Acid-Adaptation Leads to Sensitization of *Salmonella* Challenge Cultures During Processing of Air-Dried Beef (Biltong, Droëwors)

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Article

Acid-Adaptation Leads to Sensitization of *Salmonella* Challenge Cultures During Processing of Air-Dried Beef (Biltong, Droëwors)

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Abstract

US food regulatory agencies have adopted a preference for researchers and testing labs to use 'acid-adapted challenge cultures' when performing inoculated validation studies of food processes that involve acidic treatments to accustom the cultures to acidic pH so that they will not be easily affected during processing. We evaluated acid adaptation in regard to the processing of South African style air-dried beef notably, biltong and droëwors, using a mixture of five serovars of *Salmonella*. Acid adaptation was obtained by growing cultures in tryptic soy (TS) broth containing 1% glucose. Non-adapted cultures were obtained by growth in TS broth without glucose or, in TS broth with 1% glucose but buffered with 0.2M phosphate buffer. Processes included biltong (dried solid beef) and droëwors (ground, sausage-style). Each trial was performed twice and triplicate samples were examined at each sampling point (i.e., n = 6). Statistical analysis was applied using analysis of variance (ANOVA) or one-way repeated measures (RM-ANOVA) and the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). We observed that in all processes examined, treatments using acid-adapted cultures were more sensitive to the biltong and droëwors processes, giving greater reductions than when non-adapted cultures were used. We conclude that acid-adaptation leads to stressed conditions in *Salmonella* resulting in sensitization to the multiple hurdles found in biltong/droëwors processing (acid/vinegar, salt, desiccation) and the use of non-adapted cultures could actually lead to more robust bacterial conditions for testing process effectiveness.

Keywords: acid adaptation; challenge organisms; air-dried beef; biltong; droëwors; foodborne pathogens

1. Introduction

South African 'air-dried' meat products include biltong (similar to beef jerky) and droëwors (sausage sticks). Biltong is usually made from lean strips of beef marinaded in traditional spices (coriander, black pepper), salt, and vinegar dried at ambient temperature (75°F) and humidity (55% RH). Droëwors is made from residual beef and fat trimmings leftover from processing biltong, similarly marinaded, ground in a meat grinder, stuffed into casings, and dried under similar conditions as biltong to produce dried beef sticks.

In the United States, beef jerky processing is the standard for dried, shelf-stable beef product produced under United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) compliance guidelines. Many processors cite USDA-FSIS lethality performance standards (Appendix A) for meat and poultry products for their temperature targets and hold times

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during processing (i.e., 145 °F for 4 min) which state that relative humidity (RH) must be 90% or higher for at least 25% of the total cooking time, or 1 hour, whichever is longest [1]. Processors who do not adhere strictly to the USDA-FSIS Appendix A guidelines (i.e., humidity, cooking temperature) for the manufacture of dried beef or poultry jerky must provide validation that their process provides adequate pathogen reduction to ensure the product is safe and wholesome for human consumption. Validation can be done with an outside lab (or reference to acceptable peer-reviewed published literature) to validate that their process is sufficient in satisfying USDA-FSIS food safety requirements. 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' (i.e., *Salmonella*) can be achieved during processing. Since biltong and droëwors processes are significantly different than beef jerky, USDA-FSIS provided 2 alternative processes by which processors could manufacture and sell these products [2,3]:

i. Test every lot of edible ingredients 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
ii. Use a process that is validated to give ≥ 5-log reduction of a pathogen of concern (*Salmonella*).

Food processes designed to inhibit or reduce foodborne pathogens often require verification of the inhibitory capacity by using 'challenge organisms' artificially introduced into the food to determine the effect of the process on those pathogenic microorganisms. In discussions with USDA-FSIS on what they require in microbial validation studies, one of the required parameters was the use of 'acid-adapted challenge cultures' otherwise USDA-FSIS may not consider the process properly validated [2,4].

