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Effect of a phytogenic feed additive on the susceptibility of *Onchorhynchus mykiss* to *Aeromonas salmonicida*

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ABSTRACT: In recent years, feed additives have increasingly been adopted by the aquaculture industry. These supplements not only offer an alternative to antibiotics but have also been linked to enhanced growth performance. However, the literature is still limited and provides contradictory information on their effectiveness. This is mainly due to the wide variety of available products and their complex mechanisms of action. Phytogenic feed additives have been shown to have antimicrobial effects and can improve growth performance. In the present study, we investigated the susceptibility of several fish pathogenic bacteria to a phytogenic essential oil product *in vitro*. In addition, we determined the protective effect of a commercial phytogenic feed additive containing oregano, anis and citrus oils on the resistance of rainbow trout *Oncorhynchus mykiss* to infection by *Aeromonas salmonicida*. The bacterium was administered through 3 different routes: intra-peritoneal injection, immersion in a bacterial solution and cohabitation with infected fish. Mortality rates were significantly lower in infected rainbow trout that had received the feed additive: the overall mortality rate across all routes of infection was 18% in fish fed a diet containing the additive compared to 37% in fish that received unsupplemented feed. The route of infection also significantly impacted mortality, with average mortality rates of 60, 17.5 and 5% for intraperitoneal injection, immersion and cohabitation, respectively. In general, fish were better protected against infection by immersion than infection by injection.

KEY WORDS: Rainbow trout · Disease resistance · Essential oils · Oregano · Anis · Citrus oil

INTRODUCTION

The development of antibiotic resistance is arguably one of the most pressing concerns currently encountered in the field of microbiology. The issue, illustrated by the emergence of Carbapenem-resistant enterobacteriaceae, and in particular Carbapenem-resistant *Klebsiella pneumoniae* and *Escherichia coli* (Limbago et al. 2011), was at the heart of the recent annual report from the UK's Chief Medical Officer (Davies 2011). In 2006, the EU banned the use of any antibiotics for growth promotion in animal farming, and increased pressure to reduce the use of antibiotics in food animals is expected in other regions.

Development of antibiotic resistance is also a concern for the industry itself. Indeed, while the development of effective vaccines allowed the aquaculture industry to greatly reduce its reliance on antibiotics during the 1990s (Alderman & Hastings 1998), the presence of resistant bacterial strains is still a problem in fish farming (Schmidt et al. 2000, 2001). Furunculosis is a good example of this phenomenon. Its causative agent, *Aeromonas salmonicida*, is a major aquatic pathogen that has been responsible for mass

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mortalities, particularly in salmonid populations (Hiney & Olivier 1999, Cipriano & Bullock 2001, Roberts 2001). In salmonids, this bacteria causes acute septicemia as well as external and internal hemorrhages, and can enter a carrier state which makes its detection difficult (Michel & Faivre 1991, Hiney et al. 1997). Therapy has traditionally relied exclusively on the use of antibiotics; however, their application has decreased sharply, in parallel to the prevalence of the disease following the introduction of an efficient vaccine (Smith & Hiney 2000). Nonetheless, outbreaks of furunculosis still occur, and most isolates are now resistant to one or more antibiotic treatments (Inglis et al. 1991, Schmidt et al. 2000). This situation is concerning, considering the limited arsenal of antibiotic drugs currently licensed for use in food fish (4 or 5 in most members of the EU: Oxolinic acid, Oxytetracycline, Sulphonamide, Amoxicillin and Florphenicol; Alderman & Hastings 1998).

In this context, there has been interest in developing alternatives to the use of antibiotics, and in particular, a varied array of non-specific immune enhancers and natural antimicrobial substances. The most studied among these have been probiotics: supplemental live bacteria that beneficially affect their host (Gatesoupe 1999, Gomez-Gil et al. 2000, Verschuere et al. 2000, Irianto & Austin 2002, Vine et al. 2004, Burr et al. 2005, Balcázar et al. 2006, Kesarcodi-Watson et al. 2008, Wang et al. 2008). For example, addition of the probiotic *Lactobacillus plantarum* protected larvae of blue swimming crab *Portunus pelagicus* from infections by *Vibrio harveyi* (Talpur et al. 2012). Similarly, addition of *Lactobacillus acidophilus* and the yeast *Saccharomyces cerevisiae* to the diet of *Channa striata* improved immune parameters and fish survival when infected with *Aeromonas hydrophila* (Talpur et al. 2014). Other substances such as β-glucans (Talpur et al. 2014) have also gained momentum as immunostimulants alongside phytogenic feed additives (Peterson et al. 2011, Giannenas et al. 2012), all targeting the non-specific immune response (Sakai 1999).

