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Research Note

Temperature-Dependent Antimicrobial Activity of Menhaden Fish Oil In Vitro and on Pet Food Kibbles against *Salmonella*

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ABSTRACT

Fish oil inclusion into a dry pet food provides a source of long-chain omega-3 fatty acids. Polyunsaturated fatty acids in fish oil have antibacterial activity against various foodborne pathogens, such as *Salmonella* and pathogenic *Escherichia coli*. The purpose of this study was to determine the effect of temperature applied to dry pet food kibbles on the antimicrobial activity of menhaden fish oil against *Salmonella* spp. Sterile menhaden oil was inoculated with ~8 log of a *Salmonella* cocktail (~3% moisture; *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Typhimurium) and incubated at 25, 37, and 45°C. Microbiological evaluation of the water phase was done after 2 h on tryptic soy agar. Sterile kibbles were coated with fish oil (7.0%, w/w). Canola oil coating was kept as a control. One hour after coating, the kibbles were inoculated with ~9 log of *Salmonella* and incubated at the respective temperature. The microbiological evaluation was conducted at 0, 2, 6, 12, and 24 h. The oil phase of the fish oil system was negative for *Salmonella* after 2 h of incubation and confirmed by enrichment and PCR. From the water phase, 8.1 and 7.3 log were recovered at 25 and 37°C, respectively, and no *Salmonella* was detected at 45°C. On the kibble, menhaden oil had higher antimicrobial ($P \leq 0.05$) activity after 12 h at 25°C and throughout the experiment at 37°C. At 45°C, the fish oil had a superior antimicrobial activity against the *Salmonella* cocktail after 2 h. When the fish oil alone was compared at different temperatures, a higher antimicrobial activity was observed at 37 and 45°C across all time points. The results indicate antimicrobial activity of menhaden oil increases with temperature. This is an important finding to the pet food industry: a higher fat holding temperature (~45°C) and the application process may help mitigate *Salmonella* on extruded kibbles.

HIGHLIGHTS

- Menhaden fish oil has antimicrobial activity against *Salmonella* spp.
- The antimicrobial activity of fish oil increases with temperature.
- Compared with canola oil, fish oil has superior antimicrobial activity.

Key words: Different temperatures; Dog food kibble; Menhaden fish oil; *Salmonella*

Several reports have indicated that humans can acquire *Salmonella* from handling contaminated pet foods and pet treats *(1, 9, 19, 21)*. It is a common practice to coat dry dog food kibbles with fats, oils, and flavoring agents to enhance palatability and increase energy density. It has been postulated that *Salmonella* and other pathogen contamination events in pet food kibbles occurs during postprocessing steps from coating with fats and oils and flavors *(12, 24, 39)*. In a study with rendered animal products, 26.1% of the final products were found to be *Salmonella* positive *(39)*. The 2006 and 2008 outbreaks of human *Salmonella* from dried dog food were sourced back to the enrobing and flavoring room in the manufacturing plant, indicative of contamination from either coating or spraying materials, such as fats and oils or flavoring agents *(4, 7)*. *Salmonella* can survive for a long period in dry pet food kibbles, despite low moisture and water activity *(5, 20)*. Although the high temperature and pressure during extrusion could be sufficient to kill pathogens, postprocessing contamination from the environment, oil, and favoring ingredient remains an issue in dry pet food safety. The low-water-activity fats and oils can harbor *Salmonella* from the very small amount of water residue present in the fat and oil storage tanks.

The fats and oils that are added to pet foods include mammalian sources (tallow, lard, and chicken fat), vegetable oils (soybean, canola, and sunflower), and marine sources (fish oils generically and some specific sources, such as salmon oil). Most of these are coated on the outer surface of the kibble *(2)* postprocessing kill step. They supply energy, essential fatty acids, flavor, and texture and aroma and aid in the absorption of fat-soluble vitamins.