Acid tolerance was first recognized by Foster [5,6] and became more concerning as a food safety issue when it was recognized that certain organisms can become tolerant of acidic conditions [7,8]. This was significant in lieu of pathogens tolerating and surviving acidic conditions in the animal rumen and subsequently tolerating acidic rinse treatments on animal carcasses meant to eliminate them [9]. Equally disconcerting were pathogens that may tolerate acidic-processed foods [10,11]. This concept was further developed into the methodology of inoculated food studies whereby challenge cultures would be conditioned to tolerate acidic conditions (i.e., acid-adapted). Growing pathogens in the presence of 1% glucose lowers the pH, and the resulting culture would be considered acid-adapted [12–15]. Alternative approaches were to acidify a culture post-growth using inorganic or organic acids [6,16]. Acid adaptation was listed in the National Advisory Committee for the Microbial Criteria for Food (NACMCF) 'white paper' on recommended parameters for inoculated challenge study protocols [17]. They believed that acid-adapting pathogenic cultures intended for product inoculation would harden the organisms against sensitivity to acidic conditions they would encounter during processing. This would ensure that the process needs to be sufficiently robust if it were to demonstrate a significant reduction of the challenge cultures. This likely developed from studies during the 1990's to reduce *E. coli* O157:H7 and *Salmonella* serovars on beef carcasses that resulted in foodborne illness from contaminated ground beef by treatments with various acidic antimicrobials [18–21]. Acid-adapting challenge cultures for use in validation studies would harden the cultures so that they wouldn't easily succumb to acidic treatments. This was subsequently adopted by USDA-FSIS as a general recommendation for the preparation of pathogen challenge cultures in validation experiments involving acidic treatments and was requested by them when discussing biltong and droëwors processing.

USDA-FSIS has often relied on peer-reviewed published literature to affect policy on the strength of scientific data that could influence its stance on issues, such as acid-adaptation of challenge cultures. However, there have been a number of publications demonstrating the reverse effect on the use of acid-adapted cultures as had originally been proposed [22–28]. The objective of this study was to determine whether acid-adapted treatment hardens or sensitizes *Salmonella* challenge organisms to processing conditions during manufacture of biltong and droëwors.

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2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Bacterial cultures were grown in Tryptic Soy Broth (TSB, BD Bacto, Franklin Lakes, NJ, USA) in 9-mL tubes and incubated at 37°C. Fresh overnight cultures (10-20 ml) were prepared for storage by centrifugation (6,000xg, 5°C). Cell pellets were resuspended in 2–5 mL of sterile TSB containing 15% glycerol and 1% trehalose, and then aliquoted to several glass vials and stored in cryoboxes in an ultra-low freezer (−80°C). Frozen stocks were revived by transferring 100 µL of the thawed cell suspension into 9 mL of TSB, incubating overnight at 37°C, and sub-culturing before use. Microbial enumeration on other media was always compared to counts obtained on Tryptic Soy Agar (TSA, BD Bacto; 1.5% agar), plated in duplicate.

Salmonella serovars used in this study included: *Salmonella enterica subsp. enterica* serotype Thompson 120 (chicken isolate), *Salmonella enterica subsp. enterica* serotype Heidelberg F5038BG1 (ham isolate), *Salmonella enterica subsp. enterica* serotype Hadar MF60404 (turkey isolate), *Salmonella enterica subsp. enterica* serotype Enteritidis H3527 (phage type 13a, clinical isolate), and *Salmonella enterica subsp. enterica* serotype Typhimurium H3380 (DT 104 clinical isolate). These are well-characterized strains that have been used in numerous research publications involving antimicrobial interventions against *Salmonella* spp [2,4,29–32]. These *Salmonella* serovars were previously shown to be resistant to spectinomycin (5 ug/mL), clindamycin (5 ug/mL), and novobiocin (50 ug/mL) [4] and were added to enumerative and selective media to further prevent background contamination. The individual and combined levels of antibiotics did not affect enumeration when added to plating or selective media. *Salmonella* serovar I 4, 12:i:- [5] was also provided by USDA-FSIS as a test culture that might prove difficult to demonstrate reduction by the air-dried biltong process as it was isolated from dried beef. This serovar served as our first example of comparing acid-adapted and non-adapted cultures.

Acid adaptation of the *Salmonella* cultures was carried out according to Wilde et al. [15] in which they were inoculated in TSB supplemented with 1% glucose [14]. Cultures were maintained individually, harvested by centrifugation, and cell pellets were resuspended with 0.1% buffered peptone water (BPW, BD Difco) and held refrigerated until use (5 °C). For a mixed serovar inoculum, the individual resuspended cultures were mixed in equal proportions. The various processing tests in this study were performed using acid-adapted *Salmonella* cultures in TSA containing 1% glucose as described above in comparison with non-acid-adapted cultures. USDA-FSIS 'highly recommends' the use of acid-adapted cultures when such inoculum strains would be used in processes involving acidic treatments to ensure that they are not easily overcome by acidic processing conditions. Non-acid-adapted cultures were grown in TSB with 0% glucose. In some experiments, we also examined non-acid-adapted cultures whereby TSB was supplemented with 1% glucose but buffered with 200 mM phosphate buffer (pH 7.0) in order to match the metabolic availability of nutrients while buffering the media to prevent acidification during growth.

