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Can heat-killed *Gordonia bronchialis* enhance growth and immunity in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

In the present study, the efficacy of heat-killed *Gordonia bronchialis* on growth performance, immune system and gastrointestinal structure in rainbow trout (*Oncorhynchus mykiss*) were evaluated. Fish (mean weight 30 g) were fed basal diet (control), or treatment diets containing 2.48×10^8 (low dose) or 1.24×10^9 (high dose) cells kg^{-1} feed of heat-killed *Gordonia bronchialis* on a pulse basis (5 days on treatment diet; 10 days on basal diet) for 95 days. On days 95 and 105, some of the fish were sampled for analysis of mentioned parameters. On days 110 and 120, the remaining fish were injected intraperitoneally with a 20 ml L^{-1} suspension of chicken red blood cells. Results showed that growth performance was significantly enhanced in both treatment groups compared to the control group. Serum complement and lysozyme activities and hemagglutination antibody titer were higher in both treatment groups compared with controls. The length of the intestinal and pyloric caeca folds were increased in the high dose group. Meanwhile, the number of goblet cells was increased in both treatment groups. This study suggests that heat-killed *Gordonia bronchialis* has the potential to enhance growth, immunological parameters and the gastrointestinal structure in rainbow trout.

KEY WORDS: *Gordonia bronchialis*, rainbow trout, immune system, growth, gastrointestinal histology.

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50 # Introduction

51 It is widely demonstrated that farmed fish are more susceptible to disease agents than their wild
52 counterparts due to the artificial conditions posed by intensive rearing (Price 1999; Huntigford
53 2004; Salinas *et al*. 2006). When infectious outbreaks appear they may be fought by means of
54 chemotherapeutics, vaccines or immunostimulants (Salinas *et al.* 2006). The administration of
55 immunostimulants to fish through the diet has appeared as a very promising control measure in
56 fish farms. In fact, these additives augment the innate and adaptive immune responses and
57 remain the key strategies for prevention of diseases in fish aquaculture.
58 Among the aerobic, near-mycobacterial genera, within the Actinomycetales, are some species
59 with adjuvant activities and antigens very similar to those of *Mycobacterium vaccae*, but with
60 subtle differences (Stanford & Stanford 2012). More recently, a number of these species,
61 including *Gordonia bronchialis* (*G. bronchialis*), *Rhodococcus coprophilus*, and *Tsukamurella*
62 *inchonensis*, capable of exerting subtly different adjuvant or immunostimulatory activities in
63 human, veterinary and agricultural medicine have been identified (Tarrés *et al.* 2012). For
64 example, published studies with heat-killed *G. bronchialis* showed that it could limit
65 parasitaemias in rats challenged with live *Trypanosoma cruzi* (Fontanella *et al.* 2007). Pregnant
66 rats treated with *G. bronchialis* also gave birth to offspring protected from subsequent challenge
67 with *Trypanosoma cruzi* (Davila *et al.* 2011). In dogs, a preparation of *G. bronchialis* was
68 particularly effective against an allergy induced by flea-bites (Marro *et al.* 2011). Mice treated
69 with heat-killed *G. bronchialis* controlled an IgE-mediated food allergy in a mouse model
70 (Smaldini *et al.* 2013). These findings can be attributed to immune modulation resulting from the
71 effects of powerful cell wall-associated adjuvants in *G. bronchialis* (Smaldini *et al.* 2013).

72 Some studies have so far addressed the effects of heat-killed *G. bronchialis* in aquaculture. Oral
73 administration of *G. bronchialis* improved survival and weight gain in shrimp and young fish
74 and enhanced growth rate and color and reduced skeletal abnormalities in koi carp (*Cyprinus*
75 *carpio*) (Stanford & Stanford 2012). In this study, the effects of dietary heat-killed *G.*
76 *bronchialis* on some immune parameters, growth performance and gastrointestinal structure in
77 juvenile rainbow trout (*Oncorhynchus mykiss*) were evaluated.

