

FINAL REPORT TO THE AMERICAN MEAT INSTITUTE FOUNDATION  
on

**Pathogen Inhibition and Shelf-Life of Raw and Precooked Meat with Protamine**

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In the original proposal, the study was designed to accomplish the following specific objectives:

1. To determine the antimicrobial activity of different levels of protamine on *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* inoculated onto meat and poultry before and after precooking.
2. To measure the shelf-life and microbial status at 35, 40, and 50°F (abusive temperature) of precooked meat and poultry treated with protamine and packaged in vacuum and modified atmospheres (80% nitrogen:20% carbon dioxide).
3. To evaluate the flavor and odor profiles of stored precooked meat and poultry products treated with protamine through trained sensory panel determinations.

**Milestone 1**

1. To determine the antimicrobial activity of different levels of protamine on *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* inoculated onto meat and poultry before and after precooking.

In Sept-Nov, 2001, studies were conducted to determine the minimal inhibitory concentration (MIC) of protamine against *Salmonella typhimurium* and *E. coli*.

**Methodology**

The first protamine-treated ground beef experiments were carried out using low protamine concentrations (0- 5mg/g) and at room temperature (23°C). Based on the results obtained the levels of protamine were increased to between 5.5 and 10mg/g and incubation was at 2-4°C. Microbial inoculum levels of 0 and 10<sup>6</sup> cfu/g were used.

Minimal inhibitory concentration (MIC) experiments in TSB at pH 7.0 were repeated for both microbes (*E. coli* and *S. typhimurium*) and *Listeria monocytogenes* using a growth indicator dye (resazurin). Resazurin, which is blue in the oxidized state, can be reduced to resorufin (pink in color) by growing cells. The use of the growth indicator reduces erroneous deductions due to precipitation of protamine in tryptic soy

broth. Absorbance readings of resazurin treated samples serves as a quantitative means for growth determination.

#### *Ground beef studies:*

Ground beef slurries were prepared in a ratio of 5g: 5ml ground beef to 0.85% saline containing protamine of different concentrations containing no microbial inoculum or  $10^6$  cfu/ml of *E. coli* or *S. typhimurium*. Experiments were carried at refrigeration temperature and at 23°C. For the experiments at refrigeration temperature, protamine concentrations of 5.5-10 mg/ml were used. Protamine concentrations of 2.5- 5 mg/ml were used for experiments at 23°C. Samples were stored for a 24h period before bacterial enumeration using plate count agar (PCA) and enteric bacteria selective media, Levine-Eosin Methylene Blue (L-EMB) agar, for *E coli* and Salmonella. Meat pH was determined by preparing a mixture of ground beef and distilled water (1:1 ratio) and the reading obtained with a pH meter.

#### *MIC test in tryptic soy broth (TSB) using resazurin as growth indicator:*

Different concentrations of protamine in TSB were prepared and inoculated to a final microbial concentration of  $10^6$  cfu/ml of *E coli* and salmonella and  $10^5$  cfu/ml of *L. monocytognes*. Samples prepared in duplicate were incubated at 37°C for 48h and examined after 24 and 48h. Resazurin solution (440µM in phosphate buffer at pH 7 and filter sterilized) was added to MIC samples after 24h of incubation at 37°C. The samples, which contained different levels of protamine (0-10mg/ml), were re-incubated for 2h at the same temperature and the absorbance of each sample taken at 610nm. A ratio of sample absorbance to that of the blank was calculated to determine how much resazurin had been converted. Samples were further stored at 37°C for an extra 24h and observed for changes in color.

## **Results**

Results of the MIC determinations indicated that at pH 7.0, 5mg/ml protamine was required to effectively inhibit bacterial growth in tryptic soy broth (TSB). Adjusting the pH to 5.8 increased the MIC to 7mg/ml for *S. typhimurium* while *E coli* would not grow at this pH. At the acidic pH, salmonella in TSB required 48h of incubation at 35°C to grow to  $10^8$  cfu/ml. Although the pH of fresh meat is approximately 5.7, some pathogenic bacteria such as *E. coli* 157 and *S. typhimurium* are able to grow in meat and pose health hazards.

#### *Ground beef studies*

Results showed that after 24h incubation at 23°C, microbial levels in ground beef samples had increased to 8.8 log cfu/g (Figure 1). This was an increase of more than 2 log units in the inoculated samples. At 5mg/g protamine, the microbial levels in uninoculated and *E coli* inoculated samples showed reduced growth levels compared to lower protamine concentrations. Salmonella inoculated samples were 9.6-9.7 log cfu/g at protamine concentrations of 4.25 and 5mg/g. Plate counts on L-EMB agar for *E coli* and *S typhimurium* showed the same trend as the total plate count agar (Figure 2).

