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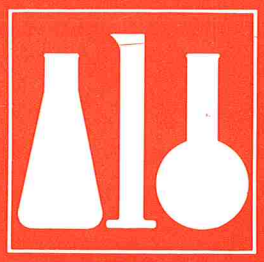

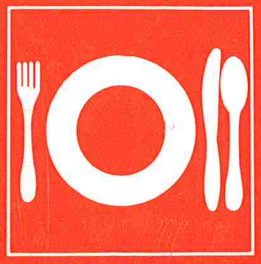

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Radiation-Sterilization of Food: Treating Scanty Data from Inoculated Packs[1]

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(Received for publication January 26, 1979)

ABSTRACT

This paper is about the mathematical methods used in calculating the 12D radiation-dose for a food which has been subjected to an inoculated pack. It concerns the case in which only one partial-spoilage data point is obtained from the experiment. A simple, partly graphical procedure is described. This method is based on binomial confidence limits and furnishes an estimate that lies above the (unknown) true 12D value with probability greater than 90%. The method is applied to an inoculated pack for low-level nitrite/nitrate ham described by Anellis et al. (*2*).

The purpose of this paper is to describe a mathematical method that may occasionally be useful in treating data on radiation-sterilization obtained from an inoculated pack. The emphasis is less on the microbiological implications of the method than upon explaining it and showing how to use it.

The specific question addressed here is, how can a "safe" dose be estimated when the data contain exactly one dose at which some, but not all, sample units (cans) are inactivated. In most inoculated packs the data contain two or more such "partial-spoilage" points, and in that event the safe (12D) dose is best estimated (in the author's opinion) by the maximum-likelihood method (*8*) or the least-squares procedures (*6*). Both these methods fail (i.e., they do not yield a unique estimate of the 12D dose) when only one partial spoilage point is obtained, as in Anellis et al. (*1,2*).

In this case the older methods of estimating a safe dose (e.g. Schmidt-Nank or Spearman Kaerber) are still usable, in the sense that they provide a unique estimate of 12D. However, they are based on the assumption that organism-inactivation follows a simple, one-parameter, exponential distribution, an assumption which may sometimes be too restrictive; see the discussion by the author (*6*). In fact, this assumption may be inconsistent with the data of the experiment, but these methods furnish no way of determining this. The procedure proposed here avoids these difficulties.

This method is based on the general probability theory for inoculated packs given by the author (*6*) and assumes that organism inactivation follows the shifted (two-parameter) exponential distribution (*5,7*). The computations are not excessively burdensome, and will lead to roughly the same 12D that would have been obtained by assuming a simple-exponential distribution if that assumption is consistent with the data. That is, the method abandons the simple-exponential assumption only when the data force it to do so.

[1]*Presented at the First International Congress on Engineering and Food, Boston, Massachusetts, August 9-13, 1976.*

METHODS

The following nomenclature will be used in describing the experimental results of the inoculated pack.

x_i = i-th experimental dose in grays
T_i = number of cans exposed at dose x_i
n_i = number of organisms per can at dose x_i
S_i = number of spoiled cans at dose x_i
K_i = number of sterilized cans at dose x_i
$S_i = T_i - K_i$

Any of several definitions of spoiled cans could be used, e.g. visual swelling or presence of toxin, but we shall assume that a spoiled can is one which exhibits microbial growth after a specified period of incubation.

This procedure is intended specifically for the situation where the data contain only one partial-spoilage point, denoted by the subscript 2. The subscript 1 refers to the highest dose for which all cans are spoiled, i.e. $S_1 = T_1$, and x_1 is the highest dose with $S_1 = T_1$; the lowest dose at which no cans are spoiled is denoted by subscript 3, i.e. $S_3 = 0$ and x_3 is the lowest dose for which $S_3 = 0$. Only these three data points play any part in the method.

Two theoretical probabilities are important in this procedure:

$G(x)$ = probability of organism inactivation (death) at dose x
$\Phi(x)$ = probability of can-sterilization at dose x.

In graphing results, the quantity

$$Y(x) = \log_{10}[1 - G(x)] \quad [1]$$

is also used. It is well known, e.g. Ross (*6*), that

$$G(x) = 1 + (1/n)\ln\Phi(x)$$

and therefore

$$Y(x) = \log_{10}[-(1/n)\ln\Phi(x)] \quad [2]$$

The 12D-dose, denoted by x_c, is the dose at which the probability of organism survival is 10^{-12}, and satisfies

$$1 - F(x_c) = 1 \times 10^{-12}$$
$$Y(x_c) = -12$$

This method is based on binomial confidence limits. A significance level, $a = .05$, is chosen first. Given that $K_2 = T_2 - S_2$ out of T_2 cans were sterilized at dose x_2, one finds from charts or tables of binomial confidence limits for proportions two numbers, Φ_{2L} and Φ_{2U}, which are the lower and upper (1-a) confidence limits for the true value of $\Phi_2 = \Phi(x_2)$. Similarly, at dose x_1 an upper $[1 - (a/2)]$ confidence limit, Φ_{1U}, is found for $\Phi_1 = \Phi(x_1)$, and at dose x_3 a lower $[1 - (a/2)]$ confidence limit, Φ_{3L} is found for $\Phi = \Phi(x_3)$. That is,

$$\text{Prob}[\Phi_1 \leqslant \Phi_{1U}] \geqslant 1 - (a/2)$$
$$\text{Prob}[\Phi_{2L} \leqslant \Phi_2 \leqslant \Phi_{2U}] > 1 - a$$
$$\text{Prob}[\Phi_3 \geqslant \Phi_{3L}] \geqslant 1 - (a/2)$$

By means of formula [2] this set of inequalities is transformed into a set of inequalities on $Y_i = Y(x_i)$,

$$\text{Prob}[Y_1 \geqslant B_{1U}] \geqslant 1 - (a/2) \quad [3]$$
$$\text{Prob}[B_{2L} \geqslant Y_2 \geqslant B_{2U}] > 1 - a \quad [4]$$

Prob $[Y_3 \leqslant B_{3L}] \geqslant 1 - (a/2)$, [5]

where

$B_{iW} = \log_{10}[-(1/n_i)\ln \Phi_{iW}]$, $i = 1,2,3,$

and W may be either U or L. Notice that the inequalities on Y_i are the reverses of the inequalities on Φ_i.

The probability that these inequalities are simultaneously satisified exceeds $1 - 2a = .90$. These inequalities are plotted on a graph of Y versus x, and a death-kinetic function, G(x), is sought such that Y(x) satisfies the inequalities at the doses x_1, x_2, x_3. G(x) is assumed to have the two-parameter exponential form $G(x) = 1 - 10^{-\lambda(x-A)} = 1 - e^{-2.303\lambda(x-A)}$

or

$Y(x) = -\lambda(x-A)$

With this assumption we are restricting ourselves to functions which give straight lines when plotted on the Y versus X graph. The constant $-\lambda$ is the slope of the straight line and A is its x-intercept or shoulder width, i.e. the dose below which the organisms experience only recoverable injury.

The 12D estimate, $\hat{x}_c$, is determined as follows:

(*a*) find the least steep line which satisfies all the inequalities [3], [4] and [5] and has $A \geqslant 0$.

(*b*) Then $\hat{x}_c$ is the x-value (dose) at which that line intersects Y = -12. Because of the way $\hat{x}_c$ has been derived, it is true that the (unknown) value of x_c satisfies

$x_c \leqslant \hat{x}_c$

with probability exceeding 1-2*a* = .90.

EXAMPLE

An inoculated pack was carried out with low level nitrite-nitrate ham as substrate, Anellis et al. (*2*), and gave the following data:

$x_1 = 17, S_1 = 100, k_1 = 0$

$x_2 = 20, S_2 = 76, k_2 = 24$

$x_3 = 23, S_3 = 0, k_3 = 100$

where $T_i = 100$ and $n_i = 2.41 \times 10^6$ for all i. The binomial confidence limits are

$\Phi_{1U} = .037; \Phi_{2L} = .16, \Phi_{2U} = .34; \Phi_{3L} = .963.$

and the bounds are found, for example, as

$$B_{1U} = \log_{10}[-(1/n)\ln \Phi_{1U}] = \log_{10}\left[\frac{-1}{2.41 \times 10^6}\ln .037\right] = -5.86$$

Similarly

$B_{2L} = -6.12$ $B_{2U} = -6.35$ $B_{3L} = -7.80$

These four bounds are depicted by arrows in the customary semi-logarithmic plot of survival fraction as a function of dose, Fig. 1. On this graph the line (R) was obtained by visually finding the least-steep straight line which passes above the arrow at x = 17, between the arrows at x = 20 and below the arrow at x = 23. This line is associated with the parameter values

A = shoulder width = 6.4

λ = - (slope) = .469

and has

$\hat{x}_c = 12D = 32.0$

In this case we assert with 90% confidence that the (unknown) true value of x_c satisifies $x_c \leqslant \hat{x}_c = 32.0$.

Notice that many lines can be found to satisfy all the inequalities. In Fig. 1 (Q) is another such line. However, the graph shows that any line steeper than (R) leads to an x_c that is lower (i.e. less safe) than $\hat{x}_c$. In choosing the least-steep line we are demanding the highest 12D value that is consistent with the data. Also, that choice leads to the line that has the smallest possible shoulder-width (least value of A) and is therefore closest to a simple

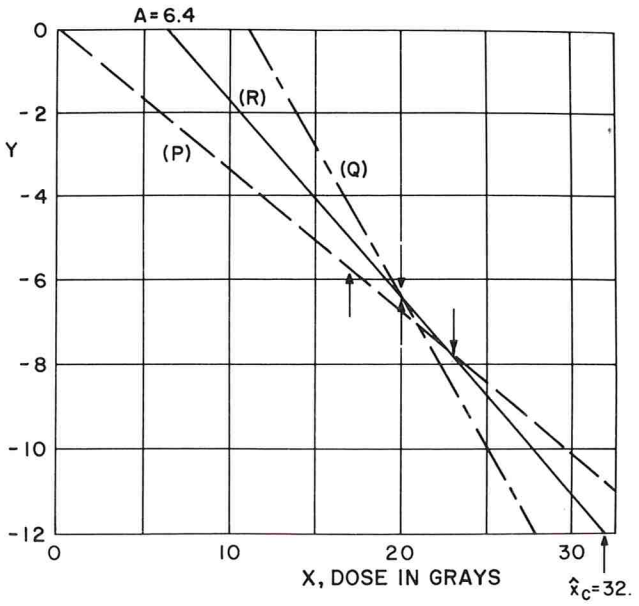

Figure 1. *Graphical determination of* $\hat{x}_c$ *= 12D based on binomial confidence limits.* $Y = \log_{10}$ *(survival probability).*

exponential among all the possible lines.

Notice also that step (*a*) of the procedure specifically excludes the possibility of a negative shoulder, $A < 0$. For, if a straight line can be found that satisfies the inequalities and has $A < 0$, then other acceptable lines can be found with A = 0, and the least-steep line of the latter family is the one that should be used. This will of course correspond to using a simple-exponential death-kinetic hypothesis.

We might ask whether a simple-exponential form of death-kinetics is possible in this case. That is, can a line be found that satisfies all the inequalities and intersects the origin, x = 0, Y = 0? The answer is no. The line (P) in Fig. 1 represents an attempt to do so; (P) and any steeper line violate the lower inequality at x = 20 and any less steep line violates the inequality at x = 23.

We may examine this question from another point of view. Suppose we assume that G(x) is a simple-exponential distribution, i.e. A = 0 and

$1 - G(x) = 10^{-\lambda x}$

$Y(x) = -\lambda x.$ [6]

The best estimate of λ is found, see Ross (*6*), by using the lone partial-spoilage point and Equation [2],

$\hat{\Phi}(20) = 24/100 = .24$

$$Y(20) = \log_{10}\left[-\frac{1}{2.41 \times 10^6}\ln(.24)\right] = -6.23.$$

Equation [6] is then solved (at x = 20) to obtain $\lambda = .311$. Hence the simple exponential function which fits the one partial-spoilage point is

$1 - G(x) = 10^{-.311x}$

$Y(x) = -.311x.$

It is easy to see that this function is inconsistent with the data at x = 23. For,

$\Phi(23) = e^{-n}[1 - G(23)]$

$= e^{-[2.41 \times 10^6 \times 10^{-.311 \times 23}]} = .844$

The probability of getting the observed experimental

result, i.e. all 100 cans sterilized at x = 23, given that the probability of getting one sample sterilized is .844, is given by the binomial theorem as $.844^{100} = 4.3 \times 10^{-8}$. That is, if G(x) is simple-exponential and consistent with the partial spoilage data, it is extremely unlikely that all sample cans would have been sterilized at x = 23. Again, we are forced by the data to conclude that G(x) is unlikely to be simple-exponential.

DISCUSSION

To use this method, one has to have charts or tables of binomial confidence limits for proportions. Such charts and tables can be found in Dixon and Massey (*4*) or Beyer (*3*) or many other sources. It is not always easy to read the charts accurately in finding Φ_{1U} and Φ_{3L}. The following approximate formulas are helpful provided T_1, $T_3 \geqslant 20$ and $a = .05$:

$\Phi_{1U} \simeq (3.7/T_1)\,[1 - (1.7/T_1)]$.

$\Phi_{3L} \simeq 1 - (3.7/T_3)\,[1 - (1.7/T_3)]$.

The procedure described here may be criticized for several reasons. Primarily, one is likely to feel uncomfortable about the stability of the estimates. For example, if the pack were repeated, we would not be surprised if k_2 were, say, 20 or 30 instead of 24; nor would it be startling if k_1 or S_3 were 1, 2 or 3 instead of zero. These could lead to substantially different values of $\hat{x}_c$. How can we rely upon these estimates in this case? Secondarily, it is unpleasantly arbitrary to base so much on the choice $a = .05$. If smaller values of a were used ($a = .01$ might be popular) larger values of $\hat{x}_c$ would result, and again we seem to be adrift.

In replying to these aspersions, several things can be said. The main point is that $\hat{x}_c$ is not exactly an estimate of the unknown true 12D dose, x_c. Rather, with probability $\geqslant .9$, it is an upper bound on x_c. This means that, if we were to repeat the entire inoculated pack many times, the true x_c would exceed $\hat{x}_c$ in less than 10% of the repetitions.

We can view this in different ways. Suppose a pack is run and the estimate $\hat{x}_c$ obtained by this procedure. Then a second pack is run and the estimate $\hat{x}_c^1$ is found, where $\hat{x}_{c'} > \hat{x}_c$. Does this mean that the higher estimate, $\hat{x}_{c'}$, must be used? Not at all. The statement that

$x_c \leqslant \hat{x}_{c'}$ with probability $\geqslant .9$

is logically consistent with the statement

$x_c \leqslant \hat{x}_c$ with probability $\geqslant .9$

because

$x_c \leqslant \hat{x}_c$ implies $x_c \leqslant \hat{x}_{c'}$.

In other words, probably $\hat{x}_{c'}$ is merely a worse (higher) upper bound than $\hat{x}_c$, and we are not forced to use the worse bound when a better one is known.

A looser, but simpler, description is merely to say that by using the $[1 - (a/2)]$ confidence limits, we are building a substantial safety-factor into the estimate $\hat{x}_c$, which provides reasonably high (90%) assurance against underestimating x_c because of random errors. In fact, we may regard x_c as roughly a 13D estimate because of this extra 90% safety factor. Naturally, if we used a smaller a-value (say .01) we would be building-in even more of a safety factor. This seems excessive and unnecessary, but the method is not changed in any essential way by a different choice of a. The only effect is that a different graph or formula must be used to find Φ_{1U}, Φ_{2L}, Φ_{2U} and Φ_{3L}.

It is clear that this method makes crucial use of information from outside the partial-spoilage range, i.e. the points x_1 and x_3. This is natural because in the present case the partial-spoilage range consists of only one point, x_2, and furnishes relatively little information.

It is perhaps distasteful to arrive at a unique estimate of 12D by introducing the extra condition that 12D itself be maximized. However, procedures of this general type are common in mathematics. For example, one of the basic steps in defining the pseudoinverse of a linear equation system is to make an otherwise non-unique solution unique by demanding that the vector solution itself be minimized. Thus, there are both theoretical and practical reasons (safety) why this extra condition is a plausible one.

In a larger context, we should recognize that this procedure probably ought to be viewed as making the best of a bad situation. An alternative procedure would be to re-run the inoculated pack, using more closely spaced doses, so that several partial-spoilage data points are obtained. The present method is intended to avoid doing this, if possible, since repeating the pack may involve considerable loss of time or money. However, the dilemma as to whether this repetition is needed emphasizes the importance of avoiding the difficulty by designing the original inoculated pack as wisely as possible. In this connection the suggestions of the author (*7*) may be helpful.

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Microbiological Safety of Radappertized Beef[1]

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ABSTRACT

To assure microbiological safety and stability of irradiated meats, inoculated pack studies are performed with each meat. These studies are designed to provide partial spoilage data for computation of that dose required to reduce the number of viable spores of a single most resistant strain of *Clostridium botulinum* by 12 log cycles. A beef inoculated pack had only one significant partial spoilage "point" at 22 kGy. Therefore, the binomial confidence limit method was used to compute a 12D dose of 41.2 kGy. This 12D dose, together with a prveious thermal treatment of inactivate autolytic enzymes, provides a wide margin of microbiological safety and stability to packaged meats stored without refrigeration.

Microbiological safety and stability of foods irradiated with high doses (>1 Mrad) is dependent upon destruction of all potential foodborne pathogenic and spoilage microorganisms during processing. This process has been designated radappertization (*23*). Once formulated, the meat is placed in cellulose casings or in metal molds, heated to an internal temperature of not less than 73 C or more than 77 C to inactivate the autolytic enzymes, chilled to -3 to 5 C, vacuum-packed (16.7 kPa or 125 mm Hg pressure) in cans or in multilayered (nylon 6; epoxy-polyester adhesive; aluminum foil, ethylene - acrylic acid copolymer adhesive; chemically bonded mylar-polyethylene) flexible pouches, frozen to about -40 C and irradiated within a temperature range of -40 C to -8 C. Cans of meats are subjected to gamma irradiation (^{60}Co) whereas flexible pouches are irradiated with either gamma rays or electrons from a linear accelerator (10 Mev). Throughout the process, meat is handled in accordance with USDA sanitary regulations (*61*).

Literature surveys (*29,30,44,47*) amply document that the indigenous bacterial spores involved in intoxication or commercial spoilage of foods are more difficult to destroy by ionizing radiation than other microbial forms which are known to cause food poisoning and/or spoilage. Among the spore formers of greatest concern to the canning industry, *Clostridium botulinum*, unlike thermal processing experience, was found to be most radiation tolerant (*42,43*) and among the serotypes encountered in foods, types A and B were apparently the most resistant (*31*). Of the 200 or more previously available strains of these two types, Anellis and Koch (*8*) examined, in a model system, the comparative sensitivities to gamma rays of 56 type A and 43 type B strains (including 3 strains which apparently lost their ability to form toxin). On the basis of computed average D values, these organisms were grouped as most resistant (D = 3.36 to 3.17 kGy or 0.336 to 0.317 Mrad), of intermediate resistance (D = 2.53 to 2.24 kGy) and least resistant (D = 1.29 kGy, one strain). Since the radiation resistance of an organism varies with the food substrate (*2,3,5,7*), five type A (33A, 36A, 62A, 77A, 12885A) and five type B) 9B, 40B, 41B, 53B, 67B) strains or 10% of the total tested, were selected to represent the most and intermediate groups found. These 10 strains are used as indicator organisms in inoculated food pack studies for establishing a prototype minimal radiation dose (MRD). The MRD has been defined (*32,33,56*) as the dose which will reduce 10^{12} spores of the most radiation resistant strain of *C. botulinum* tested to 10^0 spore, or any equivalent reduction, in the food under study. This involves the so-called "12-D concept," where D is the dose required for 90% inactivation or one $\log_{10}$ reduction.

This paper discusses (*a*) the estimation of a 12D dose using one-partial-spoilage data point from an inoculated pack study, and (*b*) the microbiological safety and stability provided to meats given a process which includes thermal inactivation of autolytic enzymes followed by a 12D irradiation dose for *C. botulinum* spores.

MATERIALS AND METHODS

Beef preparation

The beef used complied with USDA regulations (*61*). The preparation of the meat rolls and the packing into cans (211 × 101.5) were detailed elsewhere (*9*). The average percent of protein, fat, water, NaCl, brine, ash and phosphate (sodium-tripolyphosphate) in the unirradiated beef (pH 6.1) was 22.14, 13.97, 61.12, 0.58, 0.94, 2.05 and 0.28, respectively (*9*).

Inoculated pack preparation

An inoculated pack, designed to provide statistically acceptable toxicological and microbiological data for computation of a 12D dose for any desired food, was approved by the U.S. Food and Drug Administration (*21*). Table 1 shows the experimental design used for beef.

C. botulinum spore crops were produced by the biphasic technique (*4,9*). The method of enumeration of the stock spore suspensions and inoculum (mixture of 10 strains, about 10^6 spores/strain) was cited previously (*9*). One-ml portions of the inoculum (delivered by automatic syringe) were sampled for spore counting just before can inoculation and after inoculation of 400, 800 and 1200 cans, respectively. The average count was 9.8×10^6 spores/can.

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TABLE 1. *Inoculated pack experimental design for beef.*

Prototype food:	Beef formulated with 0.75% NaCl and 0.38% TPP[a]
C. botulinum strains:	A mixture of 33A, 36A, 62A, 77A, 12885A, 9B, 40B, 41B, 53B, 67B
Spore inoculum:	10^6/strain; 10^7/can
Containers:	211 × 101.5 (epoxy enamel) metal cans
Food container:	40 ± 5 g
Cans/dose:	100 replicate
Vacuum seal:	16 kPa[b]
Radiation source:	^{60}Co gamma rays
Radiation doses (kGy)[c]:	14, 18, 22, 26, 30, 34, 38, 42, 46, 50
Radiation temp:	- 30 ± 10 C
Incubation:	6 months at 30 ± 2 C
Analysis:	Swelling: daily - 1st month weekly - 2nd thru 6th month Botulinal toxin: 7th month Recoverable *C. botulinum*: 7th month

[a]Sodium tripolyphosphate.
[b]One pascal (Pa) is a unit of pressure equal to 1.333×10^2 mm of mercury (Hg) at 0 C. Thus 16.7 kPa equals 125 mm Hg pressure.
[c]Unit of absorbed ionizing radiation dose. 10×10^3 joule/kg is equivalent to one rad (which is equal to 100 ergs of energy absorbed per g of matter). One kj/kg equals 1×10^3 joules/kg. Ten kj/kg equals 10×10^3 joules/kg (formerly one Mrad).

The procedure for inoculation, can sealing, irradiation and examination for botulinal spoilage was presented elsewhere (*9*).

Computation of a 12D dose

Determination of the MRD was based upon (*a*) presence of viable botulinal cells in the cans, regardless of their inability to outgrow and produce toxin and/or can swelling (this criterion yielded the highest quantal response), (*b*) a single most resistant strain and (*c*) a shifted exponential (an initial shoulder followed by a semilog decline) rate of spore death. Extreme value statistics (*51,52*) were used to calculate the 12D dose. The two parameters (slope and shoulder) which specify the distribution were obtained by employing the binomial confidence limits technique (*13*). The theory and application of this special procedure were described by Ross (*53*).

Microbiological analysis

Random uninoculated beef, packaged in both cans and flexible pouches, was examined for microbiological quality. Each of six unirradiated and gamma (^{60}Co) irradiated (48-71 kGy) cans, and 32 unirradiated and 12 electron (linear accelerator, 10 Mev) irradiated pouches was involved in the assays.

Aerobic plate counts (*12*) were obtained from 50-g samples of meat blended with 450 ml of Butterfield's phosphate buffer with additional appropriate decimal dilutions prepared for plating. Unirradiated samples were inoculated in duplicate plates/dilution and irradiated samples were inoculated in 10 replicate plates/dilution; a duplicate series of plates was incubated in Brewer jars to acquire anaerobic plate counts. To elicit aerobic and anaerobic spore counts, the decimally diluted portions were heated at 80 C for 10 min, cooled, plated and incubated as above.

Chemical analysis

Proximate chemical analysis was conducted (*12*) on uninoculated unirradiated random cans of beef, using 10 samples, four composited cans/sample (160 ± 20 g); duplicate determinations were made on each sample.

RESULTS

Microbiological analysis

The level of aerobic and anaerobic bacteria contaminating uninoculated beef, which had been thermally treated to inactivate autolytic enzymes and then frozen (- 40 C) was minimal just before irradiation (Table 2). Subsequent irradiation (47-71 kGy) resulted in sterile beef.

Experimental sterilizing dose

Radiation doses of 26 kGy or higher produced flat, nontoxic and sterile cans of beef (Table 3). A dose of 22 kGy, however, permitted 50 of the 100 replicate cans to swell and form toxin in the beef; all of these cans had viable botulinal cells. An additional six nonswollen, nontoxic, cans harbored dormant but recoverable *C. botulinum* cells; thus, 22 kGy produced 44 sterile cans. All cans irradiated to 18 kGy yielded all three types of spoilage. Hence the experimental sterilizing dose was $22 < \text{ESD} \leqslant 26$ kGy, while the LD_{50} computed to 22.24 kGy.

TABLE 3. *Spoilage of irradiated beef inoculated with* C. botulinum *spores.*[a]

Radiation dose (kGy)	No. of cans of beef			
	Tested	Swollen	With botulinal toxin	With viable *C. botulinum*
0	100	100	-[b]	-
14	100	100	-	-
18	100	100	100	100
22	100	50	50	56
26	100	0	0	0
30-50[c]	600[d]	0	0	0

[a]A mixture of 10 strains (5 type A and 5 type B), 10^6 spores/strain or 9.8×10^6 spores/can, was used.
[b]Not tested.
[c]Doses increase in 4.0 kGy increments.
[d]100 cans/dose.

TABLE 2. *Microbial profile of uninoculated beef.*[a]

Radiation source	Dose (kGy)[b]	Samples tested[c]		Aerobic		Anaerobic	
		Type	No.	Range	Median	Range	Median
				Plate count/g[d]			
None	0	Cans[e]	6	<10-1010	55	< 10-570	< 10
None	0	Pouches[e]	32	< 10-695	75	< 10-390	2
Cobalt 60	47-71	Cans	6	< 1	< 1	< 1	< 1
Linac	47-71	Pouches	12	< 1	< 1	< 1	< 1
				Spore count/g[d]			
None	0	Cans	6	< 10	< 10	< 10	< 10
None	0	Pouches	32	< 10	< 10	< 10	< 10
Cobalt 60	47-71	Cans	6	< 1	< 1	< 1	< 1
Linac	47-71	Pouches	12	< 1	< 1	< 1	< 1

[a]Source: Mr. Edmund Powers, US Army Natick Research & Dev. Command, Natick, MA (unpublished data).
[b]Unit of absorbed ionizing radiation dose. 1 kGy (or lkJ/kg) is equal to 10^5 rad; 1 rad is equivalent to 100 ergs of energy absorbed/g of matter.
[c]Used 50 g beef + 450 ml Butterfield's buffer: inoculated 2 plates/sample/dilution of non-irradiated beef and 10 plates/sample/dilution of irradiated beef.
[d]Incubated at 35 C for 48 h.
[e]Enzyme inactivated and frozen to -40 C.

Cans of beef exposed to 18 kGy were 100% swollen in only 4 days of incubation. In the same time interval, samples of beef irradiated to 22 kGy showed 11 swollen cans out of 50 (22%), in 15 days 45 cans (90%), and in 64 days all 50 cans swelled. The maximum rate of visible spoilage of the 22-kGy cans occurred in 4 days of incubation and decreased markedly after 7 days.

Inequalities on y values

Ross has described in detail how one may estimate a 12D dose from one partial-spoilage data point (*53*). Therefore, this special procedure is merely sketched here (Table 4) to indicate how the 12D for beef was acquired. The first two columns represent the experimental data (Table 3) secured from the inoculated pack (Table 1). The third column was derived from the graph of confidence limits for the binomial probability (*13*); these entries are the limits on the true value of Φ, the fraction of sterile cans, that are satisfied with 97.5% probability. The definition $y = \log_{10}$ (spore survival probability) and the extreme value equations, $u = -\ln \Phi$ and $y = \log_{10}(u/n)$, where n is the spore inoculum, were used to find the inequalities on y, which were tabulated in column four. The data in column 4 reflected the most conservative assumption possible, viz., that of the 10-strain inoculum, only one strain ($n = 9.8 \times 10^5$ spores/can) affected can sterility because it was the most radiation resistant. The 12D dose was found by using the least steep straight line that satisfies all four inequalities on y (Fig. 1). This procedure produces an under-estimate of the shoulder width and thus causes an overestimate of the 12D, both with confidence ≥ 0.90.

TABLE 4. *Extreme value calculation of inequalities on y values using the binomial confidence limits technique.*

Radiation dose (kGy) (x)	No. of sterile cans	Confidence limits on Φ (97.5%)	Inequalities on y value[a]
18	0/100	< 0.037	> -5.47
22	44/100	> 0.347	> -5.96
		< 0.538	< -6.20
26	100/100	> 0.963	< -7.41

[a]Based on a single strain, or $n = 9.8 \times 10^5$ spores/can.

12D Dose

A series of straight lines may be constructed which would satisfy the computed results in Table 4, and which would yield 12D values anywhere between the ESD and 41.2 kGy. We selected the most conservative plot, thus producing a shifted exponential curve with a maximum slope of $y = 0.303 (X - 1.4)$ and a minimum shoulder of 1.4 kGy (Fig. 1). This curve gave a 12D dose of 41.2 kGy.

DISCUSSION

Estimation of a 12D dose by conventional statistical treatment (*10*; eq. 1, 2 or 3) of inoculated pack partial spoilage data assumes that the only microbiological probabilistic event occurring in the food can be expressed by simple exponential death kinetics (i.e., a semi-log decline, without an initial shoulder, of the

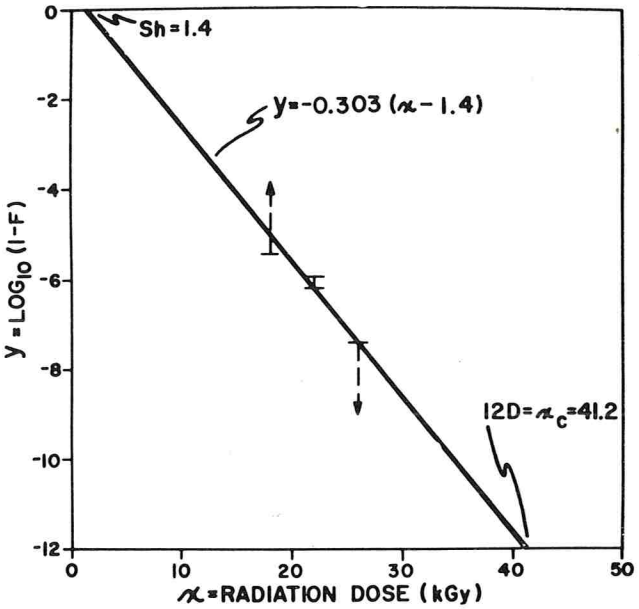

Figure 1. *Graphical estimation of 12D irradiation dose and shoulder width (Sh) for an inoculated* (C. botulinum *spores) beef pack containing only one partial spoilage point. Log_{10} of spore survival probability equals y. The equation [$y = -0.303 (x - 1.4)$] describes the theoretical relationship derived between the spore survival probability and the irradiation dose (x).*

inoculum in a can of food). However, Ross (*51,52*) has shown that two interrelated distribution functions occur simultaneously in an inoculated pack subjected to irradiation: (*a*) a spore inactivation rate (which may not necessarily be simple exponential) within a can of food, and (*b*) a can sterilization rate, where the replicate cans undergo sterilization as the dose increases; the latter rate is equal to the probability of inactivating the most resistant spore in a can of food. Extreme value statistics not only take into account both of these distribution functions, but the mathematics provide a universal relationship between Φ, the can sterilization probability, and $y = \log_{10}$ (the spore survival probability) i.e., the equations

$$u = -\ln \Phi \text{ and } \qquad u = n \times 10^y \text{ and}$$

or

$$y = \log_{10}(u/n) \qquad \Phi = e^{-u}$$

are valid regardless of the type of death kinetics assumed for the inoculum. Thus these formulas permit an accurate translation of information about Φ into information about y, and vice versa, without assuming the rate of spore kill.

The maximum likelihood (ML) technique is the best vehicle presently known for estimating all the parameters (i.e., the undetermined constants) required by the above formulas for any type of death kinetics, even with an inoculated pack containing more than one inoculum level. Moreover, it uses data outside the partial spoilage range (viz., 100% and 0% spoilage), and therefore comes closer than other procedures to using all available data. The only limitation on its use, other than for a simple exponential distribution, is that an inoculated pack must

produce at least two partial spoilage points. The beef inoculated pack, with its one point (22 kGy), was therefore subdivided previously (*9*) into five groups of 2 0 cans each, in ascending average doses, and analyzed as separate data points with the ML technique. The analysis showed, with 90% confidence, that the botulinal spore death rate in a can of beef was not a simple exponential function, but could have been either a shifted exponential, Weibull, lognormal or normal distribution. However, the resulting 12D estimate of 31.7 kGy had such wide confidence limits that it was nearly meaningless.

To correct the above shortcoming of the ML technique when only one partial spoilage point is available and the spore death rate is not simple exponential, the binomial confidence limit (BCL) method was used, with the additional conservative stipulation that the 12D estimate be maximized. This resulted in a computed 12D dose of 41.2 kGy; this value exceeds the true MRD with a confidence of $\geqslant 90\%$, i.e., the true 12D dose is almost surely < 41.2 kGy. Furthermore, Fig. 1 obtained with the BCL procedure, supports the earlier observation (*9*) that the radiation spore kill in the beef is not first order kinetics.

Dose-survival curves reported for botulinal spores suspended in water (*1,50,54,55,58,65*), phosphate buffer (*6,16,24,25,27,34,36,42,43,57,64*), broth (*19,20,25,26,57, 64*) or foods (*16,17,27,36,46,57*) followed a shifted exponential distribution, whether the organism tested was type A, B, E or F, or was a mixture of five types A and B, each, in foods (*16,46*). In view of such weighty evidence, even though Masokhina-Porshnyakova and Ladukhina (*36*) were capable of eliminating the shoulder from their single type A strain and from three out of seven type B strains, it seems much more reasonable to base the 12D estimation on the shifted exponential form of death kinetics instead of an arbitrarily agreed simple exponential function.

An MRD of 41.2 kGy for enzyme-inactivated beef provided a rigorous margin of microbiological safety for a number of reasons:

(*a*) The MRD is at least 15 kGy higher than the actual dose used to inactivate 10^6 spores/can in each of 100 cans (10^8 spores) of the most resistant strain of *C. botulinum* tested (Table 3); the ESD (somewhere between 22 and 26 kGy) is also sufficient to destroy a mixture of 10 strains containing 10^7 spores/can in each of 100 replicate cans (10^9 spores) (Table 3).

(*b*) The process (thermal + 12D radiation dose) ensures that all currently recognized food spoilage and food intoxicating microorganisms which might contaminate beef would be inactivated. This process also assures that if 10^6 highly radiation resistant botulinal spores were to contaminate each of 10^6 cans of a commercial lot of beef (or a total density of 10^{12} spores) they would be decimated to 10^{-6} spores/can, or theoretically, to one spore/million cans; botulinal spores of lesser resistances, if present in any number, would not survive this process. However, such a high initial contaminating population of *C. botulinum* spores in a commercial lot of beef is exceedingly unrealistic, as was determined by past incidence studies (*2*). In the 77-year period 1899 through September 1976 two outbreaks of botulism attributed to commercially canned beef products, potted beef (type B) and beef stew (type A), occurred in 1925 and 1974, respectively (*40,62*). Six additional outbreaks ascribed to home-canned beef items were reported in the same 77-year time period. A miscellany of uninoculated beef products (6,515 samples), both unirradiated and irradiated (1.0 to 71 kGy) were assayed by our laboratory for *C. botulinum* in the last 17 years. All samples were negative.

(*c*) Fig. 1 shows clearly that any straight line passing between the y-values at $x = 22$ kGy is admissible, and will satisfy the inoculated pack data and produce numerous 12D doses in the range between the ESD and the maximum of 41.2 kGy. In the interest of microbiological safety, we have selected the line that yielded the maximum (most conservative) possible dose.

The 41.2 kGy estimate obtained by extreme value statistics with the aid of the binomial confidence limits technique agrees with previous estimates (42-43 kGy) which were based on a simple exponential rate of spore death in the inoculated beef pack (*9*). Obviously, the reason for the close agreement in MRD values between the two types of death kinetics is due to the very small shoulder (1.4 kGy) exhibited by the shifted exponential function (Fig. 1). Other less conservative plots which would satisfy the data would produce larger shoulders with concomitant smaller 12D values.

To date samples of all beef items, various other meats, poultry and fish products, uninoculated and inoculated with ca. 10^7 *C. botulinum* spores per 35-45 g, were bacteriologically sterile following irradiation with a 12D dose. This fact further emphasizes the microbiological safety and stability of foods so irradiated. Selected *C. botulinum* spores were used in the inoculated pack studies to determine the 12D dose because they are the most radiation resistant of the common foodborne pathogenic microorganisms. With few exceptions, bacterial spores, in general, are the most radiation resistant and gram-negative spoilage bacteria (e.g. *Pseudomonas*) are the most sensitive of the microorganism. Filamentous fungi (e.g., *Aspergillus, Penicillium*) are of intermediate resistance, most yeasts are more resistant than molds and some are as resistant as the more sensitive bacterial spores (*30*). Trichinae (*Trichinella spiralis*) in pork and tapeworms (*Cysticercus bovis*) in beef can be controlled by low doses of irradiation. A dose of 0.15 to 0.18 kGy inhibited the maturation of trichina larvae in muscle whereas 7.5 kGy was required to kill the larvae (*22*). *C. bovis* in beef was completely inactivated by a dose of 5 kGy (*45*). In ground beef irradiated at - 30 C, *C. botulinum* 33A spores were much more resistant (2.9-4.1) than other foodborne pathogens such as *Salmonella enteritidis, Staphylococcus aureus,*

and *Escherichia coli* (Table 5). In this case, a 12D dose for 33A spores would provide more than adequate protection from such pathogens. Some asporogenous microorganisms, such as viruses and *Moraxella-Acinetobacter* (M-A) are more radiation-resistant in beef than *C. botulinum* spores (*38,59*; Table 5). However, because of their apparent heat sensitivity (*14,35,37,38,60*) such radiation-resistant asporogenous microorganisms should be reduced, if not eliminated, during the preirradiation thermal treatment for inactivation of autolytic enzymes. Blackwell (*14*) showed that approximately 10^4 foot-and-mouth disease viruses in milk could be inactivated when heated at 67 C for 60 sec. High temperature short time pasteurization (72 C for 15 sec) has been shown as adequate to reduce certain oncogenic viruses (*60*) and *Coxiella burnetii* (*18*) in milk by 12 and 5 log cycles, respectively. Only limited thermal inactivation data of viruses in solid foods have been reported. However, available data were used by Larkin (*35*) to plot a time-temperature profile for the inactivation of 3 logs of viruses in solid foods. According to these data, 3 logs of viruses would be inactivated in 1 min at 71 C or in 6 sec at 75 C. At 72 C, the D value for radiation-resistant isolates of M-A in minced chicken ranged between 6.6 and 7.3 min (*37*). Using beef as the substrate and averaging the results for duplicate trials with 3 radiation resistant isolates of M-A, the D value at 68 C in low-fat (average 5.2%) and high-fat (average 44.4%) beef were 9.2 and 9.3 min, respectively (*39*). Furthermore, there is evidence to indicate that the lethal effect of radiation may be enhanced by a mild pre- or post-irradiation heat treatment (*37*). In our experience, the level of aerobic and anaerobic cells contaminating enzyme-inactivated and frozen beef just before irradiation was minimal (Table 2). Some of these cells were probably heat-damaged (*15,48,49*) and others could be due to contamination subsequent to heating.

TABLE 5. *Resistance of various microorganisms in ground beef to gamma rays.*

Microorganism	Resistance values: Radiation (kGy)[a] First decimal reduction	D Value	Reference
Clostridium botulinum spores 33A	-	4.13[b]	*28*
Clostridium botulinum spores 33A	9.75	2.94	*38*
Coxsackie virus B-2	-	7.50	*59*
M-5 (#4)[c]	22.30	6.28	*38*
M-5 (#7)[c]	40.00	4.52	*38*
Salmonella enteritidis	-	1.07	*38*
Staphylococcus aureus	-	0.95	*38*
Escherichia coli	-	0.59	*38*

[a]Radiation temperature was - 30 C.
[b]D value was computed from partial spoilage data. All other D value data were computed from survival counts.
[c]*Moraxella-Acinetobacter* group of organisms.

