RESISTANCE OF *YERSINIA ENTEROCOLITICA, ESCHERICHIA COLI* O157:H7 AND NATURAL MICROFLORA AGAINST ACIDIC CONDITIONS AND FREEZING-THAWING IN FRESH SAUSAGES

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SUMMARY

The resistance of *Yersinia enterocolitica* O:9, *Escherichia coli* O157:H7 and natural microflora against lactic acid (LA), ascorbic acid (AA), and freezing-thawing in non-inoculated and inoculated fresh sausages was studied. Samples were stored at –18 °C for 28 days and thawed in microwave (MW), at room temperature (RT), in refrigerator (R) and under flowing of tap water (F) on days 7, 14, 21 and 28. Plate Count Agar (PCA), Sorbitol Mac Conkey agar (SMC) and Mac Conkey agar (MC) were used for microbial counts. A maximal reduction of 1.57 log in mesophilic aerobes and no significant changes in total and fecal coliform levels with respect to the initial counts in natural microflora were observed along storage. In inoculated fresh sausages, reductions of 1.37 log on PCA and 2.17 log on SMC were obtained in *E. coli* O157:H7 populations as compared to the control groups on day 0. Similarly, reductions of 1.69 log on PCA and 2.79 log on MC as compared to the initial level were observed in counts of *Y. enterocolitica* inoculated samples. *Salmonella* Anatum, *P. aeruginosa*, *Y. enterocolitica* B1A O:7,8-8-8,19 and *E. coli* non O157:H7 strains were recovered from the natural microflora by enrichment techniques.

Thawing in refrigerator was more frequently related to the best reductions of total mesophilic aerobe, *E. coli* O157:H7 and *Y. enterocolitica* O:9 counts than the other thawing methods. Reductions of microbial populations observed in LA treated samples were similar to those observed in AA treated samples. Although the acidic and freezing treatments might reduce the microbial levels in natural microflora of fresh sausages, they appeared to be ineffective in the total elimination of high inocula of pathogens like *E. coli* O157:H7 and *Y. enterocolitica* O:9.

Key words: fresh sausages, *Escherichia coli* O157:H7, *Yersinia enterocolitica,* natural microflora, freezing, thawing, organic acids

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INTRODUCTION

Examination of frozen foods for the presence of spoilage and pathogenic bacteria has increased in recent years because food service operations and consumers use frozen foods and food ingredients frequently (1). Freezing halts the activities of spoilage microorganisms in and on foods and can preserve some microorganisms for long periods of time. The few outbreaks of food-borne illness associated with frozen foods indicate that some but not all human pathogens are killed by commercial freezing processes (2).

In the process of fabrication and trimming of freshly slaughtered animal carcasses, the surface area of cut muscle is increased and microorganisms become distributed throughout the product. Bacterial contamination is spread further in the grinding process. This is reflected in the higher numbers of microorganisms and shorter shelf life typically observed with fresh ground meat products compared to whole-muscle meat cuts (3). Thus, freezing and subsequent thawing of ground beef may provide an margin of safety against pathogenic bacteria like *Escherichia coli* O157:H7 (4, 5) and *Yersinia enterocolitica* (6) by killing a proportion of cells that may be present.

Besides freezing and thawing procedures, a variety of methods have been developed to reduce bacterial levels on animal carcasses and retail cuts. The most current approach focuses on washing and sanitizing procedures using short-chain organic acids, alone or combined with other methods, due to the inability of microorganisms to tolerate such an environment (7, 8). Acetic, citric, and lactic acids are frequently added to water at concentrations of 2 at 5% to wash and sanitize animal carcasses after slaughter or are sprayed on carcass surfaces to reduce the microbial load and increase product shelf life (9, 10). Alternatively, organic acids contribute to food preservation either as products of natural fermentation of foods or as acidulant additives. Effects of lactic acid on retail cuts of beef (11, 12), carcass meats (13), commercial pork trim (3) and ground beef (14) have been extensively studied. Ascorbic acid has also been suggested as a potential chemical for control of pathogenic microorganisms as *E. coli* O157:H7 in potatoes, apples and juices (7). Previously, Fletcher et al. (15) observed in vitro that *Campylobacter jejuni* was protected from the deleterious action of oxidants by low concentrations of ascorbic acid and was inhibited by higher concentrations. Factors such as microbial types, initial level of contamination, concentration and temperature of application of the chemical agent, and tissue type might influence the effectiveness of these methods (16).

Fresh sausages consumed in Argentina are typically composed by a mixture of raw ground beef and pork with salt and spices. The microbial contamination of these products mainly comes

from the ground beef flora and from the machines and utensils employed in their preparation. Micrococci, significative levels of lactobacilli, pseudomonae, enterobacteria and scarce faecal enterococci have been frequently reported. Occasionally, salmonellae can be found (17). These sausages appear to be suitable for transmission of *E. coli* O157:H7 (18, 19), while the presence of pathogenic *Y. enterocolitica* strains has been demonstrated (20). It is known that organic acids have a lethal effect on organisms in frozen substances (21). Thus, combination of freezing and acid treatment would provide an effective bacterial reduction in these fresh sausages.

