S & J Laboratories, Inc.

Report To: Dr. Don Berdahl/Mr. Greg Reynhout (Kalsec®, Inc.)

From: James Lin (S & J Laboratories, Inc.)

Date: November 14, 2005

Title: Pathogen Inoculation Study of Ground Beef Under Modified Atmosphere Package (MAP) Conditions

SUMMARY

Pathogens, *Salmonella* spp. or *E. coli* O157:H7 were inoculated into fresh ground beef at a targeted level of 1,000 cfu/g or 5,000 cfu/g and packaged under a modified atmosphere containing either 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen or 82% oxygen and 18% carbon dioxide. The ground beef packaged in the oxygen-containing atmospheres were treated with rosemary extract (Herbalox® Seasoning), an ingredient commonly added to the majority of the commercial MAP ground beef sold in the US. Samples were stored at 4° and 10°C incubators for the periods of time indicated in the table. Microbiological analyses were performed periodically by S & J Laboratories, whereas the colorimeter readings (L*a*b* values), atmosphere composition analysis, and photography were conducted by Kalsec® personnel. The microbial data, the effects of incubation temperatures, and the effects of atmosphere conditions on microbial growth and appearance are presented.

MATERIALS & METHODS

Preparation of Bacterial Cultures

A cocktail of four *Salmonella* species (*S. anatum* ATCC 9270, *S. choleraesuis* ATCC10708, S. *enteritidis* ATCC 13076, and *S. typhimurium* ATCC14028) were used for the *Salmonella* inoculation study. The inocula of *E .coli* O157:H7 were a mixture of three strains (ATCC43894, CDC EH7-2, and CDC EH7-5). Each culture was inoculated into Trypticase Soy Broth and incubated for 24 ± 2 hours at 35 ± 2°C. The cell suspensions were prepared by mixing approximately equal number of cells of each species or strains. The concentration of the inoculum was determined by the spread plating method. The suspensions were further diluted in Butterfield's Phosphate Buffer dilution water to achieve the targeted concentration.

Preparation and Packaging of Ground Beef

The ground sirloin was obtained from Harding's Market in the form of chubs. Two random samples were taken from two chubs for the initial microbial profile evaluation. The ground meat was weighed into 4 pound batches and if appropriate, inoculated with culture suspension

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or treated with Herbalox® Seasoning (0.1% by weight of HT-50, code 41-19-35, lot 8348-C) before being blended for 2 minutes in a C-100 model Hobart mixer on the slowest speed. The inoculum concentrations were targeted at 1,000 cfu/g and 5,000 cfu/g for *Salmonella* spp., and 1,000 cfu/g for *E. coli* O157:H7. After Hobart mixing, the ground beef was weighed into half-pound portions and ground through a ⅛" plate (Grinder Model: TS8, OMAS USA, Inc.) before packaging. The half- pound portions were packaged into Cryovac C971 trays using an ILPRA Modified Atmosphere Packaging (MAP) machine (model Food Pack Basic V/G DGT) with barrier film. Ground beef samples were packaged in 82% oxygen/18% carbon dioxide or 69.6% nitrogen/30% carbon dioxide/0.4% carbon monoxide according to the experimental design. The MAP machine was sanitized after packaging was completed. Sponges (sterile) were used to sample all contact surfaces of MAP machine after sanitation to ensure there was no contamination of the equipment by *Salmonella* or *E .coli* O157:H7 after the study. As further confirmation of successful sanitizing, commercial samples of retail ground beef packaged after the experiment tested negative for the presence of the test pathogens, *Salmonella* and *E. coli* O157:H7.

All test ground beef sample trays were stored either at 4°C or 10°C in the dark. On the day of analysis, samples were pulled and carbon dioxide and oxygen levels were measured using a Dansensor Checkmate 9900 (data not shown). Color analyses were performed using a Minolta CR-300 hand held Colorimeter to generate CIELAB L*a*b* values. Microbiological testing including aerobic plate count, anaerobic plate count, psychrotrophs count, and *Salmonella/E. coli* O157:H7 count were performed following the color measurement on two randomly pulled samples from each package. 3M's (St. Paul, Minnesota) Petrifilm plates were used for aerobic, anaerobic and psychrotrophs count measurement. For the quantitative analysis of *Salmonella* and *E. coli* O157:H7, the pre-poured selective agar plates, EF-18 and SD-39 plates were used (Neogen Corporation, Lansing, Michigan). A Nikon cool pix 950 digital camera was used to photograph a randomly selected tray from each treatment at each analysis period. Selected photos are attached in Appendix 1.

Total of 11 treatments of samples were prepared as follows:

1. Ground beef in 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen stored at 4° C. (Control CO 4C in table and graphs)

2. Ground beef in 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen stored at 10° C. (Control CO 10C in table and graphs)

3. Ground beef in 82% oxygen and 18% carbon dioxide with 0.10% HT-50 stored at 4° C. (Control O2 4C in table and graphs)

4. Ground beef in 82% oxygen and 18% carbon dioxide with 0.10% HT-50 stored at 10° C. (Control O2 10C in table and graphs)

5. Ground beef in 82% oxygen and 18% carbon dioxide inoculated w/1,000 cfu/g *Salmonella* w/0.1% HT-50 stored at 4° C. (1000 Salmonella O2 4C in table and graphs)

6. Ground beef in 82% oxygen and 18% carbon dioxide inoculated w/1,000 cfu/g *Salmonella* w/0.1% HT-50 stored at 10° C. (1000 Salmonella O2 10C in table and graphs)

7. Ground beef in 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen inoculated w/1,000 cfu/g *Salmonella* stored at 4° C. (1000 Salmonella CO 4C in table and graphs)

8. Ground beef in 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen inoculated w/1,000 cfu/g *Salmonella* stored at 10° C., (1000 Salmonella CO 10C in table and graphs)

9. Ground beef in 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen inoculated w/5,000 cfu/g *Salmonella* stored at 10° C. (5000 Salmonella CO 10C in table and graphs)

10. Ground beef in 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen inoculated w/1,000 cfu/g *E. coli* O157:H7 stored at 10° C. (1000 E coli CO 10C in table and graphs)

11. Ground beef in 82% oxygen and 18% carbon dioxide inoculated w/1,000 cfu/g *E. coli* O157:H7 w/0.1% HT-50 at 10°C. (1000 E coli O2 10C in table and graphs)

RESULTS AND DISCUSSION

The initial sample screening indicated that the beef chubs were free of *Salmonella* & *E. coli* O157:H7 and had relatively low initial aerobic plate count, anaerobic plate count of 750 cfu/g and psychrotrophs plate count of 1,600 cfu/g. The aerobic, anaerobic, and psychtrophs plate count of all treatments at Day 0 were in the range of 1,000~5,000 cfu/g (see table).

Aerobic, Anaerobic and Psychrotropic plate counts showed very similar growth trends (graphs 2, 3, and 4). The most important variable in the rate of growth was temperature. All three plate counts reached 1.0×10^7 cfu/g or higher by Day 4 for all sample treatments stored at 10° C. Samples stored at 4° C reached similar levels (1.0×10^7 cfu/g or higher) at Day 10 or Day 12. Higher storage temperature (10° C) accelerated the microbial growth and shortened the shelf life of the ground beef. The growth of spoilage organisms appears to be slightly faster in those samples containing the 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen atmosphere, but the effect is not pronounced. The appearance (color) of the samples differed dramatically, however, depending upon the storage atmosphere. All the samples

packaged under the 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen atmosphere remained bright red in color throughout the test, even when the meat was stored at 10°C and plate counts reached over 300 million ($3x10^8$) cfu/g. This was verified instrumentally in the a* color values (see table and graph 1). In contrast, the samples stored under the 82% oxygen / 18% carbon dioxide showed loss of red color, with color fading becoming very evident before plate counts reached 1.0×10^7 cfu/g, the level regarded as indicating spoilage.

The initial *Salmonella* counts (treatment # 5-9, Day 0) were slightly lower than the targeted concentration of 1000 cfu (see table). This type of loss on inoculation has been seen in the past but does not affect the validity of the study, since starting levels were similar between samples to be compared. *Salmonella* growth at 10°C was much faster in the 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen atmosphere than in the one containing 82% oxygen and 18% carbon dioxide and reached higher levels during the study (graph 5). The samples inoculated with 5000 cfu/g experienced even higher growth. The sample inoculated with 5000 cfu of *Salmonella* reached a concentration of 80,000 cfu/g on day 10 when stored at 10 °C. Even though the *Salmonella* levels were this high and the aerobic, anaerobic and psychrotrophic counts were extraordinarily high, this sample had the appearance of fresh red meat.

Salmonella growth at 4 °C was much slower and in fact, levels peaked at fairly low levels at day 6 in the 82% oxygen and 18% carbon dioxide atmosphere and day 8 in the 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen atmosphere and then fell to lower levels (see table and graph 6).

The initial *E. coli* O157:H7 counts were higher than the target concentration (treatment # 10, 11, Day 0). *E. coli* O157:H7 growth at 10°C was faster in the 0.4% carbon monoxide, 30% carbon dioxide and 69.6% nitrogen atmosphere than in the one containing 82% oxygen and 18% carbon dioxide and reached higher levels during the study (graph 7). Just as in the case of *Salmonella*, the *E. coli* O157:H7 sample stored in the carbon monoxide containing atmosphere had the appearance of fresh red meat throughout the study.

Selected photos are attached electronically as Appendix 1. These are:

Ground Sirloin Samples	Atmosphere/ Temperature	Day
Control (3.)	High Oxygen / 4° C	0
"	"	4
"	"	6
"	"	8
"	"	10
"	"	12
1000 cfu/g *Salmonella* (5.)	High Oxygen / 4° C	0
"	"	4
"	"	6
"	"	8
"	"	10
"	"	12
1000 cfu/g *Salmonella* (7.)	Carbon Monoxide (4° C)	0
"	"	4
"	"	6
"	"	8
"	"	10
"	"	12
Control (1.)	Carbon Monoxide / 4° C	0
"	"	4
"	"	6
"	"	8
"	"	10

"	"	12
Control (4.)	High Oxygen / 10° C	0
"	"	2
"	"	4
"	"	6
"	"	8
1000 cfu/g *Salmonella* (6.)	High Oxygen / 10° C	0
"	"	2
"	"	4
"	"	6
"	"	8
1000 cfu/g *E. coli* (11.)	High Oxygen / 10° C	0
"	"	2
"	"	4
"	"	6
"	"	8
1000 cfu/g *Salmonella* (8.)	Carbon Monoxide / 10° C	0
"	"	2
"	"	4
"	"	6
"	"	8
1000 cfu/g *E. coli* (10.)	Carbon Monoxide / 10° C	0
"	"	2
"	"	4
"	"	6
"	"	8

ILPRA MAP machine was sanitized and the results of the sponge confirmation test and retail beef re-packing indicated the machine was free of any testing pathogens.

References:

Official Methods of Analysis of AOAC International, 17th edition, 2000.
Bacteriological Analytical Manual By AOAC, 8th edition, 1998 Revision A.

Table: Color and Microbial Growth in Study Samples

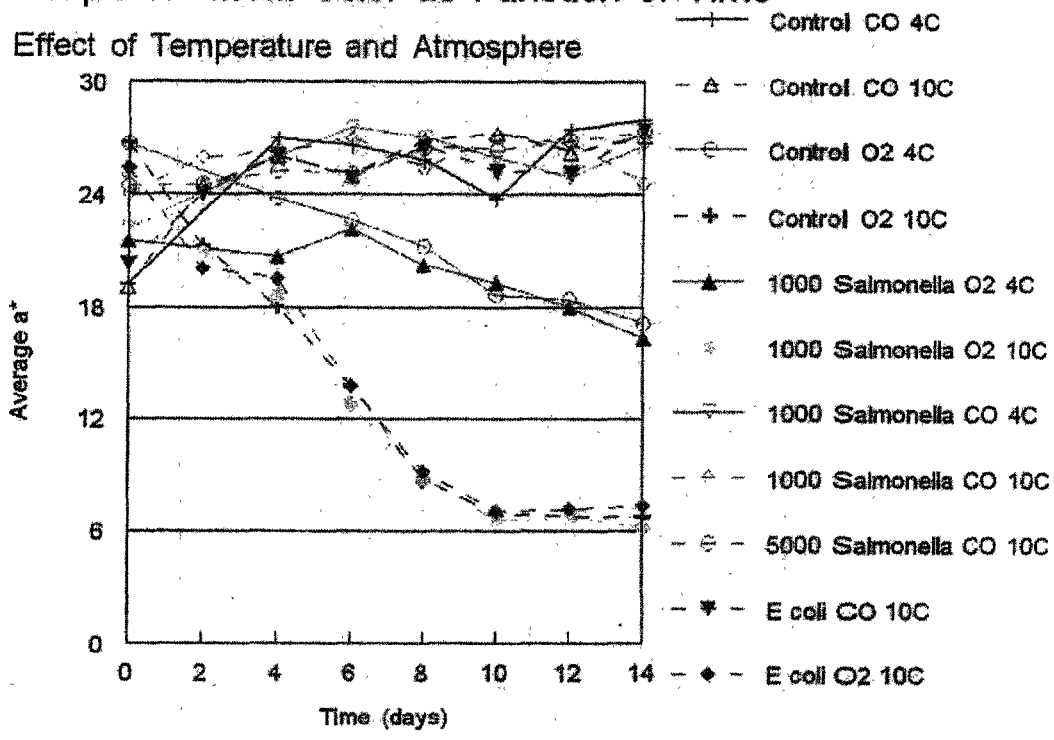
Sample	Day	a*	Aerobic Count (cfu/g)	Anaerobic Count (cfu/g)	Psychrotroph Count (cfu/g)	Pathogen Count (cfu/g)
Control CO 4C	0	19.21	1100	1300	3500	-
	4	26.96	43000	48000	73500	-
	6	26.58	125000	305000	415000	-
	8	25.81	1500000	3200000	3900000	-
	10	23.8	8000000	24000000	23500000	-
	12	27.46	14000000	41000000	70000000	-
	14	27.96	5500000	33500000	58500000	-
Control CO 10C	0	19.09	2600	1100	1300	-
	2	24.48	1015000	1250000	1450000	-
	4	26	102000000	96000000	140000000	-
	6	25.03	200000000	255000000	330000000	-
	8	26.9	170000000	300000000	230000000	-
	10	27.25	-	-	-	-
	12	26.23	-	-	-	-
	14	27.11	-	-	-	-
Control O2 4C	0	26.66	1200	1350	3250	-
	4	23.79	17500	25500	34500	-
	6	22.64	70000	115000	140000	-
	8	21.25	665000	1100000	730000	-
	10	18.63	5500000	10400000	8100000	-
	12	18.41	10500000	25000000	22500000	-
	14	17.12	12000000	30500000	22000000	-
Control O2 10C	0	26.72	1800	1150	2000	-
	2	21.27	385000	450000	600000	-
	4	18.03	82000000	78500000	105000000	-
	6	13.79	60000000	110000000	135000000	-
	8	8.71	90000000	125000000	170000000	-
	10	6.87	-	-		

	12	6.74	-	-		
	14	6.77	-	-		
1000 *Salmonella* O2 4C	0	21.56	3100	3050	2700	380
	4	20.72	23000	30000	35000	260
	6	22.18	55000	115000	170000	1400
	8	20.24	1400000	2350000	1550000	310
	10	19.32	4000000	9000000	6500000	270
	12	17.98	12500000	26000000	20000000	200
	14	16.32	9000000	29500000	13500000	200
1000 *Salmonella* O2 10C	0	25.07	1150	1300	3300	380
	2	20.91	350000	395000	425000	670
	4	18.66	83500000	83000000	120000000	4800
	6	12.72	65000000	165000000	165000000	11000
	8	8.79	160000000	170000000	105000000	12000
	10	6.58	-	-	-	11000
	12	6.73	-	-	-	-
	14	6.15	-	-	-	-
1000 *Salmonella* CO 4C	0	22.17	1200	2250	2700	530
	4	26.04	33500	46000	62000	470
	6	27.58	230000	405000	510000	420
	8	27	2150000	4200000	4650000	1300
	10	25.96	13500000	26500000	27000000	330
	12	24.91	21000000	44500000	56500000	200
	14	26.63	11500000	49000000	46000000	270
1000 *Salmonella* CO 10C	0	24.02	1250	1250	2550	500
	2	25.94	980000	1600000	1500000	2700
	4	26.3	94500000	93000000	140000000	23000
	6	26.97	205000000	210000000	320000000	23000
	8	25.38	165000000	155000000	170000000	28000
	10	27.15	-	-	-	8500
	12	26.67	-	-	-	-

	14	24.61	-	-	-	-
5000 *Salmonella* CO 10C	0	24.44	2600	2200	6250	2300
	2	24.57	455000	555000	780000	5700
	4	25.25	85000000	8800000	125000000	39000
	6	25.09	120000000	220000000	360000000	39000
	8	26.46	240000000	230000000	200000000	41000
	10	26.38	-	-	-	80000
	12	26.88	-	-	-	-
	14	27.34	-	-	-	-
1000 E coli CO 10C	0	20.31	4250	3850	5550	2900
	2	24.04	600000	595000	950000	9750
	4	26.08	64000000	78000000	125000000	14000
	6	24.94	195000000	250000000	380000000	28000
	8	26.52	215000000	225000000	205000000	6000
	10	25.2	-	-	-	6300
	12	25.13	-	-	-	-
	14	27.44	-	-	-	-
1000 E coli O2 10C	0	25.83	4200	2450	3150	2700
	2	20.06	295000	305000	375000	6100
	4	19.51	59000000	68000000	71500000	9000
	6	13.76	60000000	85000000	60000000	7000
	8	9.16	25000000	105000000	65000000	15000
	10	7.07	-	-	-	8500
	12	7.18	-	-	-	-
	14	7.35	-	-	-	-

