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Persistence of *Salmonella enterica* Serovar Agona in Oil for Fish Feed Production

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Abstract: Globally, *Salmonella* is one of the most important food borne pathogens. Even though bacteria in the genus *Salmonella* are typical intestinal organisms, they show ability to survive and even multiply in environments other than the intestine, e.g., food and feed. Furthermore, the survival of *Salmonella* sp. in stored food and feeds are enhanced by a lowered water activity (a_w). Some *Salmonella* serovars such as Agona, may occasionally be found in animal feed and its ingredients, as well as in the feed production facilities. In the present study we have examined the persistence of *Salmonella enterica* serovar Agona in an experimentally contaminated marine oil intended for fish feed production. The bacterium was added to the sterile oil in an average concentration of 2×10^6 Colony Forming Units (CFU mL^{-1}) and kept aerobically at 20°C to mimic common storage conditions. At days 0, 6, 18, 32, 54, 80, 94, 116 and 122, one sample from each of ten parallel aliquots of the oil were examined quantitatively and qualitatively. At day six after inoculation, a log increase of viable *Salmonella* cells could be observed, as the average number reached 1.9×10^7. From day 18-54 the cell numbers were stable at between 1.8 and 3.1×10^6 cells. After day 54 until the termination of the experiment the number of cells steadily decreased and the bacterium could not be detected by quantitative or qualitative methods at day 122 after inoculation. This experiment demonstrates that *Salmonella enterica* serovar Agona can survive for at least four months in a marine fish oil.

Key words: Marine oil, fish feed, *Salmonella*, persistence, becteria, Norway

INTRODUCTION

On a global basis *Salmonella* sp. is one of the most important causes of infections related to foods. The reported number of salmonellosis cases pr. 100,000 inhabitants in industrialised countries vary from 0.8-258 (D'Aoust *et al*., 2001; Adams and Moss, 2008). Transmission of salmonellosis is by the faecal-oral route, possibly after multiplication in foods. The range of foods involved in transmission of salmonellosis is large and also include seafoods as fish, shellfish and crustaceans (Huss *et al*., 2004). Due to the heterothermic conditions in seafood animals, *Salmonella* is not a constituent of the normal flora of these animals. Contamination of seafood products is therefore, a result of faecal contamination via polluted rearing water, from infected food handlers or from cross-contamination during production or transport.

Salmonella may grow in the temperature range between 5 and 46°C but as for other bacteria in the Enterobacteriaceae family the optimal growth temperature is 37°C. Under suboptimal conditions, e.g. at temperatures below 30°C, *Salmonella* tend to form biofilms on both inert and organic surfaces. In this state, bacteria are better protected against environmental stresses (Vestby *et al*., 2009; Donlan and Costerton, 2002). The bacterium is reported to grow in a pH range from 3.8-9.5 and at water activities (a_w) above 0.94 (Bell and Kyriakides, 2002).

Even though, bacteria in the genus *Salmonella* are typical intestinal organisms being able to colonize the gastrointestinal tract of animals and humans, the ability to survive and even multiply in environments other than the intestine, e.g., food and feed is well documented (D'Aoust *et al*., 2001). It is known that the ability of *Salmonella* strains to survive air drying varies considerably (Humphrey *et al*., 1995) but in frozen or dried products, *Salmonella* are reported to survive for months or even years (D'Aoust *et al*., 1993, 1994, 1995, 2001). The survival of *Salmonella* sp. in stored food and feeds are shown to be enhanced by a lowered a_w.

Some *Salmonella* serovars may occasionally be found in animal feed and feed ingredients, as well as in the feed production environment.

In a study on the prevalence of *Salmonella* in compound feed, feed materials and environmental samples from feed producers in Norway from 2000-2004, the most common serovars were found to be S. Senftenberg, S. Agona, S. Montevideo and S. Kentucky (Lunestad *et al*., 2007).

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The major components in conventional fish feeds are fish meal and fish oil, constituting between 60 and 80% of the diet. A typical salmon diet contains between 30 and 40% proteins and between 30 and 40% oil.

In the present study, we have examined the persistence of *Salmonella enterica* serovar Agona in an experimentally contaminated marine oil intended for fish feed production.

MATERIALS AND METHODS

Marine oil: The oil applied in this study originated from Spratt (*Sprattus sprattus*) and small sandeel (*Ammodytes tobianus*), mixed in a proportion of 1:9. The supplier was TripleNine, Fish Protein, Esbjerg, Denmark. The oil had a ratio of saturated, mono-unsaturated and poly-unsaturated fatty acids of 25, 41 and 29%, respectively and a water content of <0.1%. Portions of 10 mL oil were transferred to sterile reagent tubes, providing 10 parallel tubes at each sampling point included in the experiment. Prior to the experimental inoculation, the oil tested negative with respect to *Salmonella* by both the plating method and the Enzyme linked fluorescence assay described in the study.