In addition to providing essential omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic

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acid (DHA), fish oil also supports heart health, promotes a silky coat, boosts the immune system, and relieves allergies and joint pain *(3)*. The National Research Council *(31)* recommends EPA and DHA at the rate of 11 g/kg on a dry matter basis or 1.1% dry matter for adult dogs and cats and for neonatal growth. Polyunsaturated fatty acids (PUFA) from different fish species have shown antibacterial activity against *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Salmonella* Typhi, and *Salmonella* Typhimurium *(6, 8, 37)*. Menhaden fish oil is a commercially available fish oil in the United States and popular for use in pet foods. Menhaden fish oil is known to contain 14 to 30% of EPA and DHA *(42)*. The mechanism of antimicrobial action of the fish oils was reported to be a function of the long-chain omega-3 fatty acids, namely, EPA and DHA that cause cellular distortion, leading to the growth inhibition and down-regulation of virulence genes in *Porphyromonas gingivalis* and *Fusobacterium nucleatum (38)*. The PUFA in fish oils are known to kill microbes by the action on cell membranes through generation of free radicals and lipid peroxides that are cytotoxic to the cell *(11)*. Disturbing cell membrane dynamics and associated protein functions, such as electron transport and the proton gradient, is thought to be a mechanism of antimicrobial activity of fish oil *(13)*. In addition, linolenic acid from fish oil exhibited antibacterial activity against *Staphylococcus aureus* by inhibiting the FabI enzyme, a component in the fatty acid elongation cycle *(43)*.

The conditions during storage and transportation of the rendered fish oil from the rendering plant to the food manufacturing plant, as well as the application process may play important roles in limiting the postprocessing contamination of pathogens in dog foods. One of the major factors to be considered is the temperature. The antimicrobial activity of a chemical or a natural extract is known to be temperature dependent *(23, 29)*. During our comprehensive study of multiple fats and oils (chicken fat, beef tallow, pork lard, fish oil, and canola oil) against three *Salmonella* serotypes, the fish oil sample was inadvertently left at room temperature (~25°C) instead of incubating at 45°C (J.D., personal communication). Upon evaluation, a different pattern of pathogen reduction was identified for the fish oil treatment compared with our regular pattern of results at 45°C. This led us to hypothesize that the antimicrobial behavior of fish oil varies on the basis of temperature. Therefore, the objectives of this study were to determine the effect of temperature on the antimicrobial activity of fish oil against a cocktail of *Salmonella* in bulk oil, as well as postapplication on kibbles.

MATERIALS AND METHODS

***Salmonella* serotypes, menhaden fish oil, and dry dog food kibble sources.** *Salmonella* Enteritidis (ATCC 4931), *Salmonella* Heidelberg (ATCC 8326), and *Salmonella* Typhimurium (ATCC 14028) used in the experiment were maintained in tryptic soy broth (TSB)–glycerol (7:3) at –80°C. The first two serotypes in this study were selected because they are among the top 25 most prevalent serotypes in pet food *(27),* and *Salmonella* Heidelberg was included because it is among the most commonly isolated serotypes from human foods. Prior to use, the frozen cultures were streaked on tryptic soy agar (TSA) plates and incubated at 37°C for 24 h. A single colony of the *Salmonella* strain was inoculated in 10 mL of TSB and incubated at 37°C for 18 to 24 h. Rendered menhaden fish oil was provided by an established fish rendering company (Omega Protein, Inc., Reedville, VA). The dry dog food kibbles for the study were custom manufactured at Extru-Tech Inc. (Manhattan, KS) and were postdry, precoating kibbles. The moisture percentage of the kibble was 8.28%. The composition of the kibbles is provided in Table 1.

TABLE 1. *Composition of dry dog food kibbles*

Ingredients	Dry feed recipe (lb)[a]	Percentage
Chicken meal	852.42	42.621
Corn	354.67	17.733
Wheat	354.67	17.733
Rice, Brewers	354.65	17.732
Vinegar, dry	39.99	2.000
Salt	10.08	0.504
Potassium chloride	6.46	0.323
Choline chloride, 60% dry	5.04	0.252
Dicalcium phosphate	5.04	0.252
Calcium carbonate	5.04	0.252
Trace mineral premix	4.20	0.210
Vitamin premix	2.20	0.110
Fish oil, Menhaden	2.53	0.126
Taurine	2.53	0.126
Natural antioxidant preservative	0.50	0.025
Total	2,000.00	100.000

[a] 1 lb = 0.45359237 kg.

