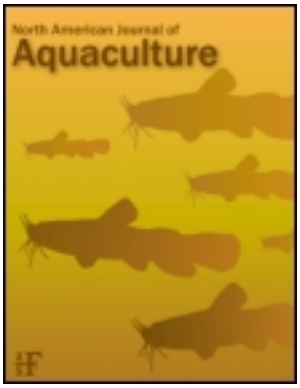

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Efficacy of Intraperitoneally and Orally Administered ProVale, a Yeast β-(1,3)/(1,6)-D-glucan Product, in Inhibiting Xenoma Formation by the Microsporidian Loma salmonae on Rainbow Trout Gills

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[Communication]

Efficacy of Intraperitoneally and Orally Administered ProVale, a Yeast β-(1,3)/(1,6)-D-glucan Product, in Inhibiting Xenoma Formation by the Microsporidian *Loma salmonae* on Rainbow Trout Gills

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Abstract.—The objectives of the research were to compare the efficacy of ProVale yeast beta-glucan (β-glucan) with that of a previously tested research-grade yeast β-glucan preparation when administered as an intraperitoneal (IP) injection and to also pilot test the effectiveness of ProVale yeast β-glucan as a feed additive for reducing *Loma salmonae* xenoma formation on the gills of rainbow trout *Oncorhynchus mykiss*. Rainbow trout received IP injections of ProVale (4, 10, or 20 mg of ProVale/kg of fish) or Sigma β-glucan (4 mg/kg). Oral challenge with *L. salmonae* occurred 1 week after IP injection with the β-glucan products. For the second objective, 400 rainbow trout were separated into tanks and duplicate groups were treated with 0, 50, 100, and 200 g of ProVale/1,000 kg of feed. Starting at 3 weeks prior to challenge and continuing 2 weeks after challenge, rainbow trout were fed the various ProVale doses daily at a feeding rate of 1% of the fish biomass. Commencing at 4 weeks postchallenge, the fish in each trial were evaluated for the presence of xenomas on the first left gill arch. The most protective IP dose of commercial ProVale was 10 mg/kg when compared with the laboratory-grade IP dose of Sigma β-glucan (4 mg/kg). Both of these intraperitoneally administered β-glucan products were effective in reducing the mean xenoma count. ProVale used as a feed coating (200 g/1,000 kg) was able to reduce the mean xenoma count by 50%.

Microsporidial gill disease of salmonids (MGDS) is caused by the obligate intracellular pathogen *Loma salmonae*. The typical disease pathogenesis is branchitis associated with the rupture of spore-rich xenomas that develop within the endothelial and pillar cells of the gills. The impact of MGDS in intensively net-pen-reared Pacific salmon *Oncorhynchus* spp. (e.g., coho salmon *O. kisutch* and Chinook salmon *O. tshawytscha*) is evident in terms of mortalities (>30% in some instances) that occur just prior to harvest, when the fish have the greatest monetary value (Kent et al. 1989; Becker et al. 2002).

The challenge in treating MGDS is the limited scope of pharmaceutical substances, none of which are approved for *L. salmonae* treatment, and the lack of a commercial *L. salmonae* vaccine. This lack of chemotherapeutants registered for use in aquaculture in North America has necessitated the use of off-label or extralabel treatments. Of the various unlicensed treatments that have been used to control *L. salmonae*, only fumagillin, albendazole, and monensin have been effective in reducing the xenoma burden (Kent and Dawe 1994; Speare et al. 1999, 2000; Becker et al. 2002). Off-label use of drugs is often contraindicated (Burka et al. 1997). For this reason, alternative management strategies to prevent or treat disease are preferred, and the need for such strategies has spurred investigation into the application of alternative therapies, including immunostimulants such as yeast beta-glucans (β-glucans).

Yeast β-glucans are the major structural components of the yeast cell wall, and they exist in arrangements of a basic β-(1,3)-linked glucose chain with small numbers of β-(1,6)-linked branches (Volman et al. 2008). Beta-glucans have been extensively used in aquaculture both as immunostimulants and as growth promoters and are generally regarded as a safe food additive by the U.S. Food and Drug Administration (Raa 1996, 2000; Schrueder et al. 1996; Galeotti 1998; Sakai 1999; Galindo-Villegas and Hosokawa 2004; Jaafar and Zueco 2004; Bricknell and Dalmo 2005; Dalmo and Bøgwald 2008).