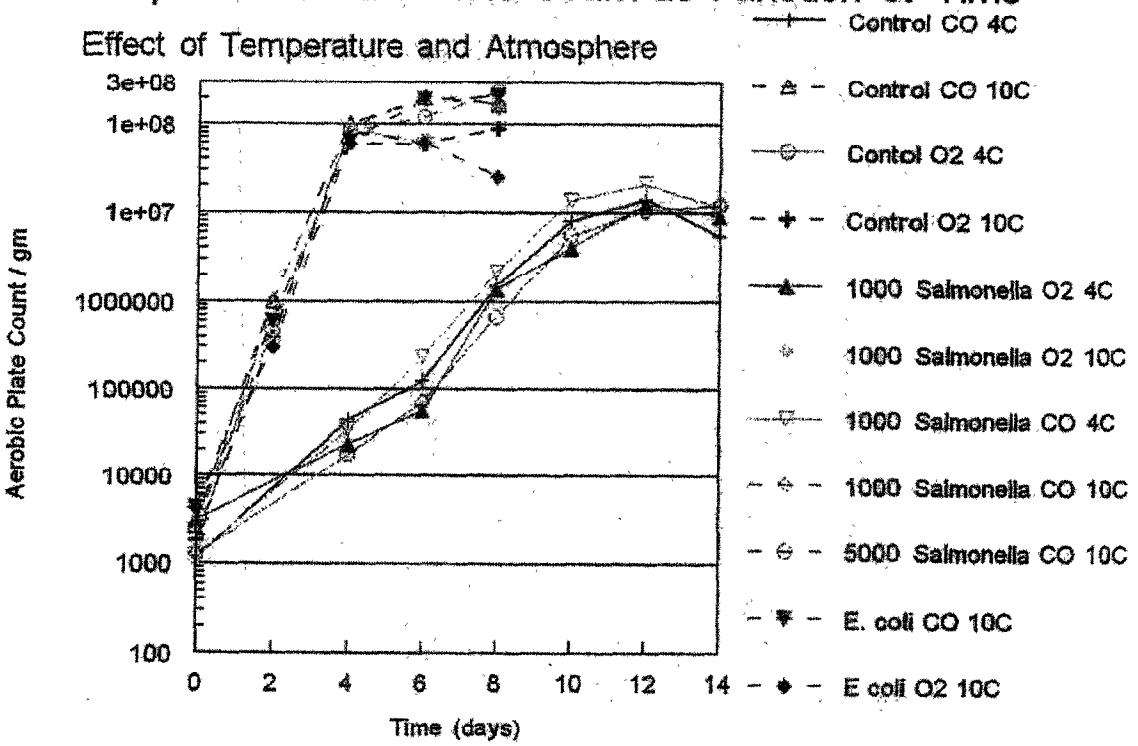
Graph 1. Meat Color as Function of Time

Effect of Temperature and Atmosphere



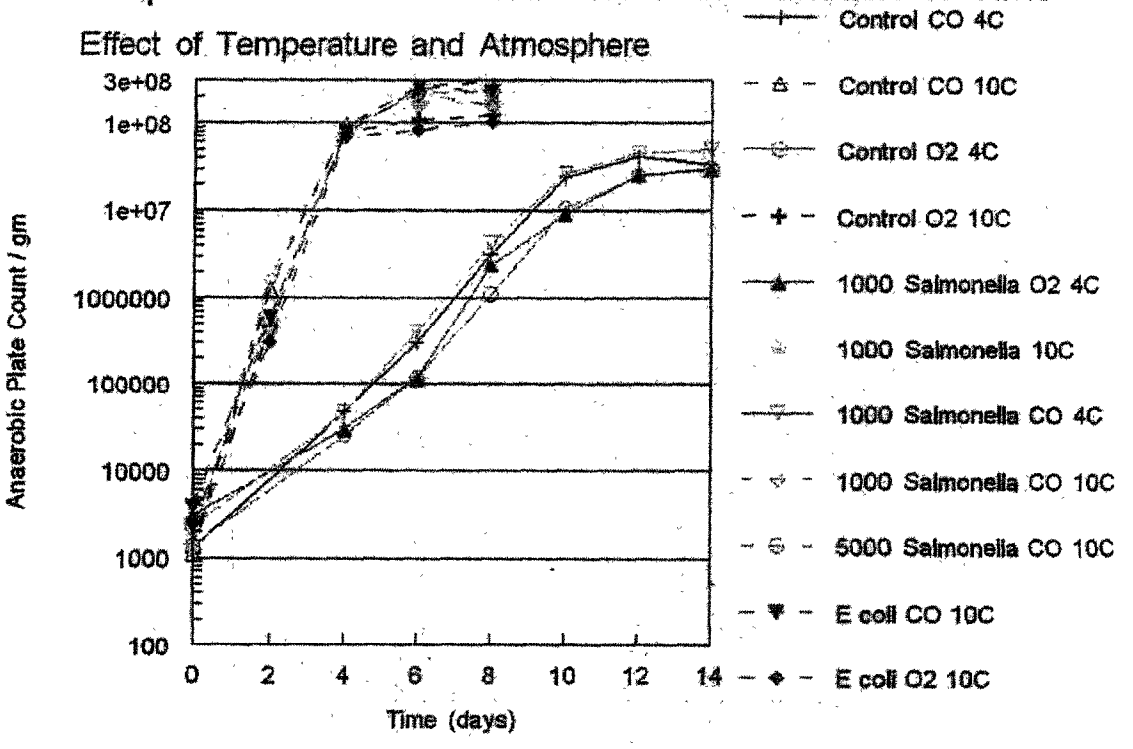
Graph 2. Aerobic Plate Count as Function of Time

Effect of Temperature and Atmosphere



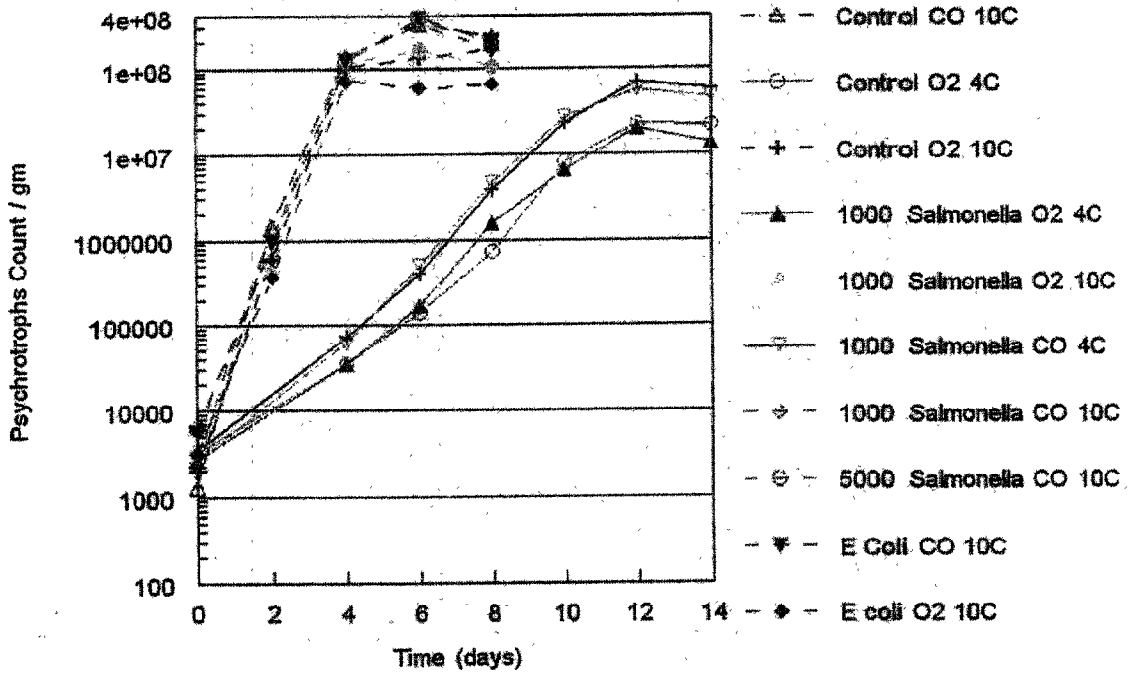
Graph 3. Anaerobic Plate Count as Function of Time

Effect of Temperature and Atmosphere



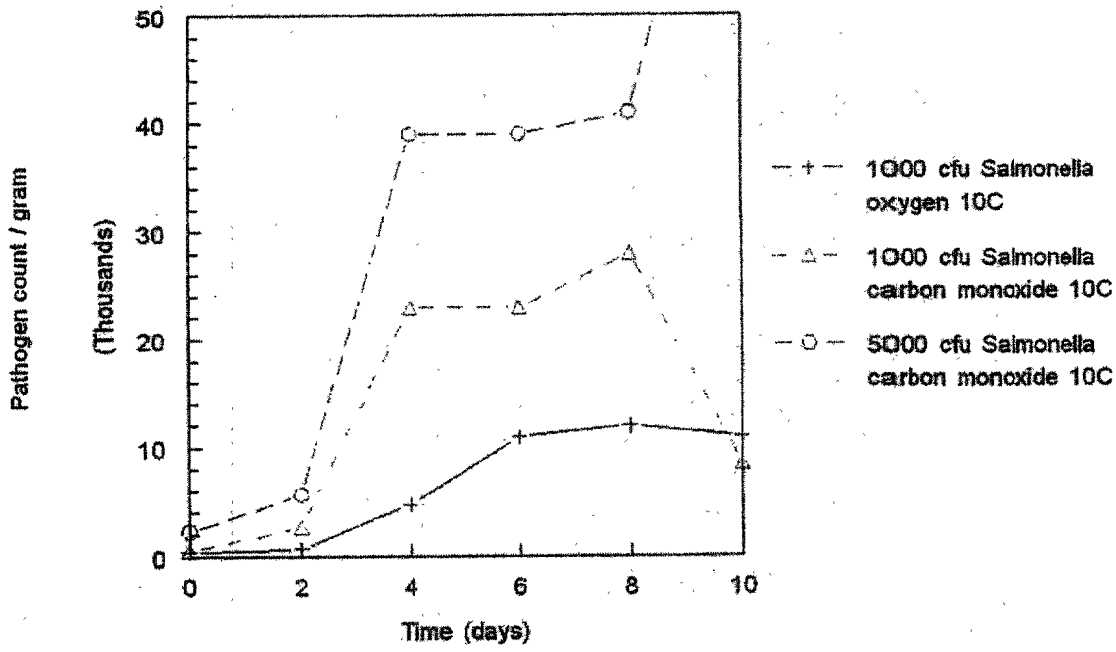
Graph 4. Psychrotrophic Plate Count as Function of Time