2.2. Evaluation of Salmonella-Selective Agar Media for Enumeration of Salmonella from Droëwors Processing

We examined and compared four selective agar media in preparation for the enumeration of non-acid-adapted *Salmonella* serovars during droëwors processing. These same media previously demonstrated significant differences in enumeration after various process stress situations with *Salmonella* encountered during biltong processing [4]. Biltong processing has an accumulation of antimicrobial factors (vinegar, salt, dessication) and bacteria at the beef surface resulting from a 60-65% moisture loss during processing. This results in stressful conditions on surface bacteria that causes a reduction of injured/stressed cells when plated on certain inhibitory selective media [4]. In droëwors, the beef/fat, inoculum bacteria, and ingredients are ground up and evenly dispersed during processing and we were interested to see if similar stresses would result in similar media-influenced lethality during quantification using various *Salmonella*-selective media. The selective

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media included TSA (non-selective), Selenite Cystine Agar (SCA), Hektoen Enteric (HE), and Xylose Lysine Desoxycholate (XLD) agars. All four of these agar media contained three antibiotics: spectinomycin (5 ug/mL), clindamycin (5 ug/mL), and novobiocin (50 ug/mL) to which the *Salmonella* serovars were resistant.

2.3. Air-Dried Biltong Beef Process

The biltong process was performed exactly as described previously [2,33,34] (Figure 1). Briefly, beef pieces were placed in a chilled stainless steel tumbler chamber, harvested/mixed challenge cultures were added and tumbled for 10 min in order to distribute the inoculum. Then the marinade of spices (corriander, black pepper), salt, and vinegar were added and tumbled again for 30 min. The beef pieces were then hung in a drying oven set at 75°F and 55% relative humidity (RH). Samples were retrieved periodicially for microbial enumeration at 0, 2, 4, 6, 8, and 10 days.

Figure 1. Biltong process: Whole bottom round; cut into ~100-gm pieces of beef and surface inoculated with gloved finger; addition of marinade ingredients; marination in a tumbler bin; beef pieces after marination; beef hanging in a humidity oven; biltong beef pieces cut in half (~5 days).

2.4. Air-Dried Droëwors Beef Process

The droëwors process was similar to the biltong process, except that fat (5%) was added to the beef, then inoculum was added and tumbled, followed by the marinade mixture of spices, salt, and vinegar and tumbled again for 30 min (Figure 2). The inoculated/marinaded mixture of beef/fat was then ground using an LEM meat grinder (8 mm grind plate) and then again through a 2nd grind (10 mm grind plate) which also had a 12 mm stuffing horn to extrude the ground beef mixture into 17 mm collagen casings. The stuffed casings were tied with twine at approximately 6-7 inches and held in the refrigerator before being placed in the humidity oven.

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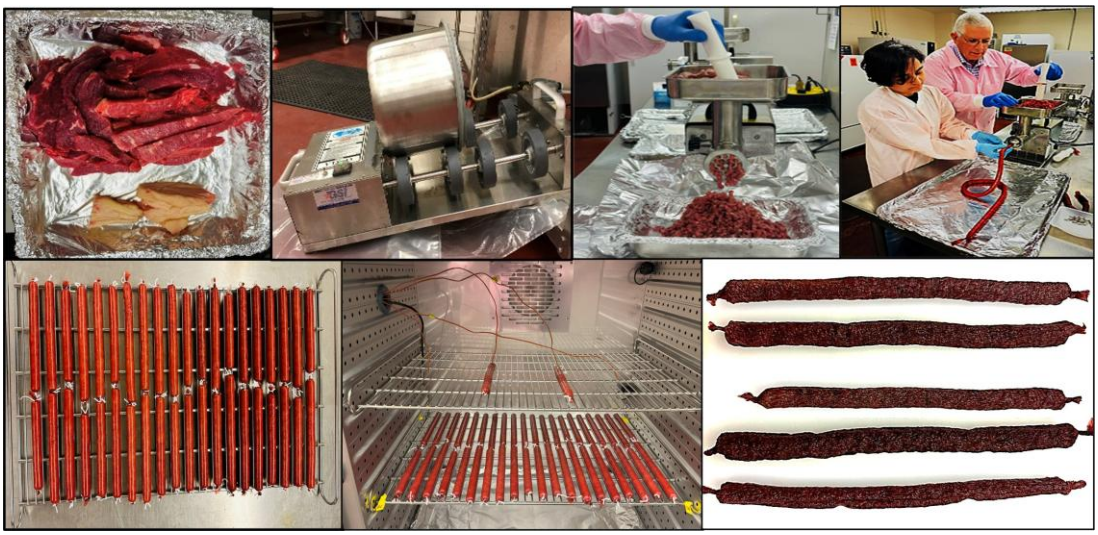

Figure 2. Droëwors process: Beef and fat trimmings leftover from biltong, tumbled first with inoculated culture (10 min) and then with added ingredients (30 min), ground twice, stuffed into collagen casings, and sectioned into sausages, dried in a humidity oven, and finished droëwors beef sticks.