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91 **Materials and methods**

92 *Fish*

93 The experiment was carried out at a fish farm in Firoozkooh, Iran. Rainbow trout, of 30g average
94 wet weight, were acclimatized for 10 days on the basal diet produced in a commercial mill
95 (Faradaneh, Isfahan, Iran). The open formula of this commercial diet is given in Table 1. Fish
96 were kept in indoor cement tanks (1.8 × 0.22 × 0.35 m) in flow-through system supplied with
97 aerated well water with following characteristics: flow rate 0.5 l s^{-1}, water temperature 12 ± 1ºC,
98 dissolved oxygen>8 ppm, NH_3 <0.01 mg l^{-1}, NO_2 <0.1 mg l^{-1}, hardness 275 mg l^{-1} and pH 7.8. A
99 total of 162 fish were randomly distributed into three experimental groups following a
100 completely randomized design.

101

102 *Experimental diet*

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103 Cultured and heat-killed *G. bronchialis* by autoclaving was obtained from BioEos Ltd (Kent,
104 UK). Experimental diets were prepared by adding 2.48×10^8 and 1.24×10^9 cells kg^{-1} feed of
105 heat-killed *G. Bronchialis* into commercial basal diet which was equivalent to 2.074 g and
106 10.370 g powder in potato starch, according to the manufacturer. The heat-killed organism was
107 added to the diet by spraying 20 ml fish oil kg^{-1} feed. A control diet was prepared by spraying 20
108 ml fish oil kg^{-1} only to the basal diet. The modified feed was stored in tightly sealed plastic bags
109 at 8 - 10ºC until they were used in the feeding experiments. Three groups in triplicate with 18
110 fish in each tank were fed the experimental diets formulated with 2.48×10^8 or 1.24×10^9 cells
111 of dead *G. bronchialis*, or the basal diet for 95 days with pulse administration of treatment diet
112 for 5 days followed by 10 days on the basal diet as following: day 1 till 5: treatment diet; day 6
113 till 15: basal diet; day 16 till 20: treatment diet; day 21 till 30: basal diet. Similar pattern was
114 followed till day 95. From day 95 till day 105, fish were reverted back to the basal diet. Fish
115 were sampled on days 95 and 105 to evaluate whether the effects of using this immunostimulant
116 would last till that time. In this study, fish were initially fed at a feeding rate of 25 g kg^{-1} of body
117 weight daily; this rate was designed to assure apparent satiation. Till the end of the trial, fish
118 were weighed once every 2 weeks to adjust the feeding during the experiment.
119
120 *Growth performance*
121 All fish in each tank were taken and weighed at the beginning and the termination of the feeding
122 trial on day 95. Feed conversion ratio (FCR) and thermal growth coefficient (TGC) were
123 determined for each tank using the following formula:
124 FCR = total feed supplied / weight gain.
125 TGC = $[W2^{1/3} - W1^{1/3} / (T2 - T_1) \times °C] \times 1000$, where W_1 and W_2 are the initial and final
126 weight (g), respectively, T2 – T1 is the number of days in the feeding period and °C is the mean
127 daily temperature (Sanden *et al.* 2006).
128
129 *Immunological parameters*

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130 On days 95 and 105 of the trial, four fish from each tank were anaesthetized in a clove oil bath
131 (50 µl L^{-1}) and blood was collected from the caudal vein. Fish bloods were allowed to clot at
132 room temperature for 1 h and stored in a refrigerator overnight. The clot was then centrifuged at
133 1500 g for 5 min. Then the serum was collected and stored in sterile eppendorf tubes at -20°C
134 until used for assays. Noteworthy, all sampling fish for immunological parameters were removed
135 from study after blood collection.

136 Serum lysozyme activity was measured according to methods described by Demers & Bayne
137 (1997). Briefly, 25 µl of individual serum samples was mixed with a 175 µl *Micrococcus*
138 *lysodiekticus* (Sigma) suspension at 75 µg ml^{-1} in 0.1 M phosphate citrate buffer, pH 5.8. After
139 rapid mixing, turbidity changes were measured every 30 s for 5 min at 450 nm at approximately
140 20°C using a microplate reader. The dilutions of hen eggwhite lysozyme (Sigma) ranging from 0
141 to 20 µg ml^{-1} in the same buffer served as the standard. The equivalent unit of activity of the
142 sample as compared to the standard was determined and expressed as µg ml^{-1} serum.