The purpose of this work was to assess the inhibiting activity of lactic and ascorbic acids as shown by i) the population changes of the natural microflora in frozen sausages, and ii) the reduction of *E. coli* O157:H7 and *Y. enterocolitica* O:9 in artificially contaminated frozen fresh sausages, stored for 28 days and thawed by four different methods. We also investigated the presence of *Pseudomonas, Salmonella, Yersinia* and *E. coli* O157:H7 species in the indigenous flora of fresh sausages.

MATERIALS AND METHODS

Strains

Two bacterial strains, *Escherichia coli* O157:H7 Sor-/βglu-/E--Hly+/ *eae*+, biotype C, producer of Stx1 and Stx2, from the collection of the National Institute of Infectious Diseases "Dr. Carlos G. Malbrán", Buenos Aires, Argentina, and *Yersinia enterocolitica* O:9 W1024 pYV+, kindly provided by Dr. G. Cornelis (Louvain, Belgium), were used to inoculate fresh sausages. Organisms were maintained at 4 °C on tryptic soy agar slants (TSA, Merck Laboratories, Darmstadt, Germany). Loop inocula were transferred to tryptic soy broth (TSB, Merck) and incubated for 24 h, at 37 °C for *E. coli* O157:H7 and at 22 °C for *Y. enterocolitica*. Consecutive loop transfers of 24-h TSB cultures were made before being used as inocula in experiments. Aliquots of *E. coli* O157:H7, and *Y. enterocolitica* were plated onto Sorbitol Mac Conkey agar (SMC, Merck) and Mac Conkey agar (MC, Merck) respectively, and the culture purity was confirmed by Gram staining and classical biochemical tests. Initial inocula were standardized at OD_{600} 0.2 (Metrolab VD40 Spectrophotometer, Lab. Rodriguez Corswant, Bernal, Argentina) and assessments of their counts were performed by spread plating onto TSA.

Acids

Lactic acid at concentration of 2% (LA, pH 2.15, Parafarm, Buenos Aires, Argentina) and ascorbic acid at 3.4% (AA, pH 2.46, Parafarm) were prepared using sterile distilled water. The acid solutions were maintained at 4 °C during storage prior to dip treatment of samples.

Sample Collection

Fresh sausages were obtained from butcher's shops in San Luis city, Argentina. The ingredients consisted of ground beef 20%, ground pork 75%, NaCl 2% and low concentrations of spices and vinegar, stuffed into bovine casings or edible membranes. The final product pH was 5.7 ± 0.2. A total of nine kilograms including nearly 90 units of 3.5 to 4 cm in diameter by 12 cm in length were cut with a sterile knife to obtain 360 samples of 25 g each and placed into individual sterile plastic bags. They were divided into the following groups: non-inoculated/nonacid-treated (NI/NT, 85 samples), non-inoculated/lactic acid treated (NI/LA, 85 samples), non-inoculated/ascorbic acid treated (NI/AA, 85 samples), *E. coli* O157:H7 inoculated/nonacid-treated (EC/NT, 17 samples), *E. coli* O157:H7 inoculated/lactic acid treated (EC/LA, 17 samples), *E. coli* O157:H7 inoculated/ascorbic acid treated(EC/AA, 17 samples), *Y. enterocolitica* inoculated/nonacid-treated (YE/NT, 17 samples), *Y. enterocolitica* inoculated/lactic acid treated(YE/LA, 17 samples), and *Y. enterocolitica* inoculated/ascorbic acid treated (YE/AA, 17 samples).

Inoculation

Samples of the EC/NT, EC/LA, EC/AA, YE/NT, YE/LA, and YE/AA groups were inoculated with 0.2 ml volume of *E. coli* O157:H7 or *Y. enterocolitica* suspensions prepared to achieve a level of approximately 8×10^5 CFU per gram. They were kept at 4 °C for 12 h to facilitate the bacterial adhesion. Later, samples were dipped in acid solution as above and they were subjected to microbial analysis (day 0) or stored at freezer a –18 °C.

Acid treatment

A 25-ml volume of the corresponding acid solution was added to each one of samples belonging to NI/LA, NI/AA, EC/LA, EC/AA, YE/LA, and YE/AA groups and softly shaken into the plastic bag to facilitate the contact with the sample. After 15 min, liquid was removed and samples were subjected to microbial analysis (day 0) or stored at freezer at –18 °C for up to 28 days.

Thawing Methods

Frozen samples were thawed on 7, 14, 21 and 28 days by the following methods: i) microwave oven 200 w for 1 min (MW), on the counter at room temperature for nearly 50 min (RT), in refrigerator at 7 °C for 4 to 5 h (R), and under flowing of tap water at 20 °C for 25 min (FW).

Microbial Analysis

Total mesophilic aerobe populations, total and fecal coliforms and, *Salmonella, Pseudomonas, E. coli* O157:H7 and *Yersinia* species were investigated in NI/NT, NI/LA and NI/AA groups on day 0 before freezing, and at 7, 14 , 21 and 28 days after freezing. At each sampling time and from each treatment, frozen samples were thawed by four methods described above. Twenty five grams of sample contained in a sterile plastic bag were submerged into 225 ml of 0.1% peptone water pH 7.2 (PW, Merck) and homogenized in a stomacher (IUL Masticator, Koningswinter, Germany) for 1 min. Five serial decimal dilutions were prepared in PW. Volumes of 0.1 ml of each dilution were spread in duplicate on agar plates. Counts of total mesophilic aerobes were performed on plate count agar (PCA, Merck) after incubation at 37 °C for 24 h.