Effect of Temperature and Atmosphere



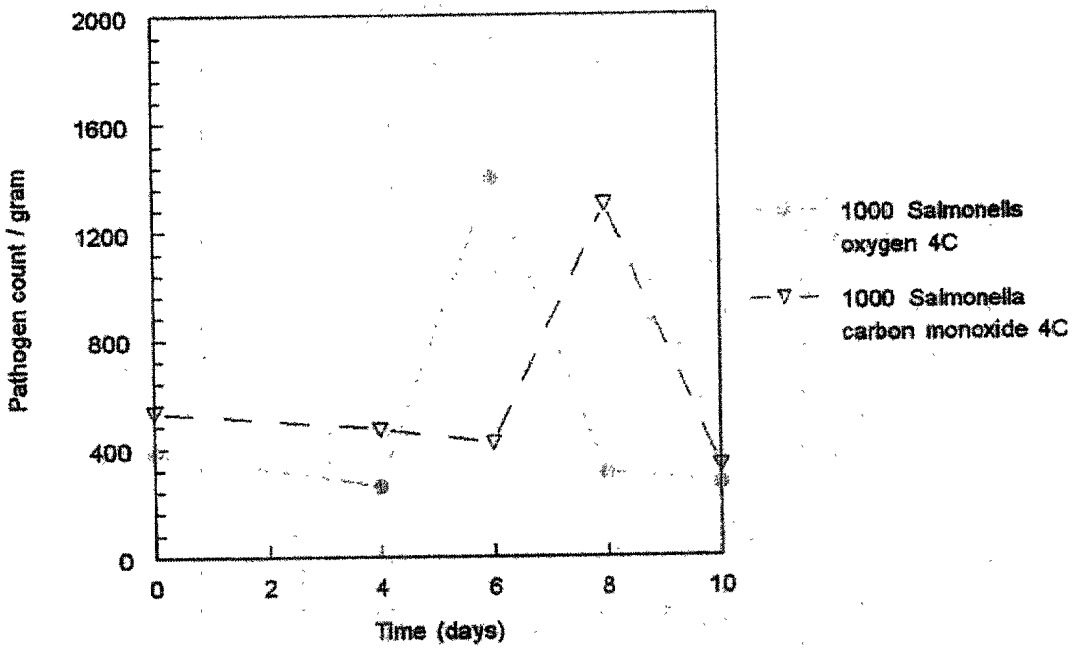
Graph 5. Salmonella Levels

Growth at 10 degrees C



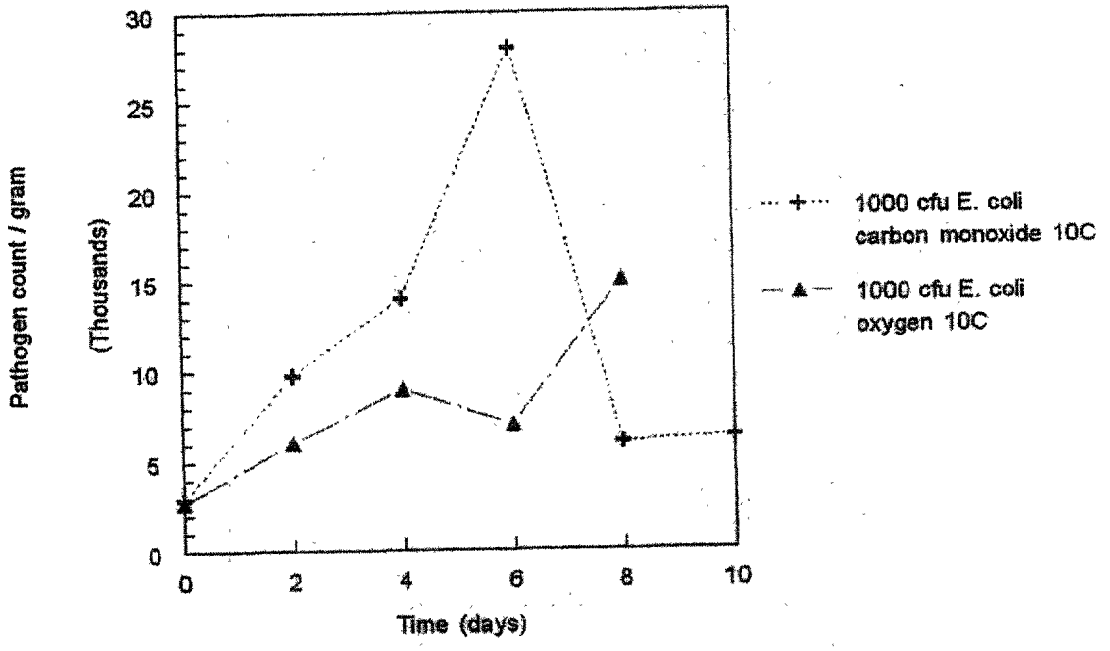
Graph 6. Salmonella Levels

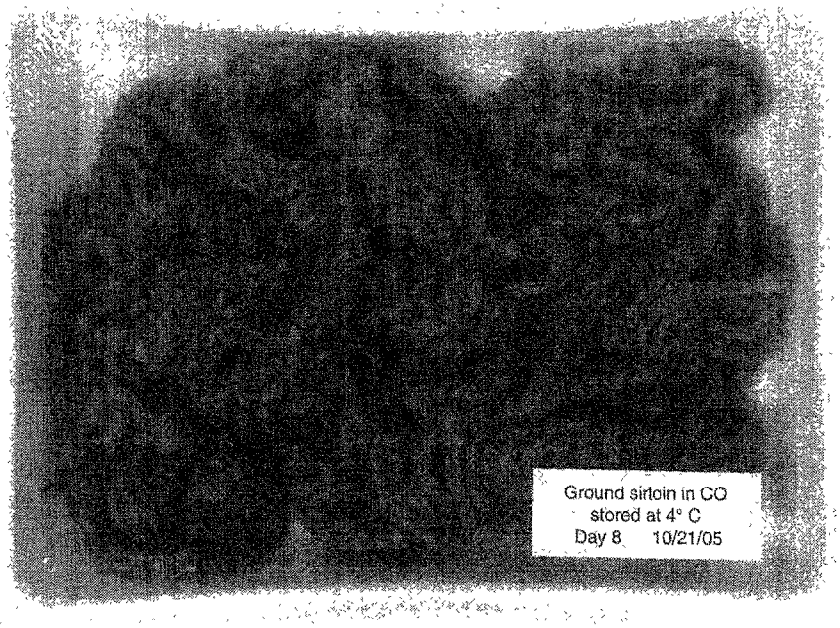
Growth at 4 degrees C



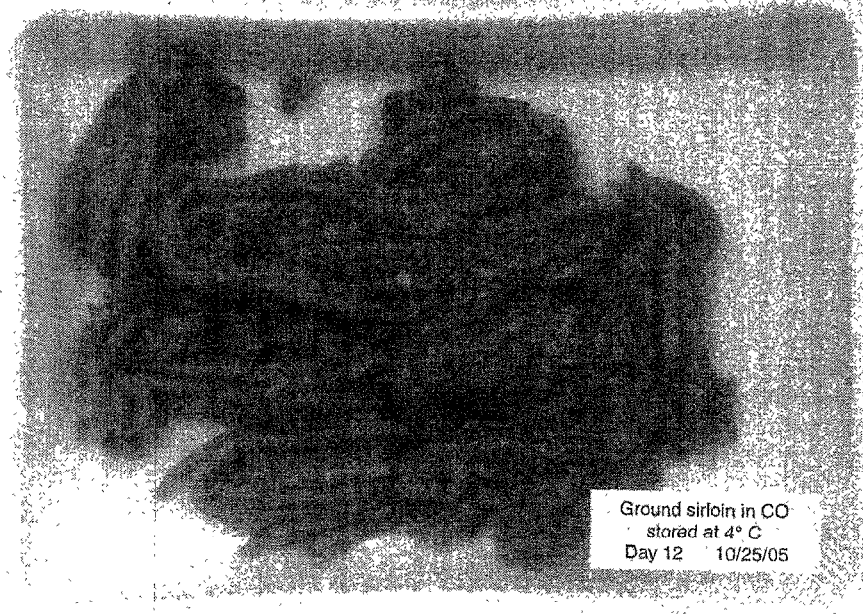
Graph 7. E. Coli O157:H7 Levels

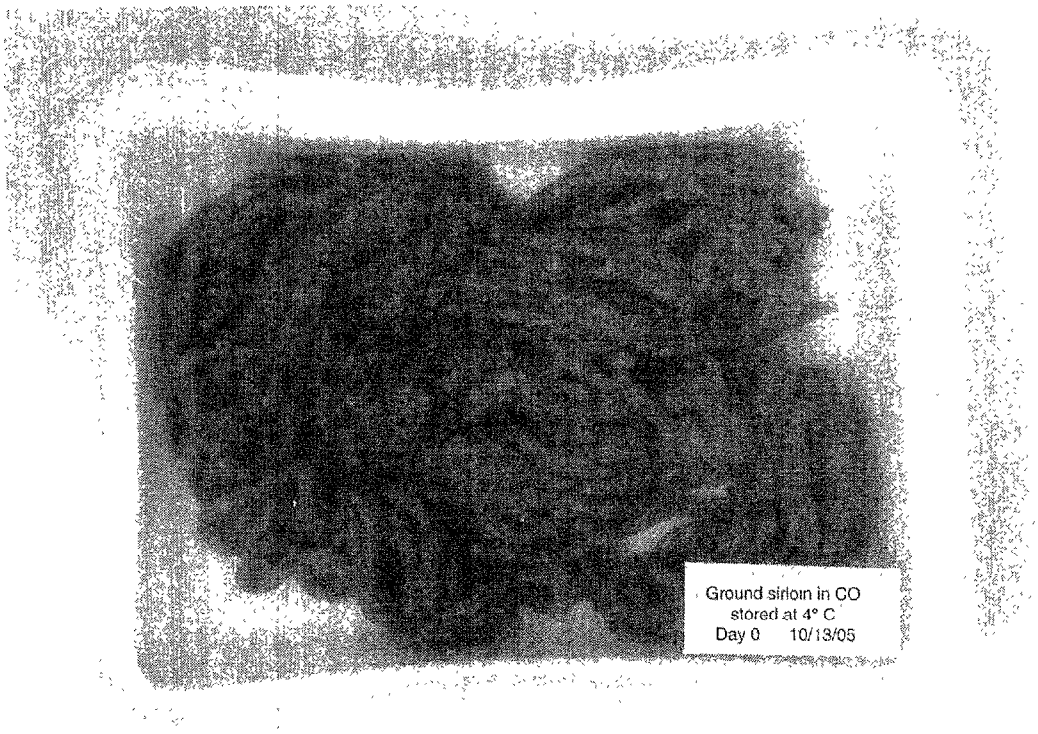
Growth at 10 degrees C

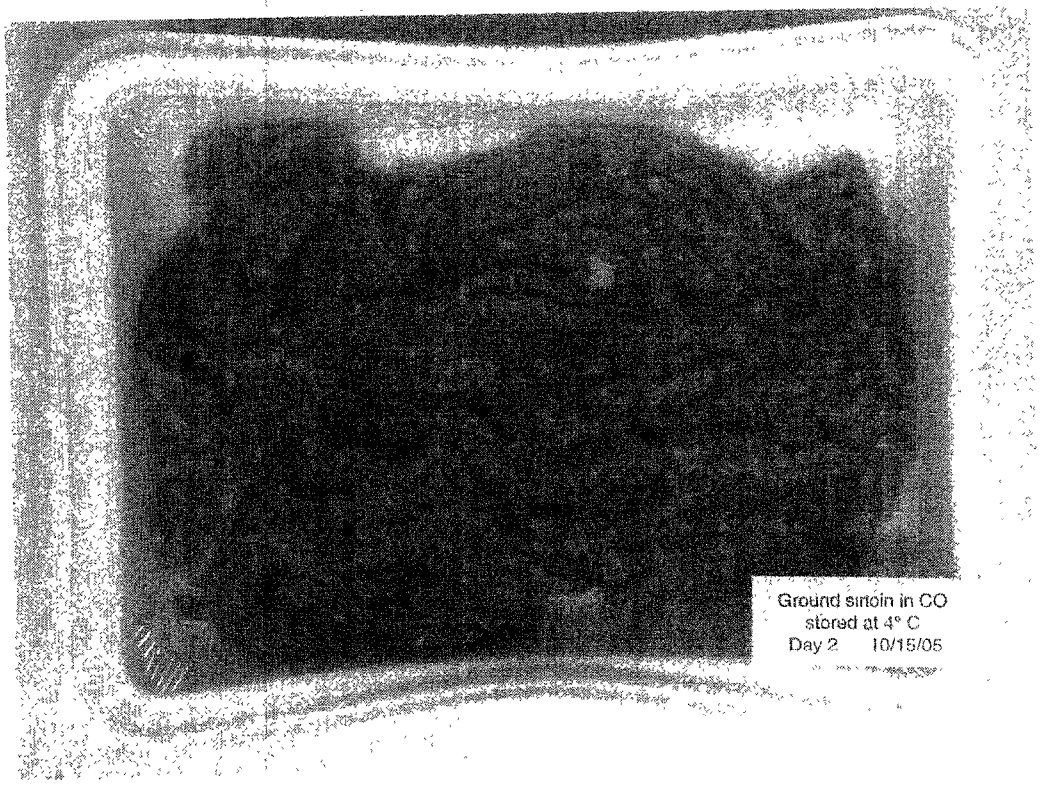



Ground sirloin in CO
stored at 4° C
Day 8 10/21/05

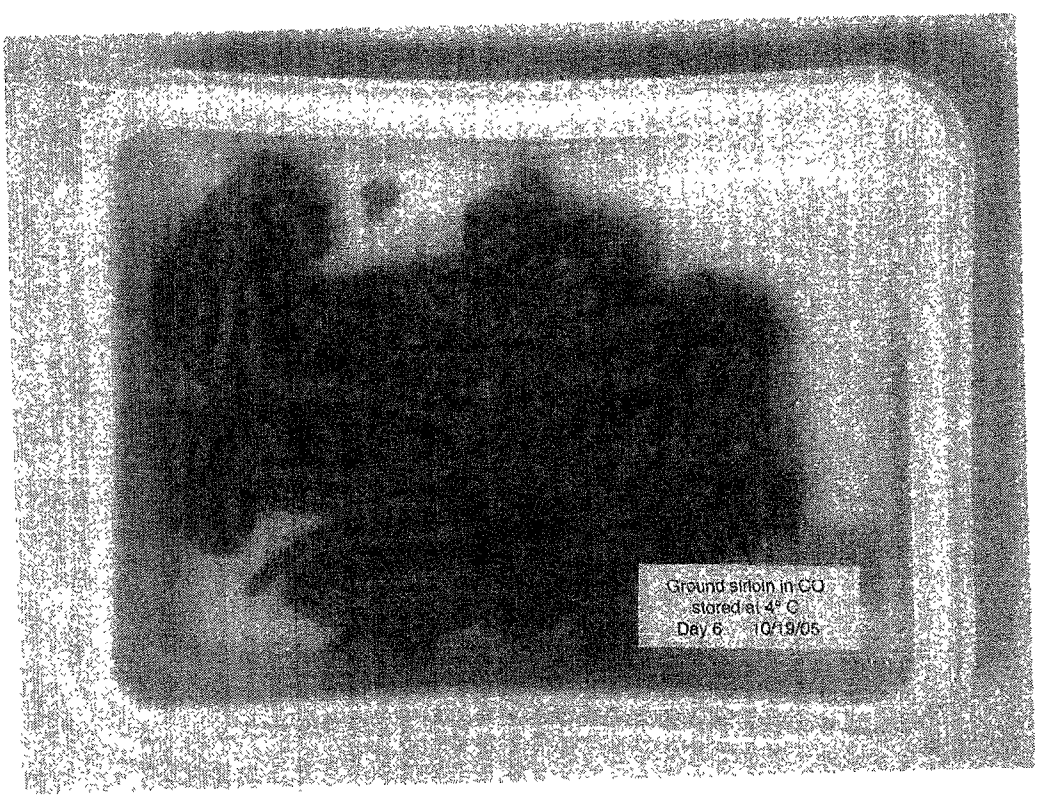
Ground sirloin in CO
stored at 4° C
Day 10 10/23/05


