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Effect of Low-Frequency Ultrasound and Elevated Temperatures on Isolation of Bacteria from Raw Milk

C. N. HUHTANEN

*Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division,
U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118*

Treatment of milk with low-frequency ultrasound significantly increased the total bacterial counts and the counts of enterococci, coliforms, and staphylococci. Warming diluted milk for about 12 min at 30 or 40 C increased the counts of some organisms, but the heat produced by ultrasonic treatment did not account entirely for its effect. The ultrasonic effect was related to the energy output of the generator and to the energy absorbed by the treated materials.

A previous report (3) indicated that ultrasonic treatment of milk caused increases in total numbers of recoverable bacteria, presumably by breaking up the clumps of bacteria which normally occur in milk. These clumps cause difficulties in determining the absolute numbers of bacteria present. The obvious manifestations of ultrasonic wave energy include mechanical agitation, caused by the rapid formation and collapse of cavitation bubbles, and heat production by the acoustic energy. The present report explores further these two ultrasonically derived effects and presents detailed analyses of the effects of ultrasound on a number of raw-milk samples collected over a period of 1 year. The effect of ultrasound on other groups of bacteria was also studied.

MATERIALS AND METHODS

Two types of ultrasonic generators were used. One, normally designed for cleaning operations, was the "Ultra Clean" model 320 LU produced by the L & R Manufacturing Co., Kearney, N.J. This instrument operates at a nominal frequency of 75 kc per sec with an output of 65 w distributed over a bath area of approximately 50 square inches. Most of the experiments with this instrument were initiated with 1 liter of ice water at 2 to 4 C in the bath; care was taken that little or no visible ice was present when sonic treatment began. Milk-dilution bottles containing 11 ml of milk and 99 ml of sterile water were placed in the bath, always in the same position. Under these conditions, the temperature rose to about 10 to 15 C.

This Ultra Clean model was also used for controlled-temperature experiments. An Eastern pump (model B-1; Fisher Scientific Co., Pittsburgh, Pa.), controlled with a rheostat, was connected to the bottom drain of the ultrasonic tank, and the water

was circulated through an 8-foot copper coil in a 20-liter circular water bath at the rate of 850 ml per min. The circular bath temperature was thermostatically controlled with a TECAM Tempunit model TUS controlling unit. Under these conditions, the temperature rise during the period of sonic treatment was less than 1 C per 10 min of operation.

The other type of ultrasonic generator used was the Branson model S125 (Branson Division, Smith, Kline and French Co., Danbury, Conn.). The Branson generator operates at 20 kc per sec at a maximum of 125 w through a 0.5-inch horn, resulting in a very concentrated sonic output. Power on this instrument could be varied. The probe was inserted in a glass rosette immersed in an ice bath. The temperature of the sonically treated sample was less than 10 C. A 10^{-1} dilution of milk was also used in experiments with this instrument.

Raw milk samples were obtained from the bulk tank-delivered supply of a local dairy and were maintained at 35 to 40 F until analyzed. The media used were Plate Count Agar for total counts, Violet Red Bile Agar for coliforms, Enterococcus Confirmatory Agar for enterococci and Staphylococcus Medium 110 for staphylococci. The counts were standard dilution counts except for the staphylococci. These were determined by streaking 0.1 or 0.5 ml of a 10^{-1} dilution on a cooled agar plate with the aid of a bent glass rod. Incubation was for 2 days at 27 C for total counts and at 37 C for the others. For statistical purposes, all colonies were counted except when the numbers exceeded 400 per plate.

RESULTS

Table 1 shows the effects of ultrasonic treatment on the numbers of enterococci, coliforms, staphylococci, and total bacteria. When all figures were considered together, they were very signifi-

