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

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ABSTRACT

Biltong is a South African dried beef product that has grown in popularity in U.S. markets over the last five years. Unlike traditional American beef jerky, biltong is dried at ambient temperature and humidity after marination. However, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) requires the use of heat lethality and maintenance of 90% relative humidity in a sealed oven to accomplish adequate reduction of pathogens on dried beef products for consumption [1]. If these parameters are not met, such as is the case with biltong processing, a microbial validation study must be provided to demonstrate sufficient bacterial reductions of a 'pathogen of concern' can be achieved [2]. Moreover, the use of acid-adapted cultures during validation studies for acidic foods are 'highly recommended' by USDA-FSIS. It is widely thought if challenge cultures are not acid-adapted prior to acidic treatment the culture may react by being overly sensitive and result in falsely high microbial reductions. With this, communication with USDA-FSIS officials indicate that research demonstrating the importance of acid-adaptation would move USDA-FSIS guidelines to require acid-adapted cultures for industry process validation. Research done in our laboratory directly addressed this issue by performing process validation studies using acid-adapted and non-acid-adapted *Salmonella* serovars and *Listeria monocytogenes* serovars for biltong processing to determine whether acid-adaptation is a necessary pre-culture treatment prior to microbial validation studies. The data using *Salmonella* serovars disproves the USDA-FSIS approach that non-acid-adapted cells would be more sensitive during an acid process treatment than acid-adapted cells. However, this relationship was not clear when using *L. monocytogenes* serovars as a biltong processing challenge culture.

1. 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 red wine vinegar then dried at ambient temperature and humidity (75 °F, 55% RH). In the United States, the USDA-FSIS requires the use of a sealed oven of steam injector with an internal temperature ≥ 160 °F (71.1 °C) and ≥ 90% relative humidity to accomplish adequate reduction of pathogens on dried beef products for consumption [1]. 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,' such as *Salmonella* spp., which has been linked to outbreaks and recalls of dried meat products, can be achieved during processing [3]. Since biltong processing is significantly different than American beef jerky, USDA-FSIS provided two 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*) [2].

Discussions with USDA-FSIS officials on their requirements for 'microbial validation studies' indicated a necessary use of 'acid-adapted cultures' with acidic foods where the presence of metabolizable sugars is important for microbial survivability in acidic environments [4]. These validation studies are 'highly recommended' by USDA-FSIS or they may not consider the process properly validated and safe for consumption. Acid-adaptation was a condition demonstrated in the 1980's whereby pathogens grown in the presence of 1% glucose would produce acid, effectively lowering the pH of the growth media and therefore, become acid-adapted.

The USDA-FSIS believes that acid-adapting cultures intended for product inoculation would fortify the organisms against acidic conditions, such as that with biltong processing, and ensure the process is sufficiently robust when targeting a 5-log reduction of a pathogenic challenge organisms. Previous research conducted at Oklahoma State University utilized *Salmonella* I 4,[5],12:i:-, an isolate from dried beef provided by the USDA-FSIS, as a pathogenic challenge organism for biltong processing and disproved the USDA-FSIS approach

that non-acid-adapted cells are more sensitive to an acid processing treatment than acid-adapted cells. This is shown in *Figure 1* below.