All evidence now available indicates that beef heated to an internal temperature of 73 to 77 C to inactivate autolytic enzymes, vacuum packed, frozen to about - 40 C and irradiated with a 12D dose of 41 kGy would be free of microbial spoilage and health hazards during an extended shelf-life without refrigeration.

Radiation sterilization is not designed to destroy aflatoxins (*41*) or bacterial toxins such as *C. botulinum* neurotoxin (*63*). Furthermore, like other methods of food manufacturing, it is important to use raw foods of good microbiological quality, proper processing procedures with adequate record keeping, good personal hygiene and sanitation practices, and adequate packaging, and that the processed foods be appropriately handled during distribution (*11*).

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Survival of *Klebsiella pneumoniae* Heated in Buffer and in Tomato Juice

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ABSTRACT

Klebsiella pneumoniae survived nonexponentially when heated in phosphate buffers at pH 7.0 and 4.3 and in tomato juice at pH 4.3. At 60 C, an initial inoculum of 10^9 cfu/ml decreased by 6 log units in 15 min, with a long tailing of the survivors to 75 min of heating. At 67 C, total destruction of *K. pneumoniae* was attained in both buffers at 25 min, and in tomato juice at 40 min. Factors influencing heat resistance of the bacterium are discussed.

Gram-negative bacteria heated in liquid media die at an initially rapid rate with an estimated short heating time to achieve the death of all cells. D-values calculated for these bacteria frequently do not describe their heat resistance, since extensive tailings of the survivor curves are not considered (*9*), and the tailings may be to 120 min for very few survivors among a large initial number of cells subjected to heat. Tailing has been observed for several types of salmonellae (*9,20*) and other bacteria (*8*). Heating to 85 C for 40 min was required to destroy *Salmonella typhimurium* in minced chicken muscle (*17*). Angelotti et al. (*1*) observed a rapid decrease in numbers of salmonellae in chicken a-la-king heated to 140 F, and their data suggest the recognition of a tailing effect, leading to a recommended heating time of 4D or 6D to ensure destruction of all salmonellae. Dabbah et al. (*11*) observed the recovery of an unidentified *Pseudomonas* species heated at 55 C for 30 min, when the proper conditions for subculture were followed.

Resistance to heat in liquids may be influenced by physiological age (*2,13,25*), solutes (*2*), concentration of the initial product (*19*), peptides and selected amino acids or sodium acetate (*20*), variation within strains (*19*), water activity (*3,7,14*), particulate matter (*2*), random distribution of critical sites (*20*), and variation of individuals in a large population, with those possessing abnormal resistance to heat in the minority (*6*). Cerf (*5*) states that tailing is a normal feature in heat resistance studies.

Klebsiella pneumoniae is a gram-negative bacterium of the nonfecal coliform group. Berry (*4*) recovered chiefly *Aerobacter* (*Enterobacter*) species from spinach and string beans, but not English peas, after scalding at 95 C for 1 to 3 min. Since in the past many nonmotile cultures that actually were klebsiellae were classified as members of the genus *Aerobacter* (*12*), it is possible that Berry's isolates were klebsiellae. *K. pneumoniae* was recovered from a number of containers of home-canned tomatoes and tomato juice (*22*). While it is possible that some of the occurrence of this organism may be attributed to post-processing contamination, the probability that this is generally true is precluded by the relatively high percent of the containers from which the bacterium was recovered.

Because of the reports of salmonellae with extremely long survival times during heating, the recovery of *K. pneumoniae* prompted a study of the resistance of this bacterium to heat. It will be shown that *K. pneumoniae* exhibits a pattern of initially rapid decrease in numbers of viable cells, followed by a prolonged period of survival of very small numbers of cells.

MATERIALS AND METHODS

Two strains of *K. pneumoniae* which had been isolated from home-canned tomatoes were used. Flasks containing 150 ml of trypticase soy broth were inoculated with 1 ml of a 24-h-old culture grown in the same medium and incubated 22 to 24 h at 37 C. The cells were concentrated by centrifugation, resuspended with agitation on a vortex mixer in 5 ml of 0.15 M phosphate buffer (PB) adjusted to the pH of the fluid in which they were to be heated, and filtered through glass wool. The suspensions were then added to the fluids in which they were to be heated.

Heating was accomplished in 100 ml of PB at pH 7.0 or 4.3 and in tomato juice at pH 4.3, sterilized 15 min at 121 C. The flasks were stirred with magnetic stirring bars which were used at the time of addition of the suspension and at each removal of an aliquot. The buffers and tomato juice were equilibrated to 60 or 67 C by immersion of the flasks in waterbaths overnight. The flasks were maintained in the baths during the heat-experiments, except for momentary removal to the magnetic stirrer as aliquots were removed.

Aliquots were taken at 0 time, and at 1, 5, 10, 15, 20, 30, 45, 60 and 75 min from flasks heated to 60 C, and through 40 min when heated to 67 C. Aliquots removed during the first 15 to 20 min were surface-plated on trypticase soy agar, and thereafter survivors were enumerated by the three-tube MPN procedure in lactose-nutrient broth. All data represent the minimum of three replicates. Identity of growths in broth was confirmed to be *K. pneumoniae* by streaking on EMB agar.

To determine the effect of anaerobiosis on outgrowth, procedures just outlined were repeated, except that aliquots were removed beginning with 15 min of continuous heating. The aliquots were serially diluted for enumeration of survivors by the three-tube MPN procedure. Duplicate series of tubes were inoculated, with one series in each replicate over-laid with sterile mineral oil. All media were incubated for 48 h at 37 C.

RESULTS AND DISCUSSION

Survival curves of *K. pneumoniae* heated in PB at pH 7.0 and 4.3, and in tomato juice, are shown in Fig. 1, 2

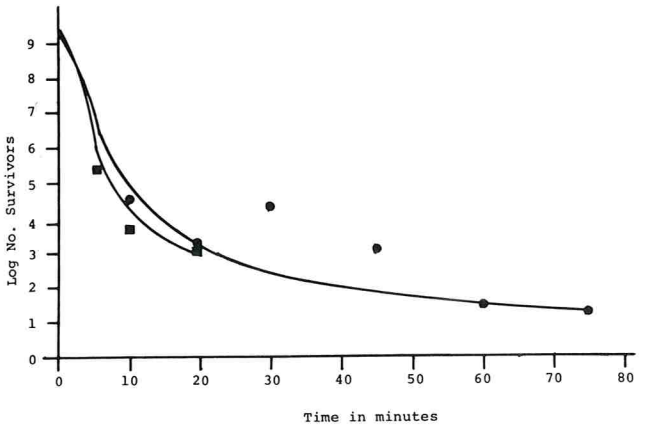

Figure 1. *Survival of* Klebsiella pneumoniae *heated in phosphate buffer, pH 7.0, at ● 60 C ■ 67 C.*

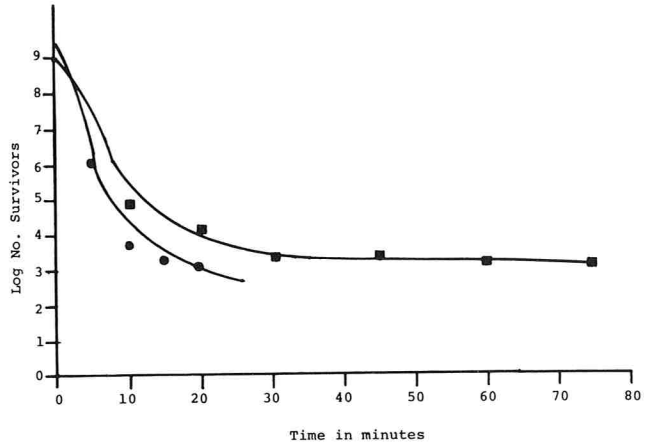

Figure 2. *Survival of* Klebsiella pneumoniae *heated in phosphate buffer, pH 4.3, at ● 60 C and ■ 67 C.*

and 3. The curves of survival at 60 C in each fluid are similar to those of Dabbah et al. obtained with milk (*9*) and with liquid whole egg homogenate (*10*). The numbers of cells are reduced by approximately 6 logs during the first 20 min of heating at 60 C in each of the three fluids. Thereafter the numbers of cells decrease very slowly to result in a long tail which extended to 75 min.

At 67 C, the number of survivors decreased rapidly during the first 10 min in both the buffers and in tomato juice. The numbers continued to decrease rapidly in each of the buffers, and less rapidly in tomato juice, with complete destruction in the buffers at 25 min and in tomato juice at 40 min. Moats et al. (*21*) noted the protective effect of organic materials when survival in broth and buffer was compared. The ability of the bacteria to survive for a longer time in tomato juice may be due to the complexing of components of the tomato with heat-sensitive protein to render it more heat stable (*19*) or to the particulate (*24*) or colloidal (*18*) nature of the juice or production of protective substances excreted by the cells (*18*).

At both temperatures in the buffers and in tomato juice the results were reproducible during the logarithmic reduction in numbers. With extended heating, variations in numbers and endpoints occurred between

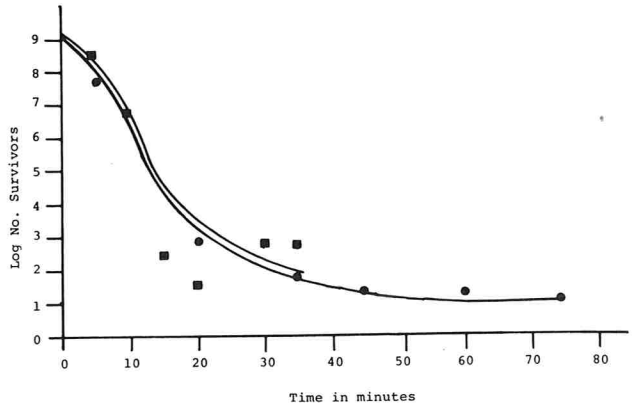

Figure 3. *Survival of* Klebsiella pneumoniae *heated in tomato juice, pH 4.3, at ● 60 C and ■ 67 C.*

replications. Graumlich and Stevenson (*15*) attributed such variations to the rapid destruction of cells during the initial period of heating. Variations may also be due to the existence of variable numbers of heat-resistant cells in successive preparations and to variation in the initial numbers of cells. The higher heat resistance of large cell populations over smaller populations provides a greater chance for the presence of organisms with different degrees of natural heat resistance (*18*). In the experiments reported here, the initial numbers varied between 1.0 and 1.3×10^9 cfu/ml, or a range of 3×10^8 cfu/ml. When the initial numbers of cells approximated 1×10^8 cfu/ml, no survivors were detected at 60 min when heated at 60 C.

Numbers and endpoints were identical or exhibited very little variation in the duplicate series of MPN tubes with and without mineral oil. Oxygen thus did not appear to affect survival of *K. pneumoniae* in these experiments.

K. pneumoniae has been isolated from most fresh tomatoes examined in our laboratory, either in higher or lower numbers than those of other nonfecal coliform bacteria. The isolation of only this member of the nonfecal group from home-canned tomatoes and juice implies a resistance to heat which is greater than that of the associated bacteria.

In this study, the bacterium was exposed continuously to the temperatures of the experiment. In home canning, it is questionable whether the equivalent amount of heat would be introduced to the centers of the container contents. Harris and Davis (*16*) attained an internal temperature around 180 F (80 C) when the initial pack temperature was adjusted to 150 F (59 C) in boiling water vessels equipped with clamped covers. In the cold-pack home-canning process, the initial temperature is more likely to be 26 to 33 C, and covered vessels are not commonly used. Coupled with the slow transfer of heat through particulate material which will impede or prevent distribution of heat uniformly by convection, Powers (*23*) has projected a final temperature of 71 C in the center of containers processed in the boiling water bath. There is also the probability that the preheated

materials are contaminated during the filling process by failure to perform interim sanitation and hand washing.

One may conclude that *K. pneumoniae* is a bacterium for which the heat required for destruction of all cells is very close to that given during the canning process. Without actual chronometric and temperature monitoring in the individual kitchens it becomes impossible to assign survival to specific conditions. That variations do exist may be inferred by the sporadic isolations of the bacterium during a large sampling of home-canned tomatoes and tomato juice.

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A Research Note

Influence of Reconstitution on Isolation of *Salmonella* from Dried Milk

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ABSTRACT

When milk powder is examined for the presence of salmonellae using a volume of preenrichment medium three times or more that of the milkpowder, false-negative results may be obtained. Reconstitution for half an hour at room temperature using an amount of medium twice the volume of the milk powder, before further dilution and preenrichment, increased *Salmonella* recovery.

Many comparative studies on isolation of salmonellae from dried products have been carried out using various preenrichment and enrichment media, but to our knowledge, the influence of the sample treatment on the performance of a method has received only limited attention (*1,6*). In comparative trials on isolation of salmonellae from rejected batches of pancreatic powder, the industry's (Organon) laboratory had a significantly higher recovery than the National Institute of Public Health (NIPH) laboratory while essentially the same method was used. The main difference appeared to be that in the NIPH laboratory the powder was mixed with the preenrichment medium, while in Organon's laboratory the powder was placed on top of the fluid without mixing. Using the latter method, the powder was soaked with preenrichment fluid for a varying period, gradually dissolving, while with the NIPH method the powder was dissolved directly.

Ray et al. (*5*) found that reconstitution of milk powder for 1 h in a small volume of preenrichment fluid (dilution ration 1:2.5) gave a higher MPN count than did reconstitution in a greater volume (dilution ratio 1:10). It has been demonstrated that the method of rehydration of freeze-dried cells may have a significant influence on their recovery (*3,4*). In light of this and our findings we started to examine the effect of rehydration procedures on isolation of *Salmonella* from products like milk powder, cocoa, and dried egg white. Moreover we tried to elaborate a sample treatment which would be easier to standardize than a "soaking" procedure.

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MATERIALS AND METHODS

Dried milk was mixed with artificially contaminated milk powder in such a way that batches with different numbers of salmonellae were obtained. Contaminated powder was prepared by spray-drying condensed milk which was inoculated with 24-h-old *Salmonella* cultures that were washed twice with phosphate buffer. Two *Salmonella* strains were used: *Salmonella typhimurium* and *Salmonella panama*. The samples were examined using the "soaking", the "shaking" and various reconstitution procedures using buffered peptone water (BPw) as preenrichment medium. In the first two techniques, the ratio of powder to BPw was 1:9 (w/v), while for reconstitution other ratios were used. In the "soaking" procedure, powder was poured carefully on the medium, avoiding mixing. In the "shaking" procedure, powder was shaken by hand for 10 sec with BPw. For reconstitution the powder was shaken by hand for 10 sec with the fluid in a ratio of 1:1.5, 1:2, 1:2.5, 1:3 and 1:5 (w/v). These reconstituted powders had water activities of 0.965, 0.973, 0.977, 0.980 and 0.984 ± 0.003, respectively. Following reconstitution at room temperature the samples were further diluted to the final ratio of 1:9 (a_w 0.991).

Screw-capped glass containers of 300 ml capacity and 6-cm inside diameter were used for shaking, soaking and reconstitution. Twenty-five grams of sample were used throughout. The preenrichment media were incubated at 37 C for 16-20 h, then 10 ml of the BPw were inoculated into 100 ml of tetrathionate bile brilliant green (TBB). Following 24 h of incubation at 43 C, plating was carried out on large (14-15 cm) brilliant green agar plates (*2*). After 24 h of incubation at 37 C suspected colonies were confirmed biochemically and serologically. The MPN count to determine the contamination level was carried out using the soaking procedure taking 5 times 100 g, 10 g, 1 g and 0.1 g of the batch. A limited number of similar experiments was carried out with egg white and cocoa powder.

RESULTS AND DISCUSSION

Some of our results (more results will be published in the near future) are presented in Tables 1 and 2. From data in Table 1 it is clear that reconstitution of milk powder in 1.5-2 times the amount of buffered peptone water leads to essentially the same results as the soaking procedure and gives a much higher recovery than the shaking procedure.

The reconstitution period is not of great importance as long as the period is not shorter than 10-20 min in batches with a low contamination rate (batch 3 in Table 2). With *S. panama* we obtained the same type of results as with *S. typhimurium* reported in Tables 1 and 2. The reconstitution effect became apparent when the contamination level was less than approximately 1

TABLE 1. *Salmonella isolation from dried milk using different dilutions for reconstitution.*

Sample treatment	Dilution ratio	Batch[a] 1	Batch[a] 2
Soaking	1:9	23[c]	23
Reconstitution[b]	1:1.5	25	24
"	1:2	25	24
"	1:2.5	20	20
"	1:3	19	22
"	1:5	8	11
Shaking	1:9	8	11

[a]25 samples of 25 g containing ca. 3.5 salmonellae per sample were examined per batch.
[b]After 2 h of reconstitution at room temperature, samples were further diluted to a final ratio of 1:9.
[c]Number of positive samples.

TABLE 2. *Salmonella isolation from dried milk using different reconstitution times.*

Sample treatment	Dilution ratio	Time of reconstitution (min)[b]	Batch[a] 3	Batch[a] 4	Batch[a] 5
Soaking	1:9		13[c]	25	25
Reconstitution	1:2	2½	8	23	20
"	"	5	9	25	23
"	"	10	15	24	22
"	"	20	14	25	ND[d]
"	"	30	15	25	25
"	"	60	ND	ND	24
Shaking	1:9		2	13	9

[a]25 samples of 25 g containing ca. 0.8 (batch 3) or ca. 8 (batches 4 and 5) salmonellae per sample were examined per batch.
[b]After the given time of reconstitution at room temperature the samples were further diluted to a final ratio of 1:9.
[c]Number of positive samples.
[d]Not done.

salmonella per gram and when the milk powder was stored at 4 C for 3 weeks or longer. We have no data for milk powder which was stored for shorter periods. The experiments with cocoa powder and egg white indicate that reconstitution using a small volume of BPw may lead to false-negative results, most probably due to the inhibitory properties of these commodities.

The effect of reconstitution on isolation of salmonellae is most probably due to dehydration of the cells caused by the low water activity (a_W) during storage. The water activity during reconstitution could therefore be a more accurate parameter than the dilution ratio but it still has to be proved. Figure 1 was drawn to relate the results of the reconstitution experiments with batch 1 and 2 (see Table 1) to water activity. For determination of the *Salmonella* isolation efficiency, the mean number of positive samples per treatment was divided by 25 and multiplied with 100. In Fig. 1 it is shown that the critical a_W was around 0.98. When the milk powder was diluted to a higher a_W, cells died. Cells remained viable when

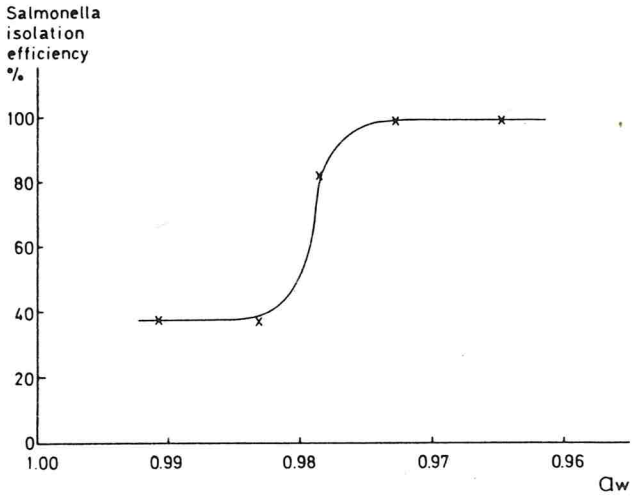

Figure 1. *Salmonella isolation efficiency related to the water activity of the buffered peptone water - milk powder mixture during reconstitution, using the data of Table 1.*

they were first brought into an environment with an a_W of less than 0.98. Soaking the powder will increase the a_W gradually to the high a_W (0.99) of the final 1:9 dilution thus protecting the cells from "osmotic shock".

Since soaking may be carried out in various ways in different laboratories, this procedure may lead to differences in results. We therefore recommend the following procedure as a standard method: reconstitution of a 25-g dried milk sample in 50 ml of buffered peptone water for a half an hour at room temperature followed by a further dilution with 175 ml of buffered peptone water and subsequent incubation for 16-20 h at 37 C.

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Significance of Psychrotrophic Strains of *Serratia liquefaciens* in Milk[1]

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ABSTRACT

Enterobacter cloacae, Citrobacter freundii, Serratia liquefaciens and *Escherichia coli* were the predominant *Enterobacteriaceae* species isolated from raw milk samples collected from refrigerated bulk tank trucks at the entry of a milk processing plant. About half of the 181 *Enterobacteriaceae* isolated were psychrotrophs and these included 80% of *E. cloacae*, 80% of *Klebsiella ozaenae* and 62% of *S. liquefaciens*. *S. liquefaciens* grew in raw and in pasteurized milk at refrigeration temperatures, causing significant pH reduction but only slight lipolytic changes. The significant proteolytic activity of *S. liquefaciens* in refrigerated milk samples is assumed to have been the cause of the unclean flavor detected.

Although *Enterobacteriaceae* are commonly regarded as mesophiles, much evidence exists about the occurrence of strains able to multiply and to cause undesirable changes in milk at temperatures below 5 C (*7,9,10,11,13*). Reports about psychrotrophic strains of *Enterobacteriaceae* isolated from raw milk refer to species of the *Klebsiella-Enterobacter* group as predominant organisms (*9,13*). Depending on the species and numbers involved, the presence of psychrotrophic *Enterobacteriaceae* in milk and dairy products has one or two points of significance: potential impairment of shelf-life, and index of poor sanitation.

The objectives of this study were to determine some of the changes caused by psychrotrophic strains of *Serratia* (*Enterobacter*) *liquefaciens* in milk held at refrigeration temperatures.

MATERIALS AND METHODS

Milk samples

Raw milk. Samples of raw whole milk were collected aseptically during a 1-month period from refrigerated bulk tank trucks at the entry of a milk processing plant. They were brought refrigerated to the laboratory and analyzed within less than 3 h.

Pasteurized milk. Aseptically drawn whole raw milk, obtained at The Volcani Center dairy farm, was pasteurized in the laboratory at 63 C for 30 min in a constant temperature bath. Total aerobic (PCA at 32 C for 48 h) and psychrotrophic (PCA at 7 C for 10 days) plate counts indicated less than one organism per ml of the pasteurized milk.

Enterobacteriaceae

Enumeration. Serial dilutions of the milk samples in 0.1% peptone were plated in double-poured plates of violet red bile agar (Difco) with 1% glucose (VRBG) and incubated for 24 h at 32 C.

Identification. All colonies giving a typical reaction on VRBG agar were isolated from plates which had 5 to 30 colonies. A total of 207 colonies was picked and streaked on plates of MacConkey agar (Difco) for purification. They were identified to the species with the Minitek system (BBL), following the manufacturer's instructions. Isolates giving positive results for glucose fermentation and nitrate reduction tests were taken as *Enterobacteriaceae*. Isolates of *Enterobacteriaceae* were also inoculated into lactose broth (Difco) in test tubes containing inverted Durham vials, and production of gas determined within 48 h incubation at 32 C. Lactose-positive strains were classified as coliforms.

The identity of four of the isolates - classified as *S. liquefaciens* by the Minitek system - was also confirmed by conventional tests, carried out according to the procedures of Edwards and Ewing (*2*) and following the system of classification and nomenclature proposed by Ewing (*3*). These four isolates were used throughout the study. Additional tests for cultural characteristics of *S. liquefaciens* were done as outlined in the *Laboratory Methods in Food and Dairy Microbiology* (*4*) and included proteolysis in skim milk - nutrient agar and lipolysis on Tween 80 agar, results being recorded after 7 days of incubation at 20 C.

As to other *Enterobacteriaceae*, no attempts were made to determine the agreement between results obtained with the Minitek system and those of conventional tests.

Psychrotrophic strains. Isolated colonies - taken from surface growth on plates of PCA after 24 h of incubation at 32 C - were suspended in 0.1% peptone to a turbidity similar to McFarland No. 3 turbidity standard. The suspension was streaked on pre-poured plates of PCA and the plates were incubated at 7 C. Strains showing significant growth within 3 days were considered psychrotrophic.

Serratia liquefaciens in milk

Growth. Before being used for inoculation, *S. liquefaciens* was subcultured at least twice in sterile skim milk (Oxoid Ltd., England; 10% solids) at 24-h intervals and incubated at 32 C. Inocula containing approximately 10^5 organisms per ml, prepared by diluting a 24-h-old culture in fresh skim milk, were added to freshly pasteurized whole milk at a concentration of 1% (v/v). The inoculated milk was divided into 10-ml portions in screw-cap test tubes (15 × 125 mm) and incubated at 2, 5, 7, 10 or 15 C in thermostatically-controlled water baths. Before inoculation, the milk samples were tempered to the selected incubation temperature. At selected time intervals tubes were taken and viable bacterial counts were performed by surface-plating 0.1-ml aliquots of serial dilutions on plates of trypticase soy agar (BBL) and incubating the plates at 20 C for 3 days.

Growth rates were calculated from the change in counts during the first 4 days of incubation, and expressed as log of CFU/ml/day.

Chemical changes. The following characteristics were measured in pasteurized whole milk inoculated with *S. liquefaciens*: pH, titratable acidity, reduction of methylene blue, degree of lipolysis and proteolysis.

pH and titratable acidity. The pH was measured with a Radiometer PHM 26 pH meter (Radiometer A/S, Copenhagen, Denmark). The titratable acidity was determined by titrating a 10-ml sample to pH 8.6 with 0.1 N NaOH; it is expressed as percent of lactic acid.

Lipolysis. The free fatty acids (FFA) content of milk was determined by the extraction-titration method described by Deeth and Fitz-gerald (*1*); it is expressed as micro-equivalents/ml.

Proteolysis. Hydrolysis of milk proteins was followed by the colorimetric method of Hull (*6*); it is measured by the increase in free

[1]*Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. No. 107-E, 1979 series.*

tyrosine plus tryptophane in milk, and converted to tyrosine equivalent (μg/ml) from a standard curve.

Methylene blue reduction. S. liquefaciens was grown in whole milk for about 9 days at 5 C and then decimal dilutions were made in milk. Methylene blue reduction as affected by increasing concentration of *S. liquefaciens*, was determined by the procedure described in the *Standard Methods* (5).

Heat coagulation. Ten milliliters of milk in screw-capped test tubes were heated for 30 min in a water bath at 63 C.

Sensory evaluations. Immediately after sampling for microbiological analysis, the samples were tempered to 20 C, and then checked for flavor and physical changes by a panel of three judges. Uninoculated samples were used for comparison.

RESULTS

From 207 colonies, isolated from 30 samples of raw milk on plates of VRBG agar and identified as gram-negative rods, 181 (87.4%) were confirmed as *Enterobacteriaceae*. On the basis of gas production from lactose, 112 (61.9%) of the *Enterobacteriaceae* were classified as coliforms.

Table 1 shows that the predominant species were (% of total isolates of *Enterobacteriaceae*): *Enterobacter cloacae* (20.4), *Citrobacter freundii* (17.7), *Serratia liquefaciens* (17.7) and *Escherichia coli* (14.9). Of 165 isolates tested, 47% were defined as psychrotrophs and included the majority of *E. cloacae, Klebsiella ozaenae* and *S. liquefaciens* (Table 1).

TABLE 1. *Distribution of* Enterobacteriaceae *isolated from raw milk.*

Species	Number of isolates	% of psychrotrophs in each species
Citrobacter diversus	5	0
C. freundii	32	21
Enterobacter agglomerans	11	54
E. cloacae	37	80
E. hafniae	11	0
Escherichia coli	27	33
Klebsiella ozaenae	5	80
K. pneumoniae	16	NT[a]
Serratia liquefaciens	32	62
S. marcescens	5	0
Total	181	

[a]Not tested.

The growth of four isolates of *S. liquefaciens* was studied in raw and pasteurized milk incubated at five different temperatures. The data obtained with one of the isolates in pasteurized milk are presented in Fig. 1. After 4 days of incubation, counts increased from 3.2 log to 3.6 (2 C), 5.0 (5 C), 5.3 (7 C), 6.8 (10 C) and 8.0 (15 C) CFU/ml. About the same results were obtained when aseptically drawn raw milk was used in place of pasteurized milk.

The growth of *S. liquefaciens* brought about an increase in titratable acidity and a decrease in pH, which were greater at higher incubation temperatures (Fig. 2 and 3).

The extent of lipolysis caused in milk by *S. liquefaciens* is shown in Fig. 4. At 7 C *S. liquefaciens* caused an increase from 0.23 to 0.24 - 0.29 and to 0.34 - 0.56 μeq FFA/ml after 2 and 5 days of incubation,

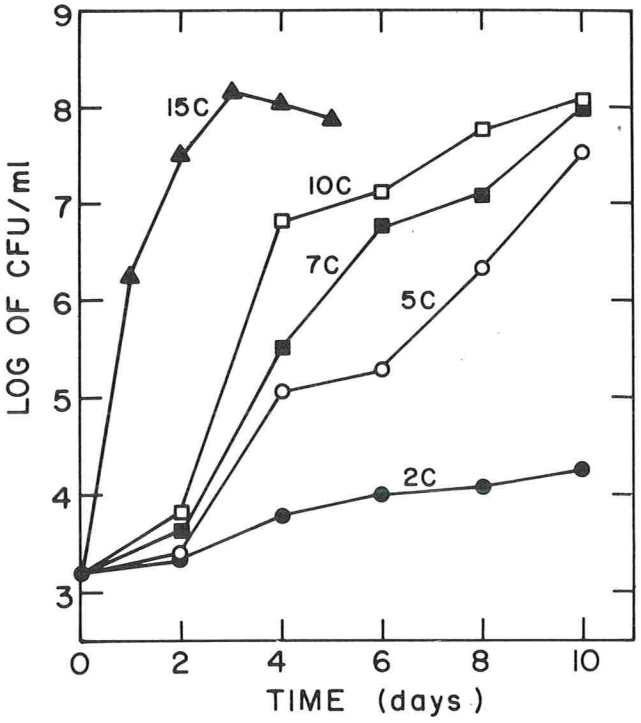

Figure 1. *Growth of psychrotrophic* S. liquefaciens *in whole pasteurized milk at five different temperatures.*

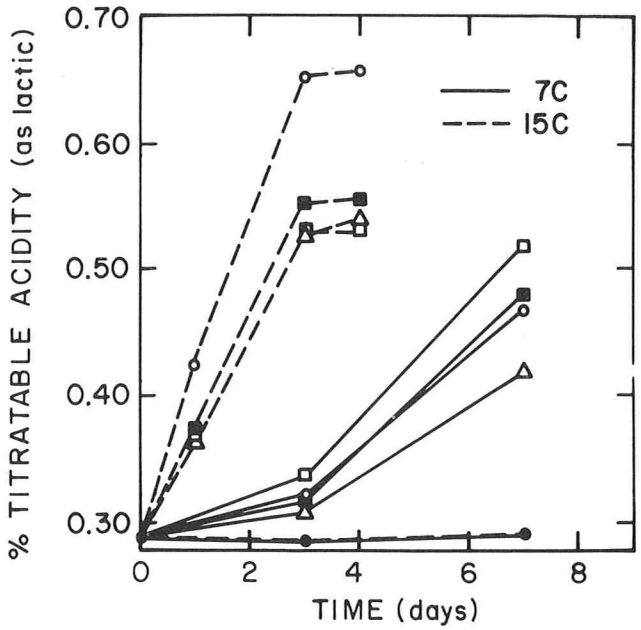

Figure 2. *Changes in titratable acidity occurring in uninoculated milk (filled circles) and in milk inoculated with each of four isolates of* S. liquefaciens *(empty triangles, empty squares, empty circles and filled squares) at 7 or 15 C.*

whereas at 15 C the FFA were raised to 0.45 - 0.55 and to 0.72 - 0.93 μeq/ml after 2 and 5 days of incubation, respectively.

After 3 days of incubation, *S. liquefaciens* caused an increase in free tyrosine plus tryptophane contents in milk from 6.0 to 16.4 - 17.0 μg/ml at 7 C and to 20.0 -21.5 μg/ml at 15 C (Fig. 5).

Heat coagulation tests showed that the higher the temperature at which pasteurized milk samples inocu-

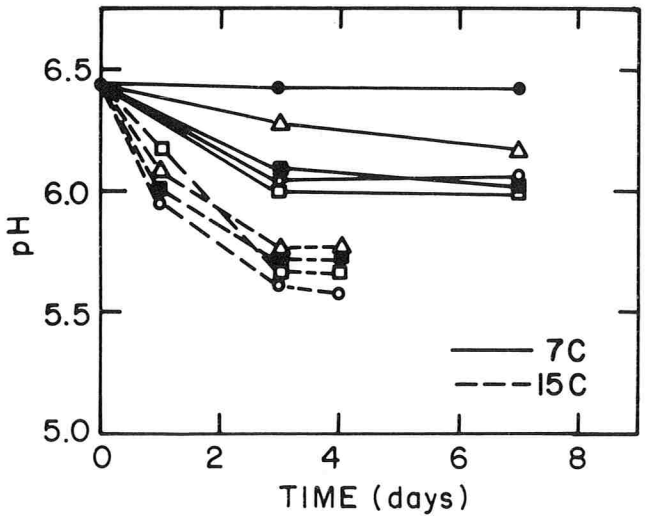

Figure 3. *Changes in pH occurring in uninoculated milk (filled circles) and in milk inoculated with each of four isolates of* S. liquefaciens *(empty triangles, empty squares, empty circles and filled squares) at 7 or 15 C.*

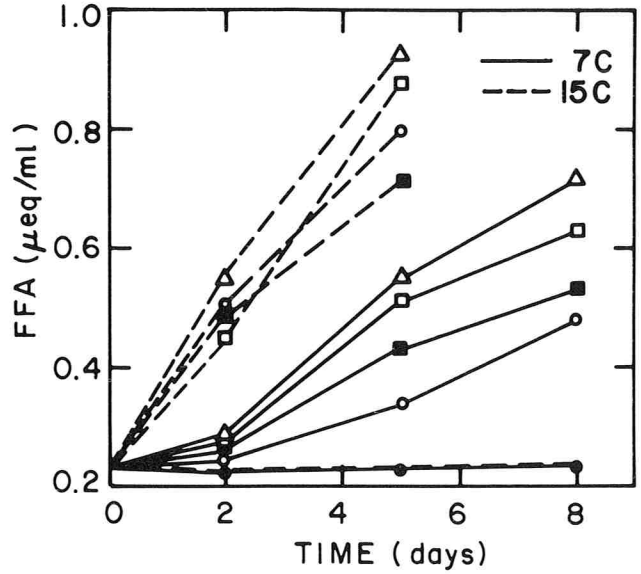

Figure 4. *Changes in free fatty acids (FFA) content in uninoculated milk (filled circles) and in milk inoculated with each of four isolates of* S. liquefaciens *(empty triangles, empty squares, empty circles and filled squares) at 7 or 15 C.*

lated with *S. liquefaciens* were held, the shorter the time necessary for the milk to coagulate after 30 min at 63 C: 2, 4, 6, 8 and 10 days of storage at 15, 10, 7, 5 and 2 C, respectively.

None of the *S. liquefaciens* isolates tested showed proteolytic activity on skim-milk nutrient agar or lipolytic activity on Tween 80 agar.

Figure 6 shows counts of *S. liquefaciens* grown in milk at 5 C plotted against methylene blue reduction times, and indicates a highly significant inverse correlation between the two series of values ($r = -0.958$; $p < 0.0001$).

In milk samples inoculated with *S. liquefaciens* and held at 2 C, flavor changes were not detected during 10 days. At 5, 7, 10 and 15 C, astringency plus an off-flavor characterized as unclean appeared after 6, 5, 4 and 2 days, respectively.

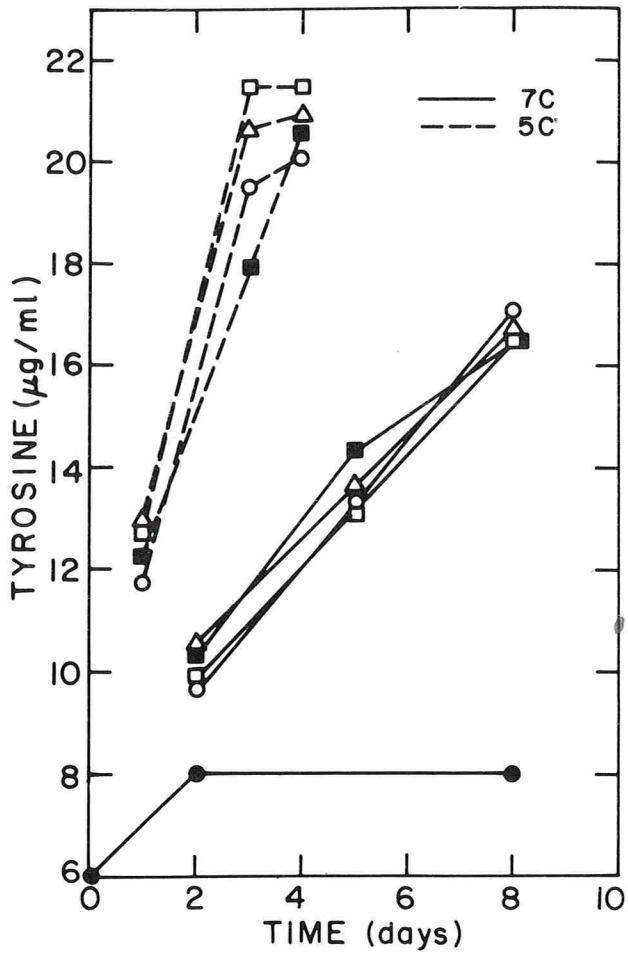

Figure 5. *Proteolytic action of four isolates of* S. liquefaciens *in milk at 7 and 15 C.*

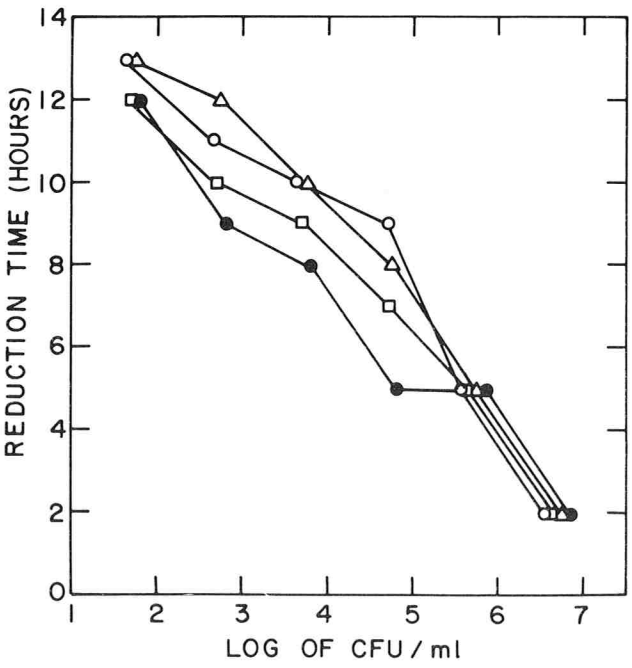

Figure 6. *Relationship between counts of four isolates of* S. liquefaciens *grown in milk at 5 C and the time taken to reduce methylene blue at 37 C.*

DISCUSSION

Samples of raw whole milk were collected from refrigerated bulk tank trucks at the entry of a milk processing plant and analyzed for *Enterobacteriaceae*. From 207 colonies of gram-negative rods isolated on plates of violet red bile glucose agar, 87.4% were confirmed as *Enterobacteriaceae*. About 62% of the *Enterobacteriaceae* isolates were coliforms.

The following arguments lend support to an enumeration of total *Enterobacteriaceae* rather than a coliform count in foods: (*a*) the first are a taxonomically - defined group and (b) the lactose-negative strains of this family do not differ from the lactose-positive ones, either in their sanitary or their public health significance.

E. cloacae, C. freundii, S. liquefaciens and *E. coli* were the predominant *Enterobacteriaceae* species isolated from the raw milk samples.

About half of the *Enterobacteriaceae* isolated in this study were psychrotrophs and these included the majority of *E. cloacae, K. ozaenae* and *S. liquefaciens.*

Panes and Thomas (*9*) found that about one-third of 108 raw milk samples contained psychrotrophic coliform strains, and that species of the genus *Enterobacter* were dominant among these populations.

Serratia (*Enterobacter*) *liquefaciens* constituted 17.7% of the *Enterobacteriaceae* isolated in this study and 62% of the strains of this species were psychrotrophs.

It is common practice for large volumes of raw milk to be kept for relatively long periods at refrigeration temperatures before being processed. During these periods, psychrotrophic bacteria can grow and cause irreversible changes.

In raw and in pasteurized milk at refrigeration temperatures *S. liquefaciens* exhibited the following growth rates (increase in log of CFU/ml/day): 2 C, 0.0 - 0.1; 5 C, 0.4 - 0.6; 7 C, 0.5 - 0.9; 10 C, 0.9 - 1.2; and 15 C, 1.2 - 1.6. Multiplication of *S. liquefaciens* brought about an increase in titratable acidity and a decrease in pH which were greater at high incubation temperatures. The reduced heat stability of inoculated milk samples is explainable on the basis of the significant pH reduction (*12*).