***Salmonella* inoculation in menhaden fish oil.** The working stock of menhaden fish oil was tested for background contamination before the start of each experiment. Aliquots of sterile menhaden oil were transferred into 50-mL sterile centrifuge tubes and stored at three different temperatures: 25, 37, and 45°C. A duplicate set of tubes was used for analysis at each temperature. To maintain ~3% final moisture in the fish oil, an aqueous phase containing sterile distilled water and a *Salmonella* cocktail (~8 log) in 0.1% peptone water (PW) were added. In a 40-mL volume, 1.2 mL was the aqueous phase. A total of 1-mL volume was plated on four TSA plates at the rate of 250 μL each, including that from the zeroth dilution, with the limit of detection as 1 CFU/mL. Control samples were maintained by treating with sterile distilled water only (no *Salmonella*). After mixing the contents, tubes were incubated at the respective treatment temperatures for 2 h. The rationale behind opting for a 2-h incubation time in this study came from preliminary evaluations in which menhaden fish oil at 45°C reduced the *Salmonella* to a nondetectable level within 2 h. The aqueous phase of the solution was processed for microbiological analysis by serial dilution in 0.1% PW and plating on TSA. The plates were incubated at 37°C for 24 h before counting the colonies. To sample the aqueous phase, the top oil phase was gently discarded by using the pipette. The aqueous phase was collected by using a long 1,000-μL pipette tip and transferred to the PW water without the pipette tip touching the edge of the tube to not introduce the oil portion.

***Salmonella* inoculation in dog kibbles coated with menhaden fish oil.** A 125-g aliquot (final weight) of sterile kibbles (8.3% moisture) of dry dog food kibbles was transferred to sterile plastic containers. Duplicate containers were maintained for analysis for each temperature. For each temperature condition,

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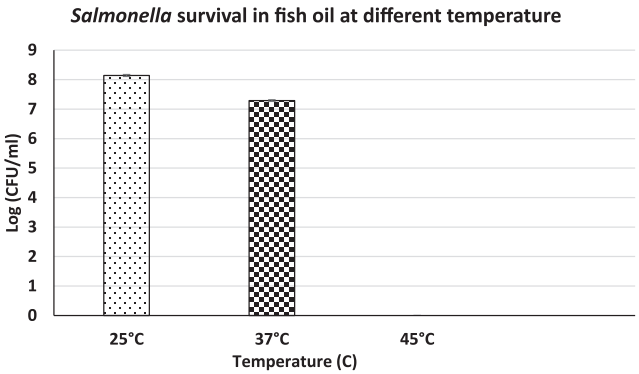

FIGURE 1. *Effects of various temperatures (25, 37, and 45°C) on survival of a* Salmonella *cocktail of* Salmonella *Enteritidis (ATCC 4931),* Salmonella *Heidelberg (ATCC 8326), and* Salmonella *Typhimurium (ATCC 14028) in a menhaden fish oil system when evaluated after 2 h of incubation. The limit of detection for the study was 1 CFU/g.*

control kibbles coated with canola oil were maintained. A negative control sample was also maintained (kibbles alone, no fish oil, and no canola oil) to determine the level of background *Salmonella* (if any). Sterile menhaden fish oil was applied to the kibbles to achieve a final ~7% (w/w) fat coating. The control kibbles were coated with canola oil. The oil-coated kibbles were stored at the respective temperatures (25, 37, and 45°C) for 1 h before inoculation. To maintain a 2% or less final additive or flavor (aqueous solution), 2.5 mL of ~9 log of the *Salmonella* cocktail was uniformly applied on the oil-coated kibbles. The *Salmonella* inoculum was spot inoculated on kibbles by manually shaking and rotating the kibble container, followed by thorough mixing after the lid closure.

