AQUATIC RESEARCH
E-ISSN 2618-6365
Aquatic Research 8(2), 130-139 (2025) • https://doi.org/10.3153/AR25013 *Research Article*

Heat inactivation of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in sous vide-cooked anchovy enriched with ascorbic acid at low temperature

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Cite this article as:
Doğruyol, H. (2025). Heat inactivation of *Escherichia coli* O157:H7 and *Salmonella* Enteriditis in sous vide-cooked anchovy enriched with ascorbic acid at low temperature. *Aquatic Research*, 8(2), 130-139. https://doi.org/10.3153/AR25013

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Submitted: 21.01.2025
Revision requested: 11.02.2025
Last revision received: 20.02.2025
Accepted: 06.03.2024
Published online: 16.03.2025

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Available online at
http://aquatres.scientificwebjournals.com

ABSTRACT

Low-temperature cooking during the sous vide process enhances sensory properties, particularly in heat-sensitive foods. While enhancing efficiency, it also raises the risk of foodborne pathogen persistence. In this study, butterfly anchovy fillets were inoculated with a low dose of *Escherichia coli* O157:H7 or *Salmonella* Enteritidis. To amplify the effect of heat treatment, ascorbic acid (AA) was incorporated into sous vide anchovies before thermal processing at 55°C. Sampling was conducted at 5-minute intervals up to 30 min, followed by longer intervals. The initial *E. coli* load was 4.49 log CFU/g. The addition of AA significantly reduced ($P<0.05$) bacterial counts at and after the 45th min compared to the untreated control (C) group. The lowest count, 1.30 log CFU/g, was observed in the AA group at 120 min of cooking. A tailing effect was noted after 30 min of heating in both groups. On the other hand, *Salmonella* counts gradually declined without statistically significant differences ($P>0.05$) between groups. No colonies (<1.00 log/g) were detected after the 30th and 45th min in the AA and C samples, respectively. *Salmonella* exhibited greater heat sensitivity than *E. coli*. Further research is needed to assess the safety of incorporating AA into low-temperature cooked sous vide seafood.

Keywords: Thermal inactivation, Food safety, Food poisoning, Pathogenic bacteria, Vitamin C

Introduction

Low temperature is being utilized progressively in the food industry and integrated into modern culinary practices (Becker et al., 2016). This approach ensures that the food consistently exhibits a pleasing texture and colour every time it is cooked. Heat-sensitive foods are frequently cooked at low temperatures to create a delicate and tender texture (Misu et al., 2024). Tenderness, juiciness, flavour, and appearance are crucial factors in evaluating the organoleptic quality of food products intended for human consumption (Christensen et al., 2012). Due to seafood's high susceptibility to quality deterioration, preserving its sensory attributes during heat treatment is essential.

Anchovy is the most popular seafood species among Turkish consumers. In 2023, 387,115 tonnes of marine fish were captured from seas in Türkiye. Anchovy accounted for 185,000 tonnes (%48) of this total, making it the most frequently wild-caught species (TUIK, 2024). Anchovy is used in a variety of dishes, including soup, rice dishes, stews, and marinades, and can be prepared using methods such as frying, steaming and grilling (Uran & Gokoglu, 2014). However, elevated cooking temperatures cause changes in myofibrillar proteins that can toughen the texture of food (Becker et al., 2016). Besides, frying results in the loss of EPA and DHA omega-3 fatty acids in fatty fish, which are essential for human health (Ansorena et al., 2010).

Fine dining restaurants have been preparing vacuum-packed, namely, sous vide foods, in temperature-controlled water baths at low temperatures (55 to 60°C) for the past decades (Mortensen et al., 2012). Sous vide is a cooking technique that involves vacuum sealing raw or pre-treated material and pasteurizing it at a precisely controlled temperature (50 to 95°C) for a predetermined time. Then the food can either be served or refrigerated. Rapid chilling prior to cold storage is crucial for ensuring safety (Coşansu et al., 2022). Mild heat treatment during sous vide cooking for an insufficient duration can pose a risk of foodborne illness, as some bacteria may thrive in the "temperature danger zone". The danger zone is the range of temperatures between 5°C and 60°C. Within this temperature range, pathogenic foodborne bacteria may reach hazardous levels, potentially leading to food poisoning (FSIC, 2014; USDA, 2017). Therefore, applying low-temperature heat treatment for an insufficient duration during sous vide cooking may compromise food safety.