Lipolytic changes occurred in milk inoculated with *S. liquefaciens* and stored at 7 C or 15 C. However, it seems that they were not large enough to explain significant changes in flavor. According to Deeth and Fitz-gerald (*1*), levels below 1.0 μeq FFA/ml usually do not produce detectable off-flavors.

On the other hand, the proteolytic changes caused in milk by *S. liquefaciens* could explain the production of the off-flavors detected. The levels of tyrosine released in milk at 7 or 15 C from the proteolytic activity of *S. liquefaciens* were higher than those reported by Mayerhofer et al. (*8*) and which were connected with the appearance of detectable flavor defects in milk containing bacterial proteases.

Psychrotrophic bacterial strains isolated from milk are often screened for potential proteolytic and lipolytic activities on agar assay media containing an adequate substrate. In spite of the proteolytic and lipolytic activities of *S. liquefaciens* demonstrated in milk, negative results were obtained on agar media. In the case of proteolysis, it may be concluded, therefore, that the screening test performed on agar media is not sufficiently sensitive to detect a significant proteolytic potential of bacteria.

Dye reduction tests in general and the methylene blue test in particular depend not only on microbial content in the milk sample but also on the relative metabolic rates of the organism(s) present. Therefore, the value of these tests - for the assessment of the microbiological quality of milk previously stored for long periods at low temperatures - has been questioned. After incubation in milk for 9 days at 5 C, a highly significant correlation was obtained between counts of psychrotrophic *S. liquefaciens* and methylene blue reduction times. Comparison of the measured reduction times with proposed standards of quality indicates, however, that in spite of high bacterial counts, the quality of a milk sample could be erroneously established as satisfactory on the basis of reduction times.

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Accuracy of Micro-ID for Identification of *Salmonella* and Other *Enterobacteriaceae* from Clinical and Food Sources

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ABSTRACT

The accuracy of a new 4-h *Enterobacteriaceae* identification kit, not previously tested with food isolates, was evaluated by using 265 known bacterial cultures. These clinical and food isolates represented 11 genera of *Enterobacteriaceae*; about 10% were atypical strains. Micro-ID test strips were inoculated from 24-h brain heart infusion agar slants and then incubated at 37 C for 4 h. With initial tests, 251 of the 265 cultures (95%) were correctly identified to species. When cultures initially misidentified were re-inoculated into Micro-ID, accuracy of identification increased to 98%. In tests to determine whether this multitest system would perform efficiently if incubated overnight at a lower temperature, 100 cultures of *Enterobacteriaceae* were inoculated into Micro-ID strips and incubated at 22 C for 16 h. Under these conditions, the system correctly identified 86 cultures to genus and 78 to species. Most of the misidentifications involved *Enterobacter agglomerans*. The degree of accuracy of Micro-ID at 22 C for 16 h was comparable to that at 37 C for 4 h, i.e., 95%, for identification to genus of the following: *Arizona, Citrobacter, Edwardsiella, Escherichia, Klebsiella, Proteus, Salmonella, Serratia, Shigella* and *Yersinia*.

Micro-ID is a new biochemical identification kit (General Diagnostics, Division of Warner-Lambert Company, Morris Plains, New Jersey), which consists of 15 biochemical tests and requires only 4 h to identify *Enterobacteriaceae*. Several clinical laboratories have recently evaluated this system. Edberg et al. (*6*) reported that Micro-ID correctly identified 98% of the *Enterobacteriaceae* isolated from blood cultures. In 1978, Edberg and co-workers (*5*) found that the agreement between Micro-ID and conventional identifications was 98% for 350 fresh clinical isolates and 94% for 50 stock cultures. In other clinical studies, Micro-ID correctly identified 95% (*7*) and 90% (*1*) of the organisms tested. In a recent study (*3*) involving 400 *Enterobacteriaceae* freshly isolated from eight different foods, the biochemical test reactions obtained with the Micro-ID system were in 97% agreement with corresponding conventional tests. In that same study, three laboratory technicians independently recording results were in complete agreement for over 99% of the 6000 Micro-ID test reactions. This indicates that the results were easy to read and interpret.

There is no evidence in the literature to indicate that the Micro-ID system has been evaluated for accuracy in identifying *Enterobacteriaceae* isolated from food. Also, the efficiency of Micro-ID has not yet been tested at a lower incubation temperature for a longer time. The objectives of this study were (*a*) to evaluate the accuracy of the Micro-ID system in identifying known enterics, many of which were isolated from food, and (*b*) to determine whether Micro-ID would perform adequately if incubated for 16 h at 22 C instead of 4 h at 37 C.

EXPERIMENTAL PROCEDURE

Test cultures

A total of 265 known stock cultures representing 11 different genera of *Enterobacteriaceae* were used in this study, and about 10% of these cultures were atypical strains. The salmonellae serotypes used are shown in Table 1. Some of the salmonellae had been isolated from poultry in a previous study (*4*) and had been sent to the Diagnostic Bacteriology Laboratory, National Animal Disease Center (Ames, Iowa) for final confirmation and determination of the serotype. Others were biochemically atypical serotypes obtained from Ms. Alma Murlin of the Center for Disease Control (CDC), U.S. Public Health Service, Atlanta, Georgia. Some of the non-salmonella cultures were also obtained from this source; others had been isolated from food and identified in previous studies in this laboratory (*2,4*). Each culture was assigned a number, and its identity was not known to the analysts who conducted the tests.

Experiment 1

Manufacturer's instructions were followed for inoculating the Micro-ID system. A sufficient amount of each culture grown for 24 h on a Brain Heart Infusion (BHI) agar (Difco) slant was emulsified in 3.5 ml of physiological saline solution to produce a suspension with a turbidity equal to or greater than a McFarland No. 0.5 turbidity standard. A 0.2-ml aliquot of this suspension was pipetted into each of the 15 inoculation wells. After inoculation each Micro-ID unit was incubated upright for 4 h at 37 C in a support rack supplied by the manufacturer. Each culture was identified by coding results of the 15 tests into a 5-digit octal number, as per the manufacturer's

TABLE 1. *Salmonella serotypes used in this study.*[a]

albany (40)	litchfield (1)
anatum (7)	manhattan (1)
bareilly (1)	montevideo (5)
blockley (5)	muenchen (1)
branderup (1)	newington (1)
bredeney (3)	newport (3)
brookfield (1)	oranienburg (1)
chameleon (1)	panama (3)
chester (2)	poona (1)
cubana (1)	pullorum (1)
derby (6)	saint-paul (5)
enteritidis (1)	schwarzengrund (1)
gallinarum (1)	senftenberg (4)
heidelberg (12)	tennessee (7)
infantis (4)	thompson (4)
java (1)	typhimurium (12)
javiana (3)	typhimurium var. copenhagen (2)
kentucky (1)	

[a]The number of strains is shown in parenthesis.

instructions, and then referring to a Micro-ID identification manual to determine the species corresponding to the code. Bacteria incorrectly identified by Micro-ID were tested a second time.

Experiment 2

Of the 265 cultures, 100 grown for 24 h on BHI slants were inoculated into duplicate Micro-ID strips by the procedure described above. One strip was incubated for 4 h at 37 C, and the second for 16 h at 22 C. Results from both were recorded and compared.

RESULTS AND DISCUSSION

Experiment 1

Table 2 shows the accuracy of the Micro-ID system in identifying the 265 organisms. The 98% accuracy agrees with previously reported accuracies of the Micro-ID in identifying clinical isolates (*5,6,7*).

The misidentifications in our study, both initially and after retesting, were caused in part by aberrant reactions in the Micro-ID system and by atypical strains. One of the *Enterobacter agglomerans* cultures was initially misidentified because of false-positive adonitol and inositol reactions and a false-negative arabinose reaction. When retested, these erroneous reactions were not observed, and therefore the correct identification was obtained. Of four *Escherichia coli* cultures initially misidentified, the error for two of them was caused by the incorrect results obtained for the arabinose, lysine and/or Voges-Proskauer tests. When retested, these cultures were correctly named. The other two *E. coli* cultures, however were classified as *Citrobacter freundii* and *Serratia liquefaciens*, both initially and after retesting. *Klebsiella rhinoschleromatis* was originally classified as *Klebsiella pneumoniae* because of false-positive results with the Voges-Proskauer and lysine tests, but was accurately identified when retested.

TABLE 2. *Accuracy of Micro-ID in identifying known enteric cultures.*

Organism	Number tested	Number correctly classified upon initial testing	Number correctly classified after incorrect determinations were retested[a]
Arizona hinshawii	3	3	3
Citrobacter freundii	7	7	7
Edwardsiella tarda	1	1	1
Enterobacter agglomerans	39	38	39 (1/1)
Enterobacter cloacae	1	1	1
Enterobacter hafniae	1	1	1
Escherichia coli	33	29	31 (2/4)
Klebsiella ozaenae	1	1	1
Klebsiella pneumoniae	3	3	3
Klebsiella rhinoschleromatis	1	0	1 (1/1)
Proteus rettgeri	1	1	1
Proteus vulgaris	1	1	1
Salmonella spp.[b]	144	140	141 (1/4)
Serratia liquefaciens	22	19	22 (3/3)
Serratia marcescens	1	1	1
Serratia rubideae	1	1	1
Shigella boydii	1	1	1
Shigella dysenteriae	1	1	1
Shigella flexneri	1	1	1
Shigella sonnei	1	1	1
Yersinia enterocolitica	1	0	1 (1/1)
Total	265	251 (94.7%)	260 (98.1%)

[a]Number of correct identification per number of organisms retested is indicated parenthetically.

[b]The Micro-ID system will identify *Shigella sonnei* and *Shigella* spp. and *Salmonella typhi, Salmonella cholerae-suis* and *Salmonella enteritidis.* Complete identification of *Salmonella enteritidis* and *Shigella* spp. requires additional biochemical and/or serological tests.

Four salmonellae were misclassified. One strain (*Salmonella brookfield*) was KCN positive, ONPG positive and H_2S negative and was identified as *E. coli* both times. When *Salmonella schwarzengrund* was initially inoculated into Micro-ID, the octal code obtained from the results was not listed in the identification manual. The code was incorrect due to a false-negative ornithine reaction. When retested, all the reactions were the same as before except that the ornithine test was positive; and with the new octal code the organism keyed out correctly. For the other two salmonellae cultures, the errors were probably caused by a combination of incorrect reactions from Micro-ID (false-positive malonate, ONPG and adonitol) and atypical characteristics of the organisms (lactose, sucrose and ONPG positive and H_2S negative).

Three of the *Serratia liquefaciens* cultures were misclassified initially, but were properly identified after retesting. One of these was originally called *Enterobacter hafniae* because of false-negative arabinose, inositol and sorbitol reactions. The second culture keyed out initially as *Enterobacter cloacae* because of a false-positive Voges-Proskauer and a false-negative lysine test reaction. The third culture exhibited negative results for all 15 tests on Micro-ID; thus it was coded 00000, suggesting *Shigella* spp. or *Yersinia pseudotuberculosis*. However, when retested, it keyed out correctly as *S. liquefaciens*. An inadequate inoculum level may have caused the initial error. A false-negative urease test resulted in the classification of the *Y. enterocolitica* culture as *Shigella*; but when retested, the organism gave a positive urease reaction and was correctly identified.

Experiment 2

Clinical or food microbiology laboratories may receive cultures to be identified near the end of an 8-h work day. In such instances, a 4-h identification system would seemingly offer very little advantage over other systems requiring more time for identification, because the culture would either have to be held until the following morning for inoculation into Micro-ID or, if it were inoculated that afternoon, someone would have to return to the laboratory later that night to read and record the results. Therefore, we believed that many laboratories would want to know whether results for Micro-ID incubations overnight (16 h) at 22 C (approximately room temperature) would be the same as those for 4-h incubations at 37 C. Table 3 shows the variances in test results that were obtained when the Micro-ID strips were incubated under these two sets of conditions. Of the 100 organisms tested, 86% were correctly identified to genus and 78% to species when incubated at 22 C for 16 h. The Micro-ID system performed with a relatively high degree of accuracy at the lower incubation temperature for all the organisms tested except for *E. agglomerans*. Nine of

TABLE 3. *Comparison of Micro-ID identifications based on incubations at 37 C for 4 hours and at 22 C for 16 hours.*

Organism	Differences in resulted associated with the incubation procedure[a]		Agreement in identification to genus
	37 C-4 h	22 C-16 h	
Citrobacter freundii	OD −, U −, Sorb +	OD +, U +, Sorb −	Yes
Citrobacter freundii	Inos −, Sorb +	Inos +, Sorb −	Yes
Citrobacter freundii	H_2S +	H_2S −	No
Enterobacter agglomerans	VP +, OD −, LD −, Sorb +	VP −, OD +, LD +, Sorb −	No
Enterobacter agglomerans	OD −, LD −	OD +, LD +	No
Enterobacter agglomerans	OD −, LD −, Inos −	OD +, LD +, Inos +	Yes
Enterobacter agglomerans	VP −, OD −, LD −	VP +, OD +, LD +	No
Enterobacter agglomerans	OD −, LD −	OD +, LD +	No
Enterobacter agglomerans	OD −	OD +	Yes
Enterobacter agglomerans	OD −, LD −	OD +, LD +	No
Enterobacter agglomerans	OD −, LD −	OD +, LD +	Yes
Enterobacter agglomerans	OD −	OD +	Yes
Enterobacter agglomerans	OD −, LD −, Sorb +	OD +, LD +, Sorb −	No
Enterobacter agglomerans	OD −	OD +	Yes
Enterobacter agglomerans	OD −, LD −, Inos +, Sorb +	OD +, LD +, Inos −, Sorb −	Yes
Enterobacter agglomerans	OD −, Sorb +	OD +, Sorb −	No
Enterobacter agglomerans	OD −	OD +	No
Enterobacter agglomerans	OD −, LD −, Inos −	OD +, LD +, Inos +	No
Enterobacter agglomerans	Sorb +	Sorb −	Yes
Enterobacter cloacae	OD −, LD −, U −, Sorb −	OD +, LD +, U +, Sorb +	No
Escherichia coli	OD −	OD +	Yes
Escherichia coli	OD −, Sorb +	OD +, Sorb −	Yes
Escherichia coli	Sorb +	Sorb −	Yes
Escherichia coli	LD +	LD −	Yes
Escherichia coli	Sorb +	Sorb −	Yes
Escherichia coli	OD −	OD +	Yes
Escherichia coli	OD −	OD +	Yes
Klebsiella ozaenae	PD −, Sorb +	PD +, Sorb −	No
Klebsiella pneumoniae	OD −, U −	OD +, U +	Yes
Klebsiella pneumoniae	OD −	OD +	Yes
Klebsiella pneumoniae	U −, Adon −	U +, Adon +	Yes
Proteus rettgeri	OD −, Inos −	OD +, Inos +	Yes
Proteus vulgaris	OD −	OD +	Yes
Salmonella albany	Inos −	Inos +	Yes
Salmonella albany	N −, I −	N +, I +	Yes
Salmonella albany	Sorb +	Sorb −	Yes
Salmonella albany	Inos −	Inos +	Yes
Salmonella albany	H_2S −	H_2S +	Yes
Salmonella brookfield	H_2S −	H_2S +	Yes
Salmonella chameleon	Sorb +	Sorb −	Yes
Salmonella chester	H_2S −	H_2S +	Yes
Salmonella manhattan	Inos −, Sorb +	Inos +, Sorb −	No
Salmonella montevideo	Inos −	Inos +	Yes
Salmonella newport	H_2S +	H_2S −	Yes
Salmonella poona	H_2S +	H_2S −	Yes
Salmonella schwarzengrund	OD −	OD +	Yes
Salmonella tennessee	ONPG +, Inos −	ONPG −, Inos +	Yes
Salmonella typhimurium	N +	N −	Yes
Serratia liquefaciens	U −, Inos −	U +, Inos +	No
Serratia liquefaciens	VP −, OD −, Arab −, Inos −, Sorb −	VP +, OD +, Arab +, Inos +, Sorb +	Yes
Serratia liquefaciens	VP −	VP +	Yes
Serratia liquefaciens	Arab −, Inos −	Arab +, Inos +	Yes
Serratia liquefaciens	VP −, Adon +	VP +, Adon −	Yes
Serratia liquefaciens	Inos −	Inos +	Yes
Serratia liquefaciens	OD −, Inos −	OD +, Inos +	Yes
Serratia liquefaciens	Arab −, Inos −	Arab +, Inos +	Yes
Serratia liquefaciens	VP −, OD −, Arab −, Inos −, Sorb +	VP +, OD +, Arab +, Inos +, Sorb −	Yes
Serratia liquefaciens	VP −, Arab −, Inos −	VP +, Arab +, Inos +	Yes
Serratia liquefaciens	VP −, OD −, Arab −, Inos −, Sorb +	VP +, OD +, Arab +, Inos +, Sorb −	Yes
Serratia liquefaciens	LD −, Arab −, Inos −	LD +, Arab +, Inos +	Yes
Serratia liquefaciens	VP −, Arab −	VP +, Arab +	Yes
Serratia liquefaciens	ONPG −, Arab −, Inos −, Sorb +	ONPG +, Arab +, Inos +, Sorb −	Yes
Serratia marcescens	Adon −, Inos −	Adon +, Inos +	Yes
Serratia rubideae	VP +, OD −, LD −, M −, Sorb +	VP −, OD +, LD +, M +, Sorb −	Yes
Yersinia enterocolitica	U −, Inos −	U +, Inos +	Yes

[a]No difference was observed in 35 strains, and these data were not included in the table.

the 14 incorrect identifications occurred with strains of *E. agglomerans* and one with *E. cloacae*. Identical octal codes were obtained at both incubation temperatures for 35% of the cultures. The other 65% had one or more different test reactions, but the majority (51/65) or these were correctly identified. Several *Serratia* cultures showed disagreement in as many as five different instances when the test results for the two incubation

conditions were compared, but still keyed out as *Serratia*. In contrast, for one *C. freundii* culture and one *E. agglomerans* culture the identifications based on the 37- and 22-C incubations differed because of disagreement in only one, obviously very critical, test.

At 22-C incubation, several of the biochemical tests were somewhat unreliable regardless of the organism being tested. For example, the ornithine decarboxylase, inositol and lysine decarboxylase tests gave 30, 22 and 13 false-positive reactions, respectively; while the sorbitol test gave 18 false-negatives. Despite such inconsistent reactions, all cultures of *Arizona hinshawii, Edwardsiella tarda, E. coli, K. pneumoniae, Proteus* sp., *Salmonella* sp. (26 out of 27), *Shigella* spp. and *Y. enterocolitica* were accurately identified to genus when incubated at 22 C for 16 h. Apparently, some of the aforementioned tests are not critical for the generic identification of these cultures.

Our data indicate that when used as directed by the manufacturer, the Micro-ID is a simple, rapid and accurate system for identifying *Enterobacteriaceae*, including many such important organisms as salmonella isolated from various foods. Incubating the system at a temperature lower than 37 C for over 4 h reduced the accuracy of the system from 95-98% to about 80%, primarily because of misclassification of *E. agglomerans*. If one expects to accurately identify to species all the members of the *Enterobacteriaceae* family, then the user should strictly adhere to the incubation conditions recommended by the manufacturer (37 C for 4 h).

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Mention of specific brand names does not imply endorsement by the authors or institutions at which they are employed to the exclusion of others not mentioned.

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The results of a more extensive experiment comparing the efficiency of fresh and old YEA for the recovery of heat-stressed (7 min, 110 C) *C. sporogenes* spores are shown in Table 4.

The data include the variation in heat destruction (tube to tube) as well as the variation due to media difference (each tube number is a separate independent evaluation). The variation in heat destruction is the difference between the results for the fresh medium and old medium test for each tube number. The results suggest that fresh medium may be better than medium prepared a few days in advance of the test day; however, it appears that the differences are not significant. Since laboratory efficiency increases if media can be prepared in advance, we conclude that this can be done without a significant decrease in the resulting spore recovery.

Yeast extract agar with additives at time of plating is a good recovery medium for heated *C. sporogenes* spores. The results suggest that the basic medium can be prepared in advance and stored at 4 C until used.

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Evaluation of Recovery Media for Heated *Clostridium sporogenes* Spores[1]

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ABSTRACT

The efficiency of four culture media for recovery of heat-activated and heated *Clostridium sporogenes* spores was studied. Yeast extract agar gave the highest spore recovery. The effect of the method of preparing the yeast extract agar on the recovery of heated spores was also evaluated. The results indicate that (*a*) a significantly lower spore recovery was obtained when the dextrose was omitted completely or when added to the medium before autoclaving, and (*b*) no significant difference in spore recovery was found between yeast extract agar freshly made or prepared and stored at 4 C up to 11 days before use.

Clostridium sporogenes spores are widely used as a heat process test organism in the food and drug industries. Historically, infusion type media have been used for recovery and enumeration of these spores. Preparing infusion media is time-consuming; therefore many workers have attempted to find other equally reliable media for recovering *C. sporogenes* (*1,2,5,6*).

Yeast extract agar (YEA) appears to be a potentially good recovery medium for *C. sporogenes* spores; however, there are several compositional and usage points yet to be resolved. These include the way YEA is prepared (*2,5,6*), the effect of added dextrose and the age of the medium at the time of use.

This study was carried out to determine if YEA can be efficiently used for recovery of *C. sporogenes* spores after heat-activation or for thermally-stressed spores, and to evaluate variation in the preparation and use of YEA, including the effect of adding dextrose to the medium.

MATERIALS AND METHODS

Spore suspensions

Spore suspensions were prepared from *C. sporogenes* PA 3679, obtained from C. F. Schmidt (Continental Can Co., Chicago, Illinois), using Beef Heart Infusion Medium (*1*).

Recovery media

Yeast extract agar (*YEA*). Yeast extract, 10.0 g; soluble starch, 1.0 g; K_2HPO_4, 2.0 g; agar, 15.0 g; distilled water, 1000 ml; pH 7.2; autoclaved for 15 min at 121 C. Before pouring plates, the following additions were aseptically made to 300 ml of molten medium: 3.75 ml of 10% sodium thioglycollate solution, 3.75 ml of 40% dextrose solution and 7.5 ml of 4% sodium bicarbonate solution. The dextrose solution and the sodium bicarbonate solution were sterilized by membrane filtration, and the sodium thioglycollate solution was sterilized by heat (121 C, 15 min).

Modified YEA. All the YEA medium ingredients, including dextrose, sodium thioglycollate and sodium bicarbonate, were added at the time the medium was prepared. The medium was then autoclaved.

Simplified YEA. Prepared as YEA, except that addition of the 40% dextrose solution at the time of plating was totally eliminated.

Pork infusion agar (*PIA*). Prepared using the procedure of Stumbo (*4*).

Trypticase soy agar (*TSA*). Commercial dehydrated medium (BBL) was prepared according to manufacturer's directions.

All media, except the YEA, were made 1 to 11 days in advance of the day of use. The YEA was prepared on the day of use except where indicated.

Heat-activation experiment

In this experiment, the objective was to evaluate the effect of culture media on the recovery of heat-activated spores. Ten μl of the spore suspension containing approximately 10^7 spores per ml were added to a tube containing 10 ml of water for injection (USP)[a)]. The tube with its contents was placed in boiling water for 8 min. At the end of the heating time, the tube was transferred to an ice water bath until spore recovery procedures were carried out.

Thermal destruction experiments

In these experiments, the objective was to evaluate the effect of culture media on recovery of thermally-stressed (heated) spores. Five minutes before the start of heating, 1 ml (containing approximately 10^7 spores) of a water suspension of *C. sporogenes* spores was added to an 18 × 150 mm screw-cap test tube containing 10 ml of water for injection (USP). The tube and contents were heated for 3 min in a miniature retort at 121 C in the first, and 7 min at 110 C in the second set of experiments. After heating, the tubes were transferred immediately to an ice water bath until spore recovery was carried out.

Recovery procedures

In a clean room, the heat-activated or the heated spore suspension was diluted in Butterfield's phosphate buffer. Five replicates of the appropriate dilution were pipetted into 100-mm diameter petri plates and about 20 ml of recovery medium was added to each plate. The dilution scheme of the heat-activated or heated spore suspension was chosen on the basis of the number of spores added to the initial tube of water for injection (USP) and the length of the heating time, to give counts in the range of 30 to 300 colonies per plate.

Media that were prepared before the day of use were stored at 4 C. On the day of use the medium was melted by placing it in an autoclave at 121 C for 5 min.

The plates were incubated for 48 h at 32 C in BBL Gaspak anaerobic jars using hydrogen and carbon dioxide Gaspak generators. Colonies were counted with the aid of a Bactronic colony counter.

RESULTS AND DISCUSSION

The average plate counts, the standard deviation and coefficient of variation of colonies recovered using four culture media for heat-activated and heat-stressed *C. sporogenes* spores are presented in Tables 1 and 2 respectively.

The data in Tables 1 and 2 suggest that the recovery of heat-activated spores was of the same magnitude for all

[1]*Paper No. 10,775, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.*

[a)]*Water for injection is water purified by distillation or by reverse osmosis that meets the USP requirements for purified water.*

TABLE 1. *Number of heat-activated*[a] Clostridium sporogenes *spores recovered using four different media.*

Subculture medium	Mean plate count[b] $\overline{N}$	Standard deviation σ	Coefficient of variation $\sigma/\overline{N}$	Percent change from YEA[c]
YEA	43.8	4.32	0.098	
Modified-YEA	48.2	6.72	0.139	+ 10.0
PIA	33.2	5.49	0.165	− 24.2
TSA	38.0	6.78	0.178	− 13.2

[a]Eight minutes at 100 C.
[b]Average of five plates.
[c]Percent change from number of colonies recovered in YEA.

TABLE 2. *Number of heat-stressed*[a] Clostridium sporogenes *spores recovered using four different media.*

Subculture medium	Mean plate count[b] $\overline{N}$	Standard deviation σ	Coefficient of variation $\sigma/\overline{N}$	Percent change from YEA[c]
YEA	191.4	19.77	0.103	
Modified-YEA	47.8	17.03	0.356	− 75.0
PIA	43.2	6.61	0.153	− 77.4
TSA	0.8	0.84	1.046	− 99.6

[a]Three minutes at 121 C.
[b]Average of five plates.
[c]Percent change from number of colonies recovered in YEA.

media tested, whereas the YEA was a better recovery medium for heated spores. These results suggest that the heat-stressed spores have a more specific nutritive requirement than the heat-activated spores, and that the YEA as used in these experiments is the best of the alternatives evaluated.

The PIA described by Stumbo (*4*) does not contain sodium bicarbonate or sodium thioglycollate. Odlaug and Pflug (*3*) reported that addition of these compounds increased recovery of heated *C. botulinum* spores. If these compounds had been added to the PIA in the experiments of this study, the recovery of *C. sporogenes* spores might have been increased.

After YEA was established as a better recovery medium for the heated *C. sporogenes* spores, four yeast extract medium variations were evaluated (Tables 3 and 4).

The number of heat-stressed (3 min, 121 C) *C. sporogenes* spores recovered using fresh and old YEA and fresh and old simplified YEA (addition of 40% dextrose eliminated) is reported in Table 3. In most instances where a comparison is possible, higher counts were obtained when dextrose was added to the medium at the time of plating. The results do not indicate any difference in spore recovery between media prepared the day of the test and media prepared ahead of time.

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TABLE 3. *Number of heat-stressed*[a] Clostridium sporogenes *spores recovered as a function of the YEA media type and age.*

Test no.	Recovery medium	Age of medium (Days)	Mean plate count[b] $\overline{N}$	Standard deviation σ	Coefficient of variation $\sigma/\overline{N}$
1	YEA	0	20.0	3.81	0.190
	Simplified YEA[c]	0	5.0	2.34	0.469
	YEA	11	48.2	2.05	0.042
	Simplified YEA	11	21.2	4.02	0.190
2	YEA	0	101.0	9.25	0.091
	YEA	10	60.6	5.32	0.088
	Simplified YEA	10	44.4	6.54	0.147
3	YEA	0	56.2	8.04	0.143
	YEA	10	46.0	6.56	0.143

[a]Heated at 121 C for three minutes.
[b]Mean count of five plates.
[c]Simplified YEA - the addition of 40% dextrose at time of plating eliminated.

TABLE 4. *Number of heat-stressed*[a] Clostridium sporogenes *spores recovered using fresh and old YEA media.*

Tube number	Fresh medium[c]		Old Medium[d]		Difference between log mean count
	Mean plate count[b]	Log mean plate count	Mean plate count[b]	Log mean plate count	(Fresh-Old)
1	118	2.0718	116	2.0644	0.0074
2	118	2.0718	94	1.9731	0.0987
3	95	1.9777	102	2.0086	- 0.0308
4	84	1.9242	84	1.9242	0
5	100	2.0000	101	2.0043	- 0.0043
6	104	2.0170	100	2.0000	0.0170
7	98	1.9912	96	1.9822	0.0089
8	111	2.0453	106	2.0253	0.0200
9	104	2.0170	84	1.9242	0.0927
10	88	1.9444	114	2.0569	- 0.1124
11	102	2.0086	112	2.0492	- 0.0406
		Arithmetic Average of Log Mean Difference			0.0051
		Standard Deviation of Log Mean Difference (σ)			0.0584

[a]Seven minutes at 110 C.
[b]Average of two counts.
[c]YEA prepared on day of plating.
[d]YEA prepared 11 days before plating.

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Correlation Between Total DDT Residues in Blood and Fat of Beef Animals[1]

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ABSTRACT

In Guatemala, where in certain regions heavily pesticide-sprayed cotton fields are interspersed with pastures for cattle, pesticide residues in beef fat represent a problem. Organochlorine pesticides are still widely used and even if the use of DDT has been decreasing over the last few years, this pesticide is still a major food contaminant. The present study was undertaken to establish if a correlation between total DDT levels in blood and fat could be found. Samples of blood and fat from 30 bovines were analyzed by gas-liquid chromatography. The "ppm in fat/ppb in blood" ratio was calculated to be 0.96 ± 0.39 (mean ± S.D.), the regression line to be Y = 2.54 + 0.61 X (Y = ppb in blood, X = ppm in fat) and the correlation coefficient to be 0.889. It was established that blood analysis may be used to estimate, before slaughter, if the residue levels in the fat are exceeding the legal limits.

In the Pacific coastal plains of Guatemala, extensive and heavily pesticide-sprayed cotton fields are interspersed with pastures for milk and beef cattle. Organochlorine pesticides are still widely used, and even if the use of DDT is decreasing because of the new pesticide law (*3*), this pesticide is still a major food contaminant. The toxic effects of organochlorines have been reported to be higher in subjects on deficient diets (*7*), an important fact in Guatemala where protein-calorie malnutrition is very high (*9*).

At present, kidney fat collected after slaughter is used for determination of pesticide residues. When high levels are found, some decision must be made on the disposition of the carcass. Rejection of the meat represents a high economic loss and, if released, it represents a potential health hazard. Estimation of the residue levels before slaughter would prevent killing highly contaminated animals. Ware et al. (*10*) investigated the correlation between total DDT in blood and kidney fat. They concluded that analysis of blood samples may be used to estimate total DDT in carcass fat before slaughter.

The present study was undertaken to establish if the same correlation would be found under local conditions in Guatemala. In developing countries, data from other countries are too often used without further confirmation. It should be taken into account, however, that local conditions may change the results and the conclusions.

[1]*Thesis work by C. E. Gutiérrez B. in obtaining the Veterinary Surgeon degree.*
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MATERIALS AND METHODS

Samples

Samples of blood and fat were collected from 30 bovines of different sex and breed in the Department of Escuintla on the Pacific Coast. Twenty ml of blood were taken by puncture of the jugular vein and collected in glass tubes containing 4 drops of heparin as an anticoagulant. The tubes, as well as all the glassware used for analysis, were previously washed with soap and water, rinsed with tap water and distilled water, dried and then rinsed with Nanograde® acetone and petroleum ether. About 1 g of fat was obtained by biopsy in the sternum region and wrapped in aluminum foil. A previous study in this laboratory showed the residue levels in kidney fat to be equal to those in the pectoral region ($r = 0.984$, $p < 0.05$) (*8*). The blood samples were kept at 4 C for a few days before they were analyzed, the fat samples at -20 C for a few weeks (*4,5*).

Analysis

The fat was rendered and filtered at 130 C and 0.25 g was analyzed as described by McLeod and Ritcey (*5*). The samples were analyzed in duplicate; the mean coefficient of variation was 10%. The mean recovery of known added quantities of DDT and its metabolites (o,p′ - and p,p′ - isomers of DDT, DDE and DDD) carried through the whole method was 80%. The results are reported in ppm (parts per million). The blood samples were analyzed as described by Brown and Chow (*1*). The mean coefficient of variation between duplicates was 8% and the mean recovery 75%. Heparin gave no interference peaks. The blood values are reported in ppb (parts per billion). The extracts were analyzed by gas-liquid chromatography (GLC) as follows: gas chromatograph: TRACOR, Model MT-220; columns: 6 ft, ¼inch OD; off-column injection, 200 C; column for identification and quantification: 1.5% OV17/1.95% QF1; column for confirmation: 4% SE30/6% QF1; injection port: 225 C; transfer line: 257 C; and detector Ni^{63}: 275 C.

RESULTS AND DISCUSSION

The results are tabulated in columns A and B of Table 1. A roughly 1000-fold ratio can be found between the two values (Column D), meaning that the ppm value in fat is roughly equal to the ppb value in blood. The relation: ppm in fat/ppb in blood was calculated as 0.96 ± 0.39 (mean ± S.D.). The same data are illustrated in Fig. 1 with the fat values in the abscissa (X) and the blood values in the ordinate (Y). The linear regression line was calculated as $Y = 2.54 \pm 0.61X$ or $X = \frac{Y - 2.54}{0.61}$ and the correlation coefficient as $r = 0.889$.

The values calculated by this procedure (ppm in fat = $\frac{\text{ppb in blood} - 2.54}{0.61}$ are reported in Column C (Table 1), and the relation between real and estimated values is illustrated in Fig. 2.

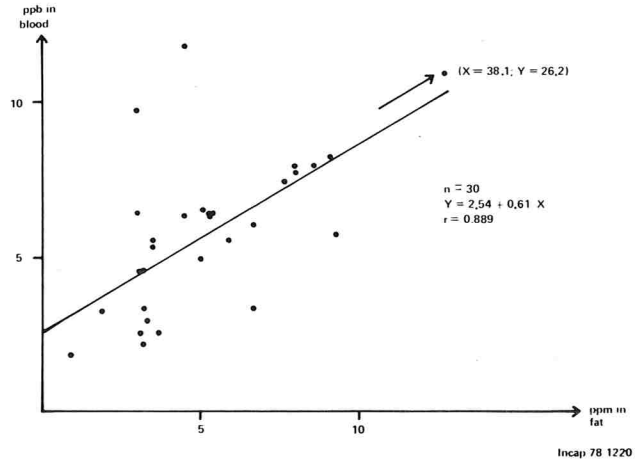

Figure 1. *Correlation between total DDT levels in blood and fat of bovines.*

TABLE 1. *Total DDT residues in blood and fat of bovines.*

No.	A Blood (ppb)	B Fat (ppm), real value	C Fat (ppm), calculated value[a]	D Ratio ppm in fat / ppb in blood
1	11.80	4.51	15.20	0.38
2	4.47	3.16	3.16	0.71
3	6.35	5.44	6.25	0.86
4	5.46	5.92	4.79	1.10
5	4.90	4.97	3.87	1.00
6	1.76	0.87	0.00	0.49
7	5.25	3.45	4.43	0.66
8	6.28	5.26	6.13	0.84
9	2.54	3.69	0.00	1.50
10	6.42	3.03	6.36	0.47
11	5.95	6.73	5.59	1.10
12	2.06	3.20	0.00	1.60
13	3.28	3.17	1.21	0.97
14	6.28	5.29	6.13	0.84
15	2.93	3.26	0.64	1.10
16	2.54	3.11	0.00	1.20
17	8.24	9.14	9.34	1.10
18	7.89	8.02	8.77	1.00
19	4.47	3.05	3.16	0.68
20	5.54	3.46	4.92	0.63
21	6.54	5.11	6.56	0.78
22	7.74	8.02	8.52	1.00
23	26.20	38.10	38.80	1.50
24	3.21	1.90	1.10	0.59
25	3.37	6.74	1.36	2.00
26	7.39	7.71	7.95	1.00
27	9.68	2.96	11.70	0.31
28	6.30	4.52	6.16	0.72
29	5.72	9.31	5.21	1.60
30	7.85	8.62	8.70	1.10
				$\bar{x} = 0.96 \pm 0.39$ (mean ± S.D.)

[a] ppm in fat $= \frac{\text{ppb in blood} - 2.54}{0.61}$

The same regression line calculated from the data of Ware et al. (*10*) is $Y = 0.27 + 1.1X$, the line having a much steeper slope and passing almost through zero (for calculation purposes 0.9 was used where < 1 ppb was reported). The reason for this difference is not clear. Breed or sex of the animals, chronicity of the contamination or climatic conditions may be contributing factors.

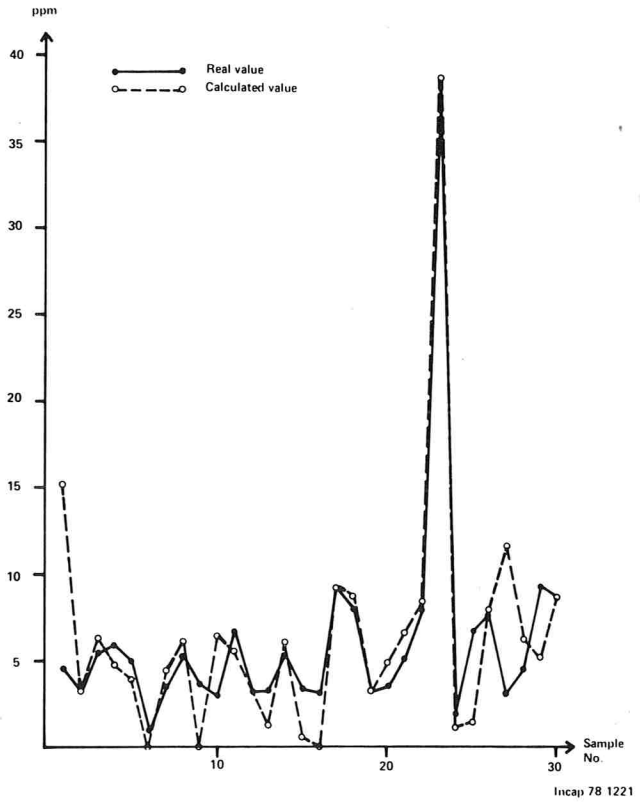

Figure 2. *Total DDT in fat of bovines. Comparison between real and calculated values.*

In Guatemala, the legal limit for total DDT in beef fat is 7 ppm, whereas meat exported to the U.S. has to meet the 5-ppm limit. Using the regression line $Y = 2.54 + 0.61X$, the critical levels for total DDT in blood were calculated as 6.8 ppb for Guatemala and 5.6 ppb for export animals. Theoretically, animals with blood levels higher than these values should be held until blood levels reach an acceptable level. Table 2 summarizes correct and incorrect conclusions using this estimation method.

Even if the possibility of error, according to this study, varies from 10 to 20%, the advantage of using the proposed method for estimating the residue levels in fat before slaughter seems obvious; economic losses would be diminished as well as the potential health hazard for the population. The answer as to the fate of the rejected animals is not easy. Several studies on detoxication of contaminated animals have been published (*2,6*), but further research is certainly needed. In Guatemala, transfer to a clean environment would probably be the immediate answer.

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TABLE 2. *Estimation of total DDT residues in 30 fat samples based on analysis of blood.*

	7-ppm limit		5-ppm limit	
	n	% of total	n	% of total
Correctly accepted	21	70	12	40
Correctly rejected	6	20	12	40
Correct conclusions	27	90	24	80
Wrongly accepted	1	3	2	7
Wrongly rejected	2	7	4	13
Wrong conclusions	3	10	6	20

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Antibiotic-resistant Bacteria in Raw Milk and Ability of Some to Transfer Antibiotic Resistance to *Escherichia coli*

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ABSTRACT

Raw milk samples were examined for number and percentage of bacteria resistant to seven antibiotics: penicillin, ampicillin, chloramphenicol, neomycin sulfate, polymyxin B sulfate, tetracycline and streptomycin sulfate. A significant negative correlation was found between the total aerobic count of the milk sample and the concentration (above 5 or 10% of the total count) of bacteria in each milk resistant to each of the antibiotics tested. Three of 42 gram-negative isolates were capable of transferring their antibiotic resistance to *Escherichia coli*. Substantial numbers of antibiotic-resistant bacteria in raw milk were found and some survived pasteurization. Inspection of farms failed to indicate a relationship between farm practices or use of antibiotics in feed or as pharmaceuticals and number of antibiotic-resistant bacteria in the raw milk.

Bacterial antibiotic resistance may arise by spontaneous mutation or extrachromosomal inheritance in man and other animals brought about by selective pressure of antibiotics used in therapy and prophylaxis or for growth promotion (*8*). Resistant bacteria are eliminated by animals and may contaminate the soil and other objects. Thus agricultural products may contain antibiotic-resistant bacteria, and if the products are consumed raw the resistant bacteria may invade man. An overview of the importance of antibiotic-resistant bacteria to food microbiology has been presented (*5*).