Total coliforms and fecal coliforms were investigated by the three-tube Most Probable Number (MPN) procedure. The sample prepared as described in the former paragraph was used as inoculum. One mililiter from each dilution tube was transferred into Mac Conkey broth (Merck) and incubated at 35 °C for 48 h. One loopful from positive tubes was transferred to brilliant green lactose broth (BGLB, Merck), incubated at 35 °C for 24 h, and to EC broth (Merck) incubated at 44.5 °C for 24 h. The tubes with turbidity and gas formation in BGLB indicated the presence of

coliforms, whereas the positive tubes of EC broth indicated the presence of fecal coliforms. Results were calculated from the MPN table. A loopful of culture from EC broth was streaked on Eosin Methylene Blue agar (EMB, Merck) and incubated at 35 °C for 24 h. Colonies with metallic sheen were picked and transferred to TSA slants. They were Gram stained, seeded on triple sugar iron agar (TSI, Merck) and assayed for IMViC and sorbitol fermentation. Sorbitol non-fermenting isolates were reserved to continue the investigation of *E. coli* O157:H7.

Presence of *Pseudomonas aeruginosa, Salmonella* sp*, E. coli* O157:H7 and *Y. enterocolitica* was investigated from remaining suspensions of samples in plastic bags by using the following enrichment techniques.

P. aeruginosa: 25 g samples were seeded in 225 ml of asparagine broth (Merck) and incubated at 37 °C for 24 h. Isolation was performed on cetrimide agar (Merck) at 37 °C for 48 h. Colonies developing green pigment were selected for confirmation. Casein hydrolysis in milk agar with green pigment production, positive oxidase and Gram negative staining were indicative of *P. aeruginosa*.

Salmonella spp: 25 g samples were seeded into 225 ml of lactose broth (LB, Merck) and incubated 24 h at 37 °C. One-ml aliquots were transferred into two tubes containing 9 ml of selenite broth (Merck) and two tubes with 9 ml of tetrathionate broth (Merck). One tube of each selective broth was incubated 24 h at 37 °C and the other one was incubated 24 h at 42.5 °C. Isolations were done on bismuth sulfite agar (BISA,Merck). Suspect colonies were assayed by Gram staining and classical biochemical tests. Serological confirmation of *Salmonella* strains was made by using polyvalent O-A and O-B antisera (National Institute of Infectious Diseases, Buenos Aires, Argentina). Positive strains were submitted to Dr. R. Terragno, National Institute of Infectious Diseases "Dr. Carlos G. Malbrán", Buenos Aires, Argentina, for definitive serotyping.

E. coli O157:H7: 25 g samples were seeded into 225 ml of EC broth and incubated 2 h at 25 °C. Then, EC was supplemented with 20 mg/l sodium novobiocin (Sigma Chemicals, St. Louis, MO) and 1.12 g/l bile salts (Merck) and enrichment continued at 37 °C for 18 h (1). Isolates were performed on SMC at 37 °C for 24 h and sorbitol non-fermenting colonies were studied by biochemical tests, challenged against O157 antiserum and examined for the presence of *stx1, stx2,* and *rfbO157* gene by multiplex PCR (18).

Yersinia spp: 25 g samples were seeded in phosphate buffered saline pH 7.6 added with 1% sorbitol and 0.15% bile salts and enriched for 21 days at 4 °C. Isolations were performed on MC for 48 h at 22–25 °C. Presumptive *Yersinia* colonies were subjected to Gram staining and biochemical tests. The serological characterization was carried out by Dr. E. Carniel, Pasteur Institute, Paris, France.

Y. enterocolitica strains isolated from NI/NT, NI/LA and NI/AA groups were assayed for autoagglutination (22) and calcium dependence for growth at 37 °C (23).

Countings of total mesophilic aerobes and the artificially inoculated pathogens, *E. coli* O157:H7 or *Y. enterocolitica,* were performed in the EC/NT, EC/LA, EC/AA, YE/NT, YE/LA, and YE/AA groups on day 0 before freezing, and on days 7, 14, 21 and 28 after freezing. At each sampling time and from each treatment, frozen samples were thawed by four methods described above. Processing of samples was carried out as described for non-inoculated groups. Total mesophilic aerobes, *E. coli* O157:H7 and *Y. enterocolitica* were enumerated on PCA at 37 °C for 24 h, SMC at 37 °C for 24–48 h, and MC at 22 °C for 24 h, respectively.

Statistical Analysis

Three replications of each experiment were done. The means of duplicate plate counts were transformed to log CFU/g. The values of MPN/g corresponding to total and fecal coliforms were also transformed to logs. Data were compared by analysis of variance with Statistix version 3.5 software and Student's *t*-test using $p < 0.05$ as level of significance.

The reductions of the initial counts for each treatment group (NT, LA and AA) as a function of time and thawing method were obtained by the difference:

$[\log CFU/g_{\text{day 0}} - \log CFU/g_{\text{day x, n thaw method}}]$, being x: 7, 14, 21 or 28 days, and n: MW, RT, R or F.

