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Persistence of Orally Administered *Salmonella enterica* Serovars Agona and Montevideo in Atlantic Salmon (*Salmo salar* L.)

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ABSTRACT

The objective of our experiments was to study the persistence and dissemination of orally administered *Salmonella* in smoltified Atlantic salmon. In experiment 1, salmon kept at 15°C were fed for 1 week with feed contaminated with 96 most-probable-number units of *Salmonella* Agona per 100 g of feed and then starved for 2 weeks. Samples were taken from the gastrointestinal tract and examined for *Salmonella* 1, 2, 8, 9, 15, and 16 days after the feeding ended. In experiment 2, *Salmonella* Agona and Montevideo were separately mixed with feed and administered by gastric intubation. Each fish received 1.0×10^8, 1.0×10^6, or 1.0×10^4 CFU. The different groups were kept in parallel at 5 and 15°C and observed for 4 weeks. Every week, three fish in each group were sacrificed, and samples were taken from the skin, the pooled internal organs, the muscle, and the gastrointestinal tract and examined for the presence of *Salmonella.* The results from the two experiments showed that the persistence of *Salmonella* in the fish was highly dependent on the dose administered. *Salmonella* was not recovered from any of the fish that were fed for 1 week with the lowest concentration of *Salmonella.* In the fish given the highest dose of *Salmonella*, bacteria persisted for at least 4 weeks in the gastrointestinal tract as well as, to some extent, the internal organs. The present study shows that under practical conditions in Norway, the risk of *Salmonella* in fish feed being passed on to the consumer of the fish is negligible.

Salmonella is a facultative intracellular bacteria that infects a wide variety of animals, including humans *(11).* It has not been regarded as a fish pathogen, with the possible exception of *Salmonella arizonae (2, 10).* However, fish may be exposed to *Salmonella* through their consumption of contaminated feed or through their residence in contaminated water. It is imperative for the quality of farmed fish that it does not harbor any *Salmonella* that may be harmful to consumers.

The occurrence of *Salmonella* in feed has for a long time been a well-recognized problem worldwide, and feed ingredients are believed to represent a major risk for *Salmonella* contamination in factories that produce feed *(5, 6, 8, 12, 14–16).* Several investigations show that fish meal, as well as raw material of vegetable origin, can be contaminated with *Salmonella (8, 12, 16).* From 1998 to 2000, *Salmonella* was isolated in four Norwegian fish feed factories *(12).* Strict control measures are performed to ensure that the fish feed delivered from Norwegian factories is not contaminated with *Salmonella.* However, the risk that farmed fish may receive contaminated feed cannot be completely ruled out.

Little is known about the persistence and dissemination of *Salmonella* in fish exposed to the bacterium via feed. Orally administered *Salmonella* has been reported to enter from the gastrointestinal (GI) tract into the internal organs and muscle tissue in several freshwater species, e.g., rainbow trout (*Salmo gairdneri*), Israeli mirror carp (*Cyprinus carpio*), and tilapia (*Tilapia aurea*) *(3, 4, 7, 9).* Similar experiments have not been reported in saltwater fish. Atlantic salmon become saltwater fish by going through the process of smoltification (a physiological adaptation to seawater enabling the fish to maintain homeostasis in a hyperosmotic environment, primarily by reversing sodium/potassium pumps over the gills). The objective of the present experiments was to study the persistence and dissemination of orally administered *Salmonella* in smoltified Atlantic salmon (*Salmo salar* L.), as well as the possible influence that water temperature and the dose of *Salmonella* administered have on these parameters.

MATERIALS AND METHODS

Fish. Smoltified Atlantic salmon (*S. salar* L.) were used. The fish were kept in tanks with a flow-through system (mean flow = 0.8 liter per kg of fish per min), with a maximum density of 20 kg of fish per m^3 and a mean salinity of 34‰. The seawater used was filtered mechanically and irradiated with UV radiation. Commercial fish feed was given ad libitum twice a day, except when the fish were starved. Before inoculation, the fish were starved for 3 days and then anesthetized with benzocain chloride (5% in propylene glycol) with a working dose of 1 ml of 5% solution per liter of water.

