

Research Note

Gas Formation in Ground Beef Chubs Due to *Hafnia alvei* Is Reduced by Multiple Applications of Antimicrobial Interventions to Artificially Inoculated Beef Trim Stock†

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ABSTRACT

Gas-forming microorganisms were isolated from gas-swollen ground beef chubs obtained from a commercial source and were phenotypically identified as *Hafnia alvei*. In situ experiments, the isolated *H. alvei* strains produced gas in inoculated irradiation-sterilized ground beef chubs. A five-strain cocktail of *H. alvei* isolates was inoculated on beef trim. The inoculated beef trim samples were treated with either a water wash (W) at 65 psi for five passes (a pass refers to the application of successive multiple antimicrobial treatments to inoculated beef trim on a moving processing conveyor belt at a speed of 1 cm/s under heat ducts or oscillating spray nozzles), W plus a 2% (vol/vol) lactic acid wash (L) at room temperature at 30 psi for three passes (W/L), or a combination treatment (COMB) consisting of W plus 82°C water for three passes plus 510°C hot air for six passes plus L, or were not treated (control). After treatment, the beef trim was ground and vacuum packaged. The numbers of *H. alvei* were reduced with water alone and with the aforementioned antimicrobial intervention treatments. For the untreated and inoculated control samples, the numbers of *H. alvei* increased from 7.03 to 8.40 log CFU/g after 7 days of incubation at 4°C. However, the numbers of *H. alvei* treated by successive antimicrobial interventions (COMB) were initially reduced to 5.25 log CFU/g and increased to just 6.9 log CFU/g after 7 days of incubation at 4°C. Gas was produced in untreated control samples after 3 days at 15°C (15 of 15 inoculated chubs). However, in meat treated with W, W/L, and COMB, gas was produced after 4 to 5, 7 to 8, and 9 to 10 days of storage at 15°C, respectively. These results demonstrate the effectiveness of multiple antimicrobial interventions in reducing *H. alvei* numbers on beef trim and subsequently delaying gas formation in the resulting ground beef chubs.

Gas formation in ground beef chubs is a product defect associated with the processing and grinding of red meat (4, 11, 15), resulting in wasted product and economic loss for meat processors. Several microorganisms have been identified as gas producers in vacuum-packaged meat (4, 6, 8, 11, 12, 14, 15, 24). Among these microorganisms, *Hafnia alvei* is listed as a potential contributor to and cause of this defect (10–12, 14). Of corollary interest is the association of biochemically classified putative *H. alvei* strains with gastroenteric disease in humans (1, 13).

Recently, our laboratory reported the development of multiple antimicrobial interventions to reduce fecal coliforms in beef trim (16, 17). These same interventions decreased total mesophilic aerobic bacteria as well as psy-

chrotrophic bacteria and coliforms in refrigerated stored beef trim (16, 17). The hypothesis of the present study was that *H. alvei* numbers and subsequent gas production in ground beef chubs could be reduced with multiple antimicrobial interventions. This hypothesis was tested by using actual gas-producing *H. alvei* strains isolated from gas-swollen ground beef chubs to inoculate beef trim and subsequently treating that trim with multiple antimicrobial interventions prior to grinding and packaging.

MATERIALS AND METHODS

Isolation of gas-forming microorganisms. Ground beef chubs with visible evidence of gas formation were obtained from a commercial beef-processing company (name withheld). Samples of ground beef (25 g) were mixed with 25 ml of buffered peptone water (BPW; Difco, Becton Dickinson Microbiology Systems, Becton Dickinson and Co., Sparks, Md.) and pummeled for 2 min using a stomacher. The stomached samples were diluted 10-fold and plated on tryptic soy agar (15 and 32°C) and deMan Rogosa Sharpe agar (15 and 32°C) (Difco, Becton Dickinson Microbiology Systems, Becton Dickinson and Co.) both aerobically and anaerobically (in Gas-Pak jars with an anaerobic gas-generator envelope [Gas-PakII, Becton Dickinson Microbiology Systems, Becton Dickinson and Co.]). The stated culture conditions were used to select a total of approximately 50 bacterial isolates for