Phytogenic supplements are plant-derived substances with proposed beneficial effects on animal performance, and encompass a variety of often ill-defined compounds such as 'essential oils' or 'spices'. These terms describe a number of molecules with varied mechanisms of action, such as increasing palatability of the feed (Windisch et al. 2008), preserving the feed's lipids through anti-oxidative action, in particular of phenolic compounds (Zheng & Wang 2001, Wojdyło et al. 2007) as well as direct antimicrobial activity. Thymol and carvacrol, for example, have been shown to disintegrate the outer membrane of the gram-negative *E. coli* and *Salmonella typhimurium* (Helander et al. 1998), as have organic acids (Van Immerseel et al. 2006). Furthermore, essential oils have been shown to have anti-inflammatory activity (Acamovic & Brooker 2005) and to improve the immune response in poultry (Hashemipour et al. 2013).

Digestarom® P.E.P. MGE (Biomin Holding) (hereafter Digestarom®) is a commercially available phytogenic feed additive specifically formulated for aquatic application. The product is matrix-encapsulated, and contains a defined blend of essential oils with active compounds including carvacrol, thymol, anethole and limonene (Peterson et al. 2014). This product has been successfully applied to aquatic species such as shrimp (Encarnacao et al. 2014), Nile tilapia (Khaled & Megahad 2014) and channel catfish (Peterson et al. 2011, 2014), but until now has not been evaluated in salmonid fish. The present study, therefore, aimed at testing the antimicrobial efficacy of phytogenic essential oils against known aquatic pathogens in a series of *in vitro* tests. Additionally, we investigated the potential of Digestarom® to improve rainbow trout *Oncorhynchus mykiss* resistance against infections by *A. salmonicida*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Yersinia ruckeri (isolate number DSM#18506) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Vibrio harveyi* was obtained from Kasetstart University. The other isolates (*Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda* and *V. parahaemolyticus*) were isolated from clinical cases by the Viennese University of Veterinary Medicine between 2005 and 2012. *A. salmonicida* A-14390 is a clinical isolate originating from an outbreak of furunculosis in an Austrian farm in 2012. It was initially identified based on its morphology on agar plates and biochemical testing using an API 20 NE test followed by diagnostic PCR (Hiney et al. 1992).

All isolates were routinely grown on tryptic soy agar (TSA; Oxoid#CM0131) or in tryptic soy broth (TSB, Oxoid#CM0129) under aerobic conditions. The halophilic species *V. harveyi* and *V. parahaemolyticus* were routinely grown in TSB and TSA supplemented with 1% sodium chloride to achieve optimal growth conditions for the marine isolates. All pathogens were cultivated at 30°C, except for *V. harveyi*

and *A. salmonicida*, which were incubated at room temperature for optimal growth. All strains used in this study are deposited in the strain collection of Biomin Holding, Austria.

Agar diffusion assay

To test the antimicrobial activity of Digestarom®, TSA (±1% sodium chloride) was prepared according to the manufacturer's instructions, sterilized and stored in a water bath at 45°C to prevent solidification. A total of 3 independent overnight cultures of each pathogen were set up. To create pathogen-supplemented agar plates, 20 ml of tempered TSA were seeded with 1 ml pathogen overnight culture and poured into a sterile Petri dish (1 plate per overnight culture; 3 plates per strain). After solidification for 2 h, the blunt end of a sterile Pasteur pipette was used to punch 2 holes in each agar plate. The holes were filled with 50 µl of pure Digestarom® P.E.P. Plates were then incubated bottom-down in a Memmert HP226 incubator set to 30°C at 500 rpm, or in an orbital shaker at room temperature. Diameters of the clearing zones around the point of inoculation were measured after 48 h. The average diameter for each strain was determined, as well as the standard deviation.