Bacteria. The bacteria used were *Salmonella* Agona (VI-j.nr.2000-01-1967-3) and *Salmonella* Montevideo (VI-j.nr.2000-01-2179) from the strain collection of the National Veterinary In-

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stitute. Both strains had been isolated from fish feed factories in Norway *(12)*. The strains were grown in buffered peptone water (BPW; CM509, Oxoid, Basingstoke, UK) at 37.0 ± 1°C for 18 to 24 h and diluted to the proper concentrations before being mixed with commercial fish feed.

Qualitative analyses of *Salmonella*. The analyses were performed according to the standards detailed in Nordic Committee on Food Analyses No. 715 ed., 1999/ISO 6579:2002. Organs or muscle tissue from sacrificed fish was incubated with BPW 1:10 (wt/vol), and skin swabs were incubated in 10 ml of BPW for 16 to 24 h at 37.0 ± 1°C. Then, 0.1 ml was transferred to 9.9 ml of Rappaport-Vassiliadis soy peptone broth (CM866, Oxoid) and incubated for 18 to 24 h at 42.0 ± 0.2°C. The cultures were streaked out for isolation on brilliant-green phenol-red lactose sucrose agar plates (1.10747, Merck, Darmstadt, Germany) and incubated for 16 to 24 h at 37 ± 1°C. From these plates, suspected colonies were subcultured on bromthymol blue lactose sucrose agar plates and confirmed biochemically (API 20E, bioMérieux, Marcy l'Etoile, France) and serologically by standard procedures. To test the sensitivity of these *Salmonella* analyses, eight fish with a mean weight of 250 g were each kept at a temperature of 15.0°C (15.0 to 15.2°C) and force-fed once by gastric inoculation with 140 CFU of *Salmonella* Agona in BPW mixed 2:1 (vol/wt) with commercial fish feed. On the following day, all of the fish were sacrificed, and individual samples of the GI tract (except for the gall bladder) and its contents were aseptically removed and subjected to qualitative analyses as described above. *Salmonella* was recovered from seven of the eight fish.

Quantitative analyses of *Salmonella*. The most-probable-number (MPN) dilution technique with 95% confidence limits was used *(1)*. When analyzing the content of the GI tract, the content was aseptically removed and mixed with BPW 1:10 (wt/vol). This mixture was equally divided into three test tubes, and a three-tube three-decimal dilution series was made using BPW. When analyzing the *Salmonella* concentration in feed, triplicate serial dilutions were made by mixing 25, 2.5, and 0.25 g with BPW (1:10, wt/vol). The sample/BPW tubes were then treated the same way as in the qualitative analyses. Each test tube in the serial dilutions was finally scored positive or negative for *Salmonella*, and the MPN units of *Salmonella* in the original sample was calculated using an MPN table.

Experiment 1. In all, 85 fish with a mean weight of 1,520 g were kept at a temperature of 15.0°C (15.0 to 15.2°C). The fish were fed for 1 week with a commercial fish feed that was mixed with *Salmonella* Agona to a concentration of 96 MPN units per 100 g of feed (95% confidence limits were 16 to 396 MPN units per 100 g of feed), after which time they were starved for 2 weeks. Fish were sacrificed at 1 ($n = 10$), 2 ($n = 7$), 8 ($n = 20$), 9 ($n = 20$), 15 ($n = 20$), and 16 ($n = 8$) days of starvation. Individual samples of the GI tract (except for the gall bladder) and its contents were aseptically removed and subjected to qualitative analyses (all samples) and quantitative analyses (samples from days 1 and 2) as described above.