Samples stored at refrigeration temperature had decreased microbial counts with increased protamine concentration. This indicated that a low temperature factor enhanced protamine activity. At 10mg/g protamine in meat, *S typhimurium* had decreased by almost 2 log units to approximately 4.6 log cfu/ml from an initial inoculum level of  $10^6$  cfu/ml on L-EMB agar plates (Figure 3). Similar trends were observed for *E coli* inoculated samples and uninoculated ground beef. *E coli* was affected by the low storage temperature and at 0mg/g protamine, *E coli* counts on EMB plates were below the initial inoculum level of  $10^6$  cfu/g (Figure 4). At 8.5 and 10mg/g protamine *E coli* counts had reduced to below 4 log cfu/g.

The inability of 10mg/g protamine to completely kill microbes in ground beef may have been due to the high inoculum levels used and the presence of mineral cations, which have been shown to prevent interaction of protamine with the microbial cell wall at high mineral ion concentrations. Meat systems contain soluble proteins such as serum albumin, used in neutralizing broths for studies on antimicrobial agents. Their presence may also interfere with the action of protamine. Other components such as lipids in meat may also exert a negative effect on protamine. It has been reported that bacteria counteract the inhibitory activity of protamine by producing proteases that degrade protamine until protamine effectiveness is nullified. Thus, the effects of natural cation chelators on effectiveness of protamine in meat systems need to be investigated. The contribution of proteases, produced by bacteria, on the stability of protamine needs to be analyzed in the presence of protease inhibitors.

#### *MIC determinations using resazurin*

MIC determinations were carried at 610 nm and pH 7 to measure the disappearance of resazurin. MIC of protamine against all three microorganisms at the concentrations used in the experiment was 3mg/ml in TSB (Table 1). For all three microorganisms, growth occurred at 1mg/ml concentration and below. On addition of resazurin at 10% of the sample volume, samples and blank containing 10mg/ml protamine immediately changed color to violet indicating that the presence of protamine in solution may have caused a reduction of resazurin. The graph of percent absorbance relative to the blank (i.e. no protamine and no microbe) showed that more than 70% of the dye was retained in the oxidized form (Figure 5). Since protamine disrupts the cell membrane of microorganisms, the enzyme systems responsible for reduction of resazurin may have been released into the broth and thus could have caused a slight reduction of resazurin. A blank sample containing 10mg/ml protamine also changed color to violet indicating that protamine may induce conversion of resazurin to resorufin in broth. In spite of the color change, the solutions of protamine and microbes at the higher protamine concentrations remained clear even after 48h of incubation.

The effect of protamine at 0-25 mg/g on total microbial level in *Salmonella* inoculated ground beef after 7 days storage was determined. Samples treated with 0-25 mg/g protamine were stored for 7 days at 2-4°C. At protamine concentration of 25 mg/g, microbial levels decreased after 24 h but increased with storage time, at the end of 7 days storage the microbial levels were higher than the level on day 1 regardless of the concentration of protamine used in the samples (Figures 6 and 7). Increased protamine concentration was not associated with high bacterial kill. The level of protamine needed

to provide complete inhibition of bacterial growth could not be achieved during this study.

## **Conclusion**

Although protamine inhibited microorganisms in meat, concentrations of 10mg/g and above are required to reduce the load by more than 2 logarithmic units. At 10mg/ml, reductions of greater than 95% of the controls were achieved. For high levels of microbes of about  $10^6$  cfu/g, this is not a significant reduction. MIC of protamine against *L. monocytogenes* was 3mg/ml at pH 7.0.

## **Prospect for the future**

Meat is a complex system containing high levels of proteins, lipids, and minerals. Potential inhibitory effects of these bioactive compounds on protamine antimicrobial activity needs to be studied. Our studies will be directed towards the inhibition of protease activities in meat followed by protamine addition to evaluate the effectiveness of protamine under such conditions. Because a complete bacterial kill was never achieved during this study and because of the cost of protamine (\$135/5g) from commercial sources, it was determined not to investigate the inhibitory effect of protamine on *E.coli*. *E.coli* is known for its ability to produce protease which may have also degraded protamine. Milestones 2 and 3 could not be contemplated during this study because milestone 1 was not completed. Although we have identified naturally occurring protease inhibitors that could eliminate the proteolytic activity from bacteria, funds were not available to pay a graduate student and purchase reagents for further investigations.

## **Milestone 2.**

To measure the shelf-life and microbial status at 35, 40, and 50°F (abusive temperature) of precooked meat and poultry treated with protamine and packaged in vacuum and modified atmospheres (80% nitrogen:20% carbon dioxide).

Because of the high concentration of protamine required to obtain one log reduction and inability of protamine to completely inhibit microbial growth, milestone 2 was not attempted.

## **Milestone 3**

To evaluate the flavor and odor profiles of stored precooked meat and poultry products treated with protamine through trained sensory panel determinations.

