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## Feeding with heat-killed *Gordonia bronchialis* affects growth performance, intestinal morphology and immunomodulation in Japanese quail (*Coturnix coturnix japonica*)

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**ABSTRACT:** Studies have shown that bacterial immunomodulators based on aerobic Actinomycetales such can switch off pre-existing Th2 preponderance and prompt Th1-mediated mechanisms, whatever the previous environmental immune priming of the individual. *Gordonia bronchialis* (*G. bronchialis*) is a Gram-positive, weakly acid-fast species of the genus *Gordonia* within the sub-order Corynebacteriaceae of the order Actinomycetales. In this study, the efficacy of heat-killed *Gordonia bronchialis* (*G. bronchialis*) on growth performance, immune system and intestinal structure in quail (*Coturnix coturnix japonica*) was evaluated. Quails (mean weight 7.8 g) were fed basal diet (control), or treatment diets containing 10<sup>5</sup> (low dose) or 10<sup>6</sup> (high dose) CFU per bird per day in food of heat-killed *G. bronchialis* continuously (for 42 days). Body weight gain (BWG) and feed consumption was recorded during grower period and finisher period. On days 7, 14, 28, 35 and 42 some of the quails were sampled for analysis of Newcastle antibody titer. Interleukin-4 (IL-4), Interferon- $\alpha$  (IFN- $\alpha$ ), and interferon- $\gamma$  (IFN- $\gamma$ ) concentrations were analyzed using ELISA kits. An indirect ELISA was performed to quantifying IgA. At the end of 14, 28 and 42 days old, three chicks from each group were selected for histopathological and histomorphometrical studies. Results showed that growth performance was significantly enhanced in both treatment groups compared with the control group. Serum anti Newcastle disease virus, IL-4 and IFN- $\alpha$  titers were higher in low dose treatment group compared with the control group. The length of the intestinal and pyloric caeca folds was increased in the high-dose group. Meanwhile, jejunum and ileum showed the most significant morphological changes in different days of sampling, particularly in high dose group. Among the evaluated factors, villous length and intestinal crypt depth demonstrated more significant differences. This study suggests that heat-killed *G. bronchialis* has the potential to enhance growth, immunological parameters and the intestinal structure in Japanese quail.

**Keywords:** Japanese quail; Growth; Newcastle disease; Interferon; *Gordonia bronchialis*

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## INTRODUCTION

Newcastle Disease (ND) is one of the most devastating diseases of the domestic fowl, which can cause high level mortality of these animals. ND is caused by Newcastle Disease Virus (NDV), an avian Paramyxovirus type 1 (APMV-1) that belongs to the genus *Avulavirus*, family *Paramyxoviridae* (Silva et al., 2010). The NDV's are classified into four pathotypes; asymptomatic enteric, lentogenic, mesogenic, and velogenic (Rehman et al., 2018).

Despite the importance of the antibody-mediated response for protecting against NDV infection, the innate immune response induced by NDV challenge remains unclear. The host innate immune system provides the first line of defense against pathogens. The innate immune system can recognize components of pathogens called pathogen-associated molecular patterns (PAMPs) recognition receptor (Zhang et al., 2018).

The commercial production of Japanese quails (*Coturnix coturnix japonica*) is extensively distributed in several countries around the world and many studies showed that this species can easily adapt to commercial management conditions, with good performance in terms of meat and egg production (Lima et al., 2004). However, there is little information available on health control programs in this species. In addition, as today happens with broilers and turkey, quails will probably be intensively produced and the high bird concentration in some areas may cause the dissemination of infectious disease.

Currently, organic farms and foods are important to humans because the excessive use of antibiotics for treatment of diseases and animal husbandry has led to drug resistance in infectious agents, raising interest in products derived from nature to prompt human and animal health. Studies have shown that bacterial immunomodulators based on aerobic Actinomycetales such as *Mycobacterium vaccae* (*M. vaccae*) can switch off pre-existing Th2 preponderance and prompt Th1-mediated mechanisms, whatever the previous environmental immune priming of the individual (Tarreset et al., 2012). Among the aerobic, near mycobacterial genera, within the Actinomycetales, are some species with adjuvant activities and antigens very similar to those of *M. vaccae*, but with subtle differences. *Gordonia bronchialis* (*G. bronchialis*) is a Gram-positive, weakly acid-fast species of the genus *Gordonia* within the sub-order Corynebacteriaceae of the order Actinomycetales. It is an environmental organism that rarely gives raise to human infections (Arenskotter et