RESULTS AND DISCUSSION

According to the Argentine Alimentary Code (AAC), the maximum value admitted for mesophilic aerobes in fresh sausages is 10^7 CFU/g. In this work, total mesophilic aerobes in non acid-treated samples were initially 7.17 log CFU/g, decreased on days 7 and 14 with respect to day 0, and increased on days 21 and 28 for matching the initial level. A maximal reduction of 1.57 log on day 7 with respect to the initial count was observed in nonacid treated samples thawed in refrigerator (Table 1).

LA and AA led to significant reduction of mesophilic aerobes on day 0, as compared with counts in non acid-treated samples. Thawing in refrigerator in combination with LA proved to be more effective than other combinations in controlling mesophilic aerobe population. Reductions of 0.61, 1.22 and 0.60 log with respect to day 0 counts were obtained on days 14, 21 and 28 respectively, in samples treated with this combination.

Prasai et al (25) observed a significant difference of aerobic plate counts on vacuum-packaged beef subprimals which received 1.5% lactic acid spray treatments and underwent 28 days of –1.1 °C storage, when compared to untreated controls. They concluded that although organic acid decontamination may not dramatically reduce bacterial contamination from meat surfaces, it can improve the microbial quality of meat.

Some non significant reductions in relation to the total coliform initial count were observed on day 7 in non acid-treated samples thawed in MW (0.70 log) and on day 28 in non acid-treated samples thawed in refrigerator (1.98 log) (Table 2).

Though AAC admits a maximum of 500 *E. coli*/g in this kind of food, presence of *E. coli* nonO157:H7 strains in these samples would be related to the deficient hygienic quality of fresh sausages. Fecal coliforms were not detected on day 28 in the LA treated group. Neither were they detected on day 21 in AA treated samples thawed under flowing of tap water nor on day 28 in AA treated samples thawed at room temperature and under flowing of tap water.

PCA significantly recovered more *E. coli* O157:H7 cells ($p < 0.05$) than SMC from samples artificially contaminated with this microorganism (Table 3). On PCA, significant *E. coli* O157:H7 reductions were observed throughout the experiment. Highest

Table 1. *Counts of total mesophilic aerobes in natural microflora of noninoculated fresh sausages*

Time (days)	Thawing method	Total mesophilic aerobes (log CFU/g) Mean/SD		
		NT	LA	AA
0		7.17/0.70^{A,1}	5.95/0.42^{B,1}	5.90/1.08^{B,1}
7	MW	6.11/0.91^{A,2,a}	5.62/0.60^{A,1,a}	5.55/ 0.42^{A,1,a}
	RT	6.20/0.18^{A,2,a}	6.15/0.89^{A,1,a}	5.93/0.96^{A,1,a}
	R	5.60/0.44^{B,2,b}	6.42/0.75^{A,1,a}	5.56/0.39^{B,1,a}
	F	6.14/0.60^{A,2,a}	6.17/0.79^{A,1,a}	6.16/0.84^{A,1,a}
14	MW	5.62/1.07^{A,2,a}	5.50/0.99^{A,1,a}	5.60/1.46^{A,1,a}
	RT	5.94/1.40^{A,2,a}	6.08/0.98^{A,1,a}	6.10/1.13^{A,1,a}
	R	5.95/1.40^{A,1,a}	5.34/0.63^{A,1,a}	5.80/1.30^{A,1,a}
	F	5.93/1.14^{A,2,a}	6.08/1.48^{A,1,a}	6.11/1.17^{A,1,a}
21	MW	6.30/0.88^{A,2,a}	5.67/0.92^{A,1,a}	5.65/1.23^{A,1,a}
	RT	6.18/0.18^{A,2,a}	6.42/0.90^{A,1,a}	6.29/1.06^{A,1,a}
	R	6.34/1.06^{A,1,a}	4.73/0.41^{B,2,a}	5.68/1.21^{A,1,a}
	F	6.85/0.33^{A,1,a}	6.08/0.55^{A,1,a}	5.70/1.40^{A,1,a}
28	MW	5.85/1.21^{A,2,a}	5.61/1.17^{A,1,a}	5.30/1.16^{A,1,a}
	RT	6.90/0.36^{A,1,a}	6.08/1.04^{A,1,a}	5.82/0.89^{A,1,a}
	R	5.94/1.06^{A,2,a}	5.35/1.01^{A,1,a}	5.68/1.29^{A,1,a}
	F	6.73/0.72^{A,1,a}	5.78/0.90^{A,1,a}	6.56/1.34^{A,1,a}

NT = nonacid treated samples, LA = lactic acid treated samples, AA = ascorbic acid treated samples. MW = microwave, RT = room temperature, R = refrigerator, F = flowing of tap water. Values are means of three trials. For a given combination of time and thawing method (row), means followed by different capital letters indicate that acid treated groups are significantly ($p < 0.05$) different from the NT group. For a given combination of NT, LA or AA treatment and thaw method (column), means followed by different number indicate significant differences ($p < 0.05$) between storage days. For a given combination of time and NT, LA or AA treatment (column), means followed by different small letter indicate significant differences ($p < 0.05$) between thawing methods

reductions were observed on day 28 in non acid-treated and LA--treated samples thawed in refrigerator, whose counts were 1.32 and 1.37 log lower than the respective initial count. On SMC, a significant decrease of 2.17 log was observed on day 21 in non acid-treated samples thawed in refrigerator. *E. coli* O157:H7 populations exhibited maximum reductions of 1.95 log by LA and 1.79 log by AA on day 21 in samples thawed in MW.