2.5. Statistical Analysis

Each experimental trial was performed in duplicate and each sampling period within a trial involved triplicate samples. The data for each experiment was presented as the mean of duplicate trials (2 trials x 3 replicates/sampling period/trial; n=6). All data were presented as the mean with standard deviation of the mean represented by error bars. Statistical analysis was done using one-way analysis of variance (ANOVA), or for timed series using one-way repeated measures ANOVA, using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). Data treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

3. Results and Discussion

3.1. Comparison of Selective Media for Quantitative Enumeration of Salmonella Challenge Cultures After Droëwors Processing

In a prior study with biltong, background meat contamination was experienced that came up among the inoculated *Salmonella* challenge organisms during plating on TSA + antibiotics, forcing us to seek a more selective media that allowed quantitative enumeration of *Salmonella* [4]. Several selective media (XLD, HE) were examined but these media have a history of inhibiting stressed, injured cells [4,35–38]. We included an agar made with selenite cystine broth that is often used as an enrichment broth for *Salmonella* and referred to it as selenite cystine agar (SCA). Our data confirmed that even with droëwors, SCA media was equivalent to TSA in enumeration of *Salmonella* during droëwors processing compared to XLD or HE selective agars (Figure 3).

The data shows significant reductions of *Salmonella* when plated on XLD and HE media compared to TSA and SCA, indicating inhibition of stressed or injured cells by those media. We have not noticed any significant background contamination from our raw beef in recent trials as observed previously as it could have been a seasonal or processor-related issue as we source our beef from a local processor who obtains it through a broker who obtain their beef from different sources. Because of the consistency in recovery, we have continued to use SCA media with the *Salmonella* challenge organisms for our studies.

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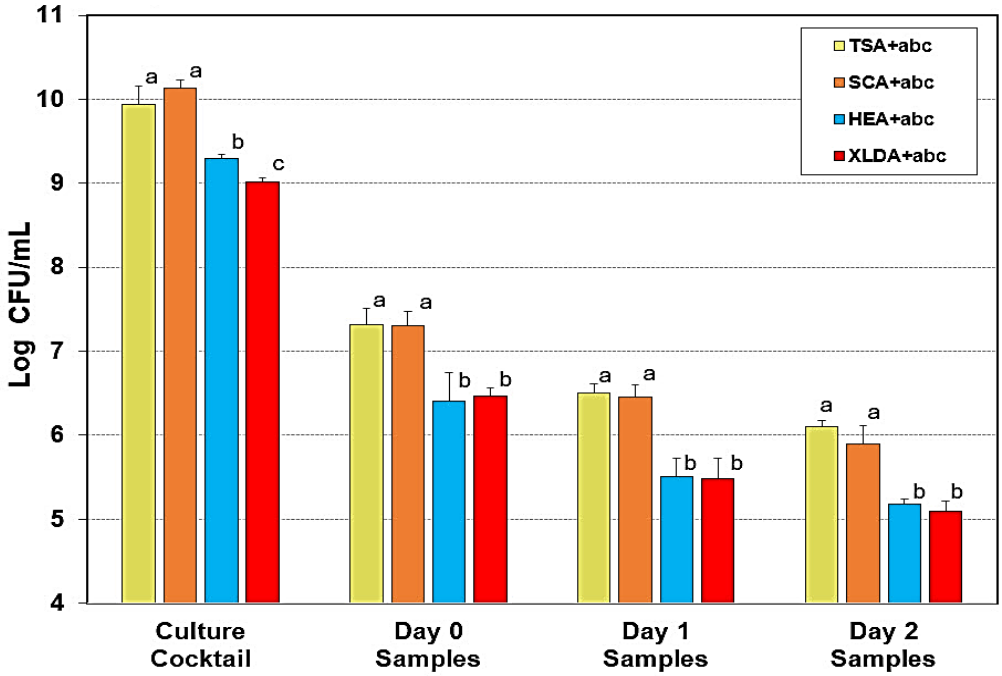

Figure 3. Comparison of selective media on enumeration of an inoculum mixture of 5 *Salmonella* serovars during droëwors processing. Droëwors-processed beef was plated on TSA, SCA, XLD, and HE (all containing antibiotics that the *Salmonella* were resistant to). Treatments within the same sampled grouping were analyzed by ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); those with the same letters show no significant difference ($p > 0.05$).