Seafood contamination resulting from improper handling and processing before cooking is a major challenge, as mild heat treatments only partially eliminate microorganisms. Although pathogenic bacteria in food do not lead to any observable changes and cannot be detected visually, the consumption of contaminated seafood poses significant health risks (Mol & Coşansu, 2022). *Escherichia coli* O157:H7 and *Salmonella* Enteritidis are foodborne pathogenic bacteria that cause infectious diseases. They are ubiquitous in water and soil environments and are part of the intestinal flora of many animals (Newell et al., 2010). *Escherichia coli* O157:H7 lead to hemorrhagic colitis and hemolytic uremic syndrome, while *Salmonella* Enteritidis typically cause gastroenteritis, characterized by symptoms such as nausea, abdominal cramps, vomiting, and diarrhoea (Coşansu, 2018; Vencia et al., 2015). Therefore, additional hurdles are needed to inhibit unwanted bacteria in food when cooked at low temperatures.

Ascorbic acid (AA) is a natural powerful antioxidant that is found in almost all fruits and vegetables. It is an essential water-soluble micronutrient in human diets. Vitamin C, its biologically active form (L-AA), supports several cellular processes that help the immune system (Davey et al., 2000; Naidu, 2003). The Recommended Dietary Allowance (RDA) is 75 and 90 mg/day for adult women and men, respectively (Institute of Medicine, 2000). AA is extensively utilized in seafood processing to inhibit lipid oxidation, decelerate spoilage, and maintain product quality by preserving colour and texture (Deng et al., 1978; Hambre et al., 2003). Besides its high reducing power, AA appears as an antimicrobial agent in various studies (Giannuzzi & Zaritzky; 1996, Sangcharoen et al., 2017; Przekwas et al., 2020). It has also been utilized in food research to investigate its potential antimicrobial effects by incorporating it into carrot juice (Tajkarimi & Ibrahim, 2011), cheese (Elafify et al., 2022), pork (Ogden et al., 1996) and sole (Zambuchini et al., 2008). In seafood processing, AA functions as an antimicrobial agent, enhancing the safety of seafood products by curbing the proliferation of microorganisms (Sanjúas-Rey et al., 2012).

By lowering the pH and acidifying the environment, AA inhibits bacterial growth through oxidative stress and membrane disruption. Oxidative stress is a disturbance in the balance between reactive oxygen species (ROS) and antioxidants. ROS such as hydroxyl radical, hydrogen peroxide and superoxide radical cause oxidative damage to bacterial proteins, lipids, and nucleic acids, disrupting vital metabolic pathways. As a ROS scavenger, AA leads to the leakage of essential intracellular compounds. Thereby, it destroys the structural stability of the cell and results in bacterial death (Betteridge, 2000; Liu et al., 2021; Ma et al., 2024).

Food safety concerns related to ready-to-eat seafood products cooked at low-temperature can be addressed through the addition of antimicrobial compounds such as organic acids. This study aimed to evaluate the antimicrobial effects of ascorbic acid in sous vide-cooked anchovy, particularly in inhibiting *E. coli* and *S.* Enteritidis that may persist due to insufficient heat treatment.

Materials and Methods

Materials

Iced fresh anchovy was purchased from Karaköy fish market, İstanbul, Türkiye. A total of 3 kg of anchovy was obtained. Fish were brought to the laboratory without breaking the cold chain. Anchovies were beheaded and gutted, and the backbones were removed to obtain butterfly fillets. Cleaned fish (ca. 1.8 kg) were taken to gastronom trays and equally divided into two groups: 1) control (C), and 2) ascorbic acid-added (AA). Food-grade ascorbic acid (Balmumcu Kimya LTD., Türkiye) was used to prepare a 10% stock solution (w/v). The AA group was prepared by adding 0.5% (w/w) ascorbic acid solution directly to the fillets and manually mixed under aseptic conditions. For the control group, the same amount of sterile distilled water was added to the fish. Ten grams of fish were placed into heat-stable polyethylene-polyamide pouches (Apack Ambalaj, Türkiye) appropriate for sous vide cooking. Each group yielded 90 bags, of which at least seventy-five were used. Then, the samples were frozen at -24 °C until use. *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis were obtained from the culture collection of the Department of Food Engineering, Sakarya University.