Micro-dilution inhibition assay

The micro-dilution method (Langfield et al. 2004) was used to determine the minimum inhibitory concentration (MIC) of Digestarom® against *A. hydrophila*, *A. salmonicida*, *E. tarda*, *V. harveyi*, *V. parahaemolyticus* and *Y. ruckeri*. Pathogens were cultivated in triplicate as mentioned above, then sub-inoculated into the same medium and grown to an optical density (OD) of 0.1. OD of the overnight cultures was measured in 2 ml cuvettes (Sarstedt #67.740) with an INULA U-2001 spectrophotometer set to 690 nm, with sterile growth medium used as a background control. Then 100 µl of overnight culture was mixed with 100 µl TSB (growth control) or TSB supplemented with Digestarom®, chloramphenicol (Sigma #23275), or oxytetracycline (Sigma #O5875) in the wells of a micro titer plate (96-well plate with lid, NUNC #, F-bottom, clear, sterile). To test its 50% MIC (MIC_{50}), the following Digestarom® concentrations of 960, 480, 240 and 120 mg l^{-1}were tested in triplicate for each strain according to the method described by Langfield et al. (2004). In addition, the MIC_{50} of chloramphenicol and oxytetracycline were evaluated in triplicate for each strain using 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32 mg l^{-1}. The 96-well micro titer plates with lid were incubated with aeration either at 30°C or at room temperature for the psychrophilic pathogens *V. harveyi* and *A. salmonicida*. Unsupplemented TSB was used for the negative control; 2 plates were prepared. The micro titer plates were measured in a TECAN Sunrise plate reader (10 s horizontal shaking before the measurement, without lid) at $OD_{690\,nm}$ to determine the optical density as a readout for growth in each well. Each pathogen and compound combination was measured in triplicate at the time of inoculation and 24 h post-inoculation to compare bacterial growth in the presence or absence of the compound. Average and SD were calculated from 2 triplicates for each pathogen and compound combination. Inhibition was calculated in percent related to the growth control which was set to 0%; MIC_{50} was determined as the lowest concentration at which growth was reduced by 50% or more.

In vivo testing

Treatment groups and fish allocation

A total of 180 specific pathogenic free (SPF) *Oncorhynchus mykiss* of approximately 8.0 ± 0.4 g were randomly assigned to 2 groups. The first group was fed a control commercial feed (CF) without any phytogenic supplement (Table 1). The second group received the same feed to which Digestarom® had been added during the extrusion process at a rate of 0.2 g kg^{-1} of feed. In both feeding groups, an amount of feed equivalent to 1.5% of the total biomass of fish was given every day, divided between 2 meals to restrict any differences attributed to feed intake variation. Three 50 l tanks were allocated to each feed group with 30 fish $tank^{-1}$.

The aquaria were aerated and alimented by flow-through at a rate of 1.5% h^{-1}. The fish were held for 175 d. During this period, the supply of warm water

Table 1. Commercial feed composition

Component	Value
Crude protein (%)	43.0
Crude lipid (%)	16.0
Crude fiber (%)	2.5
Phosphorus (%)	1.3
Total energy (MJ kg^{-1})	18.2

in the tank was progressively adjusted to increase the temperature to 17 ± 2°C. Fish were fed 2 meals totaling 1.5% of the fish biomass per day, and weighed once a month for 175 d before being subjected to the challenge with *A. salmonicida*.

Challenge with *Aeromonas salmonicida*

Prior to the start of the challenge experiment, *A. salmonicida* A-14390 was inoculated on a blood agar plate and incubated at 15°C for 2 d. A single colony from this plate was then used to inoculate a brain heart infusion (BHI) broth and grown at 15°C in a static incubator. On the morning of the challenge, the bacterial concentration was measured by spectrophotometry (OD_{600nm}) and adjusted to the 2 infection doses.

Three challenge methods were used (Table 2), as it was felt that the route of infection might affect the efficiency of the supplement. (1) Intra-peritoneal (IP) injection: washed *A. salmonicida* cells were resuspended in 1 ml of 0.9 M phosphate buffered saline (PBS) to a final concentration of 7×10^3 CFU ml^{-1}; 100 µl of this solution was injected intraperitoneally into 10 fish from each aquarium. (2) Immersion: a bacterial culture of *A. salmonicida* in BHI was added to a 50 l aquarium, to a final concentration of 10^5 CFU ml^{-1}; 10 fish from each aquarium were transferred into this aquarium for 2 h before being returned to their original aquarium. (3) Cohabitation: injection- and immersion-infected fish were returned to their original aquaria so that the remaining 10 fish in each tank would be indirectly infected via horizontal transmission. Fish subjected to each method of infection were identified by fin clipping. In addition, one aquarium from both feeding groups was set apart to be mock infected: 10 fish were injected with 100 µl sterile 0.9 M PBS, 10 fish were immersed in a solution containing sterile, un-inoculated BHI broth and 10 fish were left undisturbed to mimic the cohabitation challenge.

Table 2. Experimental setup showing number of rainbow trout *Oncorhynchus mykiss* in each challenge group (IP injection, immersion, cohabitation and mock infection) receiving either unsupplemented feed or feed supplemented with Digestarom® P.E.P.