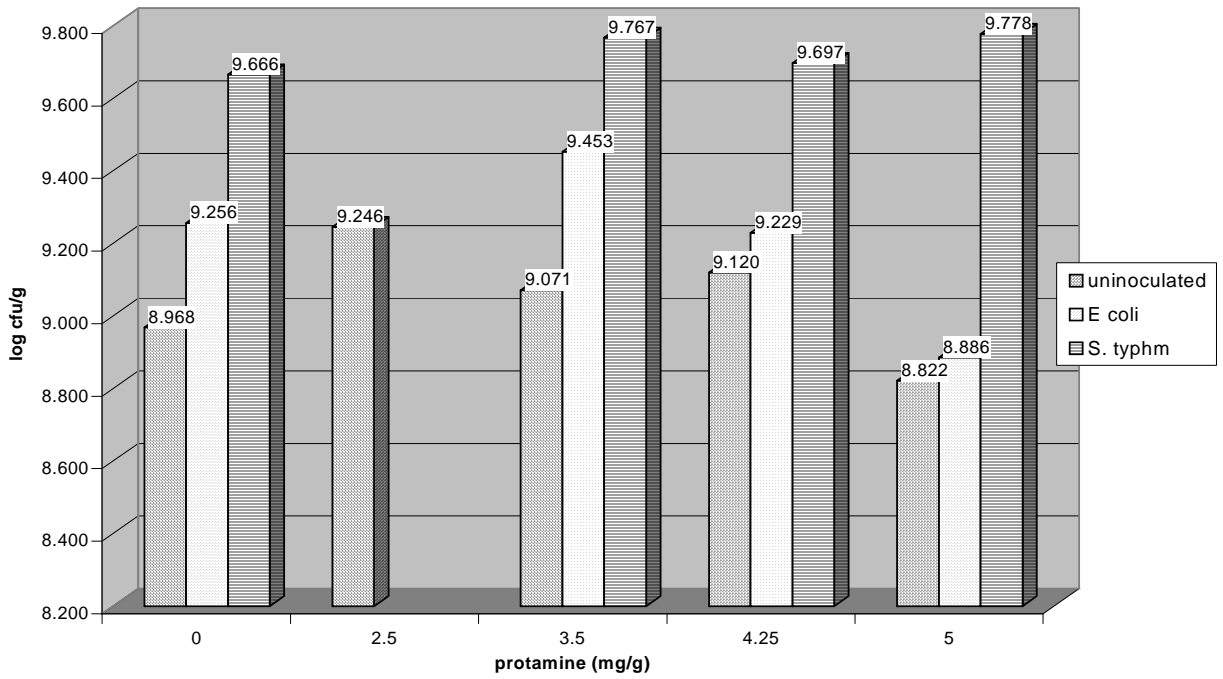
This milestone was not carried for the same reasons mentioned in milestone 2.

Table 1: MIC of protamine against pathogenic bacteria in TSB with resazurin as growth indicator (20hrs after incubation at 37°C with an extra 2hrs with resazurin)

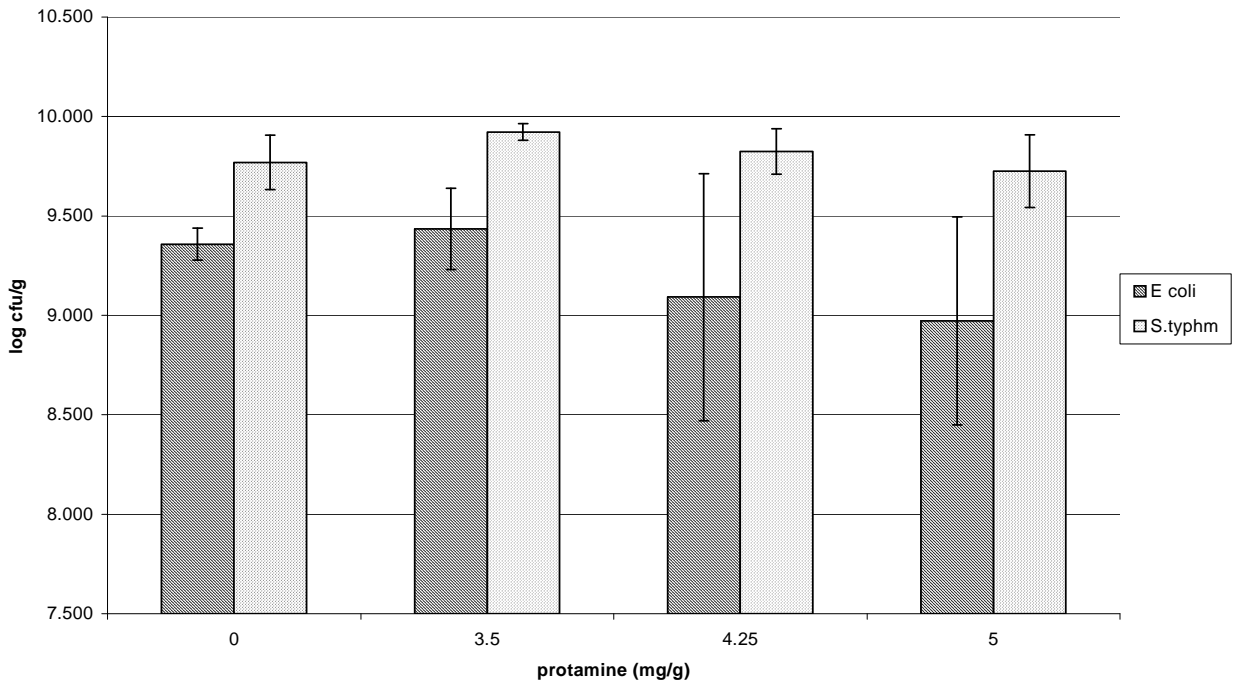
Protamine (mg/ml)	E coli		L monocytogenes		S. typhimurium	
	1	2	1	2	1	2
0	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)
0.6	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)
1	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)	G(pink ring)
3	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)
5	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)
7	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)	NG (Blue)
10	PPT(violet)	PPT(violet)	PPT(violet)	PPT(violet)	PPT(violet)	PPT(violet)
Blank 0mg/ml	NG (Blue)					
Blank 10mg/ml	PPT(violet)					