al., 2004). Killed preparations of *G. bronchialis* and some other genera within the order Actinomycetales are potent immune modulators useful in the prevention and treatment of many immune-related diseases in laboratory animal and veterinary medicine (Fontanella et al., 2007; Davila et al., 2011; Stanford and Stanford, 2012). Rats treated with *G. bronchialis* and challenged with live *Trypanosomacruzi* show significantly reduced parasitaemias and less chronic myocarditis (Stanford and Stanford, 2012). *G. bronchialis* enhances growth and immunity in rainbow trout (Sheikhzadeh et al., 2016) and decreases the malondialdehyde (MDA), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels in serum of rainbow trout (Shabanzadeh et al., 2016).

Since there is no information about the effect of *G. bronchialis* on quail, in the present study the effect of dietary inclusion of heat killed *G. bronchialis* on the serum anti NDV titer, serum Immunoglobulin-A (IgA) titer, and serum cytokines [interleukin-4 (IL-4), interferon- $\alpha$  (IFN- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ )] of Japanese quail was investigated. Furthermore the growth performance and intestinal structure of Japanese quail fed with heat-killed *G. bronchialis* was also determined.

## MATERIALS AND METHODS

### Animal care and experimental design

Unsexed Japanese quails were obtained at one day of age acclimated to laboratory surroundings for one week before immunizations and measurements were begun. Quail were housed in cages measuring 61 × 56 × 81 cm. Temperature in the animal room was maintained at 23°C. Food and water were available *ad libitum*. Quails were fed bird food containing approximately 20% crude protein that contained no coccidiostat or other medications (Supplementary file 1). Fluorescent lights provided a photoperiod of 12 h light and 12 h dark. The University of Tabriz Animal Care and Use Committee approved all protocols (FVM.REC. 1395.58).

Seventeen Japanese quails were assigned randomly to each of nine groups in a 3 × 3 factorial design with three bacterial treatments. The quails were randomly assigned to each treatment, because sexing could not be done until later development. The bacterial treatments consisted of (1) a control or no bacteria group, (2) a low dose group, and (3) a high dose group. The low- and high dose groups received 10<sup>5</sup> or 10<sup>6</sup> CFU per bird per day in food, respectively, from the first day of age.

### Growth performance and sample collection

Body weight gain (BWG) and feed consumption was recorded during grower period and finisher period. On d 14, 28 and 42, two birds closet to the median weight from each pen (6 per treatment) were randomly selected, weighed, stunned and slaughtered by exsanguination. Bursa of Fabricius, and spleen were then precisely removed and weighed separately on a sensitive digital scale. Blood samples were taken from wing vein and centrifuged for 15 min at 1250 × g and 4 °C. Serum was collected and stored refrigerated at -20 °C pending analysis.

### Immunological responses in serum

Interleukin-4 (IL-4), IFN- $\alpha$ , and IFN- $\gamma$  concentrations were analyzed using ELISA kits (Cusabio, USA) following the manufacturer's instructions. An indirect ELISA was performed to quantifying IgA. The commercial chicken IgA ELISA quantification set (Cusabio, USA) was used according to manufacturer's instructions. Antibody titers against NDV was measured by haemagglutination-inhibition (HI) test according to Sun et al. (2018), and using ELISA kits (IDDEX, USA) following the manufacturer's instructions. Haemagglutination-inhibition results were expressed as  $\log_2$  of the reciprocal of the last dilution.

### Intestinal morphology development

At the end of 14, 28 and 42 days old, three chicks from each group were selected for histopathological and histomorphometrical studies. The samples were taken from different organs including liver, kidney, heart and brain (to evaluate the hepatotoxicity, nephrotoxicity, cardiac toxicity and neurotoxicity or other side effects of *G. bronchialis*, respectively). Besides, different parts of the small intestine (duodenum, jejunum, and ileum) were obtained for histomorphometrical study. The mentioned tissues were fixed at 10% buffered formalin, embedded in paraffin, sectioned at about 5  $\mu$ m, stained with hematoxylin and eosin and studied microscopically with a light microscope (Olympus-CH30, Japan). According to previous studies (Sakamoto et al. 2000; Aptekmann et al. 2001), the measured morphometric variables included: villous height (measured from the villous-crypt junction), villous thickness (measured at mid-villous height), intestinal crypt depth (measured from the villous-crypt junction until the end of glands), intestinal crypt number, and goblet cell number.

