The Effect of Acid Adaptation on Pathogenic Bacteria Used as Challenge Organisms for Microbial Validation of Biltong Processing

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Introduction

Biltong is a popular South African 'air-dried' meat product usually made from lean strips of beef marinaded in traditional spices (coriander, black pepper), salt, and vinegar and dried at ambient temperature and humidity.

In the United States, the USDA-FSIS requires the use of ≥ 160ºF heat and 90% relative humidity to accomplish adequate reduction of pathogens on dried beef products for consumption. If these parameters are not met, as with biltong processing, a microbial validation study must be provided to demonstrate that sufficient bacterial reductions of a 'pathogen of concern' can be achieved during processing.

Since biltong processing is significantly different than beef jerky, USDA-FSIS provided 2 alternative processes by which processors could manufacture and sell biltong:

1) Test every lot of edible ingredient for *Salmonella* prior to use (must test negative) *and* use a process that is validated to provide ≥ 2-log reduction of a pathogen of concern (i.e., *Salmonella)*, or

2) Use a biltong process that is validated to give ≥ 5-log reduction of a pathogen of concern (*Salmonella*).

In discussions with USDA-FSIS on what they require in 'microbial validation studies', one of the required parameters was the use of 'acid-adapted cultures' during validation studies with acidic foods are 'highly recommended' by USDA-FSIS or they may not consider the process properly validated. Acid-adaptation was a condition demonstrated in the 1980's whereby pathogens grown in the presence of 1% glucose would produce acid and lower the pH and be 'acid-adapted'. USDA-FSIS believed that acid-adapting cultures intended for product inoculation would harden the organisms against sensitivity to acidic conditions that they would meet during biltong processing and ensure that the process is sufficiently robust when targeting a 5-log reduction of the pathogenic challenge organisms and have applied this to the validation of many food processes, including biltong.

Communication with USDA-FSIS officials indicated that research data demonstrating the importance of acid-adaptation, if proven, would move USDA-FSIS policy to require acid-adapted cultures for industry process validation. The objective of this study is to determine whether acid-adaptation desensitizes pathogenic organisms during biltong processing compared to non-adapted cultures.

Methodology

Culture Cocktail Preparation (Trials 1a and 1b). *Salmonella* serovars were inoculated into tryptic soy broth (TSB; 0% glucose) and grown at 37℃ for 24 hr. After 24 hr, cultures were transferred into 200 mL bottles containing either TSB containing 1% glucose or TSB containing 0% glucose and grown at 37℃ for 24 hr. All bottles were centrifuged for 20 min at 8000 rpm. The inoculum tested are listed in Table 1.

- *Salmonella* spp. grown in TSB containing 1% glucose represents the acid-adapted culture cocktail.
- *Salmonella* spp. grown in TSB containing 0% glucose represents that non-acid-adapted culture cocktail.

Culture Cocktail Preparation (Trials 2a and 2b). *Salmonella* spp. was inoculated into 0% TSB and grown at 37℃ for 24 hours from frozen stock. *Listeria monocytogenes* spp. was inoculated into TSB and grown at 30℃ for 24 hours from frozen stock. After 24 hours, cultures were transferred into 200 mL bottles containing either TSB containing 1% glucose or TSB containing 1% glucose and sodium phosphate buffer. Cultures were grown at 37℃ and 30℃, respectively, for 24 hours. All bottles were centrifuged for 20 minutes at 8000 rpm. The inoculum tested are listed in Table 1.

- *Salmonella* spp. and *L. monocytogenes* spp. grown in TSB containing 1% glucose represent the acid-adapted culture cocktails.
- *Salmonella* spp. and *L. monocytogenes* spp. grown in TSB containing 1% glucose and sodium phosphate buffer represent the non-acid-adapted culture cocktails.

Sodium Phosphate Buffer. The 0.05M sodium phosphate buffer used for this study was made using bibasic sodium phosphate (heptahydrate) and monobasic sodium phosphate (monobasic). The sodium phosphate buffer utilized the corresponded to the pH 7 and used four-times the concentration (i.e., 6.2gms/100 mL dibasic and 1.24gms/100 mL monobasic buffer).