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† Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

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further testing. Of this group, 14 isolates were retested for in vitro gas formation by inoculation in deMan Rogosa Sharpe broth with a Durham tube assembly. Five isolates were selected from different samples (beginning with the highest dilution) and subjected to characterization. On the basis of the Gram reaction and criteria determined with API 20E microbial identification system (bio-Merieux, Inc., Hazelwood, Mo.) results, all five selected isolates were identified as *H. alvei*. All isolates were positive for the API 20E system's *o*-nitrophenyl- β -D-galactopyranoside, alcohol dehydrogenase, lysine decarboxylase, ornithine decarboxylase, citrate, Voges Proskauer, glucose, mannose, rhamnose, and arabinose tests. All isolates were negative for the API 20E system's H₂S, urea, tryptophane deaminase, indole, gelatin, inositol, sorbitol, saccharose, melibiose, and amygdalin tests. Gene profiling tests of all five isolates by a previously described (19) polymerase chain reaction protocol for *stx1*, *stx2*, *eaeA*, and enterohemorrhagic *Escherichia coli hlyA* were negative for the aforementioned virulence factors. These isolates (coded MARC-37, MARC-54, MARC-56, MARC-73, and MARC-82) were used singly or in a cocktail mixture inoculum for the subsequent experiments.

Confirmation of in situ gas formation in the *H. alvei* isolates. Samples (100 g) of previously irradiation-sterilized and frozen ground beef in plastic bags (Advantage Food Equipment Systems Co., Omaha, Nebr.) were inoculated with 5 ml of a BPW-diluted culture (tryptic soy broth, 24 h, 32°C) to achieve a total inoculum of between 10⁵ and 10⁶ cells, resulting in 10³ to 10⁴ CFU/g. All air was manually excluded by squeezing the bag, and then the bag was vacuum packaged and heat sealed with a vacuum packager (Hollymatic, New Age Industrial Co., Norton, Kans.). The vacuum-packaged bags were incubated at 15°C for up to 7 days. Uninoculated pouches were inoculated with 5 ml of sterile BPW. Each bag was visually examined daily for evidence of gas formation. As the onset of gas formation was usually accompanied by rapid bloating of the package, a positive result was scored only when pouch swelling was visually obvious compared with the uninoculated control pouches.

Inoculation of isolated gas-producing *H. alvei* and evaluation of multiple antimicrobial interventions. The five *H. alvei* isolates were propagated individually in tryptic soy broth for 24 h under static conditions. After incubation, cells were harvested by centrifugation (Beckman Instruments, Inc., Palo Alto, Calif.) at 4,000 \times g for 20 min at 4°C, washed once with BPW, and resuspended in BPW. After serial dilution in BPW, 8 ml of the *H. alvei* five-strain culture cocktail (approximately 9 log₁₀ CFU/ml) was spoon inoculated (5, 7) onto beef trim (approximately 15 by 15 by 2 cm) as described previously. The treatments applied were described previously (16, 17). Briefly, the uninoculated and inoculated beef trim samples were marked C (control; not treated), W (water wash at 65 psi), W/L (W + L [2% (vol/vol) lactic acid wash at room temperature at 30 psi]), COMB (combination treatment: W + HW [82°C water] + HA [510°C air] + L) on a commercial type beef trim belt located at the Roman L. Hruska U.S. Meat Animal Research Center's pilot plant facility (described previously (16, 17)). Within 1 h following treatment, the beef trim was ground to 1 cm in diameter with a commercial grinder (Davpol Enterprises Inc., New York, N.Y.) that had been chemically sanitized (bleach followed by 95% ethanol) and rinsed with sterile water before reuse. After separate grinding, 100 g of each untreated or treated ground beef portion was sampled and vacuum packaged in plastic film (0% oxygen permeability; Advantage Food Equipment Systems Co.) with a vacuum packager (Hollymatic). The vacuum-packaged ground beef chubs were stored at 4°C for 7 days. Samples (before treatment, after treatment, after 2 days,