### Statistical analysis

The results were expressed as means  $\pm$  standard

error of mean (SEM) and all data were statistically analyzed by one-way ANOVA, using SPSS version 22.0 software for Windows (SPSS Inc., Chicago, IL). Differences between treatment groups were tested by LSD test, and differences were significant at  $P < 0.05$ .

### RESULTS

The decrease in dietary *G. bronchialis* content from  $10^6$  to  $10^5$  Bacilli/Bird/Day caused a significant ( $P < 0.05$ ) decline in weight gain (Table 1); feed conversion efficiency; however, was not affected by dietary *G. bronchialis* level.

The level of the IL-4 cytokine, which corresponded to Th<sub>2</sub> cytokines in birds, significantly increased following stimulation with *G. bronchialis* in low dose group compared to the control. IFN- $\alpha$  concentration was enhanced at both treatment groups (Figure 1).

The HI serum antibody results from day 1, 7, 14, 21, 28, 35, and 42 are given in Table 2. The findings by the ELISA method are given in Table 3. Although higher titers were obtained when *G. bronchialis* was used in high dose in day 42, the statistical analysis did not reveal differences between different treatments.

The effect of dietary *G. bronchialis* on serum IgA is presented in Figure 2. As the dietary *G. bronchialis* enhanced, IgA rose on day 7 (low dose group) and day 42 (high dose group), significantly.

Spleen and bursa of Fabricius were increased in relative weights as a consequence of increasing dietary *G. bronchialis* (Table 4). Both relative spleen and bursa weight increased.

Microscopically, there was not hepatotoxicity, nephrotoxicity, cardiac toxicity, and neurotoxicity or other side effects in the liver, kidney, heart, and brain, respectively. The most morphological changes were observed between low dose and high dose groups with the control group, which was seen more on day 42 of sampling in comparison. Interestingly, jejunum and ileum showed the most significant morphological changes in different days of sampling, particularly in the high dose group. Among the evaluated factors, villous length and intestinal crypt depth demonstrated more substantial differences (Figures 3 and 4). On the 42<sup>nd</sup> day of sampling, there were also significant differences in the goblet cell number between various groups. While, intestinal crypt numbers showed less morphological changes in both experimental groups.

**Table 1.** The effect of *Gordonia bronchialis* administration on performance parameters on different days in various experimental groups of Japanese quail

Days	Parameters	groups		
		Control	Low dose	High dose
1-14	BWG	53.7±0.89	58.7 ± 0.95	56.6 ± 1.50
	FI	126.41	121.19	120.51
	FCR	2.35	2.06	2.12
1-28	BWG	128.9±0.96 <sup>a</sup>	126.7 ± 1.69 <sup>a</sup>	140.1 ± 0.94 <sup>b</sup>
	FI	318.92	294.09	294.11
	FCR	2.47	2.32	2.09
1-42	BWG	208.6±1.10 <sup>a</sup>	209.4±0.92 <sup>a</sup>	219.8 ± 1.16 <sup>b</sup>
	FI	575.38	532.06	530.55
	FCR	2.75	2.54	2.41

BWG: body weight gain (mean ±SD, g/bird); FI: feed intake (g/bird); FCR: feed conversion ratio.

<sup>a-b</sup>Means within a row with no common superscripts differ significantly ( $P \leq 0.05$ )

**Table 2.** Effects of dietary heat-killed *Gordonia bronchialis* on antibody titers to Newcastle disease virus ( $\log_2$ ) in Japanese quail (Haemagglutination Inhibition test)<sup>1</sup>

Sampling day	Groups		
	Control	Low dose	High dose
d 1	3.77 ± 1.56	3.77 ± 1.56	3.77 ± 1.56
d 7	1.77 ± 0.44	1.55 ± 0.52	1.77 ± 0.66
d 14	2.55 ± 1.94	2.88 ± 2.26	2.66 ± 2.54
d 21	3.66 ± 2.59	4.88 ± 1.61	4.44 ± 2.74
d 28	4.55 ± 2.69	5.55 ± 0.72	5.77 ± 1.09
d 35	5.33 ± 2.54	5.77 ± 0.66	5.44 ± 2.65
d 42	6.33 ± 2.12	6.33 ± 0.5	7.33 ± 1.58

<sup>1</sup>Japanese quails were vaccinated with Newcastle disease virus vaccine at 10 and 20 days of age.

