Vol.64: e21200480, 2021
https://doi.org/10.1590/1678-4324-2021200480
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

Article - Human and Animal Health

Eimeria bateri Natural Infection: Oocysts Reductions in Grey Quail (*Coturnix coturnix*) Treated with *Bacillus thuringensis* var. *israelensis*

Yasmine Alves Menegon[1]
https://orcid.org/0000-0002-0871-7300

Aline Arassiana Piccini Roll[2]
https://orcid.org/0000-0002-0671-4675

Natália Berne Pinto[3]
https://orcid.org/0000-0001-6822-501X

Victor Fernando Büttow Roll[2]
https://orcid.org/0000-0002-4928-0299

Fábio Pereira Leivas Leite[1,2*]
https://orcid.org/0000-0003-0941-7286

[1]Federal University of Pelotas, Center for Technological Development, Biotechnology, Laboratory of Microbiology, Rio Grande do Sul, Brazil; [2]Federal University of Pelotas, School of Agronomy Eliseu Maciel, Department of Animal Science, Rio Grande do Sul, Brazil; [3]Federal University of Pelotas, Parasitology Graduate Program, Rio Grande do Sul, Brazil.

Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Cheila Roberta Lehnen

Received: 2020.07.27; Accepted: 2021.02.18.

*Correspondence: fleivasleite@gmail.com; Tel.: +55-53-99127-6663 (F.P.L.L.).

HIGHLIGHTS

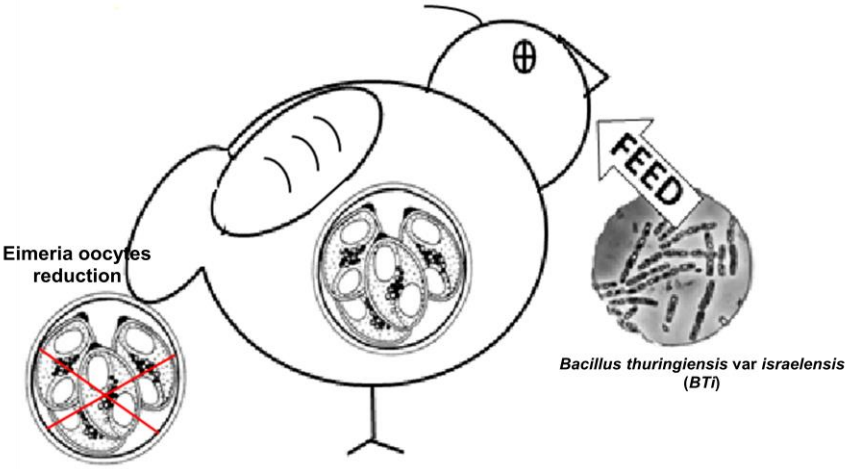
- Coccidiosis cause important economic losses for poultry industry.
- *Bti* produce proteins with high specific parasiticidal effect.
- *Bti* reduce the number of *Eimeria bateri* oocysts in quail.

Abstract: Coccidiosis, a disease caused by the parasitic *Eimeria* spp., affects birds of all ages, particularly young birds more intensely. Infected poultry presents significant economic losses. *Bacillus thuringiensis* var *israelensis* (*Bti*) is a Gram-positive, spore-forming bacterium that produces proteins with high specific parasiticidal activity against various orders of parasites. Thus, the aim of the present study was to evaluate the parasiticidal potential of *Bti* in quails that were naturally infected with *Eimeria bateri*. Twenty 12-week-old male quails (*Coturnix coturnix coturnix*), naturally infected with *Eimeria bateri*, were randomly divided into two groups of 10 birds: *Bti* treated and control. The treated group was supplemented with *Bti* (1×10^8 spores·g^{-1}) in the feed, while; the control group received the same feed without *Bti*. To evaluate the occurrence of oocysts, samples of feces were collected every week for four weeks. Significant ($P < 0.05$) oocysts reductions of 56.64% and 94.51% were noted in the *Bti* treated group at 2nd and 4th week of study, respectively. The *Bti* supplementation may contribute to the reduction of oocysts in quails and environmental contamination.