Table 1. List of strains used as challenge organisms for biltong processing in this study

Organism/Serovar	Strain	Antibiotic Resistance	Source
Salmonella I 4, [5],12:i: :-	FSIS-1	SPC, 5; CC, 5; NB, 50	USDA-FSIS
Salmonella heidelberg	F5038B91	SPC, 5; CC, 5; NB, 50	Muriana Culture Collection
Salmonella hadar	MF60404	SPC, 5; CC, 5; NB, 50	Muriana Culture Collection
Salmonella typhimurium	H3380	SPC, 5; CC, 5; NB, 50	Muriana Culture Collection
Salmonella thompson	120	SPC, 5; CC, 5; NB, 50	Muriana Culture Collection
Salmonella enteritidis	H3527	SPC, 5; CC, 5; NB, 50	Muriana Culture Collection
Listeria monocytogenes	SCA-2	S, 100; RIF, 10	Muriana Culture Collection
Listeria monocytogenes	V7-2	S, 100; RIF, 10	Muriana Culture Collection
Listeria monocytogenes	382-2	S, 100; RIF, 10	Muriana Culture Collection
Listeria monocytogenes	39-2	S, 100; RIF, 10	Muriana Culture Collection

Methodology (cont.)

Table 2. Amounts for 0.05M (1x) sodium phosphate buffer used for biltong processing in this study

pH	Dibasic	gm/100 mL	Mono-basic	gm/100 mL
6.6	0.375	0.95	0.625	0.50
6.8	0.490	1.24	0.510	0.40
7.0	0.610	1.55	0.390	0.31
7.2	0.720	1.82	0.280	0.22
7.4	0.810	2.05	0.190	0.15

Beef Sample Preparations and Inoculations. USDA select-grade (or no roll) boneless bottom rounds were obtained from a local meat processor (Ralph's Perkins, OK, USA). Beef rounds were trimmed and cut into approximately 5.1-cm wide x 1.9-cm thick x 7.6-cm long beef squares. The inoculum suspension (150 µL) was applied to each side of the beef pieces and immediately spread with a gloved finger and then allowed to incubate for 30 min at 5℃ to allow for bacterial attachment.

Biltong Processing, Marination and Drying. Following attachment, the beef pieces were then dipped in sterile water or 5% lactic acid for 30 seconds and placed into a chilled metal tumbling vessel containing a biltong marinade of 2.2% salt, 0.8% black pepper, 1.1% coarse ground coriander, and 4% red wine vinegar (100-grain; 10% acetic acid) in relation to the total meat weight. Beef pieces were vacuum tumbled (15 inches Hg) in a vacuum-tumbler for 30 min and then hung to dry in a humidity-controlled oven at 55% relative humidity and 24.9℃ (75°F) for 8-10 days.

Microbial sampling and Enumeration. Microbial enumeration of surviving bacteria was performed post-inoculation, post-marination, and after 2-, 4-, 6-, 8-, and 10 days of drying for each individual surrogate organism. At each respective time point, beef samples were stomached with 100 mL of neutralizing buffer peptone water (nBPW) then serially diluted, plated on antibiotic-containing media, and incubated at 37ºC and 30ºC, respectively, for 48 hours before enumeration. Trials were performed in duplicate replication with triplicate samples tested per sampling time and analyzed by RM-ANOVA.

Results

Acid adaptation of cultures was tested in 2 ways in this study: 1) Comparison of cultures grown in TS broth, with 1% glucose and without glucose (i.e., acid-adapted & non-adapted; Figure 1), and 2) comparison of cultures grown in TS broth, both with 1% glucose, but buffered and unbuffered (i.e., non-adapted & acid-adapted). The pH was taken of *Salmonella* serovars acid-adapted in TSB with various levels of glucose (0, 0.25, 1.0%) to determine the level of final pH. Acid-adapted cultures using TSB with 1% glucose was used for all prior biltong studies. Acid-adapted TS broth with 1% glucose gave an average final pH of 4.9 (Figure 1) which the cultures were adapted to. For non-acid-adapted, TSB without glucose (0%) gave a final pH of ~6.7 (Figure 1) and were considered not acid-adapted because of the near neutral pH. USDA-FSIS presumed that the non-acid-adapted condition would make the cultures more susceptible to acid treatment.

