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Spoilage *Pseudomonas* survive common thermal processing schedules and grow in emulsified meat during extended vacuum storage

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Abstract: Some *Pseudomonas* species are common meat spoilage bacteria that are often associated with the spoilage of fresh meat. The recently reported ability of these bacteria to also spoil cooked and vacuum packaged meat products has created the need to investigate all potential routes of spoilage they may be able to utilize. The objective of this experiment was to determine if spoilage *Pseudomonas* spp. survive thermal processing and grow during refrigerated storage under vacuum. *Pseudomonas* spp. isolates collected from spoiled turkey products were inoculated into a salted and seasoned meat emulsion that was vacuum sealed and thermally treated to final temperatures of 54.4 and 71.1°C to mimic thermal processes commonly used in the meat industry. Samples were stored for a total of 294 days at 4 and 10°C and plated using *Pseudomonas* spp. specific agar plates. *Pseudomonas* spp. concentrations were below the detection limit (0.18 $\log_{10}$ CFU/g) immediately after thermal processing and were first recovered from thermally processed samples after 14 days of storage. The final concentration was greater than 2 $\log_{10}$ CFU/g ($p < 0.05$ compared to post-thermal processing) in thermally processed treatment groups at the end of storage, indicating that these *Pseudomonas* spp. isolates were able to survive thermal processing and grow during extended vacuum storage. This raises concerns about the ability of spoilage bacteria to survive the thermal processing schedules commonly used in the meat industry and confirms that some *Pseudomonas* spp. are capable of thriving in products other than aerobically stored fresh meat.

Practical Application: Spoilage *Pseudomonas* spp. can survive traditional thermal processing schedules. Heat resistance should be evaluated for commensal and spoilage bacteria to better understand possible ways spoilage of food products may occur.

KEYWORDS
meat spoilage, *Pseudomonas*, thermal processing

1 | INTRODUCTION

Global food loss and waste costs are estimated at $900 billion annually (Hanson & Mitchell, 2017). Limiting food loss due to microbial spoilage can contribute to decreasing the economic burden in the food chain. Controlling the presence and growth of pathogenic organisms in meat and poultry products has been a public health priority for nearly 30 years, after the widespread implementation of the hazard analysis and critical control points systems. However, to achieve sustainable meat and poultry production, it is important to gain a deeper understanding of both the evolving threat of biological hazards and the population dynamics of spoilage bacteria during shelf life.

Hygienic harvest, fabrication, and further processing procedures are designed to minimize the risk of contamination at all stages of production (Rouger et al., 2017), but bacterial contamination of meat throughout production is inevitable (Wickramasinghe et al., 2019). The improvement of process control and sanitation procedures in the meat industry has drastically improved the quality and safety of meat products, but these processes have also created ecological niches for certain bacteria to survive in the meat processing environment. Research has documented the ability of bacteria that are minimally present in the processing environment to become the dominant spoilage bacteria in cooked products, implicating the postlethality processing environment as the potential source of the spoilage organism (Hultman et al., 2015; Raimondi et al., 2019; Stellato et al., 2017).

There are growing concerns that thermotolerant bacteria may be increasing in the meat supply. Thermal processing has long been used to control bacteria in meat products, and great care is taken in the industry to adequately heat and cool meat products so that vegetative and spore-forming bacterial pathogens are controlled. However, commonly used pasteurization processes that are successful for destroying *Salmonella* and preventing the outgrowth of *Clostridia* do not completely sterilize a product. A recent report has shown that bacteria native to cattle may be exhibiting an increased prevalence of extreme heat resistance (Guragain et al., 2020). The primary concern is that heat resistant traits that exist in nonpathogenic *Escherichia coli* could arise in pathogenic organisms as well. The presence of heat resistant pathogenic bacteria in meat would undoubtedly pose a threat to human health, but the increased prevalence of these traits in spoilage bacteria could contribute to a decrease in cooked product stability during storage and further complicate the meat supply chain.