**Table 3.** Evaluation of immune response to Newcastle disease virus by ELISA method in different days in Japanese quail receiving  $10^5$  or  $10^6$  bacilli of heat-killed *Gordonia bronchialis* per day (mean ± SEM)

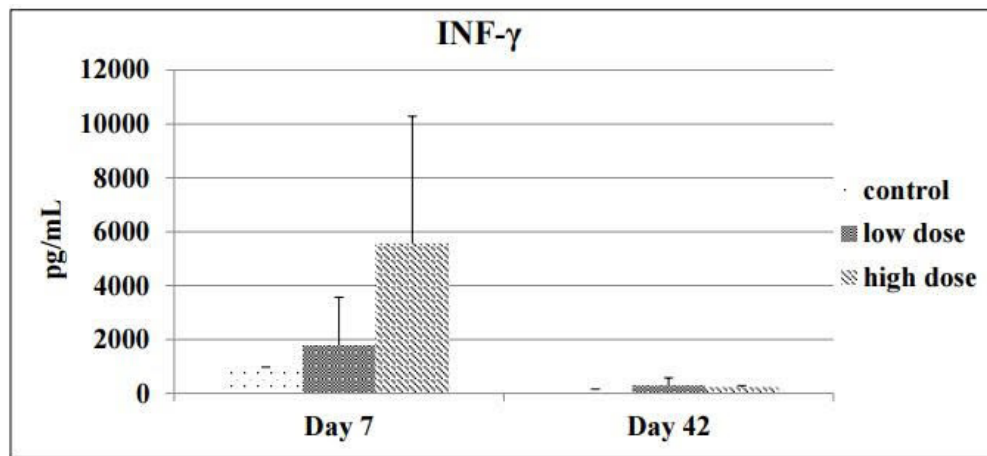
Group	Age (days)						
	D 1	D7	D 14	D 21	D 28	D 35	D 42
Control	2507.2 ± 1218.83	400 ± 0 <sup>a</sup>	920 ± 83.66	1220 ± 228.03 <sup>a</sup>	1736 ± 539.56	1779.2 ± 84.17	1916.6 ± 358.41
Low dose	2507.2 ± 1218.83	520 ± 130 <sup>a</sup>	1300 ± 300	1620.2 ± 375.1 <sup>ab</sup>	1966 ± 914.61	2164.8 ± 869.21	2187.4 ± 1413.28
High dose	2507.2 ± 1218.83	720 ± 178.88 <sup>b</sup>	1620 ± 884.3	1903.6 ± 598.52 <sup>b</sup>	2314 ± 1077	2458.6 ± 428.35	2953.8 ± 1902.85

<sup>ab</sup>: Different superscripts within columns indicate significant difference among doses of bacteria ( $P < 0.05$ ).

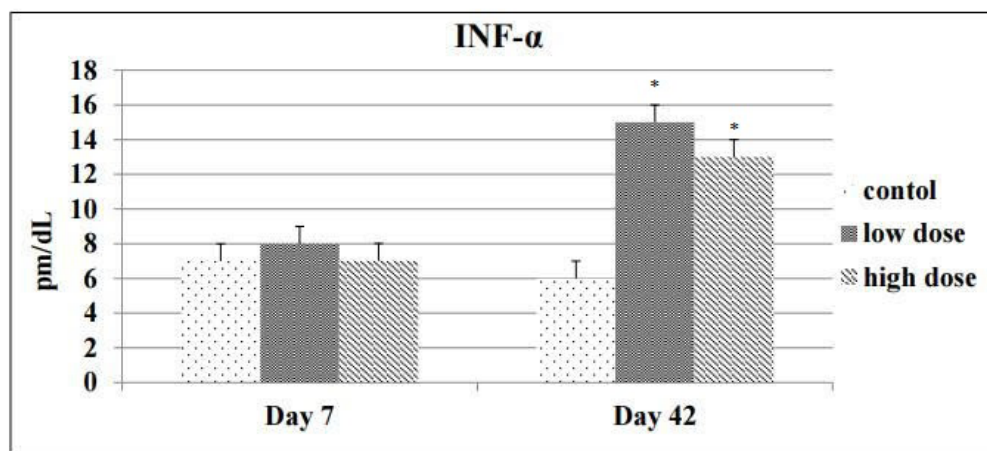
**Table 4.** Influence of varying dietary *Gordonia bronchialis* levels on relative lymphoid organ weights (% of live BW) at 14, 28 and 42 days of age (n = 4) (Mean ± SD)