Ground sirloin in CO
stored at 4° C
Day 12 10/25/05

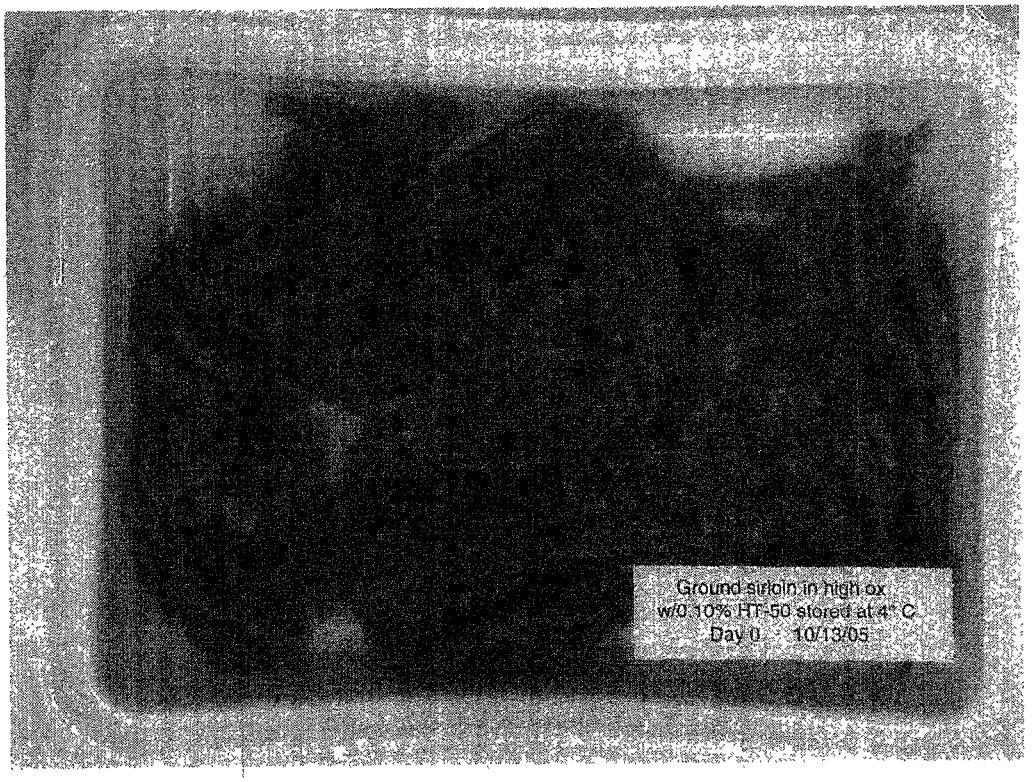

Ground sirloin in CO
stored at 4° C
Day 0 10/13/05

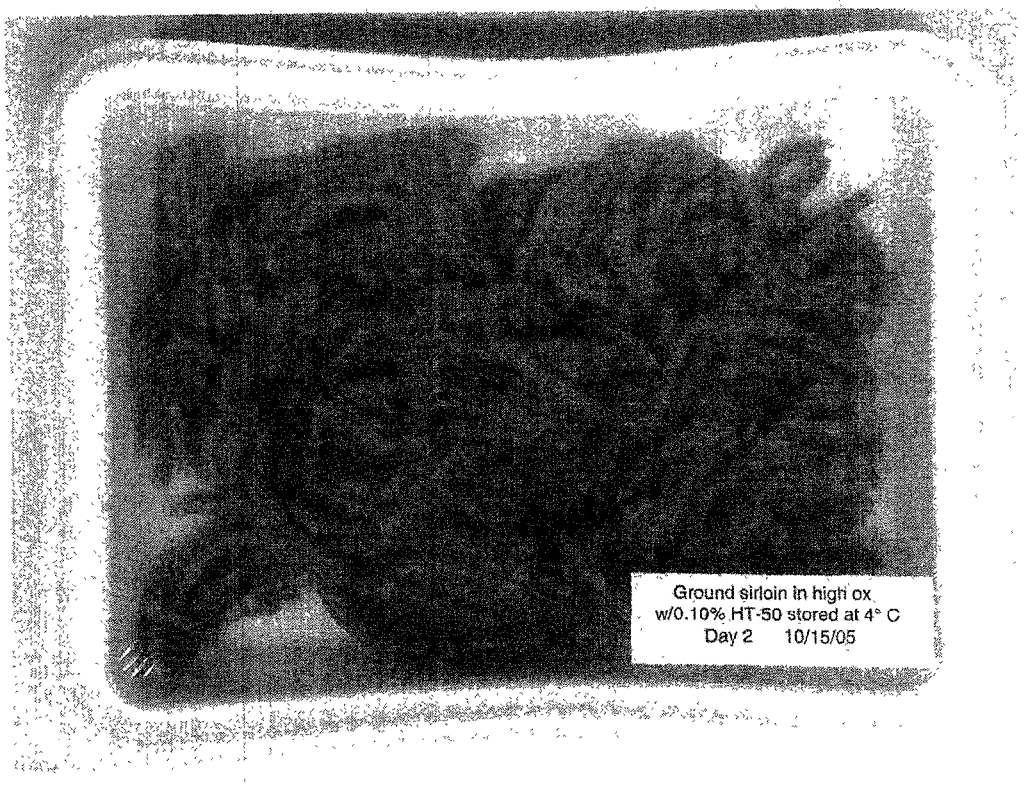

Ground sirloin in CO
stored at 4° C
Day 2 10/15/05

Ground sirloin in CO
stored at 4° C
Day 4 10/17/05

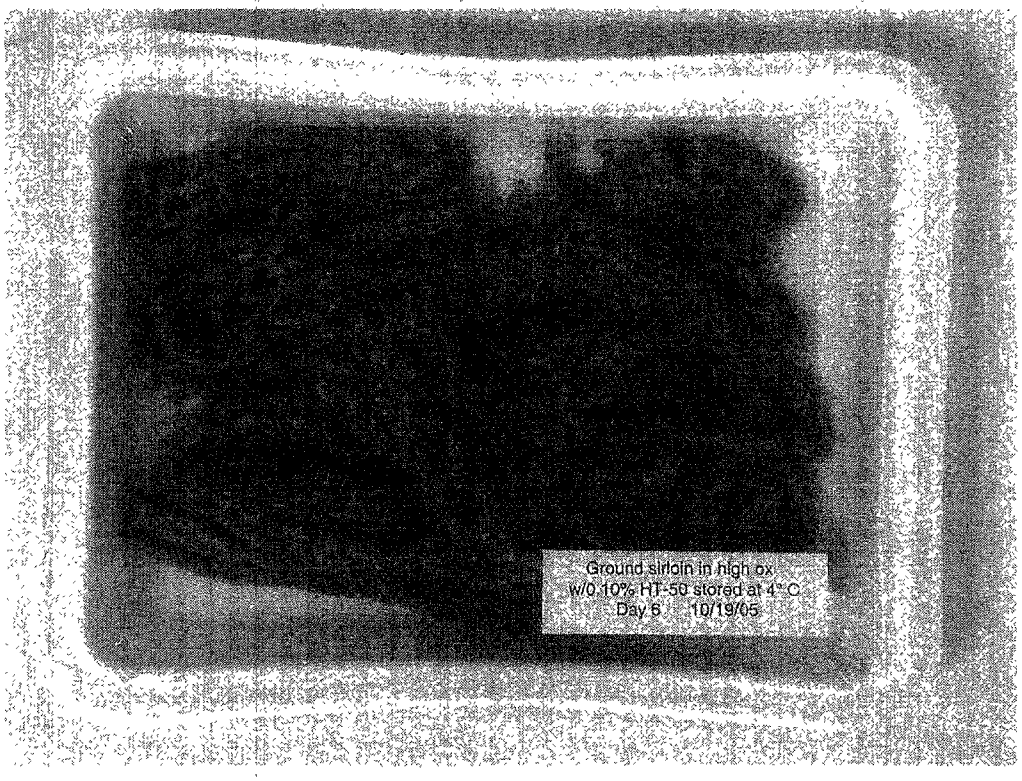

Ground sirloin in CO
stored at 4° C
Day 6 10/19/05

HI OX CONTROL 4°C


Ground sirloin in high ox
w/0.10% HT-50 stored at 4° C
Day 0 10/13/05


Ground sirloin in high ox
w/0.10% HT-50 stored at 4° C
Day 2 10/15/05

Ground sirloin in high ox
w/0.10% HT-50 stored at 4° C
Day 4 10/17/05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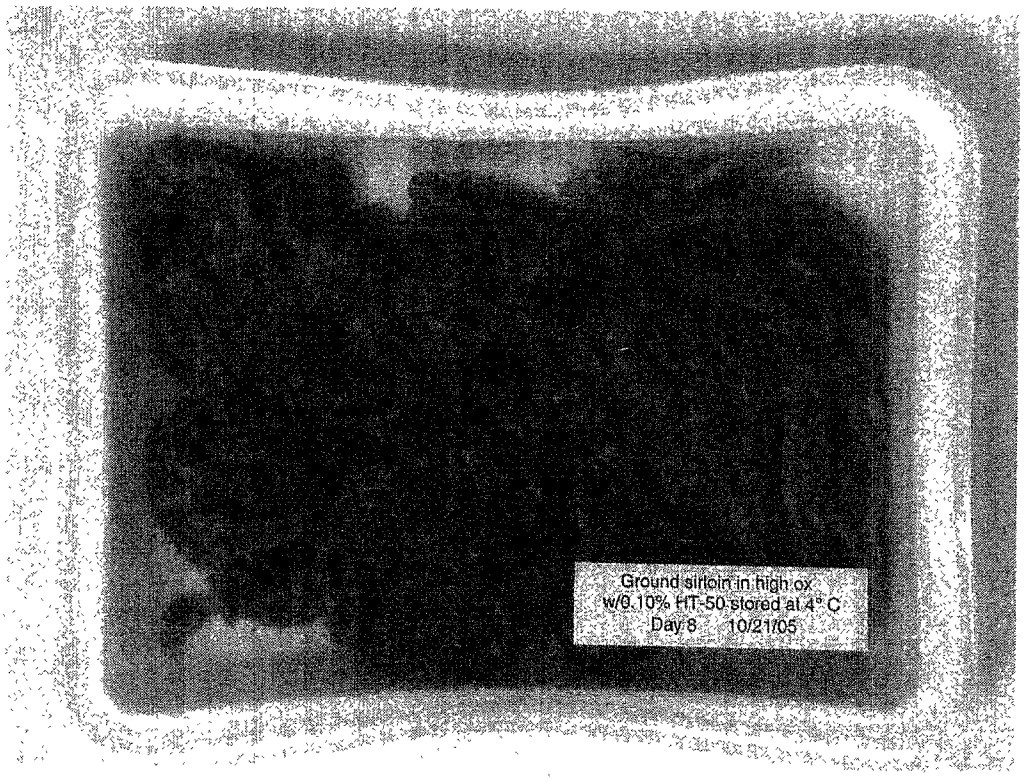
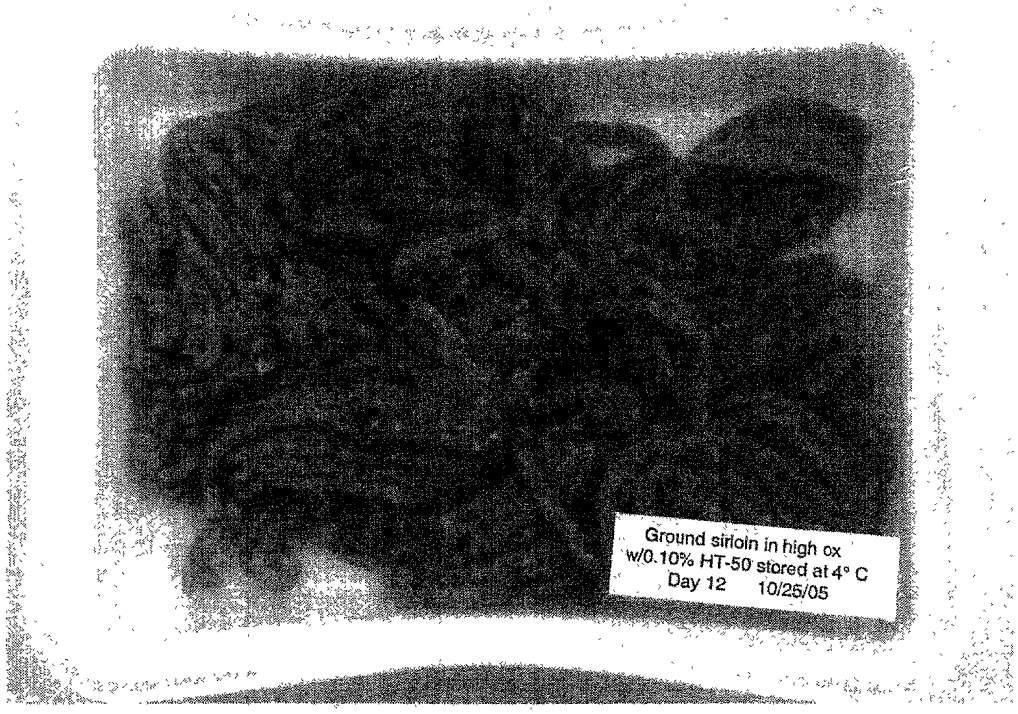
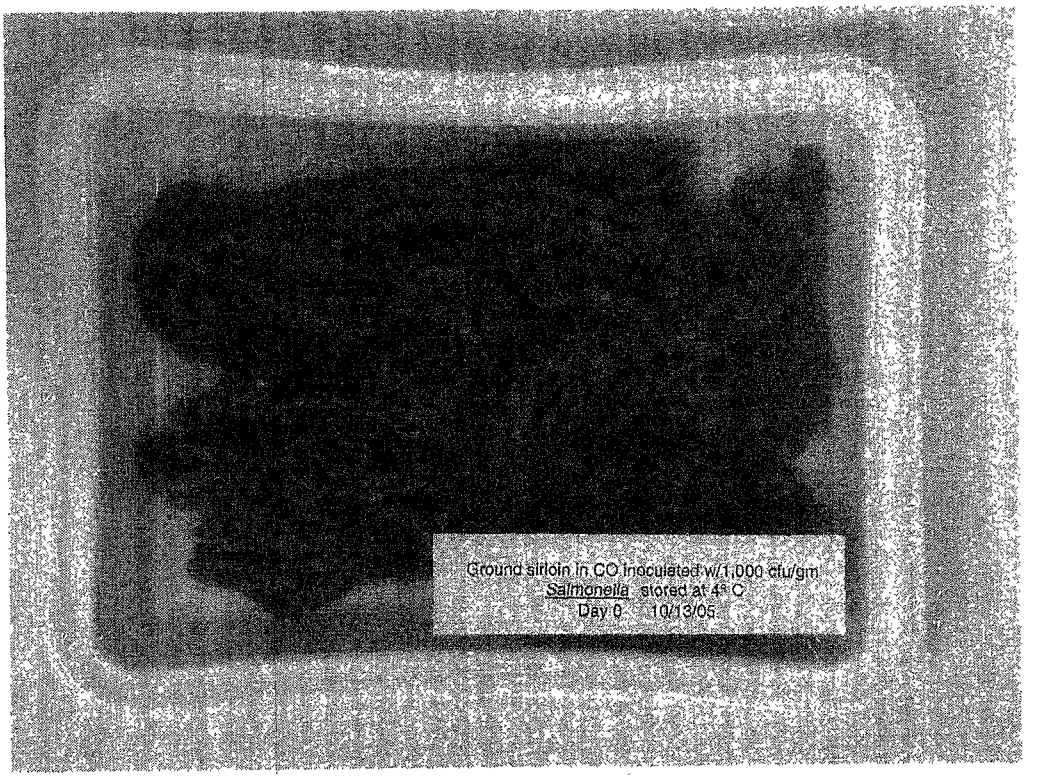
w/0.10% HT-50 stored at 4° C
Day 6 10/19/05

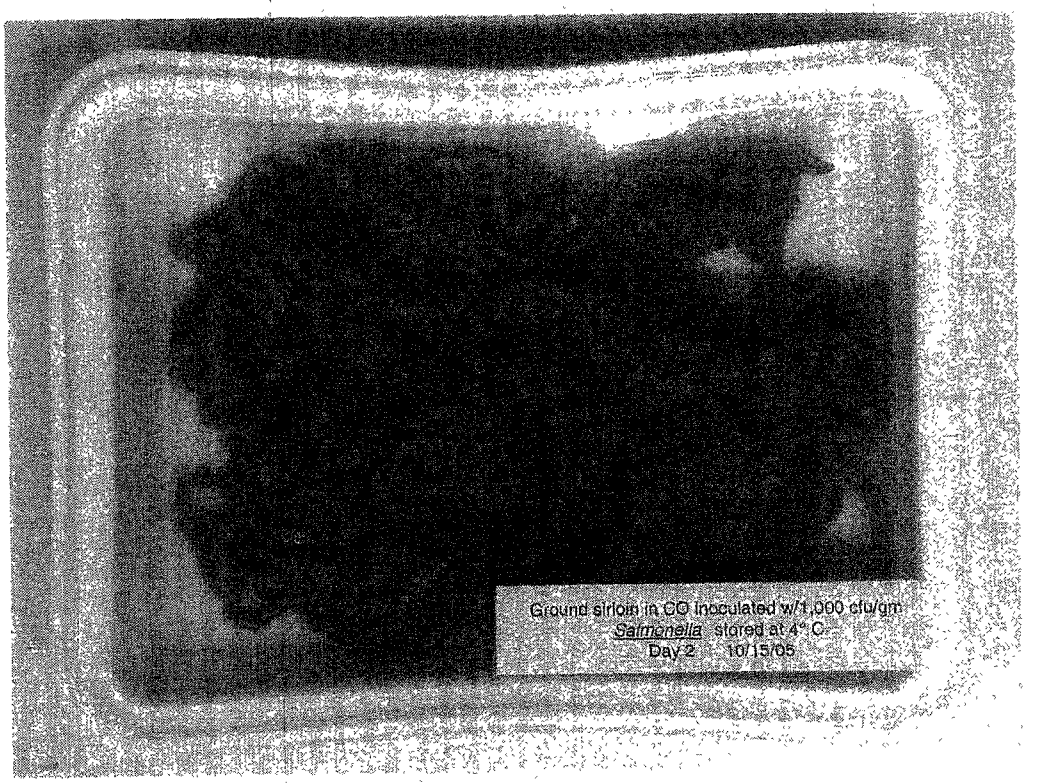

Ground sirloin in high ox
w/0.10% HT-50 stored at 4° C
Day 8 10/21/05

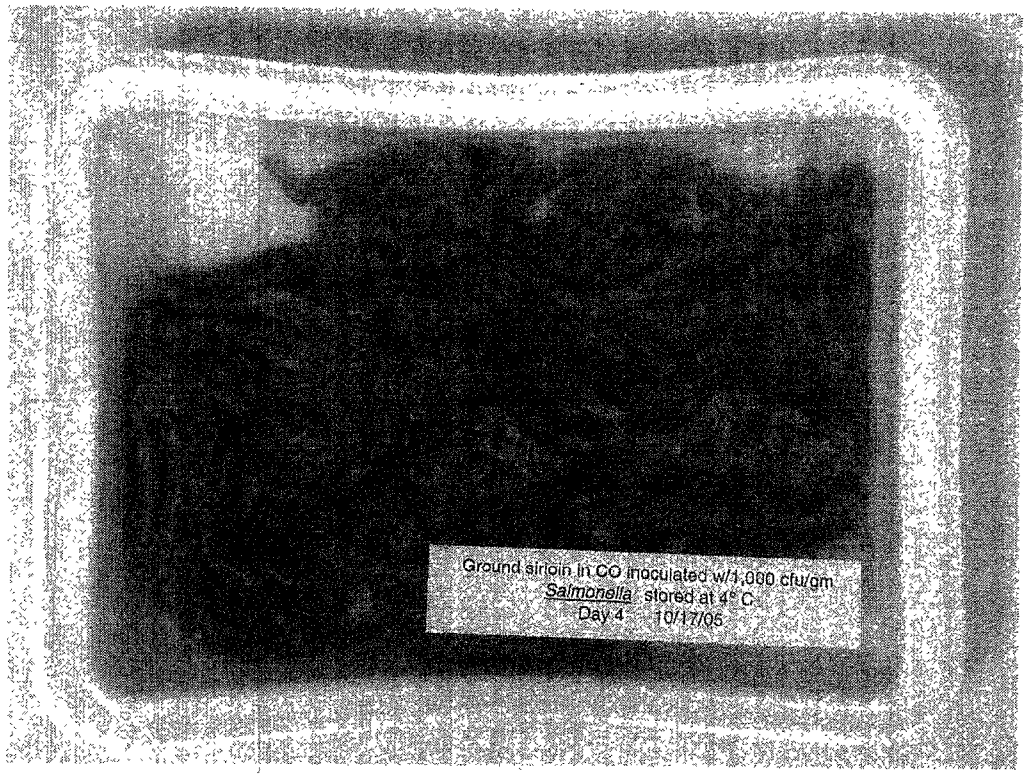
Ground sirloin in high ox
w/0.10% HT-50 stored at 4° C
Day 10 10/23/05