Pseudomonas is a taxon that includes multiple prominent spoilage organisms. Historical understanding of meat spoilage dictates that spoilage organisms from this taxon are generally responsible for the spoilage of aerobically stored meat and poultry products and that lactic acid bacteria primarily cause the spoilage of products stored under vacuum (Borch et al., 1996; Gill, 1983; Gill & Newton, 1977a, 1977b; Seideman et al., 1976). This hypothesis has been recently challenged with data from our laboratory that indicate that *Pseudomonas* spp. can cause spoilage of vacuum packaged deli meats (Bower et al., 2018; Furbeck et al., 2022). Although residual oxygen may still be present and able to aid in the growth of *Pseudomonas* spp. from these products, other reports have shown that *Pseudomonas* spp. isolated from vacuum packaged meat are able to grow under strictly anaerobic conditions and that arginine fermentation is the primary pathway anaerobic spoilage *Pseudomonas* spp. use for growth (Hilgarth et al., 2019; Kolbeck et al., 2021). Although the metabolic reasons for this phenomenon require further investigation, advances in microbial sampling techniques and proteomic analysis have generated data that rebut the traditional understanding of the role of *Pseudomonas* spp. in meat spoilage. This new understanding, in addition to concerns of thermotolerant bacteria being present throughout the meat supply, led to this investigation. We hypothesize that *Pseudomonas* spp. isolated from spoiled meat will survive common thermal processing schedules and be recovered from vacuum packaged cooked meat after thermal processing.

2 | MATERIALS AND METHODS

2.1 | *Pseudomonas* spp. isolate preparation

Pseudomonas spp. colonies were isolated from spoiled turkey using *Pseudomonas* spp. agar base (Oxoid Ltd.; Basingstoke, Hampshire, England) with Cetrimide–Fucidin–Cephalosporin selective supplement (CFC; MilliporeSigma; Burlington, MA, USA). Isolates were confirmed as *Pseudomonas* spp. by polymerase chain reaction with the Terra PCR Direct Polymerase Mix (Takara Bio USA, Inc.; San Jose, CA, USA) using *Pseudomonas* spp. specific primers Pse435F and Pse499R (Thermo Fisher Scientific Inc.; Waltham, MA, USA) as previously described (Bergmark et al., 2012). Three confirmed isolates were chosen for this experiment. Each isolate was streaked onto CFC plates (MilliporeSigma) and incubated at 32°C for 48 h to create a working stock. Prior to the start of each replication, isolates were individually inoculated in 10 mL of Luria-Bertani broth (LB; MilliporeSigma) and incubated aerobically at 32°C for 48 h to a concentration of at least

8 $\log_{10}$ CFU/mL. Inoculum of 10 mL from each isolate was combined to create a 30 mL cocktail that was used for meat inoculation.