Experiment 2. In all, 210 fish with a mean weight of 64 g were included. (To include a larger number of individuals, the fish in this experiment had to be smaller than those in experiment 1.) The fish were kept at a temperature of either 5°C (5.2 to 6.1°C) or 15°C (14.8 to 15.2°C). They were force-fed once by gastric inoculation with 0.4 ml of either *Salmonella* Agona or *Salmonella* Montevideo in BPW mixed 2:1 (vol/wt) with commercial fish feed. The doses inoculated were 1.0×10^8, 1.0×10^6, or 1.0×10^4 CFU per fish. Control fish were given the same mixture of feed and BPW without bacteria. The fish were kept in separate groups according to water temperature, *Salmonella* strain, and dose. Samples were taken at 1 day and at 1, 2, 3, and 4 weeks after inoculation. From each group, three fish were sacrificed, and swab samples were taken from the skin before muscle samples, GI tracts with their contents (except for the gall bladder), and internal organs (liver, spleen, and head kidney) were aseptically removed. Corresponding samples from the three fish in each group were pooled and subjected to qualitative analyses for *Salmonella* as described above.

RESULTS AND DISCUSSION

In experiment 1, *Salmonella* was not recovered from either the GI tract or its contents from any of the fish for starvation periods lasting from 1 to 16 days after feeding with *Salmonella*-contaminated feed ended. During the feeding period, the fish were expected to consume approximately 150 MPN units of *Salmonella*. This is an amount that should have been detected in most fish by the analyses employed, had it been present. The experiment was designed to mimic natural conditions for the Atlantic salmon in Norway during the last weeks before slaughter (relatively large fish, relatively high water temperatures, and between 1 and 2 weeks of starvation before slaughter). The fish were fed with *Salmonella*-contaminated feed with a slightly higher concentration than has been detected in naturally infected feed. In Norway, all batches of fish feed are tested for the presence of *Salmonella* before their release to the market. From 1999 to 2001, 115 of approximately 1,600 batches of feed tested positive for *Salmonella*. In 12 of these batches, quantitative analyses were performed. Eight batches contained less than 2.5 MPN units of *Salmonella* per 100 g of feed, and none of the batches had more than 4.5 MPN units of *Salmonella* per 100 g of feed *(13)*. *Salmonella*-positive batches are of course not released to the market, but the possibility that undiscovered positive batches may be released cannot be excluded. However, it is not likely that such batches have higher *Salmonella* concentrations than the discovered batches. The fish in the present experiment were fed for 7 days with a *Salmonella* concentration at least 20 to 40 times higher than what may be expected in naturally contaminated feed. These fish were *Salmonella* negative 1 day after the feeding ended.

In experiment 2, both the persistence and the dissemination of the bacteria in the fish were highly dependent on the dose administered (Table 1). The lowest dose administered was 10^4 CFU of *Salmonella* per fish. This corresponds to a feed concentration of $5 \times 10^5 - 2 \times 10^6$ CFU of *Salmonella* per 100 g of feed, since fish of this size (mean = 64 g) are expected to eat approximately 0.5 g of feed per day at 5°C and approximately 1.6 g of feed per day at 15°C. Consequently, the feed concentration was more than 10^5 times higher than what has been found in naturally contaminated feed. Even with this high concentration, most of the fish tested were *Salmonella* free after 1 week, and all were free after 2 weeks.

When the salmon received higher doses (10^6 and 10^8 CFU per fish), *Salmonella* persisted longer in the fish, primarily in the GI tract, but also to some extent in the internal

TABLE 1. *Results of qualitative analyses of* Salmonella *in pooled samples from Atlantic salmon given one gastric inoculation of different doses of* Salmonella *Agona or* Salmonella *Montevideo and kept at different temperatures*