Bacillus thuringiensis var *israelensis* appeared to be a promising complementary alternative in *E. bateri* control.

Keywords: biological control; Coccidiosis; intestinal protozoa.

GRAPHICAL ABSTRACT



INTRODUCTION

Coccidiosis is a parasitic disease that has sever adverse economic impact on the poultry industry worldwide. Most of the loss is caused due to the costs incurred in prophylactic measures, mortality, feed malabsorption, and reduction in egg production [1]. The life cycle of *Eimeria* includes extracellular/intracellular and sexual/asexual stages, with a complex immune response by the host [2]. Immunity to *Eimeria* spp. is species-specific; therefore, birds immune to one *Eimeria* species may not have protection against other *Eimeria* species. Two species of quail are breed commercially. They are "Gray quail" (*Coturnix coturnix*) a broiler quail, and Japanese quail *(Coturnix japonica*) used for laying. In this quails, different *Eimeria* species have been reported, and three of them have been described in Brazil in *C. japonica*, *Eimeria bateri*, *E. tsunodai* and *E. uzura* [3]. *Eimeria bateri* was originally described from Indian quails and is present all around the world [4]. *Eimeria bateri* can infect and develop its entire life cycle in quails, and it shed a greater quantity of oocysts during the infection. Nonetheless, *E. bateri* infection is considered an important disease since its endogenous stages and high number of oocysts in feces might be associated with intestinal lesions [5].

Currently, there are two main procedures to control coccidiosis: drugs and vaccines [6]. Drugs need to be managed regularly due to development of resistance [7]. With the development of resistance to drugs, importance has been given to vaccines. The commercially available coccidiosis vaccines are based on the principle that *Eimeria* spp. can induce a protective immunity when consecutives low dose infection occurs, and by doing so developing robust immunity [8]. Currently, the available coccidiosis vaccines can be divided basically in three groups: live virulent strains, live attenuated strains, and live strains that are relatively tolerant to the ionophore compounds. This last one gives a new prospective to the anticoccidial vaccine development [9]. The advantage of these vaccines is that they allow the use of ionophores during the first weeks when the birds are still susceptible, and immunity is not achieved. However, each one of these vaccines has its limitation, been a major drawback of live vaccines is their limited shelf life and relatively high production costs associated with attenuation [10].

Bacillus thuringiensis is a Gram-positive bacterium that produces crystal inclusions upon sporulation. These inclusions are comprised mostly of crystal (Cry) and cytotoxic (Cyt) proteins, which are toxic to a wide range of insect classes, such as Lepidopteran, Diptera, Coleoptera and Nematode [11,12]. *Bacillus* sp. strains, including the *B. thuringiensis* var*. israelensis (Bti),* has significant toxic activity against larvae of the important livestock parasite [13–15]. The toxins present in the proteins produced by *Bti* make pores in the membrane and subsequent lysis [16].

There is an important increase in the resistance of coccidia to the control drugs. And the high costs of vaccines associated with their short protection make it necessary to find alternative methods for coccidiosis controls. Nevertheless, there is scarce information of *Bti* and its possible role in the controlling of *Eimeria* species-induced infections in quails [17,18]. Thus, this study evaluated the activity of *Bti* in *E. bateri* in quails.

MATERIAL AND METHODS

Bacillus thuringiensis var. israelensis

Spores of the *Bti* strain (from the collection of the Universidade Federal de Pelotas, Departamento de Microbiologia e Parasitologia), was used in this study. Briefly, the *Bti* was cultured in a 1-L Erlenmeyer flask containing 200 mL of Nutrient Yeast Extract Salt Medium (NYSM) [19] and grown at 30 °C with rotary shaking at 150 rpm for 72 h. Then, the culture was checked for purity, sporulation, and colony-forming units per milliliter. The culture was centrifuged at 8500 × *g* for 20 min at 4 °C. The pellets were washed twice in saline (0.9% NaCl, Sigma-Aldrich, St. Louis, Missouri, USA, and purified water) to remove cell debris and secretory products, then suspended in saline (pH 7.0), and stored at 4 °C until further use.