143 Alternative complement serum activity was determined using previously reported method
144 (Andani *et al*. 2012). Rabbit red blood cells (RaRBC) were washed three times in ethylene glycol
145 tetra acetic acid–magnesium–gelatin veronal buffer (0.01 M EGTA–Mg–GVB, pH 7) and
146 resuspended in this buffer to a concentration of 2×10^8 cells ml^{-1}. A 100 µl suspension of
147 RaRBC was added to 250 µl of serially diluted serum in buffer. Samples were incubated at 20°C
148 for 90 min with regular shaking. Approximately 3.15 ml of 0.85% NaCl solution was added and
149 the tubes centrifuged at 1600 g for 10 min at 4°C. The extent of hemolysis was estimated by
150 measuring the optical density of the supernatant at 414 nm using a spectrophotometer (Awarness,
151 Palm, FA). ACH50 units were defined as the concentration of serum giving 50% hemolysis of
152 RaRBC.

153 Total antibody level in the serum was determined following the method of Siwicki *et al.* (1994).
154 The difference in total protein content prior to and after precipitation of the antibody component
155 with 12% polyethylene glycol (PEG; Sigma) was determined by the Bradford method (Kruger
156 1996).

157 The total peroxidase content present in serum was measured according to Cuesta *et al.* (2005).
158 Serum samples (15 µl) were placed in each well of a 96 well plate. 135 µl of HBSS without

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Ca+2 or Mg+2 was then added to each well. Finally 50 µl of 20 mM 3,30,5,50-tetramethylbenzidine hydrochloride (TMB) (Sigma) and 5 mM H2O2 was added. The color-change reaction was stopped after 2 min by adding 50 µl of 2 M sulfuric acid and the optical density was read at 450 nm by ELISA reader.

Gastrointestinal histological parameters

Tissues were collected from two fish in each tank on days 95 and 105 of the trial. Noteworthy, sampling fish for histology were in addition to the fish used for immunological parameters. After necropsy, macroscopic observations were performed. Then, liver, kidney, spleen, gills, skeletal muscle, intestine, pyloric caeca samples were taken, separately. Samples were fixed in buffered formalin for 48 h, dehydrated in alcohols and xylene and then embedded in paraffin. A five micron subsample was then rehydrated in alcohol and stained with haematoxylin-eosin. Length and thickness of proximal intestinal and pyloric caeca folds were measured using a graded ocular lens. The percentage of goblet cells among all epithelial cells in fish proximal intestine and pyloric caeca were also defined. Goblet cell percentage was determined by counting 20 folds in each fish and about five sections per sample. The magnification used was ×200.

Challenge test with chicken red blood cells (C-RBC) suspension

Red blood cells were collected from 28 days old, Ross strain broiler chickens raised under recommended rearing requirements (Ross Broiler Management handbook, Aviagen, 2014). Two milliliters of citrated (3.8% NaCit solution at 1:10 dilution) chicken blood was suspended in 10 ml Tris buffered saline (TBS, 50 mM TriseHCl, pH 8.0, 150 mM NaCl) and centrifuged at 2500 g for 10 min. The supernatant was aspirated without disturbing the blood cells. 10 ml TBS was added and mixed by inverting. Following the centrifugation and washing two more times, the supernatant was aspirated and 2.5% chicken red blood cell (C-RBC) suspension in TBS was prepared (Sheikhzadeh *et al.* 2012). On day 110 of trial, all remaining fish in each tank (six fish per tank) were anesthetized in a clove oil bath (50 µl L^{-1}) and injected intraperitoneally with 0.5 mL of the C-RBC suspension in PBS. 10 days later, these fish received boosting injection. On day 135 of trial, 15 days after boosting injection, blood was collected from these injected fish