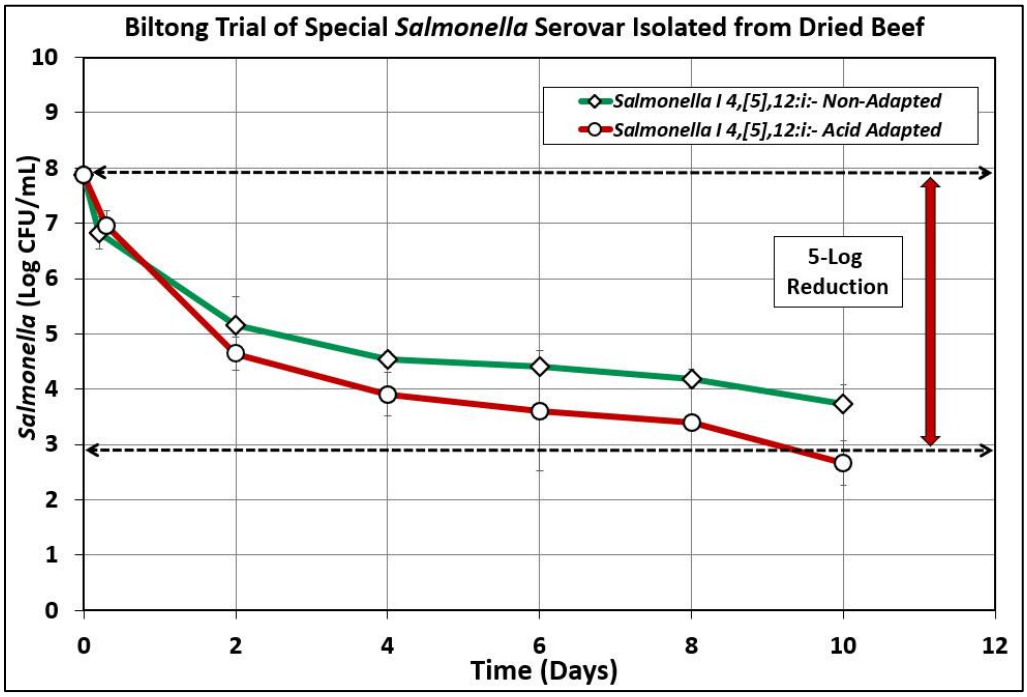

Figure 1. Biltong processing of beef inoculated with acid-adapted (1% glucose) or non-acid-adapted (0% glucose) cultures of *Salmonella* I 4,[5], 12:i:- provided by USDA-FSIS. Acid-adapted (red line) vs. non-acid-adapted (green line), both subjected to a 30-second water dip before 30-minute vacuum tumbling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

With this, 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.

2. Material and Methods

2.1. Bacterial Strains

Bacterial cultures used in this study were obtained from our laboratory culture collection as listed in Table 1.

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

Organism	Strain Designation	Antibiotic Resistance (µg/mL)*	Source
Salmonella enteritidis	H3527	SPC, 5; CC, 5; NB, 50	Muriana Culture Collection
Salmonella hadar	MF60404	SPC, 5; CC, 5; NB, 51	Muriana Culture Collection
Salmonella heidelberg	F5038B91	SPC, 5; CC, 5; NB, 52	Muriana Culture Collection
Salmonella thompson	120	SPC, 5; CC, 5; NB, 53	Muriana Culture Collection
Salmonella typhimurium	H3380	SPC, 5; CC, 5; NB, 54	Muriana Culture Collection
Listeria monocytogenes	383-2	S, 100; RIF, 10	Muriana Culture Collection
Listeria monocytogenes	39-2	S, 100; RIF, 11	Muriana Culture Collection
Listeria monocytogenes	SCA-2	S, 100; RIF, 12	Muriana Culture Collection
Listeria monocytogenes	V7-2	S, 100; RIF, 13	Muriana Culture Collection

* Antibiotic designations: spectinomycin, SPC; clindamycin, CC; novobiocin, NB; streptomycin, S, rifamycin, RIF

2.2. Culture Cocktail Preparation (Trials 1a and 1b)

The five strains of *Salmonella* serovars were individually inoculated into tryptic soy broth (TSB; 0% glucose) and grown at 37 °C for 24 hours from frozen stock. After, cultures were transferred into individual 200 mL bottles of TSB containing 1% glucose (acid-adapted) and TSB containing 0% glucose (non-acid-adapted) and grown at 37 °C for 24 hours. All bottles were centrifuged for 20 minutes at 8000 rpm, then cell pellets were resuspended with 0.1% BPW and combined to make the two mixed culture biltong inocula.

2.3.Culture Cocktail Preparation (Trial 2a)

The five strains of *Salmonella* serovars were individually inoculated into tryptic soy broth (TSB; 0% glucose) and grown at 37 °C for 24 hours from frozen stock. After, cultures were transferred into individual 200 mL bottles of TSB containing 1% glucose (acid-adapted) and TSB containing 1% glucose and 0.05M sodium phosphate buffer (non-acid-adapted) and grown at 37 °C for 24 hours. All bottles were centrifuged for 20 minutes at 8000 rpm, then cell pellets were resuspended with 0.1% BPW and combined to make the two mixed culture biltong inocula.

2.4.Culture Cocktail Preparation (Trial 2b)

The four strains of *L. monocytogenes* serovars were individually inoculated into tryptic soy broth (TSB; 0.25% glucose) and grown at 30 °C for 24 hours from frozen stock. After, cultures

were transferred into individual 200 mL bottles of TSB containing 1% glucose (acid-adapted) and TSB containing 1% glucose and 0.05M sodium phosphate buffer (non-acid-adapted) and grown at 30 °C for 24 hours. All bottles were centrifuged for 20 minutes at 8000 rpm, then cell pellets were resuspended with 0.1% BPW and combined to make the two mixed culture biltong inocula.