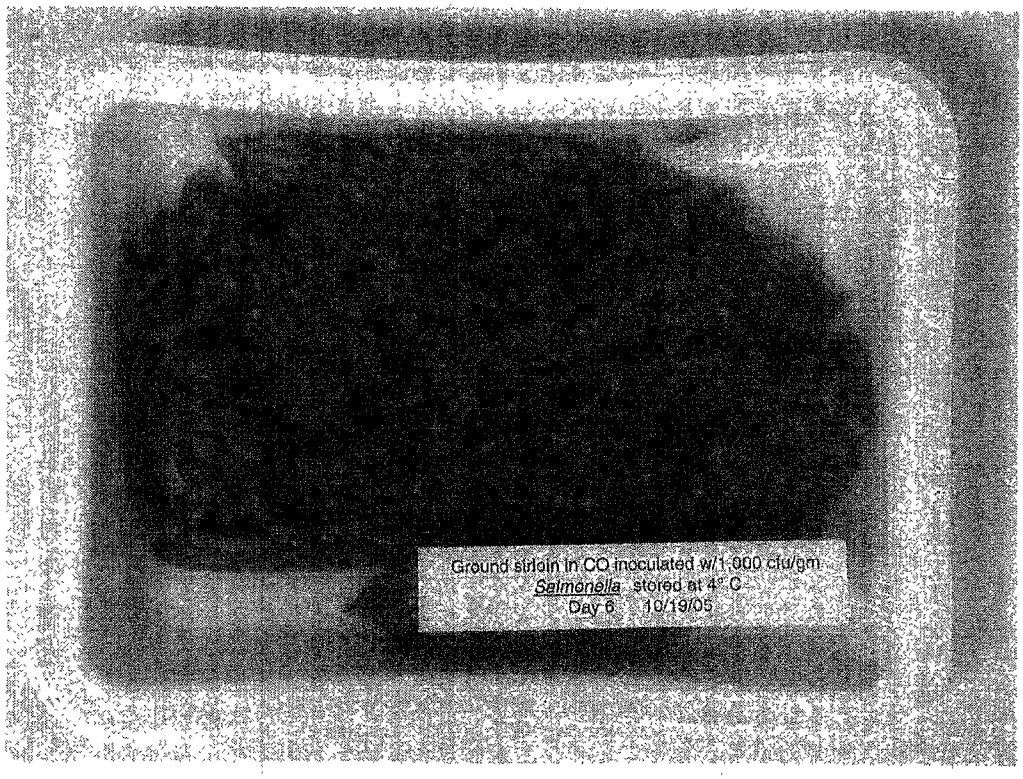

Ground sirloin in high ox
w/0.10% HT-50 stored at 4° C
Day 12 10/25/05

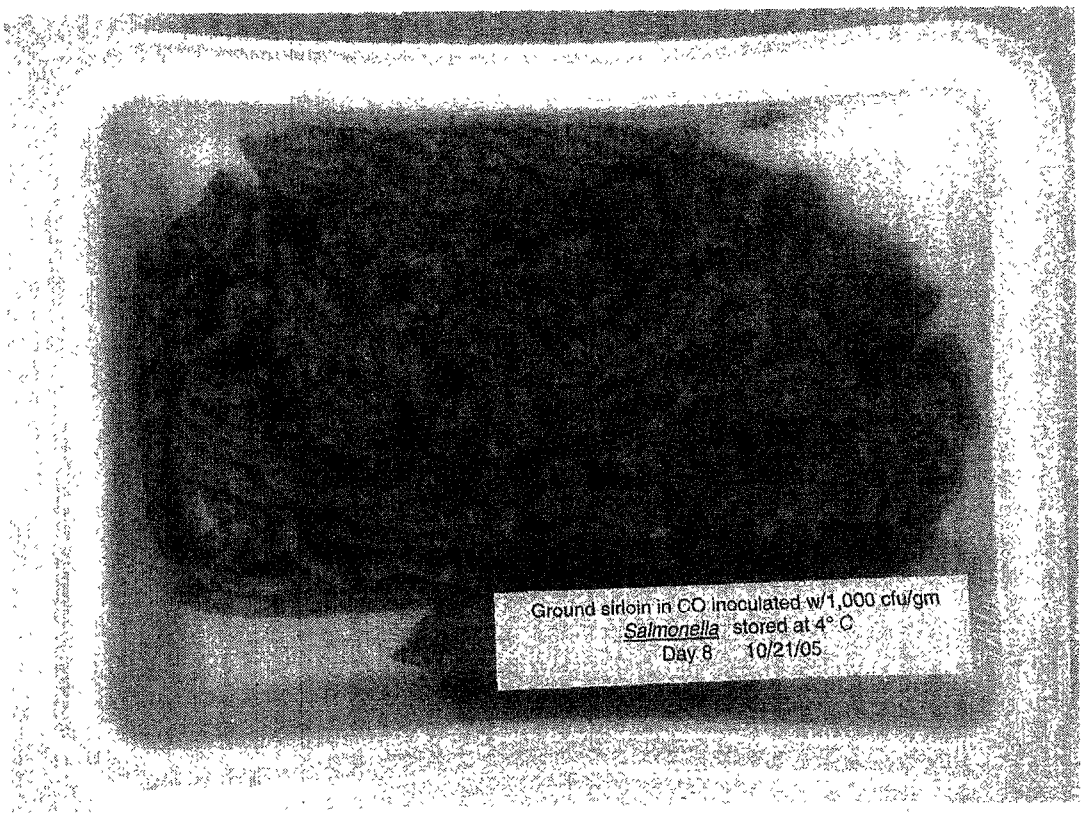
CO SALMONELLA 4°C


Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 0 10/13/05

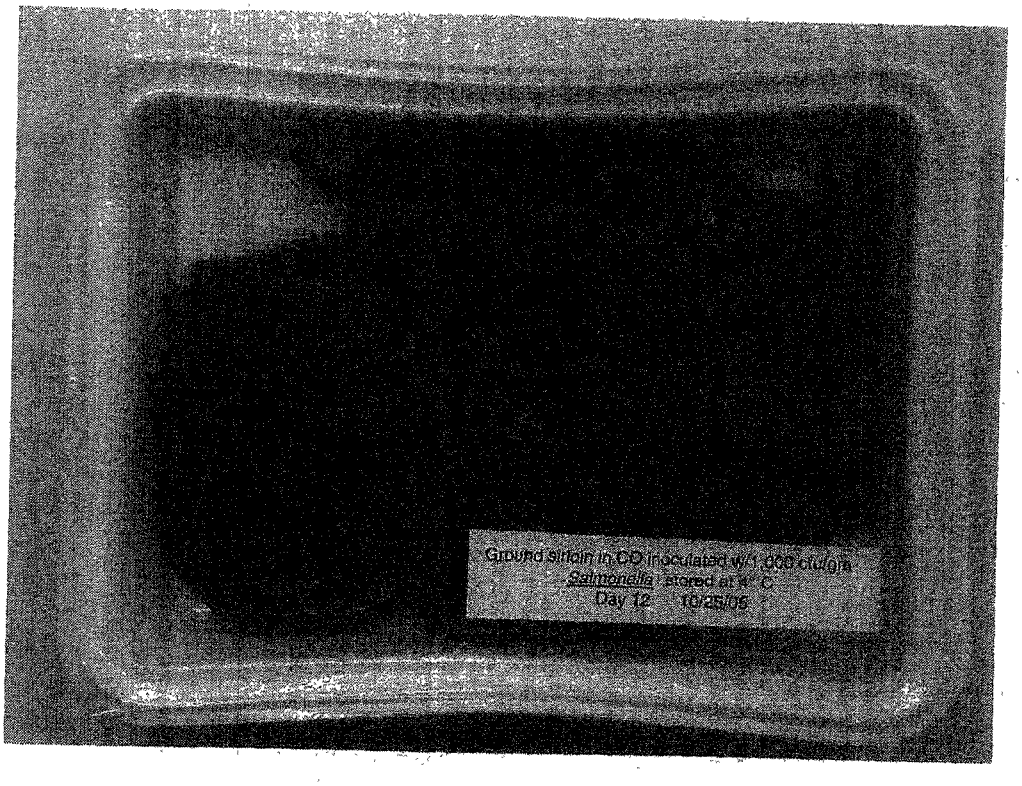

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 2 10/15/05


Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 4 10/17/05

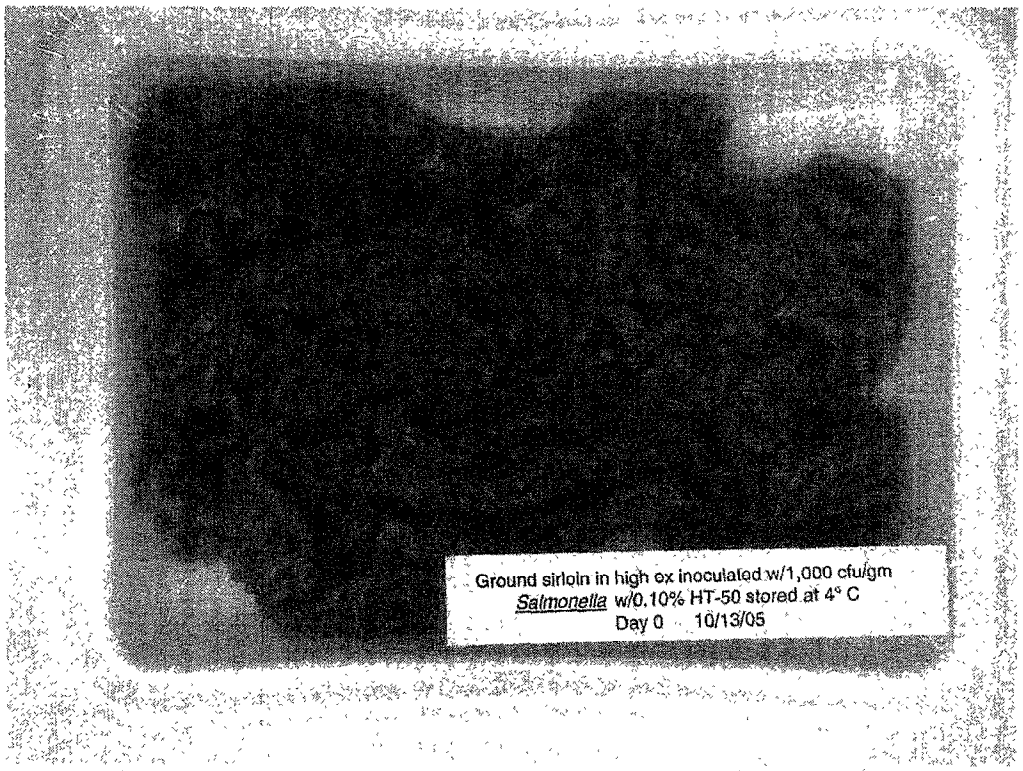

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 6 10/19/05


Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 8 10/21/05

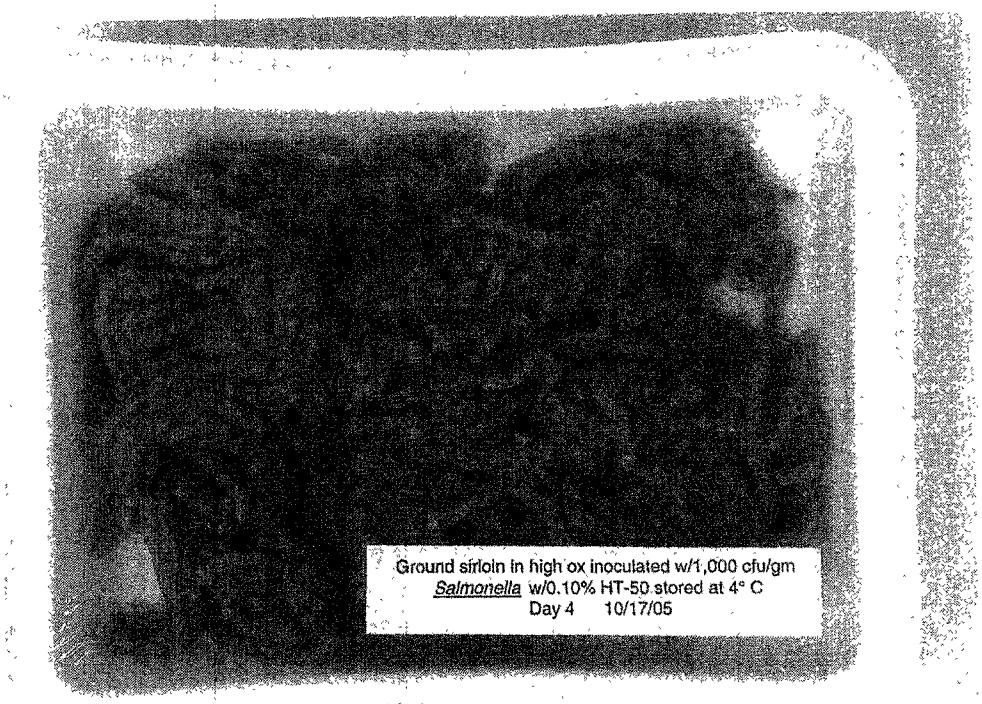
Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 10 10/25/05


Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 4° C
Day 12 10/25/05

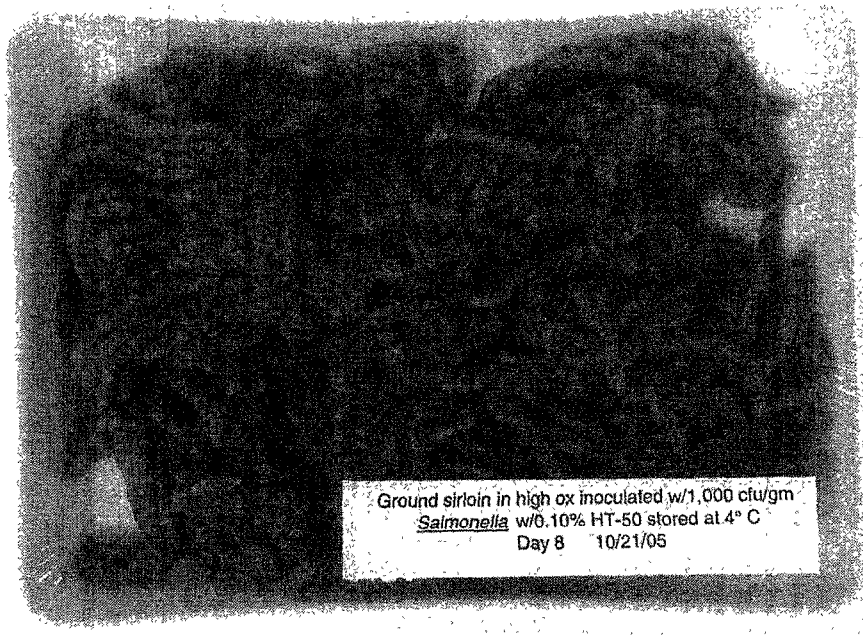
HI OX SALMONELLA 4°C


Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 0 10/13/05

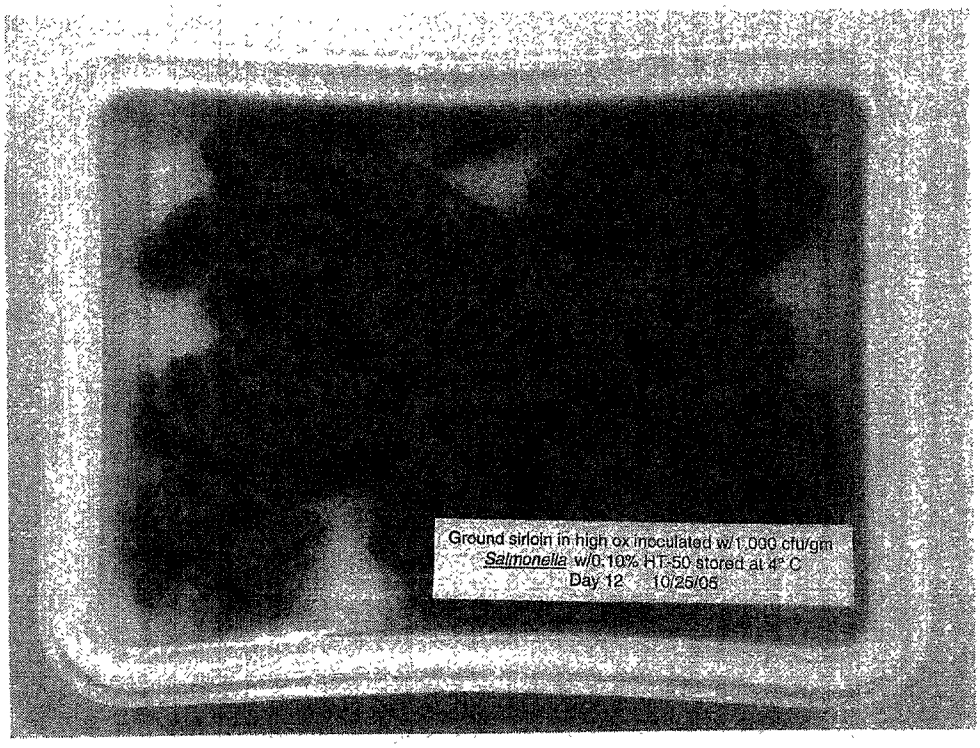
Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 2 10/15/05

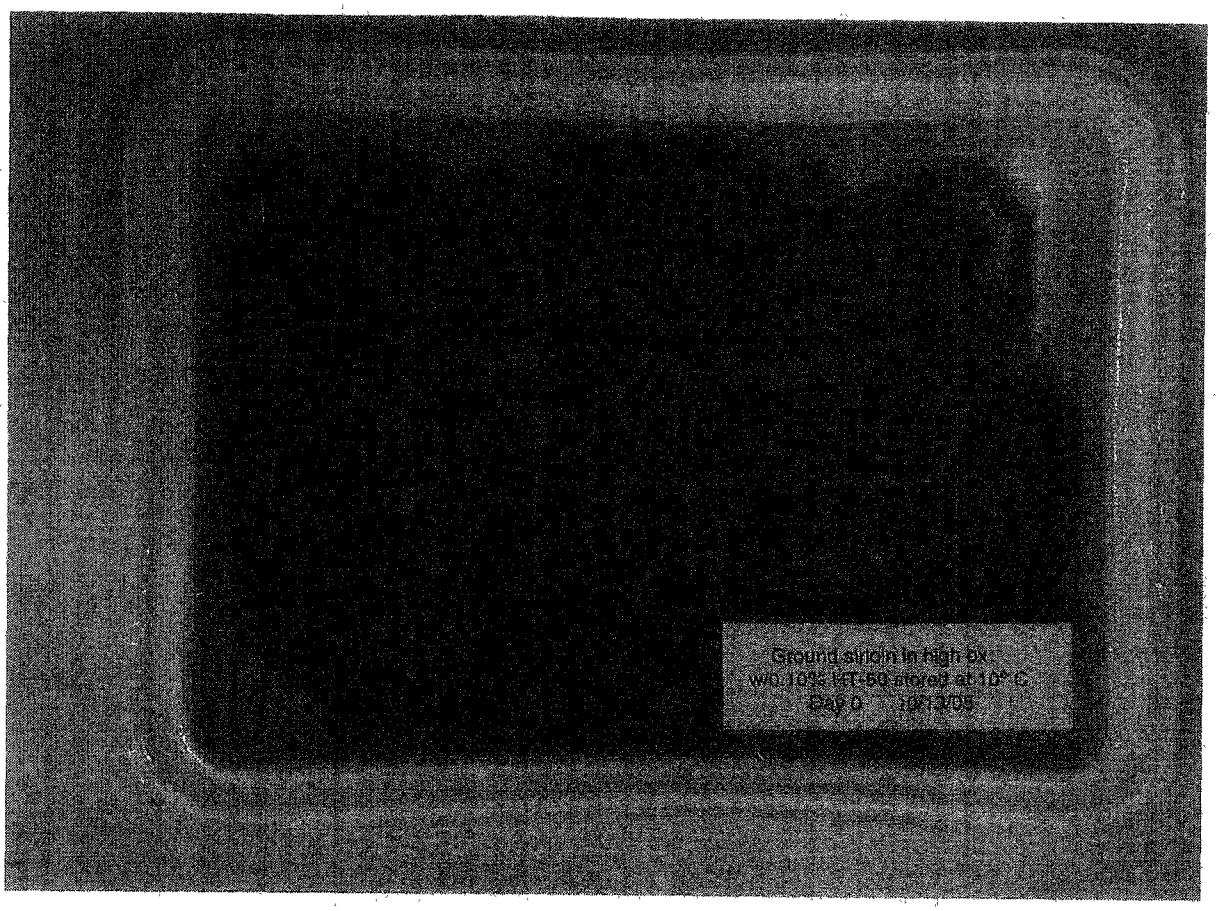

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 4 10/17/05

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 6 10/19/05

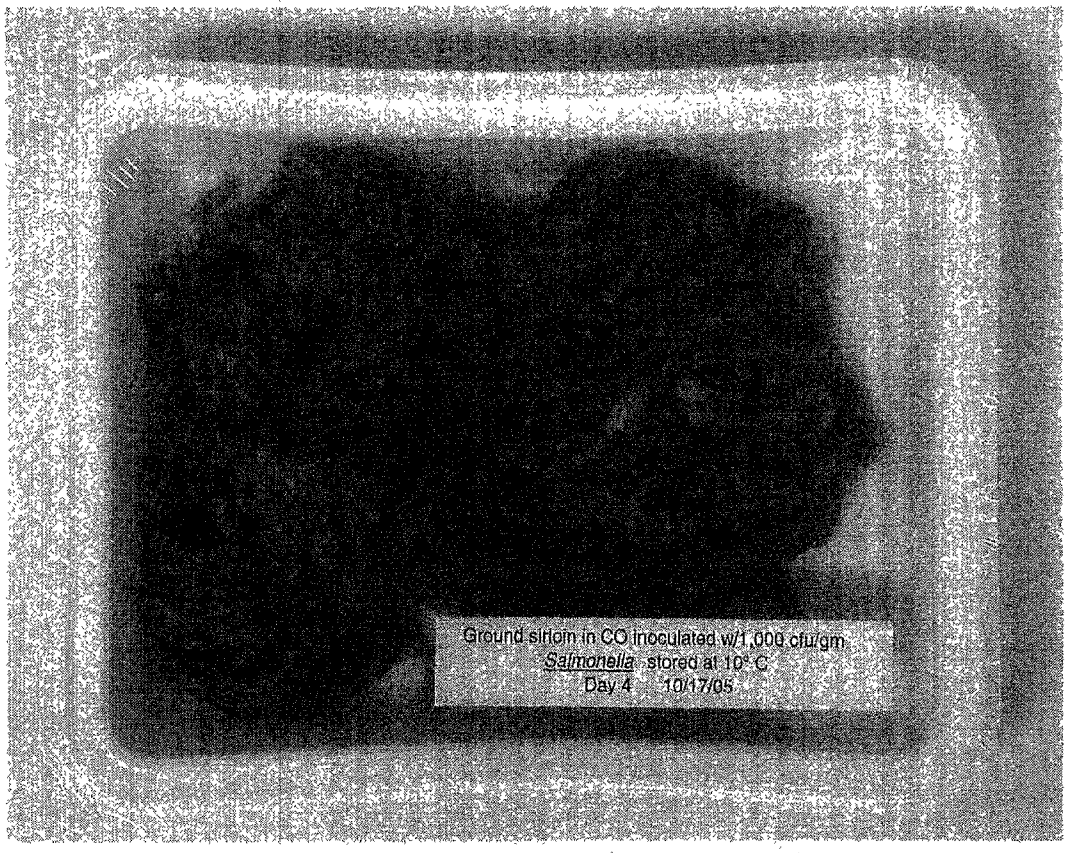

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 8 10/21/05

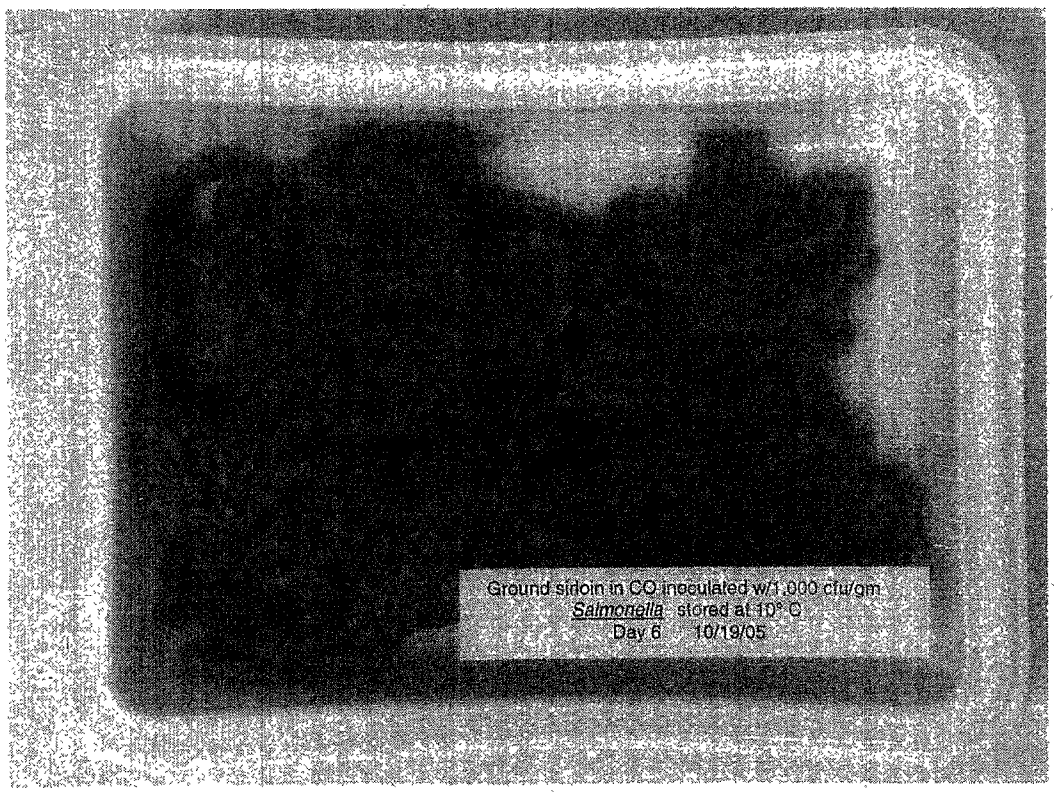
Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 19 10/23/05