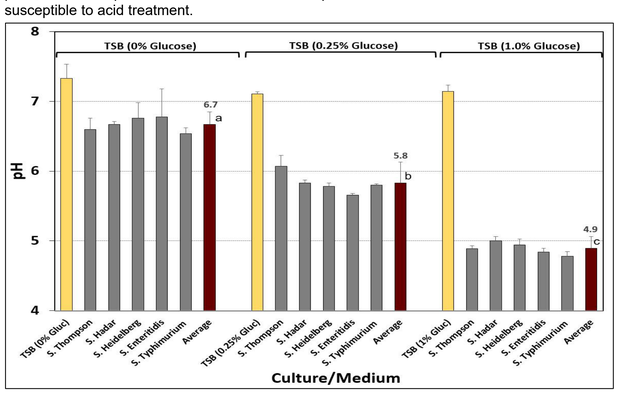


Figure 1. *Salmonella* serovars grown in TS broth at 0%, 0.25%, and 1.0% glucose and corresponding pH after growth. This method was used for acid-adapting cultures in all prior biltong studies.

USDA-FSIS (with whom we have often discussed our data and trials) were interested in seeing how the biltong process fared against a serovar of Salmonella they isolated from dried beef (Salmonella I 4,[5],12:i:-). We examined this in a biltong process using 2 batches of inoculum: Acid-adapted (1% gluc) and non-Acid-adapted (0% gluc) (Figure 2).

Results (cont.)

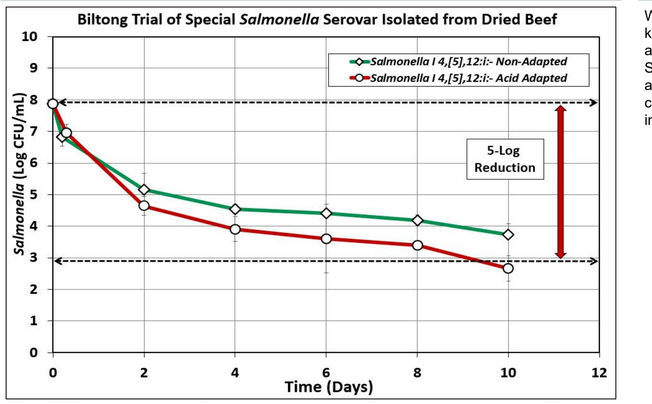


Figure 2. Biltong processing of beef inoculated with acid-adapted (1% glucose) or non-acid-adapted (0% glucose) cultures of *Salmonella* I 4,[5],12:i:- provided by USDA-FSIS. Acid-adapted (red line) vs. non-acid-adapted (green line), both subjected to a 30-sec water dip before 30-min vacuum tumbling marination with spices (coriander, black pepper), salt, and vinegar, followed by drying in a humidity-controlled oven for 8-10 days (75ºF, 55% RH).

The surprising difference of acid-adapted cultures giving larger reductions than non-acid-adapted *Salmonella* I 4,[5],12:i:- led us to consider what would happen with the same mix of *Salmonella* serovars we have used previously. The data below represents 2 trials (1a, 1b) of biltong inoculated with 5 *Salmonella* serovars. There are 4 conditions being tested: acid-adapted vs non-acid-adapted inoculum cultures and within each of these, acid dipped (5% lactic acid) vs. water dipped (both 30-sec). Both acid-adapted trials (1% glucose) using *Salmonella* spp. (water dip vs acid dip) achieved a 5-log reduction (5.3-log and 6.13-log) over an 8-day drying period. Both non-adapted trials (0% glucose) using *Salmonella* spp. (water dip vs acid dip) failed to achieve a 5-log reduction (3.91-log and 4.84-log) over an 8-day drying period (Figure 3).