Infection group	No. of aquaria & fish
Commercial feed	
Injection	2 aquaria, 10 fish each
Immersion	2 aquaria, 10 fish each
Cohabitation	2 aquaria, 10 fish each
Injection: mock infected	1 aquarium, 10 fish
Immersion: mock infected	1 aquarium, 10 fish
Cohabitation: mock infected	1 aquarium, 10 fish
Digestarom® supplemented feed	
Injection	2 aquaria, 10 fish each
Immersion	2 aquaria, 10 fish each
Cohabitation	2 aquaria, 10 fish each
Injection: mock infected	1 aquarium, 10 fish
Immersion: mock infected	1 aquarium, 10 fish
Cohabitation: mock infected	1 aquarium, 10 fish

After the challenge, fish were monitored at least twice per day. Dead and moribund fish were immediately removed, weighed and their lengths measured. A sample was taken from their kidney and inoculated on blood agar plates, which were placed in a static incubator at 15°C for 2 d. Isolated bacterial colonies were identified as *A. salmonicida* based on colony morphology and production of a characteristic brown pigment (Hänninen & Hirvelä-Koski 1997). Furthermore, isolates were subjected to PCR using primers specific for the 16S RNA sequence of *A. salmonicida* (Høie et al. 1997).

Fish were monitored until the infection had run its course (determined when no mortality occurred for 10 d), at which point the experiment was terminated (Day 35 post-infection). All remaining fish were examined for external signs of furunculosis, in particular skin lesions as well as external petechiae and hemorrhages (Hiney & Olivier 1999, Cipriano & Bullock 2001, Roberts 2001). From each aquarium, 2 fish from each of the 3 modes of infection were sampled for microbiological isolation on blood agar plates, and the plates were examined for presence of *A. salmonicida* colonies.

Survival curves were constructed for fish that received the supplemented feed and those that received unsupplemented feed. Kaplan-Meier and odds ratio analyses were performed to determine statistical differences between treatments. In addition, a regression analysis was performed to compare the growth curves between the 2 treatments, and feed conversion ratios (FCR) were calculated for both feed groups. All statistical analyses were performed using SPSS v.20 (IBM).

RESULTS

In vitro testing

Digestarom® repressed the growth of all tested pathogens in the agar diffusion assay (Fig. 1). The level of inhibition varied between bacterial species; the most sensitive pathogens were *Aeromonas*

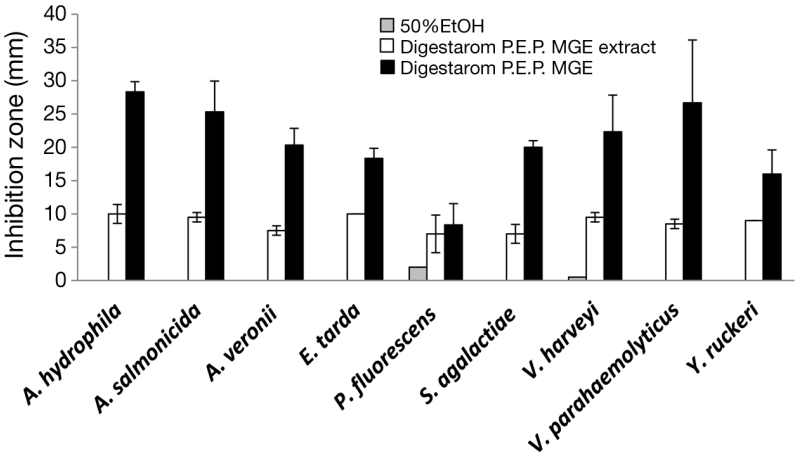

Fig. 1. Agar diffusion assay. Tryptic soy agar seeded with overnight cultures of the respective pathogen was inoculated in the center with Digestarom® P.E.P. and observed for inhibition of bacterial growth and zones of clearing at the point of inoculation. Analysis was performed in triplicate

Table 3. Inhibition zone around wells containing Digestarom® P.E.P in the agar diffusion assay. Each pathogen was assayed in triplicate

Bacterial species	Inhibition zone (mm)
Aeromonas hydrophila	28.35 ± 1.53
A. salmonicida	25.33 ± 4.62
Edwardsiella tarda	18.3 ± 1.53
Vibrio harveyi	22.33 ± 5.51
V. parahaemolyticus	26.67 ± 9.45
Yersinia ruckeri	16.00 ± 3.61

hydrophila (inhibition zone of 28.35 ± 1.53 mm), *A. salmonicida*, and *Vibrio parahaemolyticus*, followed by *V. harveyi, Edwardsiella tarda* and *Yersinia ruckeri* (inhibition zone of 16.00 ± 3.61 mm) (Table 3).