3.2. Biltong Processing: Comparison of Acid-Adapted and Non-adapted Salmonella Challenge Cultures

After having performed a variety of validations and characterizations of biltong processing [2,4,33–35], we contemplated validating whether acid-adapted challenge cultures would be more tolerant to processes involving acid treatment as had been postulated. This was especially concerning as we were about to embark on a study with droëwors to achieving a 5-log reduction with a hardy inoculum would improve the overall safety of these products.

3.2.1. Salmonella enterica Typhimurium (serovar I 4,[5] 12:i:-)

Salmonella serovar I 4,[5] 12:i:- [36] was provided by USDA-FSIS as an isolate obtained from dried beef and it was questioned whether it's desiccation resistance could serve as an ideal challenge organism for the biltong process. We examined the biltong process using both acid-adapted and non-acid-adapted culture preparations of *Salmonella* I 4,[5] 12:i:- (Figure 4).

To our surprise, the acid-adapted *Salmonella* Typhimurium serovar I, 4,[5],12:i:- inoculum showed a greater log reduction (i.e., more sensitive) in the biltong process than the non-adapted culture. The requirement to use acid-adapted cultures was supposed to make it more difficult to achieve microbial reduction over non-adapted cultures because of the tolerance to acid that growth at low pH should have provided. The implication was that the non-adapted cultures present more resistance to the processing conditions than the acid-adapted cultures and questioned whether our mix of 5 *Salmonella* serovars would demonstrate the same distinction during processing.

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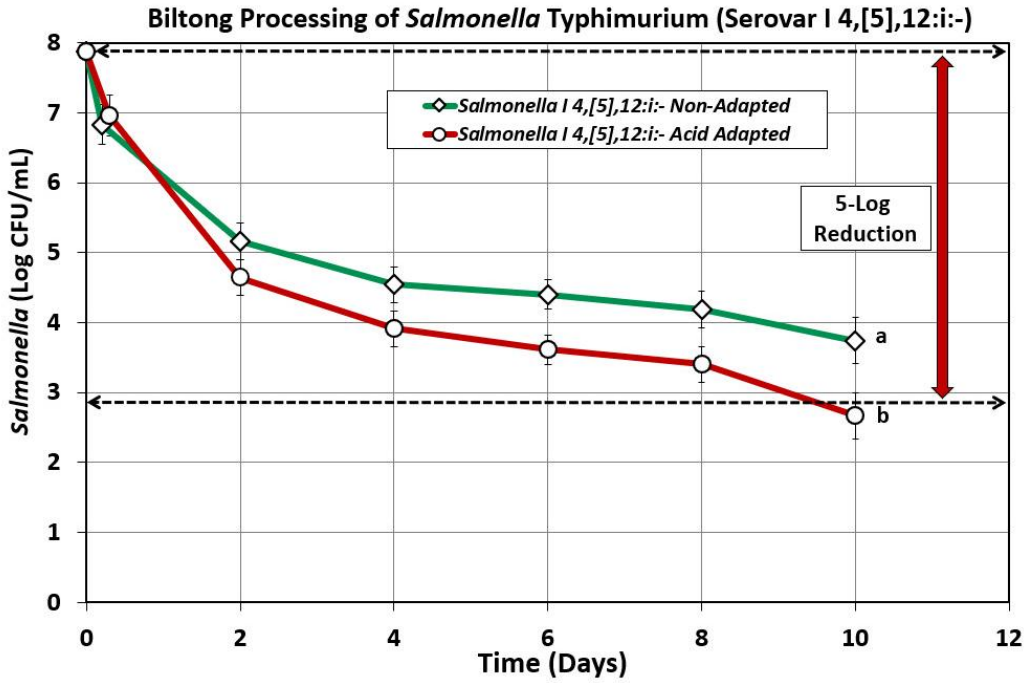

Figure 4. Comparison of biltong processing of beef inoculated with acid-adapted and non-adapted culture treatments of *Salmonella* Typhimurium serovar I, 4,[5],12:i:-. Treatments were analyzed by RM-ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$).

3.2.2. Mixture of Five Salmonella serovars: Acid-Adapted (1% glucose) vs Non-Adapted (0% Glucose)

This same comparison was then examined with the mix of five Salmonella serovars used in our prior biltong experiments and was intended for use in our upcoming study involving droëwors processing. As with *Salmonella* Typhimurium (serovar I 4,[5] 12:i:-; Figure 4), the data showed that using a mixture of 5 *Salmonella* serovars, we again demonstrated a greater reduction during biltong processing with the acid-adapted cultures than non-adapted challenge cultures (Figure 5). The data showed that for acid-adapted vs non-adapted cultures with the same dip treatment, the acid-adapted cultures provided the greater log reductions. Similarly, for trials with the same culture pre-treatments, acid-dip treatments gave significantly greater log reductions ($p < 0.05$) than water dip treatments (Figure 5).

