Effects of Peroxyacetic Acid Spray and Storage Temperature on the Microbiota and Sensory Properties of Vacuum-Packed Subprimal Cuts of Meat

Xianqin Yang,^a Hui Wang,^a Scott Hrycauk,^a Mark D. Klassen^b

^aAgriculture and Agri-Food Canada Lacombe Research and Development Centre, Lacombe, Alberta, Canada

^bCanadian Cattlemen's Association, Calgary, Alberta, Canada

ABSTRACT We investigated the impact of peroxyacetic acid (PAA; 200 ppm) spray on the microbiota and shelf life of commercial, vacuum-packed beef stored at chiller temperatures. Ribeye cuts ($n=147$) were collected from a local beef plant on the day of production for two consecutive days, with one set collected at the start of work with the PAA spray nozzles turned off (control) and during routine production with the PAA spray nozzles turned on (PAA) each day. Packs were stored at 4, 2, and -1°C for up to 34, 104, and 180 days and sampled at appropriate intervals for sensory assessment, microbial enumeration, and microbial profiling by 16S rRNA gene amplicon analysis. Treatment with PAA did not affect the initial meat pH, the initial numbers of total aerobes, lactic acid bacteria, or *Enterobacteriaceae* ($P > 0.05$) before storage; however, it delayed the onset of spoilage by 7, 21, and 54 days at 4, 2, and -1°C , respectively. Square-root models of the variation of growth rate with temperature indicated lactic acid bacteria grew faster and *Enterobacteriaceae* grew slower on PAA-treated than on untreated meat. Negative associations between pH and deterioration of meat during storage were observed for PAA-treated meat. During storage, the microbiota were primarily dominated by *Carnobacterium* and *Lactobacillus/Lactococcus* on control meat but by *Leuconostoc* on PAA-treated meat. *Serratia*, *Yersinia*, and *Clostridium* were identified by linear discriminant effect size analysis as biomarkers for control meat; *Clostridium* was found in high abundance in samples that had the highest spoilage scores.

IMPORTANCE The findings of this study show that PAA solutions applied at low concentrations under commercial settings positively modulated the meat microbiota. It did not have bactericidal effects for beef subprimals with very low microbial loads. However, it differentially impacted the members of the microbiota, which resulted in delayed onset of spoilage of vacuum-packed beef subprimal stored at all three temperatures (4, 2, and -1°C). This differential impact could be through one or a combination of the following factors: favoring the growth of lactic acid bacteria, which may in turn exert a competitive exclusion that might be due to production of antimicrobial compounds such as organic acids and bacteriocins; exerting synergistic antimicrobial effects with low temperatures against members of *Enterobacteriaceae*; and direct or indirect inhibitory effects against members of the clostridia. These findings not only advance our understanding of the microbial ecology of vacuum-packed meat stored at chiller temperatures but also suggest that bacteriostatic concentrations of antimicrobial interventions can be explored for shelf-life extension.

KEYWORDS peroxyacetic acid, shelf life, microbial community, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Clostridium*, *Enterobacteriaceae*, commercial production, vacuum-packed beef

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Address correspondence to Xianqin Yang, xianqin.yang@canada.ca.

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In response to the high-profile outbreaks of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with hamburgers, the organism was declared a food adulterant by the U.S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) in 1994. Subsequently, the USDA required all federally inspected meat processing facilities in the United States and facilities elsewhere that export to the United States to implement hazard analysis and critical control point (HACCP) systems-based preventative control plan (1). In Canada, *E. coli* O157:H7 was classified as a nationally notifiable disease in 1990 (2). Although many animals may carry STEC, cattle are regarded as the primary reservoir of the organism (3). The hides of cattle carry a large microbial load, a substantial portion of which is of enteric origin (4) and, as such, contamination from hides and fecal materials during carcass dressing is regarded as the primary source of *E. coli* on carcasses. To better control the contamination of beef carcasses with *E. coli* O157:H7, various physical and chemical decontaminating treatments have been incorporated into the preventative control plan in beef packing facilities in North America (5, 6). One such treatment gaining more popularity in recent years in both the United States and Canada is spraying carcasses and cuts with peroxyacetic acid (PAA) solutions, and in some facilities it is replacing lactic acid, likely due to its less detrimental effects on meat color (7, 8). The efficacy of PAA for treating beef has been the subject of a large number of studies, with most being pathogen driven, focusing on *E. coli* and *Salmonella* and to some extent indicator organisms (9–18).

PAA solutions used as antimicrobial interventions are mixtures of the compound PAA, acetic acid, and hydrogen peroxide, with the compound PAA being the primary active ingredient. PAA is the most potent peroxygen, and its exact mode of action is unclear; however, it is generally believed to be similar with other strong oxidizing agents, through their ability to disrupt the permeability of the cell membrane and to penetrate bacterial cell walls and disrupt cell functions by virtues of damaging sulfhydryl and sulfur bonds containing molecules on and in the cells (19, 20).