In salmonids, the intraperitoneal (IP) and dietary administration of yeast β-glucan has been shown to augment resistance to several bacterial pathogens, including *Yersinia ruckeri*, *Vibrio anguillarum*, *V. salmonicida*, and *Aeromonas salmonicida* (Robertsen et al. 1990; Nikl et al. 1991; Jørgensen et al. 1993;

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Anderson and Siwicki 1994; Siwicki et al. 1994; Anderson et al. 1995). Recently, yeast β-glucan has been utilized to manage salmonid microsporidial infection caused by *L. salmonae*. Research-grade Sigma yeast β-glucan administered intraperitoneally at doses ranging from 50 to 1,000 μg of β-glucan/100 μL of saline (2–40 mg/kg of fish) significantly reduced the number of xenomas on the gills of rainbow trout *O. mykiss* as compared with untreated control fish. Optimal results were shown in fish receiving a 4-mg/kg dose prior to or soon after experimental exposure to *L. salmonae* (Guselle et al. 2007).

The results of these trials when utilizing the optimal β-glucan IP dose of 100 μL/100 μL of saline (4 mg of β-glucan/kg of fish) are encouraging. However, costs associated with the use of a research-grade yeast β-glucan and the IP administration of the product make this approach impractical for large-scale commercial aquaculture. With this in mind, ProVale, a livestock-grade yeast β-glucan, was acquired to determine its efficacy in reducing MGDS. An initial assessment (IP treatment) of the efficacy of the commercial-grade ProVale product relative to the research-grade Sigma product (acting as a standard) was followed by a pilot study to examine whether ProVale could be effective at reducing the number of *L. salmonae* xenomas when delivered orally at 50, 100, and 200 g of ProVale/1,000 kg of feed.

Methods

Intraperitoneal treatment.—The purpose of this trial was to determine the efficacy of intraperitoneally injected ProVale at inhibiting the formation of *L. salmonae* xenomas in naïve rainbow trout.

Juvenile rainbow trout weighing 20–30 g were purchased from a certified disease-free (i.e., free of nationally notifiable pathogens) commercial hatchery on Prince Edward Island with no history of *L. salmonae* infection. Approximately 2 weeks prior to the initiation of the trial, 200 fish were reared in two 250-L, circular fiberglass tanks with water flow rates maintained at 9 L/min. Fish were anaesthetized with benzocaine (60 mg/L) prior to fin-clipping, IP injection, and nonlethal examination under a dissecting microscope. Prior to the harvest of gill material, fish were killed with benzocaine (100 mg/L). All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 2005).

The rainbow trout in the two 250-L tanks were differentially fin-clipped and allocated to five groups of 40 fish. One group of 40 fish was given a single 1-mL IP injection of particulate β-glucan refined from *Saccharomyces cerevisiae* (G5011; Sigma-Aldrich, St. Louis, Missouri) at a dose of 4 mg of β-glucan/kg of fish, and another group of 40 fish received no treatment (untreated control fish). The remaining 120 fish were placed into three groups of 40 fish that received a single IP injection of yeast β-glucan (ProVale; Stirling Products North America, Inc., Charlottetown, Prince Edward Island) at doses of 4, 10, and 20 mg of ProVale/kg of fish. After their respective treatments, the fish were returned to the 250-L holding tanks for the *L. salmonae* challenge.

Challenge with *L. salmonae* occurred 1 week after administration of Sigma or ProVale β-glucan and consisted of gill tissue containing xenomas from rainbow trout previously exposed to *L. salmonae* (Speare et al. 1998a). Feed was withheld for 1 d prior to oral challenge with infected gill material to ensure that the fish would have the appetite to fully consume the challenge material. Fish from the various treatment groups were housed together in tanks during this trial to minimize the tank effect associated with feeding challenge material.

Beginning 4 weeks after exposure to *L. salmonae*, fish were screened and sampled weekly for 5 weeks. The first left gill arch of each fish in the ProVale-injected, Sigma-injected, saline-injected, and control groups was nonlethally examined for branchial xenomas under a dissecting microscope. At this time, 10 fish from each group were randomly selected and killed by benzocaine overdose. From each euthanized fish, the first left branchial gill arch was dissected free for whole-mount observation and the numbers of xenomas were counted using a light microscope.