Microbiological analyses of the samples were performed at various predetermined time intervals (0, 2, 6, 12, and 24 h). From each treatment and control, a 25-g subsample was collected in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI), mixed in 225 mL of buffered PW, and stomached for 2 min. The mixtures were serial diluted in 0.1% PW and plated on TSA. A total 1-mL volume of the diluent was plated on four TSA plates. The plates were incubated at 37°C for 24 h, and colonies were counted. The detection limit for *Salmonella* was <1 CFU/g of the sample.

The first experiment was a complete randomized design with three temperatures as treatments. The second experiment was a 3 × 5 factorial arrangement of treatments using three temperatures and five sampling intervals. Both experiments were evaluated in triplicate. Data were analyzed with statistical software (SAS Institute, Cary, NC) using the generalized linear mixed model procedure for both studies.

RESULTS

***Salmonella* reduction in menhaden fish oil.** The recovery of *Salmonella* from the aqueous phase of the fish oil system after 2 h was 8.1 and 7.3 log from the samples stored at 25 and 37°C, respectively (Fig. 1). The recovery from samples at 25°C was higher ($P \leq 0.05$) than from 37°C. No *Salmonella* was detected from the aqueous phase of samples stored at 45°C. The oil phase of the fish oil system that did not show *Salmonella* on TSA after 2 h was subsequently processed for isolation of *Salmonella* by using a modified *Bacteriological Analytical Manual (40)* procedure and analyzed by PCR (*invA* gene), resulting as negative for *Salmonella*.

***Salmonella* reduction in dog kibbles.** When the menhaden fish oil–treated dog food kibbles were compared with the canola oil–treated controls at 25°C, there was no difference in the antimicrobial activity until 6 h. However, menhaden oil had higher antimicrobial ($P \leq 0.05$) activity after 12 h onward with 4.75-log recovery in fish oil–treated kibbles compared with 6-log recovery in canola oil–treated kibbles after 24 h (Fig. 2a).

At 37°C, the fish oil displayed a higher antimicrobial activity ($P \leq 0.05$) across all time points during the evaluation, with only a 3-log recovery in fish oil–treated kibbles compared with 5.4-log recovery for the control kibbles (canola oil treated) after 24 h (Fig. 2b). Similarly, at 45°C at all the time points beyond 0 h, the fish oil displayed a greater antimicrobial effect against *Salmonella* than canola oil. The *Salmonella* recovery from fish oil–treated samples was 3.2 log compared with 4.9 log from the control ($P \leq 0.05$) after 24 h (Fig. 2c).

Figure 2d provides a comparison of the antimicrobial activity of the menhaden fish oil at different temperatures. When the results were compared within time, the fish oil displayed a higher ($P \leq 0.05$) antimicrobial activity at 37 and 45°C when compared with 25°C at all time points. Within the treatments, at 25 and 37°C, the *Salmonella* recovery decreased ($P \leq 0.05$) across each time point until 24 h. However, at 45°C, the reductions after the 6-h time point was not different ($P > 0.05$) with 3.2-log recovery at 24 h. We observed an interaction ($P \leq 0.05$) between the types of oil and time on the reduction of *Salmonella*: menhaden fish oil provided a greater log reduction at higher temperatures than canola oil.

DISCUSSION

The reason for the higher antimicrobial activity resulting from fish oil could be due to the modification in the properties and fluidity of the cytoplasmic membranes at higher temperatures, thereby altering the sensitivity of the bacterium to the compound *(35)*. The reduction of *Salmonella* to a nondetectable level after 2 h at 45°C was a very interesting finding. The first most obvious thought is that the temperature influenced the viscosity of the fish oil and lipid membrane of the bacteria, making the infiltration and disruption more rapid. This finding in menhaden fish oil at 37°C closely resembles our previous research on chicken fat in which *Salmonella* Typhimurium was nearly constant for up to 24 h (the maximum tested time) in the water phase at 37°C *(16)*. The absence of *Salmonella* after enrichment and PCR confirmation in the oil phase was contrary to the finding by Dhakal and Aldrich *(15),* wherein *Salmonella* isolated from the inoculated control fat phase of the rendered chicken fat system was positive for retained viability. This absence of *Salmonella* in the oil phase of the control further suggests the potency of menhaden fish oil against *Salmonella* was unique to this oil and/or its fatty acid profile.

