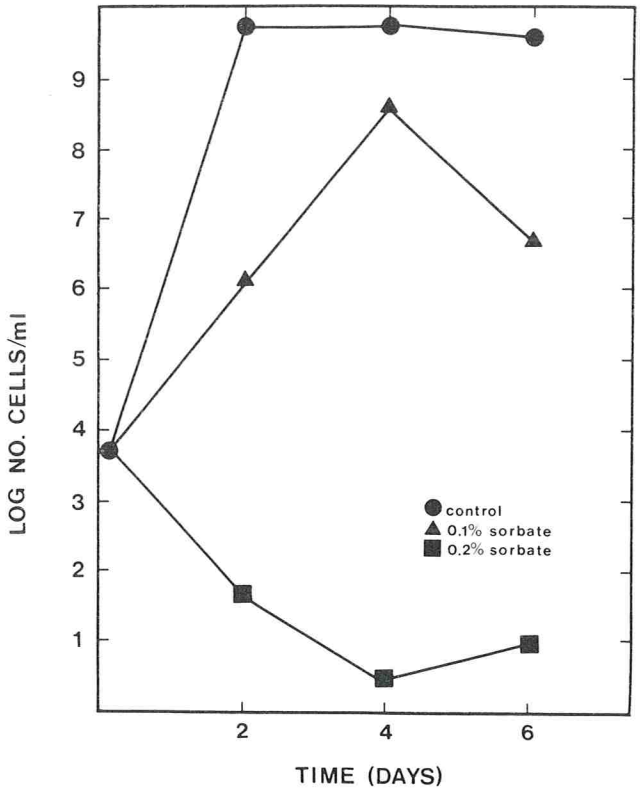

Figure 2. *Effect of potassium sorbate on the growth of* Pseudomonas putrefaciens *P5LIN in trypticase soy broth (pH 6.0) at 24 C.*

Results of this study suggest that potassium sorbate inhibits growth and inactivates *P. putrefaciens* in broth. These results also help explain the disappearance of *P. putrefaciens* from the spoilage flora of whole broilers treated with sorbate. The results obtained are in line with the report that potassium sorbate inhibits growth of *Pseudomonas fluorescens* in broth (*8*). Further studies are under way to more fully investigate the antibacterial functionality of potassium sorbate.

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Fat Test Depression During Chilled Storage of Milk Samples in Plastic Containers for Analysis by the Milko-Tester[1]

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ABSTRACT

Ambiguous depressions in fat tests were observed when milk samples were stored before testing by the Milko-Tester procedure. Fat-test depression was augmented by shipping and by chilling samples at the time of collection in an ice-water mixture. The effect was greatest in samples stored in 1-oz. plastic containers and in partially filled plastic bags. The fat-test depression was not significant in samples stored in glass bottles or in plastic containers receiving no refrigeration during the storage period.

The Milko-Tester device provides a rapid and convenient method for milkfat determination, particularly when a large number of samples are to be analyzed (*7*). The system is very accurate when calibrated and operated properly (*1,2,9,10*). The procedure lends itself well to centralized laboratory testing (*1,4,7*). Appleman and Laben (*1*) demonstrated that samples could be effectively preserved with potassium dichromate, although Minzner and Kroger (*8*) found that high initial bacterial counts could spoil processed samples when held at room temperatures. Several types of 1-oz. containers are available for economical shipping of samples to a centralized location. However, shipping conditions can vary widely in different parts of the U.S. For instance, Ginn (*4*) reported that weather extremes vary as much as 120 F in the Minnesota and Wisconsin areas. Churning of raw milk samples is the major problem encountered in shipping to a centralized location and the subsequent deleterious effects on fat tests have been demonstrated by Kroger et al. (*5*). The purpose of this study was to evaluate different containers and the effect of storage conditions on subsequent analyses for fat by the Milko-Tester procedure.

EXPERIMENTAL PROCEDURES

One gallon of raw milk from individual Jersey cows was sampled from the weigh jar during the morning milking. The samples were hand agitated (within 15 min) and aliquots taken for study of storage effects. Samples were stored in 1-oz. hi-impact plastic cups (Fill-Rite Corp., Newark, N. J.), 1-oz glass bottles, and 4-oz. plastic bags (Nasco, Ft. Alkinson, WI). All samples for tests were preserved with potassium dichromate (41 mg/oz.) added at the time of sample collection and well mixed immediately.

One set of replicate samples was chilled immediately in an ice-water mixture and then refrigerated (5 C). A second set of replicate samples was placed in a 5-C refrigerator and allowed to stabilize to that temperature without agitation. These samples will be referred to as "refrigerated" samples. Non-refrigerated control samples were allowed to come to room temperature (approximately 25 C) and were stored at that temperature until used. Samples were assigned to the treatments at random. Shipped samples were transported approximately 360 miles by bus.

All samples were warmed to 38 C and thoroughly mixed before being tested with a Mark III Milko-Tester. Results were recorded to the nearest 0.01% fat.

Acid degree values (ADV) were measured by the method of Thomas et al. (*14*). Thin layer chromatography (TLC) was done on Silplate 22 pre-coated TLC plates (Brinkmann Instruments, Inc.) without fluorescent indicator at a layer thickness of 0.15 mm. Plates were developed in hexane:ethyl ether:acetic acid (90:10:1) and were visualized by spraying with 10% phosphomolybdic acid in absolute ethanol, followed by heating at 100 C. All chemical reagents were analytical grade.

RESULTS

Milk samples collected for shipping to a centralized laboratory for testing will be subjected to unpredictable conditions of temperature and agitation. Therefore, in determining the method of sample handling for the Texas DHI Central Laboratory, comparisons were made between plastic bags and plastic cups which included samples receiving no refrigeration and samples refrigerated at 5 C for 2, 3, and 4 days before shipping. The fat-test results on milk samples from 30 cows were averaged and the data are presented in Table 1. The small differences noted between days of storage were not significant for the control samples. Fat tests were lower for each individual cow when the samples were

TABLE 1. *Effect of refrigeration and shipping on the fat test of milk samples stored in two types of plastic containers.*

Days Stored	Control		Shipped	
	No refrigeration	Refrigerated	No refrigeration	Refrigerated
	%			
	Plastic bag[a]			
2	4.76	4.74	4.76	4.70
3	4.77	4.75	4.76	4.71
4	4.78	4.76	4.76	4.74
	Plastic cup[b]			
2	4.76	4.73	4.75	4.47
3	4.76	4.72	4.72	4.38
4	4.76	4.73	4.74	4.40

[a]4-oz. Whirl-Pak.
[b]1-oz. hi-impact plastic.

[1]*Technical Article No. 11674 of the Texas Agricultural Experiment Station, College Station, Texas.*

refrigerated, and particularly in those samples which were refrigerated and subsequently shipped before testing. These effects were most significant in samples stored in 1-oz. plastic cups. Refrigerated samples stored in plastic cups, and which were not shipped, also tested low ($P < .01$).

The fat depressing effect of refrigeration is corroborated by the data presented in Table 2. These data are averages for three cows. The fat tests on the fresh milks averaged 4.01%. A slight depression occurred during the first day of storage in plastic cups. Longer storage of control and refrigerated samples in plastic cups did not cause additional fat depression. Samples which were chilled in ice water at the time of collection decreased in fat test after 3 and 6 days of storage.

No significant changes were noted upon storage of the control samples in 4-oz. plastic bags or glass jars. Slight but significant ($P < .05$) changes were noted in the refrigerated and chilled samples when compared with controls. Significance is measurable in these samples because of the low standard deviation (.009) associated with this Milko-Tester device.

To clarify relative effects of container size and composition, the shipping and cooling effect was measured in 1-oz. glass and plastic containers. Plastic bags containing approximately 50 ml of milk were also included. These were closed to the same level as if the bag were filled to capacity (approx. 125 ml) to expose milk to a maximum of bag surface. Fat depression because of shipping was least (Table 3) in glass containers and greatest in the partially filled plastic bags. The effect of decreased fat test due to cooling was again observed.

After analysis was completed, the partially filled plastic bags containing the shipped samples were emptied and flushed for approximately 1 min with cold tap water. The interior of the bag was then rinsed twice with 20-ml portions of chloroform-methanol (2:1/v:v). The washings were treated according to Folch et al. (*3*) to purify the lipids. The lipids were dried in tared vials at 80 C for 1 h under a stream of nitrogen. The weights of extracted lipid from the non-refrigerated, refrigerated, and chilled samples were 44.6, 120.3, and 200.3 mg, respectively. Thin layer chromatography of the extracted lipids indicated primarily glyceride and cholesterol ester components. No component more polar than cholesterol was observed. Thus it appears that the cooling treatment causes neutral milk fat to be released from the fat globules, with subsequent adsorption of the fat by plastic containers.

Changes in acid degree values during refrigeration treatment were observed in milk from 10 Jersey cows. The tendency of each cow's milkfat to depress during storage in 1-oz. plastic cups was also monitored. Differences were noted between animals, and the two extremes in this group of cows are represented in Table 4. The results show the fat depressing effect during storage and a concomitant increase in ADV in those samples from cow #1553. The effects were much less obvious for cow #1419. A correlation coefficient of 0.84 was observed between the amount of fat-test depression and increases in ADV.

To quantitate more closely the fat-test depressing effects during storage in plastic containers, aliquots of milk ranging from 25 to 125 ml were stored 2 days in plastic bags. The bags were closed, in a manner to exclude air pockets, to a depth which provided an exposed bag surface of 182 cm^2. Results are presented in Table 5, and show a direct relationship between fat-test depression and exposed bag surface. In addition to the cooling treatments used throughout the study, an additional set of refrigerated samples was warmed slowly to 30 C after 1 day of storage, and then cooled again to 5 C in the refrigerator. This treatment is known to be an activating treatment for hydrolytic rancidity (*6*). This treatment (Table 5) caused the greatest depression in fat tests measured by the Milko-Tester. However, all

TABLE 2. *Effect of storage and refrigeration on the fat test of milk samples.*

	Days stored								
	Control			Refrigerated			Chilled		
Container	1	3	6	1	3	6	1	3	6
	(%)								
1-oz. plastic cup	3.96	3.96	3.95	3.96	3.94	3.95	3.95	3.84	3.89
4-oz. glass jar	3.99	4.00	3.99	3.97	3.95	3.96	3.93	3.97	3.98
4-oz. plastic bag	4.02	4.02	4.02	3.98	3.99	3.95	3.99	3.97	3.98

TABLE 3. *Effect of refrigeration and shipping treatments on the fat test of milk samples in glass and plastic containers.*

	No. refrigeration		Refrigerated		Chilled	
Container	Control	Shipped	Control	Shipped	Control	Shipped
	(%)					
1-oz. glass bottle	4.90	4.88	4.88	4.76	4.89	4.88
1-oz. plastic cup	4.88	4.85	4.81	4.66	4.85	4.73
Bag (full)	4.89	4.86	4.84	4.69	4.82	4.80
Bag (1/2 full)	4.92	4.84	4.86	4.63	4.82	4.59

TABLE 4. *Effect of cooling treatments on the fat test and acid degree values in milk from individual cows.*

Days stored	Non-refrigerated	Refrigerated		Chilled	
	Fat test (%)	Fat test (%)	Acid degree value (meq/100 g fat)	Fat test (%)	Acid degree value (meq/100 g fat)
Cow #1553					
0	4.48	4.48	.5223	4.33	.5667
3	4.49	4.48	.6556	4.32	.8445
5	4.49	4.42	.8000	4.23	1.0778
Cow #1419					
0	4.75	4.70	.3040	4.71	.2660
3	4.75	4.70	.4750	4.65	.4180
5	4.74	4.73	.6080	4.72	.5320

refrigeration treatments resulted in a lower fat test, even at the lowest surface area to volume ratio. The Milko-Tester data in Table 5 were corroborated by the Babock test for milkfat.

DISCUSSION

A wide variety of containers currently are available for shipping milk samples. These results suggest that any container, and particularly those of small sample volume, should be thoroughly evaluated and a standardized procedure set before their adoption for use in a testing program.

In general, glass containers showed less change in fat test during storage and shipping. Plastic containers tested are acceptable when agitation is limited and providing the preserved sample is given no refrigeration. This would suggest problems in areas where shipping might involve exposure to extremely cold temperatures. These results also indicate a need to fill plastic containers to near maximum whenever possible.

Apparently, the fat test depressing effect of cooling treatments is due to some change in the milkfat globule which allows the release of free fat. The free fat is available for non-polar adsorption to plastics such as those included in this study. Free fat also is exposed to the action of milk lipase, resulting in the increased ADV values observed in Table 4. The fact that the effect is different between cows is in agreement with known patterns of spontaneous rancidity (*11*). These conclusions are not consistent with those of Tarassuk and Frankel (*12,13*) with respect to the rancidity activating effect of cooling milk, and suggest the need for further research in this important area of spontaneous rancidity development. A need to better understand the effects which the cooling of milk has on the physical condition of the fat globule membrane is strongly indicated.

TABLE 5. *Milk fat test results for milk stored two days in plastic bags under various conditions of temperature and a range of surfaces area/volume ratios.*

Surface area/volume ratio (cm^2/ml)	No refrigeration	Refrigerated	Refrigerated-heated	Chilled
		(%)		
1.46	5.43	5.38	5.38	5.34
1.82	5.43	5.35	5.35	5.33
2.43	5.41	5.35	5.32	5.34
3.64	5.42	5.32	5.29	5.31
7.28	5.42	5.30	5.27	5.30

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Physical and Sensory Characteristics of Pork Packaged in Various Gas Atmospheres

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ABSTRACT

One hundred and ninety-six boneless pork roasts were vacuum-packaged. Twenty-eight roasts remained vacuum-packaged to serve as controls. The remaining packages were divided into six groups of 28 packages each and injected with one of six gas mixtures: (*a*) 100% O_2, (*b*) 20% CO_2 + 80% N_2, (*c*) 50% CO_2 + 50% O_2, (*d*) 20% CO_2 + 80% O_2, (*e*) 25% CO_2 + 25% O_2 + 50% N_2 or (*f*) 51% CO_2 + 30% O_2 + 18% N_2 + 1% CO. Five packages from each packaging treatment were removed from storage after 7, 14, 21, 28 or 35 days. Data collection included gas composition of the intact packages and off-odor, surface discoloration, overall appearance, retail caselife and palatability of cuts. The most prevalent gas in vacuum packages was CO_2 with weight percentages of 62-88% during the 35-day storage period. Packages initially injected with an atmosphere containing 100% O_2 showed a gradual decrease in O_2 and an increase in CO_2 with increased storage. Roasts stored in O_2-containing atmospheres for 14 and/or 21 days had a higher incidence of off-odor and chops from such roasts had lower overall appearance ratings after 1 day of retail display and lower flavor and overall palatability ratings than comparable vacuum packaged meats. These differences were significant only for roasts stored in modified atmospheres containing high concentrations of O_2 and only after extended periods of storage. Data suggest that a modified gas atmosphere of 20% CO_2 + 80% N_2 is a suitable alternative to vacuum-packaging.

Packaging of fresh pork has received only limited attention in the last few years. Historically, wholesale fresh pork cuts have been distributed either in combo bins unwrapped or wrapped in parchment paper and then packed in fiberboard boxes. Each of these methods leads to high evaporative losses and reduced shelf-life.

Vacuum-packaging appears to be a method for packaging fresh pork cuts which could effectively reduce these losses. However, vacuum-packaging has not been used extensively for pork cuts even when extended periods of storage or transportation are anticipated, partially because of the lack of the necessary technology (*9*).

Use of modified gaseous atmospheres could create conditions that would influence meat color and limit the extent of microbial growth that occurs on fresh meat during storage (*11*). In addition, modified atmospheric packaging of pork cuts could eliminate distortion of cuts and theoretically reduce leaker rates and purge losses. It could also reduce packaging costs, since more than one cut could be stored in each package. Since previous research on the results of storing pork in gas atmospheres is limited, this research was initiated to study the effects of various gas atmospheres on the physical characteristics and palatability of packaged pork during various periods of storage.

MATERIALS AND METHODS

Packaging

One hundred and ninety-six roasts (6.4-cm thick) were fabricated from the longissimus muscles of 28 pork loins. Each loin was cut into seven roasts and assigned to one of seven treatments at a particular storage interval. Each roasts was placed in a laminated nylon/saran/polyethylene pouch with the following characteristics: Oxygen Transmission Rate (OTR) = 32 cc/m²/24 h/23.9 C/50% RH, Carbon Dioxide Transmission Rate (CTR) = 47 cc/m²/24 h/23.9 C/50% RH and a Moisture Vapor Transmission Rate (MVTR) = 0.8-1.8 g/m²/24 h/37.7 C/70% RH. Packages were evacuated of atmospheric air and sealed with a chamber-type vacuum packaging machine at the maximum capacity of the machine (747 mm of Hg).

Roasts assigned to treatment A remained vacuum-packaged to serve as controls for comparison with the other packaging treatments. Packaged roasts assigned to treatments B, C, D, E, F or G were injected with various gases, as shown in the experimental design (Table 1) to an approximate headspace to meat ratio (vol/vol) of 1 to 1. Gases were injected with a needle through a layer of dried silicone glue. Packaged roasts assigned to each of the treatments were then randomly assigned to one of six storage intervals of 0, 7, 14, 21, 28 or 35 days (Table 1).

TABLE 1. *Experimental design for number of cuts per treatment per storage interval.*

	Packaging treatment						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
0	3	3	3	3	3	3	3
7	5	5	5	5	5	5	5
14	5	5	5	5	5	5	5
21	5	5	5	5	5	5	5
28	5	5	5	5	5	5	5
35	5	5	5	5	5	5	5

Three initial packages (0-day) from each treatment were immediately analyzed for gas composition.

Gas analysis

At the termination of each storage interval, vacuum packages were injected with 50 cc of helium to create a sufficient gaseous volume to facilitate sampling of the gaseous contents. A gas sample was obtained by inserting an 18-gauge needle through a layer of dried silicone glue. The gaseous contents were purged through a 5-cc sampling loop for 30 sec at which time the loop contents were injected on the gas chromatographic column. The permanent gases (CO_2, O_2, N_2) were separated on 80-100 mesh Porapak-Q (1.52 m × 6.35 mm) and 80-100 mesh molecular sieve 5A (1.52 m × 6.35 mm) in series in a Gow-Mac series 550 gas chromatograph equipped with a thermal conductivity detector.

Subjective evaluations

Following gas analysis, each package was opened. After a 30-min bloom period (time from opening the package until evaluation) roasts were subjectively evaluated by a 2-member experienced panel for odor, using a 4-point scale (4 = no detectable off-odor; 1 = extremely detectable off-odor), surface discoloration, employing a 7-point scale (7 = no surface discoloration; 1 = extremely detectable off-odor), surface discoloration, and overall appearance according to an 8-point scale (8 = extremely desirable; 1 = extremely undesirable).

Spectrophotometry

The surface concentration of metmyoglobin (metmb) was determined on three roasts per treatment per storage interval using a Bausch and Lomb Spectronic 505 spectrophotometer equipped with a reflectance attachment employing calculation procedures described by Broumond et al. (*1*).

Retail caselife evaluations

Roasts were then cut into two equal portions. One half of each roast (3.2-cm thick) was placed in a styrofoam tray with the freshly cut surface placed against the tray, overwrapped with PVC film (Choice Wrap) and placed under simulated retail caselife conditions (1-3 C, 970 lux of incandescent light). Subjective evaluations were made after 1 and 3 days of retail display by a 3-member trained panel for surface discoloration, using a 7-point scale (7 = no surface discoloration; 1 = total surface discoloration) and overall appearance, employing an 8-point scale (8 = extremely desirable; 1 = extremely undesirable).

Palatability evaluations

The other half of each roast (3.2-cm thick) was wrapped in freezer paper and frozen at the termination of each storage interval. After the 21-day storage period, chops were randomly assigned to a cooking sequence and broiled in an electric oven (180 C) to an internal temperature of 75 C. An 8-member trained sensory panel evaluated each chop for flavor desirability and overall palatability according to 8-point scales (8 = like extremely; 1 = dislike extremely). Only chops from roasts stored for 7, 14 and 21 days were presented for palatability evaluations.

Microbiological

Data regarding the microbial flora of cuts from this study are included in the companion paper by Christopher et al. (*2*).

Statistical analysis

Analysis of data was accomplished using one-way analysis of variance comparing vacuum-packaging (control) to each of the other treatments, independently, within each of the five storage intervals. One-way analysis of variance also was done on data within each packaging treatment over the five storage intervals. When significant ($P < .05$) main effects were observed in the analysis of variance, mean separation was accomplished by Student-Newman-Keuls' (*10*).

RESULTS AND DISCUSSION

Gas analysis

The relative weight percentages of CO_2, O_2 and N_2 in the gaseous sample withdrawn from packages are presented in Table 2. Although most of these gases probably were present in the gaseous headspace of the packages, some of these gases, particularly the CO_2 in the vacuum packages, may have been dissolved in the purge and/or meat tissue during storage and upon injection of helium, were volatilized. In this respect, Taylor and MacDougall (*12*) reported that both CO_2 and O_2 dissolved in meat tissue. The weight percentage of CO_2 in the headspace of the vacuum-packaged roasts remained high and ranged from 61.81% initially to 88.59% after 35 days of storage. The percentage of CO_2 in the packages injected initially with 100% O_2 remained below 20% during the first 3 weeks of storage. In the

TABLE 2. *Relative weight percentages of carbon dioxide, oxygen and nitrogen from packages containing pork cuts stratified according to packaging treatment and storage interval.*

		Packaging treatment						
		A	B	C	D	E	F	G
Storage interval (days)	Type of gas	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
Initial	CO_2	61.81^{d}	0.0^{c}	20.80^{a}	48.23^{a}	21.21^{a}	25.80^{b}	52.58^{a}
	O_2	10.14^{a}	100.0^{a}	0.0^{e}	51.77^{b}	78.79^{ab}	24.20^{a}	29.19^{a}
	N_2	28.05^{a}	0.0^{b}	79.20^{c}	0.0^{b}	0.0^{c}	50.00^{e}	18.23^{d}
7	CO_2	71.53^{c}	7.84^{bc}	19.37^{a}	38.79^{b}	19.66^{a}	23.69^{bc}	41.49^{b}
	O_2	0.0^{d}	92.16^{ab}	0.0^{e}	61.21^{a}	80.34^{a}	22.52^{a}	33.99^{a}
	N_2	28.17^{a}	0.0^{b}	80.63^{bc}	0.0^{b}	0.0^{c}	53.79^{d}	24.52^{c}
14	CO_2	85.80^{a}	9.12^{bc}	17.48^{b}	37.78^{b}	19.46^{a}	22.18^{c}	37.66^{c}
	O_2	1.48^{c}	89.17^{ab}	0.91^{b}	57.04^{ab}	77.92^{bc}	22.47^{a}	33.31^{a}
	N_2	12.72^{c}	1.71^{ab}	81.61^{b}	5.18^{b}	2.62^{b}	55.35^{d}	29.03^{c}
21	CO_2	87.82^{a}	14.60^{bc}	16.83^{b}	32.12^{b}	20.34^{a}	21.32^{c}	34.24^{c}
	O_2	0.0^{d}	82.00^{ab}	1.26^{a}	52.92^{b}	72.95^{de}	20.13^{a}	30.67^{a}
	N_2	12.18^{c}	3.40^{ab}	81.91^{b}	14.96^{a}	6.71^{a}	58.55^{c}	35.09^{b}
28	CO_2	78.47^{b}	22.59^{b}	14.77^{c}	33.52^{b}	21.90^{a}	24.16^{bc}	36.78^{c}
	O_2	2.75^{b}	74.99^{b}	0.62^{c}	60.61^{ab}	75.93^{c}	14.52^{b}	29.47^{a}
	N_2	18.78^{b}	2.42^{ab}	84.61^{a}	5.87^{ab}	2.17^{b}	61.32^{b}	33.75^{b}
35	CO_2	88.59^{a}	44.78^{a}	15.99^{bc}	37.34^{b}	21.63^{a}	31.75^{a}	37.44^{c}
	O_2	1.40^{c}	50.72^{c}	0.42^{d}	56.53^{ab}	74.58^{c}	2.13^{c}	21.36^{b}
	N_2	10.01^{c}	4.50^{a}	83.59^{a}	6.13^{ab}	3.79^{b}	66.12^{a}	41.20^{a}

^{abcde}Mean weight percentages for the same gas in the same column bearing a common superscript do not differ ($P > .05$).

packages injected initially with 20% CO_2 + 80% O_2, the CO_2 percentage of the headspace ranged from 19-22% during 5 weeks of storage. In packages initially injected with 100% O_2, the percentage of O_2 decreased continuously during storage and this was accompanied by an increase in the relative weight percentage of CO_2. In all other packaging treatments, except for packages injected with 50% CO_2 + 50% O_2, there was a continuous increase in the percentage of N_2 during storage. This probably occurred by permeation through the packaging film. Meat tissue enzymes and bacteria both respire and convert O_2 to CO_2 (*5*). Levels of O_2 and CO_2 of pork packaged in gas impermeable film have been reported to change for up to 4 days in packages stored at 2 C (*3*). The present study suggests that continuous changes in gas composition occur during storage. These changes are likely caused by diffusion of gases through the package film which will depend upon the film permeability. In addition, activities of microorganisms and meat tissue enzymes and dissolution of gases into tissue fluids are likely involved.

Subjective evaluations of roasts

Roasts stored in modified atmospheres initially containing not more than 25% O_2 were not significantly different in odor scores from those of vacuum-packaged roasts. However, roasts stored in modified atmospheres initially containing more than 25% O_2 exhibited an increased incidence of off-odor at some period after 14 days of storage (Table 3). The incidence of off-odor significantly increased between 14 and 21 days of storage for pork packaged in modified atmospheres initially containing O_2, but it did not occur until after 28 days of storage for vacuum-packaged pork.

Few significant differences in surface discoloration scores were observed on vacuum-packaged roasts and roasts stored in various gas atmospheres during the first 28 days of storage. However, after 35 days of storage, roasts packaged in modified atmospheres initially containing 20% CO_2 + 80% N_2 or 20% CO_2 + 80% O_2 had significantly less surface discoloration than vacuum packaged roasts (Table 4). Because of the light color of pork, it was very difficult to identify areas of discoloration which would have been apparent on darker meats. This probably contributed to the few significant differences due to packaging treatment or storage interval. However, it was noted that vacuum packaged roasts generally had dark brown lines of discolored tissue where a crease or residual air pocket over the meat surface had been present. Also, roasts stored in O_2-containing modified atmospheres usually had light green areas of discoloration at early storage periods and tan to light brown discoloration at later periods of storage. As suggested by Seideman et al. (*8*), the brown lines of discolored tissue on vacuum-packaged pork are believed to be due to metmyoglobin formation resulting from low oxygen concentrations present in residual air spaces. The green discoloration on pork stored in O_2-containing modified atmospheres is believed to result from separation of the heme ring from the globin portion of myoglobin (*6*).

TABLE 3. *Mean values for odor[a] evaluation of pork roasts stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
7	4.0[c]	3.8[c]	4.0[c]	3.9[c]	3.8[c]	4.0[c]	4.0[c]
14	4.0[c]	3.8[c]	4.0[c]	3.9[c]	3.8[c]	4.0[c]	3.8[c]
21	3.8[c]	2.7[d]	3.7[c]	3.2[d]	3.3[d]	3.3[d]	2.8[d]
28	3.5[d]	2.7[d]	3.8[c]	2.9[d]	3.7[cd]	3.5[cd]	3.0[d]
35	3.8[c]	3.2[d]	3.6[c]	3.3[d]	3.3[d]	3.8[cd]	3.0[d]

[a]Means based on a 4-point scale (4 = no detectable off-odor; 1 = extreme off-odor).
[b]Means within a common storage interval that are underlined are significantly different ($P < .05$) from means obtained for vacuum packaged cuts.
[cd]Means in the same column bearing a common superscript do not differ ($P > .05$).

TABLE 4. *Mean values for surface discoloration[a] of pork roasts stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
7	6.3[c]	6.3[c]	6.5[c]	6.1[c]	6.4[c]	6.5[c]	6.5[c]
14	5.4[cd]	5.7[cd]	5.9[c]	5.5[c]	5.7[c]	5.8[cd]	6.5[c]
21	6.1[c]	5.8[cd]	5.1[d]	5.3[c]	5.8[c]	5.4[cd]	5.6[d]
28	4.8[cd]	5.3[d]	5.5[cd]	5.8[c]	5.2[cd]	4.8[de]	5.3[d]
35	3.8[d]	4.0[e]	5.5[cd]	2.3[d]	4.1[d]	3.9[e]	2.9[e]

[a]Means based on a 7-point scale (7 = no surface discoloration; 1 = total surface discoloration).
[b]Means within a common storage interval that are underlined are significantly different ($P < .05$) from means obtained for vacuum packaged cuts.
[cde]Means in the same column bearing a common superscript do not differ ($P > .05$).

The effect of packaging roasts in various gas atmospheres on overall appearance ratings was similar to that described for its effect on surface discoloration scores (Table 5). After 35 days of storage, only roasts stored in a modified atmosphere initially containing 20% CO_2 + 80% N_2 were significantly more desirable than vacuum packaged roasts. Overall appearance ratings of roasts stored in O_2-containing atmospheres usually decreased significantly between 14 and 28 days of storage, whereas the overall appearance ratings of vacuum-packaged roasts did not significantly decrease until 28 to 35 days of storage.

Spectrophotometry

The percentage of metmyoglobin (metmb) usually tended to be slightly higher for roasts from O_2-containing modified atmospheres stored for 14 or more days as compared with those values for roasts stored in vacuum packages (Table 6). Roasts stored for 28 and 35 days in 20% CO_2 + 80% N_2 had low metmb values. The percentage of metmb on roasts stored in O_2-containing atmospheres tended to reach its maximum after 21 days of storage and usually increased only slightly after this time. In a recent study on pork (*7*), the use of high concentrations of O_2 resulted in a significant decrease in the rate of metmb formation and the surface concentration of metmb was less than 30% even after 15 days of storage in an 80% O_2 + 20% CO_2 modified atmosphere. In addition, metmb formation was found to be independent of CO_2 concentration (*7*).

Retail Caselife Evaluations

Mean values for surface discoloration of retail pork chops after 1 and 3 days of retail display are presented in Table 7. After 1 day of display, retail chops derived from roasts stored in O_2-containing modified atmospheres for 21 or more days of storage tended to have significantly more surface discoloration than chops from vacuum-packaged roasts. After 3 days of retail display, chops from roasts stored in modified gas atmospheres containing 100, 80, 25 or 30% oxygen for 21 days had significantly more surface discoloration than chops from vacuum-packaged roasts. Increased surface discoloration on chops from roasts stored in O_2-containing modified atmospheres may have been caused by aerobic gram-negative spoilage bacteria, such as *Pseudomonas* spp. (*2*) and/or prolonged exposure of surface myoglobin to O_2.

Mean values for overall appearance of retail pork chops after 1 and 3 days of retail display are presented in Table 8. After 1 day of retail display, chops from roasts stored for more than 14 days in O_2-containing modified atmospheres usually had significantly lower appearance ratings than chops from vacuum-packaged roasts. After 3 days of retail display, chops from roasts stored in four of the six modified atmospheres for 14 days received significantly higher overall appearance ratings than chops from vacuum-packaged roasts.

Palatability evaluations

Flavor desirability ratings were significantly lower for those chops from roasts stored for 14 days in modified atmospheres initially containing 80% or more O_2 as compared with chops from vacuum-packaged roasts

TABLE 5. *Mean values for overall appearance[a] of pork roasts stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
7	6.9[c]	7.0[c]	7.4[c]	6.8[c]	7.2[c]	7.3[c]	7.4[c]
14	6.2[cd]	6.2[c]	6.4[cd]	6.6[c]	5.6[cd]	5.5[c]	6.6[c]
21	6.3[cd]	4.4[d]	5.0[d]	5.0[cd]	5.1[de]	3.7[d]	4.8[d]
28	4.4[de]	4.0[d]	5.3[d]	4.6[d]	3.9[de]	3.1[d]	4.4[d]
35	3.1[e]	3.1[d]	5.2[d]	2.0[e]	3.1[e]	3.6[d]	2.5[e]

[a]Means based on an 8-point scale (8 = extremely desirable; 1 = extremely undesirable).
[b]Means within a common storage interval that are underlined are significantly different ($P < .05$) from means obtained for vacuum packaged cuts.
[cde]Means in the same column bearing a common superscript do not differ ($P > .05$).

