Efficacy of ozonated water use as antimicrobial intervention in beef primal and subprimal fabrication

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Beef primals and subprimals were subjected to spray treatment with ozonated water [1 ppm dissolved O_3, at 10 pound per square inch (PSI) pressure or less, with a time of contact of approximately 10 seconds]. Enumeration of total aerobic bacteria population revealed mean surface reduction of 2.26 log_{10} CFU/100 cm^2 for the antimicrobial intervention. Also, this treatment reduced the total coliforms by 2.31 log_{10}CFU/100 cm^2 and the Enterobacteriaceae counts by 2.56 log_{10}CFU/100 cm^2. These data indicate that ozonated water treatment of beef primal and subprimal cuts applied before mechanical tenderization or moisture enhancement can significantly and effectively reduce the surface microbial contamination on these beef cuts.

INTRODUCTION

Currently, the food industry in general and the meat industry in particular, is in great need of more powerful and convenient antimicrobial interventions, suitable for general surface decontamination and effective against foodborne pathogens. In the beef industry, only slaughter facilities are currently required to use a validated antimicrobial intervention to reduce surface contamination of *Escherichia coli O157:H7* (Federal Register, 2002). Nevertheless, the beef industry and the U.S. Department of Agriculture – Food Safety Inspection Service (USDA - FSIS) continue to investigate the prevalence and prevention measures to combat foodborne pathogens in all stages of beef production. In this ongoing food safety quest, ozone can be an efficient and environmentally friendly tool to consider and adopt.

In Europe and Japan, ozone has been applied effectively in the food industry for decades, and in water treatment and food applications for over a century in some cases. In France and Germany for example, ozone has been the primary sanitizer for public water systems (Graham, 1997). Despite the worldwide successful applications of ozone, the U.S. has a more recent and modest history of its use. In 1997 ozone was designated generally recognized as safe (GRAS) in food processing, followed by Food and Drug Administration (FDA) and USDA recognition of ozone as a secondary direct food additive in 2001 (Federal Register, 2001).

Ozone (O_3) is activated (enriched) oxygen, and it exists as a gas at room temperature. The gas is colorless with a pungent odor detectable by humans at concentrations as low as 0.02 to 0.05 ppm (by volume), which is below concentrations of health concern. Ozone is a powerful oxidant, second only to the hydroxyl free radical, among chemicals typically used in water treatment. Therefore, it is capable of oxidizing many organic and inorganic compounds in water (EPA, 1999).

The antimicrobial activity of ozone is believed to be based on its powerful oxidizing effect, which causes irreversible damage to the lipids in the cell membrane and to cellular macromolecules, such as proteins, and DNA (Fetner and Ingols, 1959; Hoffman, 1971). Moreover, the damage to the cell membrane is due to the high oxidation-reduction potential (ORP) / voltage of ozone. The stronger the oxidizing character, the stronger the sanitizer pulls electrons away from the cells membrane, causing destabilization and leakage. Ozone has one of the highest oxidation-reduction potential (ORP=+2.07 V), lower only than the fluorine atom (ORP=+3.06 V), hydroxyl radical (ORP=+2.80 V), and oxygen atom (ORP=+2.42 V) (Qiu et al., 2001). The standard oxidation-reduction potential is a standard for sanitizing in many countries. In 1968, the German Health Ministry was the first to introduce this standard for water treatment, when it proved that

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disinfectant solutions with ORP ≥ + 650mV will kill *E.coli* on contact. In 1982, U.S. Environmental Protection Agency (EPA) and World Health Organization (WHO) recognized that solutions with ORP levels of + 650 mV instantaneously kill harmful microorganisms (McPherson, 1993).