Statistical comparisons were made using the mean xenoma count per gill arch (XCPGA) as the outcome variable. All comparisons were made against the untreated control group within a specific time interval. One-way analysis of variance was used to compare the treatment means, and this was followed by Bonferroni's multiple comparison test to identify where differences occurred.

The statistical analyses were performed using STATA version 9 (Stata Corporation, College Station, Texas), and a P-value of 0.05 or less was considered significant. In addition, treatment-associated XCPGA reduction for each week was expressed in proportion to the XCPGA of untreated control fish as follows:

$$\text{Percent reduction} = 1 - \frac{\text{XCPGA}_{\text{treated}}}{\text{XCPGA}_{\text{control}}} \times 100.$$

Oral treatment.—The purpose of this pilot study was to examine the efficacy of ProVale at inhibiting *L. salmonae* xenoma formation when administered per os to naïve rainbow trout subsequently challenged with *L. salmonae*. Two weeks prior to the initiation of the trial,

400 rainbow trout (20–30 g) were placed into ten 70-L tanks (40 fish/tank; 2 duplicate tanks/treatment group) with the water flow rate maintained at 2 L/min. The commercial feed used was HiPro 3-mm extruded pellets (Corey Aquafeeds, Fredericton, New Brunswick) fed daily based on a feeding rate of 1% of the fish biomass. Starting at 3 weeks prior to challenge with *L. salmonae*, the fish treatment tanks were fed the 3-mm extruded pellets, which had been gelatin coated with ProVale to obtain a dose of 0, 50, 100, or 200 g of ProVale/1,000 kg of feed. The other two tanks of fish received no ProVale and were not subsequently challenged with *L. salmonae*.

Fish were challenged orally with gill material containing *L. salmonae* xenomas as described for the IP treatment trial. Feed was withheld for 1 d prior to challenge to ensure sufficient appetite for full consumption of the challenge material. After the challenge, fish in treatment tanks were fed the ProVale-treated food for 14 d and then were fed untreated HiPro 3-mm extruded pellets at 1% fish biomass for the remainder of the trial. Screening and sampling ($n = 26$ fish/week) for xenomas commenced at weeks 4 and 7 postexposure, respectively. Data collection and morphometry were the same as for the IP treatment trial.

Comparisons were made using the XCPGA as the outcome variable. The comparison for each time interval was made against the XCPGA of the *L. salmonae*-exposed group receiving no ProVale (control fish). Proportional XCPGA reduction due to treatment for each week was calculated by the same method as described for the IP treatment trial.

Results

Intraperitoneal Treatment

The administration of ProVale by IP injection was as efficacious as the Sigma β-glucan in reducing the number of *L. salmonae* xenomas that formed on the gills of naïve rainbow trout exposed to *L. salmonae* spores.

At each weekly sampling time during the injection trial (6–9 weeks after *L. salmonae* exposure), ProVale administered at 4 mg/kg of fish ($P \leq 0.005$) and at 10 mg/kg ($P \leq 0.015$) was able to significantly reduce the mean XCPGA in comparison with the untreated control fish. Treatment with the Sigma product at 4 mg/kg of fish was also able to significantly reduce the XCPGA ($P \leq 0.003$) in comparison with untreated control fish. Interestingly, treatment with ProVale at 20 mg/kg ($P = 1.000$) did not significantly reduce the XCPGA relative to that in the positive control fish (Figure 1; Table 1).

Examination of the percentage reduction of xenomas indicated that IP administration of ProVale at dosages of 4 and 10 mg/kg of fish was able to consistently reduce xenoma formation on gills of challenged rainbow trout by over 64.5% and 72.8%, respectively (Table 2). During the trial, the percentage reduction of xenoma formation in fish receiving Sigma β-glucan at 4 mg/kg was greater than 92.5%. The high dose of ProVale (20 mg/kg) was only effective in reducing xenoma formation at week 8 (27.8% reduction) and week 9 (51.7%) postexposure (Table 2).