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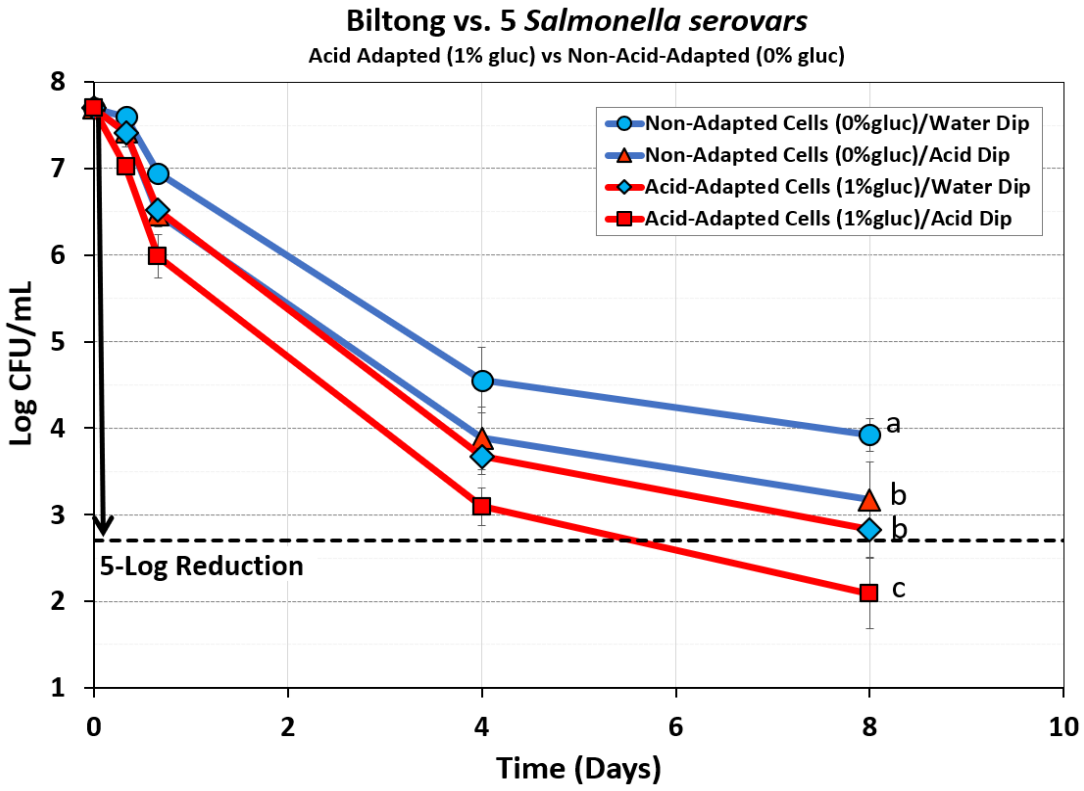

Figure 5. Comparison of processing of biltong beef inoculated with acid-adapted or non-adapted culture treatments of 5 *Salmonella* serovars (*S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380. Additionally, inoculated beef was dipped in either water or 5% lactic acid for 30 sec before marination. Treatments were analyzed by RM-ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); those with the same letters are not significantly different ($p > 0.05$). Acid-adapted cultures are represented by red lines, acid-dipped biltong is represented by red-filled symbols, and water-dipped biltong as blue-filled symbols.

3.2.3. Mixture of 5 Salmonella serovars: Acid-Adapted (1% glucose) vs Non-Adapted (1% Glucose + Buffer)

The differences in the impact of acid-adapted vs. non-acid adapted cultures shown in Figures 4 and 5 was concerning, and it was thought that the additional 1% glucose added to the acid-adapted cultures would provide a significant nutritional difference compared to the cultures in media with only 0% glucose. We therefore examined whether a non-acid adapted culture treatment by adding 1% glucose along with buffer would provide a more balanced nutritional comparison, yet buffering to maintain media pH at near initial levels would be an improved variation of non-acid-adapted culture pre-treatments. However, this comparison provided an even greater disparity between the biltong processes using acid-adapted and non-adapted culture pre-treatments and further reduced the log reduction obtained by the non-adapted treatments even when extended to 10 days of processing (Figure 6). As observed earlier, acid-dipped treatments gave greater reductions (not significantly different) compared to water-dipped treatments for both acid-adapted and non-adapted (buffered) culture treatments (Figure 6).