2.2 | Beef emulsion production

Lean beef from the inside round, outside round, and eye of round was sprayed with 70% ethanol, and the surface was trimmed to reduce background microflora. Meat was coarse-ground through a 1.27 cm plate, vacuum sealed, and refrigerated until use. At the start of each replication, approximately 2 kg of coarse-ground beef was inoculated with 0.5 mL of the *Pseudomonas* spp. cocktail to a target of 5 $\log_{10}$ CFU/g and emulsified in a Hobart FP41 food processor (Hobart Corporation; Troy, OH, USA) with 10% ice, 2% salt, 0.5% dextrose, 0.3% black pepper, 0.15% garlic, 156 ppm sodium nitrite, and 550 ppm sodium erythorbate on a meat block basis. Raw meat batter samples were individually scooped into bags (3 mil; Clarity, Koch Supplies, Riverside, MO, USA) and vacuum sealed. Each sample weighed approximately 20 g and was less than 1.5 cm in thickness. Samples were then split into one of three heat treatments: (a) uncooked control; (b) cooked to 54.4°C internal temperature and held for 121 min; and (c) cooked to 62.8°C internal temperature for 60 min, 68.3°C internal temperature for 30 min, and at 79.4°C until reaching 71.1°C internal temperature. Uncooked controlled were used to monitor any impact of the emulsification process on the inoculum and to monitor concentration changes throughout storage in *Pseudomonas* spp. cells not exposed to heat. Water baths (Anova Precision Cooker, Anova Applied Electronics, Inc., San Francisco, CA, USA) were set to the target internal temperatures, and cooking schedules were chosen to best replicate low temperature, long time cooking and stepwise cooking used for emulsified and restructured meat products, respectively. Endpoint internal temperatures and holding times were based on United States Department of Agriculture Food Safety and Inspection Service guidance documents for ready to eat meat and poultry products (U.S. Department of Agriculture, 2021). Samples were cooked in preheated water baths, and internal sample temperature was monitored using Type T thermocouples TC-08 Data Logger (Omega Engineering Inc., Norwalk, CT, USA). For each water bath, the temperature was monitored in one vacuum sealed sample by running the thermocouple wire through a small hole the vacuum bag and sealing the hole with silicone prior to adding the sample and vacuum sealing the bag. Samples from cooked treatments were cooled in ice baths immediately upon completing the cooking schedule, and then all samples were split into either 4 or 10°C for long-term storage (Fisherbrand Isotemp RPLA, Thermo Fisher

Scientific Inc.). Temperatures of the refrigerators were monitored with analog thermometers (Fisherbrand Verification Thermometer, Thermo Fisher Scientific Inc.). Two storage temperatures were used to determine if storage temperature had an impact on changes in *Pseudomonas* spp. concentration during long-term storage.

2.3 | Sampling procedures

Duplicate samples were taken at multiple points: after inoculation, after emulsifying for uncooked control samples or after cooking for cooked samples, and after 14, 28, 56, 112, and 294 days of storage. Ten grams of each sample was homogenized for 90 s with 20 mL of buffered peptone water (Becton, Dickinson, and Company; Franklin Lakes, NJ, USA), serially diluted, and plated onto CFC agar plates (MilliporeSigma). Plates were incubated at 32°C, and colonies were counted after 48 h. The detection limit was 0.18 $\log_{10}$ CFU/g, and samples that did not result in recoverable *Pseudomonas* spp. were reported as the detection limit.

2.4 | Isolate confirmation

Sequencing of the V4 region of 16S rDNA gene was used to confirm the presence of *Pseudomonas* spp. in a subset of samples. Samples stored at 4°C taken on days 0, 112, and 294 were sequenced (54 samples, 6 negative controls). DNA was extracted by spinning 2 mL of plating homogenate at 10,000×*G* (accuSpin Micro 17R Microcentrifuge; Thermo Fisher Scientific, Inc.) for 10 min, mixing with 500 μL of QuickExtract DNA Extraction Solution (Lucigen Corporation; Middleton, WI, USA), and holding for 10 min at 65°C and 2 min at 98°C. DNA was amplified with PCR and sequenced on the Illumina MiSeq platform (Illumina, Inc.; San Diego, CA, USA) using methods adapted from Ribeiro et al. (2021). Sequence data were assigned amplicon sequence variants (ASVs) using the DADA2 pipeline in R 4.0.3 (RStudio, Boston, MA, USA). The presence of *Pseudomonas* spp. in the sequenced samples was confirmed by matching the subsample ASVs to the ASVs from the inoculum isolates.

2.5 | Statistical analysis

The experiment was conducted in three independent replications with $n = 6$ total samples per treatment and time combination. *Pseudomonas* spp. concentrations are reported as $\log_{10}$ CFU/g and were analyzed using PROC GLIMMIX with LSD means separation in SAS 9.4 (SAS

TABLE 1 Concentration of *Pseudomonas* spp. ($\log_{10}$ CFU/g $\pm$ SE) in emulsified beef during thermal processing and 4°C refrigerated storage.