Commercially ozone can be applied as a disinfectant instead of chlorine and other sanitizers. Ozone has a major advantage over other antimicrobial agents, since it does not leave residual, harmful chemicals behind. While chlorine produces dangerous carcinogenic by-products, such as chloromides, chloroform and trihalomethane (Greenberg, 1980), ozone's by-products are oxygen O_2, carbon dioxide CO_2 and water. This makes ozone absolutely environmentally friendly and ideal for food processing applications and potable water treatment. Also, ozone has been shown to be a more powerful disinfectant than chlorine, deactivating of a very large number of organisms (Graham, 1997). Many other studies have reported the advantages and superior bactericidal properties of ozone as compared to chlorine (Greene et al., 1993; Kim et al., 1999). Other important advantages of the ozonated water over other sanitizers are: 1. It can be generated on site, eliminating transport, storage and handling of antimicrobial agents; 2. Chemical reaction of ozone with organic matter occurs at a very rapid rates and short reaction times, which prevent microorganisms from developing tolerance to ozone (Kim, 1998); 3. The precursors of ozone (O_2 and H_2O) are abundant and inexhaustible; 4. The treatment does not require heat and hence saves energy (Khadre et al., 2001).

Numerous studies on the use of gaseous ozone for increasing storage life, and aqueous ozone for disinfecting surfaces of vegetables, fruits, meat, poultry, eggs, seafood, fruit juices, spices etc. (Rice et al., 1982), support the position that ozone is a powerful disinfectant effective against a wide range of bacteria, viruses, yeast, molds and protozoa (Farooq et al., 1983; Graham, 1997; Güzel-Seydim et al., 2004; Restaino et al., 1995). Gram-positive bacteria showed more sensitivity to ozone than gram-negative bacteria (Kim et al., 1999).

Several experiments conducted on meat and poultry applications, have shown ozone to be an effective sanitizer in meat processing applications.

Dondo et al. (1992) evaluated ozone usage for refrigerated beef. It appeared that ozone stopped the growth of the surface contaminants of beef during several days of refrigerated storage and improved sensory quality. A study on beef that was heated showed decreased resistance of vegetative cells and spores of *C. perfringens* to ozone treatment (Novak and Yuan, 2004). The conclusion of the study was that microorganisms surviving ozone treatment where less likely to endanger food safety as compared to the organisms surviving sub-lethal heat treatments.

Kaess and Weidermann (1968) sprayed fresh beef with *Pseudomonas*, *Candida scottii*, *Thamnidium*, and *Penicillium* and then exposed to 0.15 to 5µg/L of gaseous ozone. They reported that count of *Pseudomonas* and *Candida scottii* decreased significantly at >2µg/L. Greer and Jones (1989) studied effects of ozone on beef carcass quality. The beef carcasses were continuously ozonated (0.03 ppm) under 95% RH and 1.6 C for up to 9 days of aging. The results showed that in that study ozone prevented bacterial growth on carcass surface.

Yang et al. (1979) reported that ozonation extended shelf-life of refrigerated broiler parts. Sheldon et al. (1985) conducted a few studies to validate the use of ozone in poultry. Their findings demonstrate ozone to be suitable treatment for reducing spoilage and levels of pathogenic bacteria on broiler carcasses and in chilled waters.

The efficacy of any antimicrobial intervention in food processing is ideally tested by inoculating targeted microorganisms on the surface of food, and applying the sanitizer at conditions that simulate normal processing. Alternatively, indicator microorganisms with resistance to the treatment are recommended. The indicator is ideally similar biologically to the targeted microorganism, but it should not be pathogenic if the study is carried out in the processing facilities (Banwart et al., 1981; Khadre et al., 2001). This is the case of the present experimental design, which was conducted within Wolverine Packing steak fabrication plant, in a real-time conditions environment. Castillo and al. (1997) determined that log reduction of generic *E.coli*, *Enterobacteriaceae*, and Coliforms on beef carcasses were not significantly different from reduction seen in *E.coli O157:H7*.

Given the limited data available in the literature on the specific application of aqueous ozone to decontaminate beef cuts, the goal of the present research was aimed at studying the efficacy, using indicator organisms, of ozonated water on reducing microbial counts on beef primal and subprimal cuts. The objective of this study was to evaluate the effectiveness of ozonated water as an antimicrobial intervention for beef primal and subprimal cuts in a steak cut operation.