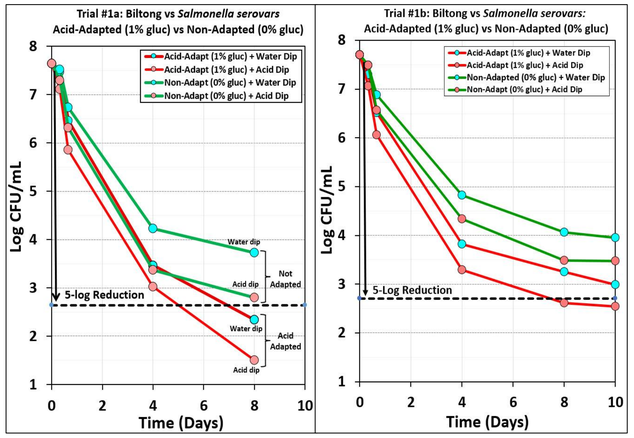


Figure 3. Two (2) trials of acid-adapted vs. non-acid-adapted cultures (mixture of 5 *Salmonella* serovars), each acid-dipped (5% lactic acid) or water dipped for 30-sec. Red lines = acid-adapted; green lines = non-acid-adapted. Blue symbols = water dipped; red symbols = acid-dipped.

SIGNIFICANCE: The data obtained in Trails 1a and 1b were the opposite of what USDA-FSIS expected from the reasoning behind using 'acid-adapted' cultures. This may be attributed to the different nutritional levels in the acid-adapted (1% glucose) media vs non-adapted (0% glucose) media. We next examined using the same carbohydrate level in growing all inoculum cultures, and the possibility of using sodium phosphate buffer to maintain the pH of non-acid-adapted cultures (Figure 4)

Results

We determined the level of sodium phosphate buffer to add to TSB containing 1% glucose to keep the media pH near physiological pH (pH 7). This growth media was used as the non-adapted culture treatment, to compare with acid-adapted (TSB 1% without buffer) cultures. Sterile, uninoculated TSB 1% and TSB 1% buffered has an average pH of 6.88 and 6.99 after autoclaving, respectively. The average pH of the 5 *Salmonella* serovars grown in TSB containing 1% glucose at 37ºC for 24 hours was 4.74, and 6.62 for *Salmonella* serovars grown in TSB 1% glucose/buffered (Figure 4).

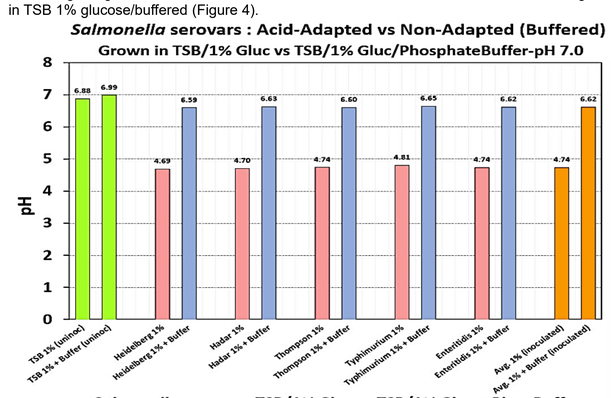


Figure 2. The pH values obtained using TSB containing 1% glucose vs. TSB containing 1% glucose and 0.5 M sodium phosphate buffer

We next performed additional biltong trials using the non-acid-adapted buffered method with 1% glucose to provide a similar level of carbohydrate during growth. The data below represents the 2 combined trials (Figure 5A) of biltong inoculated with 5 *Salmonella* serovars. Both acid-adapted trials using *Salmonella* spp. achieved a 5-log reductions over the 10-day drying period (Figure 5A). Both non-adapted trials (1% glucose, buffered) using *Salmonella* spp. failed to achieve a 5-log reduction (3.34-log, 3.51-log reduction) over the 10-day drying period (Figure 5A). Our first trial with *Listeria monocytogenes* does not show the disparity of acid- vs non-acid-adapted observed with *Salmonella* ser. but demonstrates that acid-dipping provides greater reduction more quickly than water treatment (Figure 5B).