Antibiotic resistance can also be transferred from one bacterium to another as genetic elements on plasmids. Plasmids may mediate their own conjugal transfer or be cotransferred with another plasmid (*9*).

In this study we examined the number of bacteria, as well as the proportion of the total count found in raw milk, that are resistant to seven antibiotics. We also demonstrated that some gram-negative bacteria isolated from raw milk and resistant to a specific antibiotic may transfer this resistance to *Escherichia coli* via cell to cell contact, presumably by conjugation.

MATERIALS AND METHODS

Samples

Raw milk was collected at farms in 1-oz. sterile containers from refrigerated holding tanks by collectors licensed by the Connecticut Department of Agriculture and using prescribed procedures (*2*).

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[3]*Deceased March 24, 1979.*

Media and counts

A total aerobic count was made by spreading 0.1 ml of appropriately diluted sample on previously poured and hardened Standard Methods agar (BBL, Cockeysville, MD). Plates were incubated at 30 C for 48 h. Counts of antibiotic-resistant bacteria were made in the same manner, using media containing antibiotics described below.

Antibiotic-containing media were prepared by adding sterile antibiotic solutions to melted and tempered (48-50 C) Standard Methods agar. The antibiotics used and the final concentration per ml in the test media were as follows: penicillin G (Benzylpenicillin, sodium salt, Sigma Chem. Co., St. Louis, MO), 500 and 10 units; ampicillin (Sigma), 500 μg; chloramphenicol (Chloromycetin, crystalline, Sigma), 100 μg; neomycin sulfate (Sigma), 50 μg; polymyxin B sulfate (Sigma), 500 and 50 units; tetracycline hydrochloride (crystalline, Sigma), 10 μg; streptomycin sulfate (B grade, Calbiochem, San Diego, CA), 100 μg. All antibiotic solutions were prepared in concentrated aqueous solutions and filter-sterilized so that when added to the autoclaved and tempered medium there was not more than a 1% change in medium concentration. Preparation of media containing antibiotics and their use has been described (*1,3,4*).

Transfer of antibiotic resistance

E. coli strain C600*nal* (a nalidixic acid-resistant strain obtained from Dr. R. B. Sparks, Jr., Genetics Department, this Station) and strain *E. coli* X-705 (a streptomycin-resistant strain obtained from Dr. R. Curtis III, University of Alabama) were used as the recipients for conjugal plasmids derived from the bacteria isolated from raw milk.

Cell to cell contact necessary for the conjugal transfer of plasmids was established on Plate Count agar (Difco, Detroit, MI) by a modification of a previously described technique (*6,7*). Plasmid recipient strain *E. coli* C600*nal* was streaked on the central portion of agar plates. Each antibiotic resistant, gram-negative bacterium obtained from the raw milk was streaked on the same agar plate at right angles to the recipient. After incubation for 18 h at 30 C, the cells on the agar surface were suspended in 2 to 3 ml of sterile water and 0.01, 0.1 and 1.0-ml portions of the cell suspension were placed in empty petri dishes and suspended in tempered Plate Count agar fortified with antibiotics. Putative plasmid transconjugants of *E. coli* C600*nal*R$^+$ were selected from the population of bacteria growing in the agar by their ability to grow in the presence of nalidixic acid (50 μg/ml of medium) as well as the antibiotic to which the gram-negative bacterium (donor) obtained from the raw milk was originally resistant.

As further evidence for infectious plasmid-like transfer, *E. coli* C600*nal*R$^+$ transconjugants were mated with *E. coli* X-705. Selection of *E. coli* X-705R$^+$ transconjugants was based on resistance to streptomycin sulfate (250 μg/ml in the medium) and the antibiotic whose resistance was conferred by the putative plasmid. Control plates were included to detect any spontaneous mutants among the recipient or donor bacteria to antibiotic resistance.

RESULTS AND DISCUSSION

Resistant bacteria in raw milk

A wide range in the total number of antibiotic-

resistant bacteria as well as in the percentage resistant to any single antibiotic was observed in the 114 raw milk samples examined (Table 1). The average total bacterial count of the samples was 18,000 per ml. The average percentage of the total bacterial count resistant to any of the antibiotics was less than 12.5% except when polymyxin at the 50-unit level was used. However, the percentage of resistant bacteria ranged from 0 to 100% for individual samples. Fewer bacteria were resistant to chloramphenicol than to any other antibiotic. Decreasing the penicillin concentration in the medium from 500 to 10 units per ml doubled the average number of resistant bacteria. Decreasing the polymyxin level in the medium from 500 to 50 units per ml increased the average number of resistant bacteria more than 11-fold.

TABLE 1. *Average number of aerobic bacteria in raw milk resistant to seven antibiotics.*

Antibiotic in medium	Amount per ml medium	No. samples	Total count (log avg per ml)
None	—	114	17,500
Penicillin	10 units	38	597
	500 units	114	282
Ampicillin	500 μg	114	194
Chloramphenicol	100 μg	113	80
Neomycin	50 μg	114	285
Polymyxin	50 units	92	7,850
	500 units	61	696
Streptomycin	100 μg	114	504
Tetracycline	10 μg	114	407

Concentration of resistant bacteria in each milk sample

One measure of the relative concentration of bacteria able to resist an antibiotic is shown by the proportion of samples in which bacteria resistant to a given antibiotic equals or exceeds an arbitrary level of 5 or 10% of the total count (Table 2). For example, in only 4.4% of the samples were more than 10% of the total count resistant to chloramphenicol while 93.5% of the samples had more than 10% of the total count resistant to polymyxin (50 units/ml). The second and third highest percentages of samples containing a population at least 10% resistant were in the tests containing 500 or 10 units of penicillin. Most of the bacteria resistant to penicillin appeared to be fluorescent pseudomonads. When ampicillin, another form of penicillin, was used instead of penicillin, the proportion of resistant bacteria fell from 21.9 to 10.5%.

However, when the minimum percentage of resistant bacteria in any sample was set at 5%, the percentage of samples with resistant bacteria above this value increased about two- or three-fold over that with the 10% minimum for all antibiotics except penicillin and polymyxin (50 units/ml). For penicillin this phenomenon was not unexpected since one criterion for resistance to penicillin is production of β-lactamase. Therefore, resistance to penicillin is generally an all or none phenomenon. Resistance to polymyxin was overcome by increasing the concentration of this antibiotic in the medium (see polymyxin 500 level, Table 2).

Concentration of resistant bacteria and intercorrelations

If the proportion of bacteria resistant to a given antibiotic in a sample of raw milk was at least 5% (or 10%) of the total count, then that milk was considered to have a meaningful concentration of bacteria resistant to that antibiotic. Thus each sample of milk could have from none to seven meaningful concentrations or groups of antibiotic-resistant bacteria. Therefore, another method for assessing the resistance pattern of bacteria in raw milk is obtained.

Figure 1 shows the relation of total aerobic count of samples with at least 5 or 10% of the total count resistant to none or more antibiotics (groups of antibiotic-resistant bacteria). The regression of the logarithm of the total aerobic count of each milk sample on the number of groups (0 to 7) of antibiotic-resistant bacteria was calculated. The points are the mean aerobic counts at each level. Despite considerable variation, both regressions are highly significant ($p \leq .01$) and their slopes are negative. This indicates that bacteria from raw milk with a low total count were, in general, resistant to a wide range of antibiotics. That is, the lower the total count of the sample, the more antibiotics the bacteria resisted.

Similarly, when the proportion of bacteria in a sample resistant to an antibiotic was correlated with the total

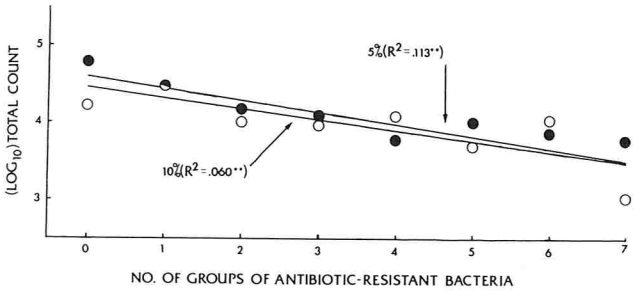

Figure 1. *Regression of total aerobic bacterial count on number of groups of antibiotic-resistant bacteria in raw milk. An antibiotic-resistant group comprises at least 5% or 10% of the total aerobic count for each sample. Each milk was assigned a numerical value of zero to seven corresponding to the number of groups of antibiotic-resistant bacteria. The points are the mean aerobic count values at each level. Solid circles = 5% or more of resistant bacteria, open circles = 10% or more of resistant bacteria, ** significant at $p \leq .01$. For 5% level, $F = 14.27^{**}(n = 114)$; for 10% level, $F = 7.09^{**}(n = 114)$.*

TABLE 2. *Percentage of raw milk samples with at least 5 or 10% of the total aerobic count resistant to the indicated antibiotic.*

Proportion of total count	Antibiotic								
	PEN[1]		AMP	CAM	NEO	PLM		STR	TET
	10U	500U	500 μg	100 μg	50 μg	50U	500U	100 μg	10 μg
	(% of samples)								
≥ 5%	44.7	27.2	18.4	10.6	18.4	96.7	41.0	39.5	29.8
≥ 10%	34.2	21.9	10.5	4.4	8.8	93.5	14.8	16.7	16.7

[1]Abbreviation: PEN, penicillin; AMP, ampicillin; CAM chloramphenicol; NEO, neomycin sulfate; PLM, polymyxin B sulfate; STR, streptomycin; TET, tetracycline. Concentrations are designated as units (U) or μg per ml of medium.

count of that sample all the correlations except that with neomycin were negative (Table 3, top row). Thus, as the total count increased there was a decrease in the proportion of bacteria in that total population resistant to a specific antibiotic. Although some of the correlations are statistically significant they account for only 5 to 26% of the observed variability.

Correlations were also calculated between the proportion of bacteria resistant to the individual antibiotics (Table 3). Several apparent anomalies in the correlations were noted. Although a significant correlation between the 10 and 500 unit levels of penicillin was observed, as expected, only the 500-unit level of penicillin and ampicillin were correlated. A correlation was found to exist between tetracycline and chloramphenicol ($r = .34$, $p \leq .01$, $n = 113$), antibiotics that inhibit protein synthesis. However, no correlation was found between neomycin and streptomycin ($r = .05$, $n = 114$) even though both antibiotics are chemically similar and inhibit protein synthesis. The lack of correlation between antibiotics with similar modes of action must assume either multiple resistance mechanisms in the same bacterium, or more likely, different populations of bacteria, each resistant to a different antibiotic.

Resampling of raw milk and examination of farms

Four months after the initial sampling, raw milk from 19 farms was resampled to determine if the antibiotic resistance patterns of the bacterial flora had changed. Although the proportion of the total count found to be resistant to any single antibiotic changed in some instances, a statistical analysis (t-test) revealed no significant difference in proportion of resistant bacteria between the two dates except for tetracycline. For this antibiotic the proportion of resistant organisms declined significantly ($t = 2.80$, $n = 38$) at the second sampling. Thus it appeared that the bacterial flora in raw milk from a specific farm remained essentially unchanged during 4 months, as determined by the proportion of the total count resistant to a specific antibiotic, except for tetracycline.

In an attempt to ascertain the reason for the variability among farms in the proportion of the total count resistant to an antibiotic, five farms from the same geographical area were selected for further study. On the first sampling, these five farms had raw milk containing high, medium and low proportions of the total bacterial count resistant to specific antibiotics. On-site inspection of these dairy farms was made to determine if antibiotics were present in either livestock feed or veterinary supplies. Use of these materials could lead to direct (intestinal selection) or indirect (manure contamination) enrichment for antibiotic-resistant bacteria. We found no antibiotic-containing feed. However, all the farms had variable but considerable quantities of penicillin-containing veterinary preparations used to treat mastitis. There was no obvious correlation for the proportion or number of antibiotic-resistant bacteria in raw milk with the presence of antibiotics used for therapy or prophylaxis.

Resistant bacteria in pasteurized products

We also tested seven commercially pasteurized dairy products for bacteria resistant to antibiotics. Although much variation was observed, bacteria resistant to penicillin, ampicillin and polymyxin were found. It was not possible from these few samples to ascertain whether the resistant bacteria survived pasteurization or were post-pasteurization contaminants.

To test this aspect, we laboratory-pasteurized 20 raw milk samples and examined them for bacteria resistant to antibiotics. Ten of the 20 laboratory-pasteurized samples contained no bacteria resistant to antibiotics. For the remaining 10 samples more bacteria were found

TABLE 3. *Correlation (r) of total aerobic bacterial count of raw milk [TCNT] with percentage of total count resistant to specific antibiotics and correlation (r) between percentage of total count resistant to each antibiotic.*

	Antibiotic								
	PEN 10[1]	PEN 500	AMP	CAM	NEO	PLM 50	PLM 500	STR	TET
TCNT	-.516**[2]	-.319**	-.175	-.259**	.053	-.236*	-.079	-.217*	-.068
	(38)[3]	(114)	(114)	(113)	(114)	(92)	(61)	(114)	(114)
PEN 10		.82**	.21	.44**	.55**	.30	—	.47**	.12
		(38)	(38)	(38)	(38)	(38)	(0)	(38)	(38)
PEN 500			.70**	.53**	.10	.06	.33*	.11	.16
			(114)	(113)	(114)	(92)	(61)	(114)	(114)
AMP				.60**	.05	-.01	.41**	.13	.23*
				(113)	(114)	(92)	(61)	(114)	(114)
CAM					.19*	.16	.05	.24**	.34**
					(113)	(91)	(61)	(113)	(113)
NEO						.24*	.10	.05	.03
						(92)	(61)	(114)	(114)
PLM 50							.25	-.04	.21*
							(39)	(92)	(92)
PLM 500								.09	-.02
								(61)	(61)
STR									.54**
									(114)

[1]Abbreviations: PEN, penicillin; AMP, ampicillin; CAM, chloramphenicol; NEO, neomycin sulfate; PLM, polymyxin B sulfate; STR, streptomycin, TET, tetracycline. See Table 1 for amounts per ml of medium.

[2]* significant at $p \leq .05$, ** significant at $p \leq .01$.

[3]Number in parentheses indicates number of samples.

resistant to streptomycin, tetracycline and polymyxin than to the other antibiotics. With streptomycin, the proportion of resistant bacteria averaged 26.5% of the total count. Before pasteurization of these same 10 samples, the average percentage of streptomycin resistant bacteria was only 4.9%. Thus it appears that bacteria resistant to streptomycin, tetracycline and polymyxin can survive laboratory pasteurization. Such information is important in light of the data presented below on R^+ plasmid transfer from bacteria in raw milk to *E. coli*.

Transfer of R^+ plasmids from antibiotic-resistant bacteria in raw milk to E. coli

From the bacteria in raw milk resistant to antibiotics, 42 gram-negative bacteria were chosen to study their ability to transfer their antibiotic resistance. Each appeared to be the predominant colony type on the medium from which it was isolated. Of these bacterial isolates 12 were resistant to penicillin, six to ampicillin, seven to streptomycin, five to neomycin, nine to tetracycline, and three to chloramphenicol. Only three isolates among the 42 selected were capable of transferring antibiotic resistance to *E. coli* C600*nal.* Two of the isolates were originally resistant to penicillin and one to tetracycline. All three *E. coli* C600*nal*R^+ transconjugants transferred resistance to *E. coli* X-705. No spontaneous mutants to antibiotic resistance were noted when *E. coli* C600*nal*. *E. coli* X-705 or isolates from raw milk were plated at similar concentrations in selective media.

Thus the data indicate that under our conditions, antibiotic resistance probably resident on conjugal plasmids can be transferred, but it was not the most prevalent type of resistance found among the gram-negative antibiotic-resistant bacteria isolated from raw milk. Further, none of the three putative antibiotic resistance plasmids carried resistance to a second or third antibiotic (among the seven antibiotics used in this study).

ACKNOWLEDGMENT

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CONCLUSIONS AND RECOMMENDATIONS

Blood analysis may be used to estimate total DDT residues in beef fat before slaughter. It is considered that the proposed method can be used to infer whether a given sample will pass the limit or not. Analysis in triplicate is recommended for samples with residue levels close to the legal limit. It is considered that the proposed method can be used to infer whether a given sample will pass the limit or not.

Comparison of the present data with others published on the same subject indicates variations in the results, but the factors causing these variations are unknown.

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Salmonella on Broiler Carcasses as Affected by Fresh Water Input Rate and Chlorination of Chiller Water

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ABSTRACT

Broiler carcasses, each inoculated with about 1000 cells of a marker strain of *Salmonella typhimurium*, and uninoculated carcasses were prechilled and chilled in a simulated commercial chilling process. For each experiment, fresh water input was either 1.90 liters (0.50 gal) or 0.95 liters (0.25 gal) per carcass, and the chlorine level was 0, 20 or 50 ppm. The rate of fresh water input had no significant effect on either cross-contamination (uninoculated carcasses showing contamination with marker organisms after chilling) or elimination of *Salmonella* from the inoculated carcasses. Fewer uninoculated carcasses showed marker *Salmonella* contamination after chilling with 50 ppm of chlorine than 0 ppm, but cross-contamination was not eliminated. Chlorine in the chilling water decreased rapidly due to the effect of organic matter.

Fresh water input rate required for chilling broiler chicken carcasses has been 1.9 liters 1 (0.50 gal) per bird since the inception of regulations applied to continuous chilling (*20*). The U.S. Department of Agriculture has recently published a proposed amendment to allow a reduction in that rate to 0.95 liter (0.25 gal) per carcass, provided the incoming water contains 20 ppm of available chlorine (*21*). Wesley (*23*) concluded that the reduction of chiller water input to 0.95 liter (0.25 gal) per broiler carcass had no detrimental effect on the quality of carcasses or chiller water, and would result in substantial savings to the processing industry.

Carcass shelf-life has been found to be extended by chlorine in the chill water (*3,6,9,14,15,16*). However, no reduction in total counts on chicken drumsticks treated with 20 ppm of chlorine has also been shown (*22*). About 50 ppm of chlorine, maintained in a continuous chiller with 5 liters (1.3 gal) of fresh water added per carcass, was found to reduce bacteria in the water to insignificant numbers (*1,10*). Chlorine at 18-25 ppm in the input water in commercial chillers was also found to reduce both total and psychrotrophic bacteria counts (*8*). In other studies (*4,12,18,19*), it was shown that chlorine could significantly reduce or eliminate artificially inoculated salmonellae on chicken carcasses, but it has also been shown that chlorine may reduce salmonellae only slightly on chicken meat (*22*).

The purpose of this study was to determine the effects of input rate of fresh supply water, either with or without chlorine, on the persistence of salmonellae on broiler carcasses, and on the transfer of salmonellae from contaminated to uncontaminated carcasses (cross-contamination) during simulated commercial chilling.

MATERIALS AND METHODS

The rates of fresh water input and levels of chlorine added were those deemed practical from commercial and regulatory view-points. A cell suspension of a marker organism (naladixic acid-resistant *Salmonella typhimurium*) of about 5000 cells/ml was prepared. The count of a suspension of this *Salmonella* had been determined to be 2×10^8 cells/ml at an O.D. of 0.2 at 540 nm in a Bausch and Lomb Spectronic 20 spectrophotometer; the desired concentration was thus prepared by serial dilutions. Concentrations of suspensions were verified by plating on brain heart infusion agar (Difco).

Eighty broiler carcasses, processed completely except for chilling and packing, were obtained at a local processing plant. Each of 10 carcasses was inoculated with 0.2 ml of the suspension. Drops were pipetted onto the breast, a thigh, under a wing and in the body cavity, then rubbed into and spread over these areas with a sterile bent glass rod. This level of contamination (1000 cells per carcass) is somewhat higher than the average which might normally be found on *Salmonella*-contaminated carcasses in commercial channels (10,13,17). The 10 inoculated carcasses, together with 30 uninoculated carcasses, were placed in an experimental prechill unit (40-carcass capacity). One hundred and fifty liters (40 gal) of water at 18 C (65 F), the maximum temperature allowed by U.S. Department of Agriculture (*20*), was added in accordance with the usual commercial ratio on initial filling of about one gallon per broiler. Sodium hypochlorite solution was added to produce a 20 ppm level of available chlorine in the supply water. Sufficient hydrochloric acid was added to adjust the water to pH 6.0, since chlorine from a gaseous source produces solution at about this pH, and chlorine solutions are significantly more bactericidal at pH 6.0 than at higher pH values (*24*). Concentrations of chlorine were determined initially and monitored during the prechilling and chilling periods by the thiosulfate titration method (*6*). Water temperatures were also monitored. Paddles which agitated the carcasses in the prechill unit revolved at a rate of 4.5 revolutions per min (rpm) for 10 min. This is the average rate in a type of commercial continuous chiller widely used in processing plants. The carcasses were then transferred to a chill unit of the same design as the prechill unit. They were agitated at 4.5 rpm for 20 min in slush ice (1:6 crushed ice to water). The supply water was adjusted to the same chlorine concentration and pH as those of the water in the prechill unit. These carcasses, which served to "condition" the prechill and chill waters so that their content of organic matter was similar to that of water in commercial chillers, were then discarded. Seventy five liters (20 gal) of the water in the prechill unit (corresponding to 1.90 liters per bird) was then drained, discarded and replaced by an equal volume pumped from the chill unit. Fresh water, adjusted to pH 6.0 and containing the level of chlorine under study, was then added with ice to refill the chill unit. This procedure simulated the commercial practice of delivering fresh water into the chill unit, channeling overflow from the chill unit to the prechill unit, then discarding overflow from the prechill unit. Ten

additional inoculated carcasses were labeled for identification, placed in the prechill unit with 30 uninoculated carcasses, then prechilled and chilled in the conditioned waters. The following combinations of chlorine level and fresh water input rate were also tested, each with a separate lot of 80 carcasses: no chlorine, 1.90 liters input (control); 50 ppm of chlorine, 1.90 liters input; 20 ppm of chlorine, 0.95 liter input; 50 ppm of chlorine, 0.95 liter input. The entire series of tests of each combination was then duplicated, for a total of 800 carcasses utilized, 400 to condition the chilling system, and 400 for further evaluation. The prechill unit, chill unit, pump and hoses were cleaned and sanitized between each lot.

The 40 carcasses chilled in the conditioned waters for each combination in each test were examined to determine whether the naladixic acid-resistant *Salmonella* survived on inoculated carcasses, and whether the uninoculated carcasses had become contaminated with these marker organisms. To sample, each carcass was placed in a plastic bag with 270 ml of sterile water and shaken vigorously. The carcass was removed and 30 ml of selenite cystine broth (10 ×), which yielded a single strength final concentration, was added to the rinse fluid. The bag with the rinse fluid was then incubated for 24 h at 35-37 C. A loopful of this broth was then streaked onto MacConkey Agar containing 100 ppm of naladixic acid, and after 24-h incubation at 35-37 C, isolated colonies were picked and then tested serologically to confirm that they were the marker *Salmonella*. This procedure had been shown to be extremely sensitive; carcasses contaminated with low numbers of *Salmonella* could be consistently detected (*2*). Sodium thiosulfate (100 ppm) was added to the sampling fluid to neutralize residual chlorine which might otherwise have interfered with bacterial growth on the selective media.

Analysis of variance and the multiple range test (*5*) were applied to the data.

RESULTS AND DISCUSSION

Neither reduction in the rate of input of fresh supply water from 1.90 to 0.95 liter per broiler carcass, nor addition of 20 ppm of available chlorine to the supply water throughout simulated commercial chilling, significantly affected the persistence of marker salmonellae organisms inoculated onto the carcasses (Table 1). Addition of 50 ppm of chlorine tended to reduce the incidence of contaminated carcasses, from among those which has been inoculated with the marker salmonellae, after chilling (this reduction statistically significant between p = 0.10 and 0.25). Adding 20 ppm of available chlorine to the fresh supply water tended to reduce cross-contamination, but a level of 50 ppm was required to reduce cross-contamination to a statistically significant extent, in comparison to supply water with no chlorine added. Cross-contamination was not eliminated, however, at any level of added chlorine studied.

TABLE 1. *Percentage of broiler carcasses testing positive for inoculated* Salmonella typhimurium *after chilling with specified rate of fresh water input and chlorine added to supply water.*

Fresh water input rate liters (gal)	ppm Chlorine added to supply water	Positive for *Salmonella* after chilling of carcasses: Inoculated before chilling	Positive for *Salmonella* after chilling of carcasses: Not inoculated before chilling (cross-contamination)
1.90 (0.5)	0	100%a[1]	80%c
1.90 (0.5)	20	75%a	33%ab
1.90 (0.5)	50	65%a	10%a
0.95 (0.25)	20	95%a	58%bc
0.95 (0.25)	50	40%a	8%a

[1]Percentages within a column were not significantly different (p = 0.05) when followed by the same letter, according to analysis of variance and the multiple range test (*5*).

Total available chlorine in the chilling water during chilling declined until the end of the second prechill period (Table 2), probably because of the organic matter from the carcasses. A buildup of organic nitrogen in chlorinated water has been shown to rapidly reduce the level of available chlorine (*24*). Fresh chlorinated supply water added at the start of the second chill stage temporarily elevated the total available chlorine of the chilling water to a level about 85% of that of the chlorinated supply water. To maintain a level of about 20 ppm of total available chlorine in the chilling water, which was the level effective in significantly reducing cross-contamination, about 50 ppm of chlorine would be required in the fresh supply water.

Researchers working near the chilling system noted sinus and eye irritation when chlorine was added to the supply water, particularly at the 50-ppm level. Excessive corrosion of water pipes and equipment, and possible reaction of chlorine with organic matter to produce undesirable compounds in the poultry meat, should be considered when the advisability of adding chlorine to the fresh supply water for chilling poultry is evaluated.

TABLE 2. *Average ppm total available chlorine in chilling waters at various stages of chilling with different rates of input and levels of chlorination of fresh supply water.*

Input rate of fresh supply water (liters per bird)	0.95		1.90	
ppm Chlorine added to fresh supply water	20	50	20	50
Stage of chilling				
End of 1st prechill	11	25	12	26
End of 1st chill	8	27	8	29
Start of 2nd prechill	7	23	10	25
End of 2nd prechill	5	17	5	16
Start of 2nd chill	10	40	15	43
End of 2nd chill	8	20	9	25

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Mention of specific brand names does not imply endorsement by the authors or the U.S. Department of Agriculture to the exclusion of others not mentioned.

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A Research Note

Identification of *Enterobacteriaceae* Isolated from Seafoods[1]

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ABSTRACT

Fifty-three retail samples of seafood were examined for members of the family *Enterobacteriaceae*, using violet red bile agar with 1% glucose. Isolation and identification of 99 typical colonies showed them all to be members of the family *Enterobacteriaceae. Enterobacter* species were most frequently isolated with *Erwinia, Klebsiella, Hafnia, Proteus, Serratia, Escherichia* and *Yersinia* recovered with decreasing frequency.

The total *Enterobacteriaceae* count (*6*) is simple, rapid and capable of indicating both enteric contamination and organisms of public health significance in the absence of coliforms (*8*). It differs from direct plating for coliforms in that glucose is added to the enumeration medium to detect all *Enterobacteriaceae*, not just those that ferment lactose. The method has been suggested as an indicator system (*1,8*); however, limited information is available regarding the numbers and kinds of organisms recovered from various foods by this procedure.

While many questions still remain unanswered concerning applicability of this type of analysis, it seemed to have sufficient merit for routine monitoring of certain foods that we undertook a study of its use in analyzing retail samples of seafood products. This study was primarily concerned with isolation and identification of organisms forming typical colonies on the *Enterobacteriaceae* plates.

[1]*Florida Agricultural Experiment Stations Journal Series No. 1679.*

METHODS

Samples were obtained from retail stores in Gainesville, Florida (Table 1). Dilutions were prepared as recommended (*1*). Duplicate pour plates were prepared with violet red bile agar containing an added 1% glucose (VRBG) (*6*), followed by incubation at 35 C for 24 h. In addition, a three-tube MPN series was prepared in parallel for analysis of coliforms (*1*).

Typical "coliform type" colonies were picked for identification from the VRBG plates. If there were less than five colonies on the duplicate plates, all colonies were picked for identification. When the number of colonies exceeded five, the square root of the number of colonies on the plates was used to determine the number of isolates to be picked.

Identification of the isolates was by standard microbiological procedures and followed accepted characteristics (*3*).

RESULTS AND DISCUSSION

All isolates recovered from the VRBG agar plates were members of the family *Enterobacteriaceae* (Table 1). Four of the 10 species identified are unable to ferment lactose and therefore would not have been detected by using the standard coliform test. Since many of the

TABLE 1. *Seafood samples used, coliform counts, and* Enterobacteriaceae *isolated by using violet red bile glucose agar.*

		Isolated organisms											
Samples	No. pos/No. samples	*Erwinia herbicola*	*Klebsiella pneumoniae*	*Enterobacter aerogenes*	*Enterobacter cloacae*	*Hafnia alvei*	*Proteus inconstans*	*Serratia marcescens*	*Escherichia coli*	*Yersinia enterocolitica*	*Klebsiella ozaenae*	Coliform MPN range	VRBG range
		(No. times isolated)											
Deviled crab	7/7	7	13	5	4	3	4	1	2			3.6- > 1100	0-10,500
Breaded shrimp	6/6	5	2	4	3	4						3.6-93	0-1400
Breaded fish	5/5	6		1	4			1			1	3.6-43	0-145
Fish fillets	11/14	3	3	4				1	1	1		0-75	0-120
Raw shrimp	3/5	1				5						0-43	0-190
Cooked shrimp	1/3		1									0-15	0-5
Breaded scallops	1/2	1			2		1					0-23	0-25
Deviled clam	1/1											0-3.6	0
Stuffed flounder	1/1	1		4								0-93	0-245
Shrimp sticks	1/2											0-3.6	0
Breaded oysters	1/1											0-3.6	0
Fish sticks	0/4											0	0
Breaded clams	0/2											0	0
Totals	38/53	24	19	18	13	12	5	3	3	1	1		

Enterobacteriaceae, including at least one *Erwinia* species, are suspect regarding their pathogenicity (*7,9*), inclusion of as many of this family as possible in an indicator system would seem to have merit. This is particularly true when organisms such as *Yersinia enterocolitica* are detected, because this organism is gaining increased recognition as a cause of gastroenteritis in man (*5*). *Y. enterocolitica* was recovered from a frozen grouper sample in our study and was probably present at a level of less than one organism per gram.

The samples used in this study were of various backgrounds representing different degrees of processing, with most of them not having received any heat treatment. Identification of large numbers of *Erwinia herbicola* can be accounted for on the basis of the change in the taxonomic status of this organism. Previously classified as *Enterobacter agglomerans*, this organism has been reclassified as *Erwinia herbicola* (*3*). *Hafnia* was also included within the genus *Enterobacter* in earlier classification schemes; however, it is now a separate genus (*3*). Ten species and eight genera were recovered from the 53 samples. *Edwardsiella, Citrobacter, Salmonella* and *Shigella* were not found.

In a study of broiler carcasses (*4*), a more restrictive group of organisms was found during total *Enterobacteriaceae* counts. *Escherichia* and *Enterobacter* were the principle genera isolated. In vegetables, 85% of the salads tested were found to contain *E. agglomerans* (*10*). In addition, *Klebsiella, Enterobacter* and *Serratia* were recovered with high frequency.

Although Mossel et al. (*6*) indicated that certain organisms would mimic the *Enterobacteriaceae* on VRBG, none were encountered in this study. Addition of 1% glucose to VRB agar results in much more easily recognizable colonies than on VRB alone and this may account for our success in picking colonies of the *Enterobacteriaceae*. Total *Enterobacteriaceae* counts were generally higher than coliform counts; however, six samples were negative for *Enterobacteriaceae* by the VRBG method, but positive for coliforms using the MPN procedure (Table 1). This occurred mainly with samples that contained small numbers of coliforms and may have been due to use of a 3-ml sample per dilution with the MPN procedure and only 2 ml by the pour plate method. In that coliform numbers are generally low in seafood products (*2*), use of only duplicate plates in this procedure is not recommended.

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Ultrafiltration of Skim Milk at High Temperature

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ABSTRACT

Raw skim milk was selectively concentrated at 60 C by ultrafiltration and diafiltration procedures in an Abcor UF-22S unit equipped with high flux membranes. The thermal effect on flux rate, microbiological quality of the retentate and whey protein denaturation were observed under this processing condition. The higher processing temperature increased flux rate, improved microbiological quality and expanded the possibility of denaturing whey proteins, particularly with diafiltration. Composition of the product was normal and alkaline phosphatase negative.

Milk ultrafiltration generally has been conducted at 17-50 C. Higher temperatures are advantageous because less bacterial growth results, leading to a potentially better quality product, an improved flux rate and a lower energy requirement. Membrane stability and denaturation of the heat-labile milk proteins dictate maximum temperature but new, better designed, more heat resistant ultrafiltration membranes are providing greater flexibility than do those made of cellulose acetate.

Four major factors that contribute to heat denaturation of milk proteins are temperature, total solids, pH and time. Whey proteins are more readily denatured in retentate than in normal milk, according to Pierre et al. (*10*). However, Gernedel and Kessler (*3*) observed no significant denaturation of whey protein in skim milk ultrafiltered at 62-65 C for 2 h.

The present objective was to ultrafilter and diafilter skim milk at 60 C through membranes of higher heat resistance than cellulose acetate and observe permeate flux rate, microbial quality of the retentates, and whey protein denaturation.

MATERIALS AND METHODS

Skim milk concentration

Lots of 240 kg of raw skim milk from the Cornell Dairy Plant were ultrafiltered at 60 C in a UF 22S Abcor unit. The ultrafiltration unit consisted of 12 high-flux membranes with a total area of 1.23 m^2, a total liquid handling capacity of 9.08 l, and inlet and outlet pressures set at 3.21 and 1.07 kg/cm^2. Finished retentates were stored at -20 C in plastic containers until required for cheesemaking.

In double diafiltration trials, tap water, equivalent to twice the retentate weight, was added gradually when the skim milk weight was reduced by two-thirds. In other experiments, 2% Marlac #9 lactic starter (Marshall Laboratories, Madison, WI) was inoculated into the milk at 32 C. When the retentate pH dropped to 6.2, the temperature was raised to 60 C to arrest further fermentation.

Compositional analysis

Total solids, fat, protein, ash and pH of the retentate and permeate were determined as described by Kosikowski (*6*). Mineral analysis was conducted by the photoelectric spectrometric method of Kenworthy (*5*); lactose in the retentates and permeates was measured in an Enzymax - lactose/glucose analyzer (Leeds and Northrup, Pa.); refractometric indices were measured in a Bausch and Lomb Abbe type refractometer at 17.5 C. All analyses were made in duplicate.

Microbiological studies

Total plate counts were obtained on SPC agar according to *Standard Methods* (*1*). The dialysis phosphatase test of Kosikowski (*7*) was used to evaluate pasteurization in retentates and permeate during ultrafiltration.

Protein aggregation studies

Whey protein aggregation in retentates from ultrafiltering and diafiltering skim milk at 60 C was observed by Sephadex gel filtration. Skim milk retentates, diluted with distilled water to their original total nitrogen level, were centrifuged at 93000 × *g* for 2 h at 4 C. The clear supernatant fluids were decanted and freeze-dried for whey protein fractionation by gel filtration. A glass column (45 × 2.5 cm) packed with Sephadex G-100, fine was eluted at room temperature with 6.98 pH sodium phosphate buffer (I = 0.1, $NaHPO_4 \cdot H_2O$ 1.06 g, Na_2HPO_4 2.02 g, and NaCl 2.92 g/l; flow rate 1.9 ml/min.). The protein content in different fractions was monitored by measuring absorbance at 280 nm using a Beckman spectrophotometer (model Acta II).

RESULTS

A typical composition of retentates obtained by direct ultrafiltration, direct ultrafiltration with simultaneous fermentation, and double diafiltration with simultaneous fermentation is presented in Table 1. Diafiltered retentates were markedly higher in protein and lower in lactose and total solids when compared to direct ultrafiltered retentates. With the progressive concentration of retentate, the dry weight protein content increased from 35.0 to 76.03%, lactose content decreased from 52.47 to 13.83% and ash content decreased from 9.26 to 7.19%, Table 2, Fig. 1. Total solids in the permeate rose from 5.43 to 6.44%. Figure 1 also illustrates the continuous reduction in permeate flux with increasing milk concentration. The time required to concentrate 240 kg of skim milk at 60 C by different ultrafiltration processes is listed in Table 3.

Changes in standard plate counts of retentates and permeates with time are given in Table 4. The F factor of the finished retentate, 0.02, was low and the finished retentates and permeates were phosphatase negative. A high microbial count developed during the initial part of the simultaneous fermentation trial, but was reduced by a factor of 4 log cycles at 60 C (Table 5).

Approximately 90, 58 and 18% β-lactoglobulin was heat-denatured after double diafiltration with simultaneous fermentation, direct ultrafiltration with simul-

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TABLE 1. *Composition of skim milk retentates obtained by various UF processes at 60 C.*

Trial	Total solids (%)	Protein (%)[a]	Lactose (%)[a]	Ash (%)[a]	Fat (%)[a]
Direct ultrafiltration	29.71	68.0	13.7	7.2	2.2
Direct ultrafiltration + fermentation	31.85	64.7	13.3	6.1	9.2
Direct diafiltration + fermentation	24.85	91.1	1.9	6.3	—[b]

[a]Dry basis.
[b]— = Did not do.

TABLE 2. *Composition of direct ultrafiltration retentate and permeate at different time intervals during concentration at 60 C.*

Process time (h)	Total solids		Protein	Lactose		Ash	
	Retentate	Permeate	retentate	retentate	permeate	Retentate	Permeate
	(Percent)			(Percent dry basis)			
0	9.51	—	35.27	52.47	—	9.26	—
0.25	—	5.43	—	—	94.30	—	—
1.0	11.09	5.43	43.73	43.19	93.10	8.63	9.20
2.0	13.91	5.53	53.92	34.08	90.90	7.28	8.91
3.0	19.10	5.72	64.97	24.03	89.60	8.49	8.28
4.0	26.06	6.08	73.94	16.31	88.00	7.63	8.23
4.75 (final)	29.71	6.44	76.03	13.83	87.70	7.19	8.24

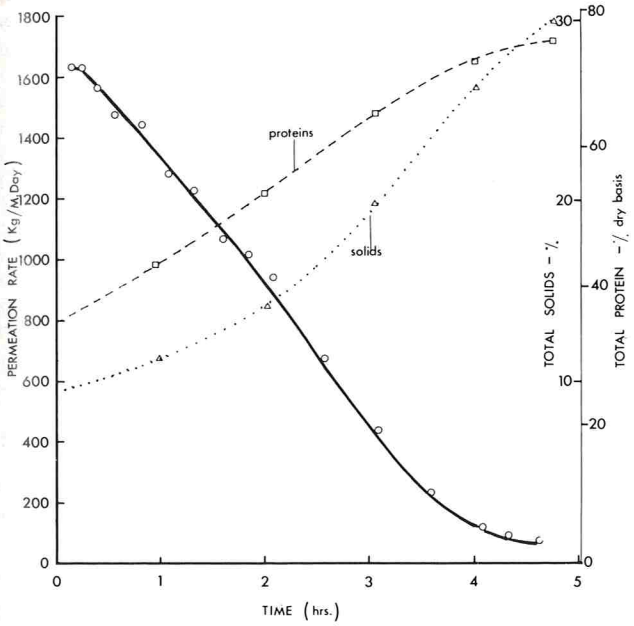

Figure 1. *Total solids and protein contents of retentate and the permeation rate at different time intervals during direct ultrafiltration at 60 C.*

TABLE 3. *Total time taken for skim milk concentration by various processes at 60 C.*

	Direct UF	Direct ultra-filtration fermentation	Double dia-filtration fermentation
Quantity of skim milk (kg) concentrated	240	240	240
Length of trial (h)	4.50	6.00	8.50
Capacity (kg/m²/h)	43.2	32.6	23.0

[a]Membrane used: (HFM), Total area: 1.23 m², Temperature: 60 C, Inlet and outlet pressure - 3.21 and 1.07 kg/cm².

taneous fermentation or direct ultrafiltration (Table 6). The losses of *a*-lactalbumin were 74%, 45%, and 18%. Whey protein losses during the initial 3 h of a double diafiltration trial - milk temperature 33 C - were minimum (Fig. 2). When the fermentation was arrested by raising milk temperature to 60 C, losses of *a*-lactalbumin and *β*-lactoglobulin occurred and continued through to the end of concentration.