and after 7 days) were taken by weighing 50 g of ground beef using alcohol-flamed forceps (9). Samples were placed in filtered stomacher bags (Spiral Biotech, Inc., Bethesda, Md.) with 100 ml of BPW with 0.1% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) and were then pummeled for 2 min in a Model 400 Stomacher (Tekmar, Inc., Cincinnati, Ohio). Appropriate sample dilutions were made in BPW, and *H. alvei* cells were enumerated by plating on a *Hafnia*-selective agar (deoxycholate lactose sucrose sorbitol) and incubating at 32°C for 48 h (2).

Evaluation of gas production in resulting ground beef chubs. The treated beef trim was ground and vacuum packaged as described above and stored at 15°C for 10 days to monitor gas production at temperatures elevated above refrigeration temperature. Each experiment involved five samples for each treatment and was replicated three times. A total of 15 samples for each treatment were visually evaluated to determine gas production.

Statistical analysis. Bacterial numbers for each treatment were converted to log₁₀ CFU/g and analyzed statistically by analysis of variance using the SAS General Linear Models procedure (23). Means were separated by using the least significant difference test (PROC MIXED) at the 0.05 probability level. Experimental variables were the C, W, L, W/L, and COMB treatments. Each experiment, including those involving gas formation in chubs and population determination, was replicated three times, and duplicate determinations were made for each analytical unit.

RESULTS AND DISCUSSION

Five bacterial isolates from gas-swollen chub-packaged ground beef samples were classified as strong gas producers and identified as *H. alvei* on the basis of morphological and biochemical profiles. All five of these isolates were capable of gas formation in 100-g inoculated chubs of irradiation-sterilized ground beef held at 4°C (data not shown). Isolation of gas-producing bacteria identified as *H. alvei* from both spoiled and nonspoiled meats has previously been documented (10, 18).

By the approach previously described for multihurdle interventions (16, 17), we applied the COMB treatment along with W or W/L sprays to beef trim artificially inoculated with known gas-forming *H. alvei* isolates in an effort to reduce gas formation in the resulting ground beef chubs. Table 1 shows the growth patterns of *H. alvei* in ground beef chubs made from beef trim after treatment with C, W, W/L, and COMB. After the initial treatment, the numbers of *H. alvei* decreased from approximately 7.00 log CFU/g to 7.23, 6.24, 5.81, and 5.24 log CFU/g for the C, W, W/L, and COMB treatments, respectively. While there were no initial differences between the results of the W treatment and those of the W/L and COMB treatments ($P > 0.05$), by day 7, differences in *H. alvei* populations were observable between COMB-treated samples and W- or W/L-treated chubs. On day 7, even though the W/L-treated chubs had *H. alvei* populations that differed from those of the COMB-treated samples by less than 0.5 log CFU/g, gas formation was evident in 10 of 15 W/L-treated samples, compared with 0 of 15 of the COMB-treated chubs (Tables 1 and 2). Under these experimental conditions, the multiple antimicrobial treatments (W/L and COMB) reduced gas-forming *H. alvei* in beef trim to a greater degree than did the application of a single ambient-temperature water spray.

TABLE 1. Counts of *H. alvei* in ground beef chubs made from inoculated sterile trim and then stored at 15°C following single or multiple antimicrobial treatment as determined from deoxycholate lactose sucrose sorbitol agar plating

Treatment ^a	Count (log ₁₀ /g) at incubation time ^b			
	Before treatment	0 days	2 days	7 days
C	7.03 ± 0.05 Aa	7.23 ± 0.13 Aa	7.39 ± 0.05 Aa	8.40 ± 0.40 Ab
W	7.15 ± 0.21 Aa	6.24 ± 0.37 Bb	6.41 ± 0.29 Bb	8.30 ± 0.41 Ac
W/L	7.02 ± 0.16 Aa	5.81 ± 0.14 Bb	6.01 ± 0.33 BCb	7.37 ± 0.27 Ac
COMB	7.36 ± 0.20 Aa	5.25 ± 0.39 cb	5.75 ± 0.49 cc	6.94 ± 0.15 Bd

^a C, control (not treated); W, water wash at 65 psi for five passes; W/L, W + L (2% [vol/vol] lactic acid wash at 30 psi for three passes); COMB, W + HW (82°C water at 30 psi for three passes) + HA (hot air at 510°C for six passes under hot-air gun) + L.