Fresh meat is primarily traded in the form of chilled, vacuum-packed primal cuts domestically in Canada and internationally, and it has been estimated more than one million tons of beef in this form is traded internationally each year (21). It is not surprising the shelf life of chilled, vacuum-packed meat has been the subject of many studies (22–26). In general, the storage life of vacuum-packed beef is substantially affected by both the number and the composition of the microbiota on meat at the time of packaging (27). Bacteria on beef can derive from chilled carcasses, the processing environment, and processing equipment (28–30) and are very diverse, including more than 15 genera from both Gram-positive and Gram-negative groups (25, 26). Information on how PAA spray may impact the microbiota and subsequently the shelf life of meat is lacking. In addition, the efficacies of decontaminating treatments applied in commercial practice may differ significantly from those obtained in laboratory settings, arising from factors such as the physiological conditions of the target organisms, the number and composition of the accompanying bacteria, the ways in which treatments are applied, and the manner in which bacteria are attached to meat surfaces (5, 31). The objectives of this study were to evaluate the impact of PAA spray under commercial settings on the shelf life of vacuum-packed beef subprimals stored at various temperatures by determining the dynamics of the microbiota using both conventional microbiological methods and 16 rRNA gene amplicon sequencing, as well as sensory assessments.

RESULTS

pH, drip, visual, and sensory assessments. The initial mean meat surface pH values were 5.48 ± 0.08 and 5.41 ± 0.07 for the control and PAA group, respectively, and they were not significantly ($P > 0.05$) different. For all three storage temperatures, meat surface pH was affected by both storage time and PAA treatment ($P < 0.05$). The overall mean pH for PAA-treated meat stored at 4, 2, and -1°C was lower by 0.13, 0.10, and 0.16 U, compared to the control group, and these differences were significant ($P < 0.05$). No differences in pH values between the PAA and control group at equivalent times were noted throughout the

storage at 4°C, but the PAA group had lower pH values than the control group during storage at the other two temperatures, for 41 and 55 days at 2°C and 41 days at −1°C (see Fig. S1 in the supplemental material). Despite these statistically significant changes, the overall change of pH was mostly within 0.4 U.

None of the packs lost vacuum to blown pack throughout the storage, as evidenced by pack integrity score of being 4 or less. The volume of rinsate increased ($P < 0.05$) with storage time except for the control group at 4°C, which did not change significantly ($P > 0.05$) over time (see Fig. S2). There was no significant difference ($P > 0.05$) in rinsate volume between the control group and the PAA group at equivalent times at any of the three temperatures, with the exception of meat stored at −1°C for 125 days, where the rinsate from PAA group was significantly higher ($P < 0.05$) than that from the control group. Overall, the mean volume of rinsate throughout the storage did not differ significantly between PAA-treated and control meat at 4 and 2°C, but at −1°C the mean rinsate volume from PAA-treated meat was higher ($P < 0.05$) than control meat by 50 ml.

As expected, the scores for both odor and overall acceptability deteriorated (increased) over time, and the variability among samples from the same group increased with storage time for all three temperatures (Fig. 1; see also Fig. S3). Compared to the control group, the PAA group had delayed the onset of significant changes ($P < 0.05$) in acceptability by 4, 42, and 76 days at 4, 2, and −1°C, respectively (Fig. 1). The trend for odor was less consistent than that for acceptability where the same (2°C), delayed (−1°C), or faster (4°C) change over time was noted for the PAA group compared to the control group. Based on sensory assessments, the shelf lives were 24, 76, and 104 days and 31, 97, and 160 days for the control and PAA groups stored at 4, 2, and −1°C, respectively. The development of sensory properties over time did not follow the sigmoid shape (see Table S2) and, as such, they did not fit well into the Baranyi-Roberts model.

Microbiological analysis as determined by plating. Most of samples from either group (83 and 88% for the PAA and control group, respectively) did not yield *E. coli* at the detection limit of 0.9 log CFU/1,000 cm², and when *E. coli* was recovered the numbers were mostly <2 log CFU/1,000 cm² (see Table S1). Interestingly, no *E. coli* were recovered from any of the samples stored at 4°C for ≥20 days, 2°C ≥69 days, or −1°C for ≥83 days.