Sampling time	Uncooked control	54.4°C cooked	71.1°C cooked
Inoculated raw beef	4.93 ± 0.05^a	5.01 ± 0.04^a	5.06 ± 0.04^a
After cooking or emulsifying (control)	4.75 ± 0.07^a	$0.18 \pm 0.00^{f,*}$	$0.18 \pm 0.00^{f,*}$
14 days storage	3.73 ± 0.06^{bc}	0.44 ± 0.09^f	0.39 ± 0.09^f
28 days storage	3.81 ± 0.10^b	0.23 ± 0.04^f	0.69 ± 0.21^f
56 days storage	3.55 ± 0.17^{bc}	0.57 ± 0.25^f	0.67 ± 0.24^f
112 days storage	3.84 ± 0.20^b	2.38 ± 0.67^{de}	1.97 ± 0.68^e
294 days storage	2.17 ± 0.45^e	3.11 ± 0.16^{bcd}	3.04 ± 0.29^{cd}

[a-f]Means across all sampling times and treatments within the table with different superscripts differ ($p < 0.05$).

*0.18 $\log_{10}$ CFU/g was the limit of detection.

TABLE 2 Concentration of *Pseudomonas* spp. ($\log_{10}$ CFU/g $\pm$ SE) in emulsified beef during thermal processing treatments and 10°C refrigerated storage.

Sampling time	Uncooked control	54.4°C cooked	71.1°C cooked
Inoculated raw beef	5.07 ± 0.04^a	5.03 ± 0.03^a	4.99 ± 0.02^a
After cooking or emulsifying (control)	4.69 ± 0.04^a	$0.18 \pm 0.00^{j,*}$	$0.18 \pm 0.00^{j,*}$
14 days storage	4.09 ± 0.19^b	$0.18 \pm 0.00^{j,*}$	0.58 ± 0.17^{ij}
28 days storage	3.75 ± 0.06^{cd}	0.28 ± 0.04^j	0.49 ± 0.04^{ij}
56 days storage	3.41 ± 0.16^{cde}	0.70 ± 0.27^{ij}	0.58 ± 0.23^{ij}
112 days storage	2.76 ± 0.42^{ef}	1.03 ± 0.34^{hi}	1.76 ± 0.69^{gh}
294 days storage	1.75 ± 0.45^{gh}	3.13 ± 0.16^{de}	2.28 ± 0.46^{fg}

[a-j]Means across all sampling times and treatments within the table with different superscripts differ ($p < 0.05$).

*0.18 $\log_{10}$ CFU/g was the limit of detection.

Institute Inc.; Cary, NC, USA). Statistical comparisons between samples stored at 4 and 10°C were not evaluated. Since only a subset of the samples was sequenced for 16S identification, further statistical analysis was not conducted on the sequence data.

3 | RESULTS AND DISCUSSION

Pseudomonas spp. were first recovered in cooked samples after 14 days of storage at both 4 (Table 1) and 10°C (Table 2) and were recoverable from all cooked sample groups stored at 4 and 10°C on days 28 and 56, respectively. For traditional plate counting experiments, an increase greater than 1 $\log_{10}$ is considered an indicator of true microbial growth (National Advisory Committee on Microbiological Criteria for Foods, 2010). Compared to the samples taken immediately after cooking, true growth was observed on day 112 of storage at 4°C ($p < 0.05$) for samples heated to either 54.4 or 71.1°C. Samples heated to 71.1°C and stored at 10°C also showed a greater than 1 $\log_{10}$ increase after 112 days of storage ($p < 0.05$), and the samples heated to 54.4°C and held for 121 min did not demonstrate true microbial growth until 294 days of storage ($p < 0.05$). In control samples stored at 4 and 10°C, total decreases in *Pseudomonas* spp. concentration greater than 2.5 $\log_{10}$ CFU/g were observed ($p < 0.05$). Competition with other native bacteria present in meat as well as general nutrient depletion are potential explanations for this phenomenon. Importantly, the presence of *Pseudomonas* spp. at concentrations greater than 4 $\log_{10}$ CFU/g from uncooked samples taken immediately after emulsification supports that the large decrease in *Pseudomonas* spp. in cooked samples is primarily due to the heat treatments. 16S sequencing was used to confirm the presence of *Pseudomonas* spp. in control and some heated sample groups stored at 4°C. Sequence reads present in the inoculated raw beef samples directly aligned with reads present after 112 and 294 days of storage, and the relative abundance chart of ASVs (Table 3) shows a numerical increase in *Pseudomonas* spp. abundance from day 0 to days 112 and 294 in cooked samples.