Preparation of Inoculum and Inoculation

Stock cultures of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis were activated in 10 mL Tryptic Soy Broth (TSB) at 35°C for 24 h, separately. A 100 µL of culture from the tube was transferred to TSB again and incubated for another day. Then, the culture solution was centrifuged at 4000 rpm for 10 min. After discarding the supernatant, the pellet was washed twice with 10 mL of 0.1% peptone water (PW) (Üçok Alakavuk et al., 2021). Finally, the precipitation was mixed with 10 mL of PW. This culture solution was serially diluted to obtain 5-6 log CFU/mL inoculum for each bacterium.

Fish samples in pouches were thawed overnight in a refrigerator (4°C). Two hundred microliters of inoculum were inoculated into pouches. The transfer of bacteria to the flesh was allowed for over 20 min. The samples were then vacuum packaged in preparation for heat treatment.

Heat Treatment

Heat treatment trials of *E. coli* and *S.* Enteritidis in sous vide anchovy were performed separately. For sous vide cooking, a water-circulating heater bath (Daihan, WBC 22, S. Korea) was used. Low-temperature cooking was carried out at 55 ±0.5°C. The inner temperature of anchovies, which were not inoculated with bacteria, was monitored with a needle temperature probe. Sampling was performed at 0, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min of cooking. Once the predetermined cooking time was up, each pouch was immediately submerged in an ice water tank to cool down.

Enumeration

Ten grams of fish sample were diluted with 10 mL Maximum Recovery Diluent (MRD) and homogenized in sterile filter bags. By transferring 1 mL, serial dilutions were prepared in 9 mL MRD from each group. A 100 µL of appropriate dilutions were spread plated on Tryptic Soy Agar (TSA) plates in duplicate. For the recovery of the heat-injured bacteria, plates were incubated at 25°C for 2 h. Subsequently, plates were overlaid with a 10-12 mL layer of selective media. For *E. coli*, Sorbitol MacConkey (SMAC) Agar supplemented with Cefixim-Tellurite (CT) solution was used, while xylose lysine tergitol-4 (XLT4) agar supplemented with XLT4 Agar Supplement solution was utilized for *Salmonella*. All plates were incubated at 37°C for 24 h, and the counted colonies were expressed as logarithmic colony forming units per gram (log CFU/g) (Brashears et al., 2001; Lee & Kang, 2009).

Statistical Analysis

Two independent trials were carried out for the predetermined samplings of each bacterium. All data were presented as means ± standard deviation. The differences among the groups were determined by independent samples t-test and performed by using SPSS v21.0 software (IBM Inc., IL). The significance level between the means was considered $P<0.05$.

Results and Discussion

Conventional cooking, which aims for a specific core temperature, involves a higher temperature and leads to a decline in sensory quality due to increased toughness and reduced juiciness (Becker et al., 2016). The Sous vide technique allows cooking at low temperatures without deteriorating the key qualities of sensitive foods such as fish (Misu et al., 2024). On the other hand, inadequate cooking cannot eliminate the risk of pathogenic bacteria that may be present in food. Contamination of food products with *E. coli* and *Salmonella* spp. is associated with numerous cases of foodborne diseases (Interagency Food Safety Analytics Collaboration, 2022).

The incorporation of ascorbic acid into seafood that is cooked at low temperatures, can enhance food safety by inhibiting foodborne pathogenic bacteria. AA is a water-soluble antioxidant that is directly influenced by the cooking conditions. A reduction in the AA content of food is expected depending on the cooking process. Ascorbic acid undergoes rapid oxidation to dehydroascorbic acid, hydrolysis to 2,3-diketogulonic acid, and ultimately polymerization to other non-nutritive components. For instance, boiling red, orange and yellow paprika for 5 min resulted in the loss of AA content due to the leaching into boiling water. On the other hand, AA reductions were not significantly different from those of the raw peppers after microwave heating (Chuah et al 2008). Vacuum packing the anchovy before sous vide cooking, minimized AA loss, resulting in a more nutritious ready-to-eat meal.