Factors contributing to the *E. coli* O157:H7 survival may be related to the protein content of the food. Food proteins exert a cryoprotective effect by forming hydrogen bonds with microbial proteins, thus stabilizing and protecting them against denaturation (26).

Regarding *Y. enterocolitica* artificially contaminated samples, PCA significantly recovered ($p < 0.05$) more cells than MC (Table 4). On PCA, maximum reductions were obtained on day 28 in LA-treated samples thawed in MW (1.64 log with respect to the initial count for this group) and in refrigerator (1.69 log reductions). On MC, best reductions were observed in non acid--treated samples thawed in MW on day 28 (2.79 log), and in AA treated samples thawed at room temperature on day 21 (2.21 log) as compared with the respective control on day 0.

Seven *Salmonella anatum* strains, eleven *P. aeruginosa* strains, three non-autoagglutinable and non-Ca^{2+} dependent growth *Y. enterocolitica* B1A O:7,8-8-8,19 strains and several *E. coli* strains not belonging to the O157:H7 serotype were recovered from the indigenous flora of sausages by enrichment techniques.

AAC recommends tolerance zero for *E. coli* O157:H7 and *Salmonella* spp in fresh sausages. *Salmonella anatum* isolated in this study might be potentially pathogenic since this species has been associated with different foodborne outbreaks (27, 28). Sorensen et al (29) have reported *Salmonella anatum* isolates from ground beef, a component of fresh sausages.

P. aeruginosa is a food spoilage microorganism and an opportunistic pathogen for human that could be affected in frozen foods

Table 2. *Enumeration of total and fecal coliforms in natural microflora of noninoculated fresh sausages*

Time (days)	Thawing method	Total coliforms (log MPN/g)			Fecal coliforms (log MPN/g)		
		NT	LA	AA	NT	LA	AA
0		3.66/0.37^{A,1}	2.74/0.70^{B,2}	2.80/0.41^{B,2}	2.42/0.86^{A,2}	1.83/0.20^{AB,3}	1.53/1.33^{AB,2}
7	MW	2.96/0.01^{B,2,b}	2.34/0.96^{C,2,b}	3.54/0.20^{A,1,a}	2.64/0.30^{A,2,a}	2.20/0.75^{AB,2,a}	2.37/0.70^{AB,2,a}
	RT	3.30/1.01^{A,1,a}	3.31/0.42$^{A,1–2,a}$	3.67/0.63$^{A,1–2,a}$	2.51/0.88^{A,2,a}	2.64/0.21^{A,2,a}	2.61/1.23^{A,2,a}
	R	3.46/0.20^{A,1,a}	3.17/0.76$^{A,1–2,a}$	3.07/0.54$^{A,1–2,ab}$	2.72/0.32^{A,2,a}	2.65/0.30^{A,2,a}	2.12/0.45^{B,2,a}
	F	3.40/0.24^{A,1,a}	2.97/0.34^{AB,2,a}	3.17/0.76$^{A,1–2,ab}$	2.15/0.73^{A,2,a}	2.11/1.83$^{A,2–3,a}$	2.33/0.28^{A,2,a}
14	MW	3.41/1.76^{A,1,a}	3.01/0.92^{A,1,a}	2.50/1.26^{A,2,ab}	2.37/1.09^{A,2,a}	2.28/0.97^{A,2,b}	2.50/1.26^{A,2,a}
	RT	2.61/1.43^{B,1,ab}	2.47/2.08$^{B,1–2,a}$	3.33/0.51$^{A,1–2,a}$	2.01/0.76$^{A,2–3,a}$	1.73/1.04^{A,3,b}	2.33/0.51^{A,2,a}
	R	3.13/0.24^{B,1,a}	3.71/0.46^{A,1,a}	2.77/0.85^{C,2,b}	2.44/0.15^{A,2,a}	3.33/0.99^{A,1,ab}	2.77/0.85^{A,1,a}
	F	3.48/0.17^{A,1,a}	3.18/1.21$^{A,1–2,a}$	2.41/0.65^{B,2,b}	2.25/0.42^{A,2,a}	2.63/0.44^{A,2,b}	1.92/0.62^{B,2,ab}
21	MW	3.22/0.81^{A,1,a}	2.98/1.25^{A,1,a}	2.98/0.92$^{A,1–2,a}$	3.09/0.64^{A,1,a}	2.45/0.83^{A,2,a}	2.15/0.60^{A,2,a}
	RT	3.41/0.64^{A,1,a}	2.46/0.^{71AB,2,b}	2.91/0.63$^{A,1–2,a}$	2.96/0.01^{A,1,a}	2.25/0.99^{A,2,a}	1.27/1.80^{A,2,a}
	R	3.78/1.11^{A,1,a}	2.95/0.51$^{A,1–2,a}$	2.37/0.69^{B,2,a}	2.78/0.18$^{A,1–2,a}$	2.36/0.24^{B,2,a}	2.37/0.70$^{B,1–2,a}$
	F	2.02/2.85$^{A,1–2,a}$	2.25/0.42^{A,2,b}	1.68/2.37$^{A,2–3,a}$	1.58/0.50^{A,3,b}	0.98/1.38^{A,3,ab}	ND
28	MW	3.36/0.53^{A,1,a}	3.48/1.03^{A,1,a}	3.50/0.47^{A,1,a}	3.37/0.37^{A,1,a}	3.08/0.67^{A,1,a}	2.97/0.46^{A,1,a}
	RT	3.32/0.77^{A,1,a}	2.21/1.05^{AB,2,ab}	2.67/0.28^{B,2,b}	3.13/0.24^{A,1,a}	2.21/1.05^{A,2,ab}	ND
	R	1.68/2.37$^{A,1–2,b}$	2.04/1.77$^{A,1–2,ab}$	1.77/1.56^{A,2,b}	1.40/1.98$^{A,2–3,ab}$	1.58/1.38$^{A,2–3,b}$	1.50/1.33^{A,2,a}
	F	2.83/0.29^{A,1,ab}	1.27/1.80$^{A,2–3,b}$	1.68/2.37$^{A,2–3,b}$	2.40/0.32^{A,2,ab}	ND	ND