^b Means with different small capital letters in the same row are significantly different ($P < 0.05$). Means with different lowercase letters in the same column are significantly different ($P < 0.05$).

As reported previously, multiple antimicrobial interventions effected an approximately 2.0-log₁₀ CFU/g reduction in fecal coliforms in regular-sized beef trim after COMB treatment (16). In this study, a reduction of about 2.1 log₁₀ CFU/g was achieved by the COMB treatment.

Following the application of multiple antimicrobial treatments, gas production was evaluated at 15°C for 10 days (see Table 2). For the untreated control, the onset of gas production was observed after just 2 days of storage at 15°C, while the onset of gas production occurred sooner for the W-treated samples (3 to 5 days) and the W/L-treated samples (5 to 8 days) than for the COMB-treated samples (8 days before any observable gas was produced). For the uninoculated samples, gas production was not observed after 10 days of storage at 15°C. On the basis of the data obtained, we would infer that initial levels of gas-forming bacteria that were lower than the high-level experimental inocula used in this work would respond to the trim interventions similarly. However, such an inference could only be tested under actual conditions of use, which are only simulated to a degree under the pilot scale conditions used here.

From these results, we conclude that multiple antimicrobial treatments for beef trim are an effective means of

retarding the growth of *H. alvei* and subsequent gas production in chub-packaged refrigerated ground beef held at the abusive temperature of 15°C. Gamage et al. (10) demonstrated that medium-dose irradiation (2.2 to 2.4 kGy) and low-temperature storage (2°C) of ground beef chubs delayed the onset of spoilage (gas formation) significantly, including cases in which the dominant spoilage bacteria were homofermentative lactococci and *H. alvei*. Another measure reported to control *H. alvei* on muscle foods is hot smoking. Bremer et al. (3), using traditional histamine-producing *Hafnia* in hot-smoked kahawai (Australian salmon), reported thermal death times of 1.42 to 0.20 min for storage at 54 to 57°C.

The isolation of *H. alvei* species of bacteria from gas-swollen ground beef chubs has previously been reported (10). Considering the biology and the fecal reservoir of this organism, it is certainly not an unexpected beef contaminant, and along with other known gas-forming spoilage agents of chub-packed ground beef, including lactic acid bacteria and species of the genus *Clostridium*, *H. alvei* is considered at least one potential cause of this economically important defect (14).

In the present study, isolates were obtained from commercially prepared beef chubs that were swollen, presum-

TABLE 2. Gas production in *H. alvei*-inoculated ground beef chubs made from treated and untreated beef trim following storage at 15°C over a 10-day period

Storage time (days)	Gas formation in uninoculated control samples ^a	Gas formation in <i>H. alvei</i> -inoculated samples (%) ^b			
		C	W	W/L	COMB
0	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)
1	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)	0/15 (0)
2	0/15 (0)	5/15 (33)	0/15 (0)	0/15 (0)	0/15 (0)
3	0/15 (0)	15/15 (100)	5/15 (33)	0/15 (0)	0/15 (0)
4	0/15 (0)	—	10/15 (67)	0/15 (0)	0/15 (0)
5	0/15 (0)	—	15/15 (100)	5/15 (33)	0/15 (0)
6	0/15 (0)	—	—	5/15 (33)	0/15 (0)
7	0/15 (0)	—	—	10/15 (67)	0/15 (0)
8	0/15 (0)	—	—	15/15 (100)	0/15 (0)
9	0/15 (0)	—	—	—	15/15 (100)
10	0/15 (0)	—	—	—	—

^a Number of samples producing gas/total tested samples (experiments were repeated three times with five samples).