TABLE 6. *Mean metmyoglobin values of packaged pork roasts stratified according to packaging treatment and storage interval.*

	Packaging treatment[abc]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
0	6.7	6.7	6.7	6.7	6.7	6.7	6.7
7	15.7	19.3	22.6	17.0	16.3	16.3	7.0
14	17.4	23.0	24.7	21.5	32.3	21.7	11.3
21	19.0	32.5	32.5	37.7	43.4	34.7	29.3
28	25.3	36.0	23.7	34.3	29.3	40.0	36.3
35	30.7	41.4	15.3	42.0	41.3	43.0	45.0

[a]Each value represents the mean percentage of 3 samples.
[b]Spectrophotometry readings taken 30 minutes after opening packages.
[c]Statistical analyses were not performed on these data.

(Table 9). Flavor desirability decreased significantly with increased storage for chops from roasts stored in atmospheres initially containing 80% or more O_2. Ordonez and Ledward (7) reported development of rancidity (as measured by TBA value) within 6 to 19 days in pork stored in modified atmospheres containing 80 to 90% O_2. They claimed that lipid oxidation — rather than bacterial spoilage or metmb formation — may be the limiting factor in the use of O_2-containing atmospheres for storage of pork.

Mean values for overall palatability of pork chops are presented in Table 10. The only significant differences in overall palatability were observed for roasts stored for 21 days in atmospheres initially containing 80 or 100% O_2. Few significant differences were observed during storage. Data presented in a companion paper (2) on the microbiological characteristics of roasts stored in vacuum packages and in various gas atmospheres can provide information regarding the causes of changes in quality attributes during storage. Lower ratings for

TABLE 7. *Mean values for surface discoloration[a] of retail pork chops stratified according to packaging treatment, storage interval of pork cuts in atmosphere packages and day of retail display.*

		Packaging treatment[b]						
		A	B	C	D	E	F	G
Day of retail display	Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
Day 1	7	6.3^{c}	5.9^{c}	5.4^{c}	5.7^{c}	5.6^{c}	5.1^{c}	6.0^{c}
	14	4.7^{d}	4.6^{c}	5.1^{c}	4.9^{c}	3.9^{de}	4.1^{cd}	4.9^{cd}
	21	5.1^{cd}	2.1^{d}	4.9^{c}	4.6^{c}	5.0^{cd}	3.9^{cd}	3.2^{e}
	28	4.7^{d}	2.7^{d}	3.6^{d}	3.0^{d}	3.0^{e}	2.4^{d}	3.9^{de}
	35	4.7^{d}	1.9^{d}	4.6^{c}	2.2^{d}	2.8^{e}	3.5^{cd}	3.4^{de}
Day 3	7	5.6^{c}	4.3^{c}	4.8^{c}	4.4^{c}	4.3^{c}	5.0^{c}	4.5^{c}
	14	3.3^{d}	2.7^{d}	4.9^{c}	3.9^{c}	2.6^{de}	3.9^{cd}	4.1^{cd}
	21	4.1^{d}	2.4^{d}	4.1^{cd}	3.2^{cd}	2.7^{de}	2.6^{d}	2.8^{de}
	28	3.2^{d}	2.8^{d}	2.4^{e}	2.3^{d}	2.9^{cd}	2.4^{d}	2.4^{e}
	35	2.7^{d}	2.0^{d}	2.9^{de}	1.7^{d}	1.3^{e}	2.7^{d}	1.8^{e}

[a]Means based on a 7-point scale (7 = no surface discoloration; 1 = total surface discoloration).
[b]Means within a common storage interval and day of retail display that are underlined are significantly different ($P < .05$) from means obtained for cuts from vacuum packages.
[cde]Means in the same column and for the same day of display bearing a common superscript do not differ ($P > .05$).

TABLE 8. *Mean values for overall appearance[a] of retail pork chops stratified according to packaging treatment, storage interval of pork cuts in atmosphere packages and day of retail display.*

		Packaging treatment[b]						
		A	B	C	D	E	F	G
Day of retail display	Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
Day 1	7	6.4^{c}	5.5^{c}	5.5^{c}	5.4^{c}	5.7^{c}	5.7^{c}	5.7^{c}
	14	5.6^{cd}	4.7^{c}	6.2^{c}	5.2^{c}	4.0^{cd}	4.9^{cd}	5.2^{c}
	21	5.9^{cd}	2.0^{d}	4.9^{cd}	4.4^{c}	5.1^{c}	3.8^{cde}	3.0^{d}
	28	4.5^{d}	2.5^{d}	3.5^{d}	2.2^{d}	3.0^{d}	2.4^{e}	3.9^{cd}
	35	4.8^{cd}	2.0^{d}	4.6^{cd}	1.8^{d}	2.3^{d}	3.1^{de}	2.7^{d}
Day 3	7	5.7^{c}	4.9^{c}	4.9^{c}	4.7^{c}	5.1^{c}	5.8^{c}	5.4^{c}
	14	3.3^{d}	2.6^{d}	5.5^{c}	4.3^{c}	3.1^{d}	4.4^{cd}	4.6^{cd}
	21	4.7^{cd}	2.4^{d}	4.6^{c}	3.3^{cd}	2.7^{de}	2.9^{de}	2.8^{de}
	28	3.5^{d}	2.9^{d}	2.6^{d}	2.2^{d}	3.2^{d}	2.3^{e}	2.3^{e}
	35	3.0^{d}	1.9^{d}	2.7^{d}	1.6^{d}	1.1^{e}	2.7^{de}	1.6^{e}

[a]Means based on an 8-point scale (8 = extremely desirable; 1 = extremely undesirable).
[b]Means within a common storage interval and day of retail display that are underlined are significantly different ($P < .05$) from means obtained for cuts from vacuum packages.
[cde]Means in the same column and for the same day of display bearing a common superscript do not differ ($P > .05$).

TABLE 9. *Mean values for flavor desirability[a] of pork chops stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]					
	A	B	C	D	E	F
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2
7	4.3^{c}	4.1^{c}	4.0^{cd}	4.4^{c}	4.4^{c}	4.3^{c}
14	4.1^{c}	3.5^{cd}	3.8^{d}	3.9^{c}	3.9^{cd}	3.8^{c}
21	4.3^{c}	3.2^{d}	4.5^{c}	3.7^{c}	3.4^{d}	3.8^{c}

[a]Means based on an 8-point scale (8 = like extremely; 1 = dislike extremely).
[b]Means within a common storage interval that are underlined are significantly different ($P < .05$) from means obtained for vacuum packaged cuts.
[cd]Means in the same column bearing a common superscript do not differ ($P > .05$).

TABLE 10. *Mean values for overall palatability[a] of pork chops stratified according to packaging treatment and storage interval.*

Storage interval (days)	Packaging treatment[b] A Vacuum packaged	B 100% O_2	C 20% CO_2 80% N_2	D 50% CO_2 50% O_2	E 20% CO_2 80% O_2	F 25% CO_2 25% O_2 50% N_2
7	4.3[c]	4.0[c]	3.8[c]	4.3[c]	3.8[c]	4.3[c]
14	4.1[c]	3.6[c]	3.9[c]	3.9[c]	3.9[c]	3.6[c]
21	3.9[c]	3.4[c]	4.3[c]	3.9[c]	3.4[d]	3.8[c]

[a]Means based on an 8-point scale (8 = like extremely; 1 = dislike extremely).
[b]Means within a common storage interval that are underlined are significantly different ($P < .05$) from means obtained for vacuum packaged cuts.
[cd]Means in the same column bearing a common superscript do not differ ($P > .05$).

certain quality attributes during storage of roasts in packages initially injected with O_2-rich atmospheres likely is related to the continued growth and activities of *Pseudomonas* spp. After 21-35 days of storage, *Pseudomonas* spp. made up 47.7-90.3% of the microbial flora of roasts stored initially in 100% oxygen but only 7 to 16% of that of vacuum-packaged roasts. With increased numbers of *Pseudomonas* spp., more extensive proteolytic and/or lipolytic activities resulting in organoleptic defects can be expected (*4*). Results of the present study suggest that gas mixtures containing O_2 would not appear to be a satisfactory method for storage of pork. In this study, the use of a 20% CO_2 + 80% N_2 modified atmosphere was superior to vacuum-packaging in retaining the natural appearance of pork. However, it is possible that other combinations of these two gases might be even more effective in this connection. Further research in this area is needed.

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Microbiology of Pork Packaged in Various Gas Atmospheres

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ABSTRACT

Boneless pork roasts were vacuum-packaged; one group remained in vacuum-packages (controls), other vacuum-packages were injected with one of six gas mixtures: (*a*) 100% O_2, (*b*) 20% CO_2 + 80% N_2, (*c*) 50% CO_2 + 50% O_2, (*d*) 20% CO_2 + 80% O_2, (*e*) 25% CO_2 + 25% O_2 + 50% N_2, or (*f*) 51% CO_2 + 30% O_2 + 18% N_2 + 1% CO. Roasts were stored from 0-35 days at 1-3 C and chops from each treatment were observed under retail conditions for 5 days. Differences, both in psychrotrophic and lactobacillus counts, between roasts stored in modified gas atmospheres and those stored in vacuum-packages were rarely statistically significant. Psychrotrophic counts of pork chops from roasts stored for 21-35 days in modified gas atmospheres were somewhat higher than those prepared from comparable vacuum-packaged roasts. However, few of these differences were statistically significant. The initial microbial flora of the roasts consisted of *Pseudomonas* and *Lactobacillus* spp. *Pseudomonas* spp. remained a significant part of the microflora of roasts stored in 100% O_2, whereas lactic acid bacteria (*Lactobacillus* and *Leuconostoc* spp.) predominated on roasts after 1 week in all other atmospheres.

Various studies have shown that vacuum-packaging of meat can suppress the growth and metabolic activity of common gram-negative, aerobic meat-spoilage bacteria and hence extend its shelf-life (*3,8,18,22*). The residual oxygen in these packages is converted to CO_2 by the respiration of meat tissue and by microbial activity. Under these conditions, facultative anaerobes such as lactic acid bacteria often become predominant. The effect of these bacteria on appearance and palatability of meat is still not certain. Several investigators have reported some color loss associated with meat packaged under vacuum (*6,20,26*). Others have reported off-flavors and -odors of meat after prolonged storage in vacuum packages (*8,13,14,22*).

Some investigators have suggested storage in controlled gas atmospheres as an alternative to vacuum-packaging of meat (*4,10,11,17*). Theoretically, carbon dioxide will inhibit aerobic spoilage bacteria while oxygen will keep the surface of the meat oxygenated. The effect of an inert gas such as nitrogen on bacterial counts of meat is not certain. Experiments using combinations of all three gases have not been conclusive (*2,10,11,19*). The present paper reports on the effect of different gas atmospheres as compared with vacuum-packaging on the level and type of microbial flora of roasts stored for up to 35 days at 1-3 C and chops from these roasts after 5 days storage under retail conditions.

EXPERIMENTAL

Samples

One hundred and eight boneless roasts (6.4-cm thick) were prepared from the longissimus muscle of 25 pork loins and randomly assigned to seven treatments. All roasts were vacuum-packaged by the use of a chamber-type vacuum-packaging machine at the maximum capacity of the machine (747 mm of Hg) in heat-sealable barrier bags with the following characteristics: Oxygen Transmission Rate (OTR) = 32 cc/m^2/24 h/23.9 C/50% RH, Moisture Vapor Transmission Rate (MVTR) = 0.8-1.8 g/m^2/24 h/37.7 C/70% RH, and Carbon Dioxide Transmission Rate (CTR) = 47 cc/m^2/24 h/23.9 C/50% RH. Roasts in treatment A remained vacuum-packaged to serve as controls. In the other treatments, the bags were injected with 500 cc of a gas mixture (head space to meat volume ratio of 1 to 1) corresponding to the following treatments: (B) 100% O_2, (C) 20% CO_2 + 80% N_2, (D) 50% CO_2 + 50% O_2, (E) 20% CO_2 + 80% O_2, (F) 25% CO_2 + 25% O_2 + 50% N_2 or (G) 51% CO_2 + 30% O_2 + 18% N_2 + 1% CO.

Bacterial counts were made on three roasts before packaging and were used as initial counts for roasts in all treatments (Table 1). Roasts from each treatment were randomly assigned to storage periods of 7, 14, 21, 28 or 35 days (Table 1) at 1-3 C (three roasts per storage interval). At the end of each storage period, leaker packages were separated from intact packages and discarded. The intact packages first were subjected to gas analysis. They were then opened and the roasts were examined for microbiological, physical and sensory characteristics. Details of the procedures concerning injection of gases,

TABLE 1. *Number of samples examined bacteriologically stratified according to packaging treatment and length of storage.*

	Packaging treatment						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged (control)	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
0	—	—	—	3[a]	—	—	—
7	3	3	3	3	3	3	3
14	3	3	3	3	3	3	3
21	3	3	3	3	3	3	3
28	3	3	3	3	3	3	3
35	3	3	3	3	3	3	3

[a]Counts at 0 day were made on 3 roasts.

gas analysis and measurement of physical and sensory characteristics are presented in a companion paper by Seideman et al. (*21*). Chops (3.2-cm thick) were removed from each roast, placed in styrofoam trays and overwrapped with polyvinyl chloride film. The chops were displayed for 5 days under simulated retail conditions (1-3 C with 970 lux of incandescent light).

Microbiological

Sampling for microbiological analyses was carried out by swabbing a 12.9-cm² area of muscle with a sterile dacron swab wetted in sterile 0.1% peptone broth. The stick then was broken and the swab was placed in 10 ml of sterile 0.1% peptone broth. The sample jar was shaken 25 times and appropriate dilutions were made with sterile 0.1% peptone broth. Psychrotrophic bacterial counts were made by spreading 0.1-ml amounts of appropriate dilutions onto Plate Count Agar (Difco) with plate incubation at 7 C for 10 days. Lactobacilli were enumerated by use of Lactobacillus MRS broth (Difco) plus 1.5% agar with plate incubation at 25 C for 4 days. Psychrotrophic counts of chops after 5 days of retail storage were made in the manner described for counts of the roasts.

Distribution of the psychrotrophic microbial flora after the various storage periods was determined by picking 30-40 colonies from countable plates and placing them on Trypticase Soy Agar (BBL) slants. Incubation of slants was at 25 C for 2-3 days. Diagnostic schemes and procedures to identify the isolates were those published by Vanderzant and Nickelson (*25*).

Bacteriological count data were analyzed using one-way analysis of variance comparing vacuum-packaging (control) to each of the other treatments independently within each of the five storage intervals. One-way analysis of variance was performed on data within each packaging treatment over the five storage intervals. When significant ($P < .05$) main effects were observed in the analysis of variance, mean separation was accomplished by use of the Student-Newman-Keuls test (*24*).

RESULTS

Psychrotrophic plate counts of pork roasts stored in various gas atmospheres were frequently slightly higher than those of comparable vacuum-packaged control roasts (Table 2). However, in only a few cases were these differences statistically significant. Increases in psychrotrophic counts were greatest between 7 and 14 days of storage for the following packaging treatments: control roasts (A) and for roasts stored initially in 20% CO_2 + 80% N_2 (C), 50% CO_2 + 50% O_2 (D), 25% CO_2 + 25% O_2 + 50% N_2 (F), and in 51% CO_2 + 30% O_2 + 18% N_2 + 1% CO (G). For roasts stored initially in 100% O_2 (B), and in 20% CO_2 + 80% O_2 (E) the greatest increase in psychrotrophic counts occurred between 0 and 7 days storage. After 21 days of storage, psychrotrophic counts of roasts initially stored in atmospheres containing 80-100% O_2 (treatments B and E) were significantly higher than those of comparable vacuum-packaged roasts. After 35 days of storage, the increase in counts of vacuum-packaged control roasts was 4.45 logs while the increase in count for roasts stored in the various gas atmospheres ranged from 4.76-5.98 logs.

Lactobacillus counts of pork roasts after 1 and 2 weeks of storage (Table 3) were in most cases slightly lower than the initial (about 10^3/in.²) lactobacillus count. However, large increases in lactobacillus counts occurred after storage for 21, 28 and 35 days. In most cases, the lactobacillus counts of roasts stored in various gas

TABLE 2. *Mean values for psychrotrophic bacterial counts[a] of pork roasts stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged[c]	100% O_2[c]	20% CO_2[c] 80% N_2	50% CO_2[c] 50% O_2	20% CO_2[c] 80% O_2	25% CO_2[c] 25% O_2 50% N_2	51% CO_2[c] 30% O_2 18% N_2 1% CO
7	3.84[e]	4.42[f]	3.59[e]	3.69[g]	4.88[e]	3.97[g]	3.78[f]
14	6.55[d]	6.10[e]	6.72[d]	6.79[f]	6.51[e]	6.61[f]	8.27[de]
21	6.60[d]	7.86[d]	6.48[d]	7.05[ef]	7.38[c]	7.21[def]	7.01[e]
28	7.20[d]	7.82[d]	7.19[d]	7.84[de]	7.54[d]	7.31[de]	7.34[de]
35	7.05[d]	8.58[d]	7.65[d]	7.99[d]	7.36[d]	7.89[d]	8.31[d]

[a]Counts (log_{10}) per 6.45 cm² (1 in.²).
[b]Counts within a common storage interval that are underlined are significantly different ($P < .05$) from counts obtained on vacuum packaged control roasts.
[c]Initial count (log_{10}) = 2.60.
[defg]Means in the same column bearing a common superscript do not differ ($P > .05$).

TABLE 3. *Mean values for lactobacilli counts[a] of pork roasts stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged[c]	100% O_2[c]	20% CO_2[c] 80% N_2	50% CO_2[c] 50% O_2	20% CO_2[c] 80% O_2	25% CO_2[c] 25% O_2 50% N_2	51% CO_2[c] 30% O_2 18% N_2 1% CO
7	1.37[e]	2.06[e]	1.83[f]	2.09[e]	2.24[f]	2.48[fg]	2.02[f]
14	2.24[e]	2.35[e]	2.07[f]	3.62[e]	2.39[f]	2.30[g]	1.76[f]
21	4.17[d]	3.59[de]	2.85[f]	3.25[e]	3.25[e]	3.48[e]	3.73[ef]
28	4.54[d]	4.58[d]	4.38[e]	4.18[e]	4.17[d]	4.42[e]	5.17[de]
35	5.05[d]	5.24[d]	5.57[d]	6.73[d]	4.58[d]	5.66[d]	6.46[d]

[a]Counts (log_{10}) per 6.45 cm² (1 in.²).
[b]Counts within a common storage interval that are underlined are significantly different ($P < .05$) from counts obtained on vacuum packaged control roasts.
[c]Initial count (log_{10}) = 3.07.
[defg]Means in the same column bearing a common superscript do not differ ($P > .05$).

atmospheres were not statistically different from counts of comparable vacuum-packaged control roasts. After 35 days of storage, increases in lactobacilli were largest on the roasts stored in atmospheres containing 50% CO_2 (treatments D and G).

Psychrotrophic counts of chops (Table 4) usually were higher (34 of 35 samples) than counts of roasts (Table 2) from which they were prepared. Counts of chops from roasts stored for 21-35 days in various gas atmospheres were almost always higher than those of chops from comparable vacuum-packaged control roasts. These differences were 0.08-1.14 logs after 21 days of storage, 0-1.37 logs afters 28 days and 0.12-0.59 logs after 35 days. However, these differences were seldom statistically significant.

The distribution of the psychrotrophic microbial flora of roasts is presented in Table 5. Immediately before packaging the microbial flora was dominated by *Pseudomonas* spp. (73.1%) and to a lesser extent by *Lactobacillus* spp. (26.9%). The *Pseudomonas* sp. found most often on pork in this study was *Pseudomonas fragi*. For the vacuum packaged control roasts and those stored in atmospheres initially containing 20-50% CO_2 (treatments C through G), lactic acid bacteria (*Lactobacillus* and *Leuconostoc* spp.) became a dominant part of the microbial flora after 7 days of storage. For roasts stored initially in 100% O_2, *Pseudomonas* spp. remained a substantial or dominant part of the microflora. When lactic acid bacteria became a substantial part of the microflora of roasts, *Leuconostoc* spp. were frequently more prominent on roasts during the early phases of storage (7-21 days) whereas *Lactobacillus* spp. became more dominant after 28-35 days.

DISCUSSION

Microbiological and physical characteristics of pork stored in various gas atmospheres have been studied

TABLE 4. *Mean values for psychrotrophic bacterial counts[a] of pork chops after 5 days of retail display stratified according to packaging treatment and storage interval.*

	Packaging treatment[b]						
	A	B	C	D	E	F	G
Storage interval (days)	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
7	7.09[d]	7.32[d]	5.74[d]	6.65[d]	6.91[d]	7.32[c]	6.19[d]
14	8.78[c]	8.71[c]	7.95[c]	8.39[c]	8.32[c]	8.38[c]	8.43[c]
21	8.10[cd]	9.24[c]	8.23[c]	8.22[c]	8.31[c]	9.09[c]	8.18[c]
28	8.54[c]	9.91[c]	8.72[c]	9.14[c]	9.18[c]	8.95[c]	8.54[c]
35	7.99[cd]	8.52[c]	8.39[c]	8.58[c]	8.11[c]	8.54[c]	8.58[c]

[a]Counts (log_{10}) per 6.45 cm^2 (1 $in.^2$).
[b]Counts within a common storage interval that are underlined are significantly different ($P < .05$) from counts on chops (after 5 days of retail display) from vacuum packaged (control) roasts.
[cd]Means in the same column bearing a common superscript do not differ ($P > .05$).

TABLE 5. *Percentage distribution of microorganisms[a] on pork roasts stratified according to packaging treatment and storage interval.*

		Packaging treatment						
		A	B	C	D	E	F	G
Storage interval (days)	Type	Vacuum packaged	100% O_2	20% CO_2 80% N_2	50% CO_2 50% O_2	20% CO_2 80% O_2	25% CO_2 25% O_2 50% N_2	51% CO_2 30% O_2 18% N_2 1% CO
7	*Pseudomonas*	7.0	32.4	0.7	9.6	33.0	14.6	3.9
	Lactobacillus	6.5	—	2.8	0.9	12.8	28.2	4.3
	Leuconostoc	86.5	67.6	96.5	89.5	53.4	57.2	91.8
	Others[b]	—	—	—	—	0.8	—	—
14	*Pseudomonas*	14.9	47.7	35.2	13.1	39.2	6.1	4.2
	Lactobacillus	13.6	29.7	22.7	7.4	9.6	4.6	7.3
	Leuconostoc	71.5	22.0	42.1	79.5	51.2	89.3	88.5
	Others[b]	—	0.6	—	—	—	—	—
21	*Pseudomonas*	7.0	90.3	0.3	0.9	3.9	11.7	6.5
	Lactobacillus	36.4	9.7	17.7	39.5	5.0	1.1	0.2
	Leuconostoc	56.6	—	82.0	59.6	91.1	87.2	93.3
	Others[b]	—	—	—	—	—	—	—
28	*Pseudomonas*	16.0	47.7	2.1	7.5	19.9	18.0	—
	Lactobacillus	82.3	52.3	91.2	66.9	23.7	82.0	100.0
	Leuconostoc	1.7	—	6.7	25.6	56.4	—	—
	Others[b]	—	—	—	—	—	—	—
35	*Pseudomonas*	7.4	83.2	3.6	1.2	15.2	13.0	10.4
	Lactobacillus	92.6	5.7	37.4	6.2	84.8	87.0	89.2
	Leuconostoc	—	11.1	58.4	92.6	—	—	—
	Others[b]	—	—	0.6	—	—	—	0.4

[a]Each percentage is based on 3 analyses. (Initial flora: *Pseudomonas* = 73.1, *Lactobacillus* = 26.9).
[b]These included species of *Micrococcus, Corynebacterium, Moraxella* and molds.

previously (*2,7,10*); however, few have compared these characteristics directly with those of vacuum-packaged pork. Huffman (*10*) reported that pork chops stored at -1.1 C in 100% CO_2 or in 70% N_2 + 25% CO_2 + 5% O_2 had significantly lower aerobic plate counts during the entire storage period than chops stored in air, 100% O_2 or 100% N_2. No significant differences in counts were observed among chops stored in air, O_2 or N_2. According to this author, there were no significant differences in the number of lactic acid bacteria on chops stored in air, O_2, N_2, CO_2 or in 70% N_2 + 25% CO_2 + 5% O_2 during the first 2 weeks. Also, at 21 days post-slaughter, counts of lactic acid bacteria of the chops in CO_2 or in the gas mixture were consistently higher than in air, O_2 or N_2.

The inhibitory effect of CO_2 on aerobic gram-negative meat spoilage bacteria is now well documented (*10,11,17,23*). It is most likely caused by the effect of CO_2 on decarboxylating enzymes such as isocitric and malate dehydrogenases (*15,16*). Information on the effects of N_2 on bacterial growth on meats often has been contradictory. According to Huffman (*10*), aerobic plate counts of pork chops stored in N_2 were similar to those stored in air. However, Newton et al. (*17*) reported that counts of lamb chops stored in N_2 (O_2-free) were much lower than those of chops stored in air, $O_2 + N_2$ (80:20) or in air + CO_2 (80:20). Lack of agreement may be caused in part by differences in (*a*) nature of samples (beef versus lamb), (*b*) composition and conditions of plate incubation (2 days at 32 C on plate count agar versus 0 C on APT agar), (*c*) sampling methods (rinse versus swab method) and (*d*) composition of the gases (about 100% N_2 versus O_2-free N_2).

Based upon psychrotrophic and lactobacillus counts of roasts in this study, storage of roasts in six different gas atmospheres did not offer any specific advantages over storage in vacuum packages. Psychrotrophic counts of roasts stored in the various gas atmospheres frequently were somewhat higher than those of comparable vacuum-packaged control roasts. The rapid increase in psychrotrophic counts in the first week on roasts packaged in atmospheres of 80-100% O_2 likely reflects the rapid growth of psychrotrophic gram-negative bacteria such as *Pseudomonas* spp. Psychrotrophic counts of these roasts after 21 days were significantly higher than those of comparable vacuum-packaged control roasts. Between 21 and 35 days of storage little change in psychrotrophic count occurred on the roasts stored in 20% CO_2 + 80% O_2 but counts continued to increase on roasts stored in 100% O_2. The rapid increase of psychrotrophic bacteria on roasts stored initially in O_2-rich atmospheres is supported by data on the gas analysis of the head space in the companion paper (*21*). The relative weight percentages of CO_2 and O_2 for the roasts packaged initially in 100% O_2 were 7.84 and 92.16% after 7 days and 9.12 and 89.17% after 14 days. For the vacuum-packaged roasts these figures were 71.53 and 0% after 7 days and 85.80 and 1.48% after 14 days. In addition, the weight percentages of CO_2 in the headspace of vacuum-packaged roasts were consistently higher than those of the roasts stored in various gases.

During the first 2 weeks of storage, lactobacillus counts remained low and then increased rapidly between 21 and 35 days. A slow rate of growth of these organisms at refrigeration temperatures during the early phase of storage may be responsible in part for this observation. In addition, changes in physical chemical characteristics and in available nutrients may have occurred during this time on the meat (through microbial or tissue enzyme activity), which may have created an environment more conducive for development of lactobacilli. Furthermore, the composition of the gas mixtures in this study represents initial concentrations. Data in the companion paper (*21*) show that extensive changes occurred in the weight percentages of CO_2, O_2 and N_2 in the headspace during storage. Development of lactic acid bacteria on meats in atmospheres where competing gram-negative aerobic bacteria are suppressed is well documented (*3,7, 10,11,12,17,23*).

Psychrotrophic counts of chops after 5 days of display usually were related to the counts of the roasts from which they were prepared. According to Hudson and Roberts (*9*), counts of retail beef cuts after a 24-h display showed a significant correlation with counts of vacuum-packaged primal cuts from which they had been prepared. Based upon the level of the psychrotrophic counts of the chops, storage of roasts in the various gas atmospheres did not seem to offer any advantage over storage in vacuum-packages.

The initial microflora of pork roasts consisted of *Pseudomonas* (73.1%) and *Lactobacillus* spp. (26.9%). *Pseudomonas* spp. remained dominant only on roasts initially stored in 100% O_2, likely because of the lack of an inhibitory CO_2 concentration. According to Shaw and Nicol (*23*), *Pseudomonas* spp. were not inhibited by 100% O_2 but were affected by O_2 concentrations below 0.8%. *Pseudomonas* spp. were detected on lamb chops held in low-O_2 atmospheres but not in O_2-free atmospheres (with or without CO_2). This indicates the significance of O_2 limitation in suppressing growth of these organisms (*17*). Clark and Lentz (*5*), however, reported that O_2 concentrations above 50% could affect growth of *Pseudomonas* spp. In the present study, inhibition of *Pseudomonas* spp. and predominance of lactic acid bacteria on roasts held in 20-50% CO_2 and less than 50% O_2 likely are caused by the inhibitory activity of CO_2 on gram-negative aerobic bacteria. Increased significance of lactobacilli in prepackaged pork stored for 2 weeks at 2 C was reported by Gardner et al. (*7*). In that study, the microflora after 2 weeks consisted of 49% *Pseudomonas-Achromobacter,* 17% *Microbacterium thermosphactum,* 30% lactobacilli, 2% *Enterobacter-Hafnia* spp. and 2% unclassified. The relatively high concentration of *Pseudomonas-Achromobacter* spp. may have resulted from a less than optimum inhibitory gas atmosphere (average 12.3% CO_2 + 11.5% O_2 + 74.52% N_2). In the present study *Leuconostoc* spp. appeared as

large mucoid colonies on plate count agar and were dextran-producing strains of *L. dextranicum* and *L. mesenteroides* (*1*).

In the companion paper, Seideman et al. (*21*) compared the physical and sensory attributes of vacuum-packaged roasts and chops prepared from such roasts with those stored in various gases. They reported a decrease in quality attributes of pork initially stored in oxygen-rich atmospheres. This observation is, as shown in this study, likely related to the continued growth and activities of *Pseudomonas* spp. in such atmospheres.

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A Research Note

Mold in Jellied and Whole-Berry Styles of Cranberry Sauce

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ABSTRACT

A survey was made to determine the level of mold contamination in jellied and whole-berry style cranberry sauce. The analytical data obtained were representative of the cranberry sauce at the national retail level. The mean and range of Howard mold counts for each style were as follows: jellied, 4.1% (0-98%); and whole berry, 1.6% (0-10%). The percentage of samples of each style found to contain mold were: jellied 85.8% and whole berry 64.9%.

Cranberries are exposed to a number of fungi while in their natural growing environment. Improper handling and storage conditions cause infection of the cranberry fruit by fungal mycelia and spores, resulting in rotten berries (Table 1). Cranberry processors cull out these berries, using both mechanical and hand-sorting techniques. Regardless of the care taken to remove defective fruit, however, some rotten berries find their way into the finished product. Although the rotten fruit is no longer visible to the naked eye, once it is incorporated into the cranberry sauce, the mold mycelia can be detected microscopically.

The concern of the Food and Drug Administration (FDA) about the quality of cranberry sauce dates back to 1941 when Dr. B. J. Howard, an FDA microbiologist, issued an internal memo describing correlation studies between rot in whole cranberries and cranberry sauce mold counts. From these early studies it was determined that mold counts in excess of 35%, as determined using the Howard mold count procedure, indicated the use of excessively rotten cranberries for sauce production. After additional studies in 1947, the regulatory action limit on cranberry sauce was reduced to a 30% mold count. In 1950, when the dilution of the mold count preparation was changed, the action limit was adjusted from a 30% to a 20% mold count. In 1973, a retail market survey was conducted to develop a current data base on mold counts in cranberry sauce as it was offered to the consumer. The sampling details and analytical findings of that survey are presented in this paper.

