



Pathogens Associated with Biltong Product and Their *in vitro* Survival of Hurdles Used during Production

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ABSTRACT

Biltong is a traditional South African spiced and dried, ready-to-eat meat product, and is an increasingly popular commodity worldwide. As few studies have evaluated its safety, this study evaluated 150 samples of South African biltong for aerobic bacteria, *Enterobacteriaceae*, coliforms, *Escherichia coli*, coagulase-positive *Staphylococcus*, *Salmonella* and *Listeria monocytogenes*. Selected strains of potential pathogens were further identified by use of 16S rDNA gene sequencing methods. In addition, the *in vitro* antimicrobial properties of each primary ingredient component of the biltong-making process was tested against selected bacterial isolates. Plate counts were the highest for aerobic bacteria (ca. 7 log CFU/g), followed by *Enterobacteriaceae* (ca. 4 log CFU/g), coliforms (ca. 3 log CFU/g), presumptive *Staphylococcus* (ca. 3 log CFU/g) and *E. coli* (ca. 1 log CFU/g) counts. All samples tested negative for *Salmonella*, while 2 samples tested positive for *L. monocytogenes* and 3 samples for enterotoxin-producing *Staphylococcus* strains. Results also showed that 25% of the isolates grew in the presence of up to 20% NaCl. Apple cider vinegar and brown spirit vinegar inhibited the growth of 63 and 50% of the isolates, respectively, while all 8 isolates (2 *L. monocytogenes*, 3 *S. aureus* and 3 *S. pasteurii*) showed the same growth patterns in the presence and absence of spices traditionally used in manufacturing biltong. Overall, strains of *L. monocytogenes*, *Staphylococcus aureus* and *Staphylococcus pasteurii* showed the most growth in all assays conducted. Results from this study highlighted biltong as a potential reservoir for foodborne pathogens, which have implications for foodborne illness.

A peer-reviewed article

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INTRODUCTION

The preservation of meat has always been important to the survival of humans. For example, one of the first references to dry-cured pork was reportedly recorded on Sumerian tablets in 2000 BC (28). Historically, cured, fermented and dried meat products are regarded as microbially safe ready-to-eat (RTE) foods because of their low water activity (a_w) and low pH as well as the presence of curing salts (15). These products have been consumed throughout history, and often have strong cultural associations. For example, pemmican was a dried meat product that provided Native Americans with protein in the lean months of winter. Similarly, carne seca and machaca in New Mexico, jerky in the United States, charqui in South America, kilshi in Sahel, rou gan in China and biltong in South Africa are popular RTE meats in modern times (16, 29).

RTE meats are often produced from meats such as beef, lamb, pork and poultry or mixtures of such meats (15). The Shiga-toxin producing *Escherichia coli* (*E. coli*) O157:H7, *Listeria monocytogenes* (*L. monocytogenes*), *Salmonella* and *Staphylococcus aureus* (*S. aureus*), have been detected in these types of meat products (22). Indeed, several outbreaks linked with *E. coli* O157:H7 and *Salmonella* in RTE meat products have been recorded (15). For example, there have been 9 recorded foodborne illness outbreaks and 5 recalls have been associated with jerky to date (1, 26).

Biltong is a traditional South African RTE dried and spiced meat product that is easy to produce (12, 24). All that is required is a selection of beef, game, chicken or ostrich meat, which is then cured with several basic flavoring agents (salt, black pepper, dried and roasted coriander and brown sugar) and vinegars (apple cider, brown spirit, wines), after which it is dried at ambient temperatures for several days (12, 20). As a result, biltong production is often a home industry, and the safety of this commodity is of concern (19). In addition, there are several new international markets for biltong, including Australia, Portugal, the UK and the US (2). However, very little updated survey data has appeared in the literature on the safety of this RTE meat (27, 29). Thus, the aims of this study were to update current knowledge on the

prevalence of bacterial foodborne pathogens associated with this product and to evaluate the *in vitro* response of selected isolated pathogens to some of the hurdles used during the biltong preparation process.