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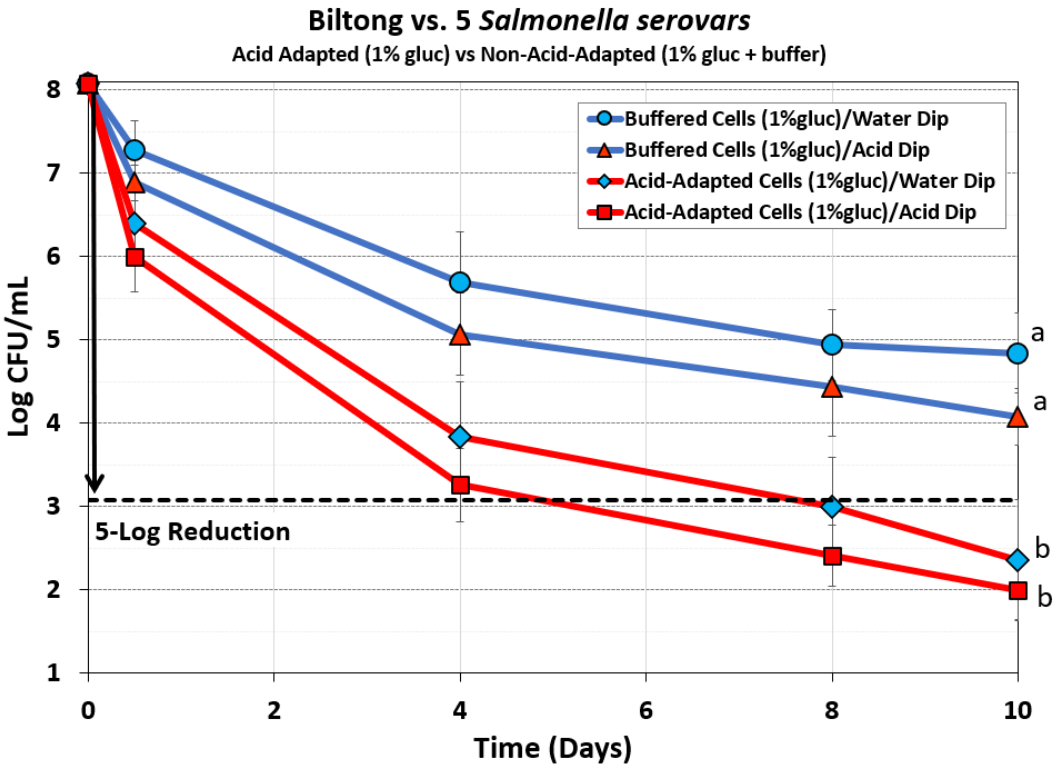

Figure 6. Comparison of processing of biltong beef inoculated with acid-adapted (1% glucose) or non-adapted (1% glucose + buffer) TS broth culture treatments of 5 *Salmonella* serovars (*S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380. Additionally, inoculated beef was dipped in either water or 5% lactic acid for 30 sec before marination. Treatments were analyzed by RM-ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$); those with the same letters are not significantly different ($p > 0.05$).

3.3. Droëwors Processing: Comparison of Acid-Adapted and Non-Adapted Salmonella Challenge Cultures

Droëwors is a sausage-like beef stick often made from leftover pieces of beef/fat trimmings from the biltong process that often complements products such as biltong. Acid-adapted and non-adapted *Salmonella* challenge cultures were also examined in droëwors processing to determine whether the effect would be different than that observed for biltong processing. The results would determine the manner in which we would approach culture preparation in lieu of upcoming droëwors processing experimentation.

Mixture of 5 Salmonella serovars: Comparison of Acid-Adapted (1% Glucose) vs Non-Acid-Adapted (0% Glucose) During Droëwors Processing.

Similar to what was observed with biltong processing, enumeration of *Salmonella* recovered during droëwors processing again demonstrated that beef inoculated with acid-adapted cultures resulted in significantly greater reductions than that obtained with non-adapted cultures (Figure 7). The use of non-acid adapted cultures of *Salmonella* provided only a 3-log reduction during 10 days of desiccation in the humidity oven (55% RH, 75°F), far short of the 5-log reduction needed to achieve USDA-FSIS approval if not testing for presence of *Salmonella* in every lot of edible ingredients used in droëwors manufacture.