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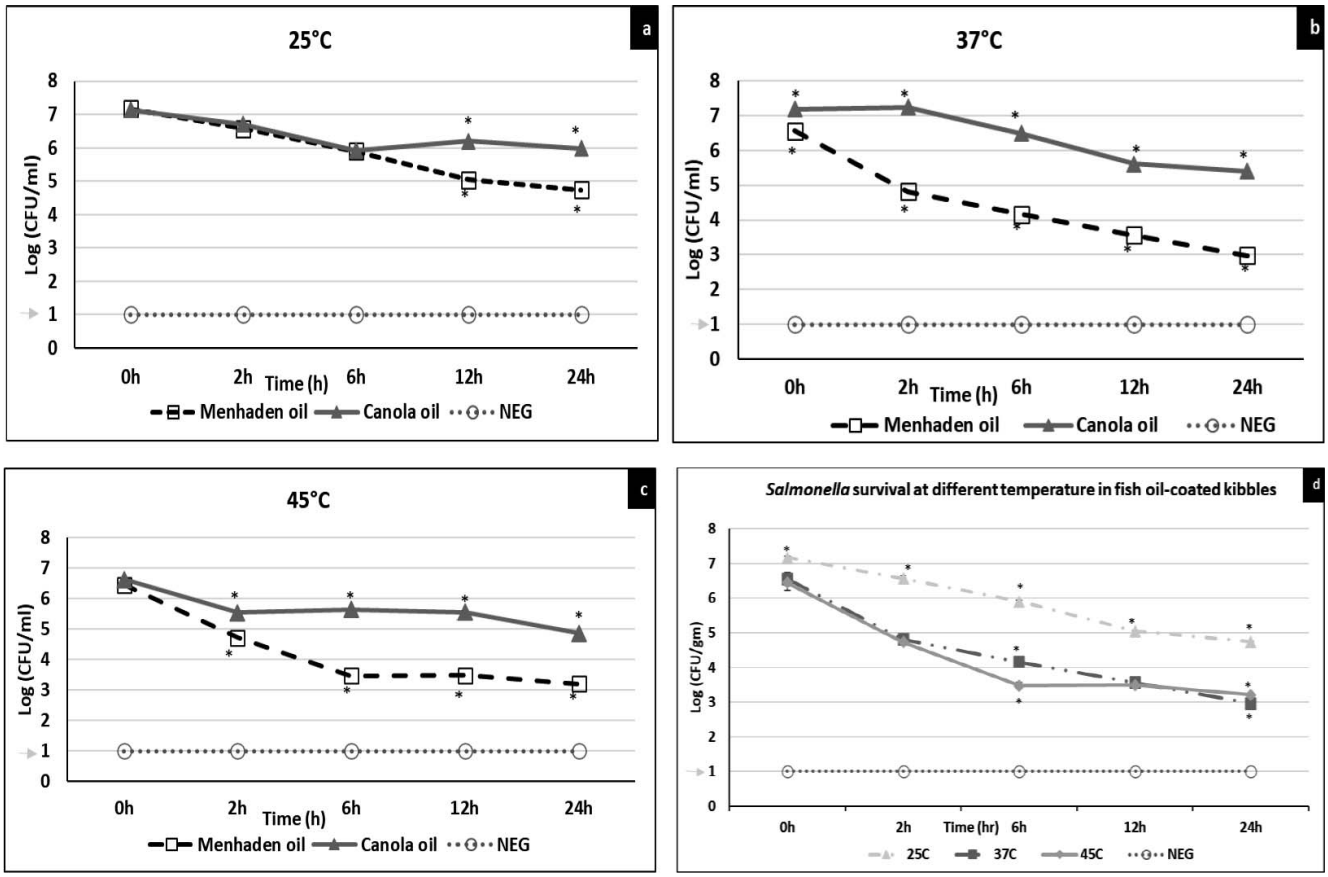