MPN = Most Probable Number, ND = no development. Other references: as described in footnote of Table 1.

Table 3. *Enumeration of total mesophilic aerobes and E. coli O157:H7 in inoculated fresh sausages*

Time (days)	Thawing method	Total mesophilic aerobes (log MPN/g)(PCA)			*E. coli* O157:H7 (log MPN/g) (SMC)		
		NT	LA	AA	NT	LA	AA
0		6.63/0.28^{A,1}	5.90/0.22^{B,1}	6.00/0.48^{B,1}	6.44/0.20^{A,1}	5.60/0.32^{B,1}	5.69/0.26^{B,1}
7	MW	6.19/0.32^{A,2,a}	5.72/0.41^{B,1,b}	5.43/0.24^{B,2,c}	6.23/0.37^{A,1,a}	4.96/0.34^{B,2,a}	5.11/0.46^{B,2,a}
	RT	6.67/0.75^{A,1,a}	6.33/0.82^{A,1,a}	5.74/0.60^{B,1,b}	5.97/0.25^{A,1,a}	5.01/0.31^{B,2,a}	5.24/0.16^{B,2,a}
	R	6.30/0.68^{A,1,a}	5.40/0.85^{B,1,c}	5.22/0.16^{B,2,d}	5.77/0.61^{A,2,ab}	4.92/0.43^{B,2,a}	4.84/0.32^{B,2,ab}
	F	6.68/0.89^{A,1,a}	6.41/0.61^{A,2,a}	6.51/0.62^{A,2,a}	5.92/0.47^{A,1,a}	4.40/0.67^{C,2,a}	5.18/0.26^{B,2,a}
14	MW	6.03/0.42^{A,2,a}	5.38/0.64^{A,1,a}	5.58/0.55^{A,2,a}	5.30/0.39^{A,2,a}	4.56/0.17^{A,2,a}	4.53/0.27^{A,3,a}
	RT	5.60/0.90^{A,2,a}	5.35/0.81^{A,2,a}	5.48/0.92^{A,1,a}	5.10/0.23^{A,2,a}	4.58/0.89^{A,2,a}	4.94/0.32^{A,2,a}
	R	6.33/0.81^{A,1,a}	5.50/0.82^{A,1,a}	5.79/0.73^{A,1,a}	5.10/0.61^{A,2,a}	4.43/0.10^{A,3,a}	4.63/0.36^{A,2,a}
	F	6.10/0.58^{A,1,a}	4.81/0.38^{B,3,a}	5.75/0.61^{A,1,a}	5.30/0.30^{A,2,a}	4.32/0.31^{B,2,a}	5.05/0.19^{A,2,a}
21	MW	6.39/0.60^{A,1,a}	5.63/0.60^{A,1,a}	5.66/0.93^{A,2,a}	4.46/0.37^{A,3,bc}	3.65/0.40^{B,3,a}	3.90/0.43^{B,4,a}
	RT	6.07/0.30^{A,2,a}	4.97/0.27^{B,2,a}	4.54/0.49$^{B,2–3,b}$	5.30/0.13^{A,2,a}	3.98/0.33^{B,2,a}	4.10/0.16^{B,4,a}
	R	5.60/0.27^{A,2,b}	5.91/0.89^{A,1,a}	5.43/0.59^{A,2,a}	4.27/0.72^{A,2,c}	4.19/0.40^{A,3,a}	4.20/0.47^{A,2,a}
	F	5.58/0.29^{A,1,b}	4.92/0.26^{B,3,a}	5.23/0.34^{A,2,a}	4.78/0.39$^{A,2–3,b}$	3.97/0.11^{B,2,a}	4.38/0.35^{A,3,a}
28	MW	5.64/0.45^{A,2,ab}	5.10/0.48^{AB,2,a}	5.34/0.31^{A,2,a}	4.58/0.86$^{A,2–3,a}$	3.92/0.27^{B,3,a}	4.35/0.35^{A,3,a}
	RT	6.16/0.89^{A,2,a}	5.10/0.93^{B,2,a}	4.98/0.53^{B,2,a}	4.82/0.74^{A,2,a}	4.23/0.06^{A,2,a}	4.32/0.27^{A,4,a}
	R	5.31/0.32^{A,2,b}	4.53/1.03^{A,2,b}	4.85/0.99^{A,2,b}	5.38/0.47^{A,2,a}	4.20/0.23^{B,3,a}	4.21/0.56^{B,2,a}
	F	5.97/0.32^{A,1,a}	5.23/0.47^{A,3,a}	5.32/0.72^{A,2,a}	4.83/1.01$^{A,2–3,a}$	3.95/0.70^{A,2,a}	4.25/0.52^{A,3,a}

PCA = Plate Count Agar, SMC = Sorbitol Mac Conkey agar. Other references = as described in footnote of Table 1.