TABLE 1. *Common cranberry fungus rots that develop in storage (2).*

Common name	Scientific name
End rot	*Godronia cassandrae*
Early rot	*Guignardia vaccinii*
Bitter rot	*Glomerella cingulata vaccinii*
Blotch rot	*Acanthorhynchus vaccinii*
Ripe rot	*Sporonema oxycocci*
Fruit storage rot	*Diaporthe vaccinii*
Black rot	*Centhospora lunata*

MATERIALS AND METHODS

Samples were collected by FDA inspectors from 25 standard metropolitan statistical areas. These 25 areas were randomly chosen from the 276 areas defined by the Bureau of the Census as integrated economic and social units with a recognized urban population nucleus of substantial size (*3*). In each metropolitan area, products in three chain stores and two independent stores were selected for sampling. Four retail units of cranberry sauce, two whole berry style and two jellied style, were collected at each of the sampling locations. Whenever possible, different brands of products were selected in a given store; however, if brand duplication was necessary, different production codes were collected. The collection plan was designed to ensure random sampling that would provide adequate lot representation nationally.

Analyses were conducted by the FDA's Division of Microbiology. Mold contamination in the samples was determined by the Howard mold count, method 44.082 of the Association of Official Analytical Chemists (*1*). The Howard mold count technique is used to estimate the amount of rotten fruit processed into cranberry sauce based on a mold mycelial fragment count. Analytical results are expressed as "% Howard mold count," a figure based on the total number of microscopic fields observed compared to the number of fields containing mold filaments. Data were obtained on 492 samples.

RESULTS AND DISCUSSION

This survey found mold decomposition to be a defect in both jellied and whole berry cranberry sauce. Table 2 presents a frequency distribution of Howard mold counts of jellied cranberry sauce. The Howard mold count in jellied sauce ranged from 0 to 98%. Approximately 86% of the jellied-style samples examined contained mold mycelial fragments and showed a broad distribution. The median mold count was 1.0% and the mean was 1.6%. Ninety-five percent of the jellied-style samples had mold counts of less than 11%.

Table 3 presents a frequency distribution of Howard mold counts of whole berry cranberry sauce. The mold count ranged from 0 to 10%. Approximately 65% of the whole berry-style samples examined contained mold mycelial fragments. The median mold count was 2.0% and the mean was 4.1%. Ninety-five percent of the whole berry-style samples had mold counts of less than 6%.

Table 4 provides a statistical summary of mold counts for both styles of cranberry sauce. The analytical data presented in this paper suggest that the quality of

TABLE 2. *Frequency distribution of Howard mold counts in jellied style cranberry sauce.*

% HMC[a]	Number of samples	% samples	Cumulative %
0	36	14.2	14.2
1	45	17.8	32.0
2	62	24.4	56.4
3	29	11.5	67.9
4	21	8.3	76.2
5	21	8.3	84.5
6	11	4.3	88.8
7	6	2.4	91.2
8	2	0.8	92.0
9	4	1.6	93.6
10	4	1.6	95.2
11-15	6	2.4	97.6
16-20	2	0.8	98.4
21-30	0	—	
31-40	1	0.4	98.8
41-50	0	—	
51-60	1	0.4	99.2
61-70	0	—	
71-80	1	0.4	99.6
81-90	0	—	
91-98	1	0.4	100.0

[a]HMC = Howard Mold Count.

cranberry sauce on the current retail market has improved significantly since 1947 when the previous mold count action limit was set as a result of an industry study.

ACKNOWLEDGMENTS

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TABLE 3. *Frequency distribution of Howard mold counts in whole berry style cranberry sauce.*

% HMC[a]	Number of samples	% samples	Cumulative %
0	84	35.1	35.1
1	59	24.8	59.9
2	50	20.9	80.8
3	16	6.7	87.5
4	6	2.5	90.0
5	11	4.6	94.6
6	3	1.3	95.9
7	5	2.1	98.0
8	2	0.8	98.8
9	1	0.4	99.2
10	2	0.8	100.0

[a]HMC = Howard Mold Count.

TABLE 4. *Statistical summary of Howard mold count data in cranberry sauces.*

	% HMC[a]	
Value	Jellied sauce	Whole berry sauce
N	253	239
Mean	4.1%	1.6%
Median	2.0%	1.0%
Minimum value	0%	0%
Maximum value	98%	10%

[a]HMC = Howard Mold Count.

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Survival of Microorganisms in Stored Pasta

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ABSTRACT

Numbers of *Staphylococcus aureus, Saccharomyces cerevisiae* and *Penicillium expansum* in artificially contaminated pasta declined exponentially during storage at room temperature; corresponding D values ranged from 18-21 days, 40-45 days and 130-160 days. In contrast to the rapid death kinetics of *Aspergillus repens* and *Escherichia coli, Streptococcus faecium* survived after 180 days of storage. These results suggest that streptococci are more reliable than *E. coli* as indicators of fecal contamination in pasta. Detection of *Salmonella infantis* and *Salmonella typhimurium* after 360 days indicates that prolonged storage of pasta is not effective for decontamination of infected products.

Although low water activities found in dry foods, such as pasta, inhibit active growth of microorganisms (*14*), the condition will not effectively reduce populations of certain bacterial pathogens which can survive the sublethal heat treatments used in commercial pasta processing (*10,11,17,18,20*). A recent survey conducted by the National Microbiology Monitoring Laboratory (Health and Welfare Canada) involving 654 samples of commercially prepared pasta reported aerobic colony counts in excess of 10^4 cells per g in 19% of the samples, *Staphylococcus aureus* in excess of 10^3 cells per g in 1% of the samples, and *Salmonella* in 3 samples (unpublished data). In most of the pasta examined, coliforms, yeasts, molds and coagulase-positive staphylococci were either not detected or present in very low numbers. Lack of information on the ability of these organisms to survive prolonged storage at ambient temperatures precludes conclusions on the probable presence of pathogenic microorganisms in stored pasta and the hygienic conditions under which the product was manufactured. The presence of *S. aureus* in pasta is of concern because staphylococcal enterotoxins are stable at temperatures lethal to vegetative cells. Enterotoxin A could be detected in dried pasta after 1 year of storage at room temperature, whereas populations of *S. aureus* declined from 10^9 cells to 10^3 cells per g after 90 days, and no organisms could be detected after 180 days of storage (*9*). Salmonellae are even more resistant than *S. aureus* and can survive prolonged storage in environments of low water activity (*4,12,20*).

This study was undertaken to determine death kinetics of selected microorganisms during storage of dried pasta.

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MATERIALS AND METHODS

Microorganisms and cultural methods

Bacterial cultures were grown for 18 h at 35 C in Trypticase Soy broth (BBL), except salmonellae which were grown in Nutrient broth (Difco). *S. aureus* S6 and R220 which produce enterotoxin B and C, respectively, were isolates from food poisoning outbreaks. Enteropathogenic strains of *Escherichia coli* 711 and H10407 obtained from Dr. Gyles (University of Guelph, Canada) produce both heat-stable and heat-labile enterotoxins. *Streptococcus faecalis, Streptococcus faecium, Salmonella infantis* and *Salmonella typhimurium* were obtained from the Laboratory Centre for Disease Control (Health and Welfare Canada) and were grown without shaking. *Saccharomyces cerevisiae* was grown in Sabouraud broth (BBL) at room temperature. Mycotoxin-producing cultures of *Penicillium expansum* and *Aspergillus repens* obtained from Dr. Harwig (Health Protection Branch, Canada) were grown at 25 C for 5 to 7 days on Potato Dextrose agar (PDA) in Roux bottles.

Microbial cultures were harvested by centrifugation, washed once and suspended to the desired cell density in sterile distilled water, using standard curves relating optical density (600 nm) to viable counts. Spores of molds were harvested by adding 25 ml of sterile 0.05% (v/v) Tween 80 to each Roux bottle and shaking; the process was repeated and spore suspensions pooled. Spores were enumerated using a Neubauer counting chamber.

Manufacture of artificially contaminated pasta

Pasta dough, made from durum semolina with and without added whole egg powder (5.5%, w/w), was inoculated with high and low levels of each test organism corresponding to 100- to 500-fold difference between levels of inoculation. Bacterial cultures were added singly, whereas *S. cerevisiae* was added with either *P. expansum* or *A. repens*. Microbial cells or spores were added at a constant rate to the mixing chamber of De Maco S-25 laboratory extruder. The moisture content of pasta extruded at 50 C ranged from 30 to 34% (w/w). Pasta was dried at 45 C over an 18-h period during which the relative humidity of the drying chamber was lowered from 97% at the beginning to 50% at the end of the drying cycle; moisture content of the final product was approximately 12% (w/w).

Enumeration of microorganisms

Artificially contaminated pasta stored at room temperature in plastic bags was tested on the day of manufacture and after 10, 20, 40, 90 and 180 days of storage; samples infected with salmonellae were also tested after 360 days of storage. All test organisms, except salmonellae, were enumerated from triplicate 11-g samples of pasta; samples were blended for 1 min in 99 ml of cold 0.1% peptone water, left to stand 2 h at 4 C and blended again for 2 min. Preliminary work indicated that this sample preparation procedure produced highest counts presumably because of improved dispersion of sample. *S. aureus* was enumerated on Baird-Parker agar (Difco), *P. expansum, A. repens* and *S. cerevisiae* were enumerated on PDA containing 0.01% (w/v) each of chloramphenicol and chlortetracycline. One set of PDA plates was incubated at room temperature for 5-7 days for mold growth and another set of PDA plates was incubated at 35 C for 48 h for yeasts. The faster-growing yeasts were easily differentiated from the molds. *S. faecalis* and *S. faecium* were enumerated on m-Enterococcus agar (Difco) and *E. coli* by the membrane technique of Anderson and

Baird-Parker (*1*) modified as follows to promote growth of cells injured during processing. Membrane filters placed on the surface of Trypticase Soy agar (BBL) plates were impregnated with diluted portions of pasta homogenates and incubated at 35 C for 4 h; the filters were then transferred to the surface of Tryptone Bile agar (*1*) and incubated at 44.5 C for 24 h in a water-jacketed incubator.

Populations of *Salmonella* in stored pasta were enumerated using the 3-tube Most Probable Number (MPN) technique. Replicate samples of pasta (100, 10 and 1 g) were preenriched in Nutrient broth for 18 h at 35 C. Portions of the preenriched cultures were selectively enriched overnight in Tetrathionate Brilliant Green (43 C) and Selenite Cystine (35 C) broths and streaked on Bismuth Sulfite and Brilliant Green Sulfa agars. All media were obtained from Difco. Plates were incubated at 35 C for 24 h and presumptive isolates were screened biochemically and serologically.

Enterotoxin analysis

Pasta contaminated with *S. aureus* and stored for up to 30 days was tested for enterotoxins B and C by the double antibody radioimmunoassay technique (*13*).

RESULTS

Analysis of durum semolina and whole egg powder used in the manufacture of pasta did not reveal the presence of any of the test organisms. Aerobic colony counts of semolina and egg powder determined separately on Trypticase Soy agar incubated at 35 C for 24 h ranged between 10^3 and 10^4 cells per g.

Survival curves for *S. aureus* R220 in pasta and egg pasta are presented in Fig. 1. Viability declined logarithmically with increasing storage time and after 180 days counts decreased to less than 100 viable cells per g in all products. D values, the amount of time required for a 90% decrease in population, ranged from 18 to 21 days; similar D values were obtained for *S. aureus* S6. Although the original experimental design had provided for a 100-fold difference in counts between the low- and high-level contaminated pasta, this condition was not achieved because both strains of *S. aureus* in the low-level contaminated products grew in the drying chamber, probably during the first 4 to 5 h when the temperature was below 45 C. No enterotoxin was detected in any of the products tested.

Exponential decreases in populations of *S. cerevisiae* (Fig. 2) and *P. expansum* (Fig. 3) were also observed in stored pasta. D values for *S. cerevisiae* and *P. expansum* ranged from 40 to 54 and 130 to 160 days, respectively. Growth of *S. cerevisiae*, but not *P. expansum*, occurred in the drying chamber, presumably because longer incubation periods are required for germination and growth of mold spores.

Some of the test organisms proved to be particularly sensitive to storage (Table 1). After 20 days, levels of *A. repens* in pasta and egg pasta decreased to less than 10^2 cells per g. Survival of *E. coli* 10407 during drying was markedly better in egg pasta than in pasta where populations decreased by 1 and 3 $\log_{10}$ units, respectively. In constrast, *E. coli* 711 could not be detected in freshly dried pasta and no viable cells could be recovered from egg pasta stored for 10 days (data not shown). Agreement between the results obtained with the membrane filter and MPN techniques confirmed the

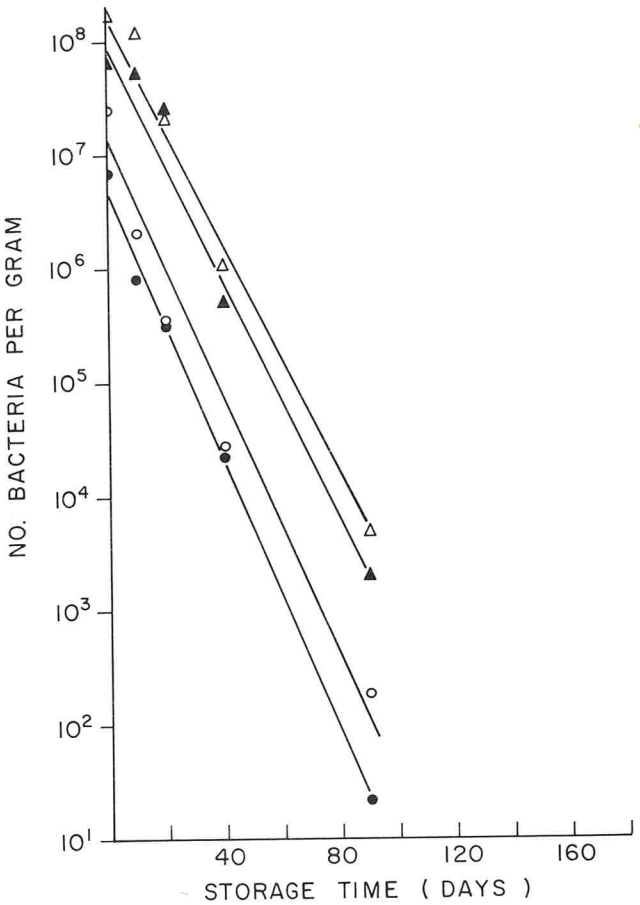

Figure 1. *Survival curves for* Staphylococcus aureus *R220 in pasta and egg pasta stored at room temperature.* ○, *high level contaminated pasta (wet product contained* 2.3×10^7 *cells for g);* ●, *low level contaminated pasta (wet product contained* 2.1×10^4 *cells per g);* △, *high level contaminated egg pasta (wet product contained* 1.8×10^7 *cells per g);* ▲, *low level contaminated egg pasta (wet product contained* 7.3×10^4 *cells per g).*

hypersensitivity of strain 711 to the pasta drying process. Good survival of *S. faecalis* was obtained in pasta but not in egg pasta where cells could not be detected after 20 days of storage (Table 1). These results contrast sharply with those for *E. coli* which survived better in egg pasta. Further studies with *S. faecium* showed good survival in both types of product where populations decreased by less than 1 $\log_{10}$ unit after 180 days of storage. Growth of *S. faecium* during drying was also noted in low level contaminated pasta (Table 2). D values for *A. repens, E. coli* and streptococci could not be determined because of rapid loss of viability or non-exponential death kinetics.

Results on the survival of *Salmonella* are presented in Table 3. The small differences in counts between the wet and freshly dried pasta are not consistent with active growth of the organisms in the drying chamber and presumably reflect the inherent inaccuracy of the MPN technique. In contrast to *S. infantis*, presence or absence of egg in the product did not affect survival of *S. typhimurium.* Although *S. infantis* was readily detected in all samples stored for 360 days, recovery of *S. typhimurium* was limited to samples initially contaminated with high numbers of organisms. Estimated D

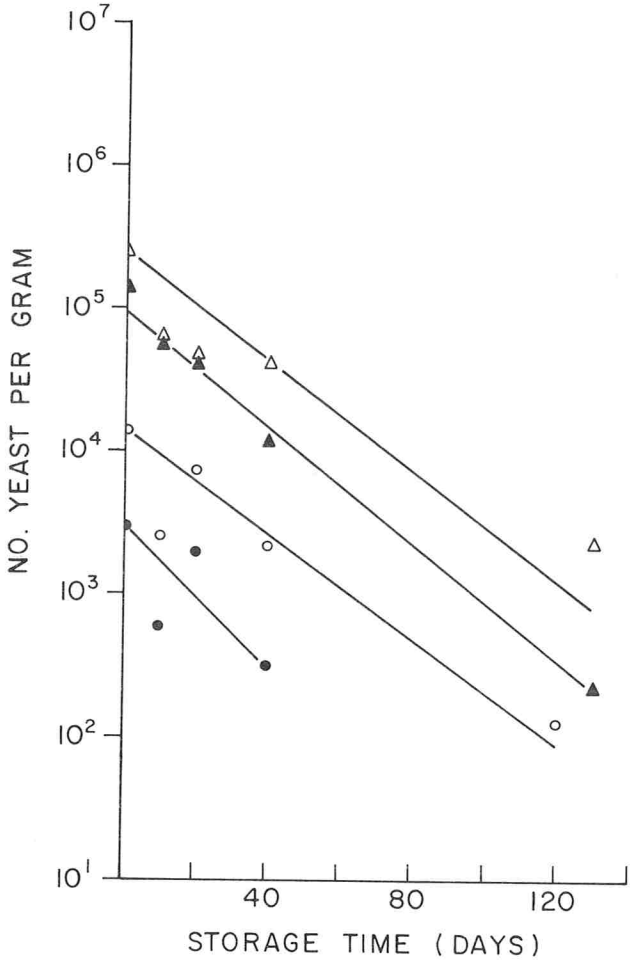

Figure 2. *Survival curves for* Saccharomyces cerevisiae *in pasta and egg pasta stored at room temperature.* ○, *high level contaminated pasta (wet product contained* 3.5×10^4 *cells per g);* ●, *low level contaminated pasta (wet product contained* 1.2×10^3 *cells per g);* △, *high level contaminated egg pasta (wet product contained* 3.5×10^4 *cells per g);* ▲, *low level contaminated egg pasta (wet product contained* 5.7×10^3 *cells per g).*

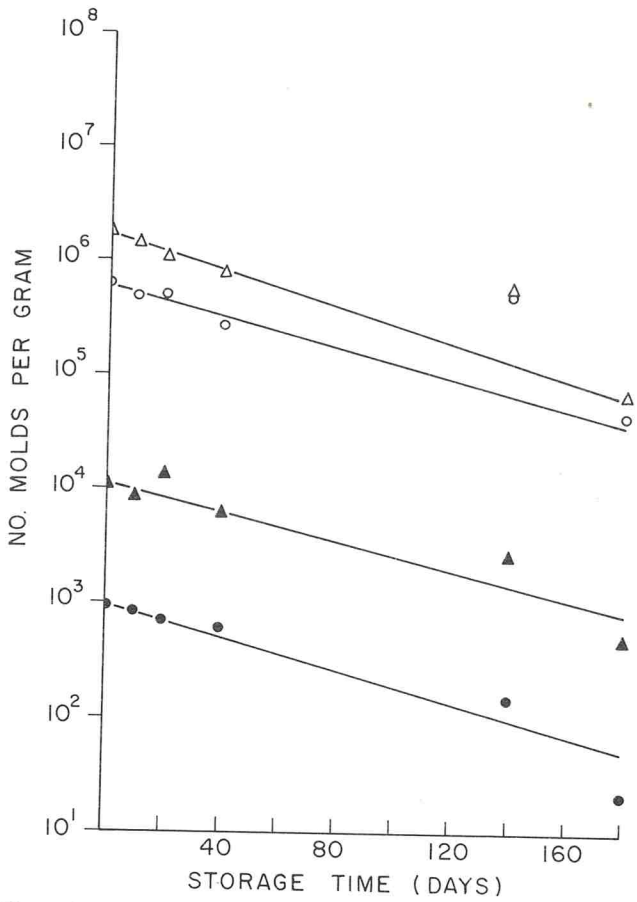

Figure 3. *Survival curves for* Penicillium expansum *in pasta and egg pasta stored at room temperature.* ○, *high level contaminated pasta (wet product contained* 4.5×10^5 *cells per g);* ●, *low level contaminated pasta (wet product contained* 2.6×10^3 *cells per g);* △, *high level contaminated egg pasta (wet product contained* 5.6×10^5 *cells per g);* ▲, *low level contaminated egg pasta (wet product contained* 2.6×10^3 *cells per g).*

values for *S. typhimurium* in pasta and egg pasta were 90 and 180 days, respectively; corresponding D values for *S. infantis* were 360 and 90 days.

DISCUSSION

S. aureus, an important foodborne pathogen, has frequently been identified in pasta products (*5,10,11,16*). Although growth of this organism during drying could not be associated with enterotoxin production in our studies, the presence of toxigenic strains in wet pasta and drying for prolonged periods at sublethal temperatures could result in a finished product containing toxic levels of heat-stable enterotoxin (*9,18,19*). Our results on the ability of *S. aureus* to survive storage at ambient temperature for 90 days or more concur with earlier findings (*9,19*).

In contrast to *A. repens, S. cerevisiae* and *P. expansum*

TABLE 1. *Survival of sensitive microorganisms in high level contaminated pasta.*[a]

Storage time (days)	*Aspergillus repens* (cells per g)		*Escherichia coli* 10407 (cells per g)		*Streptococcus faecalis* (cells per g)	
	Pasta	Egg pasta	Pasta	Egg pasta	Pasta	Egg pasta
0	5.0×10^3	2.7×10^5	1.7×10^2	3.7×10^4	2.2×10^7	1.0×10^2
10	5.0×10^2	4.0×10^2	1.7×10^2	1.0×10^4	1.4×10^7	6.6×10^1
20	$< 1.0 \times 10^2$	1.8×10^2	1.3×10^2	4.1×10^3	4.3×10^6	$< 1.0 \times 10^1$
40	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	0.5×10^1	4.1×10^3	9.0×10^6	ND
90	ND[b]	ND	ND	2.0×10^1	3.3×10^4	ND
180	ND	ND	ND	ND	7.0×10^3	ND
Wet Pasta (cells per g)	1.1×10^4	3.0×10^5	1.4×10^5	5.4×10^5	7.5×10^6	4.0×10^4

[a]Average of triplicate samples.
[b]ND = Not determined.

were found to be particularly stable in dry pasta and showed respective D values 3- and 10-fold greater than that for *S. aureus*. The presence of molds in commercial products (*16*, HPB unpublished data) is of public health significance because of possible growth and production of mycotoxins.

The value of microorganisms, such as *E. coli* and streptococci, as reliable indicators of fecal contamination in water and food has yet to be fully recognized (*3,6,15*). Our results and those of others (*2,8*) have shown that *E. coli* rapidly dies in foods of low water activity and therefore does not constitute a reliable marker. The greater longevity of streptococci in pasta (Tables 1 and 2) support their use as indicator organisms. The mechanism responsible for the marked differential rates of death of *S. faecalis* and *S. faecium* in egg pasta has yet to be elucidated.

Our results on the thermostability of *Salmonella* during drying do not support earlier work reporting substantial death during drying (*7,20*); differences in experimental design may account in part for the discrepancies in results. Ability to recover *Salmonella* from stored pasta artificially contaminated with low and high numbers of organisms (Table 3, and *9,20*) suggest that prolonged storage of pasta at room temperature is not effective for decontamination of an infected product.

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TABLE 2. *Survival of* Streptococcus faecium *in stored pasta*[a].

Storage time (days)	Pasta		Egg Pasta	
	H[b]	L[b]	H	L
0	1.6×10^7	2.7×10^7	1.9×10^6	3.7×10^5
10	2.0×10^7	5.4×10^7	1.7×10^6	5.0×10^4
20	5.5×10^6	9.2×10^6	1.6×10^6	4.9×10^3
40	7.4×10^5	2.0×10^7	9.5×10^5	2.1×10^4
90	2.3×10^5	4.5×10^6	6.1×10^4	3.8×10^4
180	2.1×10^6	4.0×10^6	3.9×10^5	5.6×10^4
Wet pasta (cells per g)	1.1×10^7	8.0×10^4	9.3×10^5	2.0×10^4

[a]Average number of cells per g from triplicate samples.
[b]High (H) and low (L) level contaminated product.

TABLE 3. *Survival of salmonellae in stored pasta*[a].

Storage time (days)	*S. infantis* (cells per 100 g)				*S. typhimurium* (cells per 100 g)			
	Pasta		Egg pasta		Pasta		Egg pasta	
	H[b]	L[b]	H	L	H	L	H	L
0	430	1.5	91.0	9.3	930	24.0	230	0.4
5	430	4.3	93.0	4.3	230	4.3	930	1.5
10	430	9.3	9.1	0.9	230	1.5	230	0.7
20	230	1.5	46.0	2.3	230	4.3	230	0.7
40	430	15.0	21.0	1.5	230	0.9	230	0.4
90	150	4.3	9.3	0.9	91	2.3	91	0.9
180	460	2.3	4.3	0.4	9.1	0.4	240	0.4
360	23	1.5	0.9	0.4	0.4	< 0.3	1.5	< 0.3
Wet Pasta (cells per 100 g)	230	1.5	91	1.5	430	0.36	230	0.4

[a]Enumerated by the 3-tube MPN technique.
[b]High (H) and low (L) level contaminated product.

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Resuscitation of Stressed Fecal Coliforms and Their Subsequent Detection by Radiometric and Impedance Techniques

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ABSTRACT

Approximately 99% of cells in a heat-stressed *Escherichia coli* culture were injured and were able to grow on trypticase soy agar (TSA) but not on Violet Red Bile Agar (VRBA). Employing a surface-overlay or pour-overlay method, complete recovery of this thermally-stressed population required incubation of the TSA plates at 35 C for 6 h before overlaying with VRBA and incubating at 45 C. While the surface-overlay technique provides a more accurate index of injury than the pour-overlay method, there appears to be little difference in plating procedures with respect to recovery of injured cells. Heat-stressed cells were inactivated by the bile salts mixture in the conventional EC broth and by incubation temperatures of 42-45 C. Most of the heat-stressed cells were able to recover in 3 h at 35-37 C, without any evidence of replication, in EC broth minus the bile salts mixture (EC-B), adjusted to pH 6.1-6.5. Under similar conditions freeze-stressed cells recovered without multiplication within 1 h and the replication of an unstressed population of *E. coli* was evident in 2 h but not in 1 h of incubation. Both radiometry and impedance show promise as rapid (17-18 h) screening techniques for determining if a cooked food meets the microbial criterion of 0 fecal coliforms/g. A resuscitation period of 3 h was essential for reliable detection of thermally-stressed fecal coliforms by either radiometry or impedance. An impedance based-MPN procedure (18 h or less) compared favorably with a TSA/VRBA pour-overlay method (24 h) and a conventional 3-tube most probable number technique (48-72 h) for enumerating freeze-injured and uninjured fecal coliforms.

Rapid detection and enumeration of bacteria is of primary importance for monitoring sanitation during food processing and determining the microbiological quality of the finished product. The rapid detection of microbiological problems is essential for early corrective action. Conventional methods such as the Standard Plate Count and the Most Probable Number (MPN) technique for determining total aerobic mesophiles and fecal coliforms, respectively, are laborious and time consuming, requiring incubation times of 24 to 96 h. Radiometry or impedance may be used for rapid screening of various foods to determine if they meet a microbial criterion of $\leqslant 1.0 \times 10^5$ total aerobic mesophiles/g (*8,15*). The time required for analysis is 5-6 h whereas the conventional plate count requires 24-48 h.

To be of maximum value these rapid assays should be capable of determining if foods meet the various microbial criteria specified for indicator microorganisms. Such microbiological criteria are often established as one means of indicating that processed foods have been prepared under good sanitary conditions, proper time-temperature profiles and that the food is free of fecal contamination and the presence of enteric pathogens. Rapid screening tests for determining microbial acceptability of cooked foods should be less laborious and time consuming and at least as reliable as conventional methods.

The most sensitive of approved methods used for determining low numbers of fecal coliforms in foods is the MPN technique employing lauryl sulfate trypticase broth at 35 C and EC broth at 45.5 C (*3*). This laborious technique requires 48 to 96 h and the 95% confidence limits for the MPN may cover a 13-fold range (*19*). In addition, sodium lauryl sulfate in the initial broth medium may be inhibitory to the recovery of injured cells (*13*). Processed foods may contain viable, but physiologically-injured, cells (*1,11,14,17*). Under the proper environmental conditions, these cells have the potential to recover and eventually replicate in the food. Therefore, unless a method established to estimate indicator microorganisms includes a resuscitation period to allow recovery from physiological injury, the method is likely to fail to detect injured but viable cells. The pour-overlay plating techniques for enumeration of coliforms (*14,18*) and fecal coliforms (*12*) allow a resuscitation period and are effective methods for enumeration of freeze-injured and uninjured cells within a 24-h period. However, detection of small numbers of injured and uninjured fecal coliforms by radiometric and impedance techniques requires a broth medium for resuscitation.

All cooked, frozen foods prepared at Francis E. Warren Air Force Base, Cheyenne, WY for distribution to missile sites must meet specified microbiological criteria, i.e., aerobic plate count (APC) $\leqslant 10^5$; coliforms $\leqslant 10^2$; and fecal coliforms 0/g. At a facility such as Warren Air Force Base a large number and variety of cooked foods are tested to determine their microbial acceptability and only a few fail to meet the specified microbiological criteria. Of 8,125 production lots tested in 1976, only six failed to meet the established criteria. Five lots (two fried fish, two sliced ham and one fried

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chicken) were rejected because of the presence of fecal coliforms. The rejected fried chicken also had excessive coliform counts as did one lot of plain white cake. One lot of the rejected ham, positive for fecal coliforms, also had an APC $> 1.0 \times 10^5$/g whereas the other lot met both the criteria for APC and coliform counts.

The long range objective of this investigation was to study the feasibility of using radiometry and electrical impedance as rapid screening methods for monitoring the microbial acceptability of foods prepared at Warren Air Force Base. One phase of this study, reported here, was concerned with heat- and freeze-stressed fecal coliforms, optimum conditions for their recovery and the use of radiometry and/or impedance for their detection.

MATERIALS AND METHODS

Organisms

Escherichia coli ATCC strain 11775, was maintained (4 C) on trypticase soy agar (TSA, Difco Laboratories, Detroit, MI). Cultures for experiments were routinely obtained by inoculating cells from a slant culture into trypticase soy broth (TSB) and incubating at 35 C on a rotating shaker at 200 rpm for 18 h.

Stressed cells

Heat-stressed cultures were prepared by heating 18-h-old cultures, diluted 1:10 in sterile skim milk, at 57 C for 3 min in a water bath and immediately cooling in an ice-bath. Approximately 70% of the cells survived this treatment and about 99% of the survivors were injured-unable to form colonies on violet red bile agar (VRBA, Difco).

Freeze-stressed cells were prepared by freezing 5 ml of an 18-h-old culture in TSB at - 20 C for 7 days. Approximately 40 to 50% of the cells survived this freeze stress; ca. 99% of the survivors were injured.

Enumeration

Total fecal coliforms were enumerated by either surface plating on TSA or using the methods (surface-overlay or pour-overlay) developed for counting the number of freeze-injured and uninjured coliforms (*14,18*) and used by Powers and Latt (*12*) for enumerating fecal coliforms. In the pour-overlay technique 1 ml of sample was pour plated with a primary medium, TSA, incubated at 35 C for a minimum of 2 h to allow resuscitation, overlaid with a secondary medium, VRBA, allowed to solidify and incubated at 45 C for 24 h. In the surface-overlay technique 0.2 ml of sample was spread on TSA, incubated at 35 C, overlaid with VRBA and subsequently incubated at 45 C as with the pour-overlay method.

Detection of $^{14}CO_2$

Septum-stoppered 50-ml serum vials, containing an EC broth medium with a proprietary ^{14}C-labeled substrate (Johnston Laboratories, Inc., Cockeysville, MD) were inoculated and incubated at 45.5 C in a PRECISION Coliform Incubator/Bath. At specified times, the evolution of $^{14}CO_2$ was detected with the BACTEC Model 301 (Johnston Laboratories, Inc., Cockeysville, MD). A reading of $\geq 20\%$ (≥ 5.0 nCi) of full scale on the BACTEC instrument was considered as positive evidence for $^{14}CO_2$ production by fecal coliforms.