There were minimal observed significant differences in *Pseudomonas* spp. concentrations between the two heating temperature treatments. In samples stored at 10°C, the 54.4°C treatment was 0.85 $\log_{10}$ greater than the 71.1°C treatment after 294 days of storage ($p < 0.05$). This lone difference between the heat treatments is less than one log of growth and is most likely not biologically relevant.

TABLE 3 Relative abundance (%) sorted by genus of amplicon sequence variants from selected emulsified meat samples inoculated with three *Pseudomonas* spp. isolates.

	C	71.1°C	54.4°C
Day 0			
Pseudomonas	67.0	61.7	61.5
Lactobacillus	8.9	15.6	5.6
Carnobacterium	11.8	15.2	24.7
Other	12.3	7.5	8.3
Day 112			
Pseudomonas	9.4	91.6	76.9
Lactobacillus	70.2	2.3	2.1
Carnobacterium	20.1	5.4	20.3
Other	0.4	0.7	0.7
Day 294			
Pseudomonas	68.7	100.0	97.3
Lactobacillus	21.4	0.0	0.5
Carnobacterium	3.8	0.0	1.9
Other	6.1	0.0	0.4

Note: The subsets of samples were processed to 54.4 or 71.1°C final temperature alongside uncooked control (C) samples and then stored at 4°C for 0, 112, and 294 days.

This indicates that the final heating temperature used in this experiment had minimal impact on the survival of these *Pseudomonas* spp. isolates during the heating process. It should also be noted that although differences between samples stored at 4 and 10°C were not evaluated statistically, samples from both groups followed similar numerical trends. It was expected that storage of samples at 10°C could have resulted in growth of *Pseudomonas* spp. compared to no growth in samples stored at 4°C. Further evaluation of *Pseudomonas* spp. growth rates and nutrient utilization in meat is necessary. Although these isolates did successfully survive heating and grow in vacuum packaging, the final concentrations did not reach the levels that would be considered indicative of meat spoilage.

4 | CONCLUSION

The results of our study show that two common thermal processing schedules did not result in destroying all *Pseudomonas* spp. from the samples and that these isolates can grow with little to no oxygen in the sealed vacuum pouch that is not opened post-thermal processing. Further investigation of the genetic and proteomic composition of these isolates is needed to determine the mechanisms of action for the phenomena observed here. Further investigation is also needed to determine the overall prevalence of heat resistance of other bacteria present on meat and in the processing environment. Although concentrations of recovered *Pseudomonas* spp. here are considerably lower than concentrations present in fully spoiled meat products, it is possible for spoilage to occur in this manner.

AUTHOR CONTRIBUTIONS
Samuel C. Watson: Conceptualization; writing – original draft; methodology; writing – review and editing; formal analysis; investigation; **Rebecca A. Furbeck**: Writing – review and editing; formal analysis; **Samodha C. Fernando**: Resources; writing – review and editing; **Byron D. Chaves**: Resources; writing – review and editing; supervision; conceptualization; **Gary A. Sullivan**: Writing – review and editing; supervision; resources; conceptualization.

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