The initial numbers for total aerobes, lactic acid bacteria and *Enterobacteriaceae* were 2.2 ± 0.4 , 0.9 ± 0.7 , and 0.2 ± 0.5 log CFU/cm² and 2.1 ± 0.8 , 0.8 ± 0.5 , and 0.3 ± 0.3 log CFU/cm² for the control and PAA groups, respectively. The numbers for all three bacterial groups did not differ ($P > 0.05$) between the two meat groups, but within each group the numbers for *Enterobacteriaceae* were significantly lower ($P < 0.05$) than those for total aerobes and lactic acid bacteria. All three bacterial groups increased with increasing storage time at all three storage temperatures (Fig. 2). There was no significant difference ($P > 0.05$) between the PAA-treated and control groups in numbers of the three bacterial groups at equivalent times for the great majority of the sampling points (see Fig. S4 to S6). The exceptions were for meat stored at 4°C for 13 and 34 days and at 2°C for 13 and 55 days, where the PAA group had higher numbers ($P < 0.05$; 0.5 to 1.5 log units) of lactic acid bacteria, and for meat stored at 2°C for 69 days, where the PAA group had a lower ($P < 0.05$; 1 log unit) *Enterobacteriaceae* count. The difference between *Enterobacteriaceae* and total aerobes/lactic acid bacteria was maintained throughout the storage and widened with decreasing storage temperature (Fig. 2). The maximum numbers (y_{end}) estimated by the Baranyi-Roberts model for aerobes and lactic acid bacteria ranged from 7.5 to 7.8 log CFU/cm², irrespective of meat being treated with PAA or not, or storage temperatures (see Table S2). The estimated maximum numbers attainable by *Enterobacteriaceae* were 6.0 ± 0.2 and 5.4 ± 0.3 , 5.5 ± 0.1 and 4.5 ± 0.2 , and 3.8 ± 0.2 and 3.1 ± 0.2 log CFU/cm² for the control and PAA groups at 4, 2 and −1°C, respectively (see Table S2). The square root growth rate as a function of storage temperature followed linear regressions for all three bacterial groups (Table 1), with the values for PAA group being 78, 109, and 72% of those for the control group for total aerobes, lactic acid bacteria, and *Enterobacteriaceae*, respectively.

Correlations between bacterial counts, sensory assessments, and pH. The numbers of total aerobes, lactic acid bacteria, and *Enterobacteriaceae* were strongly and positively

FIG 1 Sensory scores determined by a five-member panel for vacuum-packed ribeye subprimals during storage at 4°C (A), 2°C (B), and -1°C (C) for up to 34, 104, and 180 days, respectively. The meat had not (control) or had been sprayed with peroxyacetic acid (PAA) when prepared. *, the point where the assessment score significantly differed from that at day 0 for the same group. The 5-point scale used for both odor and overall acceptability assessments was as follows: 1 = strongly acceptable, 2 = acceptable, 3 = cannot decide, 4 = slightly unacceptable, and 5 = strongly unacceptable.

FIG 2 Numbers of total aerobic count (TAC), lactic acid bacteria (LAB), and *Enterobacteriaceae* (ENT) on vacuum-packed ribeye subprimals during storage at 4°C (A), 2°C (B), and -1°C (C) for up to 34, 104, and 180 days, respectively. The meat had not been treated (control) or had been sprayed with PAA when prepared.

TABLE 1 Equations describing the variations with temperature of the growth rates of aerobes, lactic acid bacteria, and *Enterobacteriaceae*^a

Bacterial group	Control		PAA	
	Equation	R ² value	Equation	R ² value
Aerobes	$y = (0.0492x + 0.3693)^2$	0.98	$y = (0.0382x + 0.4268)^2$	0.99
LAB	$y = (0.0432x + 0.4025)^2$	1.00	$y = (0.0469x + 0.5102)^2$	0.97
ENT	$y = (0.0499x + 0.3041)^2$	0.99	$y = (0.0361x + 0.3466)^2$	0.84

^aEquations are presented that describe the variations with temperature of the growth rates of aerobes, lactic acid bacteria (LAB), and *Enterobacteriaceae* (ENT) on vacuum-packed meat that had not been treated (Control) or had been sprayed with PAA.

associated at all three temperatures (Fig. 3). Of all potential associations between bacterial counts and sensory properties, only that of *Enterobacteriaceae* and acceptability at 4°C was strong (~0.7). The associations between *Enterobacteriaceae* and sensory properties decreased with decreasing temperature and were weak at -1°C (≤0.3). The decrease in the degree of association between bacterial counts and sensory properties was less for aerobes/lactic acid bacteria than that for *Enterobacteriaceae*. Interestingly, moderate to strong negative

FIG 3 Pearson correlation coefficients between bacterial counts, sensory properties, and pH for vacuum-packed meat that had not been (control) or had been sprayed with PAA during chiller storage. (A, C, and E) Control group stored at 4, 2, and -1°C, respectively; (B, D, and F) PAA group stored at 4, 2, and -1°C, respectively. TAC, total aerobic counts; ENT, *Enterobacteriaceae*; LAB, lactic acid bacteria.

FIG 4 Principal-coordinate analysis based on Euclidean distance of the composition of amplicon sequence variants from vacuum-packed ribeye cuts before storage (NA) and during storage at -1 , 2 and 4°C . The meat had not (control) or had been sprayed with peroxyacetic acid (PAA) when prepared. (A, B, C, and D) Overall analyses for all three temperatures and for -1 , 2 , and 4°C individually, respectively.

associations between pH and deterioration of sensory scores at all three storage temperatures and between total aerobes/lactic acid bacteria at -1°C were noted for the PAA group (Fig. 3).