Temp	Dose (CFU/fish)	Sample	Agona					Montevideo				
			1 day	1 week	2 weeks	3 weeks	4 weeks	1 day	1 week	2 weeks	3 weeks	4 weeks
5°C	10^4	Muscle	−	−	−	−	−	−	−	−	−	−
		Organs	−	−	−	−	−	−	−	−	−	−
		Skin	−	−	−	−	−	−	−	−	−	−
		GI tract	+	−	−	−	−	+	−	−	−	−
	10^6	Muscle	−	−	−	−	−	−	−	−	−	−
		Organs	+	−	−	−	−	+	−	−	−	−
		Skin	+	+	−	+	−	+	+	−	−	−
		GI tract	+	+	−	+	−	+	+	+	+	−
	10^8	Muscle	+	−	−	−	−	−	−	−	−	−
		Organs	+	+	+	−	−	+	−	−	−	−
		Skin	+	+	−	+	−	+	+	−	−	−
		GI tract	+	+	+	+	+	+	+	+	+	+
15°C	10^4	Muscle	−	−	−	−	−	−	−	−	−	−
		Organs	+	−	−	−	−	−	−	−	−	−
		Skin	−	−	−	−	−	−	−	−	−	−
		GI tract	+	−	−	−	−	+	+	−	−	−
	10^6	Muscle	−	−	−	−	−	−	−	−	−	−
		Organs	−	−	−	−	−	+	−	−	−	−
		Skin	+	−	−	−	−	−	−	−	−	−
		GI tract	+	+	+	+	−	+	+	+	+	+
	10^8	Muscle	−	−	−	−	−	−	−	−	−	−
		Organs	+	−	+	−	−	+	−	+	+	+
		Skin	+	−	−	−	−	+	−	−	−	−
		GI tract	+	+	+	+	+	+	+	+	+	+

organs. There are very few comparable earlier studies, and these are performed only in freshwater fish with high doses of *Salmonella.* However, these studies suggest that the persistence and dissemination of high doses of *Salmonella* are analogous in saltwater and freshwater fish. Heuschmann-Brunner *(9)* reported that *Cyprinidae* was positive for up to 60 days when fed once with *Salmonella* Enteritidis–contaminated meat and for up to 50 days when fed once with *Salmonella* Typhimurium–contaminated meat. The doses used in this experiment are not reported, but the bacteria were cultured for ''some hours in room temperature.'' Hagen *(7)* reported that rainbow trout fingerlings fed for 24 h with ''strongly contaminated fodder,'' i.e., feed moistened with an 18-h culture of *Salmonella* Typhimurium, had *Salmonella*-positive gut samples for the following 5 days. Baker and Smitherman *(3)* force-fed *T. aurea* with 3×10^9 viable cells of *Salmonella* Typhimurium per fish and recovered *Salmonella* from viscera samples at 15, but not 30, days after inoculation.

Experiment 2 in the present study was performed both at 5 and 15°C, because the sea temperature in which the Norwegian salmon are farmed varies mainly within these temperature limits. In the groups given 10^4 and 10^6 CFU of *Salmonella* Montevideo per fish, *Salmonella* persisted 1 week longer when the fish were kept at 15°C than at 5°C. There was no such difference in the *Salmonella* Agona–inoculated groups. This may indicate that the persistence after administration of high doses, at least for some serovars, is influenced by temperature, but the differences are too small to be conclusive. However, Heuschmann-Brunner *(9)* reported that *Salmonella* Enteritidis (when fed three times) persisted longer in *Cyprinidae* at 16 to 17°C (110 days) than at 9 to 12°C (68 days).

Rigorous controls ensure that if *Salmonella* is present in Norwegian fish feed at all, the concentrations in naturally infected feed are very low. Furthermore, these farmed Atlantic salmon are not fed for the last 1 to 2 weeks before slaughter to minimize the number of bacteria that would normally be found in the digestive tract, because these bacteria would contribute to the spoilage of the salmon meat and the deterioration of product quality. The results from the present study show that under these circumstances, the risk of *Salmonella* in fish feed being passed on to the consumer of the fish is negligible.

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