TABLE 4. *Total microbial counts in retentates and permeates processed at 60 C at different time intervals during concentration.*

Process time (h)	CFU/ml		"F" Factor[a] in retentate
	Retentate	Permeate	
0	4000	—	1.0
0.25	—	2600	—
1	4000	2000	0.9
2	1000	660	0.11
3	1500	930	0.10
4	1300	250	0.06
4.75 (Final)	540	1600	0.02

[a]$F = \frac{\text{Plate count}}{\text{Original plate count} \times \text{concentration factor}}$

TABLE 5. *Total microbial counts in ultrafiltrated retentates with simultaneous fermentation processed at 60 C.*

Process time (h)	CFU/ml		Temperature (C)
	Retentate	Permeate	
0	1 800[a]	—	4
1	55×10^5	120×10^2	32
2	100×10^5	99×10^2	32
3	160×10^5	110×10^2	32
4	57×10^2	47×10^2	60
5	31×10^2	—	60
6.45 (final)	28×10^2	—	60

[a]Raw skim milk.

TABLE 6. *Residual a-lactalbumin and β-lactoglobulin in various skim milk retentates concentrated at 60 C.*

Process	*α*-la	*β*-lg
	%	%
Direct ultrafiltration	81.5	82.6
Direct ultrafiltration + fermentation	42.3	54.6
Double diafiltration + fermentation	10.5	25.6

DISCUSSION

Advent of polystearine type membranes for concentration of skim milk by ultrafiltration has made it possible to realistically process at 60 C and higher.

The composition of retentates and permeates obtained by different procedures at 60 C were similar to those reported previously for skim milk ultrafiltered at 50 C (*2,9*) and the increased temperature did not influence casein retention as electrophoretic analysis of the permeate showed no traces of casein.

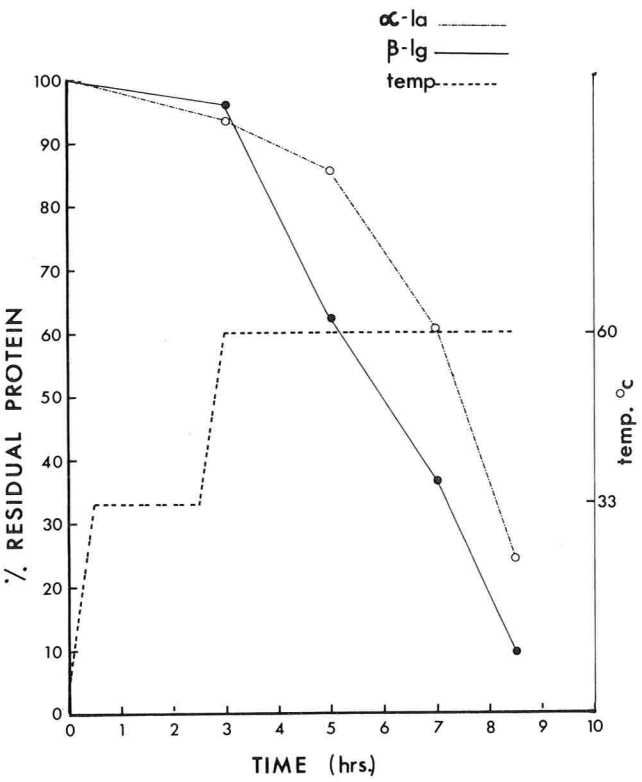

Figure 2. *Levels of a-lactalbumin and β-lactoglobulin in retentate at different time intervals during double diafiltration at 60 C.*

Conducting ultrafiltration at 60 C reduces the processing time for milk retention. A positive linear relation between the temperature of operation and permeate flux was reported earlier (*4,8*). However, the increase in operating temperature was beneficial only in accelerating the permeate flux rate and had a minimal effect on the total solids of the finished retentates. Apparently, formation of the compressible protein layer deposited at the membrane surface which limits the permeate flux rate, is independent of the operating temperatures, within limits.

Microbiological problems associated with ultrafiltration concentration of skim milk have been studied (*2,11*). The latter reported the possibility of reducing the bacterial multiplication factor (F) below 1.0 while operating UF at 50 C. Rash (*11*), however, noted that with prolonged processing times, the F factor increases many times, giving an undesirable microbiological quality to the final product. Pasteurization of retentate before usage was suggested.

An F factor as low as 0.02, observed in the present study, reflects a drastic reduction in microbial flora of the skim milk. Nevertheless, there is some possibility that toxin producing anaerobic thermophilic bacteria may grow at higher temperatures (*8*).

The finished direct ultrafiltered retentate originating from raw skim milk was phosphatase negative. Alkaline phosphatase enzyme apparently does not pass through UF membrane of 20000 daltons cut-off point and a heat treatment of 60 C during 4 h of concentration sufficed to destroy this enzyme in the retentate.

Direct ultrafiltration at 60 C for 4-5 h showed minimal denaturation of the whey proteins, an observation in agreement with Gernedel and Kessler (*3*). Ultrafiltration with simultaneous fermentation results in more whey protein denaturation, possibly due to lower pH (6.2) and an extended operation time (6.5 h). Whey proteins were largely denatured during the double diafiltration trial but as the temperature during the first 3 h was maintained at 33 C, protein aggregation was minimal. A rapid decrease in *β*-lactoglobulin and *a*-lactalbumin levels was observed when the temperature was raised to 60 C. Since Turner et al. (*12*) reported that lactose and its degradation products are complexed with milk proteins during heating of skim milk and Pierre et al. (*10*) confirmed that lactose provides protection to whey proteins from heat denaturation, it is suggested here that stripping lactose out of retentate during diafiltration accelerates the heat denaturation of whey proteins.

Other factors that contributed to whey protein denaturation during the double diafiltration trial were higher protein concentration (23%), extended time (9 h) and lower pH (6.2).

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Effect of Hydrogen Peroxide and Sodium Chloride on Enumeration of Thermally Stressed Cells of *Staphylococcus aureus*

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ABSTRACT

Evidence is presented suggesting that the decreased enumeration of heat-stressed *Staphylococcus aureus* cells on selective media is the result of accumulation of metabolic H_2O_2. It accumulates due to the decreased activity of catalase caused by the synergistic effects of heat and NaCl. Heated cells enumerated anaerobically on tryptic soy agar (TSA) containing 6.5% NaCl (TSAS 6.5) exhibited a 200-fold increase compared to cells enumerated aerobically on the same medium. The anaerobic counts on TSAS 6.5 were similar to the aerobic counts on TSA. Increases in both death and injury occurred when *S. aureus* was propagated in tryptic soy broth (TSB) plus 10% NaCl (TSBS) instead of TSB before thermal injury. Addition of catalase to TSA and TSA containing 7.5% NaCl (TSAS) increased the count to approximately the same levels on TSA and TSAS as that found following thermal injury after propagation in TSB. Catalase activity was 12-fold higher in stationary phase cells propagated in TSB than in TSBS. Indirect evidence indicates that toxic levels of H_2O_2 accumulated rapidly, causing one to two log decreases in enumeration after 30 to 60 min incubation on TSAS.

Accumulation of hydrogen peroxide (H_2O_2) has been implicated in the reduced colony-forming ability of injured microorganisms (*2,6,10,15*). Microorganisms may be subjected to a number of physical and chemical stresses (heating, freezing, drying, etc.) during food processing (*1,3,5,9,16,20*). These treatments may not be severe enough to kill microorganisms of public health concern, resulting in injured cells. Given the proper environment, the cells will recover (*21*). Two facts of general significance have been observed concerning injury (*21*): first, bacteria subjected to sublethal treatments produce repairable lesions; and, second, bacteria subjected to sublethal stress become hypersensitive to secondary stresses, such as those often encountered in selective media. For example, following thermal stress, *Staphylococcus aureus* loses its salt tolerance resulting in decreased enumeration on standard selective media containing NaCl (*12*).

Flowers et al. (*6*) demonstrated that addition of catalase to tryptic soy agar plus 7% NaCl (TSAS) and other selective media increased the enumeration of thermally stressed *S. aureus* up to 1100-fold. These cells were more sensitive to H_2O_2 than normal cells, particularly in the presence of NaCl. Addition of catalase or pyruvate (another agent causing H_2O_2 decomposition) to tryptic soy broth with 10% NaCl (TSBS) increased the enumeration of normal and thermally injured *S. aureus* using a Most Probable Number technique (*2*). Addition of catalase onto selective media increased the enumeration of *Salmonella typhimurium, Pseudomonas fluorescens* and *Escherichia coli* subjected to a variety of stresses (*15*) but did not increase the enumeration of thermally stressed *Streptococcus faecium*, an organism lacking catalase (*17*). Accumulation of peroxides in various anaerobic media has been observed after exposure to atmospheric oxygen (*4,10*). Addition of catalase to such media resulted in increased enumeration of *Clostridium perfringens*. Martin et al. (*15*) have suggested that H_2O_2 accumulation in or around the cell may be a universal phenomenon in injured cells, due to decreased catalase activity. This study was undertaken to further explore the relationship between catalase and the secondary stresses of NaCl and H_2O_2 following thermal injury.

[1]*Deceased, May, 1979.*

MATERIALS AND METHODS

Growth and stress conditions

S. aureus MF-31, obtained from the stock culture collection of Z. John Ordal was the organism used in this study. Cultivation and stress conditions were similar to those described by Iandolo and Ordal (*12*). Cultures were grown at 35 C for 12 h in tryptic soy broth (TSB) or in TSB containing 10% NaCl (TSBS) for 14-16 h. Cells were concentrated by centrifugation, washed in 100 mM potassium phosphate buffer, pH 7.2 (PPB), centrifuged and suspended in the same buffer. When lower numbers of cells were desired, an appropriate dilution of the unheated cell suspension was prepared in PPB. The cell suspensions were heated for 20 min at 52 C and enumerated on the media described below.

Media

Media used for enumeration in all aerobic experiments included tryptic soy agar (TSA), TSA containing 7.5% NaCl (TSAS), and TSAS with catalase (TSASC). Bovine catalase (13,000 units/mg; Sigma Chemical Co., St. Louis, MO) was solubilized in distilled water, filter-sterilized and spread onto previously poured TSAS plates giving approximately 780 units per plate. All platings were done in triplicate and incubated at 35 C for 24 h for TSA and TSASC and 48 h for TSAS.

Prereduced anaerobic media for the roll tube technique (*11*) were prepared under an atmosphere of 100% N_2. Cysteine hydrochloride (0.5 mg/ml) was added as a poising agent and resazurin (1 μg/ml) was added as an oxidation-reduction indicator. All media were adjusted to pH 7.0 with 1 N NaOH before sterilization (121 C, 15 min). Uninjured and injured cells were enumerated anaerobically on TSA and TSA containing 6.5% NaCl (TSAS 6.5) either by the standard surface plating procedure or the roll tube technique. All anaerobic enumeration media were supplemented with 0.1 mM uracil, 10 mM sodium acetate and

0.25% agar (*7,18*). Triplicate anaerobic plates in a 100% N_2 atmosphere and roll tubes were incubated at 35 C for 48 h for TSA and 72 h for TSAS 6.5.

Anaerobic enumeration of thermally injured cells

Thermally injured cells were enumerated aerobically and anaerobically from a common aerobically prepared decimal dilution series in 0.1% peptone. Two procedures were used for the anaerobic enumeration. The first procedure employed the roll tube technique and the Virginia Polytechnic Institute Anaerobic Culture System (*11*) using the prereduced media previously described. The second procedure utilized a Coy anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Michigan). The plates were equilibrated in the chamber for 72 h under an atmosphere of 95% argon and 5% hydrogen. After surface plating in the chamber, the plates were transferred to a 100% N_2 atmosphere and incubated as described above.

Effect of time on catalase addition

Ten-milliliter aliquots of appropriate dilutions of a heat-injured suspension were collected on sterile 0.45-μm membrane filters (Millipore Corp., Bedford, MA). The filters were placed on TSA, TSAS or TSASC and incubated at 35 C. After appropriate incubation on TSAS, replicate sets of filters were aseptically transferred to TSA, TSAS or TSASC. The plates containing the filters were incubated for a total of 24 h (TSA) or 48 h (TSAS and TSASC) at 35 C.

Lysis conditions

Frozen cell pellets of *S. aureus* were thawed and suspened in TM4 buffer [10 mM tris (hydroxymethyl) aminomethane, pH 7.6, 0.5 mM $MgCl_2$]. For lysis, 0.4 ml of a lysostaphin solution (0.5 mg/ml TM4; Sigma Chemical Co.) per ml of cell suspension was added. After 15 min of incubation at 35 C, DNAase (200 μg/ml TM4; Sigma Chemical Co.) was added at a concentration of 0.1 ml per ml of cell suspension. After incubation at room temperature for 1 min, the cell suspensions were centrifuged at 10,000 × *g* for 20 min. The supernatant fluid was retained and stored in an ice bath.

Catalase assay

The colorimetric assay of Sinha (*19*) was used. This method is based on reduction of dichromate in acetic acid to chromic acetate when heated in the presence of H_2O_2. The volume of lysate equivalent to one absorbance unit at 260 nm (Gilford Model 222 Spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, OH) was determined and added to the catalase-H_2O_2 reaction mixture. Samples were taken periodically and mixed with the dichromate/acetic acid mixture stopping the reaction. The remaining H_2O_2 was determined by measuring the absorbance of the chromic acetate after the mixture had been heated to stabilize the color. Catalase activity was calculated at each time period and the velocity constant of catalase (K_O) at 0 min determined by extrapolation. Activity was recorded as K_O per g of protein. The protein concentration of the lysate was determined using the Folin-Ciocalteau assay with lysozyme as a standard (*14*).

RESULTS

Anaerobic enumeration of thermally stressed cells

Accumulation of H_2O_2 has been implicated as a secondary stress in the decreased enumeration of heat-injured *S. aureus* (*6,15*). To test this hypothesis, thermally stressed cells were enumerated anaerobically to prevent generation of H_2O_2 due to aerobic respiration (*13*). Supplementation of the enumeration media with uracil and acetate was required for anaerobic growth (*18*). The enumeration of heated cells incubated anaerobically on TSA and TSAS 6.5 exhibited increases of 2- and 200-fold, respectively, compared to aerobically incubated cells on the same medium (Fig. 1). Stressed cells enumerated aerobically on TSAS and TSAS 6.5 exhibited the same degree of injury (data not shown).

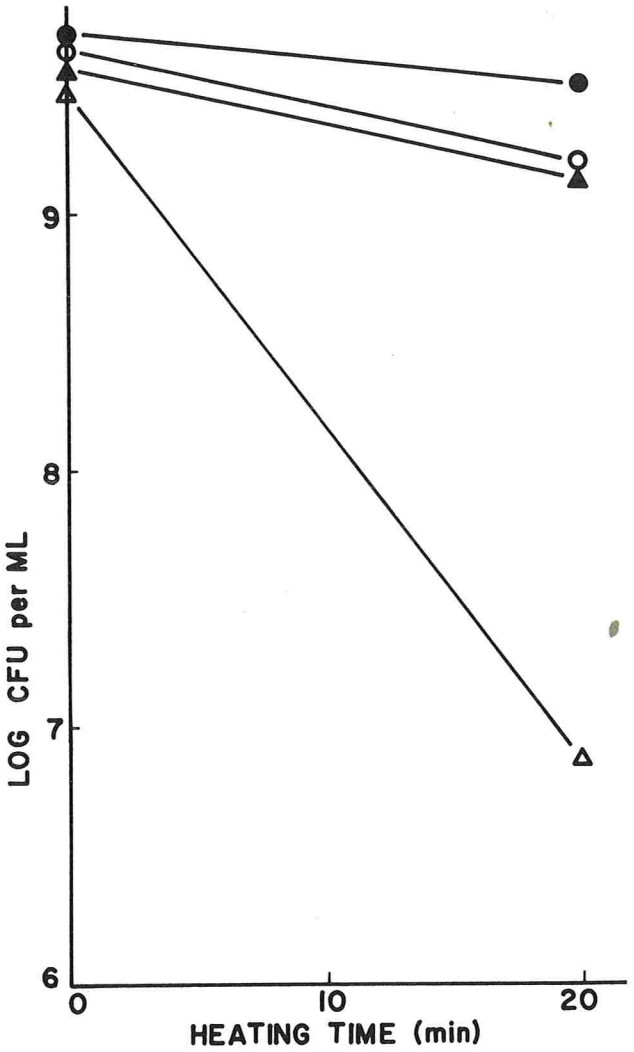

Figure 1. *A comparison of the aerobic and anaerobic enumeration of thermally stressed cells of* S. aureus *MF-31. A stationary phase culture suspended in 100 mM potassium phosphate buffer (pH 7.2) was heated at 52 C for 20 min. Cells were enumerated anaerobically either on plates in a Coy anaerobic chamber or in roll tubes using the Virginia Polytechnic Institute Anaerobic Culture System as described in the Materials and Methods section. The anaerobic data were pooled and averaged. All platings were done in triplicate; plates and roll tubes incubated under 100% N_2 at 35 C.*
Symbols: ○ aerobic enumeration on TSA, △ aerobic enumeration on TSAS 6.5, ● anaerobic enumeration on TSA, ▲ anaerobic enumeration on TSAS 6.5.

Enumeration of stressed cells incubated anaerobically on TSAS 6.5 and aerobically on TSA was similar.

Effect of delayed catalase addition on enumeration

In an attempt to determine the effect of time on both the exposure of thermally stressed *S. aureus* cells to NaCl in the selective medium (TSAS) and the resultant accumulation of H_2O_2, thermally stressed cells were collected on membrane filters and transferred to TSA, TSAS or TSASC following various periods of incubation on TSAS (Fig. 2). A similar pattern was observed when stressed cells incubated on TSAS for various time intervals were transferred to either TSA or TSASC. Enumeration of cells transferred to TSA or TSASC decreased with increased incubation on TSAS. A one to

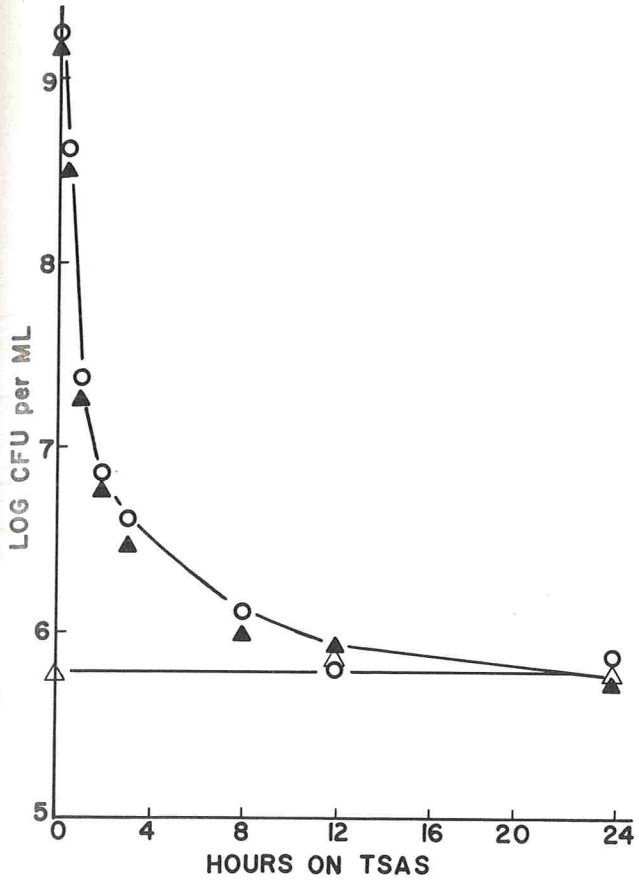

Figure 2. *Effect of delayed catalase addition on enumeration of thermally stressed cells of* S. aureus *MF-31. A stationary phase culture suspended in 100 mM potassium phosphate buffer (pH 7.2) was heated at 52 C with samples taken at 0 and 20 min. Samples were collected on membrane filters which were placed on the surface of TSAS plates and incubated at 35 C. After various periods of incubation on TSAS, the filters were transferred to TSAS (△), TSAS spread with 780 units of catalase (▲) or TSA (○).*

two log cycle decrease in enumeration occurred when the stressed cells were incubated on TSAS from 30 to 60 min before transfer to TSA or TSASC. The decrease in enumeration of transferred cells was more gradual after 2 to about 12 h of incubation on TSAS, after which there was no difference in enumeration.

Effect of propagation in TSBS on thermal injury

The effects of thermal stress on cells propagated in TSBS was examined (Fig. 3). Following standard thermal stress conditions, 99.7% of the cells were unable to form colonies when plated on the nonselective TSA. However, addition of catalase increased enumeration by 151-fold on TSA and 32-fold on TSAS. The counts of thermally stressed cells on TSA and TSASC were similar. The catalase activity in terms of K_O/g protein of stationary phase cells propagated in TSB and TSBS was 1745 and 146, respectively.

DISCUSSION

Accumulation of H_2O_2 has been implicated as a major factor for the decreased enumeration of thermally stressed cells on selective media (*6,15*). The synergistic

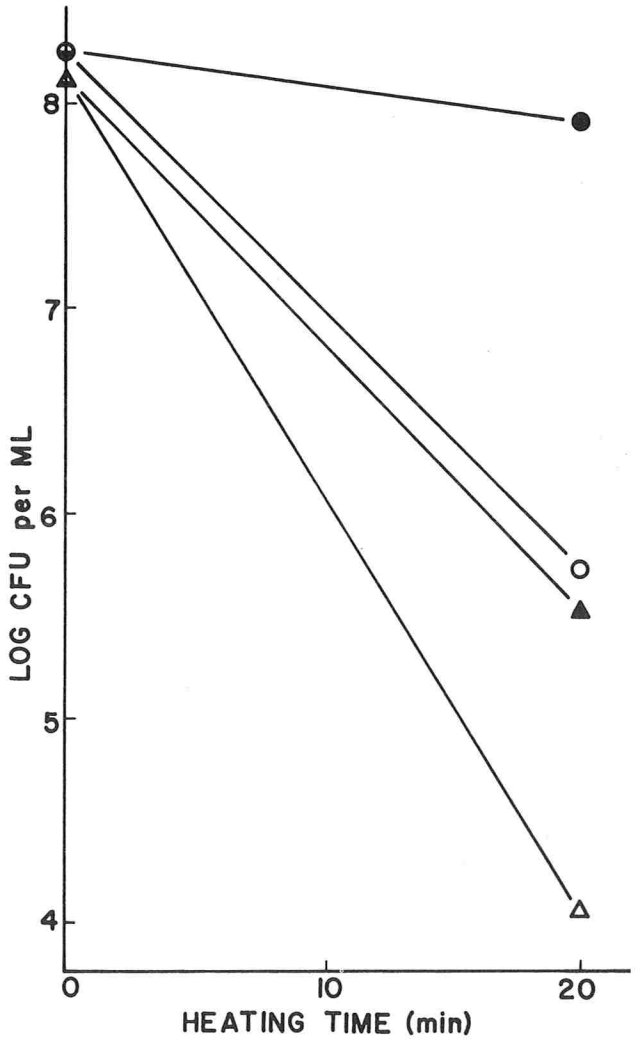

Figure 3. *The enumeration of thermally stressed cells of* S. aureus *MF-31 propagated in TSBS. A 14-16 h culture grown in TSBS was suspended in 100 mM potassium phosphate buffer (pH 7.2) and heated at 52 C for 20 min. Enumeration was on TSA (○), TSA + 780 units catalase per plate (●), TSAS (△), TSAS + 780 units catalase per plate (▲).*

effects of heat and NaCl resulted in the decreased activity of catalase in stressed cells, allowing the accumulation of H_2O_2 and eventually cell death. Instead of adding an exogenous H_2O_2 decomposer such as catalase, accumulation of H_2O_2 can be prevented by incubating the stressed cells anaerobically, since H_2O_2 is a product of aerobic respiration (*13*). The anaerobic incubation of stressed cells on TSAS 6.5 resulted in a 200-fold increase in enumeration over cells incubated aerobically on the same medium. Furthermore, no difference was observed if stressed cells were enumerated aerobically on TSA or anaerobically on TSAS 6.5. These data support the theory that accumulation of H_2O_2 results in decreased enumeration of stressed *S. aureus*.

Stressed cells were transferred to TSASC and TSA after various periods of incubation on TSAS to determine how rapidly the stressed cells became sensitive to NaCl and accumulation of H_2O_2. Toxic levels of H_2O_2 apparently accumulated rapidly in stressed cells placed

on TSAS, as evidenced by a one to two log cycle decrease in enumeration when the stressed cells were incubated on TSAS from 30 to 60 min before transfer to TSASC. After 12 h of incubation on TSAS, the toxic effects of H_2O_2 were not overcome by addition of catalase. A similar pattern was observed when cells were transferred to the non-selective medium TSA. These results are in contrast to those reported by Goff et al. (*8*) for a mixed flora from raw milk thermally stressed in TSB. They observed only a small difference in counts of thermally stressed cells collected on membrane filters and placed on TSB and cells transferred to TSB after 48 h of incubation on TSBS.

Effects of NaCl in the growth medium before thermal injury were examined. Catalase activity of stationary phase cells propagated in TSB was 12-fold higher than in TSBS. Increases in both death and injury occurred when *S. aureus* was propagated in TSBS instead of TSB (data not shown for TSB propagated cells) before thermal injury (Fig. 3). Addition of catalase to TSA and TSAS increased the enumeration to the levels normally found when *S. aureus* was propagated in TSB before thermal injury. These results indicated that the catalase activity of non-thermally stressed cells of *S. aureus* was adversely affected when *S. aureus* was grown in the presence of NaCl. The synergistic effects of heat and NaCl further decreased the catalase activity resulting in decreased colony-forming ability of thermally stressed cells on both TSA and TSAS.

In conclusion, the results of this study and others (*6,15*) indicate that accumulation of H_2O_2 resulted in a decreased enumeration of stressed cells. Toxic levels of H_2O_2 accumulated rapidly after stressed cells were plated on TSAS. Further, growth of *S. aureus* in TSBS resulted in a decreased catalase activity, with a resultant decrease in enumeration of stressed cells on TSA and TSAS compared to when grown in TSB.

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Lockhart, *con't from p. 1004*

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Sporulation and Toxin Production by *Clostridium botulinum* Type G

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ABSTRACT

A comparative study was conducted to determine the optimum conditions for sporulation and toxin production by *Clostridium botulinum* type G, strain 89. One solid and four liquid media were compared for their ability to promote sporulation. After being inoculated, the media were incubated at 35 C for 12 days, at 30 C for 16 days, and at 26 C for 21 days. Spores were harvested by centrifugation, washed 3 times and resuspended to give a 35× concentration, then counted by the MPN procedure. Spores grown on the solid medium at 35 C for 16 days gave higher counts than those grown at the same temperature in the liquid media. Toxin production was studied in eight media at 35, 30 and 26 C over a 24-day period with samplings every 2 to 3 days. Three of the media contained trypsin and five were trypsinized after growth. Toxin titers were determined by intraperitoneal injection of mice and expressed as MLD/ml of culture. Higher toxin titers were obtained at 26 and 30 C in media containing 0.4% glucose.

Type G is the most recently recognized type of *Clostridium botulinum*. Strain 89, the only known strain, was isolated by Gimenez and Ciccarelli in the Mendoza Province of Argentina (*3*). The heat-labile toxin produced by this organism causes symptoms of botulism in mice but the mice cannot be protected by botulinal antitoxins A through F. The organism is weakly proteolytic, it does not appear to ferment any sugars (*1,7*) and its toxin is potentiated 100- to 1000-fold by trypsin, as are the nonproteolytic strains of types B and F and all of type E.

Type G is a poor sporulator in traditional sporulating media and a poor producer of toxin in routine anaerobic media. Because of these characteristics, this organism may be difficult to detect. That its presence has never been established in any food poisoning outbreak or in any food may be due to a lack of suitable detection methods. The purpose of this investigation, therefore, was to determine the optimum conditions for sporulation and toxin production by *C. botulinum* type G.

MATERIALS AND METHODS

Culture

A culture of *C. botulinum* type G, strain 89, was obtained from V. R. Dowell, Jr., Bureau of Laboratories, Center for Disease Control, Atlanta, Ga. In preparation for these studies, the culture was propagated in brain heart broth (BHB) (*9*), which is a good sporulation medium for the other botulinum types.

Media

Trypticase-peptone-glucose-yeast extract (TPGY) broth with 0.4% glucose, TPGY with 0.1% glucose, BHB, Difco's dehydrated cooked meat medium (CMM), and anaerobic egg agar without the egg (AA) were compared for their ability to promote sporulation. The AA medium was slanted in 16 oz. prescription bottles, 160 ml/bottle. TPGY with 0.4% glucose, TPGY with 0.1% glucose, proteose peptone (P-P) (*1*), CMM, BHB, and the first three media with trypsin, at 0.1% final concentration added before inoculation, were compared for their ability to promote toxin production.

Spore production

Wheaton storage bottles of 500-ml volume containing 350 ml of the various liquid media were inoculated with approximately 10,000 spores each and incubated at 35 C for 12 days, at 30 C for 16 days and at 26 C for 21 days. The periods of incubation at these temperatures were determined during preliminary experiments in which maximum sporulation occurred in BHB. The anaerobic agar medium was inoculated with approximately 10,000 spores spread over the dried slant and incubated inside large GasPak anaerobic jars at 35 C for 16 days. Anaerobic requirements of the organisms were provided by the other media themselves. Spores were harvested by centrifugation, washed 3 times and resuspended in sterile distilled water to give a 35× concentration of spores, i.e., from each bottle of 350-ml medium or each prescription bottle, a 10-ml sample of concentrated spores was produced.

Spores were counted by the 3-tube MPN procedure under three conditions: as harvested; after heating to 60 C for 10 min; and after alcohol treatment at room temperature for 1 h (*4*). The supernatant fluids of all spore crops were tested for toxicity and checked against the antitoxin of types A through F. The spore crops were tested for purity aerobically and anaerobically on liver veal and AA.

Toxin production

Wheaton storage bottles of 125-ml volume containing 100 ml of medium were inoculated with approximately 1000 spores each and incubated at 35 C, at 30 C, and at 26 C over a 24-day period with samplings every 2 to 3 days. A different bottle was used for each sampling. Toxin titers were determined by intraperitoneal (IP) injection of mice and expressed as MLD/ml of culture. The cultures grown in media without trypsin were trypsinized before being injected into mice (*2*).

RESULTS AND DISCUSSION

The sporulation data on *C. botulinum* type G, summarized in Table 1, are averages of triplicate experiments. Counts of the spore suspension as harvested were generally one log higher than either the heat-treated or alcohol-treated suspensions. Heat and alcohol were found to destroy the vegetative cells in preliminary experiments, which undoubtedly resulted in the higher counts of the untreated suspensions.

The TPGY medium with either 0.4 or 0.1% glucose did not support sporulation of this organism. The spore count was approximately 3×10^5/ml in BHB and approximately 3×10^6/ml in CMM, regardless of the

incubation temperature. The highest spore counts were obtained from the slanted AA grown at 35 C for 16 days. Heat or alcohol treatment did not affect the spore counts, indicating that on this solid medium sporulation was complete with few if any vegetative cells present. A scanning electron micrograph of spores produced on this medium is shown in Fig. 1.

Toxin production by type G appeared to be affected by the temperature and time of incubation. Maximum toxin production was achieved by 7 to 10 days at 26 and 30 C. Table 2 shows that in TPGY with 0.4% glucose at 35 C (column A), the untrypsinized toxin remained constant and of low titer throughout the 24-day incubation period. When this toxin was trypsinized (column B), it was potentiated 10- to 100-fold but this was somewhat erratic. Column C shows titers of toxin produced in the same medium but with trypsin added at the time of inoculation. In this case, toxin was potentiated as it was released into the medium and its titer was higher and more uniform. The trypsin-containing medium was more sensitive in detecting the organism (5) or as a guide to complete toxin production, but was unsatisfactory for storage of toxin since trypsin activity, even at refrigeration temperature, completely degrades the toxin. At 30 C incubation the results were about the same, although trypsin gave greater potentiation of the toxin. Best results were obtained at 26 C incubation. The untrypsinized toxin titers were higher than those produced at the other temperatures, and both the trypsinized and trypsin-containing cultures gave higher and more uniform toxin titers. The lower temperature appears to favor the slow release and accumulation of toxin in the medium. There was no change in the toxin titers between days 10 and 24 and, therefore, results beyond 10 days are not shown in these tables.

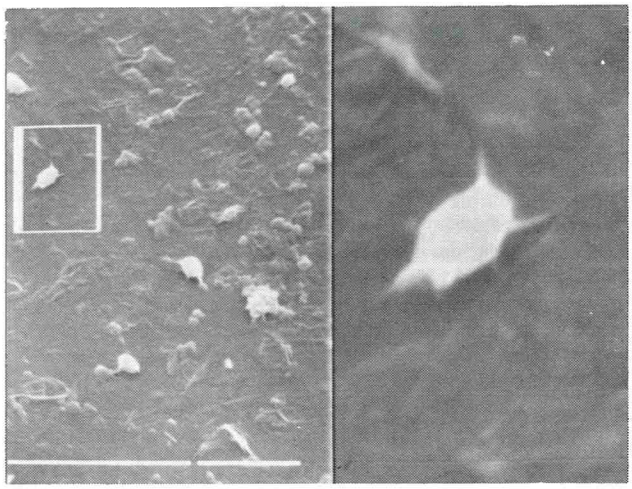

Figure 1. *Scanning electron micrograph of* C. botulinum *type G spores at 4000× (left) and 20,000× respectively. Space between bars = ½ μm.*

In TPGY with 0.1% glucose the titers of toxins produced at 35 and 30 C showed no significant difference from those produced at the same temperatures in TPGY with 0.4% glucose. However, at 26 C the amount of glucose in the medium seemed to affect both the untrypsinized and the trypsinzined toxin titers. In the P-P medium the toxin titers were comparable to those obtained in TPGY with 0.4% glucose, although the glucose content in this medium was 1%, or more than double. As shown in Table 3, the toxin titers produced in the two meat media, CMM and BHB, were approximately similar to those produced in TPGY with 0.4% glucose. These two media are used for proteolytic strains of *C. botulinum*.

Throughout this investigation of *C. botulinum* type G, the similarities of this organism with both the proteolytic

TABLE 1. *Spore counts (MPN/ml) of* C. botulinum *type G grown in various media.*

Medium	35 C/12 days			30 C/16 days			26 C/21 days		
	A[a]	B[a]	C[a]	A	B	C	A	B	C
TPGY (0.4% Glucose)	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$
TPGY (0.1% Glucose)	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$	$< 3 \times 10^1$
Beef heart broth	7.3×10^6	8.1×10^5	1.8×10^5	2.6×10^5	2.5×10^5	1.6×10^5	3.2×10^5	3.3×10^4	2.3×10^5
Cooked meat medium	3.7×10^7	1.1×10^6	1.4×10^6	3.8×10^7	4.0×10^6	3.2×10^6	3.7×10^7	1.7×10^6	3.2×10^6
Anaerobic agar slanted	7.9×10^7[b]	6.1×10^7[b]	7.7×10^7[b]	9.3×10^6	4.6×10^6	2.4×10^6	4.6×10^6	1.1×10^6	1.5×10^6

[a]A = as harvested, B = heated at 60 C/10 min, C = alcohol treated at room temp./60 min.
[b]Incubated for 16 days.

TABLE 2. *Toxin titers (MLD/ml) of* C. botulinum *type G in liquid media.*

Medium	Incubation time (days)	35 C			30 C			26 C		
		A[a]	B[a]	C[a]	A	B	C	A	B	C
TPGY (0.4% Glucose)	3	10^1	10^2	10^3	10^1	10^3	10^3	10^1	10^3	10^3
	5	10^1	10^2	10^3	10^1	10^3	10^3	10^1	10^3	10^3
	7	10^1	10^3	10^3	10^1	10^4	10^3	10^2	10^4	10^4
	10	10^1	10^2	10^3	10^1	10^3	10^4	10^2	10^4	10^4
TPGY (0.1% Glucose)	3	10^1	10^2	10^3	10^0	10^3	10^4	10^1	10^3	10^4
	5	10^0	10^2	10^3	10^1	10^3	10^4	10^1	10^3	10^3
	7	10^0	10^2	10^3	10^1	10^3	10^3	10^1	10^3	10^3
	10	10^0	10^2	10^3	10^1	10^3	10^3	10^1	10^3	10^4
Proteose-peptone	3	10^1	10^3	10^3	10^2	10^3	10^3	10^1	10^3	10^3
	5	10^1	10^3	10^3	10^1	10^3	10^3	10^1	10^3	10^3
	7	10^1	10^3	10^4	10^1	10^3	10^4	10^2	10^4	10^3
	10	10^1	10^3	10^3	10^1	10^4	10^4	10^1	10^3	10^3

[a]A = Untrypsinized, B = Trypsinized, C = In medium containing trypsin.

TABLE 3. *Toxin titers (MLD/ml) of* C. botulinum *type G.*

Incubation time (days)	CMM[a] 35 C A[c]	CMM[a] 35 C B[c]	CMM[a] 30 C A	CMM[a] 30 C B	CMM[a] 26 C A	CMM[a] 26 C B	BHB[b] 35 C A	BHB[b] 35 C B	BHB[b] 30 C A	BHB[b] 30 C B	BHB[b] 26 C A	BHB[b] 26 C B
3	10^1	10^3	10^0	10^2	10^0	10^2	10^1	10^2	10^1	10^2	10^0	10^2
5	10^1	10^2	10^1	10^3	10^1	10^3	10^0	10^2	10^1	10^3	10^1	10^3
7	10^1	10^3	10^2	10^3	10^2	10^3	10^0	10^3	10^0	10^1	10^1	10^3
10	10^1	10^3	10^2	10^4	10^2	10^3	10^0	10^3	10^1	10^2	10^1	10^2

[a]Cooked meat medium.
[b]Brain heart broth.
[c]A = Untrypsinized; B = Trypsinized.

and nonproteolytic strains of *C. botulinum* were evident. In its sporulation ability it resembles the proteolytic strains, showing the preference for the meat media or solid agar which is characteristic of that group. Like the nonproteolytic strains, its toxin production is best at lower temperatures (26 or 30 C) in liquid media, and its toxin is fully potentiated by trypsin. Because type G exhibits the characteristics of both groups into which *C. botulinum* has been divided (*1,6,7,8*), further investigations of this organism may increase our understanding of the factors which separate as well as unite the members of this species. The higher toxin titers produced by this organism in media that contain trypsin could make it a potential public health hazard when naturally occurring conditions favor its development in commercial consumer products.

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Effect of Five Surface Area-to-Sample Volume Ratios During Preliminary Incubation on The Bacterial Count of Raw Milk[1,2]

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ABSTRACT

Effects of ratio of surface area to volume of sample during preliminary incubation (PI) and of different plate incubation temperatures on bacterial counts of raw milk samples were studied. One hundred and twenty Grade A raw milk samples collected during a 10-month period were divided into five 100-ml aliquots and allotted to one of five surface area to volume ratios. The ratios during PI ranged from 0 cm^2/100 ml to 149.74 cm^2/100 ml. Following PI, pour plates of each treatment were incubated at 26, 30 and 32 C for 72 h. The Standard Plate Counts (SPC) ranged from 89×10^1 to 20×10^8/ml, with the SPC of 73.6% of the samples being less than 1×10^5/ml. Counts after PI tended to be higher as the plate incubation temperature decreased from 32 to 26 C and as the ratio of surface area to volume of sample increased. None of the differences between the counts for the 15 treatment-incubation temperatures was significant. Counts of 61 samples increased less than one log count during PI, while counts of 33 and 16 samples increased one to two log counts and over two log counts, respectively. The greater the SPC, the smaller the increase in count during PI. Of the 81 samples with SPC less than 1×10^5/ml, 29 had counts after PI that exceeded 2×10^5/ml.

Poor correlation has been reported between the Standard Plate Count (SPC) of raw milk and the prevailing sanitary conditions during production and handling of the milk (*3,5,13,15*). The present method of SPC incubation at 32 C may result in a large percentage of microorganisms which indicate poor sanitation or careless practices not being detected (*6,13*). Microorganisms which enter milk due to improper production practices grow slowly, if at all, at 32 C. Some researchers have recommended incubation at temperatures less than 32 C to obtain a more reliable index of farm sanitation, since 32 C is close to the maximum growth temperature for these contaminants (*4,8,13,14,25*).

Psychrotrophic bacteria have been used to evaluate the microbial quality of milk. The psychrotrophic count gives a good indication of sanitary conditions during production of raw milk (*5,6*). However, it has not been widely accepted because incubation at 7 C for 10 days is impracticable for routine use by the dairy industry.

The preliminary incubation (PI) test was developed as a faster method for identifying farms that had a low SPC in spite of poor sanitation (*11,12*). Raw milk samples are incubated at a temperature (12.8 C) which permits the saprophytic contaminants to grow more rapidly than the microorganisms normally present in raw milk. The PI test has been reported to give a good indication of conditions that exist during production of milk; especially where cooling has been substituted for cleaning and sanitizing (*2,12,14,15,18*). A high count after PI suggests that careless practices which allowed contamination of milk were used during production. While many tests have been developed and used to determine sanitary conditions and practices during production, the PI test seems to be the most useful and the test which has had the widest acceptance (*3,5,14*). Unlike some of the other bacterial tests, conditions have not been established for standardization of the PI test (*13,17,23*).