Shiga toxin-producing *E. coli* O157 can cause severe infections and even death. Contaminated water and food, direct contact between people, and interaction with animals or their surroundings are the transmission routes to humans. Proper food handling and adequate heat treatment are essential to prevent outbreaks (Heiman et al., 2015). In the current study, cooking at 55°C was not sufficient to destroy all *E. coli* in both sous vide anchovy groups at 120 h. The amount of *E. coli* inoculum transferred to the flesh was 4.49 and 4.43 log CFU/g in control (C) and ascorbic acid added (AA) samples, respectively. No statistically significant difference ($P>0.05$) was observed between C and AA samples until the 45th min of sampling. Thereafter, the *E. coli* counts gradually decreased over time at 45, 60, 90 and 120 min, and significant differences ($P<0.05$) were revealed between the groups. On the other hand, the greatest reduction (%) in bacterial count was achieved at 120 min when the treated group was compared to the control samples (Table 1). The combined effect of lower pH and heat compromised bacterial integrity by leading to cell death and providing an effective bacterial inactivation. The bacterial load decreased progressively over time, showing a downward trend at each sampling point. However, a tailing effect was observed, indicating that the bacteria persisted at low levels during the prolonged heat treatment at 55°C (Figure 1). In a similar study, investigating the thermal inactivation fate of *E. coli* ATCC 25922, holding inoculated sous vide cooked beef steaks at 54 °C resulted in >6 log reductions at various durations. The bacteria counts were 0.51, 0.47, and 1.01 log CFU/g at 64.5, 86, and 107.5 min, indicating a tailing effect (Hunt et al., 2021). Juneja et al. (2009) reported that the time required to eliminate *E. coli* O157:H7 in sous vide minced meat cooked at 55°C by 1 log (D value) was 67.79 ±5.48 min.

Salmonella is a vegetative pathogen frequently detected in raw meat products, which can survive low-temperature heat treatment (Hunt et al., 2023). The inoculum dose of *Salmonella* Enteritidis absorbed by the flesh was 4.45 and 4.41 log CFU/g in the C and AA groups, respectively. The *Salmonella* counts substantially decreased throughout the study period. However, the differences among groups were not statistically significant ($P>0.05$) at any duration of sampling. The *Salmonella* load in the C group was found to be 1.90 log CFU/g at 45 min, while the bacteria was undetectable (<1.00 log CFU/g) in the AA group. After the 45th min of sampling, no colonies (<1.00 log/g) were detected, suggesting the absence of viable bacteria in both groups. The highest reduction percentage was 8.33% observed at the 30th min of cooking when compared to group C (Table 2). Moreover, the comparison of time-dependent-survival curves of *Salmonella* in C and AA groups was presented in Figure 2. By these findings, Juneja (2007) stated that the count of a four-strain mixture of *Salmonella* spp. in chicken breast cooked in the bag at 55°C declined by 1.91 log within 15 min and by 4.0 log after 30 min, which were plated on TSA. Moreover, Hunt et al. (2023) studied the thermal inactivation of 7 log-inoculated *Salmonella enterica* serotypes Typhimurium, Enteritidis, and Heidelberg in sous vide-cooked beef steaks. The minimum time needed for a 5-log reduction at 54.4°C was 64.5 min, and the initial bacterial load was reduced to 0.97 log CFU/g after 107.5 min of cooking. In another study, D values of *Salmonella*, inoculated to sous vide chicken breast, were determined to be 47.65 ±3.68 min and 34.12 ±1.73 min in untreated and acidic teriyaki sauce marinated samples at 55°C. The D value of marinated samples was 28.4% lower than the control samples' values (Karyotis et al., 2017). Bacteria are more susceptible to heat when food is subjected to acid treatment. The acidic environment damages the bacterial cell membrane and alters its internal structure (Dogruyol et al., 2020).

According to the Codex Alimentarius Commission (2024), the maximum use of vitamin C (L-AA) in cooked and/or fried fish and fish products, including molluscs, crustaceans, and echinoderms is permitted under Good Manufacturing Practices (GMP). In this study, 0.5% ascorbic acid was added to anchovies prior to sous vide cooking. This AA concentration is used in various food research avoiding sensory deterioration (Giroux et al., 2001; Ouattara et al., 2002; Elafify et al., 2022). Furthermore, a 1-log reduction (6.53 to 5.70 log CFU/g) in the total bacterial count of medium-fat ground beef patties was reported during the first 4 days of cold storage, when 0.5% AA added (Ouattara et al., 2002). However, there exists a limited number of studies on the effectiveness of AA against *E. coli* and *Salmonella* in food. Elafify et al. (2022) stated that the application of 0.5% AA to artificially contaminated cheese reduced *Salmonella* Enteritidis counts significantly by 0.9 log CFU/g in comparison to untreated samples

during 4 weeks of cold storage. Aligned with the current research, Ouattara et al. (2002) reported that the counts of total coliforms and *Enterobacteriaceae* were not statistically different after the incorporation of AA to beef patties at the beginning of the study. In another study, Chiasson et al. (2004) stated that using AA along with carvacrol reduced the radiosensitivity of *E. coli* and *S.* Typhi significantly in ground beef. The incorporation of 0.03% ellagic acid in combination with 1.71% AA and 1.98% sodium ascorbate extended the shelf life of fresh sole for 2 more days and significantly delayed the growth of total aerobic, psychrotrophic and *Pseudomonas* bacteria (Zambuchini et al., 2008).