MATERIALS AND METHODS

Experimental design The intervention consisted of the application of 1 ppm ozonated water to different types of beef primals and subprimals, before mechanical tenderization or moisture enhancement. The concentration of dissolved ozone was chosen based on the optimization of previous trial experiments (data not shown), the potential loss of ozone in the spray, and its half-life.

Prior to mechanical tenderizing or moisture enhancement, the beef cuts pass through a prototype treatment tunnel (Wolverine Packing Co., Detroit, MI), on an adjustable speed conveyer belt. The speed during the experiment was set to 2.8 min per conveyer cycle, also chosen based on previous trial tests, to allow sufficient exposure to ozonated water (approximately 10 sec/piece). In the tunnel the antimicrobial agent was applied as a constant spraying via low pressure upper and lower nozzles. The aqueous ozone was produced by a DELZONE® ozone

generator (DEL Ozone, San Luis Obispo, CA), model MPI-300. The system is a corona discharge ozone generator, and produces a water flow rate of 3.3 gpm, and an ozone output range of 2.5 g/h. The pressure of the sprayed ozonated water, measured at the nozzles, was maintained at the manufacturer recommended level, at 10 PSI or less [in order to prevent gassing-off and retain the targeted concentration of 1ppm (DEL Ozone – "MPI-300 Multi-point intervention ozone sanitation system, Owner's Manual)].

The dissolved ozone concentration at the point of application was measured using Ozone *AccuVac*® kit test 0-1.5 mg/L O_3 (Hach Co., Loveland, CO), a visual indigo-based method (*AccuVac*® # 2518025 product information). Also, the oxidation-reduction potential (ORP) and pH were measured at the point of application for each run. A portable HI 98201 Redox meter (Hanna Instruments, Woonsocket, RI) was used for ORP measurement, and a HI 98108 pH meter (Hanna Instruments, Woonsocket, RI) was used to measure the water pH.

Based on the previous literature information, we decided to use generic *E.coli*/Coliforms (total coliforms count, TCC), *Enterobacteriaceae* (*Enterobacteriaceae* count, EBC), and total aerobic bacteria (aerobic plate count, APC) in this study, as indicator groups.

Sampling protocol The sampling for this study was conducted for a period of several weeks, based on a random sampling plan for the raw material entering the fabrication process. Samples (N=100) were randomly collected from the beef primal and subprimal cuts scheduled for real production, during the weeks of sampling: chucks, ribs, tenderloins, strip loins, top sirloin butts, outside rounds. The sample size was decided based on power analysis; with type I error value α=0.05, and type II error value $\beta\leq$0.1, the above mentioned sample size provides adequate power for finding statistically significant results (Cohen, 1988). Samples reflect the variability of the bacterial load of the beef cuts, given the diversity of the whole muscle parts utilized by the plant for steaks fabrication.

Sampling occurred just prior to the intervention, immediately after entering the fabrication area and after each cut has been removed from their vacuum package, yet before the treatment tunnel. The 100 untreated control samples were collected in this manner. Their corresponding treated samples were pulled after the ozonated water spraying, immediately following the mechanical tenderization of the cuts. In order to evaluate the decontamination treatment effect on the beef cuts, the treated samples were placed on a sterile table situated on-site, and swabbed one hour after the antimicrobial intervention. This timeframe was chosen based on the ozonated water half-life: 20-30 minutes in distilled water, at 20°C before reverting back to simple diatomic oxygen (Graham, 1997).

The outer surface of the beef primals/subprimals subjected to antimicrobial intervention was sampled using Biotrace Int. (a 3M Company, Bothell, WA) sponge sampling kits (sponge bags, 25ml of sterile Butterfield's Phosphate Buffer, gloves, and templates). This sampling procedure was performed as described by USDA/FSIS method for generic *E.coli* testing (FSIS, 1996); 100 cm^2 areas were swabbed before and after the antimicrobial intervention. The sponge was moistened with 10 ml Butterfield's phosphate water. Then, the sponge was used to swab 10 times horizontally and 10 times vertically over a 10 x 10 cm area, approximately on the center of each sample.