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188 and serum was obtained with the same procedure described above and kept for further use at -
189 20ºC.
190
191 *Hemagglutination assay*
192 The antigen-specific antibody response was determined by hemagglutination assay (Rao &
193 Chakrabarti 2005). Blood from chicken was collected in citrate solution and the cells were
194 washed in PBS and resuspended to 20 ml L^{-1} in PBS. Serum of control or test fish (50 µL) was
195 serially diluted in PBS in 96 well round-bottomed microtiter plates. An equal volume of 20 ml L^{-}
196 ^{1} C-RBC was added to all wells and kept for 1 h at room temperature and then overnight at 4ºC.
197 Hemagglutinating antibody titer was expressed as the reciprocal of the highest dilution giving
198 visible agglutination.
199
200 *Statistical analysis*
201 The results were expressed as a mean ± standard error (SEM). The statistical significance of data
202 was evaluated by two-way and one-way analysis of variance (ANOVA), using the statistical
203 package for social sciences (SPSS) software version 19.0. The data were analyzed by LSD
204 analysis with the least square difference to compare the means. Differences were considered
205 significant at $P < 0.05$.
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218 **Results**

219 Growth performance

220 Final weight, final length, FCR, and TGC improved significantly in both treatment groups
221 compared to the control group (Table 2).

222

223 Immunological parameters

224 No significant differences in all immunological responses between day 95 and 105 were shown
225 during this trial. Dietary intake of heat-killed *G. bronchialis* in both treatment groups increased
226 the serum complement activity compared to the control group on days 95 of the feeding trial.
227 Similar increase was also shown on day 105 of the trial. No significant impact on serum total
228 antibody and peroxidase contents in any treatment group was noted on days 95 and 105 of the
229 study. In the assay of lysozyme activity in serum of fish treated with both low and high dose of
230 *G. bronchialis*, significant elevation was observed compared with the control group on days 95
231 and 105 of the study (Table 3). After immunization, the antigen-specific response was
232 significantly higher in the high dose groups than the control group on day 130 of trial whereas no
233 significant differences were noticed between treatment groups (Figure 1).

234

235 Histological findings

236 Upon post-mortem examination, no gross lesions or microscopic changes were detected in any
237 group. No pathological changes were also noted in the intestine and pyloric caeca of fish from all
238 groups that shows the normal architectures of these tissues. In fish that received the high dose of
239 heat-killed *G. bronchialis*, the intestinal fold length increased compared to low dose group and
240 control group on days 95 and 105 of feeding. No significant change in fold thickness in any
241 group was shown on day 95 of trial where as on day 105 fold thickness increased in both
242 treatment groups in comparison with control group. Significant increase in mucous cell numbers
243 for fish in both treatment groups were shown compared to the control group on day 95 of the

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244 trial. Highest elevation was just noted in fish receiving the high dose of heat-killed *G.*
245 *bronchialis*. Meanwhile, on day 105 of the study, mucous cell numbers was only increased in
246 fish received the high dose *G. bronchialis* compared with other groups. Comparing the intestinal
247 morphology on days 95 and 105, significant differences in goblet cells number were only noted
248 between control fish (Table 4).
249 On days 95 and 105 of the trial, the fold length of the pyloric caeca increased in fish fed the high
250 dose of heat-killed *G. bronchialis* compared with control and low dose group. On day 95, higher
251 fold thickness of the pyloric caeca was observed in both treatment groups compared to the
252 control group. Whereas on day 105 fold thicknesses did not change in any treatment group
253 compared with the control group. Higher numbers of mucous cells in pyloric caeca were
254 exhibited in both treatment groups compared with the control group on days 95 and 105 of trial.
255 Additionally, highest mucous cell number was just noted in fish fed the high dose of heat-killed
256 *G. bronchialis*. Comparing the pyloric caeca morphology on days 95 and 105, similar pattern to
257 intestine were shown in a way that significant differences in goblet cells number between control
258 fish were noted (Table 5).
259
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263 **Discussion**

264 The results show that heat-killed *G. bronchialis* in both treatment groups enhanced the growth
265 performance in rainbow trout. Similar results were observed in koi carp and shrimp (Stanford &
266 Stanford 2012). In fish species, higher growth performance can happen by different mechanisms
267 (Heidarieh *et al.* 2012). Influencing nutrient especially protein digestibility by maintaining the
268 function and structure of the small intestine leads to increased digestive capacity of the gut.
269 Improved digestive enzymes, including lipase, amylase and protease could also result in better
270 growth performance. In the present study, histological examination showed some differences in
271 small intestine and pyloric caeca structures. In the high dose group, increased fold length in the
272 intestine and pyloric caeca were noted. Thicker folds in the pyloric caeca and intestine in both