Microbiological analysis as determined by 16S rRNA gene amplicon analysis. A total of 3,581,391 reads (average of 25,952 reads per sample) were analyzed. There was no significant difference in the overall alpha-diversity between PAA-treated and untreated meat before storage or during storage at any of the three temperatures. Amplicon sequence variants (ASVs) for samples from the two meat groups (PAA and control) were primarily separated into different clusters on a principal-coordinate analysis plot (Fig. 4A), along the horizontal axis, which contributes to 44.4% of the variance among samples. The microbiota from PAA-treated and untreated meat diverged more from day 0, with increasing storage time at all three temperatures (Fig. 4B to D), suggesting storage time as the second separating factor, which explains about 20% of the difference among samples. The beta-diversity, as measured by Bray-Curtis dissimilarity of the microbiota, was significantly affected by treatment ($P < 0.001$), storage temperature ($P < 0.001$), and storage time ($P < 0.001$).

Twenty-three and twenty bacterial genera each accounting for $\geq 1\%$ of the total population in at least one of the three samples were identified in the initial microbiota for the control and PAA-treated meat groups, respectively, and they accounted for 99.0 and 95.2% of the corresponding total populations. Of these genera, 12 were common between the two meat groups. An additional 13 and 24 genera at $< 1\%$ relative abundance were found in control and PAA groups, accounting for 1.0 and 4.8% of their respective total populations (see Table S3). Considering the initial microbiota being at $\sim 2 \log \text{CFU}/\text{cm}^2$, the relative abundance cutoff value was set at 1%. The relative abundance of the taxa in the day 0 samples varied largely between the three replicate samples of the same group (Fig. 5). For instance, *Carnobacterium*, *Pseudomonas*, and *Staphylococcus* were each found at 23.0, 76.3, and 29.8%, respectively, or *Leuconostoc* was found at 32.8% in one of the control and PAA groups, but each was a very small fraction or not found in the other two samples. The most predominant and prevalent

FIG 5 Relative abundance of bacterial genera on vacuum-packed ribeye cuts that had been stored at 4, 2, and -1°C for up to 34, 97, and 150 days, respectively. The meat had not (control) or had been sprayed with PAA when prepared.

taxa found in both groups were *Acinetobacter*, *Anoxybacillus*, and *Pseudomonas*, with mean relative abundances of 20.7, 31.0, and 10.2% for the PAA group and 9.8, 13.1, and 30.3% for the control group. *Clostridium* was found at 2.4 and 2.3% in the control and PAA groups, respectively. Of the lactic acid bacteria, *Lactobacillus* and *Vagococcus* were found in both meat groups, while *Lactococcus/Leuconostoc* and *Carnobacterium* were found only in the PAA-treated meat and control meat groups, respectively (Fig. 6). Despite this difference in the composition of lactic acid bacteria, the two groups of meat had similar overall proportions of lactic acid bacteria (14.4% for control group and 15.7% for PAA group). A small fraction of the microbiota was identified to belong to the family of *Enterobacteriaceae* (5.6% for the control group and 2.4% for the PAA group), with the relative abundance of *Serratia* being slightly higher in the control group (2.7%) than in the PAA group (0.9%), and no *Morganella* being found in the PAA group (Fig. 7). Of note, *Escherichia* was found in one of the three samples for both groups at a relative abundance of $\sim 1.8\%$.

The microbiota of both groups of meat were eventually predominated by lactic acid bacteria and, in general, variation among samples of the same treatment decreased as storage progressed at all three temperatures (Fig. 5 and 6). Even so, there were some marked differences between the PAA and control groups. For the control group, the predominant genus was *Carnobacterium* for most of the samples, and the fraction of *Lactococcus* increased with storage time at 4°C , and *Lactobacillus* was predominant at a later stage at 2°C and at the end of storage at -1°C . For the PAA group, the microbiota was primarily predominated by *Leuconostoc*, but its predominance was replaced by *Lactobacillus* at later storage at 2°C . At 2 and 4°C , the fraction of total lactic acid bacteria in the PAA group was mostly higher than the control group by 5 to 31%.

The relative abundance of *Enterobacteriaceae* in samples from both meat groups at the first sampling point during storage was up to 40% of the total population, a substantial increase from their respective initial relative abundance (5.7% for the control group and 2.4% for the PAA group), and thereafter generally decreased with decreasing storage temperature and increasing storage time, with the exception of PAA-treated meat stored at 2 and 4°C (Fig. 7). The former did not undergo as much of an increase as for the other conditions at the beginning of storage, and the latter had more fluctuation at later storage times. The great majority of the *Enterobacteriaceae* were *Serratia*, with *Hafnia*, *Yersinia*, and *Klebsiella* occasionally being a sizable fraction at 2 and 4°C . The fraction of *Serratia* in the PAA group was smaller than in the control group at equivalent times at early stages of storage at 2°C (≤ 55 days) and 4°C (≤ 20 days) by up to 11 to 28%.