MATERIALS AND METHODS

Sample collection

One hundred fifty biltong samples were obtained from various geographical locations in the Gauteng province in South Africa during July–September 2008. Suppliers included slaughterhouses ($n = 21$), biltong bars (small outlets that usually sell biltong as the main source of income, often found in shopping malls) ($n = 35$), convenience stores (supermarkets) ($n = 26$), biltong shacks (a biltong bar that sells both raw and dried meat in the same establishment on a small scale) ($n = 25$), home-based industries ($n = 4$), shops that sell pre-packaged product ($n = 19$) and sweet (confectionary) shops ($n = 10$).

Sample processing and enumeration

For each biltong sample, 20 g of the product (if not already sliced, then cut from the original sample, using a sterile blade) was transferred into a Whirl-Pak bag (Nasco, USA), combined with 180 ml diluent (0.1% Bacteriological Peptone {BioLab, Midrand, South Africa} + 0.85% sodium chloride {Saarchem-Merck Chemicals, South Africa}) and homogenized for 2 min with a Colworth 400 Stomacher (10). The homogenized biltong samples were serially diluted in diluent and plated in duplicate, using standard plating procedures, as outlined in Table 1. After plating, the pH of the homogenized samples was recorded by placing a pH detection probe of a laboratory pH meter (Metrohm 744) directly into each sample. In addition, biltong samples were concurrently processed with standard methods for the detection of *Salmonella*, *L. monocytogenes* and presumptive *S. aureus* (Table 1).

Duplicate plates containing between 30 and 300 colonies, or the highest number if fewer than 30 colonies were obtained, were enumerated for aerobic plate (APC), *Enterobacteriaceae* (ECB), coliform (CC), *E. coli* (EC) and coagulase-positive *Staphylococcus* (SAC)

counts and expressed as log colony forming unit (log CFU) per gram.

Presumptive pathogens were selected for further molecular identification (10). In addition, presumptive isolates of *S. aureus* were tested for enterotoxin production, using SET-RPLA TD900 kits (Oxoid, London) (20, 21).

Molecular identification of presumptive bacterial pathogens

Presumptive pathogens were further identified using 16S rDNA gene sequencing. Polymerase chain reaction (PCR) amplification was carried out as previously described, using the primer sets U1392R (5'-ACG GGCGGT GTG TRC-3') and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG\3') in combination with Fermentas 2× PCR Master Mix (Fermentas Life Science). The purified PCR product was sequenced and analyzed using BLAST against the 16S rDNA gene sequences from GenBank, and samples were submitted to obtain accession numbers (10).

Isolates selected for growth/tolerance assays

Eight isolates that were then selected for further work included strains of *L. monocytogenes*, *S. aureus* and *S. pasteurii* [accession numbers FJ160766; FJ160767; FJ392795; FJ392802; FJ392805; FJ392798; FJ392800 and FJ392804]. In order to generate working inocula, each isolate was successively cultured twice (from previously frozen stock cultures) for 24 h at 37°C in Tryptone Soy Broth (TSB) (BioLab, Midrand South Africa), streaked onto Tryptone Soy Agar plates (TSA) (BioLab, Midrand South Africa) and incubated for 48 h at 37°C. Colony morphology as well as Gram-stain reactions were examined to ensure the purity of the cultures of each isolate, and plates were stored at 4°C.

Growth in high salt concentrations

To evaluate salt tolerance, each isolate was plated by the streak plate technique, in triplicate and on four separate occasions, onto TSA plates supplemented with varying concentrations (5, 10, 15, 20, 25%) of sodium chloride (NaCl) (Saarchem, Merck Chemicals-South Africa)

TABLE 1. Culture methods, media and the aerobic incubation time and temperatures utilized in analysis of the 150 biltong samples obtained