Group	Day 14		Day 28		Day 42	
	Spleen	Bursa of Fabricius	Spleen	Bursa of Fabricius	Spleen	Bursa of Fabricius
Control	0.07 ± 0.00	0.07 ± 0.02 <sup>a</sup>	0.08 ± 0.04	0.06 ± 0.01 <sup>a</sup>	0.09 ± 0.00 <sup>a</sup>	0.07 ± 0.02 <sup>a</sup>
Low dose	0.07 ± 0.01	0.07 ± 0.01 <sup>a</sup>	0.08 ± 0.00	0.06 ± 0.00 <sup>a</sup>	0.09 ± 0.00 <sup>a</sup>	0.08 ± 0.00 <sup>a</sup>
High dose	0.07 ± 0.00	0.12 ± 0.00 <sup>b</sup>	0.09 ± 0.00	0.13 ± 0.00 <sup>b</sup>	0.12 ± 0.01 <sup>b</sup>	0.14 ± 0.00 <sup>b</sup>

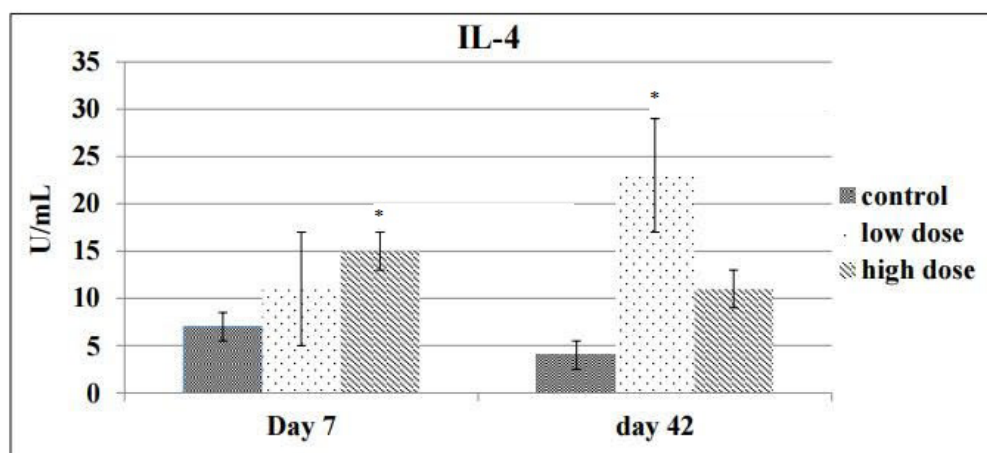
Values within a column followed by different letters are significantly different ( $P < 0.05$ ).