TABLE 1. Influence of ultrasound on recovery of raw milk bacteria^a

Sample date	Enterococci per ml		Coliforms per ml		Staphylococci per ml		Total count ($\times 10^{-3}/\text{ml}$)	
	Control	Ultrasound	Control	Ultrasound	Control	Ultrasound	Control	Ultrasound
7/12/66							1,030	650
							870	610
8/15/66							90	135
							95	120
8/22/66							1,500	1,120
							1,500	900
9/12/66							92	135
							95	115
9/19/66							180	200
							180	210
10/18/66	680	1,130	160	820	1,900	3,280	169	320
	470	900	140	750	1,800	2,200	197	298
11/2/66	740	1,650	520	1,150	1,900	2,200	2,500	3,350
	1,040	2,100	590	1,650	1,500	1,900	2,550	3,950
11/14/66	410	770	280	390	3,400	2,900	100	160
	610	860	390	450	4,100	3,200	115	175
12/1/66	5,300	10,700	1,800	2,600	4,900	5,200	1,200	1,800
	4,800	12,300	1,400	3,000	4,700	4,100	1,450	2,250
12/12/66 (A)	340	320	180	200	530	510	62	120
	200	380	60	140	520	560	82	130
(B)	340	340	140	240	510	340	65	200
	260	200	80	240	280	460	85	230
1/13/67	1,320	2,000	1,360	1,120	4,800	4,400	530	340
	1,160	1,950	1,400	1,100	3,100	5,800	360	310
1/17/67 (A)	980	1,240	380	1,220	1,240	940	2,850	2,950
	880	1,220	380	1,160	860	500	2,900	3,100
(B)	2,000	2,320	660	660	820	550	235	210
	2,160	2,100	760	760	800	680	260	225
1/30/67 (A)	640	1,640	200	380	6,500	6,500	140	175
	840	1,440	140	300	4,800	5,800	150	145
(B)	300	320	140	260	4,600	5,000	23	18
	240	480	100	180	3,700	4,700	15	19
(C)	1,700	10,600	600	1,100	6,500	8,000	19	71
	1,920	8,800	400	1,120	6,600	5,800	9	80
(D)	300	640	100	160	3,900	4,200	20	67
	400	700	120	120	2,500	3,400	25	59
(E)	620	1,260	660	1,100	3,500	4,300	15	41
	740	1,180	720	940	3,500	4,800	22	41
2/13/67	39	60	600	500			58	92
	42	46	550	600			72	75
2/28/67	700	1,600	240	740	1,400	3,000	2,140	3,200
			260	720	1,700	2,000	2,500	2,800
3/13/67	800	1,400	130	80	6,300	11,500	84	73
	800	1,150	120	110	5,800	8,000	74	75
4/28/67	510	400	490	150	640	780	37	49
	370	290	320	60	550	750	24	35
5/10/67	500	610	380	360	400	520	38	40
	570	540	460	360	510	580	25	40
5/17/67							620	790
							540	690
5/31/67 (A)	260	300	340	200	1,680	2,800	56	93
	160	180	120	100	2,000	2,760	74	105
(B)	300	540	40	0	2,400	2,900	21	28
	240	440	20	20	3,200	2,800	24	15
(C)	1,280	2,240	500	480	4,900	5,900		
	1,020	2,080	520	400	4,600	5,700		
(D)	600	940	100	0	2,100	5,000	18	44
	600	1,180	160	80	2,200	5,200	24	44
6/28/67	1,440	2,000	2,300	1,400	5,700	6,800	1,520	2,200
	1,600	2,480	2,200	800	6,300	7,500	1,200	3,000

^a Incubation: enterococci, 37 C, 2 days; coliforms, 37 C, 2 days; staphylococci, 37 C, 1 day, and 27 C, 1 day; total count, 27 C, 2 days.

TABLE 2. *Effect of ultrasound, warming, and shaking upon apparent total counts^a*

Sample	Ice-water control	Ultrasound in ice water	Warmed to 30 C	Ultrasound at 30 C	Vigorous shake
1	29	43	52	54	28
	33	59	41	48	36
2	127	340	155	312	168
	110	390	167	325	182
3 (grade A)	92	75	99	82	102
	84	81	91	75	110
4	650	570	870	1020	750
	540	490	820	950	650
5	260	390	330	310	390
	290	350	320	390	350
Averages	221*	279*	294*	363†	277*

^a Results represent replicate plates (count $\times 10^3/\text{ml}$). Ultrasound and warming for 13 min; vigorous shaking 100 times, 7-inch stroke in 15 sec. Plates were incubated at 27 C for 2 days; significant differences are indicated by dissimilar superscripts at the 5% level of significance; no differences occurred at the 1% level.

cantly increased by the treatment, although the increase was not apparent in all samples. Significantly, no decreases in counts were caused by the ultrasound, except possibly with the coliforms (5/31/67 A, B, and D, and 6/28/67, Table 1) and in a few other isolated instances. There also seemed to be no correlation between the different bacterial types and the degree of the increase in numbers caused by sonic treatment. An analysis of variance showed the increases caused by the ultrasound to be significant at the 0.05 level. The differences from one bacterial group to another were, however, not significantly different, indicating that the ultrasound was not selective in its action but was exerting its influence equally on the different bacteria.