	Incubation and growth media			
	Time (h)	Temp (°C)	Plating method	
Aerobic Plate Count (APC)	48	30	Pour (10 ¹) AND Spread	Tryptone Soy Agar (TSA) (BioLab, Midrand South Africa).
<i>Enterobacteriaceae</i> (EBC), Coliform Count (CC), <i>E. coli</i> Count (EC)	48	37	Pour	RAPID' <i>E. coli</i> 2™ Agar (Bio-Rad, France). All colonies. Blue-green colonies. Purple colonies.
Coagulase-positive <i>Staphylococcus</i> (SAC) Count	48	37	Spread	Baird-Parker Agar plus Egg Yolk Tellurite (0.5% w/v) (Scharlau, Spain). Black colonies with halo selected and Gram stained.
	24	37	Spread	Selected isolates – DNase agar (Scharlau, Spain). Flooded with 1 ml hydrochloric acid (1M) to show clearing.
	24	37	Streak	Isolates showing clearing on DNase plates selected for Rapid Staph' Agar (RSA)(Bio-Rad, France) plus Egg Yolk Tellurite (0.5% w/v) (Scharlau, Spain).
<i>Listeria monocytogenes</i> detection	24	30	Pre-enrichment	Fraser ½ (Bio-Rad, France).
	48	37	Enrichment	Fraser I (Bio-Rad, France).
	24	37	Streak	RAPID' <i>L. mono</i> ™ Agar (Bio-Rad, France), Blue colonies selected and Gram stained.
<i>Salmonella</i> detection	18	37	Pre-enrichment	Buffered Peptone Water (Scharlau, Spain).
	24	37	Enrichment	Müller Kauffmann Medium plus Brilliant Green Cycloserine supplement (1 vial/ 500 ml) plus 200 µl Gram's Iodine/ 10 ml (Scharlau, Spain).
			AND	
	24	41.5	Enrichment	Rappaport-Vassiliadis Broth (Scharlau, Spain).
	24	37	Streak	Brilliant Green Agar Modified (Scharlau, Spain). Red colonies.
			AND	
24	37	Streak	Xylose Lysine Deoxycholate Agar Scharlau, Spain). Black colonies.	

TABLE 2. Amendments made to the composition of mock biltong agar (MBA) to create variations that highlight growth susceptibilities to each component

Variation of MBA	Modification of components
MBA 1	No modifications made to original media
MBA 2	Exclusion of brown sugar
MBA 3	Exclusion of beef extract
MBA 4	Exclusion of sodium chloride
MBA 5	Exclusion of both beef extract and biltong spice
MBA 6	Exclusion of both biltong spice and brown sugar
MBA 7	Exclusion of biltong spice

(9). Inoculated plates were incubated at 37°C and qualitatively inspected every 24 h for 7 days for signs of bacterial growth.

Growth at various temperatures

A loopful of each isolate was inoculated into 20 ml of TSB, as well as plated by the streak plate technique onto TSA plates, in triplicate and on four separate occasions, and samples were incubated at 4, 25, 30, 37 and 45°C for 7 days. At 24 h intervals, TSA plates were observed for bacterial growth. In addition, a loopful from each inoculated TSB broth was streak plated onto TSA plates and incubated for 24 h at the appropriate temperature. For example, TSB-grown cultures incubated at 4°C were plated and incubated again at 4°C. These plates were also observed to confirm any bacterial growth.

Growth in the presence of organic acids

To determine if bacterial strains were tolerant to the organic acids used in the biltong manufacturing process, spot-on-lawn assays (3) were conducted in triplicate and on four separate occasions. A colony for each isolate was selected and inoculated into 50 ml of TSB, and samples were then incubated at 37°C for 18–20 h. Bacterial lawns and indicator plates were

prepared by pour and spread plating of 1 ml of this overnight bacterial culture mixed with TSA to a final colony count of ca. 10^5 – 10^6 CFU/ml (3). Plates were allowed to stand for 5 h at ambient temperature to allow drying of the surface. Indicator plates were divided into sections and 50 µl of sterile distilled water (negative control) or undiluted apple cider vinegar (Safari SAD, South Africa) or brown spirit vinegar (Safari SAD, South Africa) or 99.7% glacial acetic acid (Associated Chemical Enterprises, South Africa) (positive control) were spotted into each section. Plates were incubated at 37°C and observed every 24 h for 7 days for zones of clearing that would indicate inhibition of bacterial growth (3).