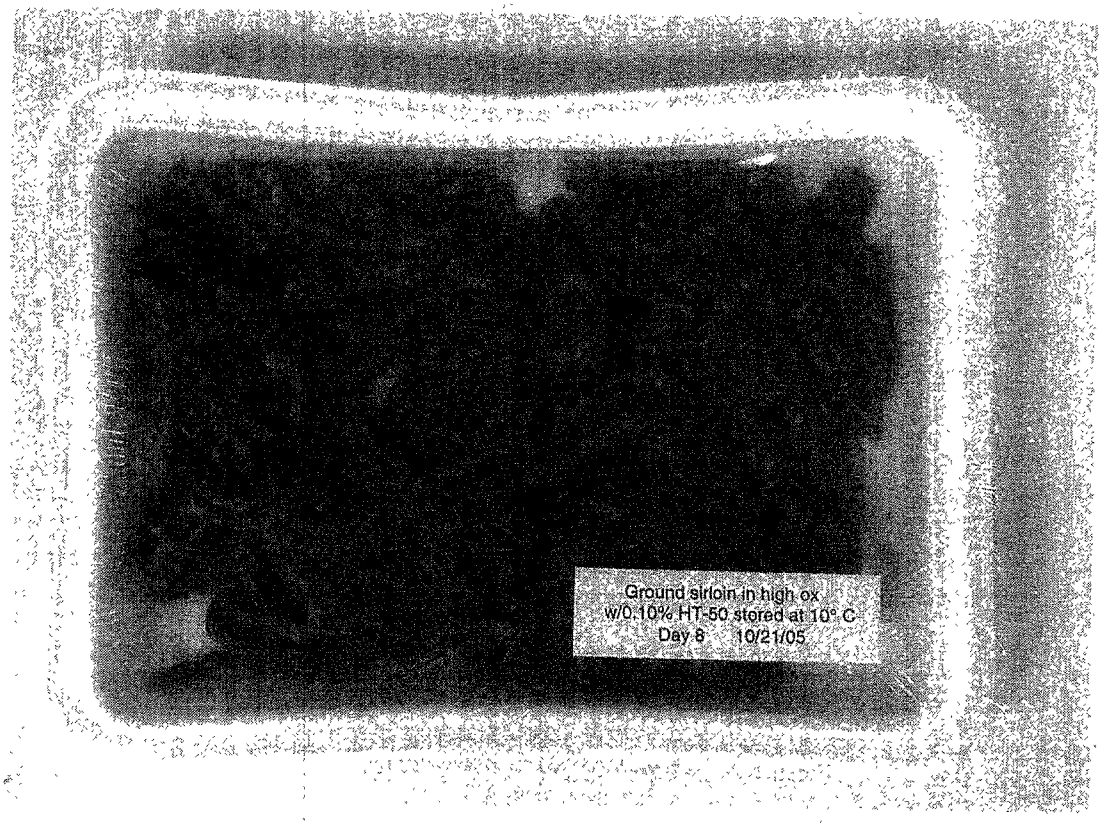

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 12 10/25/05


Ground sirloin in high ox
w/0.10% HT-50 stored at 10° C
Day 0 10/13/05

Ground sirloin in high ox
w/0.10% HT-50 stored at 10° C
Day 2 10/15/05

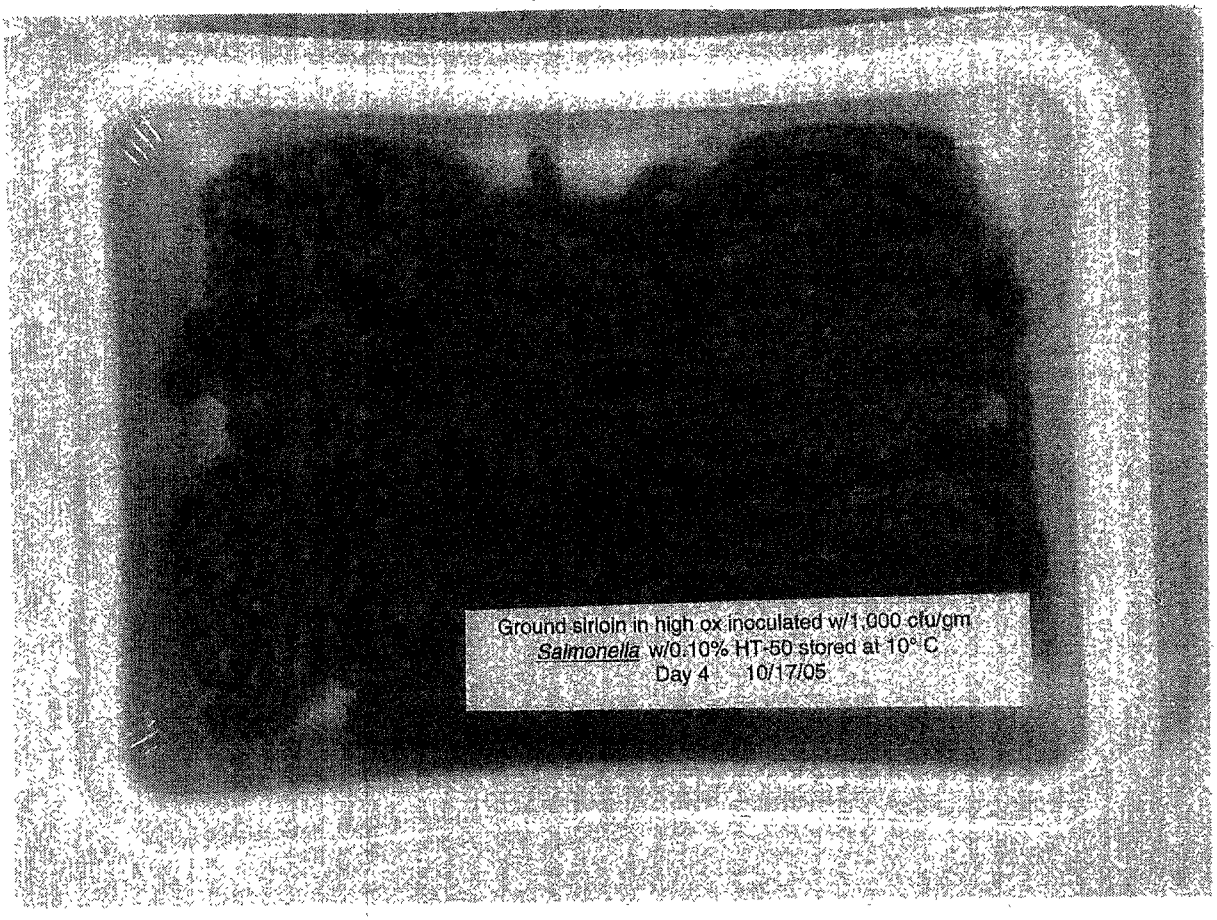

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 4 10/17/05

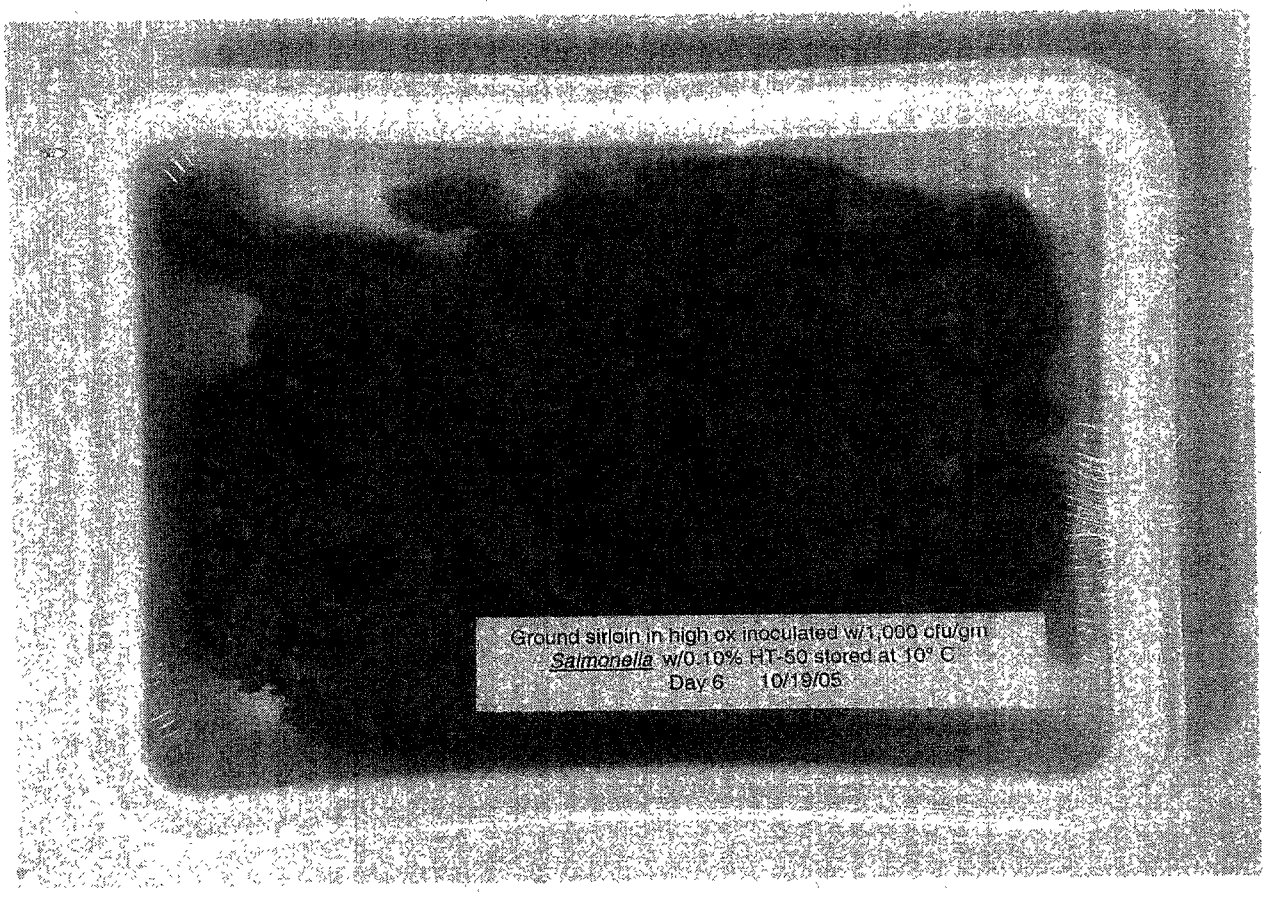

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 6 10/19/05


Ground sirloin in high ox
w/0.10% HT-50 stored at 10° C
Day 8 10/21/05

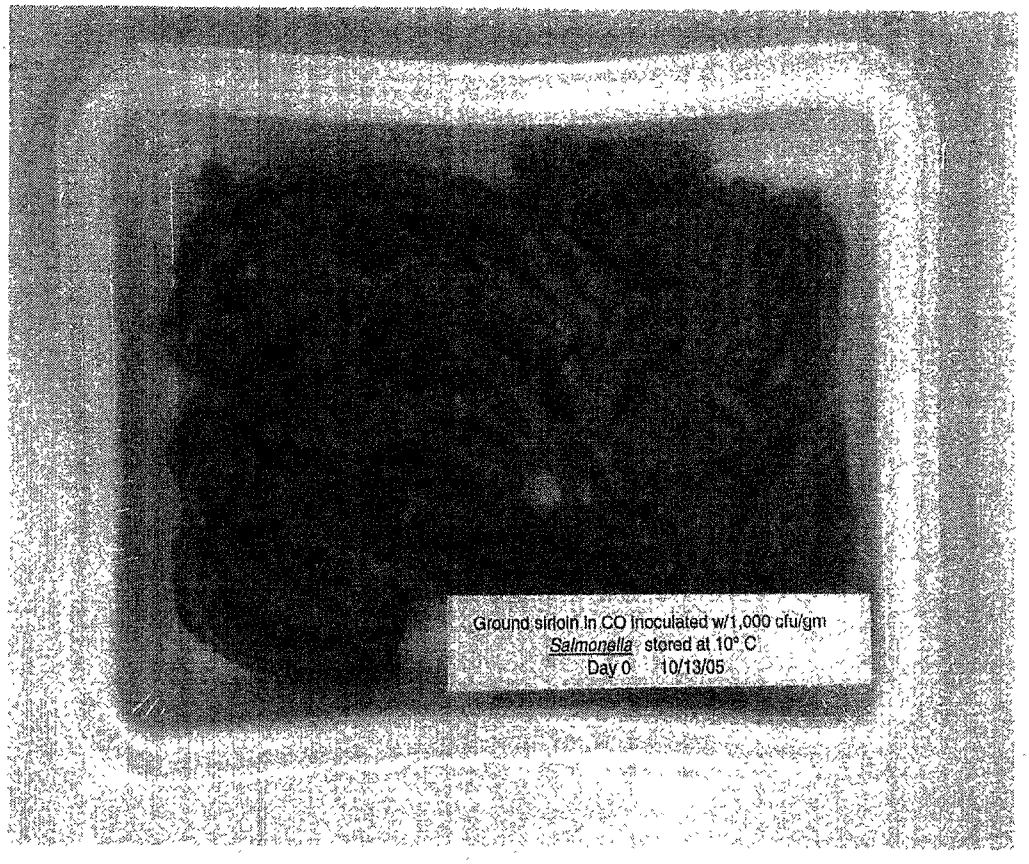
Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 10° C
Day 0 10/13/05

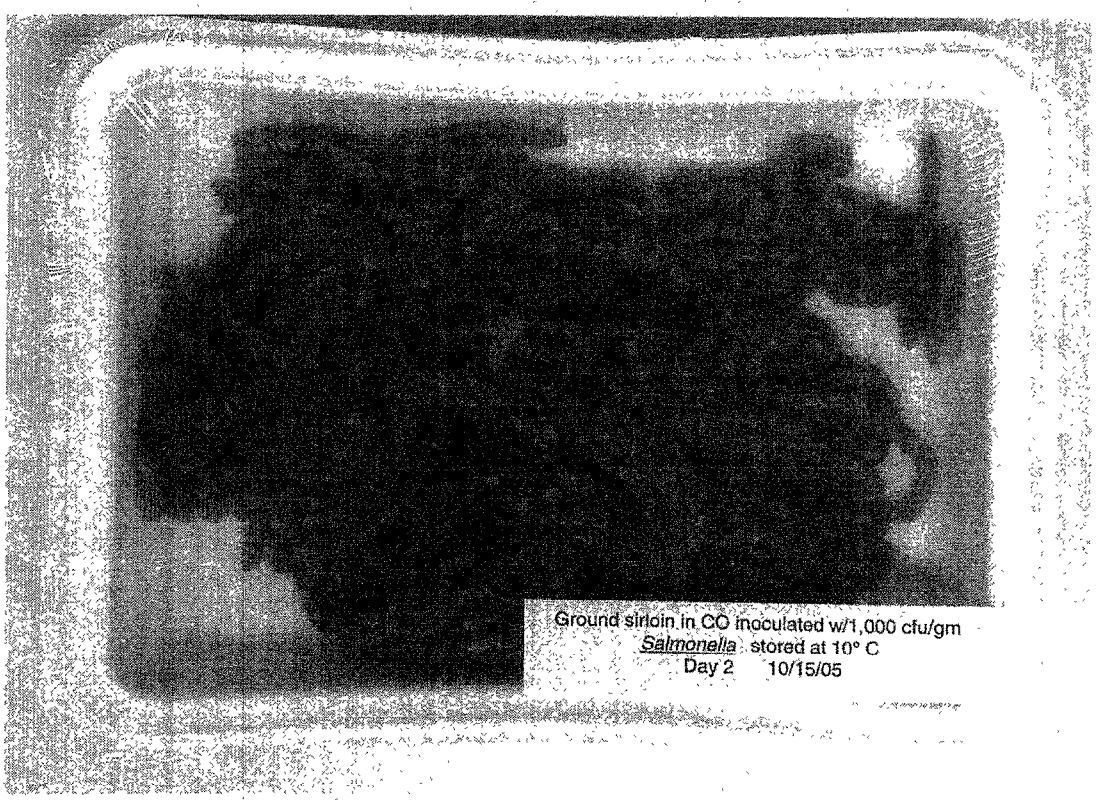
Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 4° C
Day 2 10/15/05