^b Number of samples positive/total tested samples. See footnote to Table 1 for abbreviations.

ably from the activity of gas-forming microorganisms present in sufficiently large numbers to impart this defect. Our strategy to pick suspected gas-forming bacterial isolates from only culture plates of the highest dilutions leads us to conclude that these isolates were a dominant part of the microflora of the spoiled product in question. That gas-spoilage seems to be a random occurrence in ground beef chubs would lend credence to the hypothesis that the incidence of random low-level fecal, and consequently bacterial, contamination carries through the beef trim production process and, upon the grinding, mixing, and packaging of the beef trim into chubs, manifests as gas formation.

It should be noted that several human diarrheal clinical isolates are biochemically or phenotypically classified as *H. alvei*. These characterized isolates harbor the *eaeA* gene identical to that found in enteropathogenic *E. coli* and in some instances harbor the heat-stable toxin genes and Vero cytotoxin genes (1, 18, 22). Subsequent studies of these diarrheagenic strains have found a low level of 16S rRNA sequence homology between *eae*⁺ and *eae*⁻ *H. alvei* reference strains, in addition to detecting the *phoE* outer membrane protein (thought to be unique to the genera *Shigella* and *Escherichia*) in the diarrheagenic putative *H. alvei* isolates (21). Earlier researchers have already observed two main DNA homology groups within the genus *Hafnia* (25). These and other characteristics have led to consideration of the reclassification of these *eaeA*⁺ *H. alvei* isolates as actually being members of the genus *Escherichia* (13). Of potential concern to the food microbiologist is that meat-borne isolates of these *eaeA*⁺ putative *H. alvei* and other meat isolates bearing heat-stable toxin genes (18, 21) have been documented. Ridell and Korkeala (20) also reported that *eaeA*⁺ diarrheagenic presumptive *H. alvei* strains had minimum growth temperatures on nutrient agar ranging from 10.2 to 11.5°C, while the *H. alvei eaeA*⁻ strains (from this same collection of meat isolates) and *H. alvei* reference or type strains exhibited minimum growth temperatures of 0.2 to 3.7°C. We concur with these authors that the inadequacy of the current *Enterobacteriaceae* taxonomic scheme with regard to the genus *Hafnia* is a source of confusion regarding these bacteria in meats. While it is possible that pathogenic and spoilage potential exists for the same organism, it is clear that more research delineating the contribution of potentially pathogenic *Hafnia*-like bacteria as spoilage agents is needed to distinguish those organisms that are mainly spoilage agents from those that are potential pathogens or are both pathogens and spoilage agents. Our selected isolates were both *eaeA*- and *stx*-negative and had biochemical profiles similar to those reported by Gamage et al. (10) and Ridell et al. (21) for nonpathogenic meat and food isolates of *H. alvei*.

Regardless of their correct taxonomic placement, product contamination by *H. alvei* and other gas-forming psychrotrophic bacteria remains an important problem for the meat production sector. With regard to the prevention and reduction of further transmission of feces-derived microbial pathogens and spoilage organisms in raw meats, we have previously shown that the application of multiple antimicrobial interventions to beef trim prior to refrigeration and

grinding offers a means to significantly reduce feces-derived, processing-disseminated microbial contamination present on trim from the carcass stage as well as that resulting from the extensive handling of trim in the boning process (16, 17). Here we have used this same approach to reduce a known gas-spoilage bacterium on trim and in the resulting temperature-abused ground beef chubs and to delay the onset of gas formation in ground beef from trim pieces inoculated with high levels of psychrotrophic gas-forming bacteria. The knowledge gained from this study presents trim producers with a means that, upon further testing and engineering adaptation, could reduce gas formation defects in refrigerated ground beef chubs while potentially imparting an added level of microbial safety to the final ground product.

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