Growth in the presence of biltong spice

To determine the growth of bacterial isolates in the presence of traditional biltong spice (commercially available product, www.biltongmakers.com), eight different agar combinations were prepared as follows:

- TSA was supplemented (prior to auto-claving) with traditional biltong spice (40 g/l) and was referred to as TSAB.
- Bacteriological agar (13 g/l) (Merck, South Africa) was supplemented with a combination of beef extract (10 g/l) (BioLab,

South Africa), brown sugar (3 g/l) (Selati, South Africa), sodium chloride (5 g/l) and traditional biltong spice (40 g/l) and was referred to as mock biltong agar (MBA 1).

- Six variations of MBA were also created to highlight growth or inhibition of the various components, as shown in Table 2.

Bacterial isolates were streak plated, in triplicate and on four separate occasions, onto TSAB and all variations of MBA (Table 2), incubated at 37°C and observed every 24 h for 7 days for bacterial growth.

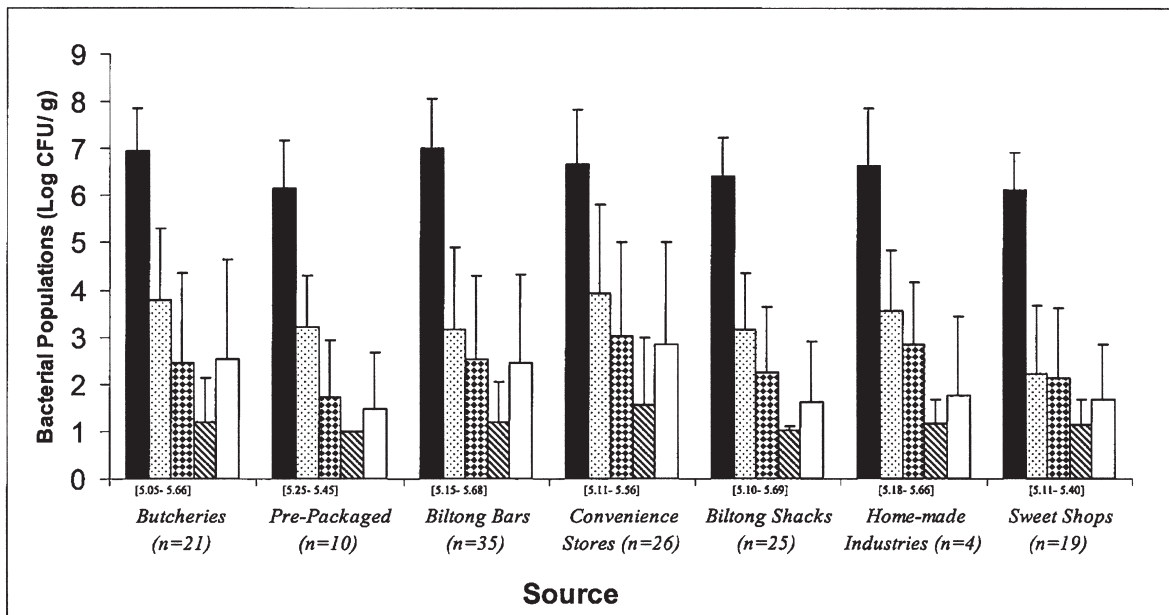
RESULTS AND DISCUSSION

Overall counts from biltong samples linked to points-of-sale

Overall, biltong samples obtained from biltong bars in this study had the highest associated APC counts (ca. 7.01 log CFU/g) (Fig. 1). This product was often sold uncovered, and handling of the product at point-of-sale may have contributed to an overall higher APC (19). In contrast, pre-packaged samples had the lowest APC counts (ca. 6.14 log CFU/g) (Fig. 1), probably because of the protective barrier provided by the packaging (14). Biltong is produced under several microbial growth-limiting conditions such as curing (salts, spices and vinegars), refrigeration and drying (19). Traditional biltong reportedly has a water activity (a_w) of 0.74–0.77 and pH of 5.5–5.8 associated with the final product (12, 29). Although these factors reduce the presence of several microbial populations, biltong thus favors the prevalence of heat-tolerant and salt-tolerant microorganisms.