In terms of inhibiting xenoma formation on the gills of naïve rainbow trout exposed to *L. salmonae* spores, treatment with ProVale (4 mg/kg of fish) proved to be less effective than treatment with Sigma β-glucan (4 mg/kg). Treatment with ProVale at an IP dose of 10 mg/kg showed promise in preventing xenoma formation. Use of the high dose of ProVale (20 mg/kg) proved to be ineffective at inhibiting xenoma formation.

Oral Treatment

In the pilot study examining the oral treatment, ProVale applied in a gelatin coating on the feed was efficacious as it reduced the number of *L. salmonae* xenomas that formed on the gills of naïve rainbow trout exposed to *L. salmonae* spores (Figure 2).

At week 7, ProVale administered at 100 or 200 g/1,000 kg of feed was effective in reducing the XCPGA in comparison with positive controls (Table 3). However, during this trial, the peak xenoma burden developed at weeks 8 and 9; at these time points, morphometry showed that the ProVale dose of 200 g/1,000 kg resulted in greater reductions in xenoma burden (Figure 2; Table 3). Overall, ProVale administered at 200 g/1,000 kg was able to consistently reduce the XCPGA by approximately 50% at all time points, whereas the lower ProVale doses (50 and 100 g/1,000 kg) failed to do so (Table 4).

Data for week 9 (Figure 2; Table 4) may indicate a dose–response effect that begins to plateau between the two upper-dose treatment levels. At a dose of 50 g/1,000 kg of feed, xenoma burden was reduced by 26.7%; at a dose of 100 g/1,000 kg, xenoma burden was reduced by 49.9%; and at a dose of 200 g/1,000 kg, xenoma burden was reduced by 58.3%.

The two tanks of fish that were not fed ProVale and that did not receive an oral challenge remained free of *L. salmonae*.

Discussion

This is the first in vivo study to directly compare the efficacy of IP administration of a commercial-grade yeast β-glucan (80% pure) with that of a research-grade yeast β-glucan (98% pure) at controlling a fish microsporidian. Prior in vivo research has examined the effectiveness of an assortment of immunostimu-