(G-growth; NG- no growth; PPT- precipitate; ( )- color after 2h with resazurin)

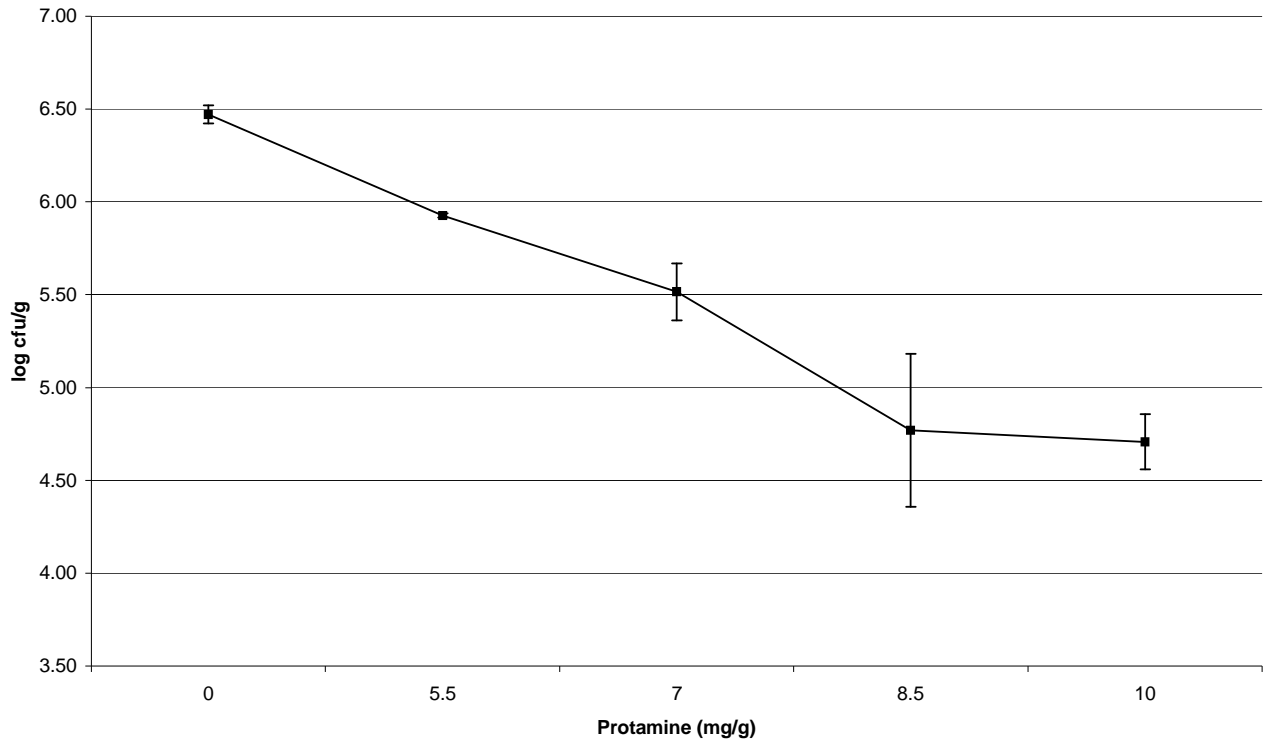
**Figure 1: Total Microbial levels in protamine treated ground beef incubated at 23°C for 24h (microbe inoculum levels of 0 or 10<sup>6</sup> cfu/g of beef slurry)**