EBC, CC and EC were used in this study to assess the overall hygiene of the production process, as high EBC and ECs are indicative of enteric pathogens (5). From results obtained in this study, it was evident that biltong samples from convenience stores showed the highest associated EBC, CC and EC counts (3.94, 3.03 and 1.57 log CFU/g, respectively) (Fig 1). In contrast, pre-packaged samples had the lowest associated EBC and CC values (2.21 and 1.73 CFU/g, respectively) (Fig. 1), and were the only samples with EC counts

FIGURE 1. Bacterial populations of aerobic bacteria (black bar), total *Enterobacteriaceae* (dotted bar), coliforms (checked bar), *E. coli* (striped bar) and presumptive *Staphylococcus aureus* (white bar) counts obtained from 150 biltong samples (lower detection limit = 1 log CFU/g). {pH ranges associated with samples.}



below the lower detection limit (Fig. 1). The presence of *E. coli*, an index organism, traditionally highlights the suspected presence of other pathogens, such as *Salmonella*, as they are capable of surviving in the same environmental niches (17). However, *Salmonella* was absent from all biltong samples tested in this study.

SAC counts showed that convenience stores, followed closely by slaughterhouses and biltong bars, were associated with samples with higher SAC counts than the other points-of-sale; indeed, all SAC counts observed were below 3 log CFU/g (Fig. 1). As *Staphylococcus* populations are often native to the human nose, throat and skin, high *Staphylococcus* counts are often indicative of poor human handling practices (15).

Presence of bacterial pathogens in biltong

S. aureus

After the screening of 159 presumptive *Staphylococcus* isolates obtained from biltong samples, 15 isolates were singled out as presumptive *S. aureus* strains and further identified with 16SrDNA sequencing. Results from molecular analysis showed that of the 15 isolates, only 3 were confirmed as *S. aureus* (accession numbers

FJ392795, FJ392802 and FJ392805). The other 12 were identified as *S. pasteurii* (FJ392791 – FJ392793, FJ392796 – FJ392799, FJ392801, FJ392803 and FJ392804), *S. saprophyticus* (FJ392800) and *Macrococcus caseolyticus* (FJ392794). All 15 isolates were also tested for enterotoxin production. Results showed that 3 isolates produced enterotoxin B, including 2 strains (FJ392795 and FJ392802) identified as *S. aureus* (99% sequence similarity to *S. aureus* ATCC 14458). Enterotoxin B-producing strains are reportedly the serotypes that are the third most common in terms of being associated with food poisoning events, after enterotoxins A, and D (4). Interestingly, a strain of *S. pasteurii* (FJ392798) (99% sequence similarity to *S. pasteurii* AF041361) also produced a positive reaction to enterotoxin B. Although enterotoxin production is often characteristic of coagulase-positive *Staphylococcus* strains such as *S. aureus*, it is not limited to these organisms (20, 25). Even though *S. pasteurii* strains are often associated with food commodities (18), there is no record of this species having been implicated in foodborne illness outbreaks.

The presence of enterotoxin producing *S. aureus* in biltong could potentially be attributed to the high concentration of salts, acidic pH (25) and increased a_w

of a moister biltong product, especially since strains of *S. aureus* are capable of growth and enterotoxin production at a_w of 0.85 (24, 25). It is reported that the modern consumer markets favor more moist biltong, which is considered more appealing to the palate (12). Traditionally, dried biltong has a water activity (a_w) of 0.77 (12); however, the favored biltong has 40% more moisture than traditional biltong and an a_w of between 0.85 and 0.93 (12, 24) which supports the growth of several pathogens.