FIG 6 Relative abundance of lactic acid bacteria on vacuum-packed ribeye cuts that had been stored at 4, 2, and -1°C for up to 34, 97, and 150 days, respectively. The meat had not (control) or had been sprayed with PAA when prepared. The data presented in Fig. 5 were replotted to highlight lactic acid bacteria.

Overall, *Leuconostoc* was associated with PAA group, whereas *Yersinia*, *Clostridium*, *Iodobacter*, *Lactobacillus*, *Serratia*, *Enterococcus*, and *Carnobacterium* were associated with the control group, as determined by linear discriminant analysis effect size (LEfSe) (Fig. 8A). The association between *Leuconostoc* and PAA was observed at all three temperatures (Fig. 8B to D), while the association between *Carnobacterium* and control was found at -1 and 2°C and that between *Yersinia*, *Clostridium*, and control was noted at 2°C . In all, there were 18 samples with relative abundance for *Clostridium* at $\geq 1\%$, with 14 from control group (one on day 0 and 13 during storage) and 4 from the PAA group (three from days 0 and 1 during storage). The samples that had odor and/or acceptability scores of >4.5 ($n=3$; one from control on day 27 at 4°C and two from control on days 83 and 97 at 2°C) all had substantial fractions of *Clostridium*, at 30.9, 56.6, and 71.8%, respectively. These ASVs had the highest similarities (99.6 to 100%) with the 16S rRNA gene of *C. algidicarnis*. The relative abundance of *Clostridium* in the other 15 samples were $\leq 11\%$ of the total population.

DISCUSSION

The effect of PAA solution as an antimicrobial intervention varies largely among studies as conditions such as concentrations, contact time, spray volumes, ways through which bacteria contaminate meat (naturally contaminated products and meat inoculated with microorganisms), level and composition of bacteria on meat, and the means by which the spray was applied differ. For beef carcasses inoculated with fecal slurry spiked with *E. coli* O157, spray with 200 ppm of PAA resulted in up to a log reduction of *E. coli* O157:H7 (11, 12). When naturally contaminated beef briskets were sprayed with 200 ppm PAA at $50\text{ ml}/400\text{ cm}^2$, the treatment was ineffective for

FIG 7 Relative abundance of bacterial genera of the family *Enterobacteriaceae* on vacuum-packed ribeye cuts that had been stored at 4, 2, and -1°C for up to 34, 97, and 150 days, respectively. The meat had not (control) or had been sprayed with PAA when prepared. The data presented in Fig. 5 were replotted to highlight *Enterobacteriaceae*.

reducing the numbers of total aerobes, coliforms, or *E. coli* for products from one beef processing plant but resulted in a 0.7-log reduction of aerobes for products from another plant (9). When applied at 200 ppm as a mist on loins in a large beef processing facility, PAA was ineffective for reducing the number of total aerobes, coliforms, and *E. coli* (16). The findings of the present study that the numbers of total aerobes and *Enterobacteriaceae* were largely unaffected by the mist applied at the beef packing plant were in agreement with published accounts under similar conditions. In a study by Brightwell et al. (18), the microbiota of PAA-treated hot-boned beef was primarily composed of Gram-positive bacteria (76% cocci and 19% rods). Since no bacteria were recovered from the corresponding control meat, the predominance by Gram-positive bacteria on PAA-treated meat could reflect the composition of the initial microflora or treatment effect. In the present study, relatively equal proportions of firmicutes (41.4% versus 52.3%) and proteobacteria (48.6% versus 44.3%) were found in control and PAA-treated meat.

The progression from a diverse initial microbiota to a microbiota primarily predominated by lactic acid bacteria is in agreement with published accounts on vacuum-packed meat stored at colder temperatures (32, 33). A variety of taxa were found in both control (36 genera) and PAA (44 genera) meat before storage, with large variation in relative abundance observed between samples of the same group. This variation is largely due to the complex nature of the meat production system and surface structure of meat and among- and within-lot variation in the initial microbial contamination of meat has been widely reported (34–36). The initial diverse taxa and the predominance of *Carnobacterium/Lactobacillus* in the microbial community on control meat with extended storage are largely in line with recent studies on shelf life of commercial vacuum-packed beef (25, 26, 36, 37). Different from the microbiota on meat that had not been sprayed with PAA, the microbiota on PAA-sprayed meat was predominated by *Leuconostoc* at all three storage temperatures. Some species of *Leuconostoc* have been found as the predominating lactic acid bacterium in chilled beef stored under a high oxygen atmosphere but not under vacuum packaging conditions (38). The authors

FIG 8 Histograms of the linear discriminant analysis (LDA) scores in logarithmic values of biomarkers (genus) found by LEfSe comparing the microbiomes of vacuum-packed meat treated with PAA and control meat and stored at different temperatures. (A) Across all three temperatures; (B) at 2°C; (C) at -1°C; (D) at 4°C.