2.5.Sodium Phosphate Buffer

The 0.05M sodium phosphate buffer used for this study was made using dibasic sodium phosphate ($Na_2\ H\text{-}PO_4 - 7H_2O$) and monobasic sodium phosphate ($Na_2\ H\text{-}PO_4 - 1H_2O$). The correct amount used to maintain physiological pH were tested before use in biltong process validation. For this study, the amounts of sodium phosphate buffer used correlated with a pH 7 and was four-times (4x) the normal concentration in order to maintain physiological pH of growth media (i.e., 6.2 g/100 mL dibasic and 1.24 g/100mL monobasic sodium phosphate buffer), as shown in Table 2.

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

pH	Dibasic	gms/100 mL	Monobasic	gms/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

2.6. Beef Sample Preparation and Inoculation

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 and held overnight on aluminum foil-lined trays wrapped in plastic bags at 5 °C. Beef pieces were inoculated the following day with respective pathogenic challenge culture depending on the trials being conducted. The inoculum suspension (150 µL) was applied to each side of the beef pieces and immediately spread with a double-gloved finger and allowed to incubate for 30 min at 5°C to allow for bacterial attachment prior to dip and marination.

2.7. Biltong Processing, Marination, and Drying

Biltong processing trials were performed in duplicate and triplicate samples were harvested at each timepoint. Following the 30-minute attachment period, the beef pieces were then placed in a plastic basket, dipped in sterile water or 5% lactic acid for 30 seconds, and drained for 60-seconds to release excess liquid. Beef pieces were then 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.

2.8. Microbial Sampling and Enumeration

Microbial enumeration of surviving bacteria was performed post-inoculation, post- water and acid dip (trials 1a and 1b), post-marination, and after 4-, 8-, and 10 days of drying for each individual trial (acid-adapted water/acid dip and non-adapted water/acid dip). At each respective time point, beef samples placed into a filter stomaching bag and stomached with 100 mL of 1% neutralizing buffer peptone water (nBPW) for 60 seconds in a paddle-blender masticator. Samples where then serially diluted in 1% BPW and plated on Selenite Cystine Agar containing spectinomycin (5 µg/mL), clindamycin (5 µg/mL), and novobiocin (50 µg/mL) for *Salmonella* and TSA containing streptomycin (100 µg/mL) and rifamycin (10 µg/mL) for *L. monocytogenes* then incubated at 37 °C and 30 °C, respectively. The filter bag was considered the 10° dilution. Trials were performed in duplicate replication with triplicate samples tested per sampling time and analyzed by RM-ANOVA.

3. Results and Discussion

3.1. Temperature and Relative Humidity during Biltong Processing

Temperature and RH measurements were recorded via computer software connected to handheld temperature and humidity recorders connected to meat samples by probe in the oven. Two temperature probes were inserted into two beef pieces to measure the internal temperature during processing. The humidity probe was placed in the center of the oven. The internal temperature and humidity of the oven was set to 23.9 °C (75 °F) and 55% RH but cycled around these set points the duration of each trial. This is shown below in *Figure 2*.