Detection of changes in electrical impedance

For detection of fecal coliforms by impedance, the sample and EC medium were placed in 40-ml vials with screw caps equipped with impedance measuring electrodes. Each vial and a reference vial (containing only sterile medium) were incubated in a 45.5-C water bath while housed in a bottle basket, linked electronically to the BACTOMATIC 32 Microbial Monitoring System (Bactomatic, Inc., Palo Alto, CA). The ratio of the impedance in the reference vial to the impedance of both the reference and experimental vial was automatically recorded on a strip chart. An accelerating impedance change of 0.8% or a full strip-chart channel width of change (*8*) was considered as positive evidence of the presence of fecal coliforms. The time of incubation at 45.5 C required to detect the accelerating impedance change was the detection time.

Standard curves

Fifty-gram samples of cooked frozen meat loaf, together with 1 ml of an 18-h-old TSB culture of *E. coli* and 199 ml of 1% peptone were blended at ambient temperature in a Waring Blendor at high speed for 2 min. Ten-fold dilutions of this meat loaf inoculated with *E. coli* were prepared by mixing 5 ml of the blended sample in 45 ml of 1% peptone containing 20% sterile meat loaf so that the amount of meat loaf in each sample would remain constant. Five ml of each serial dilution were delivered into BACTEC and BACTOMATIC vials with EC broth to yield 20 ml of EC broth with the normal concentration of the bile salts mixture. The vials were incubated at 45.5 C and the time to first detect $^{14}CO_2$ and the accelerating impedance change was recorded. Forty-three to 50 such samples were analyzed. The $\log_{10}$ of fecal coliform concentration in each serial dilution, as determined by the pour-overlay method and the detection times were subjected to linear regression analysis. This analysis provided estimates of the 95% confidence limits on the detection times and the curve of best fit when plotting $\log_{10}$ of fecal coliforms/g of sample against the time in hours to first detect $^{14}CO_2$ or an accelerating change in impedance.

RESULTS AND DISCUSSION

A variety of stresses encountered in the food industry may cause sub-lethal damage to bacterial cells (*1,10*). Some stresses may be more damaging than others. Conditions for resuscitation may, therefore, depend on the specific stress. In the surface-overlay and pour-overlay techniques recommended for recovery of freeze-injured coliforms (*14,18*) and fecal coliforms (*12*), a 1- to 2-h resuscitation period on TSA was adequate. With thermally-stressed *E. coli* cells surface plated on TSA an incubation period of 2 h at 35 C before overlaying with VRBA and incubation at 45 C did not allow maximum recovery (Fig. 1). In fact after 3 h, about 85% of the population was still injured and unable to form colonies when overlaid with VRBA. Due to the sensitivity of heat-stressed cells to VRBA and incubation at 45 C it was necessary to incubate the TSA plates at 35 C for 6 h before overlaying with VRBA or shifting to 45 C to obtain complete recovery. The resuscitation times required on an agar surface for maximum recovery of stressed cells will probably depend on the type and degree of cell injury.

Speck et al. (*18*) originally recommended a surface-overlay technique for recovery of freeze-stressed cells. More recently, Ray and Speck (*14*) found that the pour-overlay method had definite advantages over the surface-overlay when plating 1 ml or more of sample. As illustrated in Fig. 2, the incubation time required for the complete recovery of a thermally stressed *E. coli* population on the primary medium (TSA) at 35 C was about 6 h with either the surface-overlay or pour-overlay method. However, more injured cells resulting from thermal treatment are demonstrable by using the surface-overlay technique (Fig. 2). Ray and Speck showed that both 45 C and VRBA acted as secondary stresses. A medium tempered at 45 C lowered the recovery in VRBA but not in trypticase soy-yeast extract agar (TSYA). In the nonselective medium (TSYA), the cells were able to repair and form colonies. Recovery of heat-stressed *E. coli* on TSA incubated at 35 C was essentially the same whether using spread or pour plates

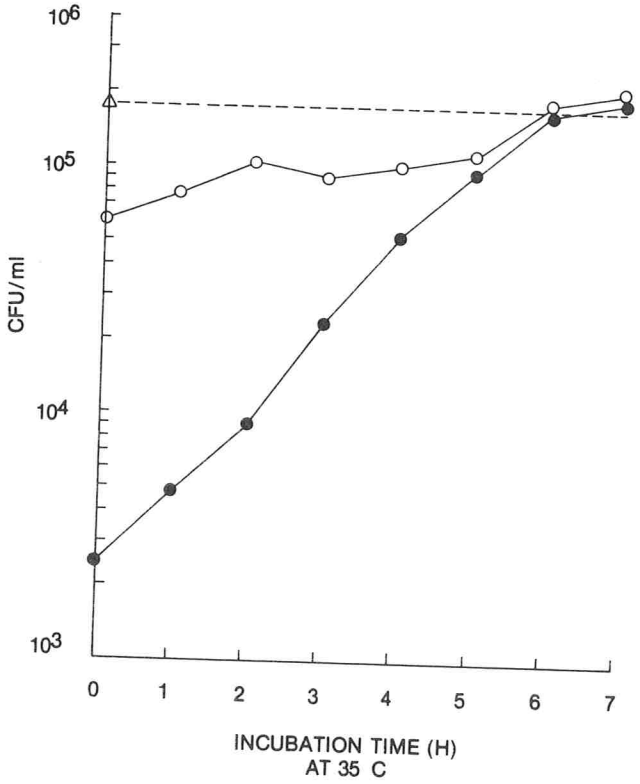

Figure 1. *Effect of incubation time at 35 C on repair of heat-stressed* E. coli *cells surface plated on agar medium. Symbols: Δ, cells plated with TSA and incubated at 35 C; 0, cells plated with TSA, incubated at 35 C for various time intervals and shifted to 45 C; ●, cells plated with the primary medium (TSA), incubated at 35 C for various time intervals, overlaid with the secondary medium (VRBA) and incubated at 45 C. Colonies were enumerated after incubation for 24 h and plotted as colony forming units (CFU) per ml of heat-stressed culture.*

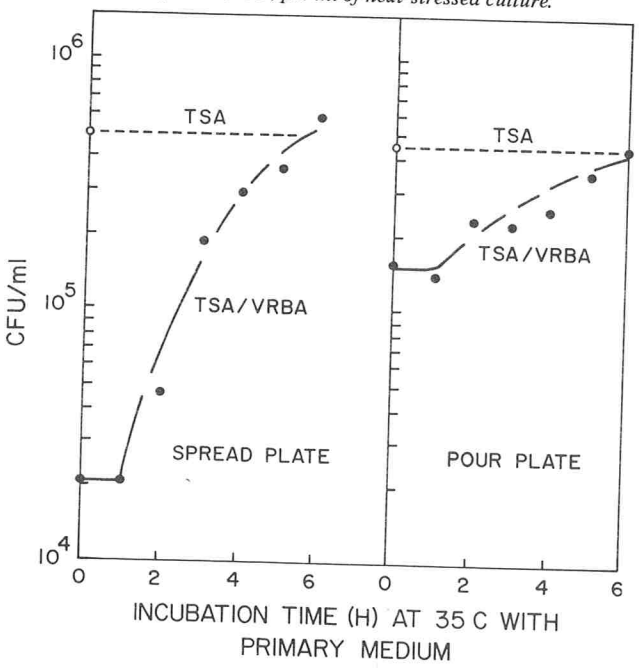

Figure 2. *Effect of plating method on the recovery of heat-stressed* E. coli *cells. Symbols: 0, cells plated with TSA and incubated at 35 C; ●, cells plated with the primary medium (TSA), incubated at 35 C for various time intervals, overlaid with the secondary medium (VRBA) and incubated at 35 C. Cell counts were made after incubation for 20-24 h.*

(Fig. 2). The short exposure of heat-stressed cells on plates to 45 C, i.e., the temperature at which the agar was poured, had little, if any effect on their recovery on the nonselective TSA. However, thermally-stressed cells spread on TSA were sensitive to longer exposure, i.e., during incubation at 45 C (Fig. 1). The fact that more cells were able to form colonies on TSA immediately overlaid with VRBA when using the pour-overlay technique as compared to the surface-overlay technique (Fig. 2) suggests that in the latter the stressed cells are in more direct contact with the selective VRBA and many are unable to repair. In the pour plate method many of the stressed cells would probably be protected from the VRBA and allowed to repair during the secondary incubation with a VRBA overlay.

In subsequent experiments, surface plating on TSA and VRBA was used to determine the number of survivors and uninjured *E. coli*, respectively. Total fecal coliform counts were made by the pour-overlay technique with a 6 h resuscitation period in a primary medium (TSA) at 35 C, followed by an overlay of the secondary medium (VRBA) and subsequent incubation at 45 C.

EC broth, as introduced by Hajna and Perry (*7*), has been successfully used at 45.5 C as a means of separating pure cultures of *E. coli* from other coliforms (*2*) and as a confirmatory medium for recovery of *E. coli* from frozen foods (*4*) and raw ground beef (*6*). Furthermore, gas formation at 45.5 C was best in EC broth when compared to lactose, maltose, sucrose, glucose, levulose and galactose broths (*2*). For rapid detection of fecal coliforms by radiometry and impedance, EC broth incubated at 45.5 C was employed.

Johnston Laboratories, Inc. produces an EC medium containing a proprietary ^{14}C-labeled substrate, which they recommend for the radiometric detection of fecal coliforms at 45.5 C. In tests to confirm the selectivity of this medium, nine coliform strains and one species of *Bacillus* were inoculated into this broth (final concentration, ca. 10^4 cells/ml) and the vials incubated at 45.5 C for 24 h. Only *E. coli* ATCC 11775 produced a positive radiometric reading (≥ 20) within the 24-h incubation (Table 1). Similarly although neither *Enterobacter aerogenes* nor *Streptococcus faecalis* inoculated into EC broth at final cell concentrations approaching 10^6/ml and incubated at 45.5 C for 24 h produced growth or electrical impedance changes, *E. coli* grew and produced a characteristic change in impedance (data not shown). Thus EC broth incubated at 45.5 C appears selective for detection of fecal coliforms by radiometry or impedance.

However, these determinations were made with unstressed cells and it is probable that, in the presence of EC broth containing bile salts at 45.5 C, stressed cells would not multiply and therefore, would not be detected by radiometry or impedance. The bile salts mixture used in VRBA prevented growth of *E. coli* cells damaged by exposure to quaternary ammonium compounds on

TABLE 1. *Comparison of $^{14}CO_2$ production in a radiometric EC broth by* E. coli *and other bacteria after 24-h incubation at 45.5 C*[a].

Organism	BACTEC reading at 24 h
Escherichia coli ATCC 11775	69
Enterobacter cloacae ATCC 13047	0
E. aerogenes ATCC 13048	18
Citrobacter freundii ATCC 8096	0
Salmonella typhimurium ATCC 13311	0
Pseudomonas aeruginosa	0
Proteus vulgaris	0
P. mirabilis	0
P. rettgeri	0
Bacillus sp.	0

[a]These data were kindly supplied by Johnston Laboratories, Inc., Cockeysville, Maryland 21030. Initial cell concentration, ca. 10^4 cells/ml of conventional EC broth with a proprietary ^{14}C-labeled substrate.

minimal agar (*16*). The bile salts in EC broth both interfered with the repair of heat-stressed *E. coli*, and reduced the number of viable cells (Fig. 3). In these experiments, heat-stressed *E. coli* cells were suspended in EC medium with (EC + B) and without (EC − B) the bile salts mixture and incubated at 37 C for 3 h. At hourly intervals, samples were withdrawn, serially diluted, surface plated and incubated at 35 C. Ninety-seven percent of the stressed population were injured and incapable of forming colonies on VRBA. During the 3-h incubation in the absence of bile salts, most of the injured cells recovered, since the counts on VRBA increased to approximate those on TSA. In contrast, when bile salts were included, the total viable cells on TSA decreased and there was very little recovery of stressed cells. In the bile salts mixture, there was a decrease in viable cells until the number of survivors approximated the uninjured population, suggesting that the thermally-injured cells were the target for inactivation by bile salts.

Thermally-stressed cells of *Staphylococcus aureus* are more sensitive to H_2O_2 than unstressed cells (*5*). Addition of catalase or sodium pyruvate, agents which enhance degradation of H_2O_2, increased the recovery of thermally-stressed cells of *S. aureus* when added to Vogel-Johnson agar (*9*). Addition of catalase did not decrease the selectivity of VJ agar (*5*). However, in our studies, addition of 0.5 to 1.5% sodium pyruvate (Sigma Chemical Co.) or 25 to 500 units of catalase (Nutritional Biochemical Co.) per ml of EC broth did not alter recovery of heat-stressed *E. coli* cells at 37 C. Thus, to allow resuscitation, the bile salts mixture must be omitted from the EC broth.

Thermally-stressed cells of *E. coli* were sensitive to incubation at 45 C. Exposure of thermally-stressed *E. coli* in a nonselective broth (EC-B) to 45 C resulted in a decrease in total viable cells and no measureable repair (Fig. 4). In 3 h, the total count on TSA had decreased and was similar to the uninjured population on VRBA. Incubation in a broth medium at 45 C for 1 h or more thus represents a lethal stress to heat injured *E. coli* cells. The optimum temperature for resuscitation of heat-stressed *E. coli* in EC-B broth (pH 6.9) was 37 C (Table 2). Recovery of heat-stressed *E. coli* cells was essentially the same at 35 and 37 C (data not shown). No replication occurred at any of the temperatures and only at 42 and 45 C was there a decrease in total viable *E. coli* cells during the 3-h test period.

The effect of pH on recovery of heat-stressed *E. coli* in EC-B broth at 37 C is illustrated in Fig. 5. Double

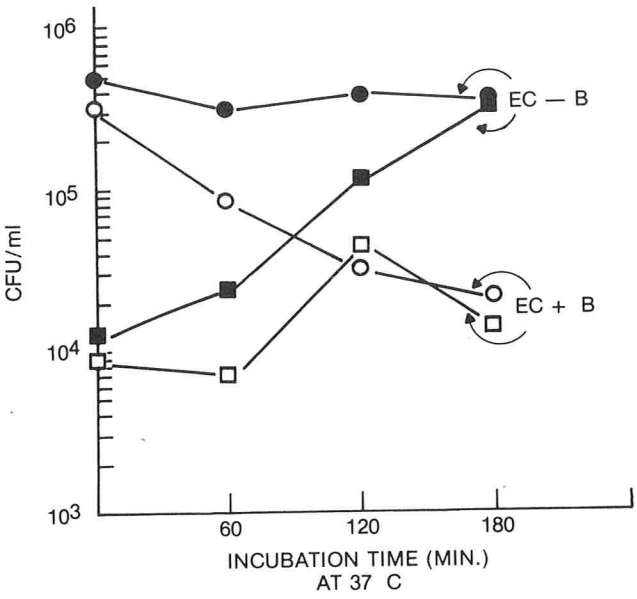

Figure 3. *Recovery of heat-stressed* E. coli *cells incubated in EC broth with (EC + B, open symbols) and without (EC − B, closed symbols) bile salts mixture. Heat-stressed* E. coli *were suspended in the appropriate broth and incubated at 37 C. At various time intervals, samples were withdrawn, diluted, surface plated on TSA (circles) and on VRBA (squares), for determination of total number of cells and number of uninjured cells, respectively, after 24 h incubation at 35 C.*

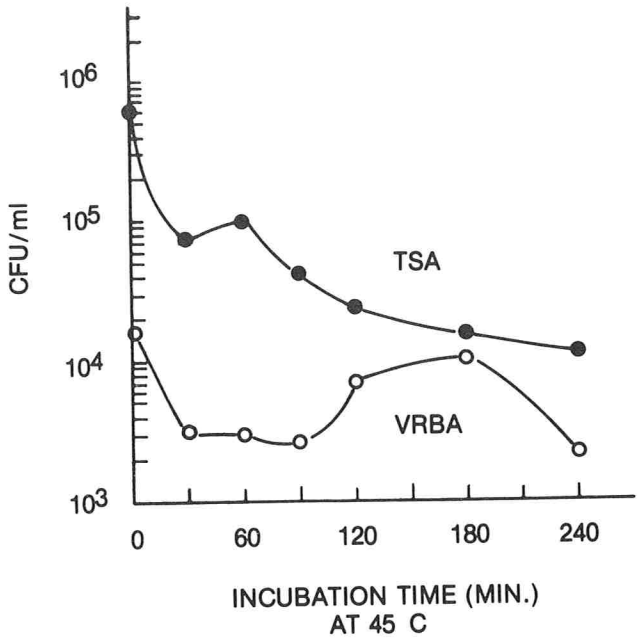

Figure 4. *Effect of incubation at 45 C on the recovery of heat-stressed* E. coli *cells. Cells of* E. coli *were suspended in EC − B broth and incubated at 45 C. At various time intervals samples were withdrawn, serially diluted, plated on TSA (●) and VRBA (0) and incubated as noted in Fig. 3.*

TABLE 2. *Effect of incubation temperature on the repair of heat-stressed* E. coli *in EC-B broth*[a].

Incubation temperature	Percent uninjured[b] at	
(C)	0 h	3 h
27	3.0	24
32	0.7	37
37	4.0	52
39	1.8	21
42	2.9	7
45	2.9	2

[a]Cells were suspended in EC broth without bile salts (EC-B) at pH 6.9 and incubated for 3 h at the indicated temperatures.
[b]Appropriate dilutions of samples, withdrawn at 0 and 3 h, were surface plated on TSA and VRBA and incubated at 35 C for 24 h. The percent of uninjured cells was determined as follows: (plate count on VRBA/plate count on TSA) × 100.

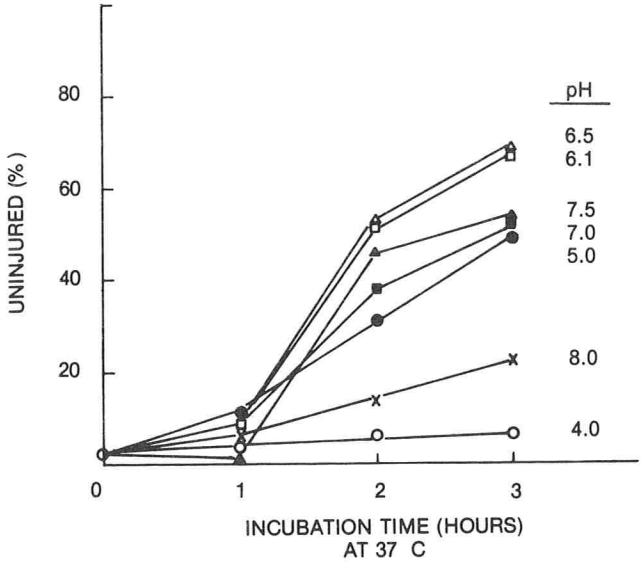

Figure 5. *Effect of pH on the repair of heat-stressed* E. coli *cells in EC – B broth.* E. coli *cells were suspended in EC – B broth, adjusted to indicated ph with 1N NaOH or HCl, and incubated at 37 C. At various time intervals, samples were withdrawn, serially diluted and surface plated on TSA and VRBA. Colonies were enumerated after 24 h of incubation at 35 C. The percent of uninjured cells was determined as follows: (plate count on VRBA/plate count on TSA) × 100.*

strength EC broth without the bile salts mixture was adjusted to the desired pH by addition of 1 N NaOH or HCl. The medium was then diluted to full volume with distilled H_2O and filter-sterilized. The percent of uninjured cells after 3 h of incubation at 37 C was the greatest in the pH range of 6.1 to 6.5, having increased from ca. 2% at 0 time to 69% at 3 h.

When using the surface-overlay plating technique for recovery of heat-stressed *E. coli*, only ca. 12 to 15% of the total population was uninjured after a 3-h incubation period at 35 C and complete recovery was obtained only after a 6-h incubation period at 35 C (Fig. 1). More complete recovery of heat-stressed pure cultures in broth could also be obtained by incubating for longer than 3 h. However, unstressed cells started to multiply within 2 h (data not shown). Therefore, to avoid the possibility of interference with the detection of low numbers of fecal coliforms by multiplication of unstressed coliforms, the resuscitation period was not extended beyond 3 h.

Freeze-stressed coliforms and fecal coliforms recovered in a 1- to 2-h resuscitation period (*12,14,18*) in a non-selective agar medium. Similarly, using EC-B broth, under our conditions of resuscitation (37 C, pH 6.1 - 6.5 3 h), most of the freeze-stressed cells of *E. coli* recovered within 1 h of incubation (Fig. 6), during which period, even an unstressed population of *E. coli* did not multiply.

Both the radiometric and impedance techniques have been used as rapid screening procedures with non-selective media for determining if various foods contain $\leq 1.0 \times 10^5$ aerobic mesophiles/g (*8,15*). The time to first detect labeled CO_2 or an accelerating change in impedance was inversely related to the concentration of bacteria in the food. Both methods should also apply to the rapid detection of fecal coliforms using selective media and conditions, e.g. EC medium and incubation at 45.5 C as recommended by Fishbein and Surkiewicz (*4*).

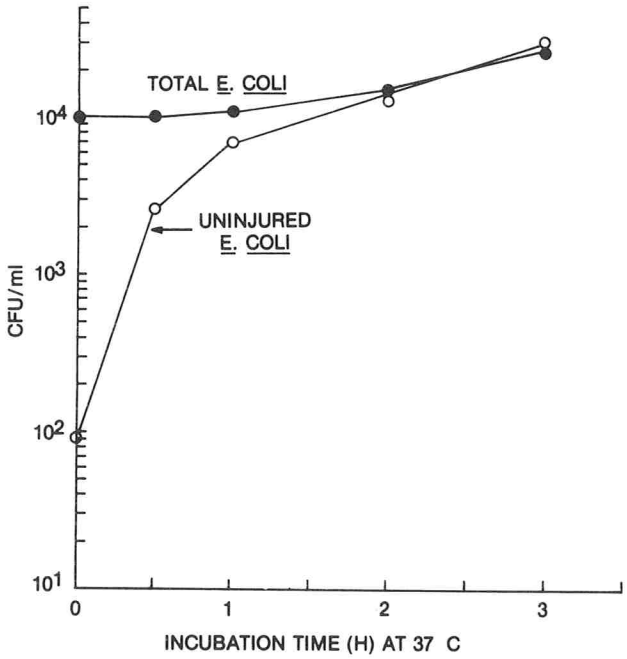

Figure 6. *Repair and growth of freeze-stressed* E. coli *cells in EC – B broth. Freeze-stressed cells were suspended in EC – B broth (pH 6.5) and incubated at 37 C. At indicated time intervals samples were withdrawn, diluted, surface plated on TSA (●) and VRBA (0) and incubated at 35 C for 24 h.*

Thus EC medium (with ^{14}C-labeled substrate for radiometry) and incubation at 45.5 C were used to prepare standard curves for cooked, frozen meat loaf inoculated with unstressed cells of *E. coli* to yield final concentrations ranging from 0 to 10^8/g. The $\log_{10}$ of fecal coliform count, as determined on VRBA spread plates, was plotted against the time in hours required for the first detection of labeled CO_2 or an accelerating change in impedance (Fig. 7). From the upper 95% confidence limits it can be seen that if no $^{14}CO_2$ or accelerating change in impedance has been detected by 14 and 15 h, respectively, one can conclude that the sample contains < 1 fecal coliform/g.

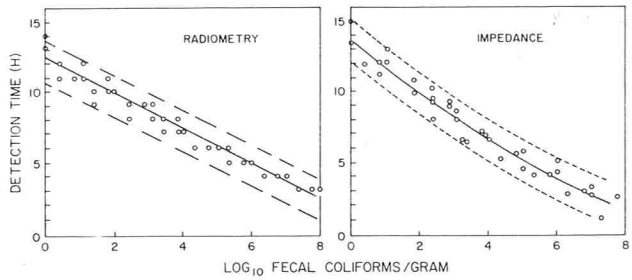

Figure 7. *Standard curves for the detection of fecal coliforms by radiometry and impedance. Detection time was the time when $^{14}CO_2$ or an accelerating change in impedance was first detected. See Materials and Methods.*

These curves do not take into account the possible presence of stressed fecal coliforms in foods. However, one should be able to detect injured as well as uninjured fecal coliforms if one uses the established optimal conditions previously outlined for resuscitation. The protocol established for the resuscitation of stressed fecal coliforms in cooked and frozen foods and their rapid detection by radiometry or impedance is as shown in Fig. 8. Following the resuscitation period, 10 ml of EC broth (pH 6.9) with double strength bile salts mixture (with ^{14}C-labeled substrate for radiometry) were added to the suspension, incubated at 45.5 C and monitored for fecal coliform growth by radiometry and impedance.

Homogenize in a Waring Blendor

50-g sample
200 ml 1.0% peptone

Add 5 ml (1.0 g) of homogenized sample to 5 ml double-strength EC – B broth; pH 6.5

Resuscitation (3 h at 37 C)

Add 10 ml of EC broth (pH 6.9) with double-strength bile salts mixture (with ^{14}C-labeled substrate for radiometry)

Incubate at 45.5 C and check for $^{14}CO_2$ or impedance change

Figure 8. *Protocol for the rapid detection of stressed fecal coliforms in cooked, frozen foods by radiometry or impedance.*

Table 3 illustrates the importance of resuscitation for detection of heat-stressed fecal coliforms in meat loaf by radiometry and impedance. A culture of *E. coli* was heat-stressed and inoculated into cooked meat loaf at various cell concentrations. Radiometric and impedance measurements were made of samples before and after 3 h of resuscitation. Although all unresuscitated samples contained *E. coli*, labeled CO_2 was detected by radiometry, within 14 h in only 2 of 8 samples. Therefore, 75% of the tests were false negative. However, following resuscitation, false negatives were eliminated. A similar situation exists with impedance and further emphasizes the essentiality of a resuscitation period for reliable detection of stressed fecal coliforms.

Both radiometry and impedance show promise as rapid screening techniques for determining if a cooked food meets the microbial criterion of 0 fecal coliforms/g. The test time should include a resuscitation period of 1 or 3 h to allow for recovery of cells stressed by freezing or heating, respectively. In the case of radiometry and impedance, one should be able to determine microbial acceptability within a maximum of 18 h. Since both methods may be automated, one may set up the test one day and have the results available the following morning. If a problem does exist, corrective action may be initiated without delay. The radiometric and impedance procedures as discussed are simple techniques for detecting injured and uninjured fecal coliforms. They are not laborious or time consuming and depending on the available instrument, 60 to 480 samples could be screened simultaneously.

Conventionally, one may quantitate fecal coliforms either by an MPN or by a pour-overlay technique, the latter yielding higher counts of fecal coliforms and more positive samples (*12*). The pour-overlay technique is a reliable method for enumerating freeze-injured and uninjured fecal coliforms within 24 h. We have shown that extension of the time of incubation at 35 C on TSA from 1-2 h to 6 h, makes the method applicable for enumeration of thermally-stressed fecal coliforms as well. We have also undertaken studies on rapid enumeration of freeze-stressed fecal coliforms by an MPN technique based on electrical impedance (Table 4). Meat loaf (50 g) was homogenized with 1 ml of a freeze-stressed *E. coli* culture and 199 ml of 1% peptone in a Waring Blendor. The blended samples were analyzed for fecal coliforms by: (*a*) pour-plating on VRBA, (*b*) pour-overlay using TSA followed by VRBA, (*c*) conventional 3-tube MPN with lauryl sulfate-tryptose

TABLE 3. *The rapid detection of heat-stressed fecal coliforms in meat loaf by radiometry and impedance*[a].

Method	Resuscitation time (h)	Test time[b] (h)	No. of samples tested	Fecal coliforms (+)	Fecal coliforms (–)	False neg. (%)
Radiometry	0	14	8	2	6	75
	3	17	8	8	0	0
Impedance	0	15	16	7	9	56
	3	18	8	8	0	0

[a]The total *E. coli* cells/g of meat loaf ranged from 79 to 3500 as estimated by the conventional three-tube MPN procedure. Ninety-three to 99% of these cells were injured (unable to form colonies on VRBA).
[b]Test time includes time of incubation in EC medium (to detect $^{14}CO_2$ or an accelerating change in impedance) plus time for resuscitation, where used.

TABLE 4. *Enumeration of freeze-stressed* E. coli *in meat loaf by impedance*[a].

Experiment	CFU/g		MPN/g	
	VRBA[b]	TSA/VRBA[c]	Conventional[d]	Impedance[e]
A	60	2.2×10^3	230	> 550
B	1	80	75	75
C	3	265	120	230
D	0.5	20	47	22
E	0.2	8	22	12

[a]Samples of cooked meat loaf were inoculated with different concentrations of freeze-stressed *E. coli* and analyzed as indicated.
[b]Pour plated on VRBA and incubated 24 h at 45 C.
[c]Pour plated on TSA, incubated 2 h at 35 C, then overlaid with VRBA and incubated 24 h at 45 C.
[d]Three-tube MPN method with lauryl sulfate-tryptose broth (incubated at 35 C for 48 h) as the primary broth and EC broth (incubated at 45.5 C for 48 h) as the secondary broth.
[e]Three-vial MPN method with EC-B broth (incubated 37 C for 3 h) as the primary broth and EC broth (incubated at 45.5 C for 18 h) as the secondary broth.

broth followed by EC broth and (*d*) 3-vial MPN impedance. In the latter case three serial dilutions were made of the homogenized meat loaf and triplicate 1-ml samples of each dilution were added to 10 ml of EC-B broth in 40-ml impedance vials with electrodes. The vials were then incubated at 37 C for 3 h to allow resuscitation. Following this incubation period 10 ml of EC broth containing double-strength bile salts mixture was added to each vial, the vials were incubated at 45.5 C and changes in impedance were monitored. As expected, fecal coliform counts were lower when cells were estimated by VRBA plates as compared to the TSA/VRBA pour-overlay method, the latter method permitting both injured and uninjured cells to form colonies. In this limited study, the impedance-based MPN method compared favorably with the conventional MPN technique and the TSA/VRBA procedure. The impedance MPN and TSA/VRBA methods may be completed within 24 h but the conventional MPN technique may require 48 to 72 h. One possible advantage of the impedance-based procedure is that the final results do not require colony counts. However, to determine reliability of the impedance-based MPN procedure, many food samples should be examined. Although this has not been tested, it should be possible to apply radiometry to an MPN procedure. In either case, the cost per assay as compared with conventional methods should be considered.

ACKNOWLEDGMENTS

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Rapid Enumeration and Identification of Stressed Fecal Coliforms

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ABSTRACT

A rapid (72 h) method for enumeration and subsequent identification of fecal coliforms is described. The procedure consists of plating a food suspension in an agar-repair medium (trypticase soy agar pour plates preincubated at 35 C for 2 h, then overlaid with violet red bile agar) incubated at 45.5 C. Fecal coliforms can be enumerated within 24 h and then confirmed as *Escherichia coli* within 48 h by subculturing typical colonies to an IMViC agar plate. Feasibility and applicability of the procedure were demonstrated by comparing the efficiency of recovery of both stressed and non-stressed cells on the agar repair medium with conventional agar and liquid most-probable-number media.

The inability of bacteria exposed to sublethal stresses to grow in selective media used for their enumeration has been reported (*1,4-9,11*). Various methods have been proposed, in recent years, to recover these injured (stressed) bacteria in both liquid and solid media (*1,4-9,11*). While these methods may have improved the sensitivity of detection, they generally did not reduce the time of analysis.

We conceived a rapid (72 h) test scheme for isolation and identification of *Escherichia coli* which was inspired by two recent developments in methods for recovery and identification of coliforms. The first development was an agar-repair method for enumeration of "total" coliforms at 35 C (*6*). By using this same method at 45.5 C, we proposed to selectively isolate and enumerate injured "fecal" coliforms within 24 h. The resulting colonies could then be confirmed as *E. coli* within 48 h on a newly devised IMViC agar plate (*3*). Completion of the test in only 72 h will reduce by as much as 7 days, the time required to identify *E. coli* by the conventional most-probable-number method (*7*). Methods previously proposed for enumeration of fecal coliforms at "elevated temperatures" (44.5 C) made no provision for recovery of injured cells (*2,10*) and they underestimated cell densities by as much as 20% (*10*).

Development of rapid analytical methods is a major area of research supported by the military because the delay of food shipments and storage of food pending analytical results is costly and causes numerous administrative and operational problems. The purpose of this study was to demonstrate the feasibility of the proposed rapid (72 h) test scheme and to compare the agar-repair medium (*6*) with conventional media commonly used for coliforms and *E. coli* in particular.