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273 treatment groups were also shown on days 95 and 105 of the feeding trial, respectively.
274 Numerous anatomical features determine the total absorptive surface area of the gastrointestinal
275 tract. For example, taller, narrower and regularly shaped fold and higher number of fold per unit
276 area are indicators that the function of the intestinal folds is activated (Heidarieh *et al.* 2013). In
277 this study, taller shaped fold in the high dose group were a good indicator but the thicker folds
278 should be evaluated further to elucidate the effects, which will happen to fish administrated with
279 heat-killed *G. bronchialis*. Comparing the effects of intestinal morphology with growth
280 performance, it is clear that most intestinal changes were exhibited with a higher dose of
281 administration. However, similar growth performance in both treatment groups was observed
282 compared to the control group. Therefore, other factors may also have affected the growth
283 performance in this study. Noteworthy, this trial was carried out on commercial fish farm;
284 different additives inside the extruded diet might also interact with heat-killed *G. bronchialis*.
285 Therefore, to elucidate the exact mechanisms through which the heat-killed *G. bronchialis* can
286 affect the growth in fish, administration of this heat-killed bacterium in a diet without any
287 additives is needed.

288 In this study, large amount of goblet cells in fish gastro-intestine after feeding dead *G.*
289 *bronchialis* were noted. Even though higher goblet cell density in fish skin and intestinal tract
290 after challenge with infectious diseases have been widely studied (Buchmann & Bresciani 1998;
291 Ringø *et al.* 2003) increase in mucus production in response to the feed additives were also
292 shown (Torresillas *et al.* 2011; Covello *et al.* 2012; Heidarieh *et al.* 2012; Xueqin *et al.* 2012;
293 Shin *et al.* 2014). Goblet cells in the gastrointestinal tract provide protection against damage by
294 forming a viscous, hydrated blanket on the surface of the mucosa which acts as a sensitive first
295 line ~~of~~ immune defense parameter in fish (Shin *et al.* 2014). Meanwhile, it was suggested that
296 goblet cells positively affect the absorption of digestible substances (Shin *et al.* 2014) that can
297 partly explain the improvement in growth performance in fish administrated with heat-killed *G.*
298 *bronchialis*.

299 Heat-killed *G. bronchialis* in both treatment groups were effective in enhancing serum lysozyme
300 and complement activities. On other hand, total antibody and peroxidase levels were not affected
301 by administration of dead *G. bronchialis*. The lysozyme and alternative pathway of complement
302 are powerful non-specific defense mechanisms for protecting fish against a wide range of

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303 potentially invasive organisms, such as bacteria, fungi, viruses, and parasites (Choi *et al.* 2008;
304 Son *et al.* 2009). In fact, the innate immune system of fish comprises several humoral and
305 cellular factors that may differentially respond to the offered immunostimulants (Shin *et al.*
306 2014). After immunization, higher antigen-specific response was shown in high dose group,
307 showing the positive effect of heat-killed *G. bronchialis* on specific immune responses in
308 rainbow trout. Immunostimulatory effects of dead *G. bronchialis* have been earlier reported in
309 laboratory animal and veterinary medicine (Fontanella *et al.* 2007; Davila *et al.* 2011; Marro *et*
310 *al.* 2011; Stanford & Stanford 2012; Smaldini *et al.* 2013). The effectiveness of preparations of
311 heat-killed Actinomycetales is attributed to highly active immunostimulatory adjuvants in their
312 cell walls, with subtle differences between species (Marro *et al.* 2011).
313 Considering the long period of fish culture, continual feeding of immunostimulants might have
314 harmful effects on the immune system (Bricknell & Dalmo 2005). Therefore, a pulse feeding
315 strategy during 95 days, with treatment diet for 5 days followed by basal diet for 10 days, was
316 evaluated. Results showed up-regulation of the immune system after administration of heat-
317 killed *G. bronchialis* on day 95 of trial. Meanwhile, fish were also sampled on day 105 to
318 analyze the effects of 10 days withdrawal time on different immunological and histological
319 parameters. In fact, on day 105 an enhanced response of the immune system and histological
320 parameters fairly similar to day 95 were noted even though goblet cell percentage in low dose
321 group returned to baseline which shows the minor decrease in immune stimulation. Noteworthy,
322 comparing the immunological and histological parameters on days 95 and 105, significant
323 differences in goblet cells number in intestine and pyloric caecum between control fish were
324 noted whereas no significant differences in treatment groups between day 95 and 105 were
325 shown during this trial. The exact reason for differences in goblet cells number between control
326 fish is unknown and needs more considerations to clarify these observations. Converse to this
327 substance, immune stimulation by feeding a probiotic is more transient and a few days after
328 cessation of probiotic administration, it decreases dramatically (Andani et al. 2012). Reduction in
329 immune responses following the administration of probiotics can be attributed to a decrease in
330 bacterial count after feeding on the basal diet (Andani et al. 2012). In general, immune
331 stimulation by feeding different substances even in withdrawal time can provide an immense
332 flexibility in fish farming especially during periods of increased disease risk.