In the in vitro studies, Verghese et al. (2017a, 2017b) reported that AA significantly inhibited the growth of *E. coli* in Tryptic Soy Broth and emphasised the possibility of incorporating vitamin C as a safe and effective antimicrobial agent, against climbing antimicrobial resistance. In another study evaluating the effect of vitamin C on the secondary contamination of food, the biofilm formation of pathogenic bacteria was observed. It was reported that the 25.0 mg/mL AA inhibited the biofilm growth of *E. coli*, *L. monocytogenes*, and *S. aureus* by 93.4%, 74.9%, and 40.5%, respectively (Przekwas et al. 2020). Ascorbic acid obtained from Japanese apricot extract also presented antibacterial activity against *E. coli* and the minimum inhibitory concentration (MIC) was determined to be 8.00 mg/mL (Gao et al., 2012). Selim et al. (2012) specified that AA eliminated *E. coli, Salmonella indica* and *Staphylococcus aureus* after 2 hours, and the minimum bactericidal concentrations (MBC) were 32 mg/mL for the above bacteria. Conversely, Sangcharoen et al. (2017) highlighted that AA alone had no inhibitory effect on the growth of *S.* Enteritidis ATCC 13076 in Mueller Hinton broth incubated at 35°C.

AA reduces reactive oxygen species (ROS) levels by improving antioxidant enzyme activity, preventing oxidative damage and controlling radical accumulation. AA exhibits antibacterial activity by inducing intracellular damage, whereas heat treatment exerts antibacterial effects by compromising the cell membrane and triggering protein denaturation (Ma et al., 2024). Consequently, the distinct mechanisms of AA and heat underpin the enhanced reduction in *E. coli* counts observed when they were used in combination. Similarly, Kang et al. (2021) emphasized that hot water and citric acid induced the synergistic generation of ROS or superoxide, resulting in excessive destruction of the cell membrane of *E. coli,* triggering the ROS leakage within the cell.

Figure 1. Comparison of the decrease and tailing effect in the *E. coli* counts over time

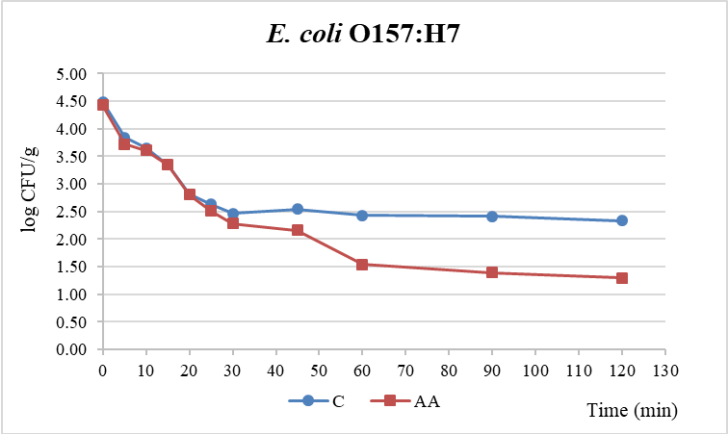

Table 1. Mean *E. coli* O157:H7 counts (log CFU/g) in control (C) and ascorbic acid-added (AA) groups at each sampling time after sous vide cooking and reduction percentage of the bacteria

E. coli O157:H7		C		AA		% Reduction
		Mean	±STD	Mean	±STD	
	Inoculum	6.67	±0.06	6.67	±0.06	
Time	**0**	4.49	±0.01[a]	4.43	±0.03[a]	1.23
	5	3.84	±0.05[a]	3.72	±0.09[a]	3.30
	10	3.65	±0.12[a]	3.61	±0.09[a]	1.34
	15	3.34	±0.02[a]	3.35	±0.01[a]	-0.04
	20	2.81	±0.03[a]	2.81	±0.13[a]	0.22
	25	2.63	±0.06[a]	2.51	±0.03[a]	4.60
	30	2.47	±0.08[a]	2.28	±0.10[a]	7.56
	45	2.55	±0.07[a]	2.16	±0.01[b]	15.27
	60	2.43	±0.09[a]	1.54	±0.09[b]	36.56
	90	2.41	±0.12[a]	1.39	±0.55[b]	42.44
	120	2.34	±0.12[a]	1.30	±0.42[b]	44.34

[a,b]: Letters indicate the statistical difference between groups at each given time point. Percentage (%) reduction was calculated by subtracting the count of AA from the count of C and then dividing by C, with the result multiplied by 100.