After swabbing, the sponge was returned to the sampling bag, and the rest of 15 ml Butterfield's phosphate water was added. Samples were sent immediately to the plant's laboratory and refrigerated for analysis within 8 h. Before proceeding to microbiological analysis, the sample bags containing the sampling sponge were stomached for 3 minutes at 200 rpm, using a Stomacher 400 (Seward, Fisher Scientific, Itasca, IL). Then, the bags were manually squeezed, in order to express from the sponge as much solution as possible. The expressed solutions were diluted to the necessary rates, using serial dilutions with Butterfield's buffer water. 1 ml of the diluted sample was inoculated in duplicate to each type of Petri film plate and incubated at 36°C for 48 h. The following 3M (3M Microbiology Products, St. Paul, MN) Petrifilm™ plates were used for enumeration of indicator bacteria: *E.coli*/Coliforms Count (PF-EC), *Enterobacteriaceae* Count (PF-EB), and Total Aerobic Count (PF-AC).

Enumeration Generic *E. coli* colonies should form on 3M plates blue precipitate and are associated with entrapped gas (approximately 1 colony diameter). Coliforms enumerated on 3M plates include *E. coli* colonies as well as red colonies associated with entrapped gas, within approximately 1 colony diameter; all red colonies were counted on these plates, regardless of their size or the intensity of their color, to determine the aerobic plate count (APC). *Enterobacteriaceae* form on 3M plates red colonies associated with gas bubbles and no yellow acid zones, or colonies with yellow acid zones and no gas production, or colonies producing both gas and acid (3M - "Petrifilm™ Interpretation Guide"). The log CFU/100 cm^2 was calculated from the total colonies counted. When no cells where detected on the lowest dilution plate, the value 1 CFU/100 cm^2 was allocated, in order to be able to converted to a log value and perform the statistical analysis ($\log_{10}1=0$).

Statistical analysis Duplicates for each sample were done, and then the number of bacterial counts per each sample was averaged. Direct plate count results were converted to $\log_{10}$ values before proceeding with the statistical analysis. Log reductions associated with the intervention were calculated by subtracting post-intervention values from pre-intervention values. This analysis was performed in order to evaluate the effectiveness of the intervention at reducing the bacterial counts ($\log_{10}$ CFU/100 cm^2 untreated, minus $\log_{10}$ CFU/100 cm^2 treated sample). Data was analyzed using "the paired t-test", and MINITAB® Statistical Software, version 15 (Minitab Inc., State

College, PA), to determine if the means of the bacterial levels (log_{10} CFU/100 cm^2) prior and after the intervention, are significantly different ($P \leq 0.05$).

RESULTS

The results demonstrate the ability of 1 ppm aqueous ozone to produce significant reduction in the total aerobic plate count (APC), total coliforms (TCC) and *Enterobacteriaceae* (EBC). While in all the 100 samples pulled before the intervention APC were enumerated, only in 56 samples were detected TCC and EBC were present in only 49 samples. The prevalence of generic *E.coli* was zero, thus we excluded this indicator from the study because of the lack of data.

The ozonated water's measured parameters had the following range values: ORP = 884 – 906 mV; pH = 7.9 – 8.2; dissolved O_3 concentration = 0.9 – 1.15 mg/L.

The lowest APC enumerated in a sample before the antimicrobial treatment, was 5.02 log_{10} CFU/100 cm^2, while the maximum bacterial load was 7.47 log_{10} CFU/100 cm^2 (Figure 1). The pre-intervention median mean was calculated as being 6.34 log_{10} CFU/100 cm^2. The sample mean before intervention is 6.36 log_{10} CFU/100 cm^2 with a standard deviation SD = 0.58.