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In conclusion, this preliminary study showed that administration of heat-killed *G. bronchialis* has beneficial effects in rainbow trout, affecting parameters like growth performance, immunity and gastrointestinal structure. Further investigations are needed to fully understand the interaction between this organism and different fish species even on the molecular level.

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Table 1 Ingredients composition of the commercial basal diet used in this study

Ingredients (g kg^{-1}, DM)	Control diet
Fish meal[a]	195
Soybean oil[b]	41
Corn flour	86.5
Soybean meal	200
Wheat	173
Poultry byproduct meal	270
Vitamin mixture[c]	5
Mineral mixture[d]	5
Zeolit	10
L-Carnithine[e]	0.3
Vitamin C[f]	0.2
Molasses	10
Lysine	0.6
Methionine	0.6
Active-mos[g]	1.5
Natuzyme-p[h]	0.3

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NaCl	1
Proximate chemical analysis (g kg^{-1})	
Dry matter	910
Crude protein	430
Crude lipid	178
Ash	90
Crude fiber	42
Phosphorus	14

.[a] North Sefid Mahi Corporation, Iran with 540 g kg^{-1} crude protein

.[b] North Sefid Mahi Corporation, Iran

[c] Unit kg^{-1} of mixture: Vitamin: retinol acetate (A), 1 600 000 IU; Cholecalciferol (D3), 400 000 IU; DL-α-tocopheryl acetate (E), 40 IU; Menadione sodium bisulfate (K3), 2 000 mg; Biotin (H2), 240 mg; Thiamin mononitrate (B1), 6 000 mg; Riboflavin (B2), 8 000 mg; Calcium d-pantothenate (B3), 12 000 mg; Niacinamide (B5), 40 000 mg; Pyridoxine hydrochloride (B6), 4 000 mg; Folic acid (B9), 2 000 mg; Cyanocobalamin (B12), 8 000 mg; Vitamin C, 60 000 mg; .Inositol, 20 000 mg; B.H.T., 20 000 mg; Carrier up to 1 kg

[d] Unit kg^{-1} of mixture: Mineral: Fe, 26 000 mg; Zn, 12 500 mg; Se, 2 000 mg; Co, 480 mg; Cu, 4 200 mg; Mn, 15 800 mg; I, 1 000 mg; Choline chloride, 12 .000 mg; Carrier up to 1 kg

.[e] Science Laboratories,Iran. Contains: 50 g L^{-}Carnithin, Carrier up to 100 g

.[f] Canavit Company, Canada

.[g] Biorigin Company, Brazil. Conatins: 100% yeast cell wall

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[h] T.F.A. Company, Iran. Conatins Unit kg^{-1} of mixture: Cellulase, 6 000 000 U; Xylanase, 10 000 000 U; β-glucanase, 700 000 U; α-amylase, 700 000 U; .Pectinase 70 000 U; Phytase, 1 500 000 U; Lipase, 30 000 U; Proteases (Acid & Neutral), 3 000 000 U