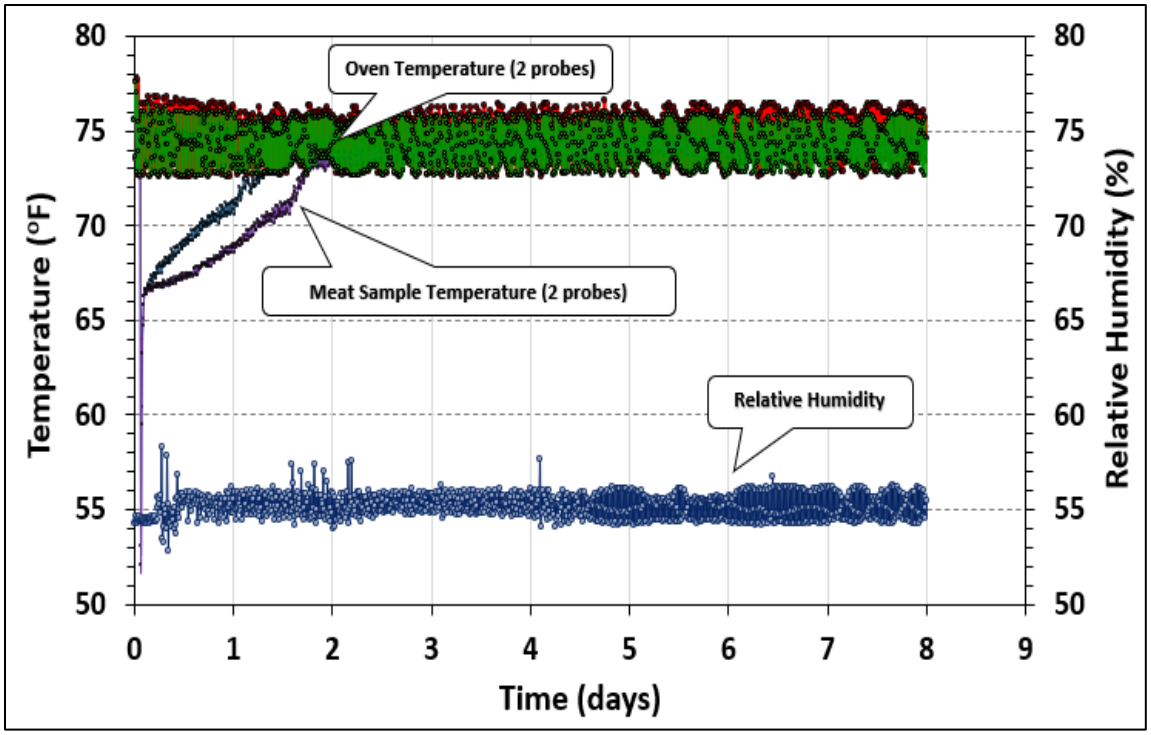

Figure 2. Oven temperature and relative humidity measurements show the typical cycling of oven control above/below the setpoints.

3.2. pH Parameters Tested Prior to Biltong Processing (Trials 1a and 1b)

Trial 1a and 1b of *Salmonella* serovars was done by growing the five strains in both TSB containing 1% glucose and TSB containing 0% glucose to achieve acid-adaptation vs. non-adapted culture inocula. To do so, the pH was taken of *Salmonella* serovars inoculated in TSB containing various levels of glucose (0, 0.25, and 1.0%) to determine the final pH after 24 hours of growth. Acid-adapted TSB containing 1% glucose was used for all prior biltong studies. Acid-adapted TSB with 1% glucose gave an average final pH of 4.9 which the cultures were adapted to for this trails 1a and 1b. For non-acid-adapted, TSB containing 0% glucose gave a final pH of approximately 6.7 and were considered non-adapted because of the near neutral pH. The USDA-FSIS presumed the non-acid-adapted condition would make the cultures more susceptible to acid treatment. These results are shown in *Figure 3*.