Table 2 shows the effects of holding at 30 C for 13 min and of ultrasound on numbers of recoverable bacteria. The temperature of the bath was maintained with the circulation pump. Again there were great variations among the samples in the effect of the treatments. In this experiment, the milk samples having the lowest numbers of bacteria showed little or no increases as a result of the treatments. Collectively, these negative results precluded a high degree of statistical sig-

nificance, although the combination of ultrasound and holding at 30 C did show significantly higher numbers of bacteria at the 5% level of significance but not at 1%. The vigorous shaking (100 times with a 7-inch stroke within 15 sec) also seemed to give results similar to ultrasound, although again the results were not statistically different. Ultrasound in ice water caused an apparent increase from 221,000 to 279,000 bacteria per ml, holding at 30 C increased numbers to 294,000, and the combination of ultrasound at 30 C increased numbers to 363,000. This last figure compares well with the additive effects of the cold ultrasound and warming (352,000 per ml). To determine whether the individual increases caused by the 30 C treatment and cold ultrasound were equal to the ultrasound at 30 C, a hypothesis was

TABLE 3. *Effect of warming to 30 C on recoverable raw-milk bacteria*

Sample no.	Incubation temp	Bacterial count ($\times 10^{-3}/\text{ml}$)	
		Ice-water control	Warmed to 30 C, 12 min
1	C	46	85
		64	113
		61	132
		52	125
		10.5	10.0
		8.0	8.0
2	2	235	440
		280	380
		275	480
		270	520
		175	142
		160	175
3	2	65	98
		75	70
		80	93
		81	126
		33	34
		29	23
4	2	330	440
		360	290
		370	540
		410	520
		15	13.5
		12	11.0
Means	2	182	240 ^a
		200	317 ^a
		55.3	52.1 ^b

^a Different at the 5% level.

^b Not different at the 5% level.

TABLE 4. Effect of different temperatures on recoverable raw-milk bacteria

Incubation temp	Test temp for 12 min	Bacterial count ^a		Increase in count	
		Ice-water control	Test		
C	C			%	
		20	77	165	104
			80	155	
		30	115	170	55
			108	175	
40	110	180	84		
	102	210			
27 ^c	20				
			85	130	58
			80	130	
		30	105	172	77
			100	190	
40	95	180	80		
	110	190			
37 ^c	20				
			2.3	2.0	0
			2.1	2.1	
		30	2.2	2.4	0
			2.8	2.3	
40	2.0	1.9	0		
	2.1	2.1			

^a For absolute counts, multiply by 10⁴/ml.

^b For 10 days.

^c For 2 days.

TABLE 5. Effect of varying intensities of ultrasound on raw-milk bacteria

Power setting	Time of sonic treatment	Bacterial count per ml	
		Enterococci	Total
	<i>sec</i>	$\times 10^{-2}$	$\times 10^{-3}$
—	0	42	65
1	5	44	84
1	20	58	94
3	5	77	101
3	20	80	70
5	5 ^a	60	100
5	20	98	49
8	5 ^a	66	98
8	20 ^a	80	102

^a Cavitation was broken at these settings. Plates were incubated at 27 C for 2 days (total count) and at 37 C for 2 days (enterococci). Apparatus used was the Branson model S125 Sonifier.

set up as follows: (30 C-treated — control) plus (cold ultrasound — control) minus the ultrasound at 30 C equals zero. Analysis of the paired differences by Student's test gave a *t* value of 1.58 which was not sufficiently great to reject the hy-

pothesis. It thus appeared that the effect of the ultrasound at 30 C was a combination of the two individual effects.

Further evidence of the effect of warming on the numbers of bacteria is shown in Table 3. In this experiment, replicate plates were incubated at the three temperatures: 2, 27, and 37 C. Significant increases in numbers of bacteria occurred in plates incubated at the lower temperatures, but no differences could be detected in the 37 C plates.

Table 4 shows the results obtained when the milk samples were warmed to 20, 30, or 40 C for 12 min and the plates were incubated at temperatures of 2, 27, or 37 C. Increases in the 2 and 27 C counts were obtained with all three temperatures; however, the 37 C counts were not affected.

The effect of a more concentrated source of ultrasound is shown in Table 5. In this experiment, 8 ml of a 10⁻¹ dilution of milk was placed in a glass rosette which was immersed in an ice bath. The 0.5-inch horn of the Branson Sonifier

TABLE 6. Effect of pH on ultrasonic disaggregation

Amt of 0.2 N KH ₂ PO ₄	Amt of 0.2 N Na ₂ HPO ₄ · 7H ₂ O	pH	Bacterial count ^a		Increase
			Control	Ultra-sound	
<i>ml</i>	<i>ml</i>				%
0 ^b	0	6.65	250	335	45
			255	395	
90	10	5.85	160	355	141
			140	370	
80	20	6.15	300	480	75
			215	420	
70	30	6.35	220	550	146
			225	540	
60	40	6.50	260	410	71
			260	480	
55	45	6.60	205	480	107
			225	410	
50	50	6.67	205	360	75
			210	365	
40	60	6.81	190	540	164
			165	400	
20	80	7.16	180	490	172
			175	480	
10	90	7.48	135	350	136
			160	350	
0 ^c	0	6.65	260	430	53
			295	420	
APHA ^d buffer		6.75	265	360	62
			205	400	

^a Plates were incubated at 27 C for 2 days. For absolute counts, multiply by 10⁴/ml.