FIGURE 2. *The reduction of* Salmonella*,* Salmonella *Enteritidis (ATCC 4931),* Salmonella *Heidelberg (ATCC 8326), and* Salmonella *Typhimurium (ATCC 14028), in dry dog food kibbles coated with menhaden fish oil (canola oil as control) over time at (a) 25°C, (b) 37°C, (c) 45°C, and (d) a comparison of reduction by menhaden fish oil at different temperatures. The limit of detection for this study was 10 CFU/g. NEG, negative control (kibbles without oil coating); *, statistical significance.*

In addition to the traditional consideration of fat and/or oil as flavor and energy sources, the potential to serve as an antimicrobial to retard the contamination with *Salmonella* is a bonus. In another study, coating dry dog food kibbles with medium-chain fatty acids had a role in mitigating *Salmonella (14)*. In the kibble-coating study, fish oil showed a general trend for higher antimicrobial potential at all the tested temperatures (25, 37, and 45°C). This could be explained by the higher amount of the omega-3 fatty acid content (∼35%) in menhaden fish oil *(32)* compared with 9 to 11% in canola oil *(10)*. Fish oils are rich in linoleic acid and PUFA *(17),* and EPA and DHA are known to exert antimicrobial effect against a number of gram-positive and gram-negative bacteria, including *Salmonella* Typhimurium and *E. coli (36)*. Oleic acids are reported to have antimicrobial activity against *Listeria monocytogenes* and *Salmonella (41)*. The high proportion of PUFA contained in fish oil makes them more efficient antimicrobial food-grade additives. In a study by Liu et al *(28),* when specific pathogen-free mice were fed diets that included PUFA for 7 days, followed by artificial infection with *Salmonella,* the incidence of the *Salmonella* infection in mice were significantly lowered. This finding is beneficial to pet food manufacturers for the dual value of protecting against potential cross-contamination during handling and packaging and inhibiting *Salmonella* in the gastrointestinal tract by lowering potential fecal shedding. Lower fecal shedding in pets likely translates to lower incidences of human pet owner salmonellosis. In addition to health benefits, feeding fish oil has been demonstrated to benefit birds challenged with coccidiosis and pigs challenged with *E. coli (30)*. Korver and Klasing *(25)* and Korver et al. *(26)* also discovered that birds could develop resistance against challenge bacterial antigens when the feed contained fish oil. Similarly, replacement of 20 to 40% soybean oil with fish oil in a rat diet resulted in significantly lower viable *E. coli* in the rat body, and the lower level was concluded to be the improved killing of the bacteria *(34)*.

Carvacrol, an essential oil, and cymene, a naturally occurring aromatic organic compound, has displayed temperature-dependent antimicrobial activity with higher activity against *Vibrio cholerae* at 25°C compared with 15 and 4°C *(35)*. In a separate study, Periago and Moezelaar *(33)* also reported a higher antimicrobial activity of carvacrol at 30°C compared with 8°C.

The high temperature at 45°C could have partly played a role in inactivating *Salmonella* in dry pet food kibbles because *Salmonella* fares best at 37°C and the rate of survival and growth decreases both above and below 37°C *(18)*. The higher recovery of *Salmonella* with 3.2 log in fish oil–coated kibbles and 4.9 log in canola oil–coated kibbles could be due to the intrinsic properties of the complex food

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matrix. The ability of *Salmonella* to grow or survive also depends on food types, as seen in a finding in which *Salmonella* Enteritidis, *Salmonella* Senftenberg, and *Salmonella* Manhattan could grow at 7°C in chicken, whereas growth was halted at 10°C in ham salad or custard *(22)*. The results from this study indicate the antimicrobial activity of menhaden oil increases with temperature, which is an important finding for the pet food industry: a higher temperature (∼45°C) during fat holding and the application process may help mitigate *Salmonella* on kibbles.

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