Growth inhibition was also determined by the micro-dilution method: pathogens were cultivated in microtiter plates containing serially diluted Digestarom® (Fig. 2, Table 4), chloramphenicol, or oxytetracycline (Table 5). At a concentration of 960 mg l^{-1}, Digestarom® was detrimental to all pathogens with the exception of *V. parahaemolyticus*, for which this concentration resulted in a 56 ± 2% growth reduction, a value close to the MIC_{50}. At 480 mg l^{-1}, Digestarom® was detrimental for *A. hydrophila, A. salmonicida* and *Y. ruckeri*, whereas *V. parahaemolyticus* and *V. harveyi* were only minimally affected (25 ± 1 and 20 ± 2%, respectively). For *E. tarda*, 480 mg l^{-1} was close to the MIC_{50} value (54 ± 3%). At 240 mg l^{-1}, Digestarom® repressed proliferation of *A. salmonicida* by 60 ± 5% compared to the control. At this concentration, a weak inhibitory effect (10 ± 8%) was observed for *Y. ruckeri*, but not for any of the other pathogens. Digestarom® was not effective at a concentration of 120 mg l^{-1} (Table 4). Results of the micro-dilution method with chloramphenicol and oxytetracycline showed that the con-

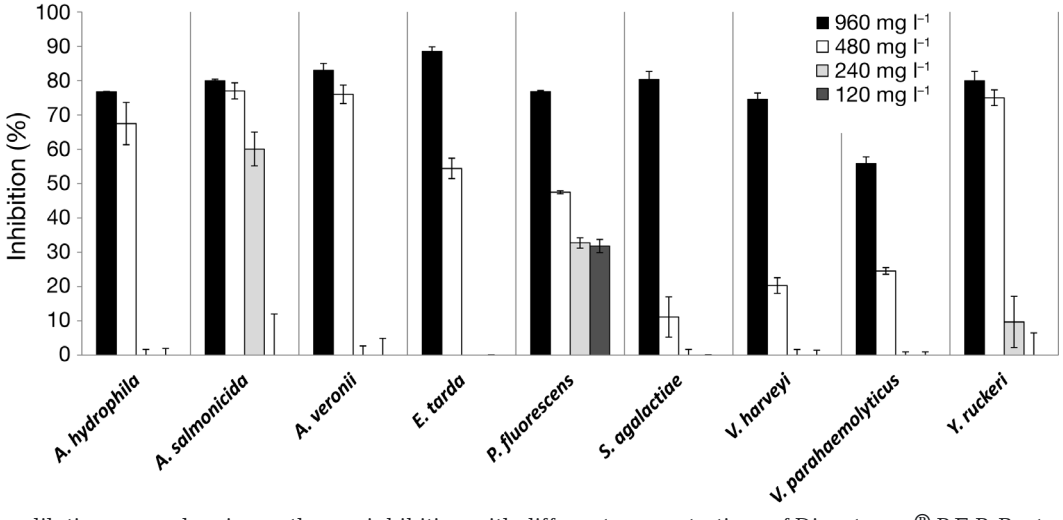

Fig. 2. Micro-dilution assay showing pathogen inhibition with different concentrations of Digestarom® P.E.P. Bacterial growth was measured in a TECAN Sunrise plate set to an optical density at 690 nm (OD_{690nm}) after 24 h incubation. Growth in the presence of different Digestarom® concentrations was compared to the control lacking Digestarom®. Inhibition is expressed in percent when the OD_{690nm} of the growth control are compared with Digestarom®. Each pathogen was tested twice in triplicate with every Digestarom® concentration (n = 6). Each pathogen culture was tested in sextuplicate

Table 4. Percent growth inhibitions for different concentrations of Digestarom® P.E.P. Values were obtained by comparing the growth of the bacteria in medium supplemented with Digestarom® to that of bacteria in unsupplemented medium. Values in **bold** indicate the minimum inhibitory concentration for that particular bacterial species, calculated at the concentration in which the growth of the bacteria was at least 50% slower than in the control. Each pathogen culture was tested in sextuplicate