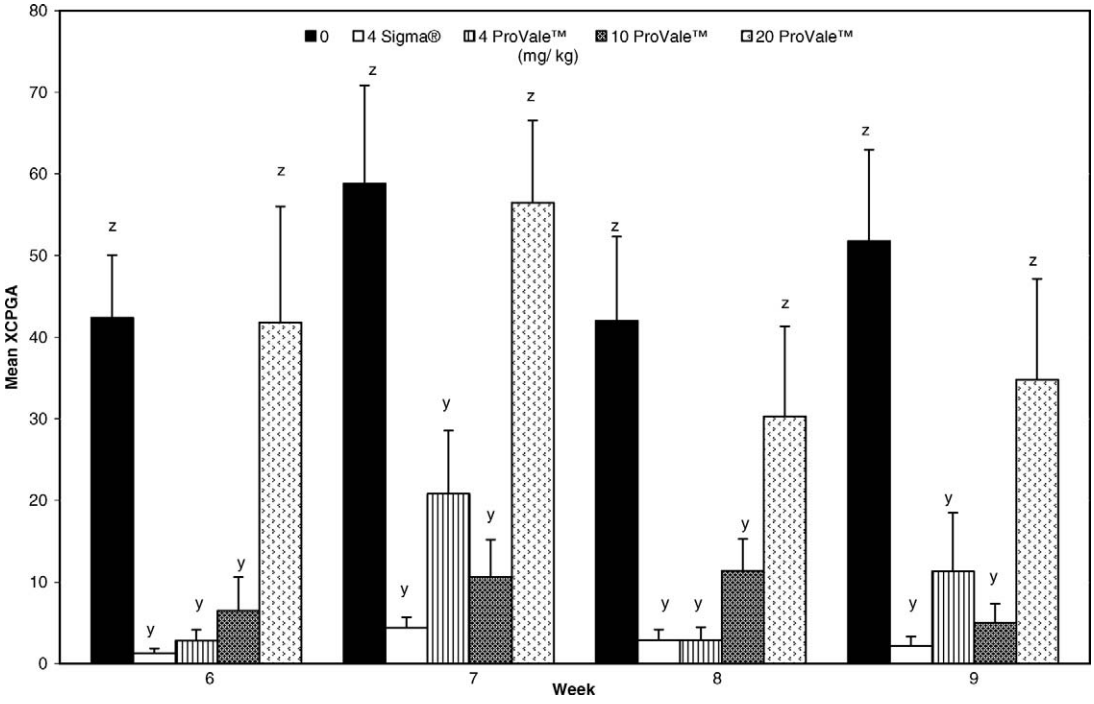

FIGURE 1.—Effect of intraperitoneal administration of beta-glucan products (Sigma at 4 mg of beta-glucan/kg of fish; ProVale at 4, 10, and 20 mg/kg) on *Loma salmonae* infection in rainbow trout. Data are expressed as mean (+SE) xenoma count per gill arch (XCPGA).

lants, including the IP administration of Chitosan (a commercial β-linked polymer of *N*-acetyl-D-glucosamine) in comparison with research-grade Sigma β-glucan (a purified β-[1,3]-glucan from barley), at reducing the mortality of brook trout *Salvelinus fontinalis* challenged with *A. salmonicida* (Anderson and Siwicki 1994; Anderson et al. 1995). These studies found that the intraperitoneally injected Sigma β-glucan was more effective than Chitosan at reducing mortalities associated with the *A. salmonicida* challenge.

In this study, IP delivery of the commercial-grade yeast β-glucan, ProVale, at 1 week prior to challenge was as effective as the research-grade Sigma yeast β-glucan in reducing the xenoma formation on gill arches of rainbow trout infected with *L. salmonae*. In fact, all ProVale IP treatments except the 20-mg/kg dose were able to significantly reduce the XCPGA relative to that in the control fish. The ProVale IP treatments at 4 and 10 mg/kg of fish reduced the peak xenoma formation by over 50%. However, only the 10-mg/kg IP dose of ProVale was considered to be acceptable as it was able to reduce over 70% of the xenomas at time points during peak xenoma formation; this result differs from a previous study that determined the optimal IP dose of Sigma β-glucan (100 μg/100 μL of saline or 4 mg/kg of

TABLE 1.—Mean (SE in parentheses) xenoma count per gill arch in rainbow trout treated with intraperitoneally administered beta glucan (β-glucan) products (mg of product/kg of fish) and challenged with *Loma salmonae*. Within a sample week, differing lowercase letters indicate significant differences between treatment groups. ($P \leq 0.05$).

	Week postexposure			
β-glucan product	6	7	8	9
None (0 mg/kg)	42.4 (7.7) z	58.8 (12.0) z	42.0 (10.3) z	51.8 (11.1) z
Sigma (4 mg/kg)	1.3 (0.5) y	4.4 (1.3) y	2.9 (1.3) y	2.2 (1.1) y
ProVale (4 mg/kg)	2.8 (1.3) y	20.8 (7.7) y	2.9 (1.5) y	11.3 (7.2) y
ProVale (10 mg/kg)	6.5 (4.1) y	10.6 (4.6) y	11.4 (3.9) y	5.0 (2.4) y
ProVale (20 mg/kg)	41.8 (14.2) z	56.5 (10.0) z	30.3 (11.1) z	34.7 (12.3) z

TABLE 2.—Percentage reduction in the number of xenomas per gill arch in rainbow trout treated with intraperitoneally administered beta-glucan (β-glucan) products (mg of product/kg of fish) and challenged with *Loma salmonae*. See Methods for calculation procedure.

	Week postexposure			
β-glucan product	6	7	8	9
Sigma (4 mg/kg)	96.9	92.5	93.1	95.7
ProVale (4 mg/kg)	93.4	64.5	93.1	78.1
ProVale (10 mg/kg)	84.7	81.9	72.8	90.3
ProVale (20 mg/kg)	0.01	0.04	27.8	51.7

fish; Guselle et al. 2006). The findings of this study are in accordance with other research showing that smaller doses of glucan provide better protection than higher glucan concentrations (Robertsen et al. 1990). It is believed that when large amounts of glucan are injected, the phagocytic cells of the fish become overloaded with glucan particles and are less able to phagocytose, leading to an increase in the susceptibility of fish to infection (Robertsen et al. 1990).