Table 4. *Enumeration of total mesophilic aerobes and Y. enterocolitica W1024 O:9 in inoculated fresh sausages*

Time (days)	Thawing method	Total mesophilic aerobes (log MPN/g)(PCA)			Y. enterocolitica W1024 O:9 (log MPN/g) (MC)		
		NT	LA	AA	NT	LA	AA
0		7.52/0.66^{A,2}	6.96/0.94^{A,1}	7.42/0.89^{A,1}	6.25/0.34^{A,1}	4.98/0.20^{B,1}	5.31/0.38^{B,1}
7	MW	7.29/0.19^{A,2,a}	7.23/0.78^{A,1,a}	7.18/1.55^{A,1,a}	5.43/0.60^{A,2,a}	4.20/0.47^{B,2,b}	4.69/0.58^{B,1,a}
	RT	7.40/1.02^{A,2,a}	7.45/1.06^{A,1,a}	7.23/1.16^{A,1,a}	5.46/0.48^{A,2,a}	4.17/0.52^{B,2,b}	4.63/0.85^{B,2,a}
	R	7.64/1.27^{A,2,a}	6.76/1.10^{A,1,ab}	7.45/1.02^{A,1,a}	4.80/0.37^{A,2,a}	4.02/0.37^{B,2,b}	4.26/0.11^{B,2,b}
	F	7.36/1.86^{A,2,a}	7.73/0.66^{A,2,a}	7.41/1.13^{A,1,a}	5.09/1.03^{A,2,a}	4.72/0.41^{A,1,a}	4.71/0.77^{A,2,a}
14	MW	6.70/2.17^{A,2,a}	6.44/0.83$^{A,1–2,a}$	7.31/0.59^{A,1,a}	4.44/0.51^{AB,3,a}	3.58/0.39^{B,3,a}	4.83/0.76^{A,1,a}
	RT	7.22/1.73^{A,2,a}	6.59/1.26$^{A,1–2,a}$	7.38/0.59^{A,1,a}	4.50/0.50^{A,3,a}	3.85/0.47^{A,2,a}	3.90/0.26^{A,3,b}
	R	7.31/1.50^{A,2,a}	6.32/0.67^{A,2,a}	7.30/1.12^{A,1,a}	4.84/0.19^{A,2,a}	3.40/0.16^{C,3,a}	4.10/0.28^{B,2,b}
	F	7.39/1.14^{A,2,a}	6.55/0.94^{A,1,a}	6.60/0.89$^{A,1–2,ab}$	4.28/0.47^{A,3,a}	3.52/0.15^{B,2,a}	3.66/0.31^{AB,3,b}
21	MW	7.61/1.80^{A,2,ab}	6.06/0.83^{A,2,a}	6.57/0.96^{A,2,a}	4.30/1.12^{A,3,a}	3.24/0.27^{A,3,a}	3.62/0.70^{A,2,a}
	RT	8.25/0.59^{A,1,a}	6.57/0.82$^{B,1–2,a}$	6.88/1.22^{B,2,a}	4.63/0.28^{A,3,a}	3.91/0.53^{B,2,a}	3.10/0.28^{C,4,a}
	R	7.70/1.41$^{A,1–2,ab}$	6.17/0.33^{B,2,a}	6.68/0.63^{AB,2,a}	4.23/0.34^{A,3,a}	3.53/0.60^{B,3,a}	3.60/0.36^{B,3,a}
	F	7.80/0.75^{A,2,ab}	6.48/0.58^{A,1,a}	6.66/0.98$^{A,1–2,a}$	4.47/0.35^{A,3,a}	3.70/0.35^{B,2,a}	3.26/0.12^{B,4,a}
28	MW	6.66/1.76^{A,2,ab}	5.32/1.02$^{A,2–3,ab}$	6.12/1.64^{A,2,a}	3.46/0.72^{A,4,a}	3.55/0.41^{A,3,a}	3.50/0.15^{A,2,a}
	RT	7.81/0.85^{A,2,a}	6.45/0.91$^{B,1–2,a}$	6.28/1.06^{B,2,a}	4.43/0.40^{A,3,a}	3.58/0.29^{B,3,a}	3.58/0.48^{B,3,a}
	R	6.32/2.04$^{A,1–2,ab}$	5.27/1.65$^{A,2–3,ab}$	6.20/1.66^{A,2,a}	3.74/0.40^{A,3,a}	3.31/0.69^{A,3,a}	3.61/0.22^{A,3,a}
	F	7.40/1.50^{A,2,a}	6.22/1.53^{A,1,a}	6.12/1.17^{A,2,a}	3.60/0.01^{A,4,a}	3.26/0.29$^{A,2–3,a}$	3.38/0.53$^{A,3–4,a}$

PCA = Plate Count Agar, MC = Mac Conkey agar. Other references: as described in footnote of Table 1.