Bacterial species	Growth inhibition (%) at Digestarom® P.E.P. dose (mg l^{-1})			
	960	480	240	120
Aeromonas hydrophila	76.72 ± 0.13	**67.46 ± 6.16**	0 ± 1.7	0 ± 1.97
A. salmonicida	80.00 ± 0.39	77.00 ± 2.36	**60.04 ± 4.92**	0 ± 12
Edwardsiella tarda	88.51 ± 1.32	**54.41 ± 2.97**	0 ± 0.56	0 ± 0.11
Vibrio harveyi	**74.51 ± 1.88**	20.29 ± 2.29	0 ± 1.67	0 ± 1.46
V. parahaemolyticus	**55.82 ± 1.92**	24.55 ± 0.96	0 ± 0.96	0 ± 0.96
Yersinia ruckeri	80.00 ± 2.68	**75.00 ± 2.3**	9.71 ± 7.47	0 ± 6.51

Table 5. Inhibition rate of the antibiotics chloramphenicol and oxytetracycline for each pathogen. Values were obtained by comparing the growth of the bacteria in medium supplemented with chloramphenicol and oxytetracycline to that of bacteria in unsupplemented medium. Each pathogen culture was tested in sextuplicate

Concentration (mg l^{-1})	Inhibition rate (%) for bacterial species					
	Aeromonas hydrophila	*A. salmonicida*	*Edwardsiella tarda*	*Vibrio harveyi*	*V. parahaemolyticus*	*Yersinia ruckeri*
Chloramphenicol						
32	108 ± 5	84 ± 2	86 ± 1	104 ± 7	112 ± 10	76 ± 1
16	110 ± 2	83 ± 2	86 ± 0	102 ± 3	121 ± 5	75 ± 1
8	109 ± 3	83 ± 3	85 ± 1	92 ± 3	117 ± 2	73 ± 0
4	105 ± 3	83 ± 2	80 ± 3	87 ± 3	106 ± 11	64 ± 4
2	88 ± 18	78 ± 2	67 ± 6	76 ± 5	96 ± 7	44 ± 8
1	73 ± 9	54 ± 2	47 ± 1	51 ± 5	77 ± 4	36 ± 2
0.5	45 ± 10	17 ± 11	38 ± 3	−39 ± 45	66 ± 3	23 ± 11
0.25	16 ± 26	−14 ± 3	15 ± 4	−96 ± 10	30 ± 27	5 ± 4
Oxytetracycline						
32	86 ± 1	108 ± 3	11 ± 10	89 ± 6	104 ± 5	93 ± 2
16	25 ± 5	106 ± 1	7 ± 7	90 ± 5	103 ± 3	89 ± 1
8	11 ± 0	104 ± 2	10 ± 1	82 ± 3	102 ± 2	83 ± 3
4	6 ± 3	105 ± 1	−19 ± 22	78 ± 4	101 ± 2	73 ± 6
2	8 ± 2	104 ± 2	4 ± 2	77 ± 4	100 ± 2	59 ± 5
1	−1 ± 2	103 ± 2	3 ± 1	61 ± 3	77 ± 13	33 ± 5
0.5	1 ± 6	52 ± 44	4 ± 1	28 ± 13	49 ± 31	24 ± 9
0.25	6 ± 7	53 ± 47	−2 ± 2	−66 ± 25	−19 ± 17	−8 ± 10

centrations of antibiotic required to block the growth of the pathogens were lower than that of Digestarom®, however, Digestarom® was efficient against all pathogens whereas *E. tarda* was resistant to oxytetracycline (Table 5).

In vivo challenge test

On average, the weight of individual fish increased from 8.0 ± 0.2 to 44.0 ± 1.3 g over the course of the experiment. Due to the restricted feeding, no significant differences were found in growth and FCR between treatments (2.05 ± 0.05).

The average mortality calculated among all infected tanks was 24%, with the highest mortality (37%) occurring in the unsupplemented control group and the lowest mortality (18%) occurring in tanks that received Digestarom® (Table 6). When a log-rank test of equality was applied to compare the 2 survival curves (Fig. 3), this difference was found to be statistically significant ($p = 0.023$). The protective effect of Digestarom® was also demonstrated when odds ratios were calculated. Fish fed the Digestarom®-supplemented diet displayed a reduced likelihood of death (odds ratio of 0.361, ranging from 0.157 to 0.833). The mortality rate varied significantly according to the infection route. Average mortality rates for IP injection, immersion and cohabitation were 60, 17.5 and 5%, respectively (Table 7). When the various routes of infection were considered separately, the protective effect of Digestarom® was found to be statistically different for the fish infected by immersion ($p = 0.041$) but not by injection ($p = 0.629$).