Although IP injection has proven to be an effective method for delivering a range of treatments (vaccines, antibiotics, immunostimulants) because it ensures that fish receive the desired therapeutic dose, it does trigger unnecessary handling stress, is impractical for use in smaller fish (<15 g), and is costly in terms of both time and labor (Galindo-Villegas and Hosokawa 2004). Similarly, immersion or bath treatment of finfish can also cause undue stress, but these approaches tend to be less expensive. Nevertheless, diligence is necessary when applying immersion treatments as bath dilution, exposure time, and levels of efficacy may not be well defined for certain products. Oral administration is the only treatment method that is economically suited to extensive aquaculture; it is nonstressful and allows mass delivery regardless of fish size (Galindo-Villegas and Hosokawa 2004).

To the best of our knowledge, ours is the first pilot study to successfully utilize a dietary β-glucan as treatment for a microsporidian parasite in any animal. In this test trial of three oral doses of ProVale, only the high dose (200 g/1,000 kg of feed) consistently reduced the XCPGA and met the criterion of decreasing the number of xenomas by at least 50% (Becker et al. 2002). The ability of ProVale at 200 g/1,000 kg to reduce xenoma burden consistently by 50% is also comparable with the effects of several known antiprotozoal agents, such as quinine chloride, monensin, fumagillin, and albendazole. However, none of these agents is likely to be licensed for use in

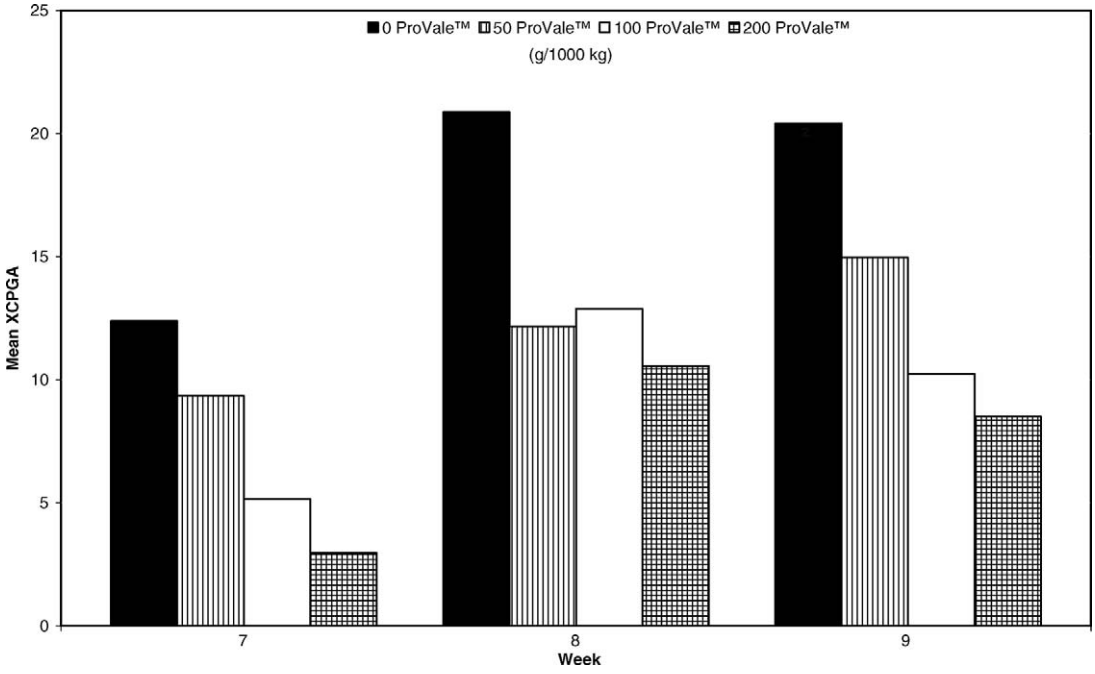

FIGURE 2.—Effect of orally administered ProVale beta-glucan (applied as a feed coating at 5, 10, 100, and 200 g of ProVale/1,000 kg of feed) on *Loma salmonae* infection in rainbow trout. Data are expressed as a mean xenoma count per gill arch (XCPGA).

TABLE 3.—Mean (SE in parentheses) xenoma count per gill arch in rainbow trout fed ProVale-coated feed (g of ProVale/1,000 kg of feed) and challenged with *Loma salmonae*.