a

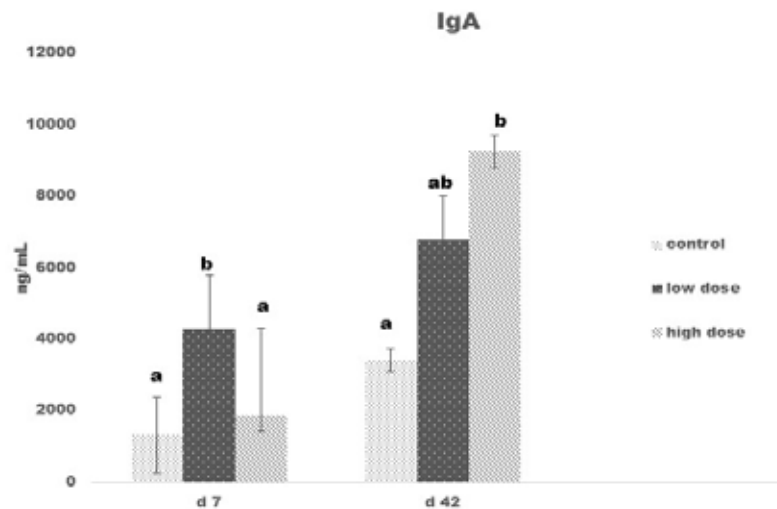


b



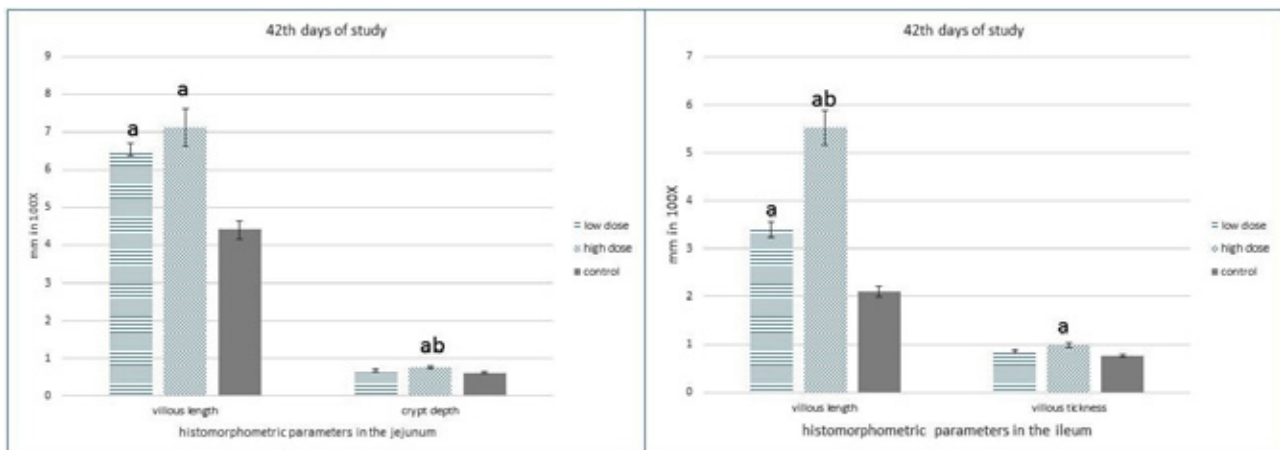
c

**Figure 1.** IFN- $\gamma$  (a), IFN- $\alpha$  (b), and IL-4 (c) productions were measured in blood. The cytokine response to heat killed *Gordonia bronchialis* was compared with control group, \* $p < 0.05$ .

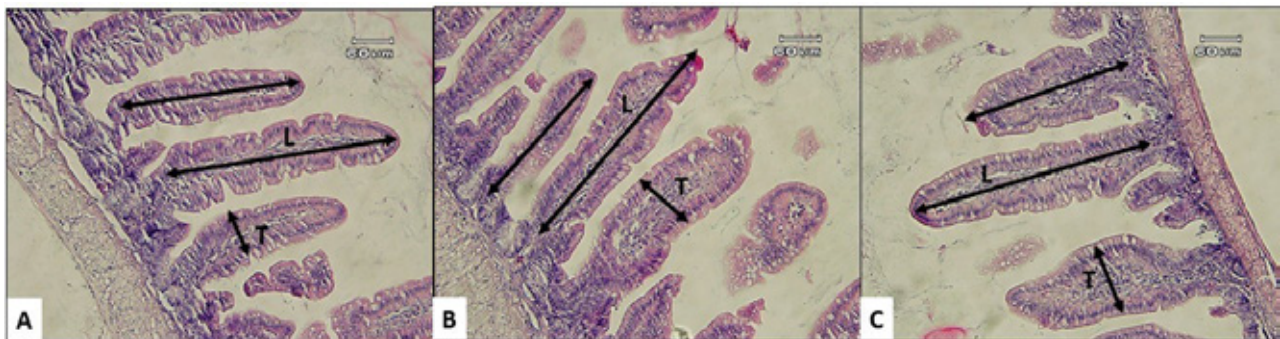


<sup>ab</sup>: Different superscripts within columns indicate significant difference among doses of bacteria ( $P < 0.05$ ).