Bacterial strain: In the present study, we have examined the persistence of *Salmonella enterica* serovar Agona. This strain was originally isolated from feed ingredients and was supplied by The Norwegian Institute of Public Health, Oslo, Norway and had the strain number 1106-4351-1.

Inoculation and incubation: The *Salmonella* strain was grown on Trypcase soya agar with 5% sheep blood (BioMerieux) at 37°C for 24 h. One well isolated colony was then transferred to 100 mL Buffered peptone water (BPV-3P, BioMerieux) that were incubated aerobically at 37°C until the cell number reached 1×10^8 mL^{-1}. The cell number in this culture was quantified by a counting chamber (Thoma, Assistent).

To each tube containing 10 mL oil, 0.2 mL of the culture was added, giving an initial average concentration of 2×10^6 cells mL^{-1} ($\log_{10}$ = 6.3±0.4). The oil was then kept aerobically in the dark at 20°C. This temperature was chosen as it is representative for common storage conditions for fish oil and fish feed, as well as the temperature in production facilities for such products.

Salmonella plate counts: At days 0, 6, 18, 32, 54, 80, 94, 116 and 122 one sample representing half of the oil (5 mL) from each of ten parallel tubes at each sampling time was examined quantitatively by agar plating. The oil sample was aseptically withdrawn and diluted in buffered peptone water (BPV-3P, BioMerieux) supplemented with 1.5% polysorbat (Tween 80, Merck) as an emulsifying agent.

From appropriate dilutions, 0.1 and 1.0 mL was plated onto *Salmonella* selective agar (Xylose lysine desoxycholat agar, XLD OXOID, CM 469) and incubated aerobically at 37°C for 24 h. Since, the oil was sterile prior to the inoculation with a pure culture of *Salmonella* Agona, all red colonies with black centres on XLD agar were counted as *Salmonella* in accordance with the instructions of the supplier.

Enzyme linked fluorescent assay: In addition to enumeration by cultivation on XLD plates, samples were also examined qualitatively by an automated Enzyme Linked Fluorescent Assay (ELFA) after resuscitation of sub-lethally injured cells. In the ELFA assay, the remaining aliquot of 5 mL from each of the ten parallel oil tubes at each sampling point, were transferred to 45 mL Buffered peptone water added 1.5% polysorbat and incubated at 37°C for 20 h for resuscitation. From the Buffered peptone water, 0.1 mL were then transferred to 10 mL Rappaport-Vassiliadis broth (RVS-T BioMerieux) and incubated at 42°C for 20 h, before transferring 1-10 mL M-broth (BioMerieux). After heating of the M-broth to 95°C for 15 min, samples were analysed by FLFA applying the MiniVidas system in accordance with instructions from the supplier (BioMerieux).

RESULTS

The results from the quantitative part of the trial are presented in Fig. 1 (The bacterium was added to sterile oil in an initial average concentration of 2×10^6 cells mL^{-1} and kept aerobically at 20°C. At days 0, 6, 18, 32, 54, 80, 94, 116 and 122 one sample from each of ten parallel tubes was examined by plating on *Salmonella* selective agar (Xylose lysine desoxycholat agar)). The Fig. 1 shows average counts of ten parallels at each sampling point and the variability among parallels given as the range of results, i.e., highest and lowest individual count. The variability among the ten parallels at each sampling point is given as the range of results, i.e., highest and lowest individual count. At the day of inoculation, the average number of *Salmonella* in the oil was found to be 2.0×10^6 Colony Forming Units (CFU mL^{-1}) ($\log_{10}$ = 6.3±0.4, n = 10). Six days after the inoculation a log increase of viable *Salmonella* cells could be observed, as the number reached 1.9×10^7CFU mL^{-1} ($\log_{10}$ = 7.3). From day 18-54 the cell counts were rather stable at numbers between 1.8 and 3.1×10^6 CFU mL^{-1} ($\log_{10}$ 6.2-6.5). From day 54 until the termination of the experiment the number of cells deceased from 3.1×10^6 CFU mL^{-1} ($\log_{10}$ 6.5) to non

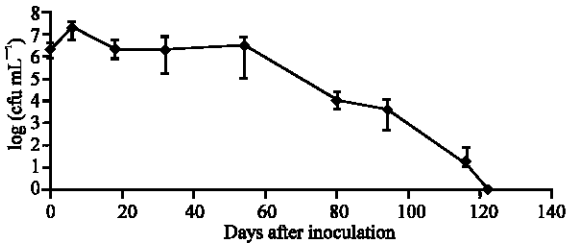

Fig. 1: Enumeration of *Salmonella enterica* serovar Agona experimentally inoculated into a commercially available mixed marine oil from Spratt (*Sprattus sprattus*) and Small sandeel (*Ammodytes tobianus*)

detectable numbers (<1.0×10^1) at day 122. The results from the qualitative enzyme linked fluorescent assay (Mini Vidas), showed that all ten parallel oil samples at each of the samplings from day 0-116 had viable *Salmonella* cells. At the sampling at day 122 however, no cells could be detected in any of the oils examined, supporting the negative findings by the plate assay.