The lowest APC enumerated in a sample after the antimicrobial treatment and the mechanical tenderization, was 2.75 log_{10} CFU/100 cm^2, while the maximum bacterial load was 5.33 log_{10} CFU/100 cm^2 (Figure 2). The post-intervention mean was calculated as being 4.13 log_{10} CFU/100 cm^2. The sample mean after intervention is 4.10 log_{10} CFU/100 cm^2 with a standard deviation SD = 0.63.

Three different graphs (Figure 3 – 5) are visual evidence of the significant log reduction, by comparing the APC (log_{10} CFU/100 cm^2) collected pre- and post-intervention.

Figure 6 supports the assumption that the samples come from a normally distributed population. Therefore, all of the statistical tests performed based on this assumption are valid.

A hypothesis test at a 5% level of significance was performed to verify and support the efficacy of the intervention.

The APC data that was used in the paired T-test is the following:

	N[1]	Mean	SD[2]	SE[3] Mean
APC log_{10} [before]	100	6.34	0.58	0.058
APC log_{10} [after]	100	4.10	0.63	0.063
Difference	100	2.26	0.22	0.022

1 - number of samples; 2 - standard deviation; 3 - standard error.

The histogram of differences (Figure 7) shows a reduction of 2 or more logs in most of the samples.

After performing the statistical analysis, we can state with 95% confidence that the actual log_{10} reduction in any beef cut sampled will be between 2.21 and 2.30 logs (Figure 7).

The same rationale and statistical analysis was applied to TCC and EBC, proving that the intervention reduces these plate counts by no less than 2 logs.

The TCC data that was used in the paired T-test is the following:

	N	Mean	SD	SE Mean
TCC log_{10} [before]	56	2.91	0.67	0.089
TCC log_{10} [after]	56	0.60	1.05	0.140
Difference	56	2.31	0.60	0.080

The EBC data that was used in the paired T-test is the following:

	N	Mean	SD	SE Mean
EBC log_{10} [before]	49	3.10	0.61	0.086
EBC log_{10} [after]	49	0.54	0.96	0.136
Difference	49	2.56	0.71	0.100

The paired t-test results indicate that this intervention method reduces the number of APC, TPC, and EBC log_{10} CFU/100 cm^2 from the sampled primal and subprimal cuts, by no less than 2 logs.

DISCUSSION

Regardless of initial bacterial population on the surface of the beef tissue, ozonated water intervention reduced the APC, TCC and EBC by at least 2 log_{10} CFU per 100 square centimeters in each sampled primal/subprimal cut. Table 1 depicts the prevalence and mean of the indicators aerobic plate count, total coliforms and *Enterobacteriaceae*, before and after the intervention. The same trend and proportion in reduction was observed in al these three indicators.

The results of our study are consistent with previous research conducted in beef applications.

Reagan et al. (1996) treated fece inoculated beef carcasses with ozone (0.3 to 2.3 ppm), or hydrogen peroxide (5%), as post-washing intervention treatments. The results indicated relatively modest reduction of aerobic plate counts by use of hydrogen peroxide and ozone, of only 1.14 and 1.30 log CFU/cm^2, respectively.

In a more recent study (Bosilevac et al., 2005), water and aqueous ozone were used to decontaminate beef hides in a simulated wash system. While the water wash reduced the total microbial count only by 0.5 logs, the ozonated water achieved a 2.1 log reduction of APC, and 3.4 logs reduction of EBC, on the hides.

Gorman et al. (1995) compared the effect of 5% hydrogen peroxide, 0.5% ozone, 12% trisodium phosphate, 2% acetic acid, and 0.3% commercial sanitizer on *E.coli* inoculated beef brisket fat tissue. The experiment was conducted using different technical conditions than the research described in this study. These researchers utilized a two-chamber

spray-wash cabinet, for water wash and subsequent sanitizer application. The results are similar to our findings. Under the conditions of this study, ozonated water and hydrogen peroxide were the most effective treatments. Spraying beef brisket fat with 0.5% ozonated water reduced bacterial contamination by 2.5 logs. While these results are in general agreement with our work, it is not clear what the parameters of ozonated water application were. The researchers report that ozonated water concentration applied on the beef samples to be 0.5%=5,000 ppm, which is far above the solubility limit for ozone in water under realistic conditions (Graham, 1997). It is possible that the authors refer to the gaseous ozone input at 0.5% (by weight) that was added to water, but the concentration of the aqueous ozone solution, which is most critical to an understanding of disinfection efficacy, was not reported in the article.