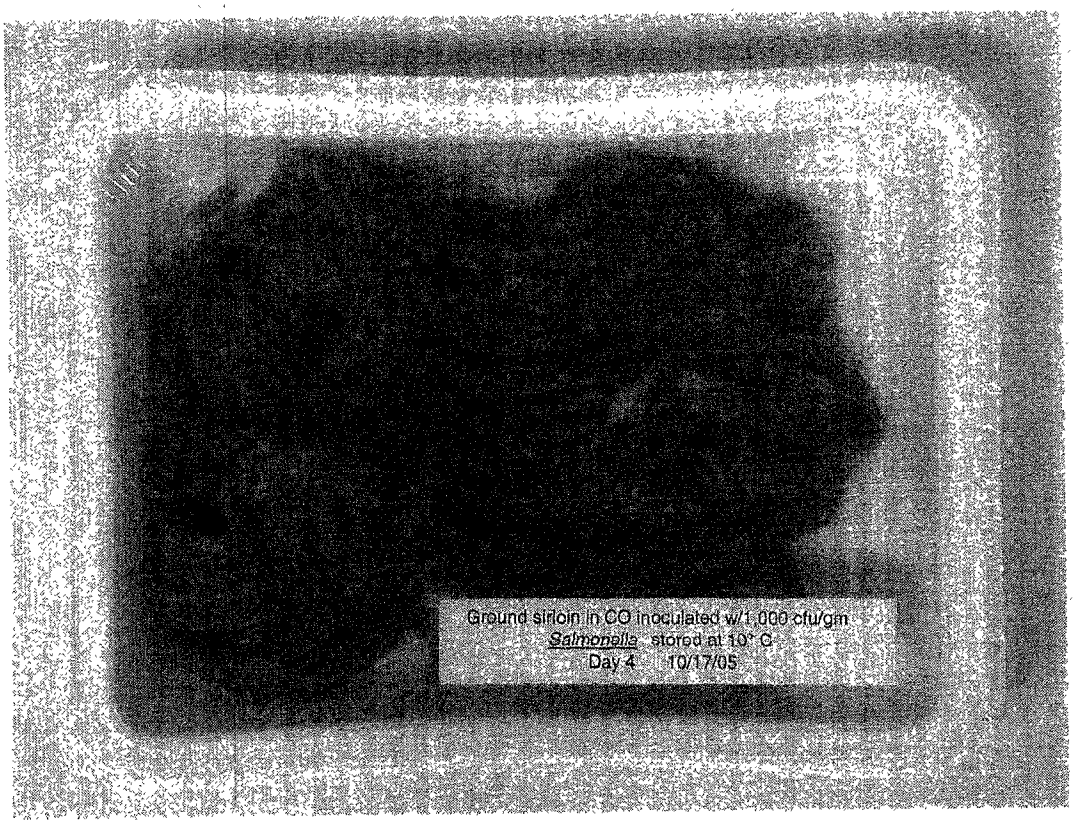

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 10° C
Day 4 10/17/05

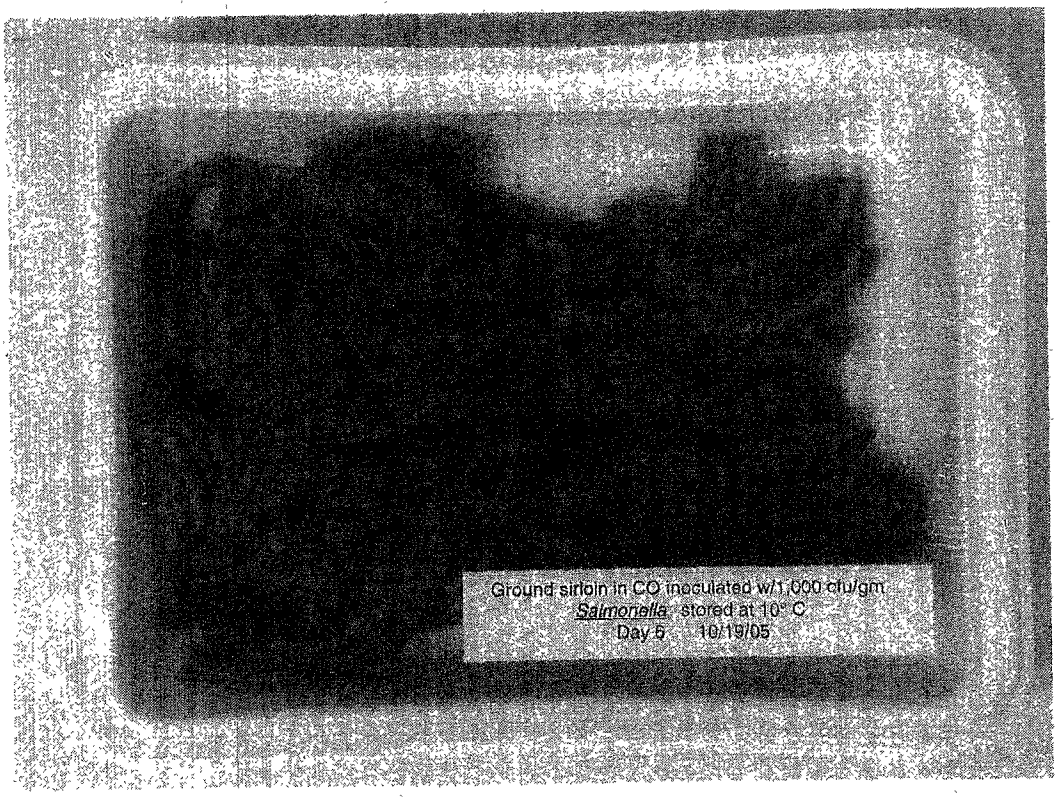

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 10° C
Day 6 10/19/05

Ground sirloin in high ox inoculated w/1,000 cfu/gm
Salmonella w/0.10% HT-50 stored at 10° C
Day 8 10/21/05

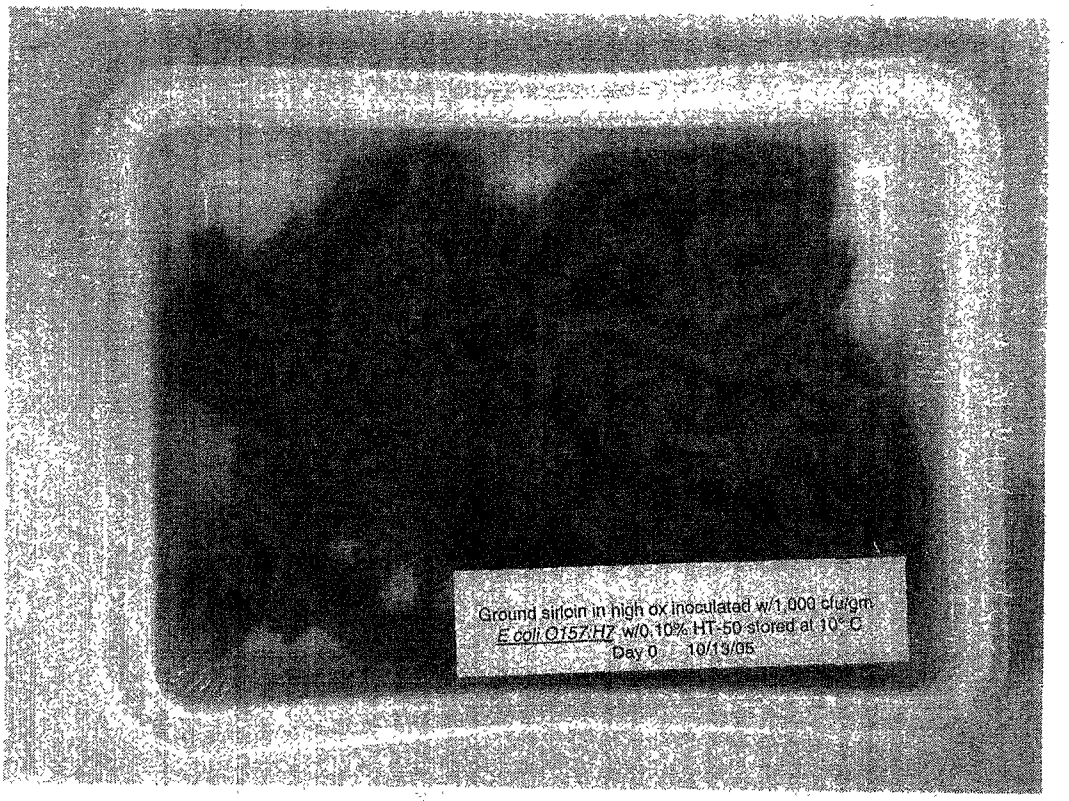

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 0 10/13/05

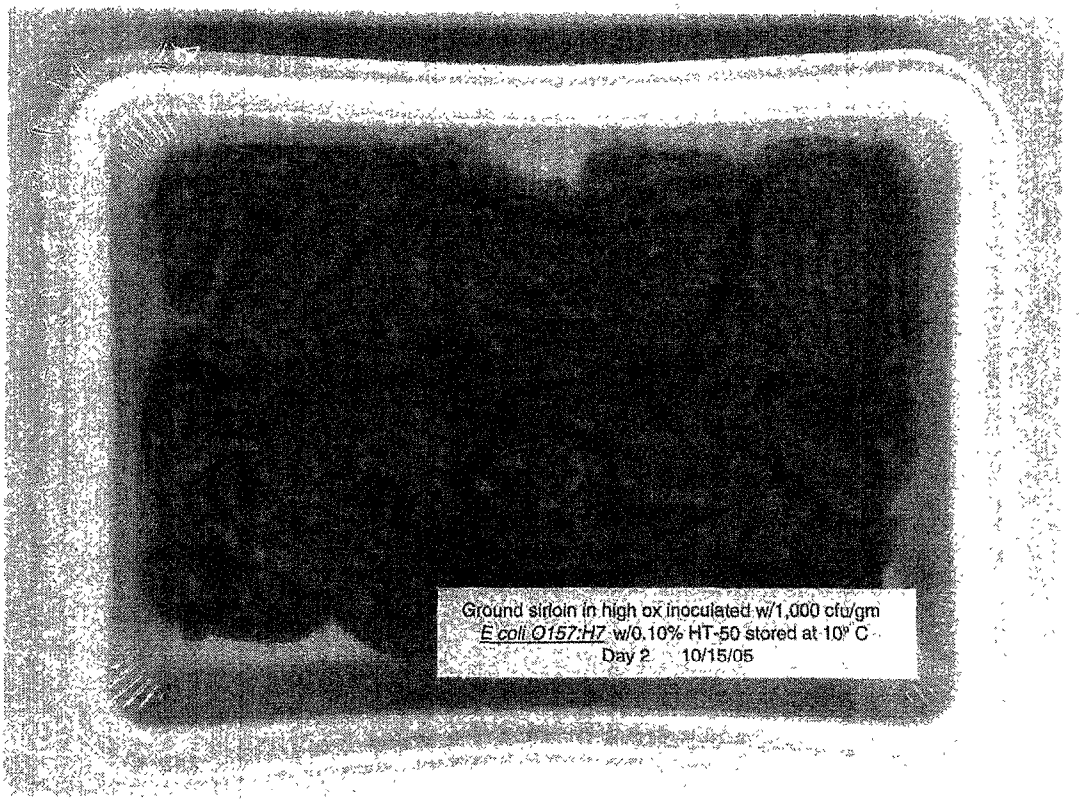

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 2 10/15/05

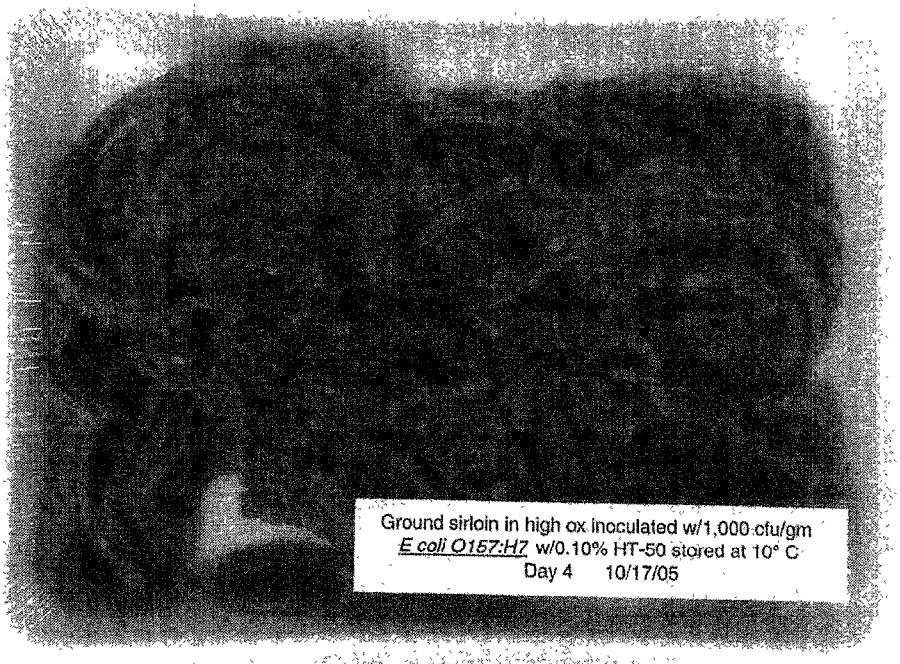

Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 4 10/17/05


Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 5 10/19/05

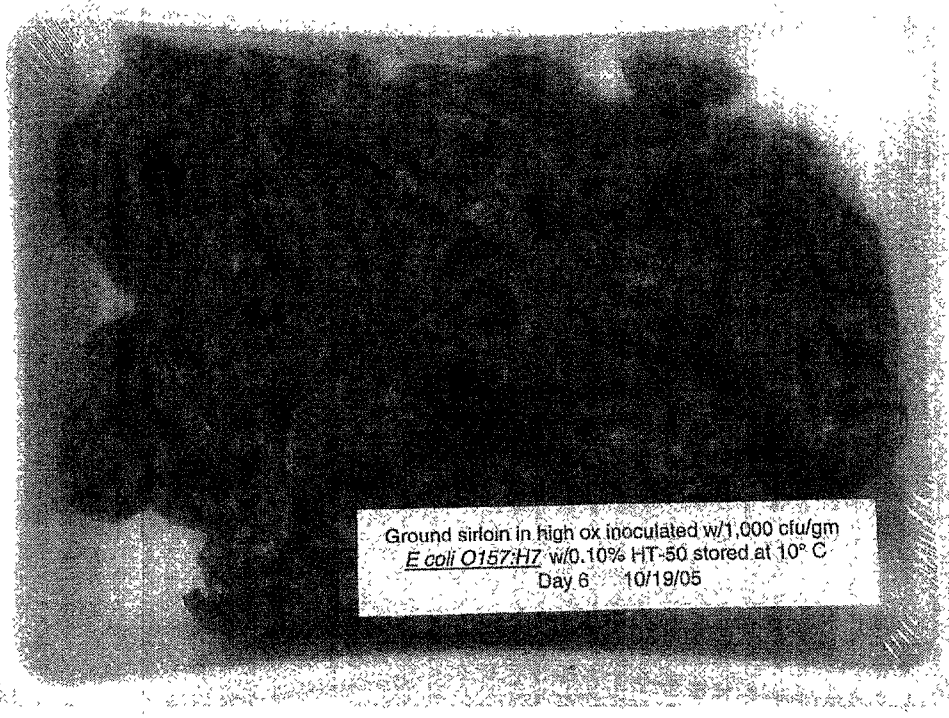
Ground sirloin in CO inoculated w/1,000 cfu/gm
Salmonella stored at 10° C
Day 8 10/21/05


Ground sirloin in high ox inoculated w/1,000 cfu/gm
E coli O157:H7 w/0.10% HT-50 stored at 10° C
Day 0 10/13/05

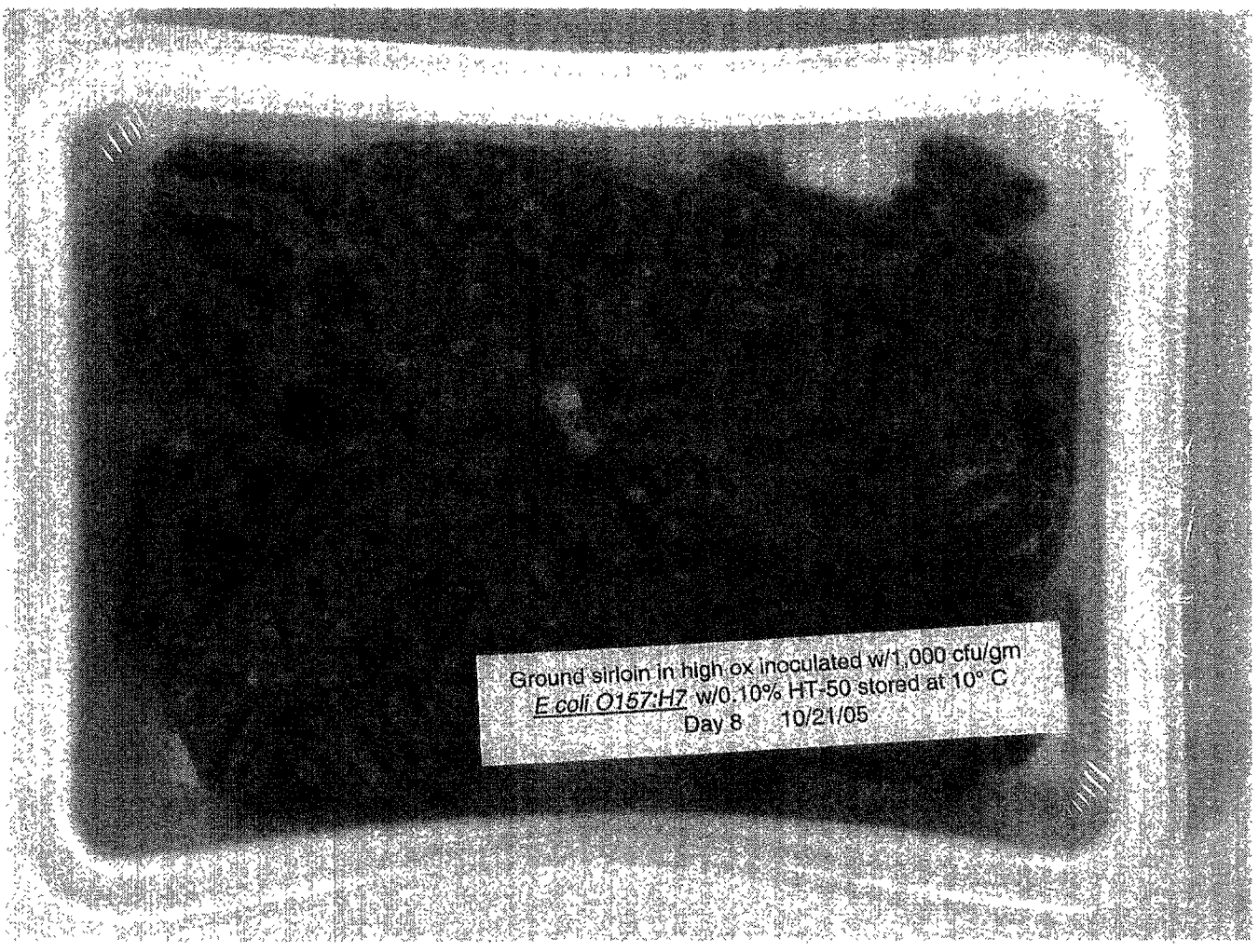

Ground sirloin in high ox inoculated w/1,000 cfu/gm
E coli O157:H7 w/0.10% HT-50 stored at 10° C
Day 2 10/15/05