**Figure 2.** Ig A levels in different days from Japanese quail receiving low dose ( $10^5$  cells) or high dose ( $10^6$  cells) of heat-killed *Gordonia bronchialis* per day (ng/mL)



**Figure 3.** Significant differences ( $P < 0.05$ ) in histomorphometric parameters between various groups on the 42<sup>nd</sup> day of sampling in the jejunum and ileum. a: significant statistical difference with the control group; b: significant statistical difference between low dose and high dose groups.



**Figure 4.** Small intestine (jejunum), Japanese quail. A: control group; B: low dose group; C: high dose group; a remarkable improvement was found in the small intestine of both treated groups. Indeed, there were the significant differences in the villous length (L) and thickness (T) of the small intestine in both *G. bronchialis*-recipient groups compared to the control group. H&E.

**Supplementary file 1.** Composition and nutrient contents of corn-soybean based diets

<b>Feed ingredients</b>	<b>1-14 d</b>	<b>15-42 d</b>
<b>Ingredient (%)</b>		
Corn	46.45	52.45
Soybean meal	48	42
Poultry fat	1.50	1.5
Dicalcium phosphate	1.2	1.2
Calcium carbonate	1.3	1.3
Salt	0.20	0.20
DL-Methionine	0.20	0.20
Vitamin premix	0.50	0.50
L-Lysine HCL	0.15	0.15
Mineral premix	0.5	0.5
Total	100	100
<b>Calculated</b>		
ME (kcal/kg)	2912	2910
CP (%)	23.1	20.2
Lys (%)	1.44	1.22
Met + Cys (%)	0.92	0.85
Calcium (%)	0.97	0.93
Nonphytate phosphorus (%)	0.49	0.47

**DISCUSSION**

With regards to immunomodulatory effects of Actinomycetes, there are some reports that Actinomycetes (*G. bronchialis*) enhances immune responses in rainbow trout (Sheikhzadeh et al., 2017). *G. bronchialis* also increases most parameters of blood profile including superoxide dismutase, GSH-Px, glutathione reductase in rainbow trout (Shabanzadeh et al., 2016). In current research, based on the absence of clinical signs, quails were considered healthy throughout the trials. This could be attributed to the low density of quails and high hygienic condition of cages used in the current study compared to commercial floor pens.

Dietary *G. bronchialis* supplementation improved weight gain. FCR value is less than control group without significant differences between treated and control group. In present study, it was observed that the use of *G. bronchialis* as an additive improved the fattening performance and there was dose response. In agreement with present observations, Shabanzadeh et al. (2016) indicated that heat-killed *G. bronchialis* in both treatment groups enhanced the growth performance in rainbow trout similar to the results reported previously for Koi carp and shrimp (Stanford and Stanford, 2012). Shabanzadeh et al. (2016) observed that *G. bronchialis* increase in fish villi height and fold height in the intestine and

pyloric caeca and therefore, may enhance the activity of digestive enzymes, resulting in higher nutrient absorption, better feed conversion ratio, and greater growth rate.

Interferons were so named due to their anti-viral properties. Our results confirmed the opinion of a number of researchers (Bailey et al., 2007; Karakolev et al., 2015), that bacterial endotoxins induce interferon synthesis after subcutaneous or intramuscular application.

The measurement of serum antibody titers to determine the potency of inactivated ND vaccines is a reliable alternative to the measurement of the protective dose 50% (PD<sub>50</sub>) of these vaccines (Mass et al., 1998). Antibody titers increased following first vaccination (live) and reached the highest level on day 42 of age (three weeks post 2<sup>nd</sup> vaccination). In this study, which was performed with a design comparable with the *T. inchoensis* trials in quails, no differences between antibody titers of quails of treated groups and those of quails of the control group were shown, but in *T. inchoensis* trial, in low dose in day 42, there was significant difference (Nofouzi et al., 2019). There was also an obvious beneficial effect of Actinomycetes as immunomodulatory against chicken RBC in mouse, probably due to activation of

macrophages, induction of transcription of cytokine genes and release of inflammatory cytokines (Nofouzi et al., 2017). Immunoglobulin A, as the major class of antibody present in the mucosal secretions of most animals, represents a key first line of defense against invasion by inhaled and ingested pathogens at the vulnerable mucosal surfaces. IgA is also found at significant concentrations in the serum of many species, where it functions as a second line of defense mediating elimination of pathogens that have breached the mucosal surface. In this work, the numerically highest serum IgA was obtained by d 42, for high dose group. In fact, we showed long-lasting serum IgA response. Both treated groups showed higher IgA titers when compared to control at varying times throughout d 7 to 42. This implies that *G. bronchialis* may stimulate the humoral immune system to produce more antibodies. Because IgA is a non-inflammatory antibody that binds complement only weakly, it protects the tissues from excessive immune-mediated damage.