attributed this to the ability of some *Leuconostoc* species to respire, thus having a growth advantage over other lactic acid bacteria. One factor that could have contributed to the predominance of *Leuconostoc* is the difference in their relative abundance in the initial microbiota, which could have resulted from the growth advantage of *Leuconostoc* on PAA-sprayed carcass surface. An additional source of oxygen for the PAA-treated meat could be from the breakdown of PAA (20). On the other hand, a higher prevalence of *Leuconostoc* in ground beef stored under modified-atmosphere packaging (MAP; 19.6% CO₂, 0.4% CO, and balancing N₂) was observed for the group derived from trimmings that had been treated with PAA or sulfuric acid and sodium sulfate (SASS) than the group that had not been treated with any antimicrobial interventions (39). This difference for the SASS group was attributed to the difference in pH values. Thus, further study is warranted to understand the role that PAA plays in the predominance of *Leuconostoc* on PAA-sprayed vacuum-packed cuts after extended chiller storage.

The samples with the highest spoilage scores were predominated by *Clostridium*, likely the species *C. algidicarnis*. In recent years, spoilage of vacuum-packed fresh meat, including beef, lamb, venison, and pork by psychrotrophic clostridia has been reported (40–42), and the spoilage is typically associated with copious amount of gas production, with *C. estertheticum* and, to some extent, *C. gasigenes* being the primary causative agents. *C. algidicarnis* has been associated with deep tissue spoilage of beef and lamb (43, 44) and spoilage of vacuum-packed cooked pork (45). The spoilage caused by *C. algidicarnis* is often associated with strong putrid/offensive, sickly spoilage odors, likely resulting from its ability of reducing sulfite to sulfide (45, 46). Different from *C. estertheticum* and *C. gasigenes*, *C. algidicarnis* is not associated with gas production, based on *in vitro* inoculation studies (43–45). *Clostridium* was identified as a biomarker

associated with the control group during storage through LEfSe (Fig. 8). Peroxyacetic acid is sporostatic and sporicidal (47, 48) and has been demonstrated to effectively inactivate spores of *C. estertheticum* *in vitro* (49, 50) and delayed the onset of blown pack spoilage of vacuum-packed beef inoculated with a high level of *C. estertheticum* (51). In addition, it has been demonstrated that *Leuconostoc* has an inhibitory effect on the development of blown pack spoilage caused by *C. estertheticum* (52). The lower abundance of *Clostridium* in PAA-treated meat during storage observed in the present study could be caused by the presence of PAA and/or the predominance of *Leuconostoc* in these samples.

Under anaerobic conditions such as those in vacuum-packed meat, lactic acid bacteria have a growth advantage over other microorganisms, and they rapidly ferment glucose in meat to produce organic acids, thus lowering the pH (53). The by-products often confer mild dairy/cheesy odors to meat compared to offensive odors from sulfide generated by some psychrotrophic clostridia and *Enterobacteriaceae* from utilizing amino acids. The findings that lactic acid bacteria grew faster, as indicated by square-root models and the higher abundance of lactic acid bacteria associated with PAA-treated meat, corroborate the general behavior of lactic acid bacteria on meat and the stronger negative association of pH and deterioration of sensory properties of PAA-sprayed meat. Lower prevalence of *Enterobacteriaceae* and higher prevalence of *Leuconostoc* has also been reported for MAP-stored ground beef derived from trimmings that had been treated with PAA than for control samples which had not been treated with any antimicrobial treatment (39). The mechanisms through which PAA treatment favors the growth of lactic acid bacteria are unclear. Synergistic effects between oxidative stress from chlorine and peroxyacetic acid and cold during spray chilling have been reported for *E. coli* and *Salmonella* (54). This may explain the slower growth of *Enterobacteriaceae*, as determined by square-root models. It is noteworthy that *Enterobacteriaceae* were mostly overrepresented in samples from meat during storage by 16S rRNA gene amplicon analysis compared to the enumeration results, regardless whether the meat had been treated with PAA or not. However, this was not observed with the day 0 samples. This could be caused by potential primer-binding bias toward some members of the community in the PCR step of 16S rRNA gene amplicon analysis (55), stressing the importance of using both culture-dependent and -independent methods when studying microbial populations.

Taken together, we evaluated the effect of PAA spray on the shelf life of commercial, vacuum-packed beef subprimal cuts stored at three temperatures using sensory assessments, enumeration of bacteria of shelf-life concerns, and microbial profiling by 16S rRNA gene amplicon sequence analysis. The findings show that PAA did not reduce the numbers of total aerobes, lactic acid bacteria, or *Enterobacteriaceae* on meat with a very low initial microbial load. However, the treatment delayed the onset of spoilage by 7, 21, and 54 days at 4, 2, and -1°C , respectively, likely through favoring the growth of some members of lactic acid bacteria and inhibiting the growth of *Clostridium* and *Enterobacteriaceae*.