DISCUSSION

Feed intended for aquaculture species may in principal be contaminated by any type of *Salmonella*. However, some serotypes dominate in these types of products. Common serotypes in vegetable or animal components for production of fish feed, or in ready to use feed are S. Agona, S. Senftenberg, S. Montevideo, S. Livingstone, S. Bloemfontain, S. Johannesburg, S. Lexington, S. Anatum, S. Cerro, S. Worthington, S. Lille and S. Oranienburg (Lunestad *et al*., 2007). The present study includes one strain of *Salmonella enteric* serovar Agona.

This serovar was imported to USA and several European countries during 1969 and 1970 by Peruvian fish meal used in animal feed production (Clark *et al*., 1973). Some clones of this serovar have shown able to persist in fish meal and fish feed factories for several years (Nesse *et al*., 2003, 2005a, b). Thus, *Salmonella enteric* serovar Agona is suitable for indicating the persistence of *Salmonella* in marine oils intended for fish feed production.

In the present study, the persistence of *Salmonella enteric* serovar Agona in a marine oil was assessed by a combination of a quantitative plate count method and a qualitative ELFA method after resuscitation in Buffered peptone water. In all cases there were good correlation between the agar based quantitative method and the qualitative ELFA method. Thus, no ELFA negative samples showed viable cells on agar or vice versa.

In the study the average number of *Salmonella* in the oil at the day of inoculation was found to be 2.0×10^6 CFU mL^{-1}. Six days after inoculation, a log increase in viable *Salmonella* cells could be observed. This temporary increase in cell numbers could result from a continuation of the log phase growth in Buffered peptone water used as inoculum. An overall decline in the number of cells could be observed for the rest of the experimental period showing negative results at day 122, as shown by both the quantitative and qualitative method.

An intermittent period with rather stable cell numbers were however seen from day 18-54, with counts between 1.8 and 3.1×10^6 CFU mL^{-1} ($\log_{10}$ 6.2-6.5). In this period, the initial log growth from the inoculum culture seems to be arrested, before a substantial die-off occurred after day 54.

It has been well documented that *Salmonella* can persist for extended periods under conditions with low water activities as those found in oils (Grau, 1994; Juven *et al*., 1984; Bell and Kyriakides, 2002). However, the available literature on persistence of relevant serovars in common marine oils for fish feed production is scarce, whereas information on the survival in other types of oil is available. The fate of different *Salmonella* serovars in vegetable oil has recently been described by Komitopoulou and Penaloza (2009). The researchers report that the *Salmonella* serovars Typhimurium, Napoli, Senftenberg, Oranienburg and Enteritidis all survived storage at 21°C for >21 days, when the experiment was terminated. On the other hand, *S.* Poona and Montevideo survived <21 days. The researchers also report cells to survive better at 5°C than at 21°C storage and that cell for inoculation of the oil prepared from a lawn culture survived better than cells from broth cultures.

Salmonella have not generally been regarded as fish pathogens, with the possible exception of *Salmonella arizonae* (Austin and Mclnotsh, 1991). Experiments have shown that even after administration of very high doses of *Salmonella*, Atlantic salmon (*Salmo salar* L.) did not exhibit any signs of disease (Nesse *et al*., 2005a, b). Furthermore, the researchers conclude that under natural rearing conditions for farmed Atlantic salmon in Norway and with low concentrations of *Salmonella* in the feed, the risk of transmission to humans via fish products is minimal. This assumption is supported by the finding that the most common serovars in Norwegian fish feed

ingredients, fish feed and fish feed factories count for only 2% of clinical *Salmonella* isolates from domestically acquired salmonellosis cases in Norway (Lunestad *et al*., 2007).

Even though a transmission of *Salmonella* from fish feed via the fish to humans is unlikely, the possibility of transfer from contaminated feed ingredients as oil or compound feed to handling personnel cannot be excluded.

The results from the current research indicate the extended persistence in marine oils of *Salmonella* serovars of fish feed relevance. More research should be done including other serovars isolated from the fish feed sector and the effect of pre inoculation conditions on the cell survival should also be considered.

CONCLUSION

Salmonella enterica serovar Agona is among the most frequently isolated *Salmonella* serovars from the fish feed sector. The present experiment demonstrates that a strain of this serovar originally isolated from fish feed ingredients can survive in a combined oil from Spratt (*Sprattus sprattus*) and Small sandeel (*Ammodytes tobianus*) for at least four months. These findings should be kept in mind, when assessing *Salmonella* contaminated oil and fish feed and its implications for the safety of personnel handling these products. The findings also demonstrate the potential for persistence of the bacterium in debris containing oil in feed production environments.

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