L. monocytogenes

The findings of this study showed that 2 of the 150 (1.33%) biltong samples tested positive for *L. monocytogenes* (accession numbers FJ160766 and FJ160767). In comparison, the prevalence of *L. monocytogenes* observed in this study was 1% higher than that observed in a study on jerky (1). Although the minimum dose of *L. monocytogenes* cells required to cause foodborne illness is variable, foodborne illness has often been coupled with elevated levels of this pathogen in a consumed food product (22).

Biltong would generally be considered a potentially unfavorable environment for *L. monocytogenes* because of its

low a_w and high salt concentrations (7, 8), and it would therefore be less likely to harbor and support its growth. However, strains of *L. monocytogenes* are often associated with raw poultry meat (7, 15). In this study, this pathogen was isolated from chicken biltong. The presence of these strains could be attributed to the contamination of biltong prior to and at production, and distribution and within the retail environments of this commodity, due to the ubiquitous prevalence of this foodborne pathogen (15).

Effect of in-process hurdles applied to biltong on selected foodborne pathogens

Overall, strains of *L. monocytogenes* ($n = 2$), *S. aureus* ($n = 3$) and *S. pasteurii* ($n = 3$) showed growth in most of the assays conducted. Seven of the 8 isolates grew in the presence of 15% NaCl. In addition, it was evident that isolates belonging to the *Staphylococcus* genus were the only isolates that showed growth at $\geq 15\%$ NaCl. This was not unexpected, as several strains of *Staphylococcus* have been shown to survive in environments containing high salt concentrations (9).

Both the plate and the broth method used in this study showed the same qualitative growth patterns. Only strains of *L. monocytogenes* grew at 4°C, while all other isolates grew optimally in the temperature range of 25–37°C. In addition, none of the 8 isolates tested showed growth at 45°C. It is important to note that during the biltong manufacturing process, meat slices are often marinated at refrigeration temperatures of ca. 4°C. Although this temperature does not favor the growth of 80% of the isolates evaluated in this study, it did support the growth of strains of *L. monocytogenes*. Such findings are not uncommon, as strains of *L. monocytogenes* are known to proliferate at refrigeration temperatures (15).

Results from this study showed that all isolates were inhibited by undiluted glacial acetic acid (positive control), while the apple cider vinegar and brown spirit vinegar inhibited the growth of only some strains. It has previously been reported that acetic acids, such as vinegars and wines, possess bacteriostatic and bacteriocidal properties (13). For example, a study conducted by Entani and associates (13) showed that even at

the lowest concentrations, vinegar had bacteriocidal properties against *Escherichia coli* O157:H7. Although apple cider vinegar exhibited enhanced antimicrobial properties compared to brown spirit vinegar, both vinegars were inadequate to cause complete growth inhibition of foodborne pathogenic and enterotoxin producing strains. Vinegar marination is an important component of the biltong manufacturing process, and survival of potential foodborne pathogens during this process is cause for concern.

Furthermore, results also showed that all 8 isolates exhibited the same growth patterns in the presence and absence of traditionally used biltong spice. Spices such as black pepper and coriander, which are the predominant spices in the traditional biltong spice mix, are known to possess weak to mild antimicrobial properties in general (6, 11).

CONCLUSION

This study highlighted biltong at point-of-sale as a potential vehicle for foodborne pathogens and showed that *S. aureus*, *S. pasteurii* and *L. monocytogenes* may also survive the hurdles used during biltong production. However, the findings in this study were based on *in vitro* results. Thus the question remains as to whether the same foodborne pathogens are able to survive the biltong manufacturing process *in situ*, a question which is currently being investigated.

ACKNOWLEDGMENTS

The authors thank the University of the Witwatersrand and the Friedel Sellschop Award, and Carnegie Corporation Transformation Programme for project funding, as well as the National Research Foundation, for student funding.

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