In a concept similar to the previous study, Castillo et al. (2002) investigated the efficacy of water wash and ozonated water treatment when applied to inoculated *Escherichia coli* O157:H7 and *Salmonella* beef carcasses. After applying ozonated water 95 ppm, at 28°C (82.4°F) and 80 PSI, the authors report no significantly differences in reduction of the pathogens ($P>0.05$), when comparing with a pressure water wash. These results are clearly different from those reported above, in our study. A possible explanation is the differences in the experimental conditions and physical parameters used in our work, when compared to the research conducted by Castillo et al. The system in this study applied 1ppm ozonated water at 10 PSI or less. Castillo et al. did not measure the ozonated water concentration at the point of application. It was only reported as a concentration in the pressurized container, where it was produced. The ozone concentration in the present study we have described was measured in the water applied to the beef cuts.

While USDA/FSIS has not mandated a level of bacterial reduction for the intervention treatments on beef carcasses and beef cuts, it is expected that any antimicrobial intervention to be efficient against general bacterial population, and against foodborne pathogens respectively. No treatment, as yet, can be relied upon to eliminate all pathogens, without affecting the sensorial quality of raw produce or meat (Kerry et al., 2002). As previously detailed, in our study we were unable to verify the effect of this antimicrobial treatment against *E.coli* O157:H7, or generic *E.coli*. However, we achieved more than 99 % (2 logs) reduction of initial indicator bacteria by using 1ppm ozonated water, which could be considered sufficient evidence for the efficacy of this antimicrobial intervention.

Even though the intervention did not eliminate completely the aerobic count, total coliforms and *Enterobacteriaceae*, significant reduction of the initial bacterial load in beef primal and subprimal cuts will increase the meat safety and subsequent quality/shelf life of the steaks which are cut from these primals and subprimals. Further studies of interest include those conducted to evaluate the treatment effect on the beef steaks' shelf life. It is important to note that there was no visible organoleptic or physical change to the surface of treated beef cuts that was observed by this research. Organoleptic and physical changes could be the object of a future research, as well.

CONCLUSION

Ozonated water intervention studied in this research shows promises as efficient and environment-friendly method for effectively reducing surface microbial contamination on beef primal and subprimal cuts. Because ozonated water is produced on-site and on demand, it can also reduce the cost of transportation and storage of other antimicrobial agents, and it may reduce the health hazard for workers manipulating chemicals. This simple and safe FDA/USDA approved technology, even though not widely embraced by the U.S. food processors, can be a valuable tool in producing safer, wholesome food products.

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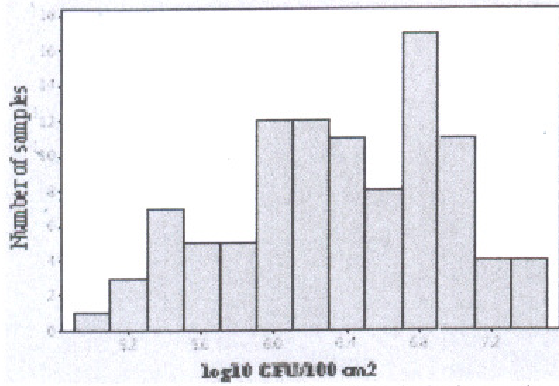

Fig. 1. Aerobic plate counts of beef primal and subprimal cuts, prior to applying the antimicrobial treatment.