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Table 2 Growth performance in rainbow trout after feeding different doses of heat-killed Gordonia bronchialis

Gordonia bronchialis in diet (cells kg^{1}	**Initial Weight (g)**	**Initial Length (cm)**	**Final Weight (g)**	**Final Length (cm)**	**Feed Conversion Ratio**	**Thermal Growth Coefficient**

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feed)					(FCR)	(TGC)
Control	31.05 ± 0.29	13.99 ± 0.03	135.82 ± 3.40[a]	22.90 ± 0.27[a]	0.91 ± 0.04[a]	1.75 ± 0.05[a]
2.48 × 10^8	30.98 ± 0.23	13.99 ± 0.03	149.76 ± 1.62[b]	24.08 ± 0.23[b]	0.80 ± 0.01[b]	1.91 ± 0.01[b]
1.24 × 10^9	30.89 ± 0.20	13.97 ± 0.03	149.83 ± 2.13[b]	24.15 ± 0.16[b]	0.80 ± 0.01[b]	1.90 ± 0.01[b]

Data are mean ± SEM. Those within a column superscripted by * are significantly different (P<0.05); for FCR and TGC n= 3 in each treatment group, for remaining parameters n = 54 in each treatment group.

Table 3 Immunological parameters in rainbow trout serum after feeding different doses of heat-killed Gordonia bronchialis

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Gordonia bronchialis in diet (cells kg^{-1} feed)	Complement titer (units mL^{-1})		Total antibody (mg mL^{-1})		Peroxidase (450 nm)		Lysozyme activity (µg mL^{-1})	
	Day 95	Day 105	Day 95	Day 105	Day 95	Day 105	Day 95	Day 105
Control	0.075 ± 0.004^{a}	0.081 ± 0.001^{a}	3.17 ± 0.20	3.10 ± 0.14	0.256 ± 0.002	0.249 ± 0.001	33.01 ± 6.77^{a}	34.89 ± 1.50^{a}
2.48×10^{8}	0.149 ± 0.009^{b}	0.138 ± 0.007^{b}	3.12 ± 0.19	2.99 ± 0.23	0.262 ± 0.003	0.260 ± 0.002	112.30 ± 19.55^{b}	115.60 ± 14.51^{b}
1.24×10^{9}	0.141 ± 0.012^{b}	0.136 ± 0.007^{b}	3.08 ± 0.20	3.09 ± 0.19	0.257 ± 0.002	0.254 ± 0.002	102.00 ± 8.92^{b}	107.09 ± 8.93^{b}

Data are mean ± SEM. Means within a column superscripted by different small letters are significantly different ($P<0.05$); Means within a row superscripted by different capital letters are significantly different ($P<0.05$); n = 12 for each treatment group.

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Table 4 Intestinal morphology in rainbow trout after feeding different doses of heat-killed Gordonia bronchialis

Gordonia bronchialis in diet (cells kg^{-1} feed)	Fold length (μm)		Fold thickness (μm)		Goblet cell percentage	
	Day 95	Day 105	Day 95	Day 105	Day 95	Day 105
Control	432.50 ± 17.10[a]	480.10 ± 19.88[a]	116.66 ± 4.97	112.08 ± 4.32[a]	33.50 ± 0.98[aA]	41.58 ± 2.00[aB]
2.48 × 10^8	465.20 ± 19.48[a]	470.08 ± 13.70[a]	122.50 ± 6.41	124.16 ± 4.99[b]	39.50 ± 0.88[b]	42.25 ± 0.87[a]
1.24 × 10^9	570.10 ± 13.25[b]	570.01 ± 12.49[b]	127.50 ± 6.97	126.66 ± 5.55[b]	46.33 ± 1.22[c]	46.50 ± 0.96[b]

Data are mean± SEM. Means within a column superscripted by different small letters are significantly different (P<0.05); Means within a row superscripted by different capital letters are significantly different (P<0.05); n = 6 for each treatment group.