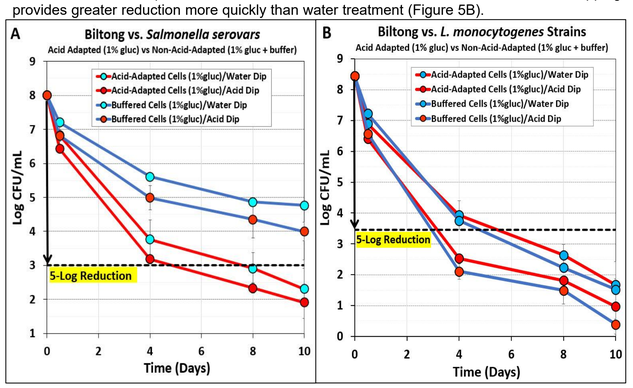
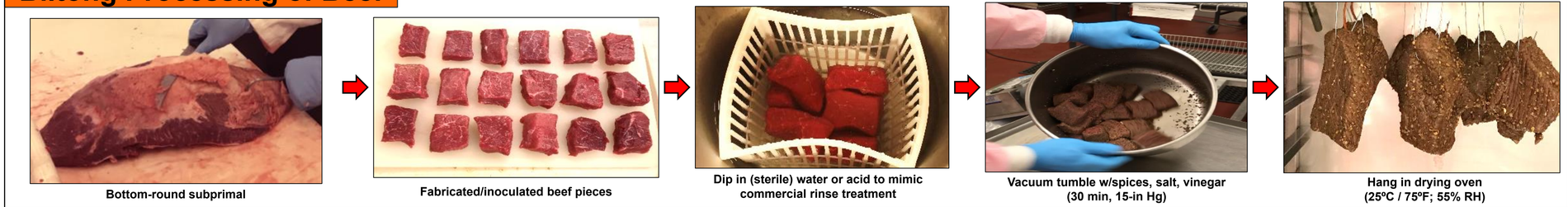


Figure 5. Biltong lethality trials vs. mixtures of 5 *Salmonella* serovars (Panel A) and 4 strains of *Listeria monocytogenes* (Panel B) comparing acid-adapted and non-acid-adapted (buffered) growth conditions as well as acid-dip (5% lactic acid) vs. water-dip treatments. The red lines are acid-adapted (1% glucose) cells while the blue lines are non-adapted (1% glucose, buffered) cultures. The red symbols are acid-dipped and blue symbols are water-dipped treatments.

Conclusions

- **The data disproves the USDA-FSIS approach that non-acid-adapted cells would be more sensitive during an acid process treatment than acid-adapted cells; this may be true for some processing conditions, but not all. This may cause USDA-FSIS to change their stance towards this requirement for validation of biltong going forward.**
- Processors can still use the alternative biltong process (test negative for *Salmonella* & use a validated process providing ≥ 2-log reduction of pathogen of concern [Note: they may be able to obtain a 'Certificate of Analysis' from their supplier of edible ingredients (spices, beef) that the lot of ingredients have tested negative for *Salmonella* to accommodate the *Salmonella*-testing requirement].
- We will be examining whether these results are also obtained for a Gram-positive pathogen of concern (either *Listeria monocytogenes* or *Staphylococcus aureus*).
- Does acid treatment affect bacterial membrane fluidity making it more susceptible to desiccation? While USDA-FSIS was concerned for the 'acid treatment' (i.e., vinegar in the marinade), it represented only a small proportion of the antimicrobial treatment, most of which was the 8-10 days of desiccation that the inoculated bacteria had to endure, not the acid.

Biltong Processing of Beef



Bottom-round subprimal

Fabricated/inoculated beef pieces

Dip in (sterile) water or acid to mimic commercial rinse treatment

Vacuum tumble w/spices, salt, vinegar (30 min, 15-in Hg)

Hang in drying oven (25ºC / 75ºF; 55% RH)