^b Beginning of experiment.

^c End of experiment.

^d American Public Health Association.

TABLE 7. *Effect of load on ultrasonic disaggregation^a*

Size of bottle	Vol of sample	Ultra-sound	Count ($\times 10^{-3}$ /ml)
	<i>ml</i>		
Regular dilution	110	—	169
			197 (183) ^b
	110	+	320
			298 (309)
	11	+	68
			34 (51)
16-oz round	11	+	76
			69 (72)
	110	+	344
			298 (321)
	550	+	300
			265 (283)

^a Plates were incubated at 27 C for 2 days. Sonic treatment apparatus was the L & R Ultra Clean model.

^b Numbers in parentheses represent the average of the two determinations.

gave a very concentrated ultrasonic discharge, compared to the relatively weak activity of the "Ultra Clean" model. This concentrated ultrasound caused increases in numbers of enterococci roughly proportional to the power applied. At the settings where cavitation was broken, however, the numbers of recovered enterococci were decreased, although they were still greater than in the untreated controls. The total count (reflecting mostly the bacteria growing at lower temperatures) showed increases up to a power setting of 3 on the instrument with a 5-min treatment. With a 20-sec treatment, the total count decreased. A further decrease, indicating some destruction of bacteria, took place at a power setting of 5 with a 20-sec treatment. Loss of cavitation at the higher power settings reversed the destructive effects.

Table 6 shows the effect of varying the pH, by use of 0.2 M phosphate buffer. A decrease in numbers of recovered bacteria occurred at pH 5.85 and at pH values higher than 6.60. The highest numbers were isolated at pH 6.50. There did not seem to be any pattern to the ultrasonic effect as related to pH, although the ultrasound with the buffer in nearly all cases resulted in a greater recovery of bacteria than occurred in the controls diluted with either water or the standard American Public Health Association buffer.

The importance of the loading factor in the ultrasonic effect was illustrated by the experiment shown in Table 7, in which a 10^{-1} dilution of milk was treated in several ways. The untreated control had an average count of 183,000 per ml. The standard treatment (110 ml in a dilution bottle) produced a count of 309,000 per ml. When the dilution bottle contained only 11 ml, the number of bacteria recovered decreased sharply to 47,000 per ml. With 11 ml of liquid in a 16-oz round bottle, a count of 72,000 resulted. Larger amounts in the 16-oz bottle showed an ultrasonic effect similar to that in the standard dilution bottle containing 110 ml.

DISCUSSION

The destructive effect of ultrasound waves on several different types of bacteria, yeasts, etc., has been shown by Kinsloe et al. (4) and Davies (2), as well as by others. It was shown previously (3) that ultrasound could also be used to increase the numbers of recoverable bacteria in milk. The nature of this increase in numbers with ultrasound is still unclear, although the present report indicates that heat can at least partially substitute for the ultrasound. The experiments with the Ultra Clean bath-type ultrasonic instrument were run in ice water with a temperature increment of perhaps 10 to 15 C. This does not preclude the possibility, however, of a greater localized temperature rise in the region of the microscopic cavitation bubble.

Keeping the 10^{-1} dilution bottles at 20, 30, or 40 C caused increases in both the 2 and 27 C counts but not in that at 37 C. This is similar to the results previously reported when raw milk was treated with ultrasound (3), and indicates that great care should be exercised in insuring rapid dilution and plating of raw-milk bacteria. The dilution bottle temperature, perhaps, should be standardized.

The experiments reported here indicate that great variations exist between different milk samples as far as the effect of the ultrasound on the bacteria is concerned. The reasons for this variation are not known, although it seems logical to assume that the types of bacteria involved would be a major factor. This is indicated by the lack of apparent correlation between the ultrasonic effect on staphylococci, enterococci, coliforms, and total counts in individual milk samples, although when samples were treated collectively the ultrasound seemed to cause changes of the same magnitude in each of these bacterial types.

ACKNOWLEDGMENT

Appreciation is expressed to W. C. Stewart of Temple University for the statistical evaluation of these results.

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