Figure 2. Comparison of the decrease and tailing effect in the *Salmonella* counts over time

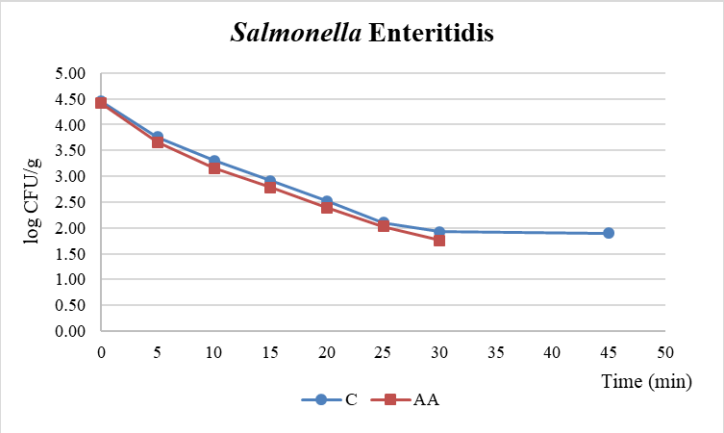

Table 2. Mean *Salmonella* Enteritidis counts (log CFU/g) in control (C) and ascorbic acid-added (AA) groups at each sampling time after sous vide cooking and reduction percentage of the bacteria

Salmonella Enteritidis		C		AA		% Reduction
		Mean	±STD	Mean	±STD	
	Inoculum	5.09	±0.49	5.09	±0.49	
Time	**0**	4.45	±0.17[a]	4.41	±0.06[a]	0.85
	5	3.76	±0.09[a]	3.66	±0.03[a]	2.68
	10	3.31	±0.01[a]	3.16	±0.05[a]	4.52
	15	2.91	±0.34[a]	2.78	±0.00[a]	4.53
	20	2.52	±0.17[a]	2.39	±0.23[a]	5.19
	25	2.10	±0.28[a]	2.03	±0.01[a]	3.32
	30	1.92	±0.38[a]	1.76	±0.09[a]	8.33
	45	1.90	±0.00	<1.00		
	60	<1.00		<1.00		
	90	<1.00		<1.00		
	120	<1.00		<1.00		

[a,b]: Letters indicate the statistical difference between groups at each given time point. Percentage (%) reduction was calculated by subtracting the count of AA from the count of C and then dividing by C, with the result multiplied by 100.

In this study, *S.* Enteritidis was more vulnerable to heat at 55°C than *E. coli* in both C and AA groups, during sous vide cooking. Contrary to the current findings, Patil et al. (2024) reported that *Salmonella* Montevideo exhibited a lower decline in bacteria counts than *E. coli* O157:NM, in sous vide cooked ground beef at 62.5°C at the 5th, 10th and 20th min of samplings. It was also stated that >4 log and 5 log CFU/g reductions were achieved after 20 min and 120 min treatments for both pathogens.

Conclusion

Ascorbic acid incorporated into sous vide anchovies prior to thermal treatment significantly reduced the *E. coli* load at and after the 45th min. However, there were no distinct differences among groups in *Salmonella* counts at any sampling times. While *Salmonella* was undetectable after the 30th min in the AA group and after the 45th min in the control group, *E. coli* was present even at the 120th min. Based on the results, it was suggested that *Salmonella* was more vulnerable to heat treatment compared to *E. coli* in both groups. Due to its advantages, such as being easy to obtain, cost-effective, and capable of reducing contamination levels in food at low concentrations, ascorbic acid holds potential as a natural additive that not only preserves but also enhances the sensory properties of foods. Further research is needed to ensure the food safety of sous vide products processed at low temperatures with the addition of ascorbic acid, particularly for heat-sensitive foods like fish.

Compliance with Ethical Standards

Conflict of interest: The author declares no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Ethics committee approval is not required for this study.

Data availability: Data will be made available on request from the author.

Funding disclosure: The author declares that no funds, grants, or other support were received during the preparation of this manuscript.

Acknowledgements: -

Disclosure: -

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