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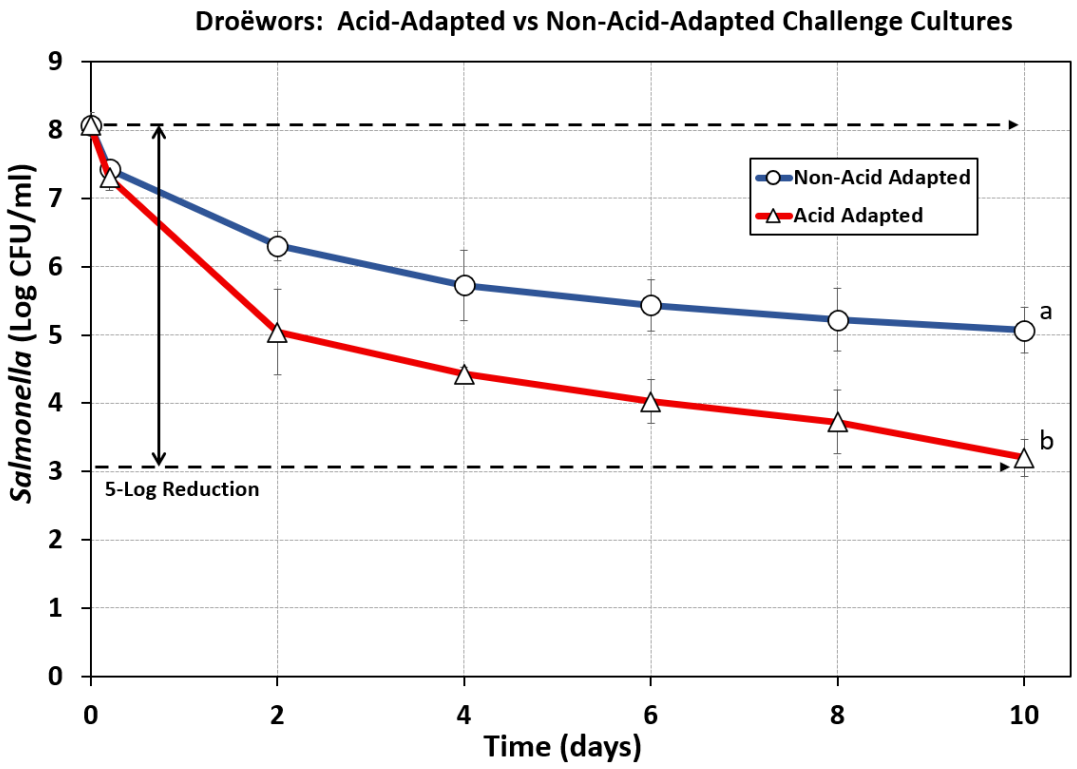

Figure 7. Droëwors processing: comparison of beef inoculated with acid-adapted (1% glucose) or non-adapted (0% glucose) TS broth culture treatments of 5 *Salmonella* serovars (*S.* Thompson 120, *S.* Heidelberg F5038BG1, *S.* Hadar MF60404, *S.* Enteritidis H3527, and *S.* Typhimurium H3380). Treatments were analyzed by RM-ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences; treatments with different letters are significantly different ($p < 0.05$).

4. Conclusions

The requirement by USDA-FSIS to use acid-adapted cultures during process validation experiments based on NACMCF recommendations may not apply to all dried beef processes involving acidic treatments. Understandably, the desire to use acid-tolerant cultures in validation studies that would not be overly sensitive to acidic treatment would be preferred in lieu of more acid-resistant strains that might naturally be present on meat/food products. Biltong and droëwors are complex processes, involving acidic treatment (vinegar in the marinade), salt, low water activity and desiccation in combination. The process of culture pre-treatment for acid adaptation may have induced stress and injury in the cultures that are impacted upon by the inhibitory conditions of the biltong/droëwors marinade and process (as evidenced by plate count differences on XLD agar), resulting in greater sensitivity by the challenge cultures to those processes as observed herein (Figure 3; [4]).

Various studies have demonstrated that acid-adaptation results in acid resistance of *Salmonella* to various processes involving acidic treatments and have likely been the justification for regulatory preference on use of acid-adapted cultures [37,38]. However, we found just the opposite, that acid-adapted cultures performed as if they were more sensitive to processing conditions in both biltong and droëwors processing, giving larger reductions than non-adapted *Salmonella*. This is similar to that found by Calicioglu et al. [14,39] comparing survival of non-adapted and acid-adapted *Salmonella* cultures during storage of beef jerky, whether they were inoculated pre- or post-drying. It may be best to have a trial run with both culture treatments in a particular process to test which stratagem is better suited to the process. Although a 5-log reduction was not obtained using the non-adapted cultures, we have subsequently identified that including a natural plant extract (pyrolyzed extract of plant material) could easily achieve a 5-log reduction with both biltong and droëwors [40].

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