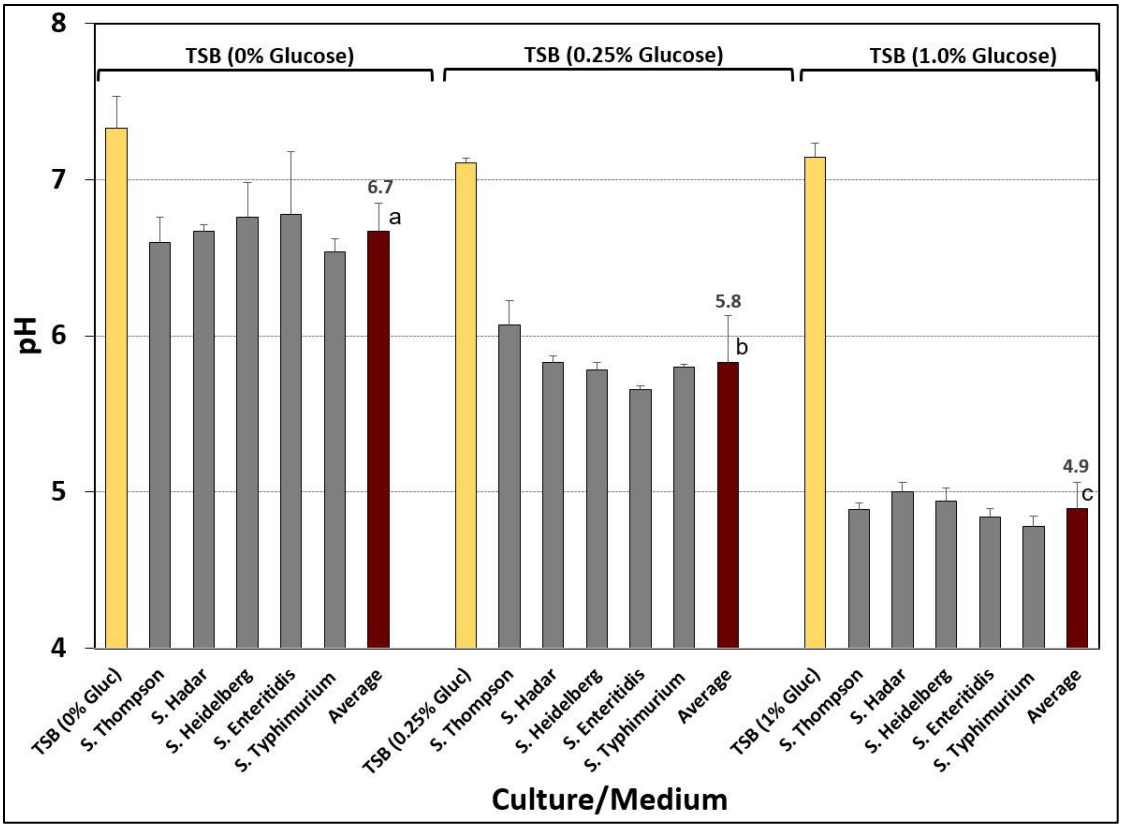

Figure 3. Salmonella serovars grown in TSB at 0%, 0.25% and 1.0% glucose and the corresponding pH after 24 hours of growth at 37 °C. This method was used for acid-adapting cultures in all prior biltong studies.

3.3. Salmonella Log Reductions during Biltong Processing (Trial 1)

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 previously used in biltong challenge studies (these serovars are listed in *Table 1*). The data below represents the average of two trials (1a, 1b) of biltong inoculated with five *Salmonella* serovars. There are four conditions being tested: acid-adapted vs non-acid-adapted inoculum cultures and within each of these acid dipped (5% lactic acid) vs. water dipped. One acid-adapted trials (1% glucose) using *Salmonella* spp. (acid dip) achieved a 5-log reduction (5.61-log) over an 8-day drying period. The acid-adapted trial (water dip) was close with a log reduction of 4.87-log. Both non-adapted trials (0% glucose) using *Salmonella* spp. (water dip vs acid dip) failed to achieve a 5-log reduction (3.77-log and 4.53-log) over an 8-day drying period. This is shown in *Figure 4*.

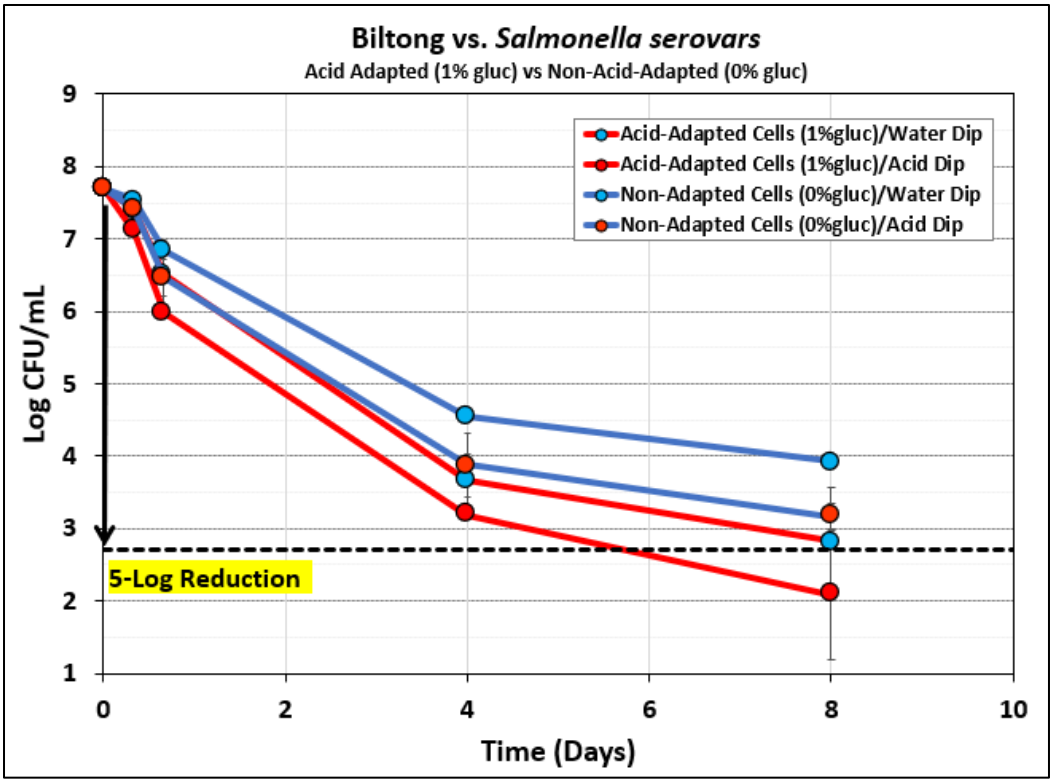

Figure 4. Two averaged trials of acid-adapted vs. non-acid-adapted cultures of 5 *Salmonella* serovars, each acid-dipped (5% lactic acid) or water-dipped for 30 seconds. Red lined = acid-adapted; blue lines = non-adapted; red symbols = acid-dipped; blue symbols = water-dipped.