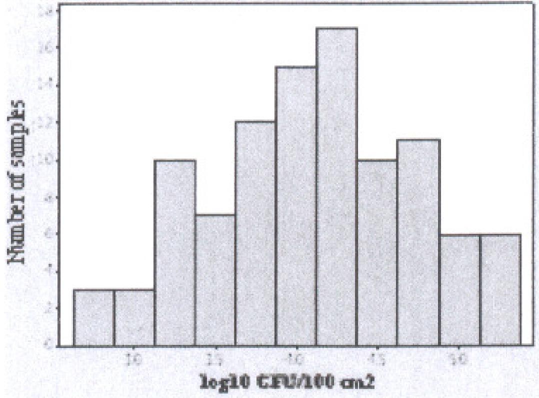

Fig. 2. Aerobic plate counts of beef primal and subprimal cuts, after applying the antimicrobial treatment.

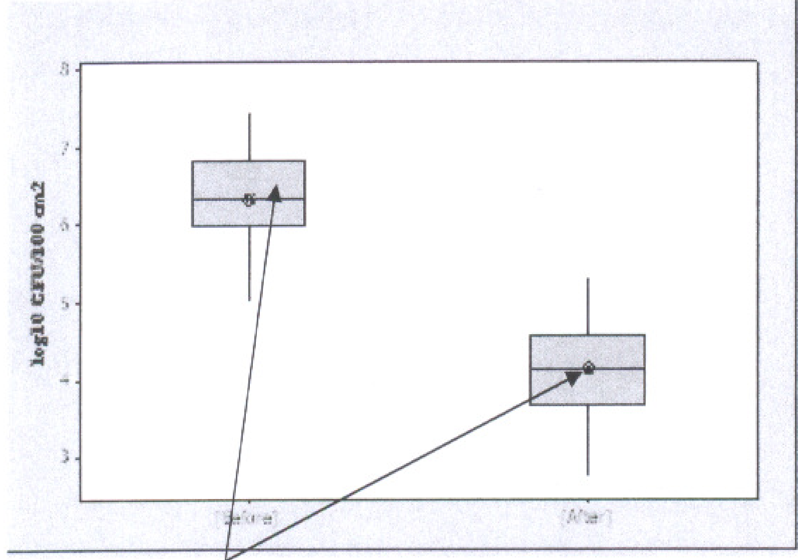

Fig.3. Visual data comparing mean (log_{10} CFU/100 cm^2) of aerobic plate counts from primal and subprimal cuts, before and after applying the antimicrobial treatment.

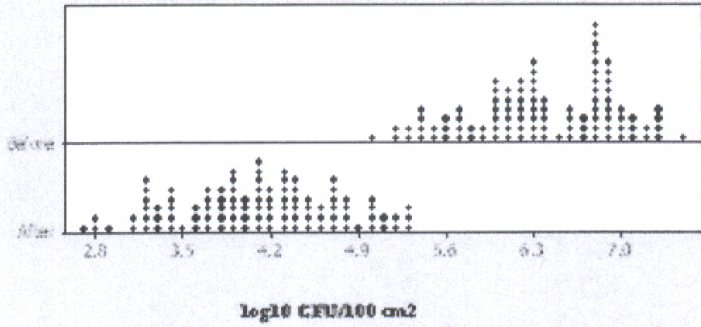

Fig.4. APC log reduction of beef primals and subprimals, as a dot plot representation.

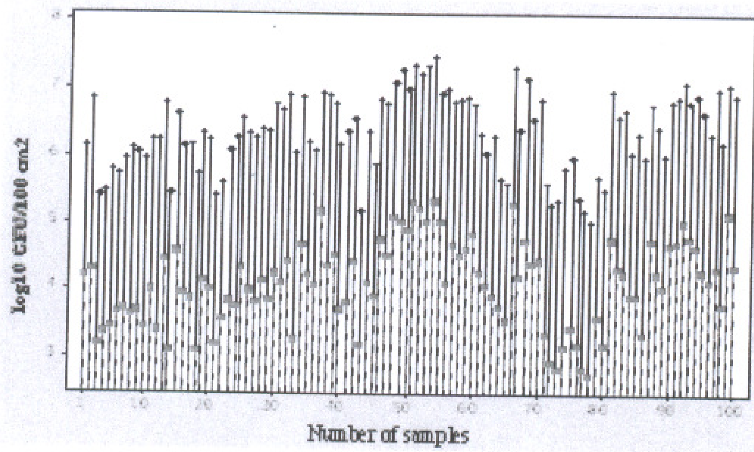

Fig.5. Visual representation of the significant log reduction in all the 100 samples of primals/subprimals.