MATERIALS AND METHODS

Collection and storage of vacuum-packed cuts. Vacuum-packed ribeye subprimal cuts were obtained from a medium-sized, federally inspected local beef packing plant in Alberta, Canada. At the plant, both sides of subprimal cuts are routinely misted with 200 ppm of PAA during preparation. One side of the cuts was sprayed when they passed through an over the belt spray cabinet in the middle of the conveyor belt. When the cuts reached the end of the conveyor belt, they were flipped over onto another conveyor where the top side was sprayed with PAA similarly. On each of two consecutive days, vacuum-packed ribeye cuts were collected from the plant at the start of work with the PAA spray nozzles being turned off (control group) and during the routine production with the PAA spray nozzles being turned on (PAA group). The carcasses from which PAA sprayed cuts were derived were also sprayed with 200 ppm of PAA right before they entered the fabrication facility. The cuts were transported in a refrigerated truck with the temperature set at 0°C to the lab, where the meat was stored in a -1°C cooler until all cuts were collected ($n = 147$). The day after all cuts were collected (the third day since the collection of the first batch of cuts), three packs selected from each group (control group and PAA group) were set aside, which were regarded as day 0 samples. The remainder packs were similarly

selected and assigned to three coolers operated at 4, 2, and -1°C . In all, 36, 51, and 54 cuts were stored in the three respective coolers. Two temperature data loggers (Sapac Temp Recorder, Argus, Auckland, New Zealand) were placed with each group of cuts to record temperatures during storage.

Sampling of cuts. At appropriate intervals (weekly for 4°C and approximately bi/triweekly for 2°C and -1°C), three packs were selected arbitrarily from the packs of each group stored at each temperature for sensory assessments and microbiological sampling. All sensory assessments were carried out by a five-member panel with previous experience of evaluation of beef odor and appearance, using five-point scales (56). Each pack selected on day 0 or after storage commenced was first assessed for the integrity vacuum packages (1 = complete adhesion, 2 = good, 3 = moderate, 4 = poor, 5 = blown pack). Indicators for 2, 3, and 4 were a few air bubbles, prevalent air bubbles, and visible air pockets, respectively. The packs were then opened aseptically by cutting the pack below the seal using a sterile scalpel. Immediately after pack opening, the odor acceptability was assessed (1 = strongly acceptable, 2 = acceptable, 3 = cannot decide, 4 = slightly unacceptable, 5 = strongly unacceptable). The meat was allowed to bloom for 1 h, after which overall customer acceptability (1 = strongly acceptable, 2 = acceptable, 3 = cannot decide, 4 = slightly unacceptable, 5 = strongly unacceptable) was assessed. The following criteria were used to determine shelf life: for both odor and overall acceptability assessments, the first sampling point where one or more samples that had an average score for either sensory property of ≥ 3.5 (the midpoint between 3 [cannot decide] and 4 [slightly unacceptable]) < 4 or the midpoint between the first two consecutive sampling points where one sampling point had scores of < 3.5 , and the other ≥ 4 was regarded as the shelf life for that condition, whichever happened first.

After the odor and overall acceptability were assessed, pH of the meat surface was measured using a flat surface portable pH meter decontaminated with 70% ethanol (Accumet; Fisher Scientific, Edmonton, Alberta, Canada), and then 200 ml of 0.1% peptone water (wt/vol; Fisher Scientific) was added to each pack. The cut was massaged from outside the bag for 2 min to spread the peptone water evenly over the entire surface of the meat. The rinse fluid, regarded as rinsate in the analyses, was transferred to 50-ml centrifuge tubes for further analysis. The total volume of the rinse fluid from each pack was recorded. Aliquots (10 ml) from the rinse fluid of each cut were centrifuged at $10,000 \times g$ at 4°C for 20 min, and the resulting pellets were archived at -80°C .

Microbiological analysis of the samples. Aliquots (1 ml) of the rinse fluid from cuts were used to prepare 10-fold serial dilutions in 0.1% peptone water. Total aerobic plate count, lactic acid bacteria, and *Enterobacteriaceae* were enumerated using the rinse fluid and appropriate dilutions, using 3M Petrifilm plates for each respective bacterial group (56). In addition, portions (10 ml) of the original fluid and appropriate dilutions were each filtered through a hydrophobic grid membrane filter (Oxoid). The filters were placed on lactose monensin glucuronate (Acumedia, Lansing, MI) plates, which were incubated at 35°C for 24 h, and then the filters were transferred to buffered MUG agar plates. After incubation of additional 2 h, the filters were examined under long-wavelength (365-nm) UV light, and squares containing blue white colonies were counted for enumeration of *E. coli* (16).