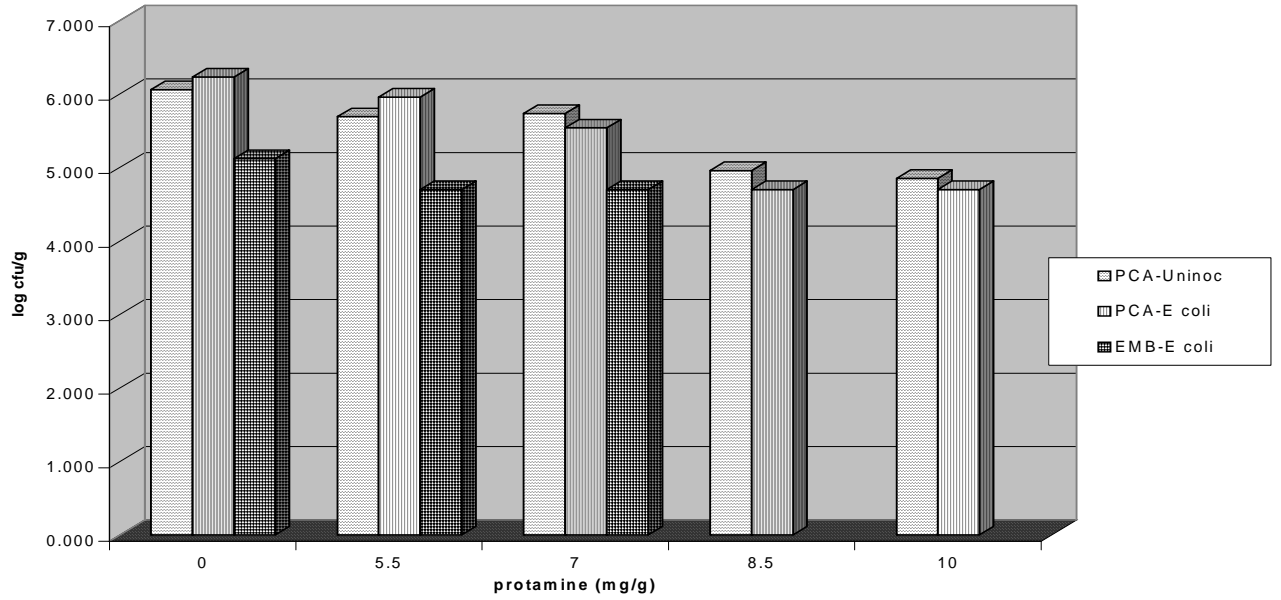
**Figure 2: Levels of E coli B and S. typhimurium in protamine treated ground beef after 24h incubation at 23°C (plated on L-EMB agar)**



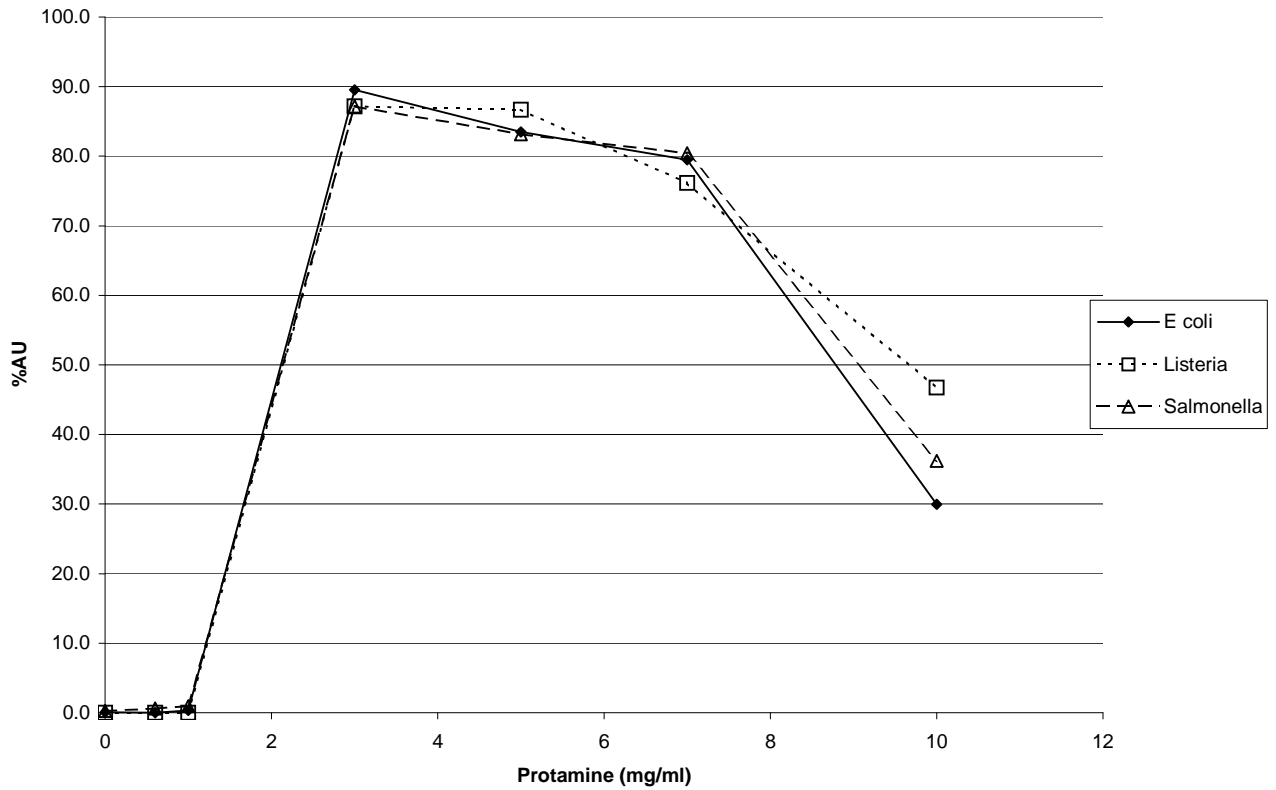
**Figure 3: Levels of *S. typhimurium* in protamine treated ground beef incubated at 2- 4°C for 24h (plated on Eosin Methylene Blue agar)**