Our results suggested that *G. bronchialis* improved the immune response of quails at low *G. bronchialis* level, probably due to the up-regulation of IL-4 and IFN- $\alpha$  production. The changes of both Th1 and Th2 cytokines in our study could be attributed to the immunological balance and cross-regulatory effects between both inflammatory and anti-inflammatory cytokines, suggestion that *G. bronchialis* could maintain immune homeostasis and prevent further activation of immune system.

The immune system guards the body against foreign substances and protects from invasion by pathogenic organisms. The immune response against viral infection may affect the host defense against virus. The immune system is affected by not only infectious disease but also the sexual cycle, stress, and growth of animals. Therefore, a better understanding of the quail's immune system may also make quail a more useful experimental animal and improve their breeding in farms.

Immune tissue development can in some cases reflect immune response and functionality. Effects of control group and *G. bronchialis* supplementation on lymphoid organs are shown in Table 4. In the present study, quails which treated with high dose *G. bronchialis* had a significant increase in bursa of Fabricius and spleen weights, in day 42. The current observation indicates that the *G. bronchialis* needs for optimum cellular immune response may be higher than those for maximum growth rate.

The results of the current study demonstrated that oral administration of *G. bronchialis* improved the development of histomorphological structure of small intestine in Japanese quail, especially in high dose. Interestingly, the improvement was more effective on jejunum and ileum particularly in crypt length and thickness. Besides, the crypt number exhibited slight alteration. Recently, some researchers reported significant increase in the crypt depth due to using alpha-mune and biomin in broiler chickens (Erfani-Majd et al., 2013) which are in agreement with the results of the present study. In recent years, it is understood that greater villous height is an indicator that the function of intestinal villi is activated (Shamoto and Yamachi, 2000). Moreover, it was stated that shortening of the villi and deeper crypts may lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, and lower performance (Xu et al., 2003). Although, villous length did not show marked differences compare with other parameters in the present study. Also, the results of the present study showed a significant increase in goblet cell number in all parts of the intestine, especially in the 42th days of sampling. Similarly, a significant increase in the number of goblet cells and in mucin secretion on the surface of the jejunum villi had been observed when feeding broilers by a mixture of carvacrol, cinnamaldehyde, and capsicum oleoresin (Jamroz et al., 2006). It has been suggested that feeding wheat-based diet (containing enzyme Endofeed W (EEW) or growth promoters<sup>®</sup> (thyme essential oil (TEO) or probiotic Primalac<sup>®</sup> (PP)) impact on intestinal histomorphology (jejunum and ileum) of broilers at 21 and 42 days of age (Khaksar et al., 2013), which is in agreement with the present results.

It seems that a period of adaptation is needed before the effects of *G. bronchialis* supplementation can be significant, because the changes in intestinal morphology and immune responses take time.

## CONCLUSIONS

The results of the current study indicate that *G. bronchialis* improves growth performance and affects immune functions, cytokine level, and intestinal mucosal morphology of quails. Body weight gain was best for both *G. bronchialis* supplemented under the experimental conditions of this study. Immune function could be modified with dietary *G. bronchialis* supplementation. The present results suggest that oral administration of *G. bronchialis* (in low dose) can improve the histomorphological structure of the small

intestine without side effects in other vital organs in Japanese quail environmental pollution, outburst of infectious disease and food safety concerns are there serious problems, which effected modern Iranian-farming industry. The current study gave us a cue of using *G. bronchialis* as an immunological stimulant of improve quail's resistance of disease. The multibeneficial effects of *G. bronchialis*, its easy access and the low cost all together made *G. bronchialis* a strong candidate in quail health feeding in Iran.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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