External signs of infection such as skin lesions were observed on several fish that died during the challenge. Necropsy results indicated different severities of internal lesions, ranging from enlarged spleen to extensive hemorrhages and ascites. Bacteria isolated from every dead and moribund fish were identified as *A. salmonicida* based on colony morphology. In all tested samples, these results were confirmed by PCR using primers specific for the 16S RNA sequence of *A. salmonicida*.

Table 6. Mortality of rainbow trout *Oncorhynchus mykiss* infected with *Aeromonas salmonicida* in aquaria receiving unsupplemented (control) or supplemented (Digestarom® P.E.P.) feed. Each aquarium contained 30 fish, infected via 3 different infection routes (intra-peritoneal injection, immersion, or horizontal transmission through cohabitation)

Food group	Aquarium	Mortality	
		%	Number
Control feed	Replicate 1	30.00	9
	Replicate 2	43.33	13
	Average	36.67 ± 4.17	22
Digestarom® P.E.P.	Replicate 1	16.67	5
	Replicate 2	20.00	6
	Average	18.33 ± 0.83	11
Average over all infected aquaria		24.44 ± 3.37	44

Table 7. Mortality of rainbow trout *Oncorhynchus mykiss* infected with *Aeromonas salmonicida* via 3 challenge routes (intra-peritoneal injection, immersion, or horizontal transmission through cohabitation), provided either control feed or feed supplemented with Digestarom® P.E.P.

Infection route	Food group	Mortality	
		%	Number
IP injection	Control feed	70.00	14
	Digestarom® P.E.P.	50.00	10
Immersion	Control feed	30.00	6
	Digestarom® P.E.P.	5.00	1
Cohabitation	Control feed	10.00	2
	Digestarom® P.E.P.	0.00	0

Finally, *A. salmonicida* was isolated from 5 of the 36 fish sampled at the time of termination, even though these individuals showed no signs of disease. No clear pattern was observable in the distribution of the carrier fish; 3 had received the Digestarom® feed and 2 had received the control feed. Similarly, 3 fish had been infected by immersion, 1 by injection and 1 by cohabitation. This suggests that the probability of the disease developing into a carrier state is not affected by the feed supplement or the infection route.

DISCUSSION

In vitro pathogen inhibition

Digestarom® was effective at inhibiting the growth of all pathogens at concentrations of 960 and 480 mg l^{-1}. Compared to the antibiotics chloramphenicol and oxytetracycline, higher concentrations of Digestarom® were required for bacterial growth inhibition; however Digestarom® was found to be active against all tested pathogens whereas oxytetracycline was