**Figure 4: Microbial levels of E coli inoculated and uninoculated protamine-treated ground beef stored at 2-4°C for 24h (plated on PCA and EMB agar)**



**Figure 5: % Retention of Resazurin after 2h incubation with 24h TSB samples containing protamine**





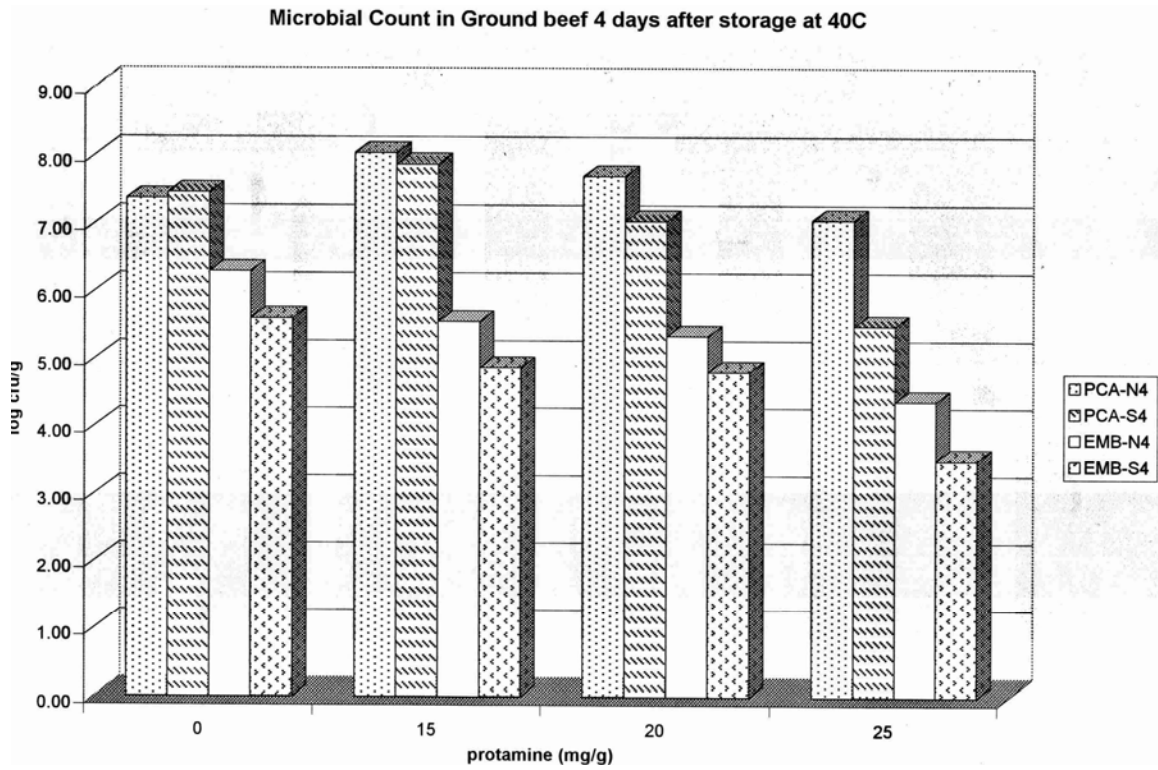


Figure 6a. Microbial count in ground beef samples 4 days after storage at 4°C (plated on PCA and EMB agar. PCA-N4 = control samples on PCA agar after 4 days at 4°C; PCA-S4 = samples inoculated with *Salmonella* on PCA agar after 4 days at 4°C ; EMB-N4 = control samples on EMB agar after 4 days at 4°C ; EMB-S4 = samples inoculated with *Salmonella* on EMB agar after 4 days at 4°C)