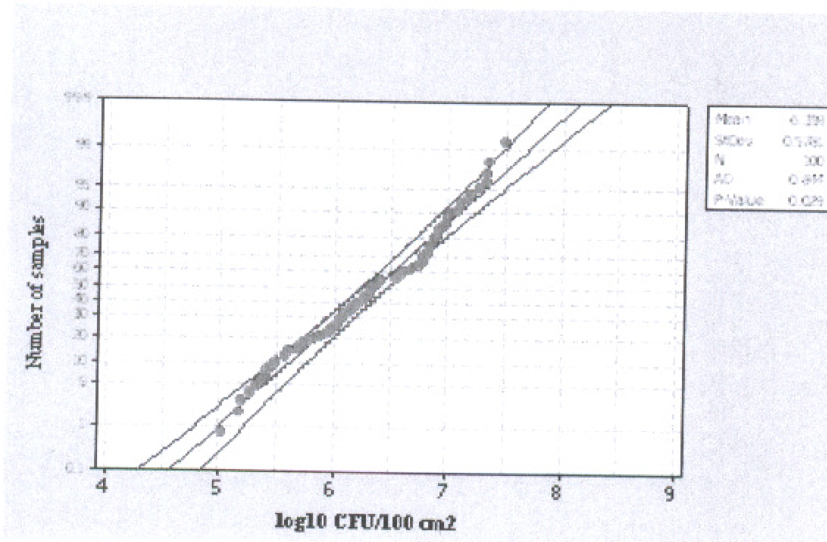

Fig.6. Normal distribution of the samples' APC pre-intervention (95% confidence interval)

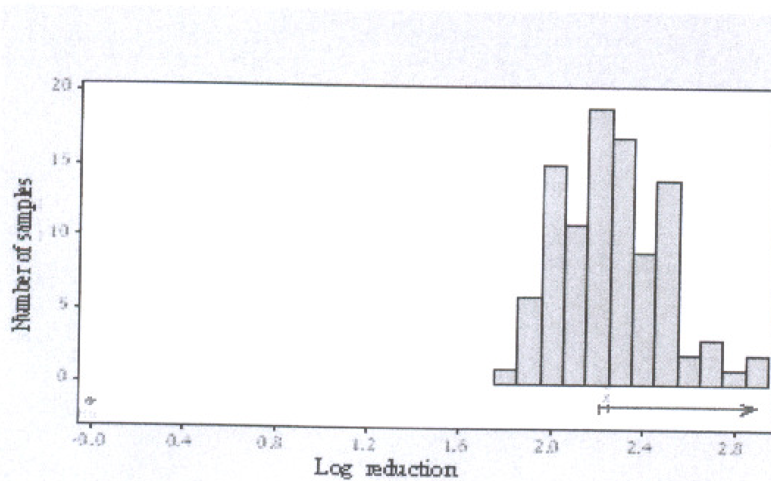

Fig.7. Histogram of difference, indicating the log reduction post-intervention (with Ho and 95% confidence interval for the mean).

Table 1 - Prevalence and mean levels of total plate counts, coliforms, and Enterobacteriaceae on beef primals and subprimals, pre- and post-intervention

	Bacterial mean					
	APC ($\log_{10}$ CFU/100 cm^2)[a]		TCC ($\log_{10}$ CFU/100 cm^2)[b]		EBC ($\log_{10}$ CFU/100 cm^2)[c]	
	Before	After	Before	After	Before	After
Mean	6.36	4.10	2.91	0.60	3.10	0.54

a - prevalent in 100% samples (P=0.000)

b - prevalent in 56% samples (P=0.000)

c - prevalent in 49% samples (P=0.000)