ProVale dose (g/1,000 kg)	Week postexposure		
	7	8	9
0	12.4 (1.6)	20.9 (2.9)	20.4 (2.9)
50	9.3 (1.9)	12.2 (2.8)	14.9 (3.6)
100	5.1 (1.3)	12.9 (2.8)	10.2 (7.2)
200	2.9 (0.9)	10.6 (2.9)	8.5 (3.2)

TABLE 4.—Percentage reduction in the number of xenomas per gill arch in rainbow trout fed ProVale-coated feed (g of ProVale/1,000 kg of feed) and challenged with *Loma salmonae*. See Methods for calculation procedure. Results from intraperitoneal (IP) administration of Sigma beta-glucan (β-glucan) are shown for comparison (from Guselle et al. 2006).

β-glucan product	Week postexposure		
	7	8	9
Sigma IP (4 mg/kg)	98.0	98.0	95.0
ProVale (50 g/1,000 kg)	24.5	41.8	26.7
ProVale (100 g/1,000 kg)	58.4	38.3	49.9
ProVale (200 g/1,000 kg)	76.1	49.4	58.3

aquaculture (Kent and Dawe 1994; Speare et al. 1998b, 1999, 2000; Becker et al. 2002). Unlike the aforementioned chemotherapeutants, ProVale is a β-glucan product extracted from *Saccharomyces cerevisiae*, which has generally been regarded as safe and can legally be used for food and pharmaceutical production (Schrueder et al. 1996; Jaafar and Zueco 2004).

The enhanced protection conferred by dietary β-glucan in this preliminary study is supported by a range of studies that have explored the efficacy of dietary commercial β-glucans at enhancing nonspecific immune responses and disease resistance to bacterial pathogens for an array of teleosts (Chen and Ainsworth 1992; Sahoo and Mukherjee 2001, 2002; Cook et al. 2003; Rodriguez et al. 2003; Bagni et al. 2005; Selvaraj et al. 2005; Palic et al. 2006; Kumari and Sahoo 2006). Early research examined the efficacy of orally administered commercial β-glucans at protecting salmonids against fish pathogens. The addition of VitaStim-Taito (0.1% and 1.0% of feed) and MacroGard (0.2 g/100 g of feed) to feed resulted in significant protection against *A. salmonicida* challenge and an overall decrease in cumulative mortalities (Nikl et al. 1993; Siwicki et al. 1994). Recently, it was demonstrated that a high dietary dose of barley β-glucan (82 g/kg of feed) was able to produce the same levels of rainbow trout resistance to infectious hematopoietic necrosis virus as a yeast β-glucan product (MacroGard at 2 g/kg of feed; Sealey et al. 2008).

It is noteworthy that treatment with the β-glucan products (Sigma or ProVale via IP injection; ProVale per os) did not completely eradicate the *L. salmonae* infection. A small number of xenomas formed on the gills of treated fish. However, the clinical consequences of infection depend on the number of xenomas that form and the subsequent degree of gill pathobiology that develops at the time of xenoma rupture. A small number of xenomas are unlikely to produce "disease." Perhaps, then, the formation of some xenomas is advantageous in that the fish will develop a strong degree of protective immunity after resolution of infection. Development of a treatment protocol that markedly regulates infection without completely abrogating the infection would prove beneficial (Rodriguez-Tovar et al. 2006).

This research indicates that IP delivery of commercial-grade yeast β-glucan (ProVale) at 10 mg/kg of fish had efficacy similar to that of research-grade yeast β-glucan (Sigma) in inhibiting *L. salmonae* xenoma formation on rainbow trout gills. Based on comparison of costs of the two products, these data suggest that ProVale is a practical and effective alternative to Sigma β-glucan. Moreover, preliminary findings indicate that dietary administration of ProVale at 200 g/1,000 kg of feed was effective at reducing the number of *L. salmonae* xenomas by nearly 50%; a higher dose of ProVale (either a higher inclusion rate in the feed or a higher delivery rate to the fish) might have an even greater effect on reducing *L. salmonae* xenoma formation. Although there are insufficient data points to develop a robust dose–response prediction, it would be worthwhile to evaluate ProVale oral dose levels of 300–600 g/1,000 kg of feed to more fully determine the maximum amount by which oral treatment can reduce xenoma development ensuing from challenge.

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