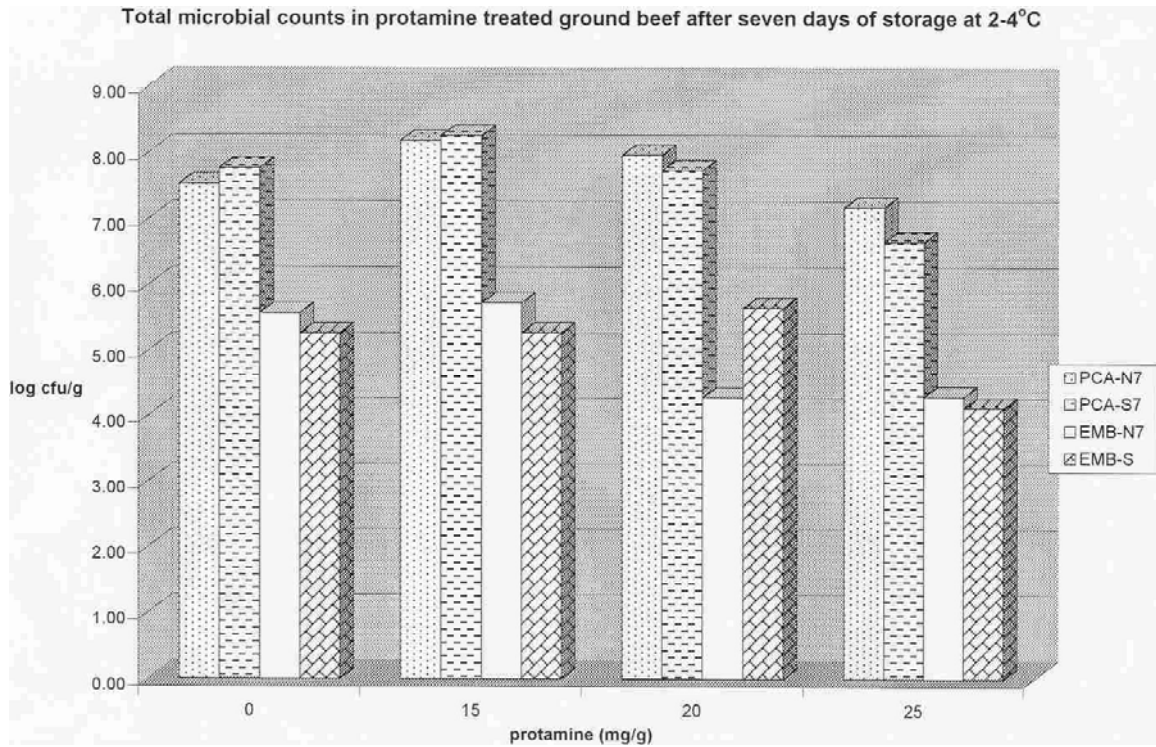


Figure 6b. Total microbial counts in protamine treated ground beef samples after 7 days of storage at 2-4°C (plated on PCA and EMB agar. PCA-N7 = control samples on PCA agar after 7 days storage at 2-4°C; PCA-S7 = samples inoculated with *Salmonella* and plated on PCA agar after 7 days storage at 2-4°C; EMB-N7= control samples on EMB agar after 7 days storage at 2-4°C; EMB-S7= samples inoculated with *Salmonella* and plated on EMB agar after 7 days storage at 2-4°C).