Statistical analysis for microbial counts and sensory analysis. All bacterial counts were converted to $\log_{10}$ CFU/cm² except that for *E. coli* which were converted to $\log_{10}$ CFU/1,000 cm². Dimensions of the cuts were taken before the packs were opened, and the estimated surface area was $\sim 2,000$ cm². The detection limits for total aerobes, *Enterobacteriaceae*, lactic acid bacteria, and *E. coli* were -1.1 , -1.1 , -0.8 , and 0.9 at their respective units. When there was no recovery for a bacterial type from a sample, 0.5 log below the detection limit was arbitrarily assigned for the bacterial type for that sample for statistical analysis purpose (57). Sensory properties, pH values, and microbial counts for each bacterial group were grouped according to conditions (PAA group and control group) and temperatures (4, 2, and -1°C). Paired *t* test and analysis of variance (ANOVA) were used to separate means for two and more sets of comparisons. Associations between bacterial counts, sensory properties, and pH values were assessed using a Pearson correlation coefficient. A significance level of 0.05 was used for all statistical analyses. Bacterial counts and sensory assessment scores were fitted into the Baranyi-Roberts model (58) using DMFIT 3.5, a Microsoft Excel add-in (<https://www.combase.cc/index.php/en/8-category-en-gb/21-tools>). To obtain equations describing the variation of growth rate with temperature, a plot of the square root of growth rates against temperatures was prepared for total aerobes, lactic acid bacteria, and *Enterobacteriaceae* under each treatment condition (control and PAA), and regression lines were fitted to the plots. Values were reported as means $\pm$ the standard deviations where appropriate.

16S rRNA gene amplicon sequencing. The 16S rRNA gene amplicon sequencing was performed for a total of 138 samples, including all samples from sampling points where cuts in both control and PAA groups were sampled. Archived bacterial pellets from rinse fluid aliquots (10 ml) were each resuspended in 2 ml of enzymatic lysis buffer containing lysozyme (20 mg/ml). An aliquot (200 μl) of each bacterial suspension was subjected to DNA extraction by using a DNeasy blood and tissue kit (Qiagen, Toronto, Ontario, Canada) according to the manufacturer's instructions for Gram-positive bacteria. Six negative extraction controls without the addition of bacterial suspensions were processed in the same manner, as were the samples. The DNA preparations were amplified using primers targeting the V4 region of the 16S rRNA gene (59), and libraries were constructed and subsequently sequenced by Genome Quebec (Montreal, Quebec, Canada) using an Illumina MiSeq instrument (Illumina, Inc., San Diego, CA) and an Illumina MiSeq reagent kit v2 (500 cycles).

16S rRNA gene amplicon sequence processing and analysis. The primers and their reverse complements were first removed by trimming paired-end reads using Cutadapt 2.10 (60). The trimmed reads were further processed using DADA2 1.14 (61) in R (v3.6.3) with the default parameters. Briefly, the forward and reverse reads were truncated to 180 and 220 bp, respectively, and merged, and the chimeras were removed. Taxonomy was assigned to these amplicon sequence variants (ASVs) using the SILVA 16S

rRNA gene database (release 138.1) (62). Contaminants were identified using the “prevalence” method of the R package decontam v1.6.0 (63) by comparing the prevalence of each ASV in true-positive samples and negative extraction controls ($n=6$) and subsequently removed from the samples. ASVs identified as mitochondria or chloroplasts were removed, along with any ASV not assigned as bacteria. ASVs that were found only in negative extraction controls were also removed prior to the downstream analysis. ASVs whose taxonomy was not assigned to the genus level were searched against the nr/nt database in GenBank using BLAST. When the query ASV had more than 99.6% similarity with sequences in the GenBank database, the taxonomy was manually reassigned to that particular ASV. The ASV counts and taxonomy tables produced by the DADA2 pipeline, and the metadata files of the samples were imported into phyloseq v1.30.0 (64) in R. The ASV counts were normalized into relative abundance which was grouped into sets according to types of treatment (PAA and control) and storage temperatures (4, 2, and -1°C). To summarize the taxonomy, the ASVs with the same taxonomy at a taxonomic rank of genus were merged. ASVs accounting for $<1\%$ relative abundance were grouped as “ $<1\%$ abund.” at the genus level.

Statistical analysis for 16S rRNA gene amplicon data. All of the samples were rarefied to 15,959 reads (the lowest number of sequence reads among 138 samples) to minimize the impact of uneven sequencing depth. The alpha-diversity was assessed using rarefied reads in R with phyloseq (64) and vegan v2.5.2 (65). Differences between the types of treatment at each storage temperature and different storage days within each treatment at each storage temperature were assessed using the Wilcoxon test and the Kruskal-Wallis test, respectively. The beta-diversity was assessed using the Bray-Curtis distance metrics based on the relative abundance at genus level and visualized using principal-coordinate analysis. Permutational multivariate analysis of variance using the adonis function with 10,000 permutations was implemented in vegan to analyze the calculated Bray-Curtis dissimilarities for each type of treatment at each storage temperature. A significance level of 0.05 was used for all statistical analysis. The LEfSe method (66) was used to determine biomarkers associated with treatment or control, with the false discovery rate set at 0.05, on a web-based platform of MicrobiomeAnalyst (67).

Data availability. All sequences were deposited into the National Center for Biotechnology Information Sequence Read Archive under BioProject [PRJNA713837](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA713837).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

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