MATERIALS AND METHODS

Media

All media were purchased from Difco Laboratories, Detroit, MI. Media were autoclaved at 121 C for 15 min except violet red bile agar (VRBA) which was steamed at 100 C. The agar repair medium (VRBA/TSA) consisted of 10-12 ml of trypticase soy agar (TSA) overlaid, after an appropriate preincubation period, with an equal volume of VRBA as described by Speck and Ray (*6*). The IMViC agar plate was prepared as cited by Powers and Latt (*3*). IMViC agar media prepared in advance were distributed into test tubes in the proper volume and refrigerated no longer than 30 days. All dilutions were made in Butterfield's phosphate buffer (*7*).

Unstressed and stressed cells

Unstressed cells were cells grown in 5 ml of tryptic soy broth (TSB) and enumerated after incubation at 35 C for 24 h. Stressed cells were cells from the remainder (4 ml) of the same cultures which were immediately frozen at - 40 C for 24 h, thawed by placing the tubes in water at 3 C and subsequently enumerated in the same manner as the unstressed cells.

Differentiation of coliforms on VRBA at 45.5 C

Fifty-six coliform cultures were streaked on the surface of VRBA with a 3-mm bacteriological loop in a manner to obtain isolated colonies. Plates were then overlaid with 4-5 ml of tempered VRBA and incubated in an air incubator at 45.5 C for 48 h. Duplicate plates were also incubated at 35 C to serve as growth controls. Plates were examined for presence or absence of growth, color and size of colonies, and presence or absence of a halo around colonies. The coliform cultures tested were the following: 26 *E. coli* strains including three enteropathogenic *E. coli*; 12 *Enterobacter* strains including four *E. aerogenes,* five *E. cloacae* and three *E. agglomorans*; four *Citrobacter* strains including three *C. freundii* and one *C. diversus*; 14 *Klebsiella* strains, including 11 *K. pneumoniae,* two *K. ozaenae,* one *K. rhinoscleromatis.* All cultures streaked on VRBA were grown in trypticase soy broth at 35 C for 24 h.

Rapid (24 h) enumeration of stressed fecal coliforms

One ml of an appropriate cell suspension was pipetted into 100 × 15 mm petri plates, in triplicate, and poured with 10-12 ml of TSA. Following a preincubation period of 2 h at 35 C, the TSA was overlaid with 10-12 ml of VRBA as recommended for total coliforms (*6*). Selective enumeration of fecal coliforms was accomplished by reincubating the agar-repair plates (VRBA/TSA) directly at 45.5 C (air incubator) for 24 h. Typical colonies (red, greater than 0.5 mm and surrounded by a halo) were counted as fecal coliforms and were transferred to IMViC agar plates (*3*) to be identified as *E. coli*.

Forty-eight-hour IMViC agar plate test

Typical colonies from the agar-repair medium incubated at 45.5 C were transferred to IMViC agar plates as previously described (*3*). IMViC agar plates were incubated at 35 C for 48 h. Following addition of appropriate reagents *E. coli* was identified by the typical IMViC

pattern (+ + − −) produced. The methyl red test was performed first, followed by the Voges-Proskauer and the indole test.

Comparative recovery of E. coli on VRBA and the agar-repair medium (VRBA/TSA)

One ml of six replicate 24 h, TSB cultures of *E. coli* ATCC 11775, diluted to obtain between 100 and 300 colonies, was plated into six sets of triplicate petri plates. For each culture, two sets of plates were poured with 10-12 ml of VRBA and four sets were poured with 10-12 ml of TSA. As soon as the agar solidified, all VRBA plates were overlaid with an equal volume of VRBA. Then one set of VRBA plates and one set of TSA plates (not overlaid) were incubated directly at 35 C and 45.5 C. Two remaining sets of TSA plates (repair plates) were preincubated at 35 C for 2 h, then were overlaid with 10-12 ml of VRBA (*6*) and reincubated at 35 C and 45.5 C. At both temperatures TSA (not overlaid) served as growth control upon which colony counts were taken as 100% recovery. Incubation at both 35 and 45.5 C was accomplished in carefully controlled air incubators. The procedure was repeated with freeze stressed cells from the same cultures.

Comparison of the agar-repair medium at 45.5 C with the most-probable-number procedure

Recovery of cells from 24 *E. coli* strains on the agar-repair medium at 45.5 C (air incubator) as described above, was compared with recovery by the conventional 3-tube most-probable-number (MPN) estimate (*7*). Both media were inoculated in parallel with unstressed and freeze (- 40 C) stressed cells grown in TSB at 35 C for 24 h. In the MPN procedure, 1 ml of appropriately diluted cultures was first inoculated into three lauryl sulfate tryptose (LST) broth tubes per dilution and incubated at 35 C for 48 h. Dilutions were calculated to yield positive and negative end points. Gassing LST tubes were subcultured to EC medium with a bacteriological loop (3 mm) and incubated at 45.5 C for 48 h in a water bath. Percent recovery on the agar-repair medium was relative to the MPN estimate in EC medium taken as 100% recovery.

RESULTS

Table 1 shows the growth of 56 coliform cultures on VRBA at 45.5 C (air incubator). Of the 26 *E. coli* cultures tested, 22 grew and produced typical colonies (red, greater than 0.5 mm in diameter and surrounded by a "halo" of precipitated bile). None of the *Enterobacter* or *Citrobacter* cultures grew and only five of the 14 *Klebsiella* cultures grew. All five were strains of *K. pneumoniae* which produced red colonies, but only three strains produced colonies surrounded by the characteristic "halo" and only one strain produced colonies larger than 0.5 mm. The other four strains produced pin-point colonies which would not be counted as coliforms on VRBA. These results show that *E. coli* will grow on VRBA at 45.5 C while the other coliforms either do not grow or produce small atypical colonies, easily differentiated from *E. coli*.

Table 2 shows the percent recovery, after incubation for only 24 h, of unstressed and freeze stressed cells of *E. coli* ATCC 11775 on VRBA, TSA overlaid with VRBA (agar-repair medium) and TSA not overlaid. The percent recoveries shown are the average of six replicate cultures and are relative to the counts on TSA taken as 100% recovery. While recovery of unstressed cells on VRBA was 78 and 84% at 35 and 45.5 C, respectively, recovery of the same cells stressed by freezing at - 40 C was only 4.3 and 10.4% at these respective temperatures. However, recovery of both unstressed and stressed cells was greater than 100% on the agar-repair medium at 35 and 45.5 C. Growth on the agar-repair medium was typical of *E. coli* except that colonies were not as large as colonies on VRBA and halos surrounding colonies were not as strong, although they were clearly discernable.

TABLE 2. *Recovery of* E. coli *cells on agar media.*

	Percent recovery[a] of *E. coli* cells[b]			
	Unstressed		Stressed[c]	
Medium	35 C	45.5 C	35 C	45.5 C
VRBA	78	84	4.3	10.4
VRBA/TSA[d]	103	109	101	100.4
TSA	100	100	100	100

[a]Average of 6 replicates cultures relative to counts on TSA taken as 100%. Incubation time was 24 h.
[b]*E. coli* ATCC 11775.
[c]24-h TSB cultures were frozen at - 40 C for 24 h.
[d]Agar-repair medium: cultures were pour plated in TSA, preincubated at 35 C for 2 h, overlaid with VRBA and reincubated for 24 h at the indicated temperatures.

Incubation for 48 h did not alter the count or the appearance of colonies on the agar-repair medium (data not shown) and is not recommended because it dries the agar to the point of splitting. There was no discernible evidence of the agar drying after incubation for only 24 h at 45.5 C.

These data indicate that the agar-repair medium (VRBA/TSA) incubated as described, will effectively recover injured fecal coliforms at 45.5 C within 24 h. While *E. coli* did grow on VRBA alone, at 45.5 C, recovery was poor, especially when cells were stressed.

Tables 3 and 4 compare the growth and average recovery at 45.5 C of 24 strains of *E. coli* on the agar-repair medium and in conventional MPN media (LST broth incubated at 35 C followed by subculturing to EC broth incubated at 45.5 C in a water bath) tested in parallel. Table 3 shows that the average count ($\times 10^7$) of

TABLE 1. *Differentiation of coliforms on violet red bile agar (VRBA) at 45.5 C.*

		Reaction on VRBA			
		Number of cultures which gave			
Coliform organism	No. of cultures	Positive growth	Halo (bile reaction)	Red or pink colony	Colony sz. > 0.5 mm
E. coli	26	22/26 (85%)	21/22 (95%)	22/22 (100%)	22/22 (100%)
Enterobacter	12	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)
Citrobacter	4	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
Klebsiella	14	5/14 (36%)	3/5 (60%)	5/5 (100%)	1/5 (20%)

TABLE 3. *Comparison of an elevated temperature agar repair method with the 3-tube MPN procedure for recovery of unstressed* E. coli.

Results	Unstressed *E. coli* cells	
	Agar repair medium[a] at 45.5 C	EC Test at 45.5 C, 3 tube MPN
Average count per ml (× 10^7)[b]	114	88
Percent recovery[c]	129	100
No. positive cultures/total	23/24	14/24
No. giving higher counts/total	16/24	6/24

[a]TSA was preincubated at 35 C for 2 h, overlaid with VRBA and reincubated at 45.5 C for 24 h.
[b]Average of 24 *E. coli* cultures.
[c]Percent recovery relative to EC test MPN taken as 100%.

TABLE 4. *Comparison of an elevated temperature agar-repair method with the 3-tube MPN procedure for recovery of stressed*[a] E. coli.

Results	Stressed *E. coli* cells	
	Agar repair medium[b] at 45.5 C	EC Test at 45.5 C, 3-tube MPN
Average count[c] per ml (× 10^7)	24	10.1
Percent recovery[d]	238	100
No. positive cultures/total	23/24	12/24
No. giving higher counts/total	18/24	4/24

[a]A 24 h trypticase soy broth culture was frozen at - 40 C for 24 h.
[b]TSA was preincubated at 35 C for 2 h, overlaid with VRBA and reincubated at 45.5 C for 24 h.
[c]Average of 24 *E. coli* strains.
[d]Percent recovery relative to EC TEST MPN taken as 100%.

unstressed cells was 114 per ml on the agar-repair medium and only 88 per ml in MPN media. The average recovery on the agar-repair medium (VRBA/TSA) was 129% relative to the MPN count in EC medium at 45.5 C, taken as 100% recovery. Twenty-three of the 24 cultures grew on the agar-repair medium (VRBA/TSA), whereas only 14 grew in the MPN media. Eight cultures were actually inhibited by the LST medium at 35 C (data not shown). Comparison of the two media showed that 16 cultures gave higher counts on the agar-repair medium (VRBA/TSA) and only six gave a slightly higher count in MPN media.

Table 4 presents similar results with freeze-stressed cells of the same 24 *E. coli* cultures. The average count on the agar-repair medium (VRBA/TSA) at 45.5 C was more than twice as high as the MPN estimate at 45.5 C. This resulted in a 238% recovery on the agar-repair medium, relative to the MPN estimate taken as 100%. Although freezing had no effect on the number of positive cultures on the agar-repair medium, two additional cultures were inhibited in MPN media when stressed by freezing. Eighteen cultures gave higher counts on the agar-repair medium whereas only four gave higher counts in MPN media. Data shown in Tables 3 and 4 clearly demonstrate the superiority of the agar-repair medium over the conventional MPN procedure and point out some disadvantages of the MPN estimate.

The effect of freezing on *E. coli* is shown in Table 5. The average percent injury ranged from 91 to 96 depending on the incubation temperature. The slightly greater percent injury at 35 C was probably due to the

TABLE 5. *Effect of freezing (- 40 C) on* E. coli *cells*[a].

Replicate[a]	% Injury[b]		% Dead[c]	
E. coli culture	35-C Incubation	45.5-C Incubation	35-C Incubation	45.5-C Incubation
1	97.4	96.8	85.7	91.4
2	95.5	84.3	79.2	87.0
3	94.1	92.7	78.5	86.0
4	97.0	90.7	73.0	81.2
5	94.0	87.3	74.4	85.6
6	97.1	91.7	79.2	85.3
Average	96	91	78	86

[a]24-h trypticase soy broth cultures of *E. coli* ATCC 11775.
[b]% Injured = [1 − (VRBA count)/(TSA count)] × 100.
[c]% Dead = [1 − (TSA count after freezing)/(TSA count before freezing)] × 100.

fact that counts on VRBA did not differ much at 35 and 45.5 C while on TSA, on the other hand, counts were considerably reduced at 45.5 C compared to counts at 35 C. The average percentage of dead cells was greater at 45.5 C (86%) than at 35 C (78%).

DISCUSSION

Previous studies (*6,8*) have indicated that the agar-repair method could be effectively used at 35 C for enumeration of "total" coliforms from food samples in which the coliforms may be present in an injured state. This study showed that the same method can be used to selectively enumerate stressed "fecal coliforms" by incubating the agar-repair medium directly at 45.5 C, following an initial repair period of 2 h at 35 C. The fecal coliform count can be completed in only 24 h by this procedure. This reduces by 72 h, the time required to estimate the fecal coliform count by the MPN method. The superiority of this method over the MPN method for recovery of fecal coliforms was clearly demonstrated with both stressed (frozen) and unstressed *E. coli* strains.

Further and more rapid identification of the fecal coliform colonies enumerated on the agar-repair medium was made possible by the recent development of the IMViC agar plate (*3*). Figure 1, for example, shows how the agar-repair medium and the IMViC agar plate can be combined to form a 72-h test scheme for isolation and identification of *E. coli*. This scheme reduces, by as much as 7 days, the time required to identify *E. coli* in foods by the conventional MPN method. The considerable saving in time eliminates many of the administrative and operational problems presented by holding of foods pending the outcome of the lengthy, tedious and inaccurate MPN procedure. The scheme presented in Fig. 1 has the additional advantage of being simple to do and with media and equipment commonly found in the smallest of laboratories.

This study also showed that fecal coliforms could not be accurately enumerated on VRBA alone at elevated temperatures as suggested by other investigations (*2*), because of the poor recovery achieved, particularly when cells were stressed (Table 2). It was clearly evident from the data presented that stressed coliforms must be allowed a period to recover before being exposed to

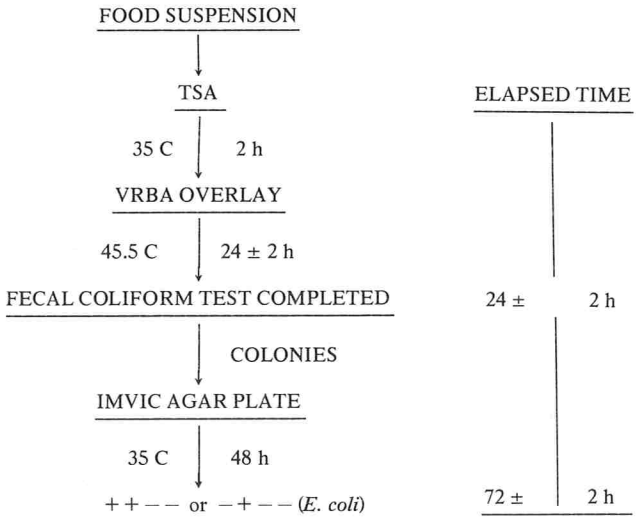

Figure 1. *Rapid (72 h) test scheme for isolation of* E. coli *from foods.*

VRBA. In so doing, however, care must be taken to prevent multiplication of cells before they are enumerated, which is a possibility when liquid media are used for repair (*6,8*). This disadvantage is avoided with solid repair media because colony forming units are immobilized.

The effect on microbiological standards, in the military and other regulatory agencies, of detecting injured organisms not previously detected with conventional methods has yet to be assessed and dealt with. Currently, our work involves evaluation of agar-repair media for recovery of "fecal coliforms" from naturally contaminated foods and improvement of media for differentiation of "fecal coliforms."

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Methods to Detect Stressed Microorganisms[1]

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ABSTRACT

A population of surviving bacteria, after a sublethal physical or chemical treatment, is composed of the stressed or injured and the uninjured or normal cells. Injured cells develop sensitivity to many chemical compounds due to damage to their permeability barriers in the cell wall and the cell membrane. They also fail to repair this damage in the presence of selective compounds and consequently fail to multiply. However, the cells can repair this damage relatively rapidly in a nutritionally adequate environment in the absence of selective agents. Repaired cells regain their resistance to the selective compound and can multiply in a selective environment. Many nonsterile or semipreserved foods are exposed to different kinds of sublethal treatments during processing, handling and storage and thus can contain indicator and pathogenic bacteria in the injured state. These organisms, because of their resistance to many selective compounds, are normally enumerated and isolated with selective media. The injured cells, due to their developed sensitivity will not be detected in these media. However, repaired cells can be detected in these media. Based on these principles two methods, designated as 'liquid-repair' and 'solid-repair' methods, have been developed in our laboratory. The liquid-repair method is effective for enumeration by the MPN technique and isolation of pathogenic and indicator bacteria from different types of semipreserved foods. The solid-repair method in principle can be used for direct enumeration of any organism which is usually enumerated by the selective plating procedure.

For a long time, microbiologists have suspected that death of microbial cells due to many physical and chemical treatments is a gradual process and if the process has not progressed too far it can be reversed in an appropriate environment. After exposure of microbial cells to low heat, irradiation and many chemicals, a certain percentage of the viable population becomes more exacting in its nutritional need (*42a,42b,63*). These cells, which require additional nutrients for their growth and multiplication, are regarded as stressed or nonlethally injured or simply injured. In 1959, Straka and Stokes (*86*) reported that freezing and thawing could inflict stress on some of the surviving bacteria cells. These cells temporarily lose their ability to multiply in a minimal medium, but not in a nutritionally complex or complete medium. To differentiate the metabolic state of the stressed cells from the unstressed surviving cells, they coined the term 'metabolic injury'. Subsequently many workers observed metabolic injury in many microbial species as a result of freezing and frozen storage (*7,57,62,65*). Several years later, other workers observed that cells of many microbial species can be stressed by different kinds of sublethal treatments, and the stressed cells temporarily lose their ability to multiply in selective media (containing one or more selective compounds) but not in nonselective complex or minimal media. Sensitivity of the stressed cells to selective media was later considered to be due to structural injury rather than metabolic injury (*69*). From the available information, we considered that these types of injury differ only in the degree of cell damage. All the injured cells have damage to their permeability barrier that renders them susceptible to many selective compounds (structural injury); some of them, in addition to this damage, have damage to the functional components that are related to their metabolic activities (metabolic injury). As long as the extent of the damage is not too great, the injury is reversible (nonlethal injury); when it goes beyond a certain level, the injury is nonreversible (lethal injury).

For about the last 15 years, many workers have been studying the molecular basis of nonlethal injury and the mechanisms of its repair. Different aspects of their findings have been intensively reviewed (*16,26,35,47,60, 69,76*). Based on the information obtained from the basic studies, several groups currently are working on development of methods to enumerate and isolate surviving microorganisms, including stressed cells, from products which have been subjected to sublethal treatments (*22,30,33,39,40,52,58,59,71,72,82,88,92*). It is anticipated that these methods will have important applications for enumeration of microorganisms from different nonsterile products. However, most of these studies have been conducted to improve the effectiveness

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of methods used for microbiological evaluation of foods. Recognizing the importance and implication of stressed microorganisms, especially the indicator and pathogenic bacteria in foods, a section has been included in the book *Compendium of Methods for the Microbiological Examination of Foods* published recently by the American Public Health Association (5).

The major objectives of this paper are to discuss the methods which have been developed in our laboratory for detection of index and pathogenic microorganisms from semipreserved foods, and to indicate their effectiveness, advantages and disadvantages. To make this more meaningful, I will discuss first the principles or basis used in the development of these methods.

PRINCIPLES USED IN DEVELOPMENT OF METHODS

Semipreserved foods, during processing, storage, transportation, and handling, are subjected to one or more types of physical and chemical sublethal treatments (Table 1). The major microbiological objectives of these treatments are to reduce the initial microbial load present in the ingredients and the products and to retard growth of the surviving population to increase the shelf-life of the finished products. It is becoming increasingly evident that among the surviving cells of bacteria, yeasts and molds, many remain in a stressed or injured condition. The numbers of stressed cells vary greatly with the nature of treatment, microbial species, composition and consistency of the foods, storage conditions and several other factors. Many species of indicator, pathogenic and spoilage bacteria, and yeasts and molds are known at present to be injured by sublethal treatments (Table 2). The major implication of microbial injury is that the stressed cells may not be detected by many of the conventional methods currently used for enumeration and isolation of various groups or species of microorganisms from foods. These methods, which are designed to detect normal cells, will detect only the uninjured survivors and not the total survivors (Fig. 1). These methods thus may have inadequacies for indicating the sanitary quality of foods as well as for insuring safety to consumers.

To overcome these problems, one needs to recognize two aspects of cell injury, viz., (*a*) how the injured and the uninjured cells differ in their characteristics, and (*b*) why the injured cells are not detected by many of the microbiological media. Many basic studies have been conducted to determine the characteristics that are

TABLE 1. *Different methods used in the processing and preservation of semipreserved foods.*

Physical
1. Low temperature: refrigeration, freezing
2. Heat (below sterilization): pasteurization
3. Drying (low moisture): air drying, freeze drying
4. High solids: sugar, salt
5. Radiation
Chemical
1. Acids: inorganic, organic
2. Preservatives
3. Sanitizers (equipment)

TABLE 2. *Microbial species known to suffer injury from sublethal stresses.*

Species	Group	Reference
Escherichia coli	Indicators	*3,6,12,13,19,45,47, 67,73,77,78*
Enterobacter aerogenes	Indicators	*65*
Streptococcus fecalis	Indicators	*10,20,28,64*
Salmonella sp.	Pathogenic	*21,25,29,66,67*
Shigella sp.	Pathogenic	*62*
Vibrio parahaemolyticus	Pathogenic	*11,15,42*
Staphylococcus aureus	Pathogenic	*8,17,37,46,49,56*
Clostridium perfringens	Pathogenic	*9,91*
Clostridium botulinum	Pathogenic	*1a*
Klebsiella aerogenes	Pathogenic	*18*
Pseudomonas sp.	Pathogenic	*7,36,41,51*
Streptococcus sp.	Associative	*57,61*
Lactobacillus sp.	Associative	*49a*
Bacillus subtilis	Associative	*55*
Saccharomyces sp.	Yeast and mold	*84*
Candida sp.	Yeast and mold	*80a*
Aspergillus sp.	Yeast and mold	*1*

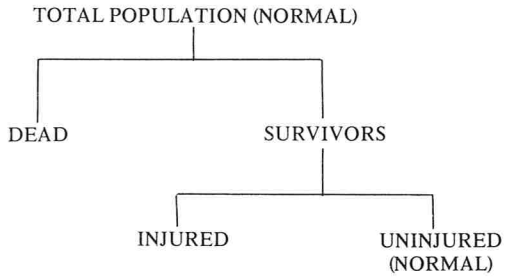

Figure 1. *Effect of sublethal treatments on microbial cells.*

different among injured and uninjured cells. Irrespective of the nature of the treatments and the microbial species, many characteristics have been found to be common among injured cells (Table 3). The injured cells lose their normal resistance and become sensitive to many chemicals; also these cells lose many small molecular cellular components to the environment. Both of these changes have been attributed to impaired permeability. In addition, these cells lose their ability to multiply; however, after repair in a suitable environment, they regain the ability to multiply. The injured cells have a longer lag period as compared to uninjured cells. These changes have been attributed to damage to many structural and functional macromolecular components (Table 4). Among these, damage to the surface structures (lipopolysaccharides, LPS, in gram-negative and pro-

TABLE 3. *Effect of sublethal treatments on characteristics of stressed cells.*

Characteristics	References
1. Increased sensitivity to:	
a. surface-active compounds	*47,60,67,70,79*
b. salt and toxic chemicals	*10,17,28,47,60,64,67-70,79*
c. antibiotics	*9,26,67-70*
d. dyes	*21,40*
e. acid and low pH	*21,56,69,73*
2. Loss of cellular materials	*47,60,67-70,76*
3. Longer lag	*47,60,67-70,76*
4. Inability to multiply (until repair)	*47,60,67-70,76*
5. Ability to repair injury in suitable environment	*47,60,67-70,76*

bably teiochoic acid in gram-positive bacteria) and the cytoplasmic membrane are reported to occur in most of the bacterial species given different treatments. Damage to these two structures, as manifested by impaired permeability and increased sensitivity to many compounds, probably plays the most important role in the failure to detect injured cells by many of the microbiological media. Probably once this damage is repaired and resistance to the selective compound is regained, cells would be able to repair their other damage in this environment. This will be evident if we recognize the ingredients which are used in the media that fail to support growth of injured cells (*4,5,31*). Basically, these media which are classified as selective media (both agar and broth), differ from the nonselective media by their content of one or more selective chemical compounds. These compounds, depending upon their properties, preferentially or selectively allow certain microbial species to grow and multiply. Cells of these species are relatively resistant to the compounds at a concentration used in the media. Depending upon their properties, these compounds can be divided arbitrarily into five groups (Table 5). Tolerance of or resistance of the organisms to these compounds is mainly because of the inability of these chemicals to enter the cells through the permeability barriers, i.e. these compounds are excluded from the cells in the resistant but not in the sensitive species. The surface structures (LPS in the cell wall of gram-negative and probably teichoic acid in gram-positive bacteria) and the cytoplasmic membrane both control their permeability in the normal cells (*26a,60*). However, when the cells are injured, their permeability barriers are impaired due to damage imposed by the treatments to the surface structures and the cytoplasmic membrane. Injured cells are no longer able to exclude the selective chemicals from entering the cells and the normally resistant cells become sensitive. Also because they cannot repair in the selective environment, the injured cells fail to multiply and thus remain undetected in the selective agar or broth media (*23,32,43,50,54,60,69,74,83,85*).

The injured cells, however, are able to repair and multiply in nonselective media. This characteristic, viz. ability of the injured cells to multiply in a nonselective but not in a selective medium, has been used to determine the percentages of injured and uninjured cells among the survivors after a sublethal treatment. Studies have indicated that the amount of injured cells varies greatly with the nature of the treatment, the microbial species, and the selective medium used. Data in Table 6 present evidence that, depending upon conditions, about 56 to 95% of the surviving population could be injured and thus could remain undetected when enumerated on a selective agar medium specific for the species. However, all the survivors formed colonies on the nonselective agar medium. To determine the number of injured cells which may remain undetected by several selective broths, the experiment was designed slightly differently. After sublethal treatment, survivors were enumerated by plating on a nonselective agar medium (Table 7). The cell suspension was then exposed to the selective broth for 5 min at room temperature; after necessary dilution, the cells were again enumerated on the same nonselective medium. Differences in counts before and after exposure to the selective broth were used as a measurement of injury. The results indicated that after only 5 min exposure in the selective broth, 25 to 85% of the survivors failed to be detected subsequently on the nonselective media. It also suggested that once exposed to the selective environment, the injured cells

TABLE 4. *Sites of damage in stressed cells due to sublethal treatments.*

Site	Reference
1. Surface structure	
a. outer membrane in gram-negative	*38,44,47,60,67-70*
b. teichoic acid in gram-positive	*45a,49a*
2. Cytoplasmic membrane	*2,11,19,47,48,49,60, 69,75,76,87*
3. Ribosomes	*2,21,36,47,60,76*
4. DNA	*6,14,34,47,60,63a,76,80,89*
5. Certain enzymes	*27,47,60,69,90*

TABLE 5. *Some selective compounds in the solid and liquid microbiological media.*

Surface active compounds
Bile salts, deoxycholate, lauryl sulfate, teepol
Salt and toxic chemicals
NaCl, LiCl, bismuth, selenite, iodine, azide, tellurite
Antibiotics
Cycloserine, polymyxin
Dye
Brilliant green, eosine
Acid
Tartaric acid, acetic acid

TABLE 6. *Inhibitory effect of several selective solid media in the enumeration of stressed bacterial cells.*

Organism	Treatment[a]	Selective media[b] (S)	% Undetected
Escherichia coli	Freezing	VRBA	90
	Heating	VRBA	56
	Acid treatment	VRBA	83
Salmonella sp.	Freezing	XLDA	90
	Freeze-drying	XLDA	95
Vibrio parahaemolyticus	Freezing	TCBS	80
	Refrigeration	TCBS	67
Staphylococcus aureus	Freezing	MSA	67
	Heating	MSA	59
Streptococcus fecalis	Freezing	KFA	88
	Heating	KFA	95

[a]Freezing at -78 C for 10 min; heating at 150 F for 15 min, acid treatment with lactic acid pH 4.0 for 10 min, refrigeration for 18 h.
[b]VRBA: violet red bile agar; XLDA: xylose lysine deoxycholate agar; TCBS: thiosulfate citrate bile salts agar; MSA: mannitol salt agar; KFA: KF agar. TSA: trypticase soy agar (with 3% NaCl for *V. parahaemolyticus*) was the nonselective medium (Ray, unpublished data).

$$\%\text{Undetected} = \left(1 - \frac{S}{NS}\right) \times 100$$

S and NS are counts on selective and nonselective media, respectively.

TABLE 7. *Inhibitory effect of several liquid media in the enumeration of stressed bacterial cells.*

Organisms	Stress	Selective[a] media	% Undetected
Escherichia coli	Freezing	BGLB	60
	Freezing	LSTB	55
Salmonella sp.	Freezing	TTB	85
	Freezing	SCB	25
Virbio parahaemolyticus	Freezing	GSTB	45

[a]Exposed to selective broth for 5 min and then enumerated on TSYA (trypticase soy yeast agar; 3% NaCl was added to it for *V. parahaemolyticus*). BGLB: brilliant green lactose bile broth; LSTB: lauryl sulfate tryptose broth; TTB: tetrathionate broth; SCB: Selenite cysteine broth; GSTB: glucose salt teepol broth. Cells were frozen - 78 C for 10 min.

$$\% \text{ Undetected} = \left(1 - \frac{\text{TSYA counts after 5 min in selective broth}}{\text{TSYA counts after freezing}}\right) \times 100$$

(Ray, unpublished data and Ray and Speck, Appl. Microbiol. 25:499-503; with permission)

lose their ability to multiply even in a nonselective environment. This could probably be due to cell death.

The injury due to sublethal treatments is reversible and can be repaired in a suitable environment such as a nonselective agar or broth medium. Repair of freeze-injury of *E. coli* cells in TSB is presented in Fig. 2. Initial difference in counts on TSA and VRBA is due to the injured cells as all the survivors formed colonies on TSA whereas only uninjured survivors formed colonies on VRBA. During incubation in TSB, counts on TSA remained unchanged but counts on VRBA increased rapidly. This increase is due to repair; the injured cells repaired the damage in their permeability barriers (which resulted in their loss of resistance to VRBA) and regained their temporary loss of resistance. Repaired cells when plated on VRBA were able to multiply and form colonies. The shape of the repair curve probably

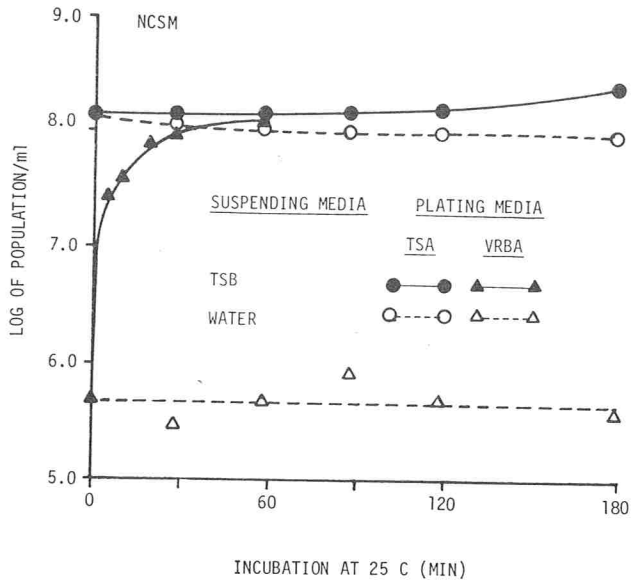

Figure 2. *Repair of freeze-injury of* E. coli *NCSM in trypticase soy broth (TSB).* E. coli *NCSM cells grown, harvested, frozen at - 20 C and thawed at 10 C. The cells were suspended in TSB and water, incubated at 25 C and at intervals surface plated on TSA and VRBA. The injured cells quickly repaired in TSB but not in water. Cell multiplication started after about 2 h (Appl. Microbiol. 26:919-924, with permission).*

indicates that the cells differed in the degree of damage to their permeability barriers. Cells with less injury repaired rapidly, while those with more injury repaired relatively slowly. Within about 90 min, most of the cells completed repair; after this period cells also started to multiply in TSB as indicated by simultaneous increase in counts on both plating media. Studies have indicated that nutritionally rich nonselective agar or broth media can support repair of this injury. Minimal media and salts of phosphoric acid (with or without Mg^{++}) have also been found to facilitate repair of frozen and freeze-dried stressed cells (67,68). Optimum repair appears to occur between pH 6 and 8, at 20 to 40 C in 1 to 3 h. The time dependency of the repair process seems to be related to the type of sublethal treatments; frozen cells seem to repair within 60 min while heated and freeze-dried cells need as long as 3 h (*47,60,69*).