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Table 5 Pyloric caeca morphology rainbow trout after feeding different doses of heat-killed Gordonia bronchialis

Gordonia bronchialis in diet (cells^{-1} kg feed)	**Fold length (µm)**		**Fold thickness (µm)**		**Goblet cell percentage**	
	Day 95	Day 105	Day 95	Day 105	Day 95	Day 105
Control	727.50 ± 21.07^{a}	780.83 ± 25.41^{a}	115.10 ± 3.37^{a}	120.83 ± 3.12	28.50 ± 1.24^{aA}	34.67 ± 1.63^{aB}
2.48 × 10^{8}	761.66 ± 17.44^{a}	728.50 ± 27.17^{a}	133.33 ± 5.41^{b}	121.66 ± 6.49	33.42 ± 0.82^{b}	37.92 ± 1.05^{b}

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1.24×10^9	833.33 ± 24.72^b	918.33 ± 28.11^b	141.66 ± 4.40^b	125.83 ± 4.51	40.50 ± 1.33^c	41.02 ± 1.41^c

Data are mean± SEM. Means within a column superscripted by different small letters are significantly different ($P<0.05$); Means within a row superscripted by different capital letters are significantly different ($P<0.05$); n = 6 for each treatment group.

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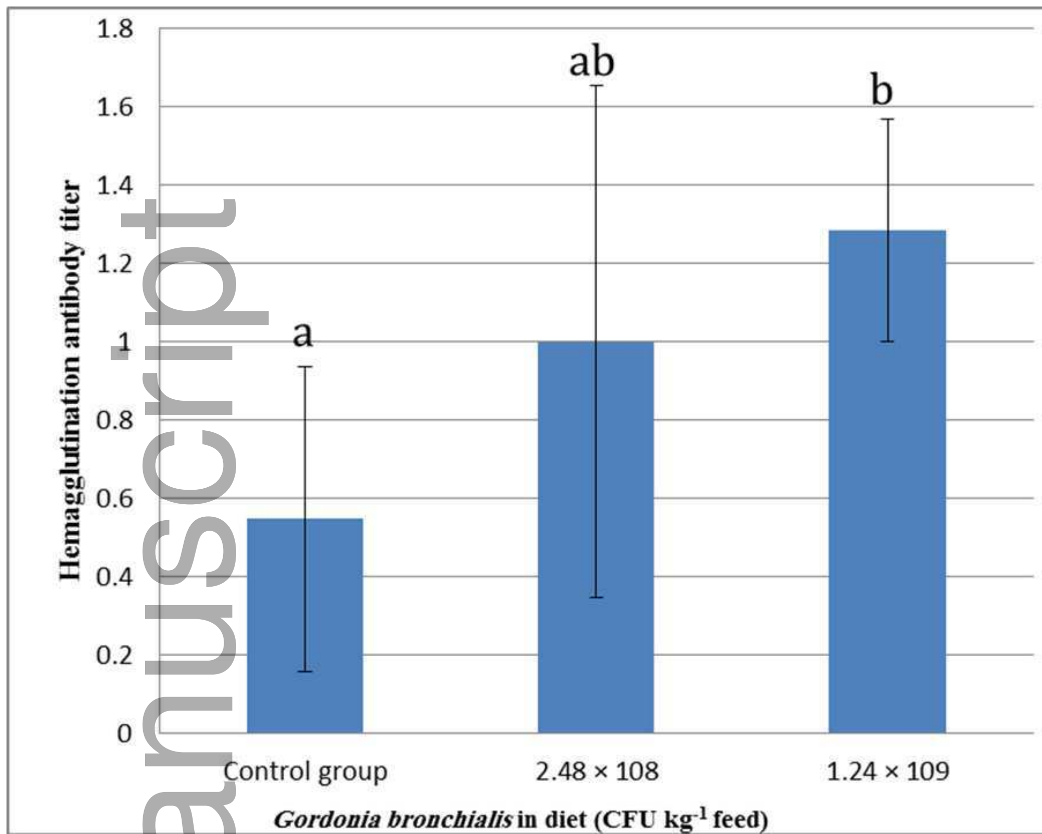

Figure 1 Hemagglutination antibody titer in rainbow trout after feeding different doses of heat-killed *Gordonia bronchialis*. Data are mean ± SEM. Those superscripted by different letters are significantly different ($P<0.05$); n = 18 for each treatment group.

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