The objectives of this study were to determine the effects that the use of different ratios of surface area to volume of sample during preliminary incubation and of different plate incubation temperatures had on recovery of microorganisms from raw milk samples.

MATERIALS AND METHODS

Samples

One hundred and twenty Grade A raw milk samples were collected during a 10-month period. Samples were obtained from farm bulk tanks by milk truck drivers after addition of the fourth milking and just before emptying of the bulk tank. Samples were collected in 907-ml, (32-oz) Whirl Pak bags (Nasco, Fort Atkinson, WI), kept refrigerated during transportation to the laboratory and held below 4 C until analyzed. Samples were analyzed within 24 h of collection.

Preliminary incubation

Each milk sample was divided into five 100-ml aliquots and randomly allotted to one of the following treatments: (*a*) 170-ml (6-oz) Whirl Pak bag folded to give a 1-cm airspace above the milk when incubated upright, (*b*) same as treatment a, except that the bag was incubated flat, (*c*) 170-ml Whirl Pak bag folded so no airspace existed above the milk when incubated upright, (*d*) 907-ml Whirl Pak bag folded to give an area twice that of the 170-ml bag when incubated flat and (*e*) 907-ml Whirl Pak bag folded to give an area four times that of the 170-ml bag when incubated flat. An empty 170-ml bag used in treatments a, b and c had an area of 6500 mm^2. The area of an empty bag used in treatment d was 13,000 mm^2, while the bag used in treatment e had an area of 26,000 mm^2. The bags in each treatment were incubated at 12.8 C for 18 ± .5 h.

Plating procedure

Except as described below, initial counts and counts after preliminary incubation were determined on Standard Methods agar (BBL) using the pour plate method (*7,17*). Initial count plates were

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incubated at 32 C for 48 h. Triplicate plates were prepared for the preliminary incubated samples and a plate from each dilution plated randomly incubated at 26, 30 and 32 C for 72 h. Plates having 30-300 colonies were counted using a Quebec Colony Counter.

Analysis of data

Data were analyzed using Statistical Analysis System (SAS) programs (*1*). The Duncan's multiple range test was used to determine if counts were significantly affected by PI treatment and/or plate incubation temperature.

RESULTS AND DISCUSSION

Standard Plate Counts (SPC) were determined for 110 of the 120 raw milk samples collected. The SPC ranged from 89×10^1 to 20×10^8/ml. Eighty-one (73.6%) samples had SPCs which were less than the maximum (1×10^5/ml) established for Grade A raw milk (*26*). Counts less than 1×10^4/ml were obtained for 27 samples, while 29 samples had SPCs which exceeded 1×10^5/ml.

Mean counts obtained after preliminary incubation (PI) for each of the five treatments at the three plate incubation temperatures are given in Table 1. Counts tended to be higher as plate incubation decreased from 32 to 26 C and as the surface area to volume ratio increased. However, none of the differences between the mean counts for the 15 PI treatments-plate incubation temperature combinations was significant. The ratios of surface area to volume of samples were 82.45 cm²/100 ml and 149.74 cm²/100 ml for treatments d and e, respectively, compared with 3.94 cm²/100 ml for treatment a and 6.48 cm²/100 ml for treatment b. The ratio for treatment c was close to zero, since as much air as possible was excluded from the bag before PI. The higher counts obtained for treatments d and e probably were due to better growth conditions for contaminants which resulted from the greater surface area to volume ratios. Bacteria which grow actively in raw milk incubated at 12.8 C for 18 h are usually aerobic (*23*). More oxygen should be available for growth of aerobic psychrotrophs as the surface area to volume of sample is increased. The increased surface along with a temperature suitable for growth of most psychrotrophs should permit growth of most contaminants and result in higher counts. These results are similar to those obtained by Reinbold et al. (*23*), except that they found the increase in count with increase in the ratio of surface area to volume of sample to be significant. They used test tubes and bottles containing 10 to 20 ml of milk rather than Whirl Pak bags.

TABLE 1. *Effect of treatment during preliminary incubation of raw milk samples on mean counts after incubation for 72 hours at 26, 30 and 32 C.*

	Incubation temperature[a]			
	26 C	30 C	32 C	
Treatment		(Log_{10}/ml)		Mean counts[b]
a	5.97	5.93	5.89	5.93
b	5.98	5.94	5.93	5.95
c	5.96	5.92	5.88	5.92
d	6.15	6.04	6.02	6.07
e	6.13	6.03	6.07	6.08
Mean counts[c]	6.04	5.97	5.96	

[a]Each value represents the means of 120 counts.
[b]Means of 359 counts.
[c]Means of 598 counts.

The higher counts obtained for plate incubation temperatures less than 32 C are similar to results obtained by others (*4,8,16,19,22,24*). Some investigators have concluded that plate incubation temperatures should not be lowered from 32 C, even though higher counts may be obtained at the lower temperatures (*9,22*). The increase in count may not justify the additional time or special incubators required for incubation at the lower temperatures.

The SPC and counts after PI along with the percentage increase in counts due to PI are shown in Table 2. Since counts after PI were not affected by ratio of surface area to volume of sample during PI or by plate incubation temperature, only the results obtained for Treatment a and plate incubation temperature of 32 C are shown. The counts of 61 samples (55.5%) increased less than one log count following PI. Increases of one to two log counts occurred for 30% (33) of the samples, while increases of over two log counts were obtained with 16 (14.5%) samples. One sample had a count after PI which was more than three log counts higher than its SPC. Other investigators have reported more than a one log count increase after PI in 48 to 76% of the samples analyzed and greater than a two log count increase in 35% of the samples (*2,11,23*).

The percentage of samples that showed more than one log count increase after PI increased as the initial counts increased up to 1×10^6/ml. These results are not surprising since milk samples with high initial counts often reflect poor or insanitary production practices (*11*). Often satisfactory SPCs are obtained for milk produced under poor or insanitary production conditions because of the effect of dilution and/or cooling (*14,15*). Such practices may result in a greater percentage of the microflora being comprised of species capable of growing during PI. Thus an increase in count following PI generally indicates poor sanitation either during production or of milk contact equipment (*11,17*).

TABLE 2. *Initial counts of raw milk samples and changes in counts after preliminary incubation (PI) at 12.8 C for 18 hours.*

Initial count/ml[a]	Number of samples	Initial Count (log_{10}/ml)		Count after PI[b] (log_{10}/ml)		Increase (%) after PI[b]		
						< 1 log	1- ≤ 2 logs	> 2 logs
$< 10^4$	27	3.70	(2.95-3.95)	4.02	(2.30-5.78)	85.2	11.2	3.7
$\geq 10^4$-$< 10^6$	66	4.63	(4.00-5.90)	5.77	(4.00-7.85)	48.5	28.8	22.7
$\geq 10^6$	17	6.96	(6.00-8.30)	8.07	(6.00-8.85)	47.1	52.9	0.0
Total	110					55.5	30.0	14.5

[a]Plates incubated at 32 C for 48 h.
[b]Based on counts of samples from treatment 1 and plates incubated at 32 C for 72 h.

Counts after PI had increased two log counts or less for the 17 samples which had SPC greater than 1×10^6/ml. The greater the SPC, the smaller the increase in count during PI. The smaller increases for samples with the higher SPC probably are due to depletion of nutrients, accumulation of toxic metabolites and/or overcrowding. Several investigators (*11,23*) have reported that counts after PI tended to increase as the SPC increased. Samples with SPC greater than 1×10^5/ml were not included in these studies.

No value has been established for the maximum allowable count after PI. Johns has suggested 2×10^5/ml as the maximum allowable count, while others have proposed 1×10^5/ml (*12,17*). In this study, 81 samples had SPCs less than 1×10^5/ml. Of these samples, 29 (35.8%) had counts after PI that exceeded 2×10^5/ml. The range of SPC for these 29 samples and the changes in counts followng PI are shown in Table 3. One sample with a SPC less than 1×10^3/ml had an increase of more than two log counts after PI. Of the 28 samples with SPC between 10^3 - 10^4/ml, the counts of 10.7% increased less than one log count during PI, while 46.4% increased more than two log counts. Of the 120 samples analyzed, 66 (55.0%) had counts after PI that exceeded 2×10^5/ml. Other researchers have found 26 to 67% of the milk samples studied had counts after PI that exceeded 2×10^5/ml (*11,20,21*).

Under the conditions of this study, the ratio of surface area to volume of raw milk during PI did not result in significant differences in counts. Therefore, the same Whirl Pak bag used to transport samples to the laboratory could be used for PI and the samples could be incubated upright during PI for maximum utilization of incubator space.

TABLE 3. *Changes in the counts of milk samples with initial counts less than 1×10^5/ml and greater than 2×10^5/ml after preliminary incubation (PI).*

Initial count[a]	Number of samples	Increase (%) after PI[b]		
		< 1 log	1- ≤ 2 logs	> 2 logs
$\leq 10^3$	1	0.0	0.0	100.0
10^3- $\leq 10^4$	28	10.7	42.9	46.4

[a]Plates incubated at 32 C for 48 h.
[b]Based on counts of samples from treatment 1 and plates incubated at 32 C for 72 h.

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Microbial Aspects of Mechanical Tenderization of Beef

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ABSTRACT

The exterior parts of mechanically tenderized outside and inside rounds and rib eye had an aerobic plate count (22 C) in the range of 1.0×10^1 to 1.5×10^4/g. The aerobic plate count of the interior parts of the same subprimal cuts was 1.0×10^1/g. Cleaning and sanitizing the tenderizer with an iodine-based sanitizer (25 ppm titratable iodine) decreased the bacterial population of both the conveyer belt and blades from 1.0×10^3 to $< 1.0 \times 10^1$/cm² or two blades (surface are area of 119.4 cm²) respectively. *Clostridium perfringens* and *Salmonella* were not detected from the exterior and interior parts of the tenderized outside round. *Staphylococcus aureus*, total *Enterobacteriaceae* and the coliform group were each at a level of $< 1.0 \times 10^1$/g. Vacuum packaging of tenderized outside round and storage at 16 C for 18-20 h did not significantly ($P < 0.05$) increase either the aerobic or anaerobic bacterial counts.

The grain crisis of 1974 and the high prices for grain lead to increasing amounts of slaughtered grass-fed beef (*2*). Grass-fed beef was found less tender than grain-fed beef (*11*). Mechanical tenderization improved the tenderness of beef in general and of lean beef in particular (*5,13,15*). Recently the process became an important part of beef fabrication (*12*). The continuous rise of beef prices will make the process a necessity.

Mechanical tenderization provides among other benefits, improved tenderness of relatively inexpensive beef and equalization of the tenderness of meat cuts containing different muscles of differing tenderness (i.e. round steak, top sirlion steak, etc.).

Mechanical tenderization may carry bacteria from the surfaces of both the meat and the tenderizer into the depth of the tenderized product. Unsanitary conditions worsen the problem. The newly introduced bacterial population, or part of it, may proliferate in the depth of the meat and increase in number (provided that adequate extrinsic and intrinsic growth factors prevail) and shorten the shelf-life of the product (*12*). It is hypothesized that a portion of the bacterial population in the depth of the tenderized product may survive the heat treatment (internal temperature of 60 C) given during the preparation of "rare" beef roast (*9*). Survival of pathogens such as *Salmonella* or *Yersinia* will pose a public health threat.

Vacuum packaging prolongs the shelf life of beef (*18*) and enhances the postmortem metabolism of "hot-boned" beef (boning before chilling the carcass) (*20*), but at the same time it may enable facultative anaerobic bacteria to proliferate (*4*). The presence of pathogens among these bacteria will reduce the wholesomeness of the product.

There has not been a great amount of work done on mechanically tenderized beef, addressing the bacterial recontamination of the depth of the tenderized product and the health hazard aspects.

This work was undertaken to study the effect of mechanical tenderization and vacuum packaging on the bacteriological quality of electrically shocked "hot-boned" beef.

MATERIALS AND METHODS

Prefabrication treatment

Six carcasses from commercial Angus and Hereford steers (290 kg average weight) were used in this study. A 15-min electrical shock was given to one side of each carcass at 30-min postmortem, as previously described by Raccach and Henrickson (*14*).

Boning of subprimal cuts

The outside and inside rounds (biceps femoris and semimembranousus muscles, respectively) and the rib eye (longissimus dorsi) were boned on-the-rail at 1.5 h postmortem from the electrically shocked side, and after 24 h at 1.1 C from the control (non-electrically shocked) side. The subprimal cuts boned from the electrically shocked side were designated as "Hot-Boned-Hot".

Mechanical tenderization

Before each use the tenderizer (Hollymatic AMT 625A Blade Tenderizer, Hollymatic Corp., Park Forest, IL) was rinsed with hot tap water, cleaned (2171 General Cleanser, Klenzade Products) and rinsed with cold water. The tenderizer was sanitized with an iodine based (25 ppm titratable iodine) sanitizer (Mikroklene DF, Klenzade Products) at least 2 min before starting the tenderization process. Special care was given to the blades and the conveyer belt of the tenderizer throughout the cleansing and sanitizing processes.

One half of each subprimal cut was tenderized twice with the top and bottom of the meat cut respectively toward the blades of the tenderizer. The second half of each subprimal cut served as a nontenderized control.

About one third of each tenderized and non-tenderized "Hot-Boned-Hot" outside round was vacuum packaged (72.5 mm Hg) in a polymylar film (E.I. duPont, Wilmington, Delaware) and stored at 16 C for 18-20 h.

Sampling

The exterior and interior parts of the outside round and the exterior parts of the inside round and the rib eye were sampled before and after being tenderized. Meat tissue (0.5-cm thick) was excised from the exterior part of each subprimal cut and cores (diameter of 5 cm) were bored from the interior part of the outside round. Sampling was done manually using sterile scalpels.

The bacteriological examinations were done on 50-g samples composited from either the sampled meat tissue or the meat cores.

Aseptic measures (lighted burner, sanitized working surfaces, frequent changes of sterile scalpels etc.) were taken throughout the sampling process to prevent contamination of the samples.

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The tenderizer was sampled for bacterial count by swabbing surface areas of 10 cm^2 of the conveyer belt and sets of two blades (each set had a surface area of 119.4 cm^2) before, in between, and after tenderization of the subprimal cuts. Each swab was dispersed in 20 ml of 0.1% peptone (Difco) water.

Bacteriological examinations

The homogenates of the meat samples were prepared by blending 50 g of meat with 450 ml of 0.1% peptone (Difco) water. Further decimal dilutions for both the meat samples and the swabbed material were prepared (as required) with the same diluent.

The aerobic plate count was done by spreading 0.1-ml aliquots of the appropriate dilutions on prepoured dried surface Plate Count agar (Difco) plates. Incubation was at 22 C for 40-48 h (*14*).

The anaerobic bacterial count was determined by the pour plate method using tryptic soy agar (Difco) plates incubated (35 C, 48 h) in a GasPak Anaerobic System (BBL). Total coliform and total *Enterobacteriaceae* were determined using violet red bile agar and MacConkey agar (supplemented with 1% glucose) respectively, incubated at 35 C for 24 h. Both media were purchased from BBL.

Clostridium perfringens, *Salmonella* and *Staphylococcus aureus* were determined according to the Bacteriological Analytical Manual for Foods (*7*).

Statistical analysis

Results were subjected to the analysis of variance and to the Least Significance Difference test (*17*).

RESULTS AND DISCUSSION

The aerobic plate count (Table 1) of the exterior parts of the "Hot-Boned-Hot" subprimal cuts before and after mechanical tenderization was in the range of 1.0×10^1 to 1.9×10^2 and 1.0×10^1 to 1.5×10^4/g, respectively. The difference in the aerobic plate counts between tenderized and non-tenderized "Hot-Boned-Hot" subprimal cuts was in the range of < 1 to 3 $\log_{10}$ with the smallest and biggest differences in the inside round and rib eye, respectively. "Hot-Boned-Chilled" cuts had a difference of 1 $\log_{10}$ across the board between non-tenderized and tenderized samples. These counts did not significantly ($P < 0.05$) change after chilling ("Hot-Boned-Chilled") for 15 h at 1.1 C. The exterior parts of the control subprimal cuts had an aerobic plate count range of 1.0×10^1 to 3.8×10^3/g. These bacterial levels are 1000-fold lower than the spoilage level (10^7 bacteria/g) (*10*).

"Hot-Boning" (boning before chilling the carcass) and on-the-rail-boning of muscles expose a small surface area to bacterial growth and recontamination. On the other hand, the processing time is shortened, limiting bacterial growth. In all treatments, the interior parts of the tenderized and non-tenderized outside rounds had identical aerobic plate counts at a level of 1.0×10^1/g (Table 1). The low aerobic plate count of the interior part of the tenderized outside round can probably be attributed to the good sanitary condition of the tenderizer. The iodine-based sanitizer (25 ppm of titratable iodine) decreased the bacterial populations on both the conveyer belt and the blades of the tenderizer from 1.0×10^3 to $< 1.0 \times 10^1$ per cm^2 of two blades (surface area of 119.4 cm^2), respectively (Table 2). The 184 blades of the tenderizer had a total surface area of 11,000 cm^2. Two-thirds of the blades were in touch with the samples but only two-thirds of each blade pierced the meat. This means that a surface area of 4700 cm^2 actually penetrated the meat. If we assume a contamination of one bacterium per cm^2 with a generation time of 20 min (under optimal conditions), we may end up with 4.1×10^3 and 2×10^6 bacteria/cm^2 or a total of 19×10^6 and 9.4×10^9 bacteria after 4 and 7 h, respectively. One must be aware of the possibility of microbial proliferation because of the availability of nutrients in the juices and debris released from the tenderized meat. This example indicates that under unsanitary conditions and lack of awareness of bacterial recontamination the tenderizer may become a bacterial "inoculating machine." The result would be a product with a short shelf-life, and if pathogens are present the product will pose a public health hazard. It is evident from the data in Table 2 that a sanitary process will prevent both economical losses and health hazards.

The combination of electrical shock, and on-the-rail "Hot-Boning" prevented bacterial growth (Table 1) and recontamination of the product especially with pathogens. *C. perfringens* and *Salmonella* were not detected in the exterior and interior parts of the outside round (Table 3), while *S. aureus*, total Enterobacteriaceae and the coliform group were each at a low level of

TABLE 1. *Aerobic plate count of mechanically tenderized beef.*

Treatment	Outside round		Inside round[c]	Rib eye[c]
	Exterior part	Interior part		
	(Count/g)[d]			
"Hot-Boned-Hot"				
BT[a]	1.9×10^2	1.0×10^1	3.8×10^3	1.0×10^1
T	1.0×10^1	1.0×10^1	6.0×10^3	1.5×10^4
"Hot-Boned-Chilled" (1.1 C, 15 h)				
BT	7.5×10^3	1.0×10^1	3.9×10^3	6.5×10^2
T	1.0×10^2	1.0×10^1	8.0×10^2	2.3×10^3
Control[b]				
BT	1.0×10^3	1.0×10^1	6.0×10^2	1.0×10^1
T	3.8×10^3	1.0×10^1	2.0×10^3	1.1×10^3

[a]BT = Before tenderization; T = Tenderized.
[b]Not electrically stimulated.
[c]The exterior part only.
[d]Each result represents the arithmetic average of six determinations.

TABLE 2. *Bacterial counts of the tenderizer.*

Sampling time	Conveyor (Count/cm^2)	Blades (Count/2 blades)[b]
Before sanitizing the tenderizer	1.0×10^3[c]	1.4×10^3[c]
Before tenderization[a]	5.0×10^1	5.0×10^2
Between tenderizations	5.0×10^1	3.0×10^1
After second tenderization	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$

[a]2 minutes after sanitizing the tenderizer.
[b]Surface area of 59.7 cm^2/blade.
[c]Each result represents the arithmetic average of six determinations.

TABLE 3. *Occurrence of pathogenic and indicator microorganisms in mechanically tenderized outside round.*[a]

Organism/Group	Count[c]
Clostridium perfringens	Absent/50 g
Salmonella[b]	Absent/50 g
Staphylococcus aureus[b]	$< 1.0 \times 10^1$/g
Total *Enterobacteriaceae*	$< 1.0 \times 10^1$/g
Total coliform	$< 1.0 \times 10^1$/g

[a]Biceps femoris muscle.
[b]Examined by the 5 tube MPN method.
[c]Each result represents the arithmetic average of six determinations.

$< 1.0 \times 10^1$/g. The absence of pathogens (and the low level of *S. aureus*) is in accordance with a proposed standard (*8*). It should be emphasized that a meat product with a bacterial level as low as 10^3/g may become potentially dangerous because pathogens (if present) may grow with less competition from the "indigenous" flora (*1,3,19*). Therefore, it is extremely important to prevent bacterial recontamination of the product, and this can be achieved (as shown in this work) using a sanitary process.

Vacuum packaging of "Hot-Boned" meat (*20*) and storage at elevated temperatures (*6,16*) enhanced the postmortem metabolism and conversion of the animal flesh to post-rigor meat. Under the conditions used in this work (72.5 mm of Hg, 16 C for 18-20 h) no significant ($P < 0.05$) bacterial growth took place either on the exterior or in the interior parts of the tenderized outside round (Table 4). An exception was a 10-fold growth of the anaerobic population on the exterior of the tenderized outside round. Nevertheless, the bacterial level of 7.0×10^3/g was still relatively low and *Salmonella* was not detected. These results indicate that if mechanically tenderized subprimal cuts are "Hot-Boned" and vacuum-stored at 16 C for as long as 20 h, the aerobic and anaerobic plate counts will not increase and will not cause deterioration of the product. One should be very cautious to adequately control the temperature of storage and the storage period.

This work showed that it is possible to obtain a tenderized meat product with a good bacteriological quality. Contamination of the tenderized product can be controlled if a sanitary process is used and environmental conditions are not abused.

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TABLE 4. *Effect of vacuum packaging on the bacteriological quality of mechanically tenderized outside round.*[a]

Treatment[b]	Aerobic plate count		Anaerobic count	
	Before storage	After storage	Before storage	After storage
			(Count/g)[c]	
Before tenderization				
Exterior part	2.2×10^3	3.6×10^3	5.0×10^2	1.6×10^3
Interior part	1.0×10^1	1.0×10^1	1.0×10^1	1.0×10^1
Tenderized				
Exterior part	2.2×10^4	1.0×10^4	4.9×10^2[d]	7.0×10^3[d]
Interior part	1.0×10^1	1.0×10^1	1.0×10^1	1.0×10^1

[a]Biceps femoris muscle.
[b]Vacuum packaged and stored for 18-20 h at 16 C.
[c]Each result represents the arithmetic average of six determinations.
[d]Significant at $P < 0.05$.

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Presence, Growth and Survival of *Yersinia enterocolitica* in Oysters, Shrimp and Crab

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ABSTRACT

Yersinia enterocolitica was recovered from 6 of 45 (13%) oyster, 2 of 50 (4%) shrimp and 12 of 58 (21%) blue crab samples. No single method of enrichment (cold mannitol broth or modified Rappaport broth for 7, 21 or 60 days) was most effective for the three types of shellfish examined. The effect of refrigerated storage on *Y. enterocolitica* depended upon the type of shellfish, condition (raw or boiled), strain of *Y. enterocolitica* and temperature and time of storage. In general, *Y. enterocolitica* counts increased in (*a*) raw oysters stored at 0-2 C for 14-21 days and at 5-7 C for 2-10 days, (*b*) in boiled shrimp stored at 3-5 C for 7-21 days and (*c*) in cooked crab meat stored at 5 C for 14 days. Freezing and heating of shrimp and crab meat caused extensive destruction of *Y. enterocolitica*. Biotypes capable of causing human illness (when inoculated into seafoods) survived and under certain conditions multiplied at refrigeration temperatures. Biochemical characteristics of the isolates from raw shellfish differed from those of clinically significant types.

Yersinia enterocolitica has been isolated from humans and animals with a wide variety of clinical manifestations. In humans, the most common syndromes are gastroenteritis, mesenteric lymphadenitis and terminal ileitis; other symptoms may include polyarthritis, erythema nodosum, septicemia and meningitis (*9,18,21*). In recent years, the number of recorded cases of *Y. enterocolitica* infections in humans has increased steadily, particularly in Europe and Canada (*20,21*). The epidemiology of *Y. enterocolitica* infections in humans is still not clear. Contaminated foods, contact with infected animals and person to person contact in an infected family are suggested as possible modes of transmission (*5,18,20*). A recent review by Bottone (*1*) provided detailed information on the taxonomy, biochemical characteristics, clinical significance and epidemiology of *Y. enterocolitica*. The occurrence of *Y. enterocolitica* in foods, including methods of isolation, survival and control of this organism was reviewed by Lee (*12*).

Numerous reports showed that *Y. enterocolitica* was frequently isolated from a wide variety of foods, including raw milk, beef, pork, lamb, chicken, turkey, fish, mussels, oysters and vegetables (*2,6,10,11,15,16,17, 19,21*). Although on several occasions contaminated foods have been suspected as the cause of human yersiniosis, only one documented outbreak has been reported in the United States (*22*). In this case, the food (chocolate milk) contained the same serotype (0:8) as that isolated from persons with the illness. Among food animals, swine are recognized in Europe, Canada and Japan as a potential source of clinically significant *Y. enterocolitica* serotype 0:3 (*20*). In addition, contaminated water has been implicated as a cause of human yersiniosis (*9*). *Y. enterocolitica* isolates from foods and water frequently differed in some biochemical characteristics from "typical" clinically significant types. The former are sometimes referred to as environmental biotypes, atypical strains or *Y. enterocolitica*-like organisms. The potential public health hazard of these types is not clear.

Although *Y. enterocolitica* has been isolated from fish and shellfish such as trout (*11*), oysters (*21*) and mussels (*19*), information on distribution of this organism in Gulf of Mexico shellfish and the potential of its survival during storage and processing is scarce. This report describes the distribution of *Y. enterocolitica* in oysters, shrimp and crab harvested along the Texas Gulf Coast and the effect of storage and preservation practices (refrigeration, freezing, heating) on growth or survival of this organism.

MATERIALS AND METHODS

Cultures

Y. enterocolitica 23715 (human isolate) was purchased from the American Type Culture Collection, Rockville, Md. Culture 2635 (isolated from chocolate milk) was obtained from the Center for Disease Control, Atlanta, Ga. Cultures 11R-A, S28R-A and C35R-A (environmental biotypes) were isolated respectively from oysters, shrimp and crab at Texas A&M University. All cultures were maintained at 25 C on tryptic soy agar slants (TSA, Difco). To inoculate shellfish samples, cultures were grown for 24 h at 25 C in brain heart infusion broth (BHI, Difco). Adjustments in the number of viable cells in the inoculum were made with sterile BHI based on established relations between optical density at 550 nm and viable cell count.

Detection and enumeration of Y. enterocolitica

Cold mannitol broth (CMB) and Modified Rappaport broth (MRB) were used as enrichment media. CMB consisted of 0.85% sodium chloride, 1% D-mannitol and 0.2% dibasic potassium acid phosphate (*3*). The medium was adjusted to pH 7.3 before sterilization. MRB was prepared as described by Feeley et al. (*4*). Bismuth sulfite agar (BS, Difco) plates were used after drying at room temperature for 2-3 h.

To examine fresh, non-inoculated oysters, shrimp and crab, a 1:10 dilution was prepared by mixing the sample with an appropriate amount of sterile 0.1% peptone (Difco) and blending it for 1 min in a Waring blender. One-ml portions of this dilution were enriched in MRB (10 ml) at 25 C and in CMB (10 ml) at 4 C for 7, 21 and 60 days. After each interval, a loopful of each broth was streaked onto BS agar.

In addition, a loopful of the 1:10 dilutions and of the blended oyster meat and liquor were streaked directly onto BS agar plates.

In the experiments in which shellfish was inoculated with *Y. enterocolitica*, enumeration was carried out by preparing appropriate dilutions of the samples with sterile 0.1% peptone. Then 0.1-ml portions of the dilutions were placed on BS agar plates and distributed over the surface with sterile bent glass rods. In some instances (low-counts), 1-ml portions of the dilutions were placed in CMB and MRB for 7 to 60 days and then streaked on BS plates.

Following incubation of BS agar plates for 2-4 days at 25 C, suspect black colonies were picked and tested for the following typical characteristics of *Y. enterocolitica:* gram-negative rods, cytochrome oxidase (−), urease (+), phenylalanine deaminase (−), ornithine decarboxylase (+), lysine decarboxylase (−) and arginine dihydrolase (−). Isolates which conformed with these test results were subjected to the following tests: bile esculin (25, 36 C); Simmon's citrate (25, 36 C); OF lactose (25 C); motility (25, 36 C); indole (36 C); nitrate reduction (36 C); MR-VP (25 C); ONPG (25 C); acid from lactose, maltose, salicin, trehalose, sorbitol and sorbose (36 C); from sucrose, raffinose, rhamnose and melibiose (25, 36 C), and from xylose (25 C). Test procedures and preparation of the media and test reagents are described in the *Compendium of Methods for the Microbiological Examination of Foods* (*4*). In a few instances, when differentiation between added and naturally occurring *Y. enterocolitica* was required, distinct differences in their biochemical characteristics (esculin, citrate, carbohydrate utilization) were used to separate the isolates.

Sample preparation - Naturally contaminated samples

Forty five samples of oysters (*Crassostrea virginica*, 35 shell stock and 10 shucked, an average of five per sample), 50 shrimp (*Penaeus setiferus*, an average of 15 per sample) and 58 samples of blue crab (*Callinectus sapidus*, an average of 20 per sample) were examined for *Y. enterocolitica*. The shellfish samples were obtained from various locations on the Texas Gulf Coast by marine agents of the Texas Agricultural Extension Service and from local seafood markets. The samples were shipped to the laboratory in foamed plastic cartons on ice. Transportation time ranged from 24 to 48 h. In the laboratory, the oysters shells were cleaned with a stiff brush under running tap water. The meat and liquor were aseptically removed and mixed in a Waring blender for 1 min. A 50-g mixture of meat and liquor was mixed with 450 ml of 0.1% sterile peptone in a Waring blender to give a 1:10 dilution. With shrimp, a 50-g sample (including head and shell) was blended with 450 ml of 0.1% sterile peptone. With blue crab, the intestines were removed and blended (Waring blender, 1 min) with an appropriate amount of sterile peptone to give a 1:10 dilution.

Sample preparation - Artificially contaminated samples

Storage of inoculated oysters at 0-2 and 5-7 C. For each of 3 strains (23715, 2635 and 11R-A), 1 gal of freshly shucked oysters was divided into six equal portions in sterile 950-ml glass jars with screw-cap lids. Three containers were stored at 0-2 C for 21 days and the others at 5-7 C for 14 days. Two of each were inoculated (one high and one low inoculum) and the other served as control. The inocula consisted of 24-h BHI cultures, diluted with sterile BHI to yield the desired concentration of viable cells. Initially and after certain intervals (at 0-2 C after 7, 14 and 21 days; at 5-7 C after 2, 5, 7, 10 and 14 days) a 25-g mixture of meat and liquor was blended with 225 ml of 0.1% sterile peptone and examined for *Y. enterocolitica*.

Storage of inoculated (surface or tail muscle) shrimp (raw or boiled) at -20, 1 and 3-5 C. Surface inoculation involved placing the shrimp (headed and peeled) for 1 min into a beaker with a 24-h-old BHI culture of *Y. enterocolitica* (23715, 2635, S28R-A) containing approximately 10^6-10^7 (storage at -20 C) or 10^5-10^6 (storage at 1 and 3-5 C) viable cells per ml. Inoculation of the tail muscle was done by injecting it with 0.3 ml of a 24-h-old culture containing approximately 10^6-10^8 viable cells per ml. Samples were stored (double-wrapped in aluminum foil) at -20 C in a freezer and at 1 C and 3-5 C in refrigerators in glass containers with screw-cap lids. Initially and after certain intervals (at -20 C, after 7, 30 and 60 days; at 1 and 3-5 C after 7, 14 and 21 days) a 10-g sample was blended with 90 ml of 0.1% sterile peptone. Examination for *Y. enterocolitica* was as described earlier.

Heating of shrimp inoculated with Y. enterocolitica. Interior inoculation of shrimp was carried out by injection of 0.3 ml of a 24-h-old BHI culture (23715, 2635, S28R-A) into the shrimp tails. Shrimp were boiled in water for 0.5 to 3 min. After cooling on ice for 10 min, 10-g samples were blended with 90 ml of 0.1% sterile peptone and examined for *Y. enterocolitica*.

Storage of inoculated cooked crab at 1 and 5 C. Meat was picked aseptically from the shells of heated (10 min at 121 C) crab. After cooling on ice, 25-g samples of meat were mixed with 1 ml of a 24-h-old BHI culture of *Y. enterocolitica* (23715, 2635, C35R-A). The samples in 50-ml closed glass containers were stored in refrigerators. Initially and after certain intervals (at 1 C, after 7 and 14 days; at 5 C after 4, 7, 11 and 14 days), a 25-g sample was blended with 225 ml of 0.1% sterile peptone and examined for *Y. enterocolitica*.

Heating of crab meat inoculated with Y. enterocolitica. Samples of minced cooked crab meat were inoculated with a 24-h-old BHI culture (23715) of *Y. enterocolitica*. The samples in closed glass containers equipped with a thermometer were heated in a water bath at 90 C. After the temperature in the geometric center reached 25, 35, 45, 55, 65, 75 and 85 C, samples (25 g) were removed, cooled on ice, blended with 225 ml of 0.1% sterile peptone and examined for *Y. enterocolitica*.

RESULTS

Isolation of Y. enterocolitica from shucked oysters.

Y. enterocolitica was recovered from 6 of the 45 (13%) samples tested (Table 1). The highest numbers of positive samples were detected by enrichment in CMB for 21 days or in MRB for 7 days. Continuation of enrichment for up to 60 days in CMB or MRB decreased the number of positive samples. With direct streaking of a loopful of blended meat and liquor on BS plates, none of the samples was positive for *Y. enterocolitica*. *Y. enterocolitica* was isolated from two samples by enrichment in CMB for 21 days and not by any of the other methods employed.

Y. enterocolitica counts of inoculated shucked oysters

The development of *Y. enterocolitica* 23715 in refrigerated shucked oysters is presented in Fig. 1. Both at the low and high level of initial concentration, *Y. enterocolitica* counts increased about 1 log after 14 days at 5-7 C or after 21 days at 0-2 C. *Y. enterocolitica*

TABLE 1. *Isolation of* Yersinia enterocolitica *from fresh oysters, shrimp and crab (intestines) by direct streaking on BS agar and through enrichments in cold mannitol (CMB) or modified Rappaport broth (MRB) and subsequent streaking on BS agar.*

Shellfish	Positive/total number of samples	Direct streaking on BS agar from		Number of positive samples after enrichment in: CMB (days)			MRB (days)		
		10^0	10^{-1}	7	21	60	7	21	60
Oyster	6/45	0	1	1	5	1	4	2	0
Shrimp	2/50	NA[1]	0	0	0	2	0	0	0
Crab	12/58	NA	0	0	1	3	7	5	3

[1]NA = not applicable.

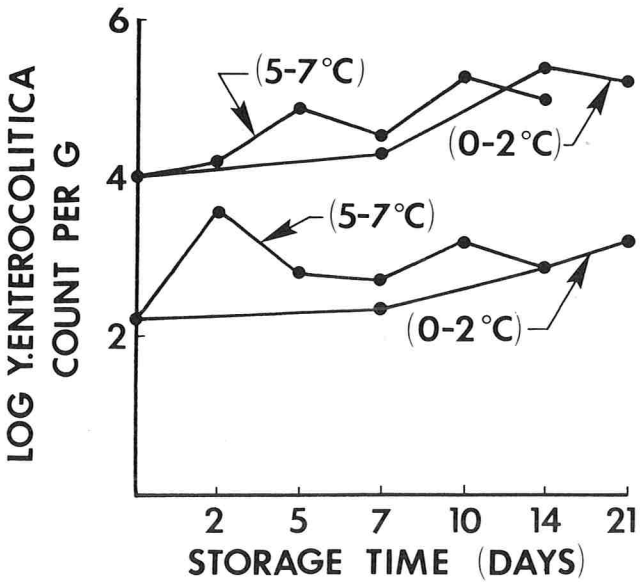

Figure 1. *Development of* Y. enterocolitica *ATCC 23715 in shucked oysters at two levels of inoculum, stored at 0-2 and at 5-7 C. (Low inoculum consisted of 1 ml of a 4.8 × 10⁴ BHI culture and high inoculum of 1 ml of a 4.8 × 10⁶ BHI culture added to 630 g of shucked oysters).*

counts in shucked oysters at 0-2 C were highest after 14 to 21 days. With storage at 5-7 C, counts increased about 1 to 2 logs after 2-5 days, after which both decreases and increases in count occurred.

Y. enterocolitica counts of shucked oysters inoculated with culture 2635 at the high level of inoculum (about 10^4 cells per g) increased approximately 2.3 logs in 14 days at 5-7 C and at 0-2 C. At the lower level of inoculation (about 57 viable cells per g of oysters), *Y. enterocolitica* was not recovered until after 5 days at 5-7 C and after 7 days at 0-2 C. Increases in *Y. enterocolitica* after 14 days at 0-2 or 5-7 C ranged from 2 to 2.2 logs. Development of culture 11R-A in shucked oysters was similar to that of culture 23715.

Isolation of Y. enterocolitica from shrimp

Only 2 of the 50 shrimp samples (4%) contained *Y. enterocolitica* (Table 1). In both instances, isolation was made only by enrichment in CMB for 60 days and subsequent streaking on BS agar.

Y. enterocolitica counts of inoculated raw shrimp stored at - 20, 1 and 3-5 C

Large decreases in *Y. enterocolitica* counts (4-5.5 logs in 60 days) occurred when raw shrimp inoculated at the surface with *Y. enterocolitica* were stored at -20 C (Fig. 2). When the inoculated samples were stored at 1 C (Fig. 3), slight increases in *Y. enterocolitica* count occurred during the first 7 days of storage. This was followed by decreases in count at 14 days, particularly for cultures 2635 and S28R-A. The samples inoculated with cultures 23715 or S28R-A showed a second increase in count between 14 and 21 days of storage. When the inoculated shrimp samples were stored at 3-5 C (Fig. 4), *Y. enterocolitica* counts increased (1.0-2.5 logs) during the first 7 days of storage. However, during the next

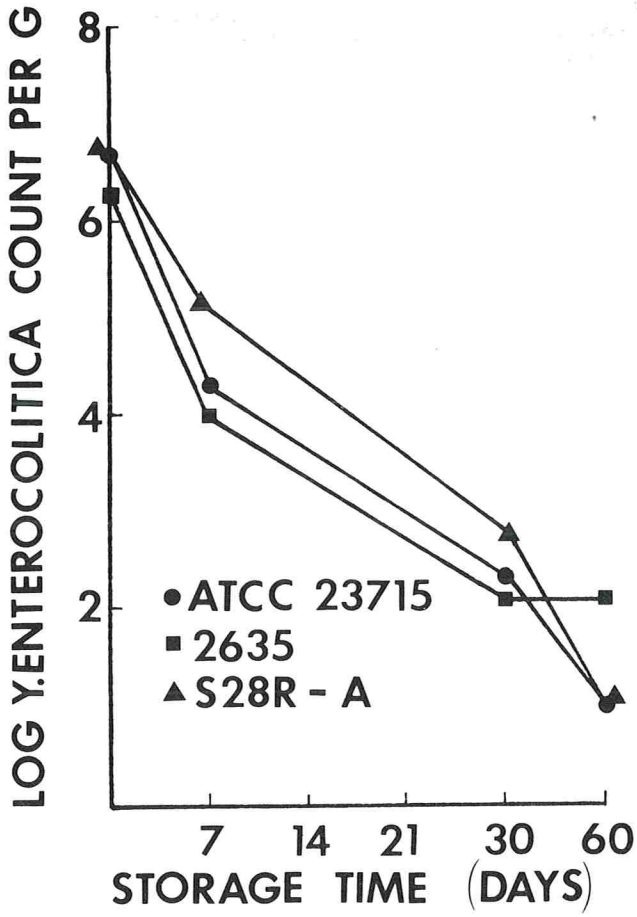

Figure 2. Y. enterocolitica *counts of raw shrimp surface inoculated with 3 different cultures and stored frozen for up to 60 days at - 20 C. [10 g of shrimp were dipped for 1 min in a BHI suspension of 1.7 × 10⁷ (23715), 6 × 10⁶ (2635), and 1 × 10⁷ (S28R-A) viable cells per ml].*

14 days of storage (after 21 days) *Y. enterocolitica* counts decreased, particularly in the sample inoculated with culture S28R-A. *Y. enterocolitica* counts of shrimp inoculated with cultures 23715 and 2635 after 21 days at 3-5 C were only about 0.5-0.7 logs higher than at day 0. The effect of freezing (-20 C) and refrigerated storage (3-5 C) on the *Y. enterocolitica* counts of raw shrimp inoculated in the tail muscle was similar to that of shrimp inoculated on the surface.