The principles which should be considered in developing methods to detect stressed microorganisms can thus be summarized as follows: (*a*) the injured cells become temporarily susceptible to many selective compounds in the media; (*b*) this developed sensitivity is due to damage of the permeability barriers in the surface structures and the cytoplasmic membranes of the cells; (*c*) the injury is reversible and can be repaired in a nutritionally rich nonselective medium; repaired cells regain their resistance to the selective compounds and also their ability to multiply; (*d*) injured cells do not repair or multiply in the presence of the selective compounds; (*e*) injured cells could be enumerated or isolated in the selective media, if they are allowed to repair in a suitable environment before exposure to the selective environment; and (*f*) the surviving population constitutes both uninjured and injured cells. Injured cells differ in their sites of damage as well as in the degree of damage of the same site and thus have different time and temperature requirements for repair. Uninjured cells under the same conditions may start to multiply long before the injured cells and can increase the counts. The repair conditions thus should be such that the increase in counts in the selective enumeration procedures is only due to cell repair and not also to cell multiplication.

METHODS TO DETECT STRESSED MICROBIAL CELLS

After undergoing repair, the injured indicator, pathogenic or spoilage microorganisms are functionally as important as the uninjured or normal cells (*24,81*). Efforts should be directed to detect all the survivors to facilitate proper evaluation of the microbiological quality of semipreserved foods and other nonsterile products. Based on the principles discussed before, we have developed two methods, designated as 'liquid-repair' and 'solid-repair' methods and have tested them for enumeration and isolation of indicator and pathogenic bacteria from commercial foods. In this section, I will describe these methods and discuss their effectiveness and advantages and disadvantages for use to detect stressed cells in foods.

'Liquid-repair' methods

In this method (Fig. 3), a food sample is mixed or blended in TSB (or any nonselective broth) in a 1:10 ratio and incubated to facilitate repair (*69*). The time and temperature of incubation during the repair phase differ with the method of detection used subsequently. After repair, samples are used for selective culturing by the procedure suggested by the recommended methods (*5,31*). This repair method could be used efficiently for isolation of pathogenic bacteria and for enumeration of pathogenic and indicator bacteria by the most probable number (MPN) technique. This method, except for regulatory purposes, can also be used for direct enumeration of pathogenic and indicator bacteria by plating the sample from TSB on selective agar media. The major objection to its use for regulatory purposes is that the increase in counts on selective plating media could be due to not only repair of the

1. Samples are blended in a nonselective broth.
2. Incubated in the broth at optimum repair conditions (eg. 1 h at 25 C for freeze-stressed *E. coli*)
3. Then transferred to selective environment for their selective growth.
4. Advantages: a) for isolation
   b) for MPN-enumeration
   c) for plating
5. Disadvantages: May not be effective for regulatory purposes especially when enumeration is done by plating.

Figure. 3. *Steps in the 'liquid-repair' method for the detection of stressed microorganisms.*

injured cells but also due to multiplication of uninjured cells; especially, when the time of incubation is prolonged. In our experience, the increase in counts of coliforms on VRBA during 1 h of incubation in TSB at 25 C seemed to be due to repair of the injured cells and not due to cell multiplication (Fig. 4). Effectiveness of this method for enumeration of coliforms was also tested on semipreserved foods (Table 8). An increase in counts on VRBA after 60 min of repair in TSB at 25 C also suggested the presence of injured coliforms in these foods and their ability to repair in TSB. The counts by the MPN method also increased during this period which is especially true for products with lower initial counts.

For selective enumeration of coliforms by the 9-tube MPN method, we used to incubate the sample for 1 h at 25 C, then transfer 3 × 1 ml of sample from each of the three decimal dilutions to each of 9 ml of selective broth (lauryl sulfate tryptose broth, LSTB) and then follow the recommended procedure (*4,5,31*). However, to avoid any doubt about cell multiplication, we have changed this method slightly. Presently the sample is blended and diluted to three decimal dilutions in phosphate buffer or in 0.1% peptone broth. Immediately from each dilution, 3 × 1 ml materials are transferred to each of 4 ml TSB, and the tubes are incubated at 25 or 35 C for 1 to 2 h. Each tube is then inoculated with 5 ml double-strength LSTB. This is followed by the recommended procedure (*4,5,31*). This method is currently being used in our laboratory.

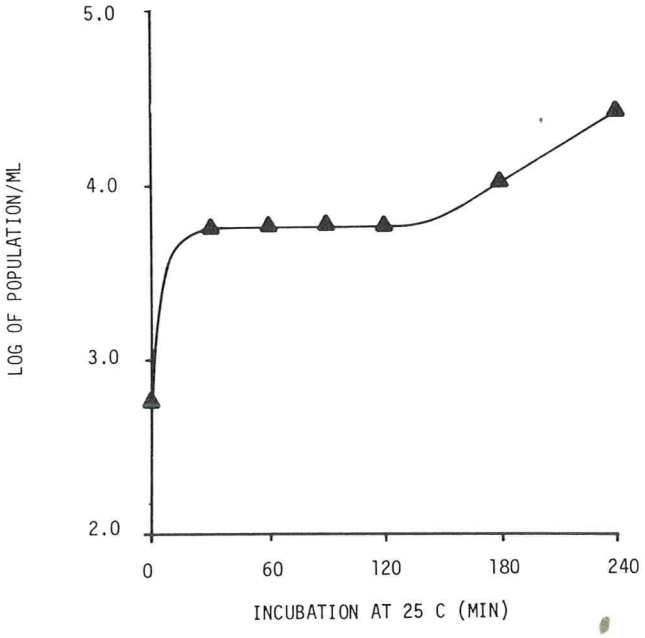

Figure 4. *Recovery of coliforms from a frozen deviled crab sample suspended in trypticase soy broth during incubation at 25 C. Frozen sample obtained from retail outlet was blended and suspended in TSB (1:10) by the method described in Figure 3. The sample was incubated at 25 C for 4 h, and at different intervals plated on VRBA. Rapid increase in counts after 30 min incubation was due to repair of injury. Cell multiplication did not start until after 2 h (Speck and Ray. 1973. Refrigeration Sci. Technol. Int. Inst. Refrigeration, Paris, BFRAAV 5:37-46).*

TABLE 8. *Enumeration of coliforms from commercial food before and after liquid-repair*[a].

	Counts/g			
	0 Min		1 h at 25 C	
Food	VRBA	MPN	VRBA	MPN
Deviled crab	500	> 1,100	5,100	> 1,100
Breaded oyster	700	1,100	5,800	> 1,100
Stuffed flounder	300	47	4,200	240
Ice cream	20	—	120	—

[a]The commercial samples were blended in 1:10 ratio in trypticase soy broth and enumerated by plating (VRBA:Pour) and by MPN (9 tubes in 3 dilution; 4, 5, 31) immediately and after 1 h incubation at 25 C (Ray, unpublished data).

The procedure of 'liquid-repair' was used in our laboratory for development of methods to isolate and enumerate *Vibrio parahaemolyticus* from seafoods (Fig. 5). For isolation, a 50-g sample is blended in 450 ml of TSB, incubated for 2 h at 35 C and then enough sterile NaCl solution (20%) is added to it to give a 3% final salt concentration. *V. parahaemolyticus*, although requiring 3% salt for optimum growth, appeared to be sensitive to it initially, probably because of injury. The material is incubated overnight at 35 C. Next morning, 10 ml of material from this preenrichment broth is transferred to 100 ml of selective enrichment broth (glucose-salt-teepol broth, GSTB) and incubated for about 6 h at 35 C. Studies with pure cultures have shown that *V. parahaemolyticus* cells in TSB + salt or in GSTB multiply rapidly and from a relatively low initial

1. Blend and then incubate sample in TSB for 2 h at 35 C.
2. Add sterile salt solution to 3% final concentration.
3. Incubate overnight at 35 C.
4. Transfer aliquot to GSTB in the morning (about 9 a.m.).
5. Incubate GSTB for 6 h at 35 C and streak on TCBS plates (about 3 p.m.).
6. Incubate TCBS plates at 35 C overnight and examine for colonies next morning.
(Total time 48 h)

Figure 5. *Liquid repair method used for the detection of* V. parahaemolyticus *from seafood.*

population (< 1000/ml) reach a stationary phase containing about 10^{8-9} cells/ml within 6 to 8 h; this is followed by a rapid decline in cell count (*72*). We expect that when a positive sample containing a very low population of *V. parahaemolyticus* is incubated overnight in TSB + salt, the population should attain > 1000 cells/ml when transferred to GSTB medium in a 1:10 ratio. These cells could reach the stationary phase of growth (> 10^8/ml) within 6 to 8 h. Because this timing will fit into the 8-h working day, the enrichment broth can be streaked on thiosulfate-citrate-bile-salt-sucrose agar (TCBS) after about 6 h of incubation. Plates are incubated at 35 C overnight and examined for characteristics colonies. The enrichment broths are further incubated overnight at 35 C, and if necessary are streaked on TCBS (especially the samples which are negative at 6 h). The additional preenrichment step did not increase the total time as compared to the recommended method due to the short enrichment step. For enumeration of *V. parahaemolyticus* by the MPN procedure, the blended sample (50 g in 450 ml TSB) is diluted to three decimal dilutions (with 0.5% saline). From each dilution 3 × 1 ml portions are transferred to each of 9 ml TSB. Other steps involving repair-incubation, addition of saline solution incubation during preenrichment and enrichment steps and streaking on TCBS are the same as in the solution method described above. Results of these studies are presented in Fig. 6 and Table 9. It can be seen that the repair method definitely is superior to the recommended method; also the 6-h enrichment step appeared to be better than the 24-h enrichment step. A similar short (6 h) incubation during enrichment is currently being studied in our laboratory for isolation of *Salmonella* from the semi-preserved foods.

'Solid-repair' method

The major drawback of the 'liquid-repair' method is in its application for enumeration of stressed organisms by the plating procedure. During incubation in broth, repair as well as cell multiplication can occur. The increase in counts due to cell multiplication is of concern especially when longer time and higher temperature are used during repair. Cell multiplication in the 'liquid-repair' method will not influence the MPN results. However, the MPN method is an indirect method and gives considerable variation in results. In addition, it is time-consuming (requires about 48 to 96 h) and

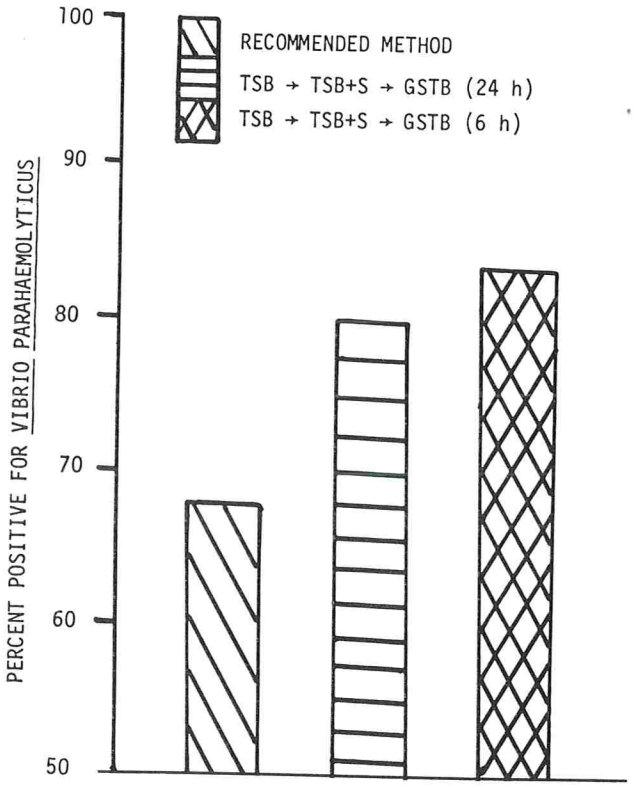

Figure 6. *Effect of three different procedures on the isolation of* V. parahaemolyticus *from naturally contaminated commercial seafoods. (Appl. Environ. Microbiol. 35:1121-1127, with permission)*

TABLE 9. *Enumeration of* V. parahaemolyticus *from seafoods by different methods.*

Food	MPN count/g by		
	Recommend method	Repair-detection 24 h GSTB	Repair-detection 6 h GSTB
Shrimp	150	210	> 1,100
Scallop	2	< 1	23
Trout	23	1	240
Clam	290	> 1,100	> 1,100
Whole crab	> 1,100	> 1,100	> 1,100

(Ray et al., 1978. Appl. Environ. Microbiol. 35:1121-1127, with permission).

uneconomical (in supplies and labor). An effective direct plating method is definitely preferred over the MPN method for enumeration of selective groups of organisms from foods. The 'solid-repair' method, developed in our laboratory, is just such a method (Fig. 7). In this method, the mixed or blended sample (with phosphate diluent) is pour-plated with 5 ml of TSA or PCA. Solidification of the medium quickly immobilizes the cells. The plates are then incubated to facilitate repair (e.g. 1 h at 25 C for coliforms). As the cells are immobilized, any cell multiplication during the repair phase will not influence the final count. At the end of repair phase, the plates are overlaid with 10 to 12 ml of the selective plating media (e.g. VRBA for coliforms), and after about 15 min are incubated for the desired time at a suitable temperature (e.g. at 35 C for up to 24 h for coliforms). During this time, the ingredients including the selective compounds from the selective

1. Blend the sample if necessary.
2. Transfer aliquot to plates (0.1 ml to 3.3 ml/plate).
3. Pour PCA or TSA about 5 ml/plate.
4. Incubate 1 h at room temperature.
5. Overlay with selective medium about 10-12 ml/plate.
6. Incubate at 35 C overnight and enumerate.

Advantages: a) Direct (so less variability).
b) Less time (24 h).
c) Economical (less supplies and labor).
Disadvantages: a) Variability at very low counts (< 10/g).
b) Some noncoliforms may form small colonies.

Figure 7. *Steps in the 'solid-repair' method for the enumeration of stressed microorganisms.*

medium will diffuse through the nonselective medium and create a selective environment throughout. As the cells have already been repaired, they are no longer sensitive to the selective environment and will be able to multiply and form colonies. Also because of the selectivity, only the specific groups of organisms (resistant to the environment) can form colonies and will be enumerated. The 'solid-repair' method, in principle, can be used for enumeration of any type of organism or group of organisms which are normally enumerated by a selective method.

We have studied extensively the effectiveness of this method for enumeration of coliforms in foods (*71,81*). We also have examined the influence of different parameters such as volumes of repair medium, time and temperature of repair-incubation and the volume and strength of the selective medium. Except the time and temperature of incubation during repair, none of the other factors had any influence. We consider that a food might contain coliforms which differ in their sensitivity to VRBA and also coliform cells with different degrees of damage. At least repair for 1 h at 25 C (preferably at 35 C) may be helpful to facilitate the repair process; especially, when only 5 ml of repair medium (TSA or PCA) is used. Instead, if the selective medium is poured soon after solidification of TSA or PCA, the selective component may diffuse rapidly through it and create a selective environment even before the sensitive cells could repair their injury. This 'solid-repair' method is very convenient, needs only 24 h, is economical and gives coliform counts directly. However, with certain types of foods, such as uncooked seafoods, hamburger, and vegetables, some noncoliforms also form red colonies. Generally these colonies are smaller and could be distinguished at 24 h from the typical coliforms with some practice. Incubation of representative colonies in brilliant green bile lactose broth (BGLB) could also be used to differentiate the coliforms from noncoliforms. Biochemical studies have indicated these noncoliforms to be *Enterobacter* (other than *E. aerogenes*), *Erwinia* and *Haffnia* species (*71*). Results in Table 10 indicate the effectiveness of this method for enumeration of coliforms from different foods. It may be seen that, depending upon the food, a large proportion of the surviving coliforms could remain in the injured state and the 'solid-repair' method (TSA/VRBA) is effective for their detection.

TABLE 10. *Enumeration of coliforms by the selective and 'solid-repair' methods.*

Food	Counts/g (or ml) on VRBA	Counts/g (or ml) on TSA-VRBA
Liquid milk	8	19
Buttermilk	24	42
Ice cream	60	240
Pimento cheese spread	900	3,000
Onion ring	380	4,000
Breaded shrimp	30	980
Cake mix	12	20
Bisquick	28	46

(Ray, unpublished data).

As indicated before, the 'solid-repair' method could be used in place of any conventional selective enumeration procedure. We have studied this method with appropriate modifications for enumeration of fecal coliforms and fecal streptococci. For enumeration of fecal coliforms, the samples were plated with VRBA and TSA/VRBA as before and the plates were incubated at 45 C in a water-jacketed air incubator up to 24 h (Table 11). Representative colonies were verified for their ability to form gas in EC broth at 44.5 C within 48 h.

TABLE 11. *Enumeration of fecal coliforms by the selective and 'solid-repair' methods.*

Sample	Counts/g by MPN	Counts/g by VRBA (45 C)	Counts/g by TSA-VRBA (45 C)[a]
Oyster (#1)	24	80	1200
Oyster (#2)	23	28	72
Clam	23	67	70
Shrimp	46	23	32
Scallop	9	19	26

[a]Colonies from plates were confirmed on EC broth. (Hackney, unpublished data)

Samples were also tested for fecal coliforms by the recommended MPN methods. None of the noncoliforms which grew on VRBA or TSA/VRBA at 35 C formed colonies at 45 C. Also, 100% of the colonies were confirmed in EC broth. Data presented here indicate that incubation of TSA/VRBA plates at an elevated temperature could be used for enumeration of fecal coliforms (also probably *E. coli*) in semipreserved foods. This currently is being studied in detail. Preliminary studies have also indicated that fecal streptococci could remain in an injured state in semipreserved foods, and that a direct plating procedure with TSA/KFA rather than KFA alone could be used for their enumeration (Table 12). Suitability of this method for enumeration of yeasts and molds, *Clostridium perfringens, Lactobacillus acidophilus* and other microorganisms needs further study.

CONCLUSIONS

The principle of using different kinds of selectively inhibitory compounds in broth and agar media for detection of pathogenic and indicator bacteria is based

TABLE 12. *Enumeration of fecal streptococci by the selective and solid-repair' methods.*

Samples	Counts/g by KFA	TSA-KFA
Scallop	10	35
Oyster	56	160
Clam	50	150
Shrimp	65	270
Crabmeat	60	80

Representative colonies were biochemically confirmed. (Hackney, unpublished data)

on the relative resistance of the microorganisms to such agents. Use of media containing these compounds helps to obtain a particular group or species of organism from a sample which contains a variety of microorganisms. However, these media will not detect indicator and pathogenic bacteria if the cells are stressed or injured by sublethal physical or chemical treatments, and thus will fail to indicate the microbiological quality of a product. With slight modification, such as incorporation of an initial repair phase, the recommended procedures can be used for more effective microbiological monitoring of nonsterile products. Both the 'solid-repair' and the 'liquid-repair' methods described here include such a repair phase and have been found to be effective for detection of indicator and pathogenic bacteria in nonsterile or semipreserved foods.

Recent sociological changes in this country as well as in many other developed and developing countries have radically changed our food consumption patterns. To cope with these changes, many types of new foods, especially semipreserved foods, have been developed. Also, many of these foods are produced in large quantities at one time and then distributed nationally and internationally. Under these conditions, proper microbiological control of the ingredients and the products is necessary to determine their sanitary quality, to ensure safety to the consumer and to reduce spoilage of the products. However, the foods during processing, storage and transportation are subjected to one or more types of preservation treatments. These are not lethal to all microorganisms and some remain injured. For effective microbiological evaluation of these products, samples should be analyzed by methods which include an initial repair phase, such as the 'liquid-repair' or the 'solid-repair' methods described herein. It is anticipated that with suitable modifications these methods will also be helpful for effective microbiological quality control of many other nonsterile products, such as pharmaceutical and cosmetic preparations, air and environmental samples, and other biologic and pathologic samples.

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Coming Events *con't from p. 369*

Sept. 18-20--WESTPACK '79. Convention Center, Anaheim, CA. Contact: Clapp & Poliak, Inc., 245 Park Ave., New York, NY 10017.

Sept. 26-27--SOUTH DAKOTA STATE DAIRY CONVENTION. Downtown Holiday Inn, Sioux Falls, South Dakota 57100. Contact: Shirley W. Seas, Secretary, Dairy Science Department, South Dakota State University, Brookings, South Dakota 57007, 605-688-5420.

Oct. 10-17--K '79 INTERNATIONAL PLASTICS AND RUBBER EXPOSITION. Fairground, Dusseldorf, West Germany. Contact: German American Chamber of Commerce, 666 Fifth Avenue, New York, N.Y. 10019, 212-582-7788.

Nov. 3-6--1979 AMERICAN MEAT INSTITUTE CONVENTION. McCormick Place and The Conrad Hilton, Chicago. Contact: Judi Winslow, American Meat Institute, P.O. Box 3556, Washington, D.C. 20007, 703-841-2431.

Nov. 11-15--FOOD AND DAIRY EXPO '79. McCormick Place, Chicago, IL. Contact: Wes Dibbern, Dairy and Food Industries Supply Assoc., 5530 Wisconsin Ave., Suite 1050, Washington, D.C. 20015, 301-652-4420.

Nov. 20-23--FIE FOODPACK, International Food Industries Exhibition, Olympia, London, England. Contact: British Information Services, 845 Third Avenue, New York, NY 10022, 212-752-8400.

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Modification of Conventional Methods for Recovery of Injured Coliforms and Salmonellae [1]

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ABSTRACT

This is a review that concentrates primarily on recent developments in the modification of conventional methods for recovery of injured coliforms and salmonellae; 43 of the 58 references were published in 1975 or later. The review encompasses four areas of activity: (*a*) most probable numbers procedures, (*b*) membrane filter procedures, (*c*) direct plating methods, and (*d*) the influence of other variables.

The purpose and format of this review are outlined in the Abstract. Other aspects of bacterial injury and resuscitation, and their practical importance, have been the subject of a number of recent reviews (*9,17,23,29,47, 50,55*). Ray has summarized the current methodology (*41*), and only certain essential details will be repeated here. Emphasis in this report will be placed on what can be done about recovering injured *Enterobacteriaceae* without drastically altering normal laboratory routines.

MOST PROBABLE NUMBER [MPN] PROCEDURES

The MPN coliform test (*1,2,3*) is an example of a rather laborious and inaccurate method to estimate the numbers of coliforms in foods, waters, and wastewaters. A presumptive test is made by using a relatively nonselective medium, such as lactose broth or lauryl sulfate (LS) broth, followed by a confirmatory test in a more selective medium, such as brilliant green bile 2% (BGB) broth. The two-step procedure is necessary because injured cells in some samples cannot initiate growth when placed directly in the BGB broth.

Various attempts have been made to simplify this two-step procedure to save time and materials. One time-saving method was to use disposable wooden applicator sticks (*2,56*) or disposable plastic loops (Carlson Scientific Co., Matteson, Illinois) to make transfers from one tube to another. These devices eliminated the necessity of flaming and cooling a loop for every transfer. Another approach was inherent in a system (*40*) that is sold by the Hach Chemical Co. (P. O. Box 389, Loveland, Colorado). The Hach procedure utilized screw-capped vials of 10-ml capacity and containing dehydrated lactose or LS broth. After addition of a liquid sample, the medium within the vial was dissolved and the vial was incubated. If gas appeared in a Durham tube, the vial was inverted to moisten the cap, and the cap exchanged with the cap from a vial full of sterile BGB broth. Thus, the confirmatory tube was inoculated from the presumptive medium by carry-over of cells on the cap liner. If a test for fecal coliforms also was desired, the presumptive tube was shaken again, and the cap exchanged with one from a vial full of EC broth to inoculate it. These may seem like mundane improvements to an existing procedure, but they have resulted in savings of valuable technician time.

Another approach to simplification of the MPN coliform test was developed by Lanz and Hartman (*32*). The idea behind this procedure was to combine the presumptive and confirmed tests so that transfer of inocula would not be necessary; both time and materials would be saved. Capsules were made, containing brilliant green and bile. When a tube of lactose or preferably LS broth was inoculated, a timed-release capsule was added. The capsule was constructed so that dye and bile salts were gradually released during incubation; the non-selective presumptive broth became selective. This afforded time for injured cells to recuperate in a nonselective environment before multiplication in a selective environment. Both presumptive and confirmed tests were conducted in the same container, and the technician had no transfers to make. Conventional and timed-release capsule methods yielded equivalent results with most water and food samples (*32*). Concentrated food samples (10 ml of a 1:10 dilution of a solid food per tube) did not do satisfactorily with the timed-release method, however, because concentrated food samples settled to the bottom of the tubes and severely retarded diffusion of the selective ingredients. Sveum (*52*) has

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solved this problem (see the section on salmonellae). The need now is for a commercial supplier of timed-release capsules and collaborative studies on the method.

Timed-release capsule methods for detection of salmonellae in foods and feeds were described by Sveum and Hartman (*53*). The objective of these studies was to combine the preenrichment and selective enrichment phases (*25*) of *Salmonella* isolation. Capsules for either tetrathionate or selenite-cystine enrichment were added to a nonselective basal medium at the time that flasks were inoculated with samples. When perfected capsules were used, there were no significant differences between the conventional and the capsule methods in recovery of salmonellae from naturally contaminated, spray-dried egg white. Containers and transfer time were saved, but the total incubation time necessary before streaking plates was not reduced. In further studies (*52*), selective agents were added by pipette at 0, 4 and 6 h after the basal medium had been inoculated. The 4-h time period was most satisfactory when both percentage resuscitation of injured cells and development of 'background' growth were considered. About 75% of heat-injured and 50% of freeze-injured cells were recovered by using the delayed selectivity procedures, and the results were comparable to those of conventional, two-step procedures. A 24-h combined tetrathionate preenrichment-enrichment procedure was highly successful in effecting recovery of injured salmonellae in the presence of large numbers of *Escherichia coli*. Furthermore, the problem of sample density was overcome because the preenrichment broth could be mixed well at the time that the selective ingredients were added.

MEMBRANE FILTER PROCEDURES

Many of the earlier publications on coliform enumeration by membrane filter procedures have been reviewed by others (see *34,46*), and only a few of the key publications will be cited here. The method of McCarthy et al. (*37*) is noteworthy because it is one of the better methods for enumeration of debilitated coliforms. A pad was placed in the lid of a plate of Endo agar, and the pad was saturated with LS broth; a membrane filter containing cells removed from a sample was placed on the pad, and the plate was inverted and incubated at 35 C for 2 ± 0.5 h. The membrane was stripped from the pad, placed onto the surface of the Endo agar, and the plate was inverted again and incubated for 22 h at 35 C. Lin (*34*) recently used this technique to estimate total coliforms from chlorinated effluents; counts were about 1.5 times those obtained by using the conventional, one-step procedure and were "...comparable in coliform recovery efficiency to the MPN procedure" (*34*). In a variation of this technique (*6*), bacteria on membranes were allowed to repair for 2 h at 35 C by using Trypticase Soy-Yeast Extract broth before transfer of the membranes to plates of m-Endo MF agar. Coliform counts from raw sewage were more than double those obtained on membranes that were placed directly on m-Endo MF.

Dufour and Cabelli (*12*) described a unique, but rather complex, assay for the component genera of coliforms in seawater (see also *5*). A special selective-differential (mC) medium was developed. After incubation of membranes on mC medium for 22 ± 2 h at 35 C, presumptive identification to genus was effected by transferring the membranes containing colonies to one or more substrate or end product indicator plates (such as urease, oxidase, and indole tests). The method seems too involved for routine use, but it is amenable to a variety of research applications when presumptive identification to genus is necessary. Furthermore, coliforms stressed in seawater grew on mC medium (*5*), but injured coliforms present in sewage and fresh water apparently did not (*14*). Thus other selective-differential media might be necessary, depending upon the situation.

Rose et al. (*45*) proposed the simplest, and probably the best, membrane filter procedure for enumeration of stressed fecal coliforms (FC). A base layer of m-FC broth-agar mixture was poured into a 50-mm-dia petri dish. After solidification, the base layer could be stored at 10 C for up to 3 days. One hour or less before use, 2 ml of lactose broth containing 1.5% agar were placed over the base layer, a membrane filter containing an appropriate inoculum was placed on the lactose agar, and the plate was incubated at 35 C for 2 h. After the 2-h period for cellular repair to occur, the temperature was increased (over a period of 45 min or less) to 44.5 C, and colonies were counted after additional incubation for 22 to 24 h. The authors proposed that, "the procedure be evaluated as an alternative to the Standard Methods fecal coliform membrane filter test in the examination of chlorinated secondary effluents, marine waters, and any natural waters that may contain pollutants with heavy metal ions." Further studies (*18*) showed that a 5-h preincubation period at 35 C was superior to a 2-h preincubation; preincubation for over 5 h resulted in excessive background growth, spreading, and lower confirmatory rates. FC counts obtained by using the 5-h preincubation approached those obtained by using the MPN procedure when chlorinated effluents were examined (*8,18*). The MPN test required 2 to 4 days; the improved MF test required only 1 day (5 h at 35 C and 17 to 19 h at 44.5 C).

An alternative to the double-layer plate method (*45*) would be to use the procedure that McCarthy et al. (*37*) used for total coliforms: enrichment on one medium and transfer to another. Lin (*33*) studied many variables in two-step M-FC procedures. Phenol red lactose broth (PRLB) was better than M-Endo broth, F-FC broth, or EC medium for enrichment. Other nonselective media, such as Trypticase Soy-Yeast Extract broth probably would be equally satisfactory (*6*). Enrichment on PRLB at 35 C was superior to enrichment at 44.5 C (*33*). An enrichment time of 4 h at 35 C was optimum; shorter times resulted in lower counts, and longer times resulted in the appearance of atypical colonies that were not fecal coliforms. There were no statistically significant differ-

ences in the mean fecal coliform values as determined by the two-step membrane filter and the MPN procedures.

A procedure (*51*) more complex than those just described (*6,33,45*) seems to offer no additional advantages (*8,45*).

The ideal situation for recovery of injured salmonellae directly on membrane filters would be to combine the three steps of preenrichment (resuscitation), enrichment (selective multiplication and colony formation), and differentiation (colony type). This has not been accomplished. Most membrane-filter studies have been with water and wastewater because the majority of liquid or semiliquid foods of interest are not readily filterable (*30,39*). Sometimes a fiber-glass prefilter was used to increase flow rate or sample size. After filtration, the membrane (*26*), prefilter (*13*), or both (*15*) were placed into preenrichment or enrichment broths; subsequent manipulations were identical to conventional MPN procedures.

In one membrane filter test for salmonellae (*28*), the sample itself was preincubated, followed by filtration of the dilution-enrichment mixture through a membrane filter, which was then placed on a selective medium. A more recent, and possibly more productive method (*26*), consisted of filtration of three aliquots of water samples. One filter from each sample was placed into a tube of lactose, dulcitol, and inositol enrichment broth, preincubated for 3.5 to 6 h, and transferred to 35 C for about 20 more hours before transfer of 1 ml from each tube to a tube of selenite cystine broth. The selenite cystine broth was incubated for 24 h at 42.5 ± 0.5 C, and then plates were streaked for isolation. The use of tetrathionate broth, in addition to or instead of the selenite cystine broth, might result in even greater success of the method.

DIRECT-PLATING PROCEDURES

Two modified direct-plating procedures for enumeration of coliforms in foods and water were published simultaneously in 1975 (*21,48*). These two procedures differ in certain respects, and a comparison and critique is presented here to provide the basis for selection of variables that deserve further study. In the original method of Speck et al. (*48*), 0.5 ml of appropriate sample dilution was surface-plated on 12 ml of prepoured and dried (room temperature, 2 days; reference *43*) Trypticase Soy Agar (TSA). The plate was incubated for 1 or 2 h at 35 C, overlaid with 10 to 12 ml of Violet Red Bile (VRB) agar, and incubated for 24 h at 35 C (*48*). Modifications of the method (*43*) included alternative use of pour plates instead of spread-plates and Plate Count Agar (TGE) instead of TSA. More recently (*39,44*), only 5 ml of TSA or TGE were used as the base layer, and 10 to 12 ml of VRB agar served as the overlay.