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. This data is shown in *Figure 5*.

3.4. pH Parameters Tested Prior to Biltong Processing (Trial 2a and 2b)

Trial 2a of *Salmonella* serovars was done by growing the five strains in both TSB containing 1% glucose and TSB containing 1% glucose plus sodium phosphate buffer to achieve acid-adaptation vs. non-adapted culture inocula. To do so, the level of sodium phosphate buffer to add to TSB containing 1% glucose to keep the media pH near neutral pH was determined. 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 five *Salmonella* serovars grown in TSB containing 1% glucose at 37 °C for 24 hours

was 4.74 and 6.62 for *Salmonella* serovars grown in TSB 1% glucose/buffered as shown in *Figure 5*. The same parameters were used for acid-adapted (1% glucose) vs. non-acid-adapted (1% glucose + buffer) *L. monocytogenes* biltong trials (*Figure 6b)*.

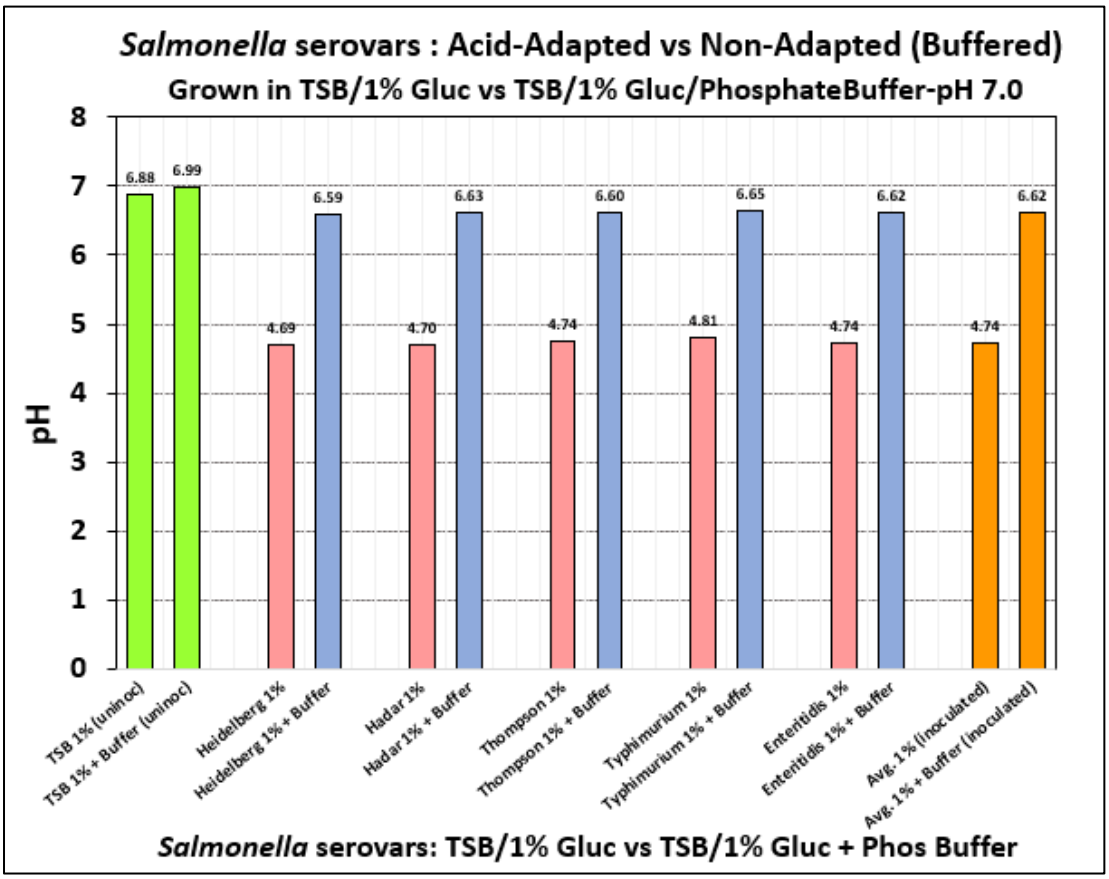

Figure 5. The pH values obtained using TSB containing 1% glucose vs. TSB containing 1% glucose and 0.05 M sodium phosphate buffer (4x).