Y. enterocolitica counts of inoculated boiled shrimp stored at -20, 1 and 3-5 C

The sharp decreases (4-5 logs) in *Y. enterocolitica* counts when boiled shrimp inoculated at the surface were stored for up to 60 days at -20 C was similar to those shown in Fig. 2 for inoculated raw shrimp. At 1 C (Fig. 5), sharp decreases (2.2-3 logs) in *Y. enterocolitica* counts occurred during the first 7 days which were followed by some increases in count over the next 14 days of storage. However, over the entire 21 day storage period at 1 C, *Y. enterocolitica* counts of the inoculated samples had decreased by 0.8 to 2.5 logs. *Y. enterocolitica* counts of inoculated boiled shrimp (Fig. 6) stored at 3-5 C increased up to 7 (cultures 23715 and 2635) or 21 days (culture S28R-A). These increases in count over a 21-day storage period ranged from 1.4 to 2.7 logs.

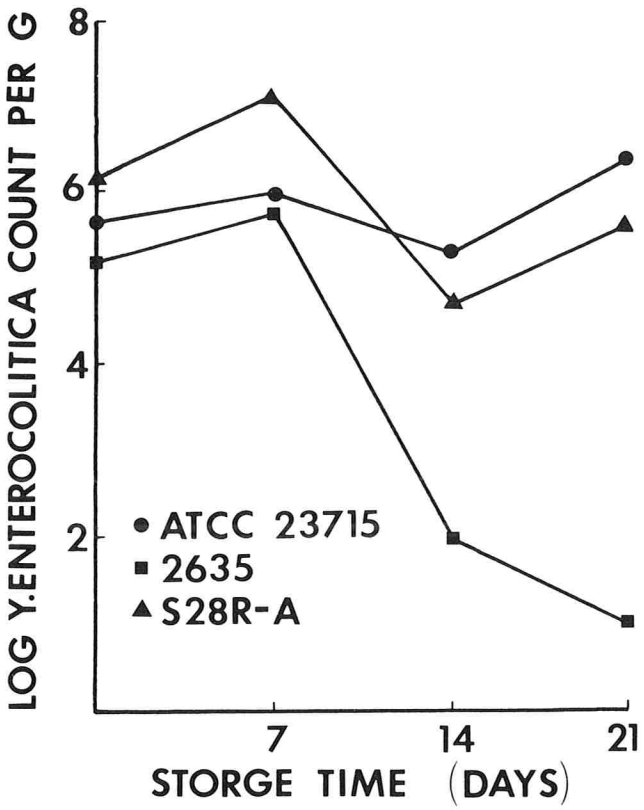

Figure 3. Y. enterocolitica *counts of raw shrimp surface inoculated with 3 different cultures and stored refrigerated for up to 21 days at 1 C. [10 g of shrimp were dipped for 1 min in a BHI suspension of* 1.7×10^6 *(23715),* 6×10^5 *(2635), and* 1×10^6 *(S28R-A) viable cells per ml].*

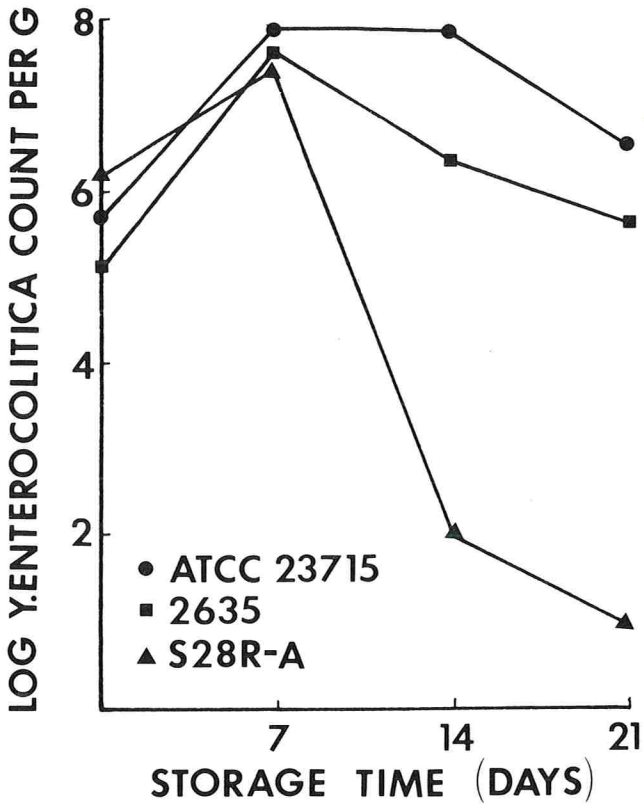

Figure 4. Y. enterocolitica *counts of raw shrimp surface inoculated with 3 different cultures and stored refrigerated for up to 21 days at 3-5 C. [10 g of shrimp were dipped for 1 min in a BHI suspension of* 1.7×10^6 *(23715),* 6×10^5 *(2635) and* 1×10^6 *(S28R-A) viable cells per ml].*

The effect of freezing and refrigerated storage on the *Y. enterocolitica* counts of shrimp inoculated in the tail muscle with cultures 23715, 2635 or S28R-A is represented by the data for culture S28R-A in Fig. 7. Holding of inoculated samples at -20 C caused sharp decreases in the *Y. enterocolitica* counts. *Y. enterocolitica* counts increased (3.5-4.3 logs) in the samples stored for 14 to 21 days at 3-5 C. The counts of samples inoculated with cultures 2635 or S28R-A and stored at 1 C initially (7-14 days) showed a decrease in count which was followed by an increase in the *Y. enterocolitica* count during the next 7-14 days. For the sample inoculated with culture 2635, this resulted in an overall decrease in count over a 21-day period of about 1.5 logs; for the sample inoculated with culture S28R-A, the result was an increase in count of almost 1 log. The *Y. enterocolitica* count of the sample inoculated with culture 23715 and stored at 1 C continued to decline.

Survival of Y. enterocolitica in shrimp heated in boiling water

Shrimp tails were inoculated with three cultures of *Y. enterocolitica,* at two levels of inoculum, and heated in boiling water for up to 3 min. No survivors were detected in the samples with the high inoculum (2-3.6 $\times 10^7$ cells per g of shrimp) after boiling for 1 min (Fig. 8). A considerable number of *Y. enterocolitica* survived 0.5 min of boiling. In the sample with the lower inoculum

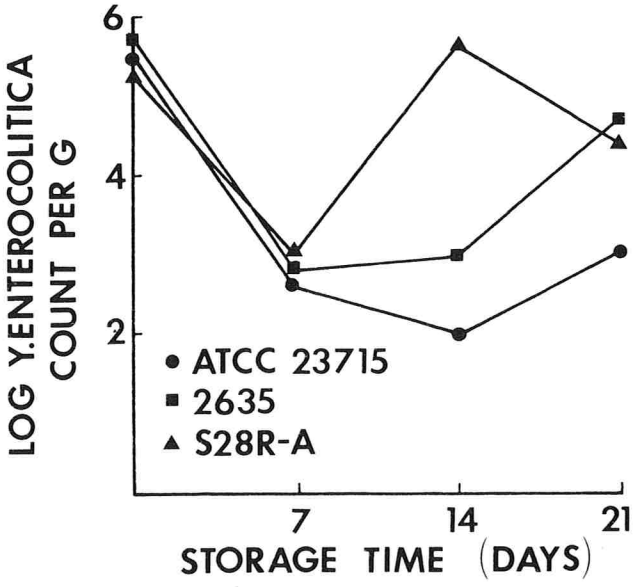

Figure 5. Y. enterocolitica *counts of boiled shrimp surface inoculated with 3 different cultures and stored refrigerated for up to 21 days at 1 C. [10 g of shrimp were dipped for 1 min in a BHI suspension of* 9.2×10^5 *(23715),* 1.6×10^6 *(2635), and* 5.4×10^6 *(S28R-A) viable cells per ml].*

(2-3.6 $\times 10^4$ cells per g of shrimp), no survivors were detected after 0.5 min (cultures 23715 and S28R-A) and 1 min (culture 2635) of boiling.

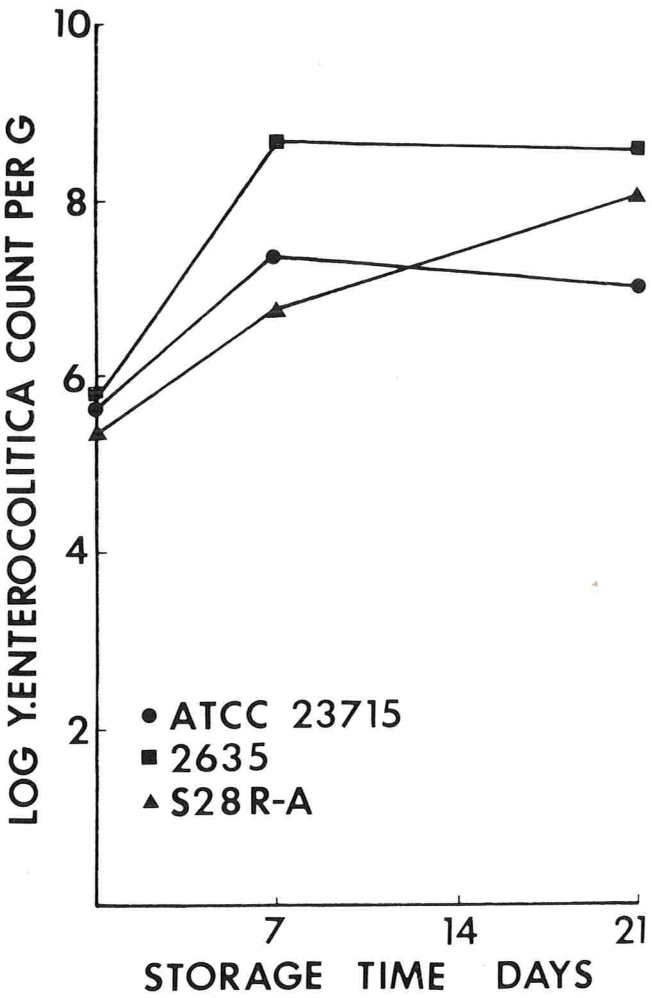

Figure 6. Y. enterocolitica *counts of boiled shrimp surface inoculated with 3 different cultures and stored refrigerated for up to 21 days at 3-5 C. [10 g of shrimp were dipped for 1 min in a BHI suspension of* 9.2×10^5 *(23715),* 1.6×10^6 *(2635), and* 5.4×10^6 *(S28R-A) viable cells per ml].*

Isolation of Y. enterocolitica from crab.

Y. enterocolitica was recovered (Table 1) from 12 of the 58 samples (21%). The highest number of positive samples was detected by enrichment in MRB for 7 or 21 days. Continuation of enrichment in MRB for more than 7 days caused a decrease in the number of positive samples. On the other hand, with enrichment in CMB, positive samples were encountered only after 21 and 60 days. None of the 58 samples were positive for *Y. enterocolitica* when examined by direct streaking on BS agar or by enrichment in CMB for 7 days. Two of the samples positive for *Y. enterocolitica* by enrichment in CMB were negative with enrichment in MRB.

Survival and/or growth of Y. enterocolitica inoculated into cooked crabmeat

Cultures of *Y. enterocolitica* were inoculated into cooked crabmeat and stored at 1 and 5 C for 14 days (Fig. 9). Marked increases in *Y. enterocolitica* count (3.5-5 logs in 14 days) occurred in crabmeat stored at 5 C. With storage at 1 C, decreases in *Y. enterocolitica* counts occurred, particularly with cultures 23715 or 2635.

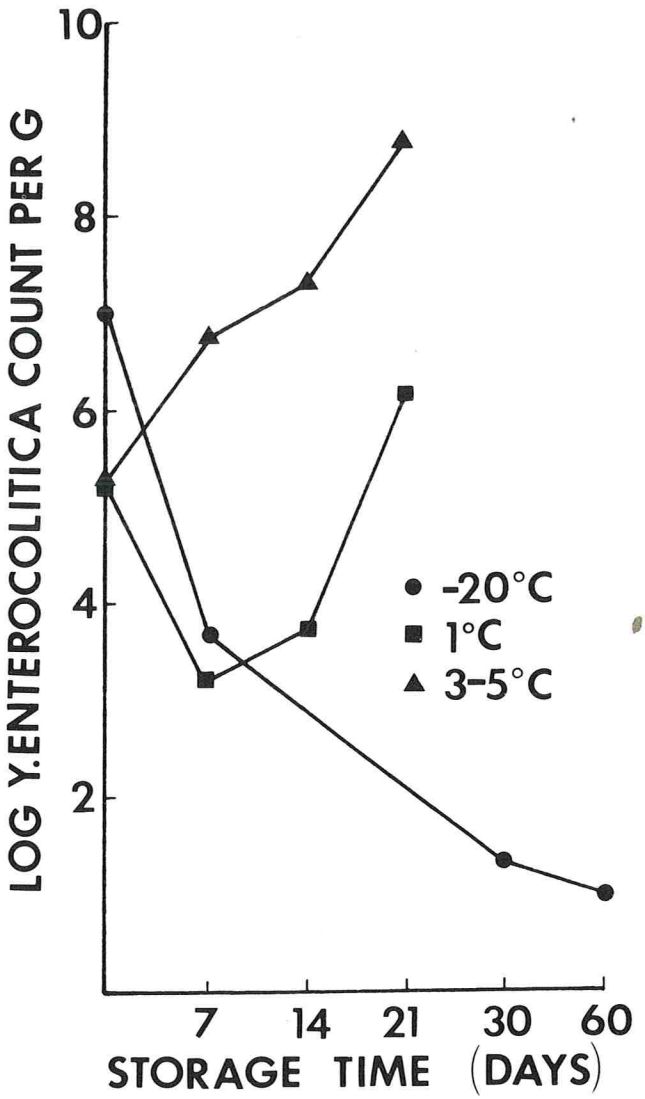

Figure 7. Y. enterocolitica *S28R-A counts of boiled shrimp inoculated in the tail muscle and stored at 3 different temperatures for up to 60 days (Low inoculum consisted of 0.3 ml of a BHI culture containing* 5.4×10^6 *cells per ml, and high inoculum consisting of 0.3 ml of a BHI culture containing* 5.4×10^8 *cells per ml added to 10 g of boiled shrimp).*

Survival of Y. enterocolitica in crabmeat during heating

Cooked crabmeat was inoculated with *Y. enterocolitica* 23715 and heated in closed glass containers in a waterbath maintained at 90 C. The results (Fig. 10) indicate that *Y. enterocolitica* could not be detected in crabmeat when the temperature of the meat had reached 65 C (after 9 min of heating). *Y. enterocolitica* was still present in the crabmeat after it had reached a temperature of 55 C (after 7 min).

Characteristics of Y. enterocolitica isolates

Forty five isolates from the fresh raw oysters, shrimp and crab samples had certain characteristics in common (Table 2). In Table 3 these isolates are grouped according to their variable characteristics (acid production from rhamnose, raffinose and melibiose at either 36 or 25 C, citrate utilization (25 C), ONPG (25 C), VP (25 C), acid from lactose (36 C) and O/F lactose (25 C).

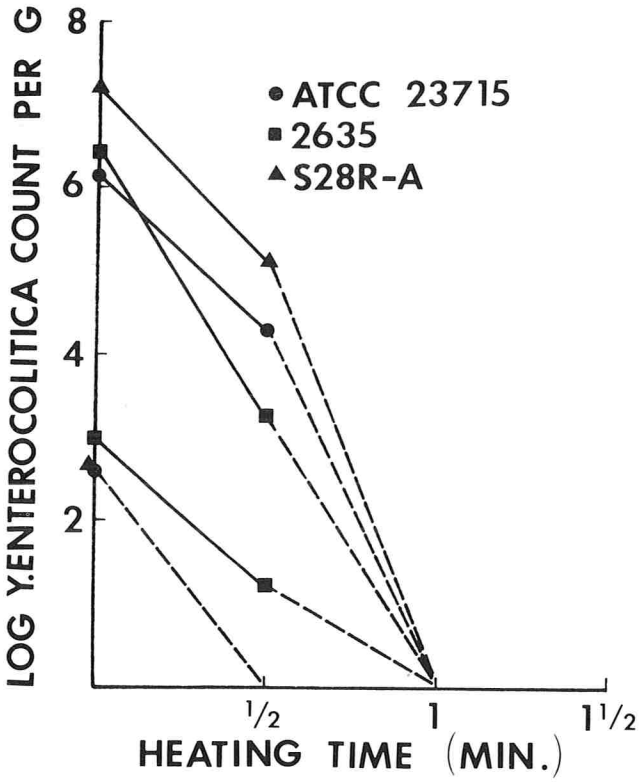

Figure 8. *Survival of* Y. enterocolitica *inoculated into raw shrimp at two levels and heated in boiling water for up to 3 min. [Low inoculum consisted of 0.3 ml of BHI cultures 23715 (2×10^5 cells per ml), 2635 (2.3×10^5 cells per ml), and S28R-A (3.6×10^5 cells per ml), added to 10 g of raw shrimp. High inoculum consisted of 0.3 ml of a BHI culture 23715 (2×10^8 cells per ml), 2635 (2.3×10^8 cells per ml), and S28R-A (3.6×10^8 cells per ml) added to 10 g of raw shrimp].*

The largest group of isolates had the following characteristics: rhamnose +, raffinose +, melibiose +, citrate (25 C) +, ONPG (25 C) +, VP (25 C) +, lactose (36 C) +, and O/F lactose (25 C) −.

DISCUSSION

Y. enterocolitica was recovered from oysters (13%), shrimp (4%) and crab (21%) from the Gulf of Mexico. Isolation by direct streaking of a loopful of the blended sample (oysters) or of a 1:10 dilution (shrimp, crab) was not very effective. This was probably caused by the relatively low numbers of *Y. enterocolitica* in the samples and the small quantity of sample (a loopful) placed on the BS plate. With respect to the enrichment methods, no single method was most effective for isolation of *Y. enterocolitica* from oysters, shrimp and crab. Isolation of *Y. enterocolitica* from oysters was more effective by enrichment in CMB for 21 days or in MRB for 7 days. With shrimp, isolation of *Y. enterocolitica* was more effective by using enrichment in CMB for 60 days; for crab, by enrichment in MRB for 7 or 21 days. Present methods for detection or enumeration of low numbers of added *Y. enterocolitica* from foods are still not very efficient and often produce erratic results (*13*). In a preliminary experiment, a 24-h-old BHI culture of *Y. enterocolitica* 23715 was mixed for 1 min with 50 g of oyster meat and liquor in a Waring blender to yield

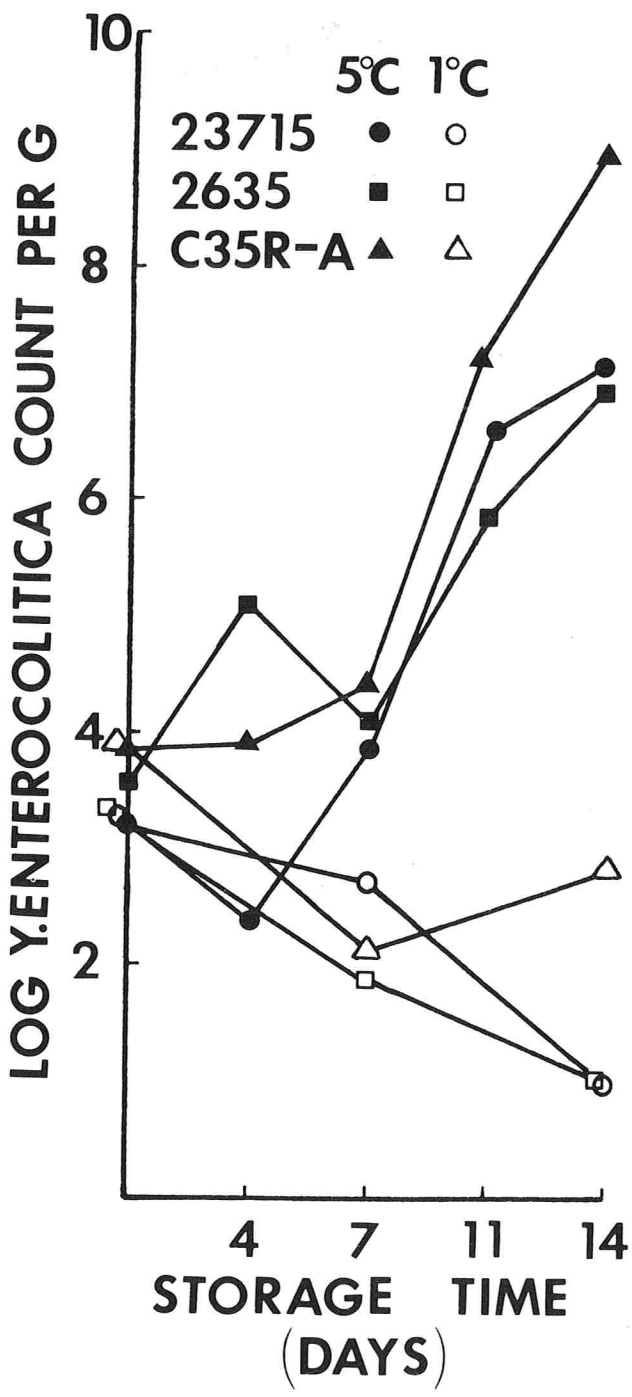

Figure 9. Y. enterocolitica *counts of cooked crabmeat inoculated with 3 different cultures and stored at 1 and 5 C for up to 14 days. [Inocula consisted of 1 ml of BHI cultures 23715 (1.4×10^5 cells per ml), 2635 (1.7×10^5 cells per ml), and C35R-A (8.1×10^5 cells per ml) added to 25 g of crabmeat].*

approximately 10^2-10^3 viable cells per g. A similar experiment was carried out with shrimp. Examination for *Y. enterocolitica* was carried out by enrichment in CMB and MRB and subsequent plating on BS agar. Recovery was best by enrichment in CMB for 21 or 60 days. *Y. enterocolitica* was recovered from oysters with a viable *Y. enterocolitica* count of 10 per total enrichment medium (10 ml), from shrimp with a count of 46 per enrichment tube.

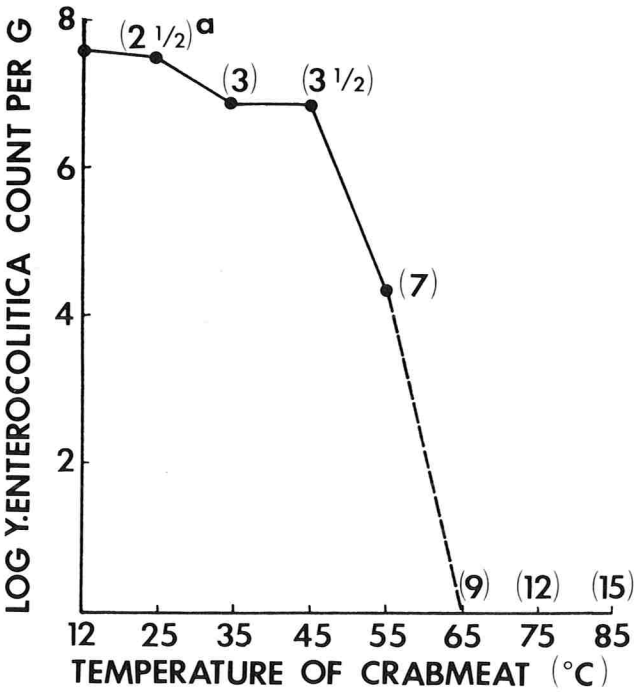

Figure 10. *Survival of* Y. enterocolitica *ATCC 23715 in cooked crabmeat heated in a waterbath at 90 C to temperatures ranging from 12 to 85 C. (Inoculum consisted of 1 ml of a BHI culture containing* 6.3×10^8 *cells per ml added to 25 g of crabmeat).*
[a](*Min*) *in the waterbath.*

Freezing of raw or boiled shrimp inoculated at the surface or in the tail muscle with *Y. enterocolitica* caused extensive destruction of *Y. enterocolitica*. A similar observation was made by Hanna et al. (*8*) for beef inoculated with *Y. enterocolitica*. Heating of shrimp and crabmeat caused extensive reduction in the *Y. enterocolitica* counts of inoculated samples. These results agree with those reported by Hanna et al. (*8*) on the effect of heat on *Y. enterocolitica* in beef roasts. They observed that no survivors of *Y. enterocolitica* were detected in beef roasts inoculated at levels as high as $3.1\text{-}3.8 \times 10^6$ viable cells per g when the final internal temperature in the center was 60 to 62 C. At 51 C, some *Y. enterocolitica* survived. Hanna et al. (*8*) also observed that decreases in

TABLE 2. *Characteristics of 45* Yersinia enterocolitica *isolates from oysters, shrimp and crab.*

Test	Result	Temperature (C)
Gram stain	–	25
Cytochrome oxidase	–	25
Urea	+	25
Phenylalanine deaminase	–	25
Ornithine decarboxylase	+	25
Lysine decarboxylase	–	25
Arginine dihydrolase	–	25
Sucrose	+	25
	+	36
Indole	+	36
Simmon's Citrate	–	36
Methyl Red	+	25
Motility	+	25
	–	36
Salicin	+	36
Bile esculin	+	25
	+	36
Trehalose	+	36
Sorbitol	+	36
Sorbose	+	36
Xylose	+	25
Maltose	+	36
Nitrate	+	36

Y. enterocolitica counts of frozen or heated samples were consistently more extensive on BS than on non-selective plating media, probably because of the deleterious effect of media constituents such as brilliant green on development of sublethally injured cells. This indicates that enumeration of *Y. enterocolitica* from heated or frozen samples by plating on or enrichment in selective media and subsequent plating on selective media may not recover all potentially viable cells. Thus, enumeration of *Y. enterocolitica* from frozen or heated foods with a mixed microbial population, particularly if low numbers of *Y. enterocolitica* are present, presents certain problems from the standpoint of efficiency of recovery. The data of the present study indicate that properly boiled shrimp and pasteurized crabmeat should not present a problem with *Y. enterocolitica* unless recontamination occurs after heat treatment.

The behavior of *Y. enterocolitica* in inoculated shellfish stored at refrigeration temperatures was not

TABLE 3. Yersinia enterocolitica *isolates grouped according to their variable characteristics [rhamnose, raffinose and melibiose done at 36 and 25 C].*

Number of isolates	Variable biochemical characteristics							
	Rhamnose 36(25)C	Raffinose 36(25)C	Melibiose 36(25)C	Simmon's Citrate 25 C	ONPG 25 C	Voges-Proskauer 25 C	Lactose 36 C	O/F Lactose 25 C
2(4)[a]	–	+	+	+	+	+	+	–
15(21)	+	+	+	+	+	+	+	–
6(1)	+	–	–	+	+	+	+	–
2(1)	+	–	+	+	+	+	+	–
(1)	–	+	+	+	+	+	+	+
(6)	–	–	–	–	+	+	–	+
10(4)	–	–	–	–	+	+	+	+
3(3)	+	+	+	+	+	+	+	+
3	+	+	+	+	+	–	+	–
(1)	+	–	–	–	+	+	+	+
1	–	–	–	+	+	+	+	–
1(1)	–	+	+	+	–	+	+	–
1(1)	–	–	–	–	+	+	–	–
1(1)	–	–	–	–	+	–	+	+

[a]Two samples belonged to this group with rhamnose, raffinose and melibiose tested at 36 C; 4 samples belonged to this group with these sugars tested at 25 C.

consistent. Results varied depending on the type of shellfish, condition of shellfish (raw or boiled), strain of *Y. enterocolitica* employed and temperature of storage. In general, at 0-2 C, *Y. enterocolitica* counts increased in oysters, but not in cooked crabmeat and infrequently in shrimp. At 5-7 C, *Y. enterocolitica* counts of oysters inoculated with cultures ATCC 23715 or 2635 increased over a 14-day storage period. In most instances, *Y. enterocolitica* counts of inoculated (surface or tail muscle) of boiled shrimp stored at 3-5 C increased over a 21-day storage period. However, the counts in inoculated raw shrimp stored under identical conditions frequently decreased. Development of *Y. enterocolitica* in oysters, shrimp and crabmeat at refrigeration temperatures (0-2, 3-5, 5-7 C) was inconsistent compared to that encountered on beef and pork at the same temperatures (*7*). On raw and cooked beef and pork, marked increases in *Y. enterocolitica* occurred at storage temperatures and during storage periods similar to those employed in the present study. The more consistent increases in *Y. enterocolitica* counts of boiled shrimp at 3-5 C as compared with those of raw shrimp may have been caused by differences in the physico-chemical characteristics of the samples such as in available nutrients, oxidation-reduction potential and presence or absence of tissue enzymes. In addition, one can assume that large differences existed in the microbial flora that developed during storage on the raw and boiled shrimp. Hence differences in microbial interactive phenomena may have taken place. Differences in development of *Y. enterocolitica* between cooked and raw beef and pork were noted by Hanna et al. (*7*).

The isolates from oysters, shrimp and crab resemble closely Niléhn's biotype 1 (*4*). Little is known about the potential public health significance of these isolates. Preliminary information indicates that the salicin-positive and esculin-positive isolates from foods do not invade HeLa cells (*14*). In addition, when a limited number of the shellfish isolates were tested for tissue invasiveness by the Serény test, all were negative (Zink, D. L., Personal communication). Although clinically significant biotypes were not detected in oysters, shrimp and crab, results of the present study indicate that those types, if present, could grow under certain conditions in these seafoods at refrigeration temperatures.

ACKNOWLEDGMENT

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A Justifiable Food-Energy-Legislative Triangle? [1,2]

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ABSTRACT

A conceptual model of the food-energy-legislative triangle within the United States food industry is presented. The scope of the triangle is pictorially represented. Each apex of the triangle is defined and examples are given to illustrate the interrelationships among them. The triangle includes all nutritious and nonnutritious foods produced; all direct and indirect energy used to produce, process, distribute and consume foods and all food-related legislation. Recent examples were chosen to illustrate how changes in one apex of the triangle affect components of the other two apexes. An analogy was drawn between the Bermuda and food-energy-legislative triangles to illustrate that the level of apathy towards solving the Bermuda Triangle cannot be tolerated for the latter triangle. Recommendations are given for using sound resource management techniques to identify all interdependencies in the food-energy-legislative triangle and thus increase the effectiveness of national policies affecting the food industry.

The interdependencies among three major components of our food industry are represented in the food-energy-legislative triangle (Fig. 1) which encompasses: (*a*) all food produced, processed, distributed and consumed or wasted, (*b*) all energy consumed for that food and (*c*) all food-related legislation. Vital economic aspects and environmental impacts are implied throughout this approach.

The purpose of this paper is to identify the need for systematically considering each triangle apex, and their interrelationships, when deliberating national policies for the food industry. To stress the importance of this concept, an analogy will be made between this triangle and the Bermuda Triangle.

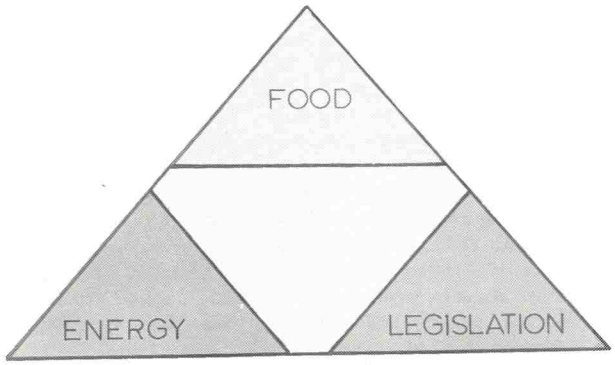

Figure 1. *Food-energy-legislative triangle.*

[1]*Based on a paper presented as the keynote address to 65th Annual Meeting, International Association of Milk, Food and Environmental Sanitarians, Inc., Kansas City, MO, August 15, 1978.*

[2]*Contribution from the Missouri Agricultural Experiment Station. Journal Series Number 8337.*

FOOD-ENERGY-LEGISLATIVE TRIANGLE: FOOD COMMODITY EXAMPLE

By illustrating the processing of olives, Fig. 2 gives a pictorial representation of the food-energy-legislative triangle. When olives are growing, their fate is partially dictated by legislation, including pesticide regulations. For their production, fossil energy is expended for many activities including manufacturing pesticides and spraying equipment and enabling field inspections. During picking operations, laborers are governed by government and labor union regulations.

During processing, olives are handled and stored according to numerous legal requirements. Furthermore, nonrenewable fossil fuels are expended for many activities, which include purifying the processing water; mining, purifying, packaging and distributing the salt which is used as a component; producing, packaging and distributing lactic acid used in processing; combining and processing the olives and ingredients; manufacturing, distributing and filling containers; distributing the processed olives and storage, etc.

Within foodservice operations, olives are used for many items, including martinis. Regulations still influence the handling of the olive. For example, the olive is placed into a glass which has been washed according to

Figure 2. *Pictorial representation of food-energy-legislative triangle.*

guidelines in the foodservice model ordinance (*9*). Thus, energy is expended from production to consumption.

In spite of the adherence to legislation and the expenditure of energy, an average medium olive only contributes 4 Kcal, .5 g of fat, 2 mg of calcium, .05 mg of iron and 10 IU of vitamin A to a daily nutrient intake (*21*). Although the author does not intend to recommend less production and use of olives, this single food commodity can be used to emphasize an important facet of the food-energy-legislative triangle. Namely, regulations are enforced and fossil fuels are expended, from production to consumption, for both nutritious and nonnutritious foods. While nutritious foods are needed in our society to satisfy physiological needs, nonnutritious foods are largely consumed to partially fulfill social needs. Thus, both nutritious and nonnutritious foods should be included in the food-energy-legislative triangle.

FOOD-ENERGY-LEGISLATIVE TRIANGLE: THE SCOPE

On a national level, all food and agricultural energy resources, and related legislation, are included in this conceptual triangle. Given the interdependent position of the United States with other countries, the scope of the triangle is global. In addition to foreign market purchases and assistance, some countries receive commodities as a result of United States food legislative actions. For example Notices of Judgment in the *FDA Consumer*, occasionally list shipment or lot of food which was not fit for human consumption in the United States, but it was suitable for shipment to Canada. However, for simplicity this paper focuses on the United States food industry. Recent situations are discussed to illustrate how changes in one apex of the triangle affect components of the other two apexes.

FOOD APEX

All foodstuffs are included in this triangle, whether or not they supply a significant level of human nutrient requirements. As food commodities progress down the food chain from production to consumption, the high-volume users of particular foods in the foodservice industry become victims of inflationary ramifications of legislation affecting these commodities. These ramifications which increase food prices should be included in impact statements for all new proposed legislation (*17*). In 1977, Hobbs (*14*) stressed insufficient attention was frequently given to the simple and economical legislative control of production processes.

In spite of the growing influence of consumer awareness and increasing concern for food protection and energy conservation, consumers are indulging in some rather energy-intensive trends. For example, Americans probably consumed more than 15 million gallons of bottled mineral water during 1978. Sales of $200 million were estimated for 1978. Consumption of imported waters from European spas leaped by 25% during 1978. Imports of these beverages amount to three million dollars per year. Reasons given for this consumption include: (*a*) fear of consuming contaminated water, (*b*) interest in health and (*c*) snob appeal (*20*).

Shipping and packaging expenses increase the cost of bottled mineral water. For example, in August, 1978, a 23-ounce bottle of Perrier cost 69 cents in New York City and 89 cents in Kansas City. The energy costs associated with this commodity have not been reported.

According to Miles (*18*), the real index of productivity within the food system is the energy usage/nutrient content ratio of foods when consumed. If excessive energy resources are expended for food items with limited nutrient content, this ratio cannot be minimized on a national basis. The extravagant consumption of mineral water is contrary to some recommendations to reduce the energy-intensiveness of lifestyles. However, it is an example of nonnutritious foods included in the social aspects of food consumption in the food-energy-legislative triangle. Thus, when nonnutritious foods are consumed to satisfy social norms, consumers should be aware of the real energy costs associated with these foods.

ENERGY APEX

All forms of electrical, mechanical, chemical, internal, kinetic and potential energy used directly and indirectly to produce, process, distribute, market and consume food items are included in the food-energy-legislative triangle. In 1976, a study by the Federal Energy Administration (FEA) (*13*) documented that energy used within the food system amounted to at least 16.5% of our national energy consumption.

LEGISLATIVE APEX

There were 38 national agencies involved in food regulation, research and international activities in 1977 (*2*). In the face of two predictions that: (*a*) the number, severity and complexity of food laws and regulations would increase, and (*b*) laws will of necessity continue to be added, deleted and altered in our continually changing society (*6*); the Office of Management and Budget was ordered in 1977 to undertake major studies to find ways to simplify federal agencies (*2*).

During the fall of 1978, the Food and Drug Administration (FDA), United States Department of Agriculture (USDA) and Federal Trade Commission (FTC) initiated a series of legislative hearings to determine what changes were needed in our national food policies regarding food labeling (*3*). Even though the Department of Energy (DOE) (*11*) has acknowledged that a proliferation of regulations, issued by federal agencies, impact upon energy usage within the food industry, DOE did not sponsor or actively participate in these hearings.

The precise impact of current food regulations upon energy usage should be determined (*10*). Food-related regulations should be modified to enable the development of energy-efficient processes which protect the

interests of consumers and the environment (*1*). Without such efforts, many technologies which have a high energy conservation potential for the food industry may not be implemented.

FOOD-ENERGY INTERACTIONS

Interactions between food and energy are pervasive throughout the food-energy-legislative triangle. Energy is expended to produce and maintain nutrient content and food quality and safety. Although linked to legislation, direct energy usage in the food supply can also be excessive because of both food waste and food distribution practices.

Twenty percent of all food produced in the United States was lost or wasted in 1974 (*8*). This startling statistic amounted to 137 million tons of food waste each year. Furthermore, 461 million equivalent barrels of oil were used to produce this wasted food on 66 million acres of land, using 9 million tons of fertilizer. Energy losses were greatest for plate waste because all the accumulative energy expenditures throughout the food system were expended without contributing to human nutritional status.

Food distribution accounts for 10% of food system energy use (*13*). Table 1 gives energy requirements for alternative methods of food distribution (*19*). Are these expenditures considered when foods are mishandled and unfit for consumption?

TABLE 1. *Efficiencies of transportation modes.*[a]

Transportation vehicles	Range of energy efficiencies (BTU's/ton mile)
Train	520 - 1340
Ship	140 - 591
Truck	2450 - 2600
Airplane	11400 - 63000
Barge	500 - 590

[a]Adapted from: National Science Teachers Association (*19*).

ENERGY-LEGISLATIVE INTERACTIONS

Energy-legislative interactions are prevalent. Three examples will be given to illustrate their broad scope. The societal effect of the first example to be discussed is limited to the foodservice industry; the effect of the other two examples, pervades throughout society.

For the first example, The Environmental Protection Agency (EPA) has established guidelines for air pollution and recommends burning the solids in exhaust from foodservice deep fat frying operations. Adherence to this procedure doubles the amount of natural gas used to deep fat fry foods (*17*). Thus consumers pay higher prices for fried menu items.

The second example involves accounting done when food ingredients are banned to protect consumers' health. When cyclamates were banned, the cost of products wasted and reformulated was estimated to be from 150 to 500 million dollars (*6*). Energy consumption was not reported as a separate entity. If proposed legislation is analyzed according to risk/benefit concepts in the future, all costs involved should be analyzed. Because energy resources are being depleted, sufficient analysis of all aspects cannot be made if energy costs continue to be "hidden costs." Thus all associated energy costs should be included when the level of risks and/or benefits is determined on a national basis.

Third, the outcome of another example which involves the nitrate controversy is unknown. The total banning of nitrites for curing pork could lead to alternative processing of billions of pounds of pork annually. Freezing, canning and drying processes are more energy-intensive than is curing. Given that 1977 energy imports amounted to 52% of our energy supply (*12*), why have consumers not been alerted to the expected increased levels of energy consumption if nitrites are banned? Where are the national figures from a cost/benefit analysis for the use of nitrites which delineate increases in health as well as energy consumption? This analysis involves a dichotomy: we must have a healthy population to survive as a productive nation and we must conserve our energy resources to ensure our survival as an industrial nation. To achieve either goal, educational efforts should enable the consumer to become aware of all ramifications of available alternatives.

Some interactions have been identified between energy consumption and environmental regulations. In 1974, the Committee on Energy and the Environment (*7*) was established by the Board on Energy Studies of the Commission on National Resources, to examine energy-environmental interrelationships. Their study, released in 1977, subsequently concluded: (*a*) inevitable trade-offs between energy and the environment are manageable, (*b*) many apparent problems can be solved or mitigated through good resource management, (*c*) mistakes in regulation can be corrected and (*d*) major risks to society can be avoided if the objectives of a rational risk/benefit concept are clearly defined within the political process. If further research activities determined these findings were also pertinent to food and energy interactions, the food industry could be assured of positive results from similar remedial actions.

Within the food industry, benefits of the risk/benefit concept are being stressed (*15*). Can this concept include all aspects of the food-energy-legislative triangle? Some partial attempts are being made at the present time. For example, the recent move by the Public Affairs Force of the Packaging Institute, USA to raise funds to disseminate factual information about food packaging within the political arena (*4*) is one example whereby policymakers will be provided with inputs for their decision-making processes.