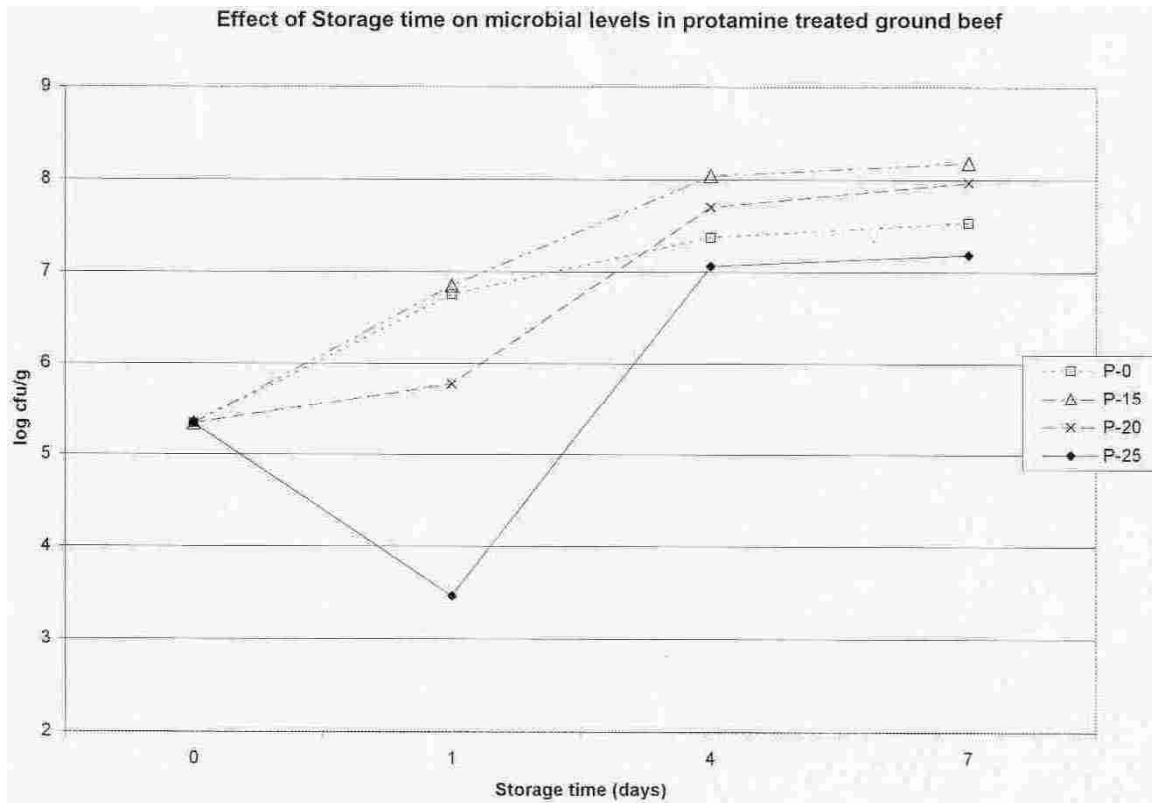


Figure 7 a. Effect of storage time (days) on microbial levels in protamine treated ground beef samples (P-0 = 0 mg/g protamine; P-15 = 15 mg/g protamine; P-20 = 20 mg/g protamine; and P-25 = 25 mg/g protamine).

Effect of storage time on total microbial levels in *Salmonella* inoculated ground beef containing protamine

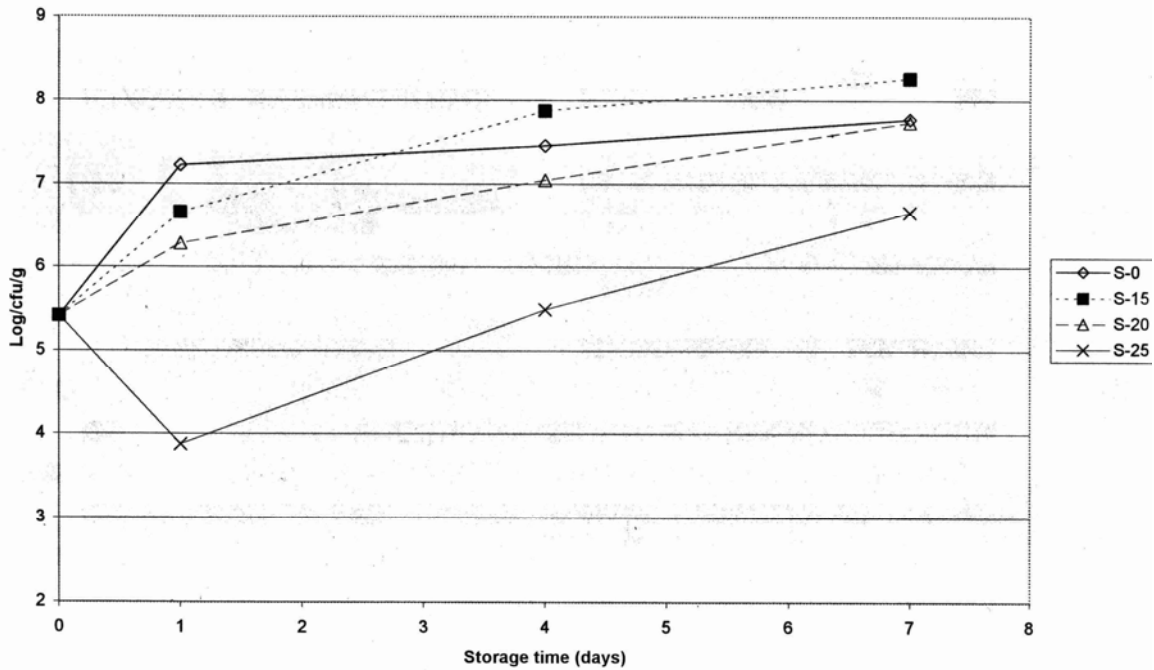


Figure 7b. Effect of storage time on total microbial levels in *Salmonella* inoculated ground beef samples containing different levels of protamine (S-0 = 0 mg/g protamine; S-15 = 15 mg/g protamine; S-20 = 20 mg/g protamine; and S-25 = 25 mg/g protamine).