3.5. Log Reductions during Biltong Processing (Trials 2a and 2b)

Additional biltong trials were performed using the non-acid-adapted buffered method with 1% glucose to provide a similar level of carbohydrate during growth. The data below represents the two combined trials of biltong inoculated with five *Salmonella* serovars. Both acid-adapted trials using *Salmonella* spp. (water dip vs. acid dip) achieved a 5-log reduction over the 10-day drying period (5.7-log and 6.09-log). Both non-adapted trials (1% glucose, buffered) using *Salmonella* spp. (water dip vs. acid dip) failed to achieve a 5-log reduction (3.24-log and 4.01-log) over the 10-day drying period. This data is shown in *Figure 6a*.

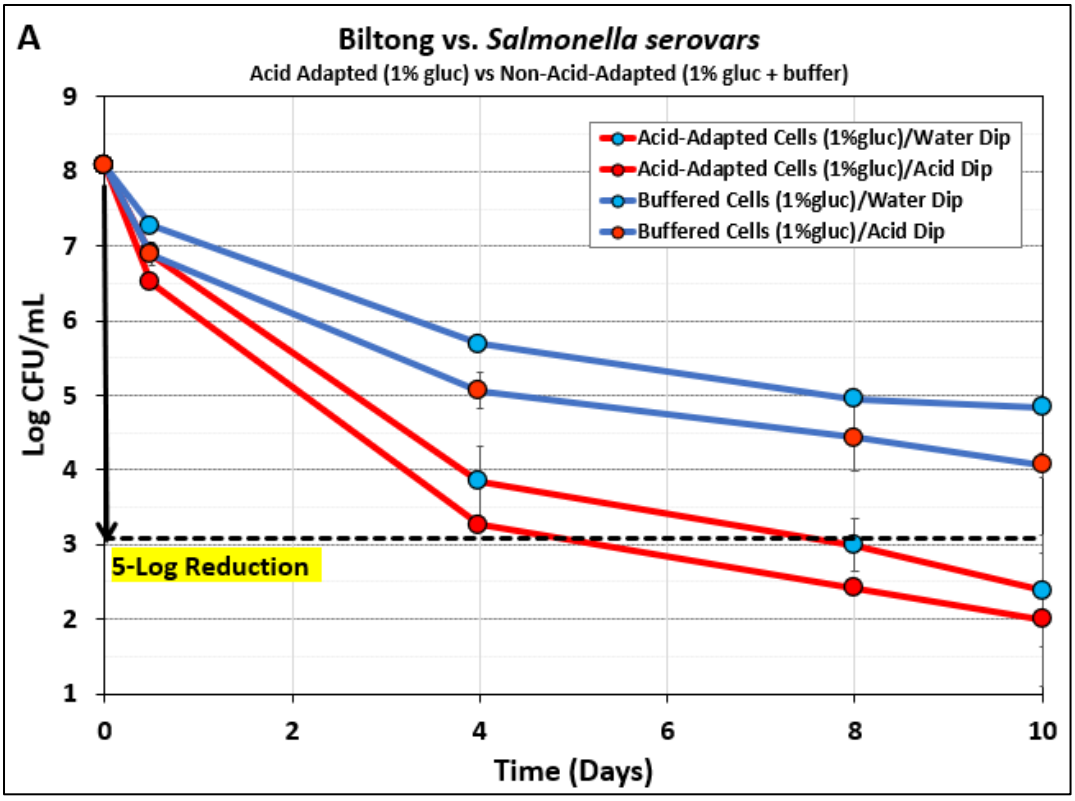

Figure 6a. Biltong lethality trials vs. mixtures of 5 *Salmonella* serovars comparing acid-adapted and non-acid-adapted (buffered) growth conditions, as well as acid-dip (5% lactic acid) vs. water-dip treatments. Red lined = acid-adapted; blue lines = non-adapted; red symbols = acid-dipped; blue symbols = water-dipped.

Our combined trials with four strains of *Listeria monocytogenes* does not show the disparity of acid- vs non-acid-adapted observed with *Salmonella* serovars but demonstrates that acid-dipping provides greater reduction more quickly than water treatment. All trials accomplished a 5-log reduction over a 10-day drying period where acid-dipped trials (acid-adapted vs. non-adapted) had greater log-reductions (7.57-log and 8.10-log) than both water-dipped trials (6.68-log and 6.89-log). This relationship is depicted in *Figure 6b*.