(30). The *Y. enterocolitica* strains isolated in this study belonged to the 1A biotype. Tennant et al. (31) have reported that some strains of this biotype might cause gastrointestinal infections in humans by mechanisms independent of the pYV plasmid or of other known virulence determinants. At least two outbreaks of gastrointestinal infection due to biotype 1A yersiniae have been reported (32, 33).

Sage and Ingham (4) investigated the lethality of freezing on *E. coli* O157:H7 in ground beef patties after thawing by different methods. These authors found that the death of the pathogen

varied depending on the strain tested, the method used to enumerate survivors, and the method used for thawing. Finally, they reported that there was seldom a statistically significant ($p < 0.05$) difference between applied thawing treatments. In the present study, thawing in refrigerator was more frequently related to the best reductions of total mesophilic aerobe, *E. coli* O157:H7 and *Y. enterocolitica* O:9 counts than the other thawing methods.

Regarding acid treatments, reductions of microbial populations observed in LA treated samples were similar to those observed in AA treated samples. As reviewed by Doores (34), lactic acid is able to inhibit the growth of many types of food spoilage bacteria, including gram-negative species of the families *Enterobacteriaceae* and *Pseudomonaceae*. The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force (35). The antioxidant properties of ascorbic acid are well known. In a study based on the antimicrobial effects of a 3.4% ascorbic acid treatment on apple slides, Burnham et al. (7) postulated that the acidity of the ascorbic acid solution was low enough to eliminate some of the weaker *E. coli* O157:H7 cells and sensitized others so they were unable to survive heat stress.

The present study suggests that organic acids combined with freezing-thawing treatment on fresh sausages might inhibit the low number of pathogenic microorganisms belonging to the indigenous flora during storage time, if they were present. Thus, *Salmonella anatum* strains were isolated on day 0 before freezing and no *Salmonella* spp. strain was subsequently recovered. Although the acidic and freezing treatments reduced the mesophilic aerobe loads of fresh sausages, they appeared to be ineffective in the total elimination of high inocula of pathogens like *E. coli* O157:H7 and *Y. enterocolitica* O:9.

These results allowed to relate the microbiological quality of fresh sausages consumed in this region with the manufacturing and preservation techniques commonly applied on these products at level of industry, butcher's shop or home. Most effective evaluation of such processes by sanitary authorities might contribute to improve the microbiological quality of fresh sausages.

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WORLD HEALTH ORGANIZATION DIRECTOR-GENERAL TOURS AFFECTED AREAS, PLEDGES SUPPORT, AS SRI LANKA VOWS TO REBUILD IN THE WAKE OF THE TSUNAMI

Colombo, Sri Lanka – Amid the ruins of a hospital in the south--eastern Sri Lankan town of Kalmunai, the World Health Organization Director-General, Dr LEE Jong-wook, again witnessed at first hand the devastation wrought by tsunami. An operating theatre torn apart, walls of brick and stone shredded as though they were made of paper, an ambulance dangling precariously from the edge of a newly-formed crater in the hospital driveway.

As he had in Aceh, Dr Lee praised the resilience with which people have responded to the tsunami and begun to rebuild shattered lives and communities. In a meeting with President Chandrika Kumaratunga, he said that Sri Lanka is a strong nation and praised the fact that even such an unprecedented disaster has "not broken the country's back".

Dr Lee was in Sri Lanka on the second stage of a visit to two of the worst-affected countries in the disaster, arriving from Indonesia. Dr Lee was accompanied by Sri Lankan Health Minister Nirmal Siripala de Silva and the WHO Representative in Sri Lanka, Dr Kan Tun. Together, they toured Kalmunai as well as the devastated southern town of Galle.

During his visit to Sri Lanka, to help assess the damage done by last week's tsunami and the health response so far, Dr Lee visited temporary relief camps set up in schools and other buildings. He witnessed the aid efforts that are taking place, including the rehabilitation of clinics and child health services.

Together with the Minister of Health, he discussed the action that WHO and its partners have already taken, and what now needs to be done. WHO's work is particularly focused on building up an early warning system for disease surveillance, which needs to be reinforced in the weeks and months ahead.

WHO's Sri Lanka action strategy targets approximately 1 million affected people, across 13 districts along the northern, eastern and southern coastline. WHO is working with the Ministry of Health and other agencies and has provided supplies to help reduce the risk of disease outbreaks, including water purification tablets, testing kits, and materials to eradicate mosquitoes and reducing the number of flies, especially at relief camps with poor sanitation. WHO is also working to rebuild vital health infrastructure, such as hospitals, clinics, pharmacies and medical stores that were washed away along the coast or badly damaged when the waves struck.

The President stressed the need to focus on mental health, and requested WHO's support in helping Sri Lanka maintain a "disaster management centre" in the future. The centre will help with crisis management in situations such as outbreaks, drought, floods, and natural disasters.

WHO has allocated more than US$1 million for Sri Lanka at this time, with a far greater sum to be allocated and raised via donors in the weeks and months to come. WHO has appealed for 12.5 million dollars to help Sri Lanka recover from the devastation of the tsunami over the next six months.

WHO thanks the many governments and others who have given their support to the relief effort. WHO also thanks the many individuals who have given donations via our website, www.who.int.

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