The procedure devised by Hartman et al. (*21*) was similar to those described in the previous paragraph (*39,43,44,48*). Pour plates were made with about 10 ml of a medium that contained, per liter: 7 g of peptone, 3 g of yeast extract, 5 g of NaCl, 10 g of lactose, and 15 g of agar. The agar was allowed to solidify, and the base medium was overlaid with about 10 ml of sterile VRB-2 agar and incubated after the overlay had solidified. The original VRB-2 agar was Difco VRB agar with double the usual concentrations of Bile Salts No. 3, neutral red, and crystal violet; Difco now sells VRB-2 agar.

After reading the paper of Speck et al (*48*), we realized the wisdom of using for the base layer a culture medium (such as TSA) that was already available in most bacteriological analytical laboratories. So we replaced the special VRB agar basal medium with TGE agar. I will return to this subject later.

When compared with the standard VRB agar pour-plating procedure, yields obtained by using the new procedures averaged 100 to 154% for dairy products (*44*), 112 to 180% for dairy products (*21*), 518% for certain ice cream samples (*48*), 130% for frozen vegetables (*21*), and 217% for surface waters (*21*). We recently completed another study (Brown and Hartman, unpublished data): Counts on hamburger by using boiled VRB-2 agar and a base layer of TGE agar were more than double those obtained by using boiled VRB agar or an LST/BGB MPN assay. Whereas 94% of the positive colonies on boiled VRB agar were verified as coliforms, only 88% of positive colonies on VRB-2 agar were verified as coliforms. Thus, the resuscitation procedure could result in a few more false-positives from some samples; in this instance, the culprits were gram-negative, oxidase-positive rods. Surface-water samples also were examined (Brown and Hartman, unpublished data), and counts on VRB-2 agar and a base layer of TGE agar were about six times higher than those obtained by using boiled VRB agar or the LST/BGB MPN. Verification rates, however, were only 22%, compared with 49% obtained by using the LST/BGB procedure, 53% of typical colonies on membranes placed on m-Endo-MF agar, and 63% when pour plates of VRB agar were used. The VRB-2 agar detected substantially more lactose-negative *Aeromonas* spp. than were detected by using the other procedures, and this may be because of the glucose in the TGE basal medium. The VRB-2 agar procedure seems applicable to any sample, including chlorinated effluents, in which injured cells are present and a sample size of 3 ml or less would be adequate. When lactose-negative bacteria are a problem, a TSA basal medium should be used.

Ray and Speck (*44*) have compared a number of variables involved in the modified VRB agar procedures, but neither the abstract that they cite (*43*) nor my notes of their oral presentation contain these results. Hopefully, these data will be published. Everyone seems agreed that, for direct-plating, repair on a solid medium is better than repair on a liquid medium (*42,57*) because injured cells vary in the time required for repair. Multiplication of some cells may occur before other cells have recovered from injury (*48*). Another variable is the plating method: pour-plates vs. surface-plating. Some data indicate that the temperature of molten agar is detrimental to

optimum yields, and surface-plating avoids lethal effects of melted (45 C) agar on injured cells (*42,48,57*). But we were unable to show that yields were better by surface-plating than by pour-plating (*21*), and Ray and Speck (*44*) recently recommended a pour-overlay method because more sample could be added per plate. They evidently observed no differences between the surface-plating and pour-overlay methods (*44*). This seems logical because bacteria on the surface of a base layer of agar are exposed to heat when the overlay is applied. Another variable is composition of the base medium. Ray and Speck (*43,44*) reported no differences between TSA and TGE. We have noticed differences (Schleifer and Hartman, unpublished data). When Difco VRB-2 agar overlays were used, counts obtained from water samples on TGE agar averaged 46% greater than those obtained on TSA agar. Also, a proportion of the colonies growing in the TSA base were not as large or as darkly colored and readily countable as those formed in TGE agar. These phenomena may not have occurred if a different brand of VRB agar had been used, but I believe that the sugar (initially 0.1% glucose) in the TGE agar may be an advantage in obtaining appropriate colony size and characteristics. Because of the growth of lactose-negative bacteria in some samples when a TGE base was used (Brown and Hartman, unpublished data), I would now recommend an enrichment comprised of TSA agar with 0.5% added lactose. The volumes of base and overlay agars used also have been varied. We observed that equal proportions of base and VRB-2 agars yielded optimum results (*21*). Initially, Speck et al. (*43,48*) used equal volumes of base and single-strength VRB agars; they recently recommended (*44*) 5 ml of base agar and 10 ml of overlay. This method does not seem to be desirable. In the first place, regular VRB agar was used (*39,44*), and the selective ingredients and dyes were diluted by one-third to one-half. The end result was a medium of different composition and selectivity after equilibration than conventional VRB agar that has a long history of use. Furthermore, our experience was that positive colonies assumed weak coloration and some were atypical if the dye concentrations were substantially reduced; more false-negatives appeared (*21* and unpublished data). The use of a 5-ml base layer has further complications. Not only does the addition of a 3-ml sample excessively dilute the agar, but the base layer is so thin that diffusion of selective ingredients is accomplished within a shorter time than when a thicker base layer is poured. This may be the reason why Ray and Speck (*44*) found that a 1- or 2-h preincubation period at 35 C (2 h at 25 C) was needed to attain maximum counts. We did not observe that preincubation was necessary in a limited trial (*21*), and we are reinvestigating this question. Preincubation definitely was not necessary when surface water samples were assayed (Schleifer and Hartman, unpublished data); food samples are presently being investigated because injured cells in these may require extended resuscitation periods (*20*). Autoclave-sterilization of VRB agar should be mentioned before leaving the subject of direct-plating procedures. Although sterilization of VRB agar sometimes resulted in substantially lowered yields when the normal procedure was used (*1,19,20*), sterilization of VRB-2 agar in the autoclave (121 C, 10 min) reduced counts on hamburger and water samples by only 5 and 11%, respectively (Brown and Hartman, unpublished data). Thus, the double-layer procedure permits sterilization of the media with small sacrifice of productivity.

A collaborative study of the new direct-plating coliform procedures should include the following variables: (*a*) composition of base (enrichment) medium, (*b*) 5 vs. 10 ml of base medium, (*c*) single vs. double-strength overlay, (*d*) preincubation vs. no preincubation before the overlay is applied, (*e*) sterilized vs. boiled overlay, and (*f*) percentage verification attainable under different situations.

Additional studies also should be conducted on the application of modified VRB agar procedures for determination of fecal coliforms. Klein and Fung (*31*) proposed a fecal coliform test wherein plates of regular VRB agar were incubated at 44.5 ± 0.5 C. Use of VRB-2 agar, and possibly preincubation for several hours at 35 C, should increase yields of fecal coliforms greatly, and we intend to study this application.

INFLUENCE OF OTHER VARIABLES

Investigators have tended to expect repair of injury in rich, nutritious media supplemented with yeast extract or various metabolites. On the other hand, there are instances in which repair occurs best in a minimal medium (*4,58*). It is imperative, therefore, to test any resuscitation procedure under the conditions of expected use.

The possibility also exists that additives might be discovered that would permit repair of cells directly on or in specific selective-differential media. D'Aoust (*10*), for example, discovered that certain additives overcame the toxicity to *Salmonella typhimurium* of Levine eosin-methylene blue-salts medium. Catalase also seems to permit repair and subsequent multiplication of some injured cells under circumstances where repair normally would not occur. Martin et al. (*35*) demonstrated that catalase increased the counts of stressed *E. coli, S. typhimurium*, and other bacteria. Bissonnette and Savio (*7*) obtained large increases in counts of *E. coli* when catalase was added to selective or nonselective media used to recover the bacterium from water collected from streams containing acid mine drainage. Total counts of natural samples also were higher on TGE agar containing catalase than on TGE agar not containing the enzyme (*7*). Catalase may increase yields still further when added to the enrichment medium in double-layer recovery methods (Hartman, unpublished data).

Media containing dyes might be reduced in toxicity to injured *Enterobacteriaceae* by using certified pre-

parations of dyes that are pretested before use. Media containing bile salts might benefit by replacement of the bile salts with a pure grade of sodium dodecylsulfate (*38*).

The dilution water used to make dilution buffer also can be important. A simplified procedure to determine the suitability of water used to make dilution buffers has been proposed by Hausler et al. (*22*). For coliforms, a culture was diluted in the water and plated, dilutions were held at room temperature for an hour, and then were plated again; toxicity, especially of certain preparations of KH_2PO_4 (*24*), was readily detected. Heavy metal toxicity also can be a problem in making buffered water for dilutions. MacLeod et al. (*36*) suggested that Mg^{+2} should be added to overcome heavy metal toxicity, and it was the intent to include adequate Mg^{+2} in the buffer formulation listed in *Standard Methods* (*2*). An error appeared, however, on page 892 (*2*); the Mg^{+2} stock solution should contain 38 g of $MgCl_2$ (not 50 g of $MgSO_4 \cdot 7H_2O$) per liter of distilled water. A correction will appear in the next edition.

The type and brand of membrane filter also affects results that might be obtained from tests when filters are used (*54*). Sample handling, especially storage, can influence the results obtained in fecal coliform analyses (*49*) and other assays.

A few final words of caution are given here about methods used to determine fecal coliform/fecal streptococcus (FC/FS) ratios in surface waters. Except in aged samples, ratios greater than 4:1 usually indicate pollution from domestic wastes, and ratios of less than 0.6 suggest pollution from farm-animal wastes or from storm-water runoff (*16*). We have already mentioned how methodology can affect the coliform count, and Dutka and Tobin (*14*) have emphasized the fact that different media and methods were each selective for different genera of the *Enterobacteriaceae*. Obtaining appropriate fecal streptococcus counts is equally important. Kenner et al. (*27*) described a KF streptococcal medium, variations of which could be used for MPN, membrane filter, or agar plate count techniques. KF streptococcal media have come into wide use (*1,2,3*), but hardly anyone is using media made according to the original (*27*) formulation. The major media manufacturers have made subtle changes in the formulation, and these media might not perform in the same manner as the original formulation. Furthermore, we have obtained differences in streptococcal counts of more than one order of magnitude on certain samples, depending on which of two brands of KF media were used (Kobilka and Hartman, unpublished data). Use of a different streptococcal medium, such as that recently described by Donnelly and Hartman (*11*), also would alter the expected FC/FS ratio.

Despite the many variables in recovering injured coliforms and salmonellae, much progress has been made. Promising techniques have been developed, and these are amenable to routine use. Yet, as in all areas of food protection, the door is open to additional creative innovations as well as to refinement and verification of existing methods.

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Current Methods to Detect Stressed Staphylococci

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ABSTRACT

Many of the operations employed in food processing can sublethally damage staphylococci, temporarily rendering the cells incapable of growth on selective media commonly employed for their enumeration. Stressed staphylococci are, under the proper conditions, capable of recovery and subsequently normal growth, including enterotoxin production by some strains. Therefore, to ensure the safety of foods, alternative enumeration methods designed to include stressed cells should be employed. Several methods have been proposed which employ a period of preincubation in/on a non-selective medium (liquid or solid) to allow recovery before exposing the cells to selective agents. In addition, recent findings have indicated that the increased sensitivity of stressed staphylococci is at least partially due to hydrogen peroxide toxicity; based on these findings direct plating and most-probable-number techniques containing catalase to degrade peroxide have been proposed. Greatly improved enumeration of stressed cells is possible, employing these methods without the use of a preincubation period.

Staphylococcus aureus continues to be a major cause of food poisoning. Although large numbers of the organism (greater than 10^6 per gram of food) are usually required to produce sufficient amounts of enterotoxin to cause disease and many strains do not produce enterotoxin, the presence of this organism in food even at low levels is regarded as a potential health hazard. In addition, the presence of staphylococci in pre-cooked ready-to-eat foods is indicative of poor sanitation practices. Therefore, considerable attention has been given to detection and quantitation of staphylococci in foods.

Staphylococci are usually found in food at relatively low levels compared to the numbers of other bacteria. Therefore, isolation requires the use of selective media which allow staphylococcal growth but inhibit growth of most other bacteria. A number of selective and differential media, employing a variety of selective agents, have been proposed for enumeration of staphylococci in foods (*28*). Use of such media is based upon staphylococci being more resistant to the selective agents than competing organisms. Thus, by the choice of an appropriate concentration of selective agent(s), growth of competing organisms is suppressed while allowing growth and quantitation of staphylococci.

Most of the selective media proposed are suitable for enumeration of normal or unstressed cells. However, many of the processes and preservatives employed to increase the shelf life of foods are known to cause sublethal damage in staphylococci. One commonly reported manifestation of this damage is the loss of resistance to certain selective agents (*22,23,43*). Thus selective media designed under laboratory conditions where cells encounter little or no stress may not be suitable for enumeration of staphylococci from processed foods.

The importance of enumerating stressed cells has been expressed by a number of investigators (*5,10,31,43*). Several studies have shown that under the proper environmental conditions stressed cells recover (*11,14, 17,23*) and are subsequently capable of growth and enterotoxin production (*11,14*). Public health concern has, therefore, been directed toward the possibility that viable food pathogens might go undetected in processed foods.

This paper discusses the problems associated with enumeration of stressed staphylococci and reviews some of the proposed methods for their detection. We have attempted to point out general phenomena regarding enumeration of stressed staphylococci. However, because of the limited number of studies involving other stresses, much of this information is based upon the characteristics of heat-stressed cells only.

CHARACTERISTICS OF STRESSED STAPHYLOCOCCI

Many of the processes employed in the preservation of food, such as, heating (*23,25*), cold storage (*24*), freeze-drying (*14*), reduction of moisture content (*32*), acidification (*29*), and combinations of these have been shown to cause sublethal damage in staphylococci (*6,29*). Several sites of damage have been reported, the most common of which are the ribosome (*1,14,21*) and the cytoplasmic membrane (*21,40*). In addition, a reduction in the activity of certain enzymes (*8*) has been reported in response to heat stress. Frequently, damage incurred upon exposure of staphylococci to a particular stress alters the growth characteristics of the organism. Altered growth characteristics of stressed staphylococci which have been reported include: (*a*) an extended lag phase before initiation of growth (*23,25,40*); (*b*) increased nutritional requirements (*2*); (*c*) alteration of the range in temperature and pH allowing growth (*30,35*); and (*d*) an increased sensitivity to secondary stress (*43*). These alterations, particularly an increased sensitivity to

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secondary stress, may prevent stressed cells from being fully enumerated.

Many of the selective media commonly used for enumeration of staphylococci employ one or more of the following selective agents: NaCl, tellurite, and/or polymyxin (*28*). As a result of sublethal stress, staphylococci have been shown to be hypersensitive to each of these compounds, preventing optimal enumeration on media containing these agents. Other procedures employed in enumeration of bacteria from foods may also subject cells to secondary stress. Pawsey and Davies (*32*) have recently observed that *S. aureus* cells stressed by exposure to a reduced water environment were not enumerated when phosphate buffer was used as a diluent. This effect was apparently due to osmotic shock upon transferring the cells from a hypertonic suspension into the hypotonic diluent. Cells from the same stress environment were enumerated when a glycerol-water solution was the diluent. Others have shown that use of too warm agar may also represent a secondary stress for sublethally damaged cells (*33*).

Characteristics of stressed cells described above in conjunction with the secondary stress imposed by some commonly employed enumeration procedures may prevent optimal enumeration of stressed staphylococci. In view of the many foods subjected to treatments which may sublethally stress staphylococci, special consideration must be given to enumeration of stressed cells.

ENUMERATION OF STAPHYLOCOCCI

Present methodology

A large number of media have been developed for the selective enumeration of *S. aureus* in foods (*28*), most of which have been shown to be somewhat inhibitory to stressed cells (*5,13,17,38*). Baird-Parker and Davenport (*5*) found that some selective media commonly used for isolating *S. aureus* were inhibitory to cells following heating or drying. Stiles and Clark (*38*) examined the relative efficiency of 15 selective media and three non-selective media for enumerating heated and unheated strains of *S. aureus*. They reported that most of the selective media were somewhat inhibitory to stressed cells, i.e., enumeration on these media gave lower estimates of viable cells than did non-selective media. In a similar study, Gray et al. (*17*) found that the productivity of selective media for enumerating heat-stressed cells varied considerably. These data are presented in Fig. 1. The efficiency of five selective agars, one non-selective agar, and the most probable number procedure (MPN; *41*) employing both trypticase soy broth (TSB) and TSB containing 10% NaCl (TSBS), were compared. All media and the MPN methods were equally capable of enumerating normal cells (counts at zero min of heating). However, the media varied considerable in quantitating stressed cells. As the heating time progressed, variations in the media became more pronounced, reflecting increased sensitization to the various selective agents. Of the several media tested, the high salt media, TSBS, Trypticase soy agar containing

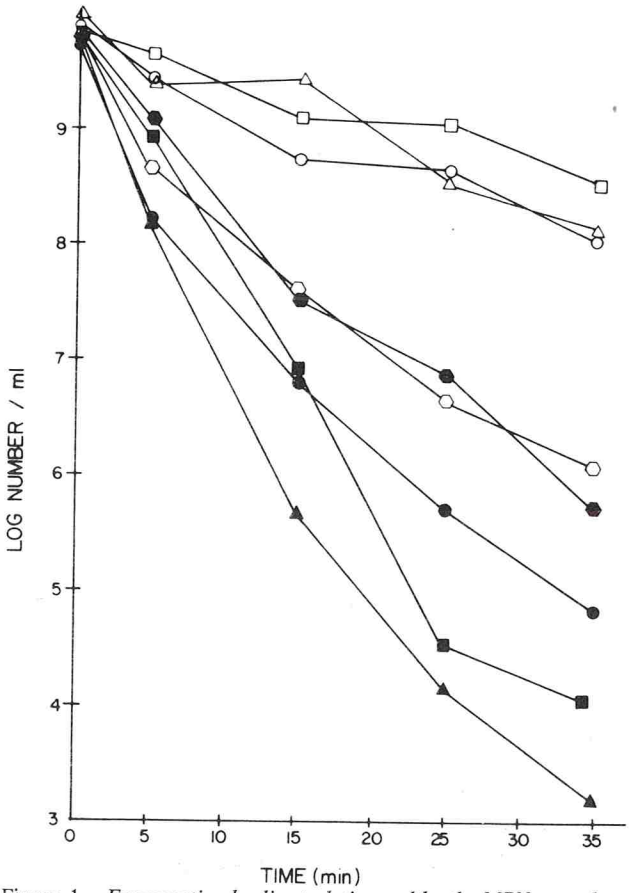

Figure 1. *Enumeration by direct plating and by the MPN procedure on thermally injured* S. aureus *MF 31. Cells were heated at 52 C in 100 mM phosphate buffer, pH 7.2 for 35 min and enumerated, at intervals, on:* □, *BP;* ▩, *Staph 100;* ○, *TSA;* ●, *TSAS;* ⬭, *TPEY;* ⬬, *VJ;* △, *MPN-TSB; and* ▲, *MPN-TSBS. All platings were done in triplicate; plates and MPN tubes were incubated at 37 C for 48 h. From Gray et al.* (17).

7.5% NaCl (TSAS) and Staphylococcus medium 110 (S110) were least productive. Vogel and Johnson agar (VJ, *42*) and Tellurite-polymyxin-egg yolk agar (TPEY, *12*) were less inhibitory than the high salt media but were also ineffective in comparison to Baird-Parker agar (BP, *4*) and the non-selective media, Trypticase Soy agar (TSA) and TSB. These data are consistent with those of other investigators (*5,13,38*). Stressed cells of *S. aureus* have been shown to be extremely sensitive to NaCl thus explaining the inefficiency of media which employ NaCl as a selective agent for enumerating stressed cells (*13,22,27*). In addition, tellurite, present in VJ, and polymyxin, contained in TPEY, have been shown to be somewhat toxic to stressed cells, although less so than NaCl at the concentrations normally employed (*22*). In general, most selective media, while suitable for enumeration of unstressed cells, have been shown to be toxic to stressed cells, giving lower estimates of viable cells than non-selective media. However, one exception exists. The egg yolk-tellurite-glycine-pyruvate agar (BP) of Baird-Parker (*4*) has been consistently shown to give equal or greater enumeration of stressed cells than non-selective media (*5,13,17,38*).

The MPN technique is generally considered more

efficient for enumeration of low numbers of organisms, or when high levels of competing organisms are present, than the direct plating method (*7,15*). The U.S. Department of Health, Education and Welfare (*41*) presently recommends an MPN procedure employing TSBS for enumeration of staphylococci in foods suspected to contain low numbers of cells (< 100/g). However, this medium has been shown to be inhibitory to stressed *S. aureus* (Fig. 1; *17*). Therefore, some alternative method of enumeration is suggested. Based on the studies described above, BP is the only medium of the many selective media suggested for the enumeration of *S. aureus* which has been demonstrated effective for enumeration of cells stressed by a variety of treatments. Use of BP is widespread throughout the United States and Europe, but the high cost of this medium makes it somewhat undesirable for routine quality surveillance. Other media are extensively used in the Soviet Union (Milk Salt agar, *34*) and West Germany (Kalium-Rhodanid-Actidone-Natriumazid-Eigelb-Pyruvate (KR-ANAP;*34*) but they have not been tested for their productivity in enumerating stressed cells.

Alternative methods

As a result of the inability of most selective media to adequately enumerate stressed staphylococci and the high cost of BP, a number of alternative enumeration methods designed to include stressed cells have been proposed. Current research has indicated that regardless of the type of stress imposed, (*a*) cellular damage is repaired when stressed cells are placed into the appropriate conditions, (*b*) the process of repair precedes cell multiplication, and (*c*) the repaired cell responds normally to selective agents (*31*). Based on this information, use of a preincubation period in a non-selective medium to allow repair of stressed cells before selective enumeration has been suggested (*17,20,31*).

Gray et al. (*17*) demonstrated the inability of several selective media to fully enumerate heat-stressed cells. However, after an appropriate preincubation period in TSB, a complete non-selective liquid medium, the productivity of all media was approximately equal (Fig. 2). This study clearly demonstrated the feasibility of using a preincubation step in the enumeration of staphylococci from processed foods. However, before employing such a procedure, the conditions (length, medium, temperature) must be carefully evaluated. The medium and temperature should be optimal for recovery, and the length of preincubation must allow full recovery of the stressed cells but not subsequent growth. Determining the length of preincubation may present a problem for the general use of this procedure. We have observed that the type and severity of the stress treatment can greatly affect the time required for recovery and subsequent growth (Flowers and Ordal, unpublished data). Thus a preincubation step of length suitable for recovery but not growth of cells from one sample, might be too short or too long resulting in incomplete recovery

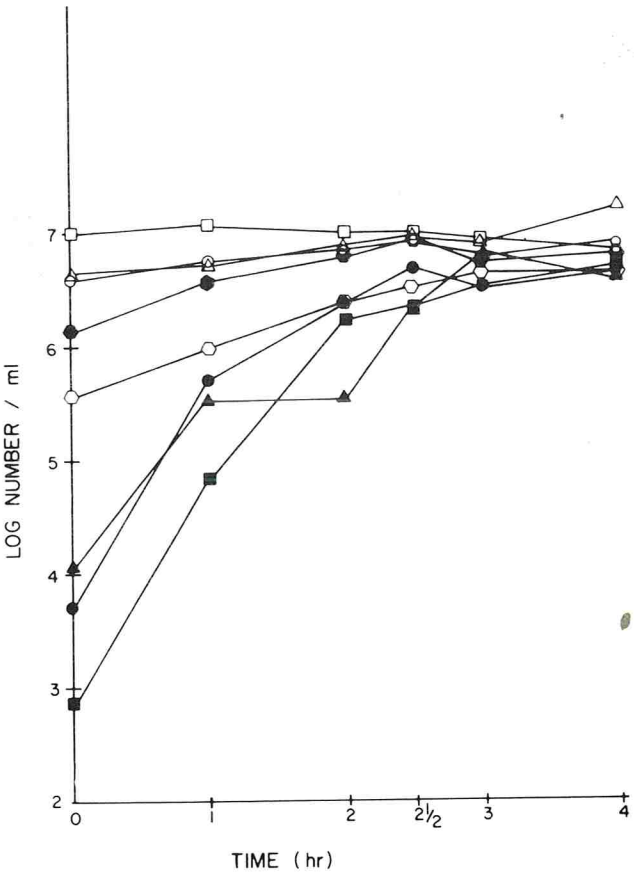

Figure 2. *Recovery of heat-injured* S. aureus *MF 31 in TSB. Cells were heated in 100 mM phosphate buffer, pH 7.2, at 52 C for 15 min. During recovery, samples were enumerated on:* □, *BP;* ■, *Staph 110;* ○, *TSA;* ●, *TSAS,* ⬭, *TPEY;* ⬬, *VJ;* △, *MPN-TSB; and* ▲, *MPN-TSBS. All platings were done in triplicate; plates and MPN tubes were incubated at 37 C for 48 h. From Gray et al.* (17).

or growth, respectively, of cells from another sample. A number of methods to alleviate this problem have been proposed. Hurst et al. (*22*) have suggested use of an inhibitor in the preincubation medium to prevent growth of recovered cells, thereby allowing the use of longer lengths of preincubation. They have shown that heat-stressed cells were capable of recovery in the presence of penicillin and that little or no cell death occurred during a preincubation period which allowed complete recovery. Of course, the use of penicillin in the preincubation medium necessitates the use of penicillinase in the selective enumeration medium. Use of these compounds, while improving the method have the disadvantage of increasing the cost. A second method which alleviates the exact determination of the length of preincubation was suggested by Goff et al. (*16*). These workers have used a membrane filter technique for recovery and enumeration of stressed bacteria. Cells from a dilution of milk were collected onto membrane filters (0.45 μ) and the filters preincubated on non-selective agar plates or filter pads soaked with a non-selective medium to allow repair of stressed cells. Since the cells were localized on the filter, some growth before formation of visible colonies can occur without loss of quantitation. The filters were then

transferred to the surface of a selective medium for enumeration. Theoretically, this procedure can be adopted for enumeration of any organism by the appropriate choice of media, temperature, atmosphere, and repair period. However, this technique has the disadvantage that it cannot be used for low dilutions of food products due to clogging of the filter by food particles. This problem may be eliminated by use of prefiltration of the suspended food particles before filtering through the membrane. However, this manipulation also may remove bacteria which are trapped in or closely associated with the food particles. A third method of preincubation has been used for enumeration of stressed coliforms (*19,36*). This technique has not been employed for enumeration of staphylococci but through the use of appropriate media and environmental conditions should be easily adapted. This method is performed by surface plating stressed cells onto a given amount of non-selective medium, preincubating the plates to allow recovery, and then overlaying with an equal amount of selective medium containing double the usual concentration of selective agents. This technique has the advantage that the length of preincubation can be extended, assuring complete recovery without the loss of quantitation.

NEW DEVELOPMENTS

Direct enumeration on a selective medium which is completely uninhibitory to stressed cells would be the most desirable method of enumeration, thereby eliminating the need for determining preincubation times, and the extra laboratory manipulations required by the above methods. Previous studies have shown that BP represents such a selective medium (*5,13,17,38*). However, as stated previously, the high cost of this medium warranted development of a less expensive but equally effective medium. The most expedient method to accomplish this task was to determine the component(s) in BP responsible for enhancing recovery of stressed cells. Baird-Parker and Davenport (*5*) suggested that this effect was due to the presence of 1% sodium pyruvate. They found that stressed cells were maximally enumerated on media containing pyruvate or blood. The common feature of these components is that they decompose hydrogen peroxide (H_2O_2). Catalase is present in blood and pyruvate reacts with H_2O_2 to form acetic acid and water (*39*).

Hydrogen peroxide is a strong oxidant with pronounced bactericidal effects. It is produced during respiration of most organisms by the action of superoxide dismutase and certain flavin-linked enzymes (*26*). In addition, presence of peroxides formed in media during preparation and storage has been reported (*18*). Thus the presence of peroxides either through respiration or those produced during preparation or storage of the medium, could be toxic to staphylococci, especially those in a debilitated state.

In agreement with Baird-Parker and Davenport (*5*), Martin et al. (*27*) found that addition of catalase to TSAS increased enumeration of heat stressed *S. aureus* by 14,000-fold. They further showed that addition of heat-inactivated catalase had little effect on enumeration. This finding confirmed that catalase activity, i.e., degradation of H_2O_2, was required for enhanced enumeration. It was suggested that the catalase activity of *S. aureus* was reduced by the stress treatment and that stressed cells therefore required an exogenous peroxide decomposer, such as catalase or pyruvate, for maximal enumeration. In support of this hypothesis it was shown that both heat and NaCl can reduce the catalase activity of a suspension of *S. aureus*, with the combination of heat and NaCl being most inhibitory. Flowers et al. (*13*) in a later study examined the effect of adding catalase to a number of selective media (Table 1; *13*). Catalase addition increased enumeration of both heat-stressed and unstressed cells on all media tested. However, the beneficial effects of catalase were most pronounced on those media least efficient in enumeration of stressed staphylococci, i.e., the high salt media, MSA, TSAS, and S110. Addition of catalase to these media resulted in increases in the enumeration of stressed cells of up to 1,000-fold. However, enumeration on these media was still somewhat less than on BP. Addition of catalase to VJ and TPEY, media not containing NaCl as a selective agent, increased the productivity of these media to a level equal to that seen on BP. These data suggested that the inability of stressed cells to form colonies on selective media was at least partially due to a decreased cellular catalase activity, and that the high salt media are most inhibitory because of the added inhibition of catalase by NaCl present in the medium. Flowers et al. (*13*) also found the requirement for an exogenous peroxide decomposer for maximal enumeration was not limited to heat-stressed cells. A similar enhancing effect of catalase was observed in freeze-dried cells and cells stressed by storage at reduced water activity (a_w) and as expected, the requirement of stressed cells for an exogenous peroxide decomposer was overcome during preincubation. These and previous findings (*5,13,27*) strongly implicated H_2O_2 as a factor in the failure of selective media to fully enumerate stressed staphylococci and demonstrated that addition of catalase or some other degrader of peroxides to these media markedly increased their productivity.

Improved enumeration procedures employing catalase

The MPN procedure currently recommended by the U.S. Department of Health, Education and Welfare for enumeration of staphylococci from foods suspected to contain less than 100 organisms per gram of food (*41*) has been shown to be inhibitory to stressed *S. aureus* cells (*9,13,17*). Flowers et al. (*13*) demonstrated that catalase addition to TSBS improved the enumeration of heat-stressed *S. aureus* cells. In a subsequent study, Brewer et al. (*9*) examined the effect of catalase or pyruvate addition on enumeration of *S. aureus* by this procedure. A number of laboratory strains of *S. aureus* were enumerated before and after heat stress

employing TSB and TSBS both with and without added catalase (200 U/ml) or pyruvate (1%). Addition of catalase or pyruvate increased enumeration of all heat-stressed strains tested. Enumeration of *S. aureus* from contaminated food samples also revealed that catalase addition had an enhancing effect. A number of contaminated egg noodle samples and low-temperature rendered ground-beef samples were tested. Catalase addition resulted in dramatic increases in the productivity of the TSBS-MPN procedure. Data obtained by TSBS-MPN plus catalase enumeration correlated well with data obtained by direct plating onto BP (*9*). It is evident from this and previous studies (*9,13,17*) that the recommended MPN procedure is ineffective for enumeration of stressed cells. The addition of catalase or pyruvate can greatly improve this procedure and is suggested, especially for enumeration of *S. aureus* from foods receiving treatments which might stress the organism.

Andrews and Martin (*3*) have employed the addition of catalase in development of an improved Vogel and Johnson agar (VJ). These workers also demonstrated an enhancing effect of phosphatidyl choline on the enumeration of stressed cells. In addition to the compounds found in VJ, the modified medium contained beef extract, deoxyribonucleic acid, phosphatidyl choline and catalase, spread onto the solidified medium before surface plating the samples. Vogel and Johnson agar, the modified VJ (PCVJ), and BP were compared for their productivity in enumeration of several laboratory strains and clinical isolates of *S. aureus*, before and after heat stress. These comparisons indicated that enumeration on PCVJ was statistically equal to or greater than that on BP. Furthermore, enumeration of *S. aureus* from naturally contaminated food samples was similar on the two media, PCVJ and BP. The findings of this study warrant further investigation of PCVJ as an alternative direct plating medium for the enumeration of *S. aureus* from foods.