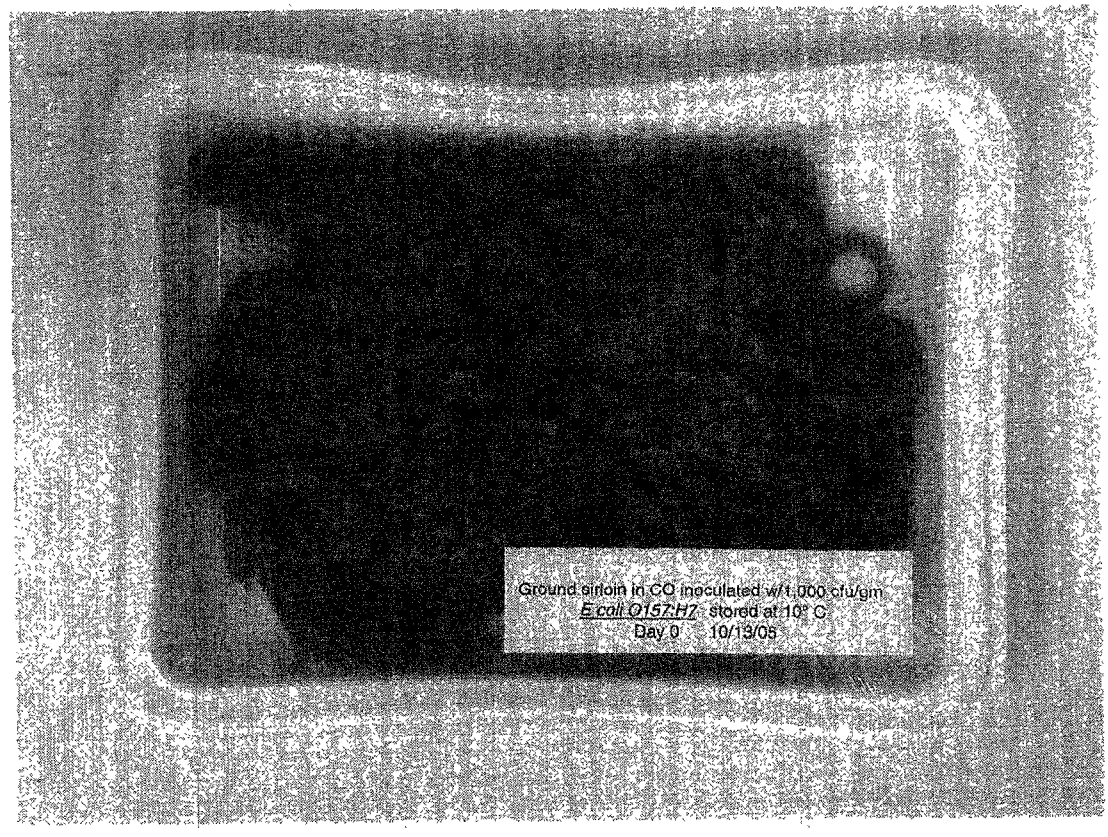


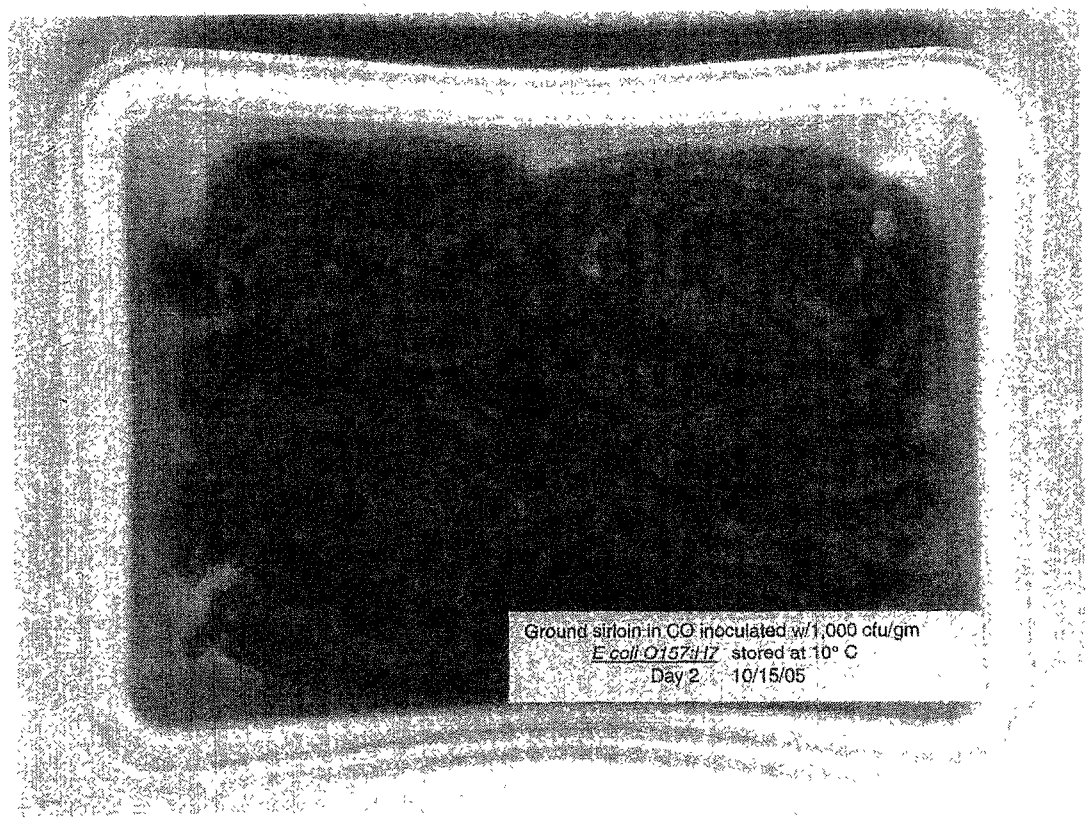
Ground sirloin in high ox inoculated w/1,000 cfu/gm
E coli O157:H7 w/0.10% HT-50 stored at 10° C
Day 4 10/17/05

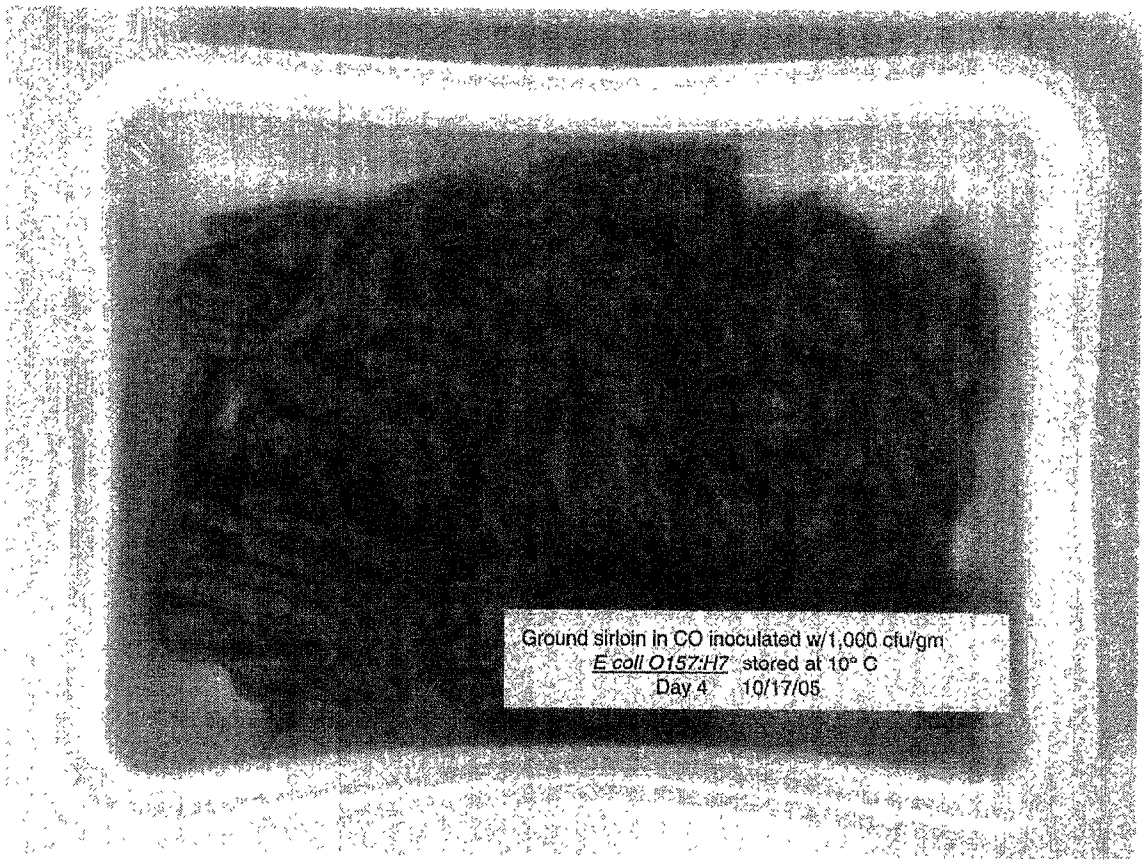


Ground sirloin in high ox inoculated w/1,000 cfu/gm
E coli O157:H7 w/0.10% HT-50 stored at 10° C
Day 6 10/19/05

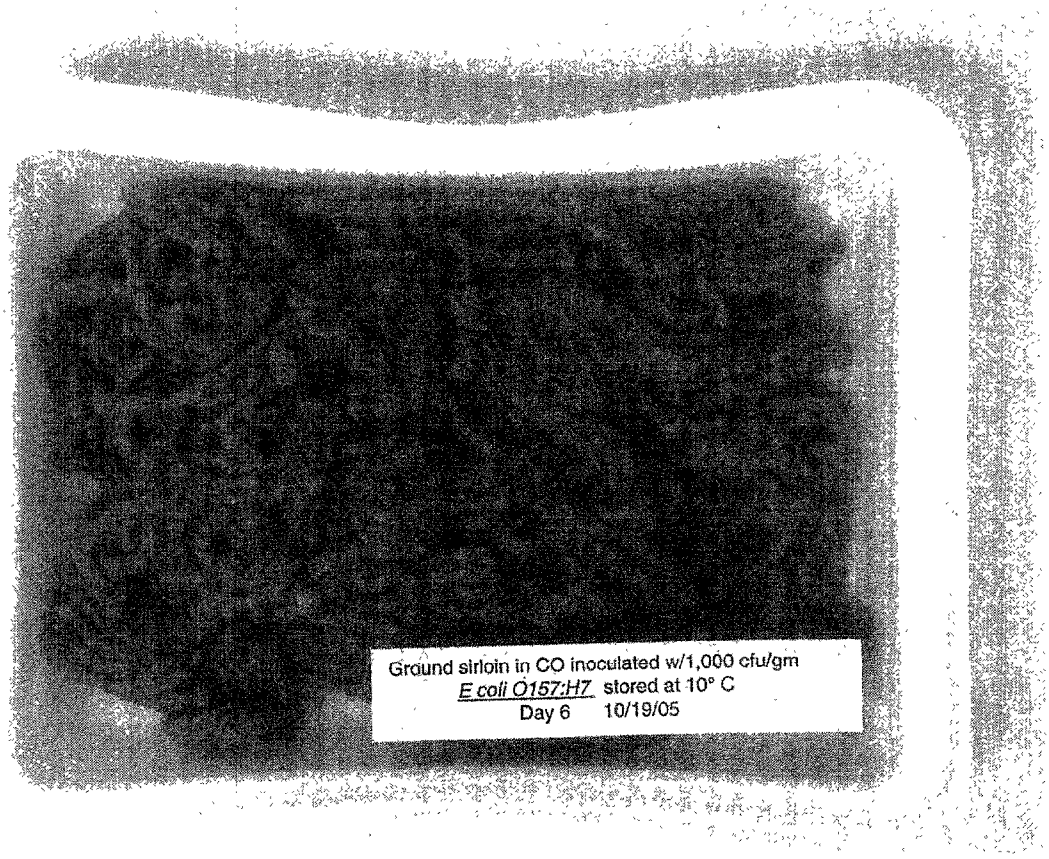

Ground sirloin in high ox inoculated w/1,000 cfu/gm
E coli O157:H7 w/0.10% HT-50 stored at 10° C
Day 8 10/21/05


Ground sirloin in CO inoculated w/1,000 cfu/gm
E coli O157:H7 stored at 10° C
Day 0 10/13/05

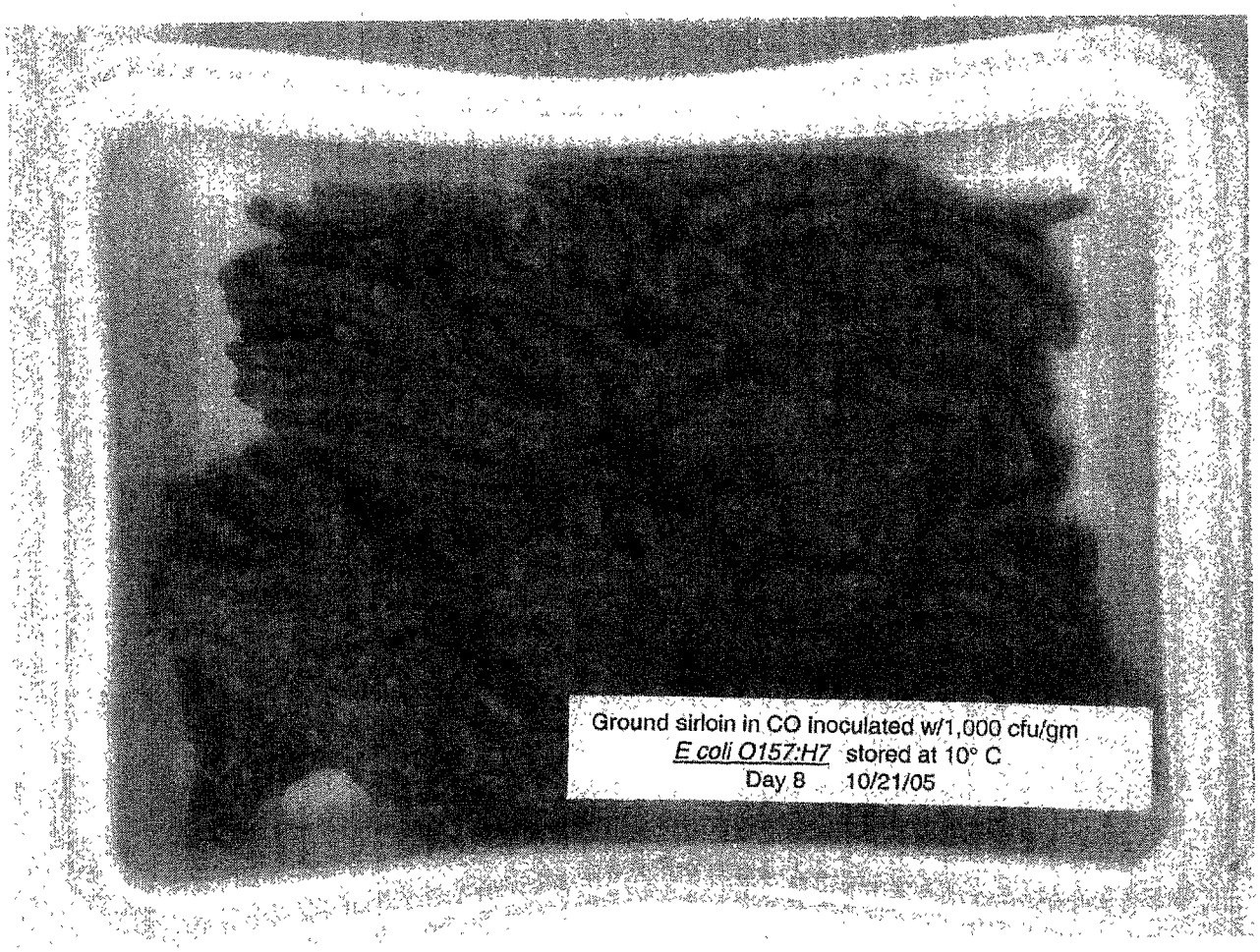

Ground sirloin in CO inoculated w/1,000 cfu/gm
E coli O157:H7 stored at 10° C
Day 2 10/15/05



Ground sirloin in CO inoculated w/1,000 cfu/gm
E coli O157:H7 stored at 10° C
Day 4 10/17/05



Ground sirloin in CO inoculated w/1,000 cfu/gm
E coli O157:H7 stored at 10° C
Day 6 10/19/05


Ground sirloin in CO inoculated w/1,000 cfu/gm
E coli O157:H7 stored at 10° C
Day 8 10/21/05