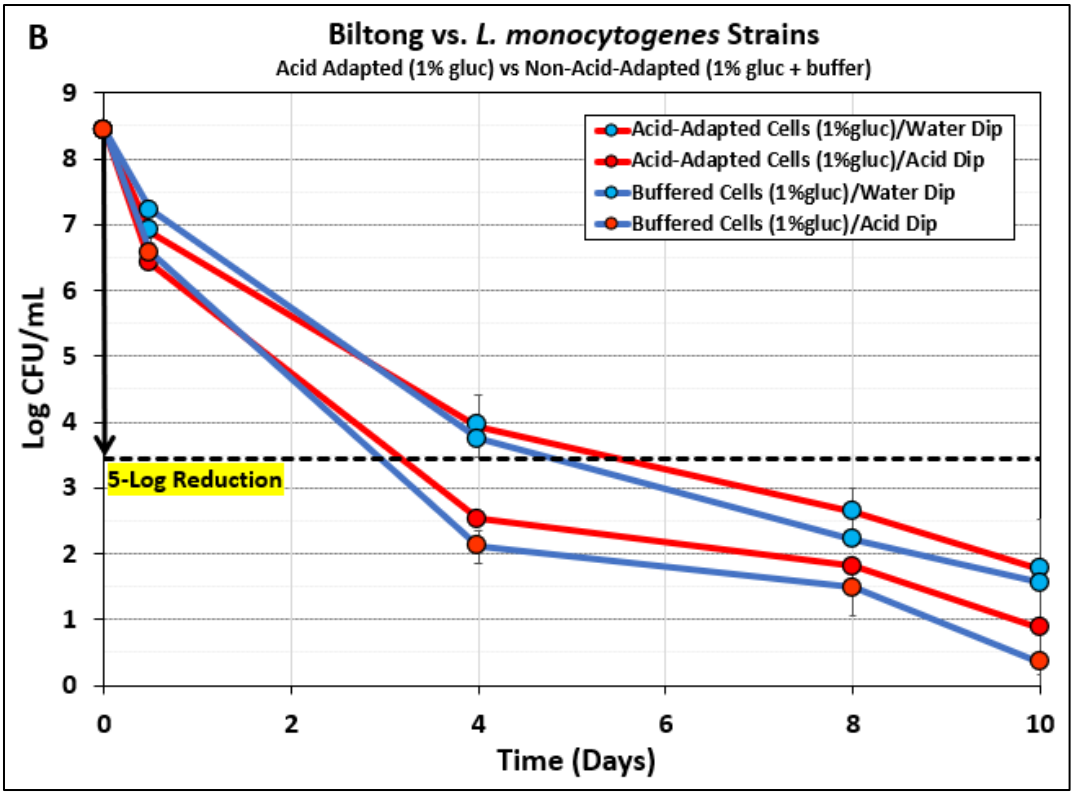

Figure 5b. Biltong lethality trials vs. mixtures of 5 *Salmonella* serovars comparing acid-adapted and non-acid-adapted (buffered) growth conditions, as well as acid-dip (5% lactic acid) vs. water-dip treatments. Red lined = acid-adapted; blue lines = non-adapted; red symbols = acid-dipped; blue symbols = water-dipped.

4. Conclusions

The lethality observed in the biltong process with *Salmonella* spp. was the opposite of what USDA-FSIS expected from the reasoning behind using 'acid-adapted' cultures for both trials comparing acid-adapted (1% glucose) vs. non-adapted (0% glucose and 1% glucose + buffer). Meaning, the acid-adapted cells were, in fact, more susceptible to the acid treatment and biltong process compared to non-adapted cells. It is important to recognize this may be true for some processing conditions, but not all. For instance, the lethality observed in the biltong process with *L. monocytogenes* spp. differed in that they did not show the disparity of acid- vs non-acid-adapted observed with *Salmonella* serovars but demonstrates that acid-dipping provides greater reduction more quickly than water-dip treatment. Still, this may cause USDA-FSIS to change their stance towards the following requirement for validation of biltong going forward: give ≥ 5-log reduction of a pathogen of concern (*Salmonella*). However, processors can still use the alternative biltong process by which they test negative for *Salmonella* and use a validated process providing ≥ 2-log reduction of a 'pathogen of concern.' This can be

accomplished by obtaining a 'Certificate of Analysis' from their supplier of edible ingredients (spices, beef) that they have tested negative for *Salmonella* to accommodate the USDA-FSIS testing requirement. Next, this same biltong process will be repeated using another gram-negative pathogen (*Escherichia coli* spp.) and another gram-positive pathogen (*Staphylococcus aureus*). Growth media preparation will follow that of trials 2a and 2b where acid-adaptation will be achieved using TSB containing 1% glucose and non-acid-adapted cells will be grown in TSB containing 1% glucose and 0.05 M sodium phosphate buffer at 4x concentration (pH 7). Moreover, it is important to note that while the USDA-FSIS was concerned for the 'acid treatment' (i.e., vinegar in the marinade), it represented only a small portion of the antimicrobial treatment. The majority of this treatment was the 8-10 days of desiccation (75 °F, 55% RH) that the inoculated bacteria had to endure, not the acid-dip or marinade.

References:

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