CONCLUSIONS

In this paper we have discussed some of the problems associated with enumeration of stressed staphylococci. It is evident that considerable progress has been made in development of enumeration methods designed to include stressed cells. However, most of these methods have been developed on the basis of heat-stressed cells only, with a minimum amount of evidence to show that these techniques are equally effective for enumeration of cells stressed by other treatments. Thus broad application of techniques developed for heat-stressed cells must be made with caution. For example, Stiles (*37*) has recently shown that the response of *S. aureus* cells stressed during ripening of Cheddar cheese to various selective media was exactly opposite to that shown previously for heated cells (*38*); i.e., the most reliable media were the high salt media, while tellurite- and azide-based media were generally unsatisfactory.

Another area involving enumeration of stressed staphylococci which has been only briefly investigated is that of doubly- or multi-stressed cells. Most studies have dealt with only a single stress. However, in actual food systems bacteria are subjected to a wide variety and number of stressful conditions. Further study in this area is warranted and should allow development of improved procedures for effective enumeration of staphylococci from foods.

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TABLE 1. *Effects of catalase on the enumeration of unstressed and thermally stressed* Staphylococcus aureus *MF-31*[a].

Medium	Unstressed		Stressed	
	CFU/ml	Enumeration[c] (%)	CFU/ml	Enumeration[c] (%)
BP	3.9×10^9	100	2.4×10^9	100
VJ	3.5×10^9	90	6.2×10^8	26
VJ + catalase[d]	4.2×10^9	108	2.9×10^9	121
TSA	3.4×10^9	87	1.9×10^9	79
TSA + catalase[d]	3.9×10^9	100	2.5×10^9	104
TSAS	3.1×10^9	79	1.8×10^7	0.8
TSAS + catalase[d]	3.5×10^9	90	1.2×10^9	50
MSA	3.0×10^9	77	2.5×10^6	0.1
MSA + catalase[d]	3.4×10^9	87	7.2×10^8	30
S110	2.2×10^9	56	2.3×10^6	0.1
S110 + catalase[d]	2.5×10^9	64	3.7×19^8	15
TPEY	4.2×10^9	108	8.0×10^8	33
TPEY + catalase[d]	4.3×10^9	110	2.5×10^9	104

[a]From Flowers et al. (*13*).
[b]Cells were heated in 100 mM potassium phosphate buffer (pH 7.2) at 52 C for 15 min.
[c]Percentage of enumeration was calculated by dividing colony-forming units per milliliter on the various media by colony-forming units per milliliter on BP and multiplying by 100.
[d]Catalase activity was about 780 units per plate.

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Detection of Stressed Microorganisms — Implications for Regulatory Monitoring

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ABSTRACT

Common food microbiology techniques as used by the food industry and the regulators range from excellent to poor when scored on the usefulness of these techniques to recover stressed microorganisms. Until demonstrated otherwise, it appears prudent to have techniques that will score stressed pathogens and indicator organisms in foods. The usefulness of a method to recover these organisms should be a consideration both in method development and in collaborative study of a method, should it be applicable to foods that might contain these organisms. Once a method is developed that recovers stressed organisms, any standards or guidelines in use should be reevaluated to determine whether they should be changed because of generally greater recovery experienced when a method that resuscitates stressed cells is substituted for a method that does not recover these cells.

It is becoming apparent that stressed microorganisms commonly occur in many of our foods such as those that are heated but not sterilized, frozen, dried, or fermented and those that contain either natural or added antimicrobials. Until data are available to the contrary, it appears prudent to assume that stressed pathogenic bacteria in foods will cause human disease at about the same concentration as their unstressed counterparts. If this assumption is accepted it follows that regulatory methods for foods must provide for the resuscitation of stressed pathogens. Similarly, since enumeration of all cells of a given kind of indicator organism, whether stressed or not, should give a more accurate assessment of the sanitary history of a food than an enumeration of only the unstressed cells, it also appears desirable to use methods that provide for the resuscitation of indicator organisms.

When our methods for bacteriological examination of foods are reviewed, it is apparent that some of the methods automatically provide for resuscitation (e.g., aerobic plate count), others are specifically designed to provide for it (e.g., lactose pre-enrichment for Salmonella), whereas still others make no provisions for it (e.g., salt broth for *S. aureus*).

This discussion will cover what I perceive to be (*a*) the most critical needs for method changes to provide for resuscitation, (*b*) changes needed in protocols used for method development, and finally (*c*) the effect on microbiological criteria for foods of adoption of methods that provide for resuscitation.

NEED CHANGES IN METHODS

One of the most common tests for a pathogen in foods is that for *Salmonella*. Basically, the methods in use for products that are generally not highly contaminated start with a step in which resuscitation can occur (e.g., pre-enrichment in lactose broth). Where the contamination is apt to be high, the blended product is added directly to selenite cystine and brilliant green tetrathionate broths to prevent the overgrowth of the non-*Salmonella* that might occur during incubation in lactose. Unfortunately, direct addition to selective enrichment broths may result in an environment where resuscitation cannot occur. An evaluation is needed to determine whether this is true. If it is, perhaps a non-selective pre-enrichment period of only 2 or 3 h would satisfy the need to resuscitate without permitting excessive multiplication of non-*Salmonella*.

Another common procedure in food microbiology is to determine the count of *Staphylococcus aureus*. Two methods are generally used: direct plating on Baird-Parker agar if the numbers of *S. aureus* are high enough to give meaningful results, and the MPN procedure using salt broth. The former method, as shown by extensive study, is excellent for recovery of stressed *S. aureus*. The latter procedure needs revision badly because of the well-known inability of stressed cells to resuscitate in a high salt environment. Once the MPN procedure is revised to provide for recovery of stressed *S. aureus*, I would anticipate better correlation between MPN and Baird-Parker agar results.

Our methods for indicator organisms also have room for improvement. The routine testing of dairy products for coliforms calls for direct plating in violet red bile agar, with an MPN procedure as an alternative. The violet red bile agar method has been demonstrated to be detrimental to stressed coliforms. Although improved methods have been developed and published, they have not been adopted as official methods for regulatory purposes. In the MPN method for coliforms in dairy products, the product is added directly to 2% brilliant green lactose bile broth. The effect of this on stressed coliforms needs to be evaluated and the method revised if indicated.

In the procedure for coliforms commonly used in routine food microbiology, dilutions of the food are

added directly to lauryl sulfate tryptose broth. Again, study of the fate of stressed coliforms including *Escherichia coli* in lauryl sulfate tryptose broth is needed and if stressed coliforms fare as I suspect, the method should be revised.

Other methods can be cited that may be detrimental to stressed foodborne microorganisms. Basically, each method that begins with the direct addition of a food or a food homogenate to a selective medium should be suspect unless specific provisions have been made for the resuscitation of stressed microorganisms or unless the method is used only for foods that do not contain significant numbers of these microorganisms.

NEEDED CHANGES IN DEVELOPMENT OF METHODS

Since stressed microorganisms are common in foods, methods development should include an evaluation of the method's usefulness for their recovery if the method is to be applicable to foods in general. All of us have been tempted to use only freshly inoculated foods in our methods development research simply because naturally contaminated foods are often very difficult to obtain. Although naturally contaminated foods are the best foods to use in methods development and evaluation, their lack should not hinder attempts to evaluate the efficacy of a method for resuscitation of stressed microorganisms; samples can be prepared in the laboratory in such a way that the stressed cell population will be similar to that in naturally contaminated foods. Many recent methods development studies have included a consideration of the recovery of stressed cells. This is a significant step forward, and this consideration should be a routine facet of methods development if the method is designed for foods that would be expected to contain stressed organisms. One word of caution may be appropriate: a quantitative technique that incorporates a resuscitation step should be so designed that any multiplication that takes place in the resuscitation step will not cause an increase in the apparent count of the food under test.

CHANGES NEEDED IN STANDARDS

Finally, if methods providing for resuscitation of stressed cells are adopted for regulatory or quality control purposes to replace other methods, any standard that was based upon the method being replaced must be reconsidered. As an example, adoption of a resuscitation method for coliforms in dairy products must involve a reconsideration of the standards for coliforms in these products if our standards are to be meaningful. The small number of microbiological criteria that exist for non-dairy foods minimizes the problem of concurrent standard change with method revision.

In conclusion, I believe that our methods in food microbiology should provide for the resuscitation of stressed microorganisms. Several of the commonly used methods may not be satisfactory for this purpose; each needs to be reevaluated and, if indicated, revised. Once this is accomplished and the modified methods are adopted for regulatory and/or quality control purposes, a revision of our microbiological criteria may be necessary.

ACKNOWLEDGMENT

Presented at the 78th Annual Meeting of the American Society for Microbiology, Las Vegas, Nevada, May 19, 1978.

News and Events

Baumgardt Named Associate Dean at Penn State

Dr. B. R. Baumgardt, professor of animal nutrition, was named associate dean for research and associate director of the Agricultural Experiment Station at The Pennsylvania State University, effective March 1.

He succeeds Dr. Walter I. Thomas, who retired in February.

Prior to his appointment, Dr. Baumgardt was head of the Department of Dairy and Animal Science in the College of Agriculture.

Dr. Baumgardt has been on the Penn State faculty since 1967 when he was named professor of animal nutrition. He became head of the Department of Animal Science in 1970 and head of the combined departments of Dairy and Animal Science in 1975.

His major research interests have been on forage utilization, dairy cattle nutrition, and control of food intake with emphasis on ruminant animals. He has taught in the areas of animal nutrition and feeding and in the field of physiology.

A native of Layfayette, Ind., he earned his bachelor's and master's degrees at Purdue University where he held a Ralston Purina Research Fellowship.

He earned the Ph.D. in agricultural biochemistry-nutrition at Rutgers University in 1959.

Dr. Baumgardt served on the faculty at the University of Wisconsin previous to joining the Penn State faculty. He was selected in 1966 by the American Dairy Science Association as recipient of its annual award for research in dairy cattle nutrition.

Coming Events *con't from p. 370*

Sept. 10-13--2nd INTERNATIONAL CONFERENCE ON FOOD SERVICE SYSTEMS DESIGN. Harrogate, England, Contact: G. Glew, Catering Research Unit, Procter Dept. of Food Science, The University of Leeds, LS2 9JT, England.

Sept- 10-14--FOOD PROCESSORS ADVANCED MICROBIOLOGY SHORT COURSE. University of California, Davis. Fee $200. Contact: John C. Bruhn, Dept. of Food Science and Technology, University of California, Davis, CA 95616, 916-752-2192.

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Coming Events

April 19--IOWA FOOD INDUSTRY CONFERENCE. Stouffer's Hotel, Cedar Rapids, IA. Contact: Bill LaGrange, Food Technology, ISU, Ames, IA 50011.

April 22-25--4th ANNUAL TROPICAL AND SUBTROPICAL FISHERIES TECHNOLOGICAL CONFERENCE. St. Petersburg Hilton, St. Petersburg, FL. Contact: W. Steven Otwell, 325 Food Science Bldg., Univ. of Florida, Gainesville, FL 32611.

April 23-24--NATIONAL SANITATION FOUNDATION SEMINARS, Washington, D.C. For more information, contact: Education Service, National Sanitation Foundation, NSF Building, P.O. Box 1468, Ann Arbor, MI 48106, 313-769-8010.

April 25-27--54th ANNUAL MEETING, AMERICAN DRY MILK INSTITUTE; 8th ANNUAL MEETING, WHEY PRODUCTS INSTITUTE. Chicago Marriott O'Hare, 8535 West Higgins Road (at O'Hare Airport), Chicago. Contact: Warren S. Clark, Jr., Exec. Director of both organizations, 130 N. Franklin St., Chicago, IL 60606.

April 30-May 4--APPLIED PROCESS PUMP TECHNOLOGY. Sponsored by the Center for Professional Advancement. Fee: $580. Contact: Mary Sobin, Dept. NR, The Center for Professional Advancement, P.O. Box H, East Brunswick, NJ 08816, 201-249-1400.

May 2-3--FDA FOOD INDUSTRY WORKSHOP - Food Storage Sanitation. Sponsored by the Food Sanitation Institute, EMA, and the FDA. Holiday Inn, Minneapolis, Minnesota. Contact: Harold C. Rowe, FSI, Executive Director, 1701 Drew Street, Clearwater, FL 33515, 813-446-1674.

May 7-10--FOOD MICROBIOLOGY LAB COURSE. American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502. Contact: Larry E. Wood, AIB, 913-537-4750.

May 8-10--34th ANNUAL PURDUE INDUSTRIAL WASTE CONFERENCE. Stewart Center, Purdue University, West Lafayette, IN. Contact: J. D. Wolszon, Purdue Industrial Waste Conference, Civil Engineering Bldg., Purdue Univ., West Lafayette, IN 47907.

May 10-11--ENVIRONMENTAL LAWS & REGULATIONS COURSE, Masters' Seminar. Philadelphia Hilton, Philadelphia, PA. Sponsored by Government Institutes, Inc., Contact: Marguerite Leishman, Government Institutes, Inc., 4733 Bethesda Ave., N.W., Washington, D.C. 20014, 301-656-1090.

May 13-16--THE NATIONAL MEAT TRADES FAIR. Exhibition Centre, Harrogate, England. Contact: British Information Services, 845 Third Ave., New York, NY 10022.

May 14-17--DELEX, Delicatessen International Exhibition. Royal Horticultural Society's New Hall, London, England. Contact: British Information Services, 845 Third Avenue, New York, NY 10022, 212-752-8400.

May 14-19--MACROPAK 1979 and INTERNATIONAL PACKAGING CONGRESS. Utrecht, the Netherlands. Contact: Royal Netherlands Industries Fair, Special Events Dept., P.O. Box 8500, 3502 RM Utrecht, the Netherlands.

May 15-17--POWDER AND BULK SOLIDS CONFERENCE/EXHIBITION. Philadelphia Civic Center, Philadelphia, PA. Sponsored by International Powder Institute of London and Chicago. Contact: Industrial & Scientific Conference Management, Inc., 222 West Adams St., Chicago, IL 60606, 312-263-4866.

May 20-22--1979 INTERNATIONAL CHEESE & DELI SEMINAR. Madison, WI. Contact: International Cheese & Deli Seminar, 801 W. Badger Rd., Madison, WI 53713.

May 20-24--CONFERENCE ON INTERSTATE MILK SHIPMENTS, Stouffer Inn, Louisville, KY. Contact: H. H. Vaux, Director, Indiana Food and Drug Division, or John Speer, Milk Industry Foundation, 910 17th St. N.W., Wash., D.C.

May 21-24--NSF SEMINARS, Seattle, WA. For more information, see entry for April 23-24.

May 21-24--NALVEX, National Licensed Victuallers and Caterers Exhibition. National Exhibition Centre, Birmingham, England. Contact: British Information Services, 845 Third Avenue, New York, NY 10022, 212-752-8400.

May 22-27--INTERNATIONAL FOOD FAIR. Copenhagen, Denmark. Contact: Bella Center A/S, Center Boulevard, DK-2300 Copenhagen S., Denmark.

May 28-31--NATIONAL CONVENTION OF THE AUSTRALIAN INSTITUTE OF FOOD SCIENCE AND TECHNOLOGY, Theme: "Food, the Consuming Interest." Adelaide, South Australia. Contact: L. Peters, Australian Institute of Food Science and Technology, 13, Bulf Parade, Brighton, South Australia 5048.

June 4-5--NSF SEMINARS, Memphis, TN. For more information, see entry for April 23-24.

June 5--PENNSYLVANIA SANITARIANS ASSOCIATION, Annual Meeting. Keller Conference Center, Pennsylvania State University, University Park Campus, State College, PA 16801. Contact: Sid Barnard, Pennsylvania State University.

June 10-13--INSTITUTE OF FOOD TECHNOLOGISTS 39th ANNUAL MEETING AND FOOD EXPO. Alfonso J. Cervantes Convention and Exhibition Center, St. Louis, MO. Contact: C. L. Willey, Institute of Food Technologists, Suite 2120, 221 N. LaSalle St., Chicago, IL 60601.

June 23-28 -- NATIONAL ENVIRONMENTAL HEALTH ASSOCIATION ANNUAL EDUCATIONAL CONFERENCE. Charleston, SC. Contact: NEHA, 1200 Lincoln St., Suite 704, Denver, CO 80203, 303-861-9090.

June 24-27--AMERICAN SOCIETY OF AGRICULTURAL ENGINEERS, Summer Meeting. Winnipeg, Manitoba, Canada. Contact: Roger R. Castenson, ASAE, 2950 Niles Road, Box 410, St. Joseph, MI 49085, 616-429-0300

July 30-Aug. 3--ADVANCES IN FOOD AND APPLIED MICROBIOLOGY. Massachusetts Institute of Technology, Cambridge, MA 02139. Program is under the direction of Anthony J. Sinskey, MIT, Professor of Applied Microbiology. Contact: Director of Summer Session, Rm. E 19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

Aug. 12-16--IAMFES ANNUAL MEETING, Sheraton Twin Towers, Orlando, FL. Contact: E. O. Wright, IAMFES Exec. Sec., P.O. Box 701, Ames, IA. 50010, 515-232-6699, or see registration form in this *Journal.*

Aug. 13-17--WORKSHOP ON EDUCATIVE PROCESSES IN FOOD MICROBIOLOGY. Sponsored by the Joint American Society for Microbiology/Institute for Food Technologists Committee on Food Microbiology Education. Quadna Resort, Hill City, MN. Contact: E. A. Zottola, Dept. of Food Science and Nutrition, 1334 Eckles Ave., University of Minnesota, St. Paul, MN 55108.

Aug. 29-31--FOURTH INTERNATIONAL IUPAC SYMPOSIUM ON MYCOTOXINS AND PHYCOTOXINS. Co-sponsored by World Health Organization and Swiss Society for Analytical and Applied Chemistry. Lausanne, Switzerland. For participation and poster presentation, contact: Prof. P. Krogh, Dept. of Veterinary Microbiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907 or Prof. D. Reymond, IUPAC, Case postale 88, 1814 La Tour de Peilz, Switzerland. *Con't. page 369*

Journal of Food Protection Vol. 42, No. 4, Pages 371-374 (April, 1979)

E-3-A Sanitary Standards for Scraped Surface Heat Exchangers

Number E-3100

Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Department of Agriculture
Poultry & Egg Institute of America
Dairy and Food Industries Supply Association

It is the purpose of the IAMFES, USDA, PEIA, and DFISA in connection with the development of the E-3-A Sanitary Standards program to allow and encourage full freedom for inventive genius or new developments. Specifications for scraped surface heat exchangers which so differ in design, material and construction, or otherwise, as not to conform to the following standards but which, in the fabricator's opinion are equivalent or better, may be submitted for the joint consideration of the IAMFES, USDA, PEIA and DFISA at any time.

A.

SCOPE

A.1

These standards cover the sanitary aspects of scraped surface heat exchangers for adding heat to or removing heat from liquid egg products.

A.2

In order to conform with these E-3-A Sanitary Standards, scraped surface heat exchangers shall comply with the following design, material and fabrication criteria.

B.

DEFINITIONS

B.1

Scraped Surface Heat Exchanger: (Referred to as SSHE through these E-3-A Sanitary Standards) shall mean a cylinder(s) with closed ends, means for heating or cooling, having a precise wiping or scraping blade(s) for removing the heated or cooled product from the cylinder wall(s), and through which the product flows continuously under pressure.

B.2

Product: Shall mean liquid egg products.

B.3

Surfaces

B.3.1

Product Contact Surfaces: Shall mean all surfaces which are exposed to the product and surfaces from which liquids may drain, drop or be drawn into the product.

B.3.2

Non-Product Contact Surfaces: Shall mean all other exposed surfaces.

B.4

Mechanical Cleaning or Mechanically Cleaning: Shall denote cleaning, solely by circulation and/or flowing chemical detergent solutions and water rinses onto and over the surfaces to be cleaned, by mechanical means.

B.5

Engineering Plating: Shall mean plated to specific dimensions or processed to specified dimensions after plating, and for these standards, the minimum thickness shall be 0.0002 inch for all product contact parts except as hereinafter specified.[1]

C.

MATERIALS

C.1

All product contact surfaces shall be of stainless steel of the AISI 300 series[2] or corresponding ACI[3] types (See Appendix, Section E.), or metal that is non-toxic and non-absorbent, and which under conditions of intended use is at least as corrosion resistant as stainless steel of the foregoing types, except that:

C.1.1

Cylinders made of the materials provided for in C.1 may be covered with an engineering plating of chromium or an equally corrosion-resistant and wear-resistant non-toxic material.

C.1.2

Cylinders may also be made of other non-toxic structurally suitable heat-exchange metal made corrosion-resistant and wear-resistant by covering the product contact surface(s) with an engineering plating of chromium or an equally corrosion-

[1] *QQ-C-320 B-Federal Specification for Chromium Plating (Electrodeposited) June 17, 1974, 40¢.*
QQ-N-290 A - Federal Specification for Nickel Plating (Electrodeposited) November 12, 1971, 20¢. Both documents available from: Business Service Center, General Services Administration, Seventh and D Streets, SW, Washington, DC.

[2] *The data for this series are contained in the following reference: AISI Steel Products Manual, Stainless & Heat Resisting Steels, April, 1963, Table 2-1, pp. 16-17. Available from: American Iron & Steel Institute, 1000 16th Street, NW, Washington, DC 20036.*

[3] *Alloy Casting Institute Division, Steel Founders' Society of America, 20611 Center Ridge Road, Rocky River, OH 44116.*

resistant, non-toxic metal. When steel other than stainless steel is used, the minimum thickness of the engineering plating shall be 0.002 inch.

C.1.3

Cylinders, bearings, springs, shafts, couplings, drive and mounting pins, seal parts, and scraping parts may also be made of non-toxic hardenable, corrosion-resistant stainless metal (AISI 400 series stainless steel or equivalent) or these materials covered with an engineering plating of nickel, chromium or an equally corrosion-resistant, non-toxic metal.

C.1.4

Solder, when used, shall have a tin content of not less than 50% and the remainder shall be lead. It shall be cadmium free and non-absorbent.

C.1.5

Silver soldered or brazed areas and silver solder or braze material shall be non-toxic and corrosion-resistant.

C.1.6

Rubber and rubber-like materials may be used for gaskets, seals and parts used in similar applications. These materials shall conform to the applicable provisions of the "E-3-A Sanitary Standards for Multiple-Use Rubber and Rubber-Like Materials Used as Product Contact Surfaces in Egg Processing Equipment," Number E-1800.

C.1.7

Plastics materials may be used for bearings, scraping parts, gaskets, seals and parts used in similar applications. These materials shall comply with the applicable provisions of the "3-A Sanitary Standards for Multiple-Use Plastic Materials Used as Product Contact Surfaces for Dairy Equipment," Number 20-08.

C.1.8

Where materials having certain inherent functional properties are required for specific applications, such as scraper parts and rotary seals, carbon and/or ceramic materials may be used. Ceramic materials shall be inert, non-porous, non-toxic, non-absorbent, insoluble, resistant to scratching, scoring and distortion when exposed to the conditions encountered in the environment of intended use and in cleaning and bactericidal treatment.

C.1.9

Single-service sanitary type gaskets may be used.

C.2

All materials having a product contact surface(s) used in the construction of an SSHE designed to be used in a processing system to be sterilized by heat and operated at a temperature of 250°F or higher shall be such that they can be (1) sterilized by saturated steam or water under pressure at a temperature of at least 250°F and (2) operated at the temperature required for processing.

C.3

All non-product contact surfaces shall be of corrosion-resistant materials or material that is rendered corrosion-resistant. If coated, the coating used shall adhere. All non-product contact surfaces shall be relatively non-absorbent, durable and cleanable. Parts removable for cleaning having both product contact and non-product contact surfaces shall not be painted.

D.

FABRICATION

D.1

All product contact surfaces shall be at least as smooth as a No. 4 ground finish on stainless steel sheets. (See Appendix, Section F.).

D.2

All permanent joints in metallic product contact surfaces shall be welded. If it is impractical to weld, they may be silver soldered or brazed, or if this is not practical, the joint may be fitted in a manner that it will be completely rigid and without pockets and crevices. All such areas of product contact surfaces shall be at least as smooth as the adjoining surfaces.

D.3

Solder may be used around blade mounting pins, bushings and bearings for flushing joints and producing fillets for minimum radii.

D.4

All product contact surfaces of an SSHE not designed to be mechanically cleaned shall be easily accessible for cleaning and inspection either when in an assembled position or when removed. Removable parts shall be readily demountable.

D.5

An SSHE that is to be mechanically cleaned shall be designed so that all product contact surfaces of the SSHE (1) can be mechanically cleaned and (2) are accessible for inspection.

D.6

All internal angles of 135° or less on product contact surfaces shall have minimum radii of 1/4 inch except that:

D.6.1

Where smaller radii are required for essential functional reasons such as sealing ring grooves, scraper blade mounting pins and parts used in similar applications.

D.6.2

The minimum radii in grooves for standard 1/4 inch O-Rings shall be not less than 3/32 inch and for standard 1/8 inch O-Rings shall be not less than 1/32 inch.

D.6.3

When for functional reasons the radius must be less than 1/32 inch, in such applications as flat sealing surfaces, the product contact surface of this internal angle must be readily accessible for cleaning and inspection.

D.7

All sanitary tubing, fittings and connections shall conform with the applicable provisions of the "E-3-A

Sanitary Standards for Fittings Used on Egg and Egg Products Equipment and Used on Sanitary Lines Conducting Egg and Egg Products," Number E-0800.

D.8
There shall be no threads on product contact surfaces.

D.9
Coil springs having product contact surfaces shall have at least 3/32 inch openings between coils including the ends when the spring is in a free position.

D.10
An SSHE designed to be used in a processing system to be sterilized with heat and operated at a temperature of 250°F or higher shall comply with the following criteria in addition to other criteria in this standard.

D.10.1
The seal(s) in an SSHE designed to be used in a processing system to be sterilized by heat and operated at a temperature of 250°F or higher shall be between the product contact surface and the steam or other sterilizing medium chamber.

D.11
The shaft(s) of an SSHE shall have a seal of a packless type, sanitary in design.

D.12
The means of supporting an SSHE shall be one of the following:

D.12.1
With legs: Legs shall be smooth with rounded ends, have no exposed threads, and shall be of sufficient length to provide a clearance between the lowest part of the base and the floor of no less than six inches. Legs made of hollow stock shall be sealed.

D.12.2
Mounted on a slab or island: The base shall be designed for sealing to the slab or island surface. (See Appendix, Section G.)

D.12.3
Mounted on a wall or column: The point of attachment of an SSHE cylinder(s) to its mounting shall be designed for sealing. The mounting, if supplied by the SSHE manufacturer shall be designed for sealing to the wall or column. The design of an SSHE with a vertical cylinder(s) to be mounted on a wall shall be such that there will be at least a 4-inch clearance between the outside of the cylinder(s) and the wall.

D.13
An SSHE designed to be installed partially outside a processing area, shall be provided with a plate or other suitable member to close the opening in the processing room wall or ceiling and shall be such that it can be sealed to the wall or ceiling.

D.14
The SSHE shall be designed so that there is at least a 4-inch space between the driving mechanism and the cylinder(s) when parts normally removed during cleaning have been removed.

D.15
Any guard(s) required by a safety standard that will not permit accessibility for cleaning and inspection shall be designed so that it can be removed without the use of tools.

D.16
Non-product contact surfaces shall be free of pockets and crevices and be readily cleanable and those to be coated shall be effectively prepared for coating.

D.17
An SSHE shall have an information plate in juxtaposition to the name plate giving the following information or the information shall appear on the name plate:
(1) The maximum temperature and pressure at which the SSHE can be operated.
(2) If the SSHE is or is not designed for mechanical cleaning.
(3) A statement that to prevent corrosion the recommendations of the SSHE manufacturer should be followed with respect to time, temperature and the concentration of specific cleaning solutions and chemical bactericides.

APPENDIX

E.

STAINLESS STEEL MATERIALS
Stainless steel conforming to the applicable composition ranges established by AISI for wrought products, or by ACI for cast products, should be considered in compliance with the requirements of Section C.1 herein. Where welding is involved the carbon content of the stainless steel should not exceed 0.08%. The first reference cited in C.1 sets forth the chemical ranges and limits of acceptable stainless steels of the 300 series. Cast grades of stainless steel equivalent to types 303, 304, and 316 are designated CF-16F, CF-8, and CF-8M, respectively. These cast grades are covered by ASTM[4] Specifications A296-68 and A351-70.

F.

PRODUCT CONTACT SURFACE FINISH
Surface finish equivalent to 150 grit or better as obtained with silicon carbide, is considered in compliance with the requirements of Section D.1 herein.

G.

SLABS OR ISLANDS
When an SSHE is designed to be installed on a slab or island, the dimensions of the slab or island should be such that the base of the SSHE will extend beyond the slab or island at least one inch in all horizontal directions. The slab or island should be of sufficient

[4] *Available from American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.*

height so that the bottom of all product connections are not less than 4 inches above the floor. The surface of the slab or island should be coated with a thick layer of waterproof mastic material, which will harden without cracking. The junction of the SSHE base and the slab or island should be sealed.

These standards shall become effective May 4, 1979.

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MAIL TO: **Jay Boosinger, Co-Chairman of Registration**
IAMFES
Florida Dept. of Agriculture & Consumer Services
Division of Dairy Industry
508 Mayo Building
Tallahassee, Florida 32304

Please check where applicable:

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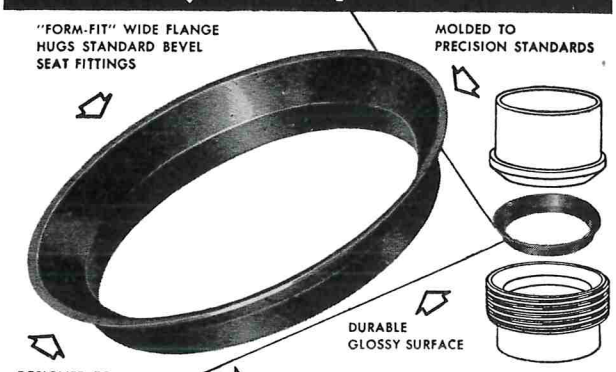

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Two Easy Steps to More Milk and Healthy Udders

By Dr. L.J. Bush
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Significant advances have been made in recent years in the design and development of milking equipment. Now the challenge is for the dairyman to take full advantage of what they can do for him. By using this new equipment in accordance with suggested milking procedures, a dairyman can help keep his cows healthy and increase profits from his herd. Two important steps in getting the most from equipment are proper adjustment of milking units and prompt removal of the units as milking is completed.

Proper Adjustment Avoids Injury and Improves Milk Flow

To do the best job of milking cows by machine, it is important that the teat cups remain in the correct position on the teats. If the teat cups are allowed to creep up on the teats as milking progresses, the passageway for milk is partially blocked due to compression of the tissue by the teat cup liners. Injury to the tissue at the base of the udder may occur. This can be prevented by providing a slight downward pull on the teat cups as the cow is milked out. With "claw" type units this usually can be accomplished only by holding down on the unit as milking nears completion, whereas suspended units can be positioned to provide the tension needed. The mere fact that the milking unit is suspended does not insure proper downward tension on the teat cups, however. It must be adjusted properly to get the job done right. Higher milk yields will result due to more complete milking, and less machine stripping time is required.

Remove Teat Cups When Milk Flow Stops

Another important step in milking is to remove the milking unit as soon as the flow of milk has stopped. Removing teat cups too early will rob you of milk. On the other hand, there is evidence that overmilking causes severe injury to teats of some cows. Hemorrhage and inflammation of the membrane lining the teat cavity often occurs, and, in some cases, the streak canal is injured. These injuries lower resistance to bacterial invasion and mastitis may result.

Cows vary in milk yield and rate of milk flow. Individual quarters of the same cow vary in the same way. Therefore, the ideal situation is for individual teat cups to be removed from the quarters as each one is milked out. Milking units designed for automatic removal of individual teat cups accomplish this with precision, if serviced regularly and used in the recommended way. It should be noted though that the same results can be achieved with conventional milking units by careful attention to good milking procedures.