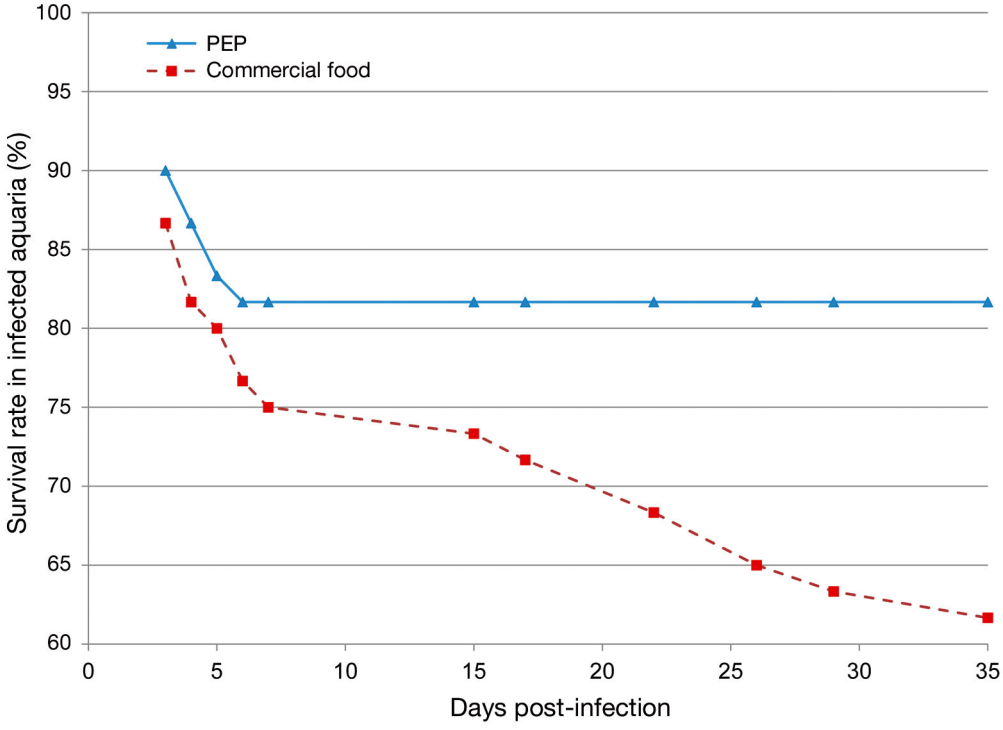

Fig. 3. Survival curves of rainbow trout *Oncorhynchus mykiss* receiving feed supplemented with Digestarom® P.E.P. versus unsupplemented feed during the challenge. Each treatment was comprised of 60 fish, split between 2 aquaria and infected via 3 different infection routes: intraperitoneal injection, immersion and cohabitation

not effective against *Edwardsiella tarda*. This highlights the resistance problem that is frequently encountered with many bacterial pathogens and the interest in providing alternatives to antibiotics (Schmidt et al. 2000, Defoirdt et al. 2007).

In vivo challenges with *Aeromonas salmonicida*

The results of the present study show that addition of Digestarom® had a positive effect on fish survival of the during *A. salmonicida* infection. Supplementation of the feed with Digestarom® was correlated with a statistically significant reduction in mortality, from 37 to 18%. This protective effect was confirmed by analysis of Kaplan-Meyer survival curves as well as calculation of the odds ratios: fish fed a diet supplement with Digestarom® displayed a lower likelihood of death (odds ratio of 0.361, ranging from 0.157 to 0.833). To the best of our knowledge, this is the first report that Digestarom® can improve resistance against bacterial infections in fish. The mechanisms through which this occurs are still unclear. As demonstrated in the present study, Digestarom® has direct antimicrobial activity, and some of its compounds can improve immune parameters in vertebrates (Acamovic & Brooker 2005, Hashemipour et al. 2013). However, elucidating the exact mechanisms of this protection requires further study.

Interestingly, when considering the various routes of infection separately, the protective effect of Digestarom® was only found to be statistically significant in the fish infected by immersion ($p = 0.041$). In the fish infected by injection, although the mortality was still lower in fish fed the supplemented feed, the difference was not statistically significant ($p = 0.629$). The precise reason for this difference is unknown. However, as demonstrated earlier in this study, Digestarom® has a direct antimicrobial effect, and because it was delivered as a feed supplement it seems likely that this effect would be most efficient within the digestive track. *A. salmonicida*, on the other hand, can infect the fish through the intestine (Jutfelt et al. 2006), gills or epithelium (Svendsen & Bøgwald 1997, Ferguson et al. 1998). Therefore, the antimicrobial effect of Digestarom® is likely to play an important protective role in fish exposed through natural routes of exposure, whereas injecting the bacterial solution intraperitoneally would bypass this protective effect.

In several cases, bacteria were re-isolated from asymptotic fish even when no mortalities had been recorded for several weeks (up to 31 d in the case of one aquarium in the Digestarom® group). It is already well established that *A. salmonicida* can establish covert infections, and that apparently healthy fish can act as asymptotic carriers (Cipriano & Bullock 2001) but there was no correlation between these isolations and supplementation with Digestarom®.

Compared to antibiotics (which are currently still the most common means to control outbreaks), Digestarom® is advantageous because of the antibiotic resistance that is common in farming environments (Alderman & Hastings 1998, Schmidt et al. 2000, Defoirdt et al. 2007), and because most isolates of *A. salmonicida* are resistant to tetracycline compounds (Inglis et al. 1991, Schmidt et al. 2001). Resistance to oxolonic acid is also widespread, as are instances of multiple resistances (Barnes et al. 1991, Inglis et al. 1991, Schmidt et al. 2001). In addition, isolates of *A. salmonicida* have been shown to harbor the plasmid paS4, which contains genes involved in resistance against multiple antimicrobial compounds, including sulphonamides (Vincent et al. 2014).

CONCLUSIONS

In recent years, a wide array of feed additives have been developed to improve performance as well as offset the reliance of the farming industry on antibiotic drugs. Here, we investigated one phytogenic feed additive, Digestarom® P.E.P. MGE, for its potential to improve the resistance of *Oncorhynchus mykiss* to infections with *Aeromonas salmonicida*, and found that addition of this phytogenic product significantly improved survival following challenge with *A. salmonicida*. Our results suggest that this additive can be a promising alternative to the antibiotics currently used, helping to avoid the increase in resistant bacterial phenotypes and the transmission of resistant genes.

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