FOOD-LEGISLATIVE INTERACTIONS

Although examples throughout this paper have alluded to food-legislative interactions, one example illustrates the grave nature of this interrelationship.

When diethylstilbesterol was banned, the cost to the food industry in lower meat yields was estimated to be between 0.5 and 1.0 billion dollars per year. In addition, actually replacing the meat lost by banning this chemical required an additional 3 to 5 million acres of corn (*6*). When new legislation is proposed, the food industry could be assisted greatly, if all the potential ramifications from that legislation were known by policymakers and included in impact statements.

ANALOGY WITH THE BERMUDA TRIANGLE

Figure 3 depicts the Bermuda Triangle, an imaginary line connecting Bermuda, Florida and Puerto Rico. Although triangles have been included in folk-lore for centuries, the Bermuda Triangle has been defined as one of today's most perplexing phenomena of nature. Mysterious occurrences continue to be reported there, and the sequence of events has grown into a sea mystery (*16*).

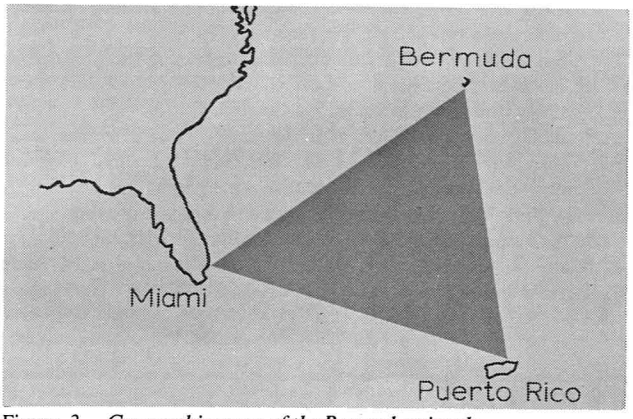

Figure 3. *Geographic scope of the Bermuda triangle.*

Although very different in scope, both triangles can serve as societal consciousness-raising factors. Both should alert society to the fact that something is wrong. Within the Bermuda Triangle, claims are still being submitted for the loss of human resources within that configuration of ocean. Within the food-energy-legislative triangle, legislation designed to protect human resources is excessively depleting energy resources while enabling food protection. Several examples have been included in this article.

In Table 2, both triangles are compared according to (*a*) unanswered questions, (*b*) proposed causes, (*c*) societal responses, (*d*) consequences and (*e*) required action.

Limited scientific investigation of the Bermuda Triangle has resulted in numerous proposed theories for bizarre and continuing human disappearances. The forces causing the losses have not been scientifically proven because the prevailing philosophy has been to ignore phenomena which cannot be easily explained (*5*). Proposed causes have included, but have not been limited to, the weather, mechanical failures and human errors.

In the latter part of the Twentieth Century, time pressures will not enable us to adopt a similar attitude towards identifying all interactions of the food-energy-legislative triangle. Several interactions were presented in this article only as examples to the numerous examples which can be revealed when the three apexes of the triangle are studied. Currently, our societal response to the food-energy-legislative tends to be a promulgation of new regulations in response to pressure from special interest groups when changes become necessary within the scope of the triangle.

What are the immediate consequences? In response to the rather nonscientific approach to solving the Bermuda Triangle, bizarre situations involving human lives continue. In response to the failure to identify all interrelationships of the food-energy-legislative triangle when new regulations are established, we continue to disregard the wastage of some of our precious resources, including energy.

Information in Table 2 emphasizes that the Bermuda Triangle situation, whatever the real reason for that troubled area, could be used as an example to mankind today. Specifically, we cannot afford to have the ramifications of the food-energy-legislative triangle linger for years, as have those from the Bermuda Triangle.

In the long term, identifying all the interactions in the food-energy-legislative triangle will involve the principles of sound resource management. All values that are perceived to be in conflict should be identified. Conflicts should be minimized through informed and preventive action. All theories and opinions should be separated from facts, and reasonable priorities for decisive actions should be rationally established (*7*). Obviously this approach has not been used to solve the Bermuda

TABLE 2. *Factors comprising the analogy between the two triangles.*

Factors	Bermuda triangle	Food-energy-legislative triangle
Unanswered questions	What forces exist?	What interactions exist among the apexes?
Proposed causes	Weather conditions Equipment failures Human errors	Consumer health and safety Protection from fraud Equipment failures Human errors
Societal responses	Numerous proposed theories with little scientific proof	Promulgate new regulations Respond to pressures from special interest groups
Consequences	Loss of human resources	Loss of energy resources
Required action	Use as an illustrative example	Scientific disclosure of all interdependent factors for use in establishing national policies

Triangle to the satisfaction of the curious consumer.

Within the food-energy-legislative triangle, the key role of the regulator is to prevent something undesirable from occurring in society. Thus all aspects of risk/benefit concepts involving food, energy, legislation, inflation, competition, productivity and the environment must be considered (*6*). The end result should be the scientific disclosure of all interdependent factors within the food-energy-legislative triangle. Once all the unbiased facts are revealed, such scientific data should greatly facilitate effective decisions regarding food, energy and legislation.

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Journal of Food Protection Vol. 42, No. 12, Pages 987-988 (December, 1979)

Report of the Editor Journal of Food Protection 1978-1979

REVIEW OF VOLUME FORTY-ONE

Publication of Volume 41 was completed with appearance of the December 1978 issue of the *Journal of Food Protection.* This was the first volume to exceed 1,000 pages and it contained more papers than any earlier volume of the Journal. There were somewhat fewer (127 vs. 136) research papers in Volume 41 than 40 but the number of review papers was greater (42 vs. 25). The number of non-technical papers and number of pages devoted to equipment standards were similar in Volumes 41 and 40. Details on the make-up of Volume 41 and of Volumes 30, 36, 37, 38, 39 and 40 are in Table 1.

Of the 180 papers in Volume 41, approximately 20% considered dairy-related topics, whereas the remainder dealt with other foods or with subjects not specifically dairy-oriented. Thirty-five percent of the papers in Volume 40 were dairy-oriented. Thus the trend of the last few years continues; interest in research on dairy products continues to decrease because of the lack of adequate funding. The Journal continues to grow because researchers on foods other than dairy products find the Journal to be a suitable medium to publish their research findings. Furthermore, the change in title of the Journal clearly identifies the major thrust of the Journal, and this has had a salutary effect on the flow of manuscripts--both research and review papers.

PRESENT STATUS OF VOLUME FORTY-TWO

The first six issues of Volume 42 (1979) consisted of 564 pages, including covers. This compares with 524, 436, 456 and 264 pages in the first six issues of Volumes 41, 40, 39 and 30 (1967), respectively. The first six issues of Volume 42 contained 73 research papers, 25 technical general interest papers and 2 non-technical general interest papers. This compares with 61, 28 and 7 papers in these same categories in Volume 41. Thus far Volume 42 contained 100 papers of all types compared to 96 for the same issues of Volume 41.

Awaiting publication on July 1, 1979 were 70 research papers, 27 technical papers of general interest and 4 non-technical papers of general interest. This compares with 71, 16 and 4 papers in the same categories on July 1, 1978. Furthermore, on July 1, 1979 there were 42 research papers undergoing review or revision. Because of financial constraints several issues of Volume 42 have been limited to 80 pages. It will be necessary to publish some larger issues during the remainder of 1979 to assure reasonably prompt publication of papers. Volume 42 is likely to again exceed 1000 pages.

INTERNATIONAL CHARACTER OF THE JOURNAL

An increasing number of authors from outside of the U.S. are choosing the Journal as a medium for publication of some of their research findings. Volume 41 contained papers by authors from the following countries outside of the U.S.: Brazil, Canada, Finland, India, Iran, Israel, The Netherlands and Turkey. Thus far Volume 42 has contained papers by authors from Australia, Canada, Denmark, Iran, Israel, Japan and The Netherlands in addition to the United States. Among papers awaiting publication are those by authors from Brazil, Canada, Guatemala, India, Ireland, Israel, Iraq, Japan, South Africa, Switzerland, The Netherlands and Turkey. It is hoped that authors from beyond the borders of the United States will continue to choose the Journal as a medium for their research and review papers.

EDITOR BOARD

The Editorial Board currently consists of 58 U.S. and Canadian scientists in academic, governmental and industrial laboratories.

TABLE 1. *Summary of contents of* Journal of Food Protection *(1977, 1978) and* Journal of Milk and Food Technology *(1967-1976).*

Item	Volume 30 (1967)	Volume 36 (1973)	Volume 37 (1974)	Volume 38 (1975)	Volume 39 (1976)	Volume 40 (1977)	Volume 41 (1978)
1. Total pages, including covers	512	752	752	868	948	944	1,080
2. Total papers published	64	108	102	136	134	175	180
3. Research papers							
a. Number	30	65	72	100	94	136	127
b. Pages	137	284	330	402	400	532	546
c. Percent of total pages	26.7	37.7	43.9	46.3	42.2	56.4	50.6
4. General interest papers-technical							
a. Number	11	31	21	26	28	25	42
b. Pages	47	208	160	178	188	125	254
c. Percent of total pages	9.2	27.7	21.2	20.5	19.8	13.2	23.5
5. Equipment standards							
a. 3-A, pages	9	17	41	25	32	22	23
b. E-3-A, pages	—	—	—	2	18	—	—
c. Percent of total pages	1.7	2.2	5.4	3.1	5.2	2.3	2.1
6. General interest papers-nontechnical							
a. Number	23	12	9	10	12	14	11
b. Pages	72	49	29	46	57	48	48
c. Percent of total pages	14.1	6.5	3.8	5.2	6.0	5.0	4.4
7. Association affairs							
a. Pages	64	84	75	67	65	52	46
b. Percent of total pages	12.5	11.2	9.9	7.7	6.8	5.5	4.3
8. News and events							
a. Pages	51	4	0	26	36	39	44
b. Percent of total pages	9.9	0.5	0.0	3.0	3.7	4.1	4.1
Percent of pages-technical material, including standards	37.6	67.6	70.5	69.9	67.3	71.9	76.2
Percent of pages-nontechnical material	36.5	18.2	13.7	16.0	16.7	14.6	12.8
Percent of pages-covers, adds, index, etc.	25.9	14.2	15.8	14.1	16.0	13.5	11.0

Members of the Board were appointed, for 3-year terms, effective January 1, 1979. Persons from the previous Board who agreed to serve for 3 more years include: J. C. Acton, J. A. Alford, L. R. Beuchat, W. A. Bough, F. L. Bryan, L. B. Bullerman, F. F. Busta, W. S. Clark, Jr., F. M. Clydesdale, N. A. Cox, R. W. Dickerson, W. J. Dyer, J. A. Elliott, D. Y. C. Fung, S. E. Gilliland, H. S. Groninger, L. G. Harmon, W. J. Hausler, Jr., D. M. Irvine, J. A. Koburger, D. H. Kropf, R. V. Lechowich, R. T. Marshall, J. R. Matches, D. W. Mather, E. M. Mikolajcik, N. F. Olson, H. Pivnick, D. S. Postle, W. D. Powrie, R. B. Read, Jr., G. W. Reinbold, G. H. Richardson, J. R. Rosenau, W. E. Sandine, D. F. Splittstoesser, J. A. Troller, B. A. Twigg, C. Vanderzant, J. H. von Elbe, H. W. Walker and E. A. Zottola.

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The Editor thanks members of the current Editorial Board, persons who recently completed service on the Board and persons not on the Board but who reviewed one or several manuscripts during 1978-1979. The voluntary efforts of all these persons serve to bring high-quality scientific information to readers of the Journal. Thanks also go to C. K. Johns for reading proofs of all papers. His efforts continue to help us reduce typographical and other errors in the Journal.

Respectfully submitted,

ELMER H. MARTH
Editor
Journal of Food Protection

Book Review

Food Processing Enzymes: Recent Development. Nicholas D. Pintauro. 1979. Noyes Data Corporation, Park Ridge, N. J. 420 pp. $42.00.

This is a well organized and informative book for researchers in Food Science, Microbiology, Biochemistry, Fermentation, Enzymology, and Product Development. Nicholas D. Pintauro reviewed 190 recent (since February, 1970) U.S. patents on Food Processing Enzymes and classified them into twelve categories: Lactase and Milk Clotting Enzymes; Cheese and Cheese Flavors; Starch, Cellulose and Corn Syrup Sweetners; Bread and Baked Goods; Soy and Vegetable Protein; Meat and Fish Products; Flavorings: Fruit and Vegetable Processing; Soluble Tea Production; Beer and Alcohol Production; Preservation and Antioxidant Methods; and By-Product and Waste Utilization and Animal Feed Production. He did a commendable job of "translating" and organizing these important patents for the readers. The publishers should have identified his institutional affiliation and position somewhere in the book.

Dr. Pintauro first gave background information on the enzymes in narrative form then focused on the uniqueness of the patent. Flow diagrams and tables were used to illustrate some of the processes. Experimental methods and procedures of some processes were presented with sufficient detail that an investigator probably could repeat the experiment. However, the author warned that a license must be obtained from the owner of a patent before performing any experiments with it.

I noticed that of more than 300 international inventors listed about 25% were Japanese. Of the 100 "Companies" to which patents were assigned, only two were universities (Cornell and University of California).

Judging from the topics reported, such as bacterial spore germinating method or preliminary starters culture for milk clotting, much research done in academic institutions might qualify for patents. I suggest that university patent officers (if available) be included in internal scientific paper review processes so that patentable works might be recognized and proper actions taken to protect university researchers and their institutions.

The book deserves a place on the book shelves of all Food Scientists.

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Coming Events

Dec. 18---PUBLIC MEETING ON STRUCTURE OF AGRICULTURE, Lafayette, IN, Howard Johnson's East, 4343 State Rd., 26 East.

Jan. 2-11---31st ANNUAL ICE CREAM MANUFACTURING SHORT COURSE. Rutgers University, New Brunswick, NJ. Contact: Norma Wanson, Office of Short Courses and Conferences, Cook College, Rutgers University, P.O. Box 231, New Brunswick, NJ 08903.

Jan. 7-17---ANNUAL PENNSYLVANIA STATE UNIVERSITY ICE CREAM COURSE. Registration Fee: $65 for Pennsylvanians, $75 for non-Pennsylvanians. Contact: Office of Short Courses, 306 Agricultural Administration Bldg., Pennsylvania State University, University Park, PA 16802.

Jan. 19---MIDWEST MEAT PROCESSORS SEMINAR. Kansas State University. Contact: Dave Schafer, Extension Specialist, Weber Hall 115, KSU, Manhattan, KS 66502.

Feb. 3-5---SANITATION THROUGH DESIGN, FDA Food Industry Workshop. San Diego, CA, Hanalei Hotel. Contact: Food Sanitation Institute, Environmental Management Association, Harold C. Rowe, Executive Director, 1701 Drew St., Clearwater, FL 33515 813-446-1674.

Feb. 10-13---1980 INTERNATIONAL EXPOSITION FOR FOOD PROCESSORS. St. Louis, MO. Contact: T. J. Gorman, Food Processing Machinery and Supplies Association, Suite 700, 1828 L St., N.W., Washington DC 20036.

Feb. 10-13---NATIONAL FOOD PROCESSORS ASSOCIATION, Annual Meeting. Convention Center, St. Louis, MO. Contact: National Food Processors Association, 1133 20th St. NW, Washington, DC 20036.

Feb. 12-13--OREGON DAIRY INDUSTRIES CONFERENCE, Valley River Inn, Eugene, OR. Contact: Mary K. Moran, ODI Secretary, Room 100, Wiegand Hall, Oregon State University, Corvallis, OR 97331, 503-754-3131.

Feb. 24-29---TENTH ENVIRONMENTAL ENGINEERING IN THE FOOD PROCESSING INDUSTRY CONFERENCE. Asilomar Conference Grounds, Pacific Grove, CA. Sponsored by the Engineering Foundation. Fee: $250, double occupancy, $300 single occupancy. Contact: Engineering Foundation, 345 E. 47th St., New York, NY 10017, 212-644-7835.

Mar. 24-28---MIDWEST WORKSHOP IN MILK AND FOOD SANITATION, Ohio State University, Columbus, OH. Contact: John Lindamood, Dept. of Food Science & Nutrition, 2121 Fyffe Road, Ohio State University, Columbus, OH, 43210.

Mar. 26-28---CONFERENCE ON WASTEWATER TREATMENT TECHNOLOGIES FOR THE CONTROL OF TOXIC/HAZARDOUS POLLUTANTS. Stouffer's Cincinnati Towers, Cincinnati, OH. Contact: Kenneth A. Dostal, IERL-Ci, EPA, Cincinnati, OH 45268.

Mar. 27-28---1980 MEAT INDUSTRY RESEARCH CONFERENCE. Ramada O'Hare Inn, Chicago, IL. Contact: Dr. John Birdsall, Director of Scientific Activities, American Meat Institute, P.O. 3556, Washington, DC 20007.

Mar. 31-April 2---AMERICAN CULTURED DAIRY PRODUCTS INSTITUTE ANNUAL TRAINING SCHOOL AND JUDGING CONTEST, Hilton Airport Plaza Inn, Kansas City, MO. Contact: C. Bronson Lane, ACDPI, P.O. Box 7813, Orlando, FL 32854.

April 14-16---5th ANNUAL FOOD SERVICE SYSTEMS SEMINAR AND EXPOSITION. Sheraton O'Hare Hotel, Chicago, IL. Contact: G. E. Livingston, Food Science Associates, Inc., 595 Fifth Avenue, New York, NY.

April 21-25---SCANNING ELECTRON MICROSCOPY/1980. McCormick Inn, Chicago, IL. Contact: O. Johari, Scanning Electron Microscopy Inc., P.O. Box 66507, AMF O'Hare, IL 60666.

April 30---USE AND ABUSE OF FOOD SUBSTANCES---TECHNOLOGICAL AND HEALTH IMPLICATIONS, Pick Congress Hotel, Chicago. Sponsored by Illinois State Medical Society, Chicago Nutrition Association and Chicago Section of Food Technologists. Contact: Therese Mondeika, 312-751-7624.

May 6-10---5th INTERNATIONAL DLG DAIRY ENGINEERING EXHIBITION 1980. Hall 5, Frankfurt Fair Ground, Frankfurt, Germany. Contact: Deutsche Landwirtschaft-Gesellschaft Zimmerweg 16, 6000 Frankfurt, Am Main 1, Germany.

May 11-13---1980 INTERNATIONAL CHEESE & DELI SEMINAR, MECCA, Milwaukee, WI. Contact: W. T. Reese, Executive Secretary, P.O. Box 5528, Madison, WI 53705.

May 12-15---SOUTH DAKOTA ENVIRONMENTAL HEALTH ASSOCIATION, Annual Educational Conference, Huron, SD. Contact: Cathy Meyer, Secretary-Treasurer, SDEHA, Box 903, Mitchell, SD 57301.

May 20-22---AMERICAN SOCIETY FOR QUALITY CONTROL, 34th Annual Technical Conference. Hyatt Regency Hotel, Atlanta, GA. Contact: D.C. Schmidt, American Society for Quality Control, 161 W. Wisconsin Ave., Milwaukee, WI 53203.

June 16-20---9th INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION AND ARTIFICIAL INSEMINATION, Madrid, Spain. Contact: Prof. Dr. Tomas Perez Garcia, INIA, CRIDA-06, Departamento de Reproduccion Animal, Avda. de Puerta de Hierro, s/n, Madrid-3, Spain. Phone: 449-11-76 or 449-16-50.

Here's Your Chance to See Your Work in Print. . .

Interested in being one of the first authors in the new IAMFES magazine? We can use your help writing or soliciting practical articles for the first few issues of the publication, slated for regular production in 1981. We'll be running a test issue in June 1980, however, and can use help for that issue, too.

Send ideas of articles you'd like to see. Better yet, send the article itself, or at least "raw material" from which Jan Richards, Assoc. Managing Editor of the *Journal of Food Protection,* will write an article.

Think about it — it's a good opportunity!

1980-81 Officer Nominees Candidates for Secretary-Treasurer

Archie Holliday and Lee Lockhart

Archie C. Holliday
Chief, Bureau of Dairy Services
Virginia Dept. of Agriculture and Consumer Services

Archie C. Holliday is Chief of the Bureau of Dairy Services in the Division of Animal Health and Dairies of the Virginia Department of Agriculture and Consumer Services. The Bureau of Dairy Services was created to broaden service programs available to Virginians who are involved with the dairy industry. As Chief, Archie is responsible for assuring that milk, milk products, ice cream and frozen desserts available to Virginia consumers are safe, pure and wholesome and produced according to standards mandated by law.

He was born in Newport News, Virginia on July 30, 1930. Archie received the B.S. degree in dairy husbandry in 1953 from Virginia Tech (VPI & SU). After serving two years in the U.S. Army he joined a dairy processing company where he supervised quality control for a local plant. After three years he joined the Virginia Department of Agriculture and has been employed in that agency since that time, 1959. He held positions of dairy inspector, dairy field supervisor, and dairy law supervisor prior to being appointed Chief of the Bureau. He is a Registered Sanitarian in the State of Virginia.

Archie has been a member of IAMFES and the Virginia affiliate for twenty-one years. He has served on several committees of the Virginia affiliate, the Virginia Association of Sanitarians and Dairy Fieldmen and also served one term as president of the association. He has been a

con't p. 1006

Leland (Lee) H. Lockhart
Chief, Bureau of Milk and Dairy Foods Control
California Dept. of Food and Agriculture

Leland (Lee) H. Lockhart, Chief, Bureau of Milk and Dairy Foods Control, California Department of Food and Agriculture, Sacramento, was born and raised on a diversified farm, which included dairying, at Santa Rosa, CA.

Lee graduated from the University of California, Davis, with a major in dairy industry. Upon graduation from college, Lee served two years as an employee with the milk processing plant at the University of California, Davis.

His job at the plant included instruction of students in the processing of milk and dairy products. He was later employed with private industry in processing and quality control of milk products.

Lee started as a dairy inspector with the Department of Food and Agriculture 36 years ago. He was later promoted to specialist in milk and milk products, district supervisor, regional administrator, and in 1974 was appointed Chief of the Bureau of Milk and Dairy Foods Control.

Some of his achievements with the Department of Food and Agriculture include development of improved methods of sampling milk, with more rapid and precise methods of testing for milk components where payment to producers is based on component test. He was instrumental in the development of a routine program for the detection of salmonella in raw milk sold to consumers.

Lee is a past president of the California Association of Dairy and Milk Sanitarians.

He has participated in the Interstate Milk Shippers Program as a state milk sanitation rating officer, a state milk laboratory evaluation officer, and a state milk sampling surveillance officer. Lee is a past chairman of the IMS Single-Service Container and Closure Committee

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News and Events

Small Loop May Help Prevent Mastitis

A USDA animal scientist says that a small plastic loop inserted into each teat of a cow's udder may prevent mastitis. Mastitis, one of the dairy industry's costliest problems, results in losses to U.S. dairymen of up to a billion dollars a year.

The principle behind the loop's effectiveness is that the loop stimulates the cow's natural disease-fighting mechanism. Increased numbers of leucocytes or white blood cells are sent to site of the loop, which becomes irritated. The leucocytes then destroy bacteria which cause mastitis.

Leucocytes normally take 24 hours to build up to a point where they can effectively fight bacteria. By this time, however, an infection may already have been established. If leucocytes are already present, due to the loop's presence, the bacterial build-up may be prevented or the udder may be better able to respond to an infection.

Changes Proposed in Meat Inspections

Several changes have been proposed in inspections of processed meat and poultry products.

Assistant Secretary of Agriculture Carol Tucker Foreman has proposed a system which would trim the number of Federal inspectors needed to keep up with the growth of the processed food industry. There would be less on-site inspection and more analysis of manufacturing plant records of quality control and laboratory analyses.

USDA inspectors would have access to data collected from firms participating in plant quality control systems. In these systems, critical points in production would be monitored and results recorded.

Seiberling Opens New Regional Office

Seiberling Associates, Inc. has opened an Eastern Regional Office in Columbus, Ohio. It will be managed by John R. Miller, a graduate of Ohio State University (B.S. & M.S.). He has been an Associate with the Seiberling firm since its beginnings in 1976.

The new office will provide consulting services related to the design of process piping and control systems requiring a high degree of sanitation.

Seiberling's other offices are in South Beloit, IL, and San Mateo, CA.

U.S. Ashworth Dies

Ural Stephen Ashworth, 74, professor emeritus of food science at Washington State University, died October 5 following a lengthy illness.

Widely known by his initials, U.S., Ashworth was a member of the WSU faculty from 1939 until his retirement in 1971. He was noted for research and in 1962 won the Borden Award for outstanding dairy research leading to flavor enhancement and longer shelf life for powdered whole milk.

The former WSU faculty member also was a nationally recognized authority on milk proteins and developed tests to determine the protein content of milk.

Ashworth was co-editor of the *Journal of Dairy Science* and served as president of the local chapter of Sigma Xi, national scientific honorary. He also was a member of the American Chemical Society and the Institute of Food Technologists.

Ashworth earned a bachelor's degree in chemistry and a doctorate in biochemistry at the University of Missouri, Columbia. He held a doctoral fellowship at Missouri from 1933-35, then a two-year fellowship at the Yale Medical School, New Haven, Conn. He was an assistant professor of biochemistry at the University of Arkansas Medical School, Little Rock, for two years before coming to WSU in 1939.

He was born Sept. 11, 1905, at Walla Walla and grew up in Missouri.

Surviving are his wife, Louise, two daughters, and three sons, and three grandchildren.

Mark R. Fry, 1950-1979

Mark R. Fry, 29, an Indiana sanitarian, was killed September 1 while inspecting a pipeline in a trailer park. He was standing in a six-foot-deep trench, checking repair work on the pipeline when the trench caved in.

Fry, the treasurer of the Indiana affiliate, was a 1972 graduate of Indiana University. He joined the Health and Hospital Corporation of Marion County, Indiana, in 1972 as an inspector in the Food Section. He assumed the position of sanitarian in the General Sanitation Department in July, 1976.

He was a member of IAMFES, the National Environmental Health Association, Indiana University Alumni Association, and the Knights of St. John Commandery 227.

Commended shortly before his death by members of the WINC Community Organization, Fry was highly eulogized by co-workers and supervisors. Said Albert L. Klatte, chief, Bureau of Environmental Health, "The fact that Mark was working on his day off, and that he was servicing a complaint which he could have referred to another employee or to the State Board of Health is an indication of the type of employee that Mark has been for a number of years.

Holliday, *con't from p. 1004*
member of the IAMFES awards committee for the past two years and is currently serving on the Farm Methods Committee. He is also secretary of the Affiliate Council.

In other activities, Archie is Virginia's representative to the Dairy Division of the National Association of State Departments of Agriculture and is currently serving as president of the Southern regional division of that organization. He is a member of the board of directors of the National Mastitis Council and is a member of the American Dairy Science Association and the Association of Food and Drug Officials. He is a delegate from Virginia to the National Conference on Interstate Milk Shipments.

In activities within the Commonwealth of Virginia Archie is an associate member of both the Virginia State Dairymen's Association and the Virginia Dairy Products Association. He is an elected member of the Virginia Dairy Science Advisory Board.

Archie has been married for 27 years to Evalyn Scott Holliday. They have three daughters, each a graduate of Virginia Tech.

Above, left to right— Roxanne Rathert, Membership Secretary, Earl Wright, Executive Secretary, IAMFES, Jan Richards, Assoc. Exec. Secretary, IAMFES, and Esther Stark, Secretary and Bookkeeper.

IAMFES and the *Journal of Food Protection*™

Dr. Elmer H. Marth, Editor
Journal of Food Protection

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Single Service milk sample tubes. For further information and a catalog, write: Dairy Technology, Inc., P.O. Box 101, Eugene, OR 97401.

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THE ONLY *Approved*
SANITARY METHOD OF APPLYING
A U. S. P. LUBRICANT
TO DAIRY & FOOD
PROCESSING EQUIPMENT

Haynes Spray

U. S. P. LIQUID PETROLATUM SPRAY

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CONTAINS NO ANIMAL OR VEGETABLE FATS. ABSOLUTELY NEUTRAL. WILL NOT TURN RANCID — CONTAMINATE OR TAINT WHEN IN CONTACT WITH FOOD PRODUCTS.

SANITARY—PURE

ODORLESS—TASTELESS

NON-TOXIC

This Fine Mist-like
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should be used to lubricate:

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MACHINE PARTS
and for ALL OTHER SANITARY
MACHINE PARTS which are
cleaned daily.

The Modern HAYNES-SPRAY Method of Lubrication Conforms with the Milk Ordinance and Code Recommended by the U. S. Public Health Service

The Haynes-Spray eliminates the danger of contamination which is possible by old fashioned lubricating methods. Spreading lubricants by the use of the finger method may entirely destroy previous bactericidal treatment of equipment.

PACKED 6-12 oz. CANS PER CARTON
SHIPPING WEIGHT—7 LBS.

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HAYNES-SPRAY INGREDIENTS ARE APPROVED ADDITIVES AND CAN BE SAFELY USED AS A LUBRICANT FOR FOOD PROCESSING EQUIPMENT WHEN USED IN COMPLIANCE WITH EXISTING FOOD ADDITIVES REGULATIONS.

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LOW COST...RE-USABLE

LEAK-PREVENTING

NEOPRENE GASKET for Sanitary Fittings

Check these SNAP-TITE *Advantages*

Tight joints, no leaks, no shrinkage
Sanitary, unaffected by heat or fats
Non-porous, no seams or crevices
Odorless, polished surfaces, easily cleaned
Withstand sterilization
Time-saving, easy to assemble
Self-centering
No sticking to fittings
Eliminate line blocks
Help overcome line vibrations
Long life, use over and over

Available for 1", 1½", 2", 2½" and 3" fittings.
Packed 100 to the box. Order through your dairy supply house.

THE HAYNES MANUFACTURING CO.
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Haynes SELF-CENTERING SNAP-TITE® Gaskets

SIZES 1" - 1½"
2" - 2½" - 3" - 4"

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"The Sophisticated Gasket"

THE IDEAL UNION SEAL FOR BOTH VACUUM AND PRESSURE LINES

Gasket Color . . . slightly off-white

SNAP-TITE self-centering gaskets of TEFLON are designed for all standard bevel seat sanitary fittings. They SNAP into place providing self-alignment and ease of assembly and disassembly.
HAYNES SNAP-TITES of TEFLON are unaffected by cleaning solutions, steam and solvents. They will not embrittle at temperatures as low as minus 200° F. and are impervious to heat up to 500° F.

FOR A FITTING GASKET THAT WILL OUT-PERFORM ALL OTHERS...

Specify . . . HAYNES SNAP-TITES of TEFLON

- *TEFLON ACCEPTED SAFE FOR USE ON FOOD & PROCESSING EQUIPMENT BY U. S. FOOD AND DRUG ADMINISTRATION*

* Gaskets made of DuPont TEFLON ® TFE-FLUOROCARBON RESINS

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A HEAVY DUTY SANITARY LUBRICANT

Available in both
SPRAY AND TUBE

All Lubri-Film ingredients are approved additives and can be safely utilized as a lubricant for food processing equipment when used in compliance with existing food additive regulations.

ESPECIALLY DEVELOPED FOR LUBRICATION OF FOOD PROCESSING AND PACKAGING EQUIPMENT

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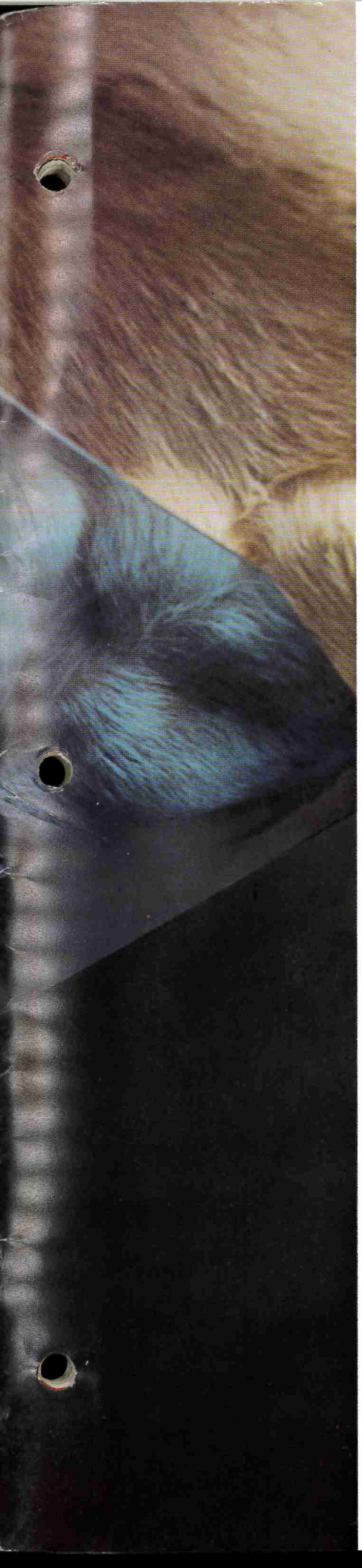
BLU-GARD™



BLU-GARD® kills both Gram-negative and Gram-positive mastitis-causing bacteria within 30 seconds after contact.

A.O.A.C. Germicidal and Detergent Sanitizer Test

	Strep. Ag.	Staph. aureus	E. Coli
BLU-GARD[2]	passes[1]	passes[1]	passes[1]
Iodophor[3]	passes[1]	passes[1]	passes[1]
Quaternary Ammonium cpd.[3 4]	fails	fails	fails
Bronopol[3]	fails	fails	fails
8-Hydroxyquinoline Sulfate[3]	fails	fails	fails

[1]Passes: 99.999+% kill after 30 seconds contact time.
[2]BLU-GARD also passes against all organisms in the presence of 10% milk.
[3]Product name available on request.
[4]"Alkyl dimethylbenzyl ammonium chloride plus alkyl dimethylethyl ammonium bromide".

Introducing BLU-GARD.® The new non-iodine teat dip from Klenzade.™

A teat dip with fast, broad-spectrum germicidal activity plus residual anti-microbial action between milkings.

BLU-GARD teat dip provides the long-lasting, anti-bacterial activity you should expect from a teat dip.

Broad-Spectrum Activity.
The active ingredient in BLU-GARD, "LAS" for short, insures a quick kill of the Gram-positive organisms, *Strep. ag, Staph. aureus, Strep. uberis and Strep. dysgalactia.* It also provides protection against the Gram-negative organisms, *E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia and Enterobacter aerogenes.*

Long-lasting.
BLU-GARD provides more than the quick kill of ordinary teat dip products such as Iodophors and Chlorine. In fact, tests indicate that BLU-GARD maintains anti-bacterial activity for up to 12 hours, from time of dipping until it's washed off at the next milking.

Non-irritating.
BLU-GARD contains no iodine, so it's less irritating to both the cow and the dairyman. A high level of glycerin (12%) and an emollient base helps protect the cow from chapping in cold, wet weather. This also helps keep the cow's teat ends soft and pliable. You know the importance of teat dipping in controlling new mastitis infections. For broad-spectrum activity, with lasting anti-bacterial action, recommend BLU-GARD from Klenzade.

KLENZADE™

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St. Paul, Minnesota 55102
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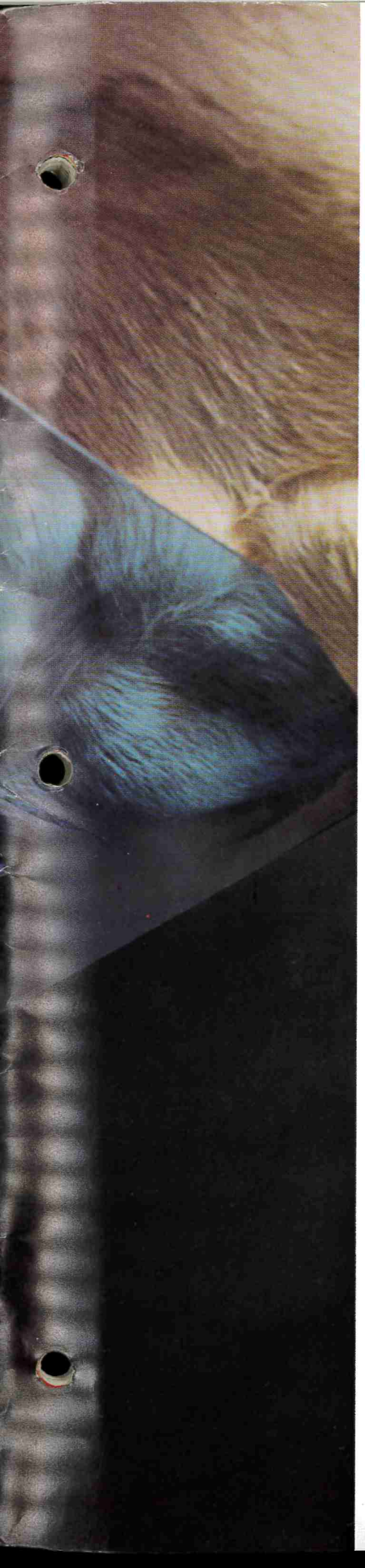

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BLU-GARD[2]	passes[1]	passes[1]	passes[1]
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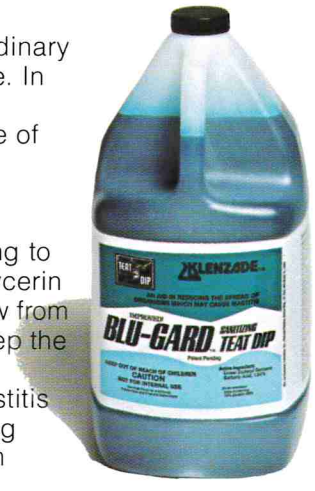

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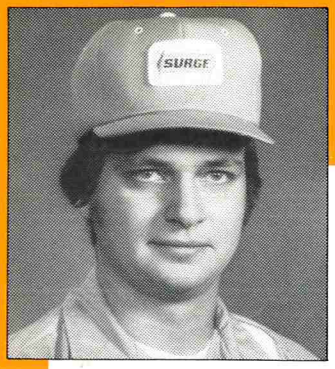
SOMEONE YOU SHOULD KNOW IN THE DAIRY INDUSTRY



Richard Athey, Dairy Equipment Serviceman

Richard Athey grew up at his father's dairy equipment dealership in Berlin, Pennsylvania. He has attended the Surge Training Center near St. Charles, Illinois, on three separate occasions, receiving performance certificates in both Basic and Advanced Refrigeration and in Advanced Dairy System Trouble Shooting. While in high school, Mr. Athey worked weekends and summers as a dairy equipment serviceman and has continued this profession on a full-time basis for the past six years.

"Dairymen have a lot on their hands these days just trying to manage their operations for better performance. The last thing they want to worry about is the efficiency of their milking equipment. They have a right to expect this important system to operate at top efficiency, and that's where a serviceman can make a big difference."

Preventing Trouble Before it Starts

"Along with emergency service and repairs, my activities include checking the entire milking system periodically to find small problems before they become major breakdowns. The regularity of these preventative maintenance inspections is determined by the dairyman and vary from one dairy to the next. However, one way or another, certain things must receive regular, periodic attention to assure the system is working properly.

"These include:

- Vacuum System—Checking vacuum pump oil level, pump oilers, regulator and filters is essential to consistent vacuum operation.
- Milking System—Since this system can have a direct affect on udder health, it is most important to examine inflations, pulsator ratios, pulsator timing and milking vacuum levels.
- Sanitation Equipment—There is a direct relationship between milk quality and cleanliness so making checks of pipe line washers, hot water heaters, as well as analyzing the water help protect the milk from contamination.
- Refrigeration—Keeping tabs on the cooling time and blend temperatures can help prevent a major loss of milk through cooling breakdowns.

"No matter if these things are all checked by the serviceman, or some of them are accomplished by the dairyman, they are necessary to keep the system working right and prevent major problems from developing. For a car or tractor to work right, it must be kept in proper tune with all lubricants provided in sufficient quantity. The milking system is even more important than these machines, and that means it must be tuned properly to perform right.

"I firmly believe that if we compared a group of dairies with regular maintenance programs against a group without it, we would find that most of our major equipment outages and emergency calls would go to the last group. The investment in regular, preventative maintenance is money well spent because it protects the dairyman and his herd from unexpected and often costly emergencies.

"Why is this so? Because the milking system is the only equipment on the farm that works on live tissue, namely the cow's udder and teats. If the pulsation goes bad or improper vacuum level or fluctuating vacuum occurs, the herd could develop very costly udder problems. This just doesn't need to happen if the equipment is checked and adjusted regularly. After checking the system with our testing equipment such as the Levograph, we can correct it right away before it affects the herd."

Why We Care

"I care about the job I do for the dairyman. He has a big investment and you have to help him protect that investment. It's needless to say we are like partners. If his operation suffers, it won't be long before I'm suffering along with him. If he's out of work, so am I, and that means one thing to me: I'll do everything in my power to help the dairyman succeed. Working together, we do the best we can to improve what we do, and that helps the dairy industry succeed in the process."