Quails

The experiment was conducted in the Laboratório de Ensino e Experimentação Zootécnica e Prof. Renato R. Peixoto (LEEZO) at Departamento de Ciência Animal – FAEM – UFPel, with gray quails which has been developed of in the same department by individual selection for body weight. Twenty gray quail's male twelve weeks old, weighting ~ 297.2 grams and naturally infected with *E. bateri* were used in this study. The birds were individually housed in metal cages equipped with gutter-type metal feeders and nipple drinkers, in the same room, with controlled temperature around 25 ºC and cycles of 17 h of light and 7 h of darkness. During the experimental period, the birds received water *ad libitum* and the feed was provided daily. Two groups of 10 birds each were separated randomly to form a *Bti*-treated group and a control group. The birds were weighted at the beginning (day zero) and at the last day of the experiment. The control birds were fed on commercial feeds without any antimicrobial agent (MigCodor, Mig-Plus agroindustrial, RS, Brazil), while the *Bti*-treated group was fed on the same feed supplemented with $1\times10^{8}\,g^{-1}$ of viable *Bti* spores daily. The birds have been kept in sanitary and well-being conditions within international animal production standards, with a Veterinarian supervision.

Fecal samples

Feces were collected directly from the cages, every week, and the fecal samples were placed into plastic bags, identified, for later processing at the Laboratory of Parasitic Diseases, School of Veterinary medicine, Federal University of Pelotas. For oocysts preparation, three aliquots of each sample were separated and diluted in 2.5% aqueous potassium dichromate ($K_2Cr_2O_7$) and kept in Petri dishes for sporulation at room temperature. After sporulation, the oocysts were recovered by centrifugation with a saturated sugar solution as described by Duszynski and Wilber (1997) [20] and were used in subsequent analysis. For the counting of the oocysts, a Carl Zeiss binocular microscope with immersion objective (100 x) was used. The number of oocysts per gram of feces was determined according to the technique described by Menezes and Lopes [21]. The *Eimeria* characterization was base in its sporulated oocysts morphology following Teixeira and coauthors and Berto and coauthors [22,3]. Briefly, oocysts subspherical to ellipsoidal, bi-layered and smooth, ~1.0 thick were characterized as *E. bateri*.

All analyzes were performed in triplicate and the protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 7087) of the Universidade Federal de Pelotas (UFPel). The UFPel-CEEA agreement has been approved by the Brazilian National Council for Animal Experimentation Control (CONCEA).

Statistical analysis

The number of oocysts per group (*Bti*-treated and control) was analyzed using Statistix 8.0®, (Statistix, Tallahassee, FL, USA). The difference in the quantity between the groups were determined using the Shapiro-Wilk Normality Test, followed by Tukey's HSD all-pairwise comparisons, with $P < 0.05$. For the weight difference between groups a Student t test with a $P< 0.05$ were used. The figures were drawn in GraphPad Prism 5.0 program (GraphPad Software Inc., San Diego, CA, USA). In order to evaluate the efficacy of treatment by the end of the fourth week, following equation was used:

E (%) = 100 × (the number of oocysts in control – the number of oocysts in *Bti*-supplemented group) / number of oocysts of control.

RESULTS

In fecal exams, a sporulated oocysts subspherical, ovoid or ellipsoid were observed. The oocyst wall was smooth, double layered, with brownish inner layer and colorless outer layer. In the first week of the experiment there was no statistical difference in the number of oocysts between the control group (n = 5683) and the group *Bti*-treated (n = 7900).

From the second week of treatment onwards, a progressive reduction in oocyst numbers was observed in the *Bti*-treated group. In the second week this reduction was 37.73% (n = 3550), in the third week it was 43.96% (n = 1316), and in the fourth week 80.76% (n = 433), compared to the control group (Figure 1A).

Compared with infection at the beginning of treatment, a significant reduction ($P < 0.05$) in oocytes of the *Bti*-treated group was observed amounting to 56.64% and 94.51% at the second- and fourth-weeks post-treatment, respectively (Figure 1B).

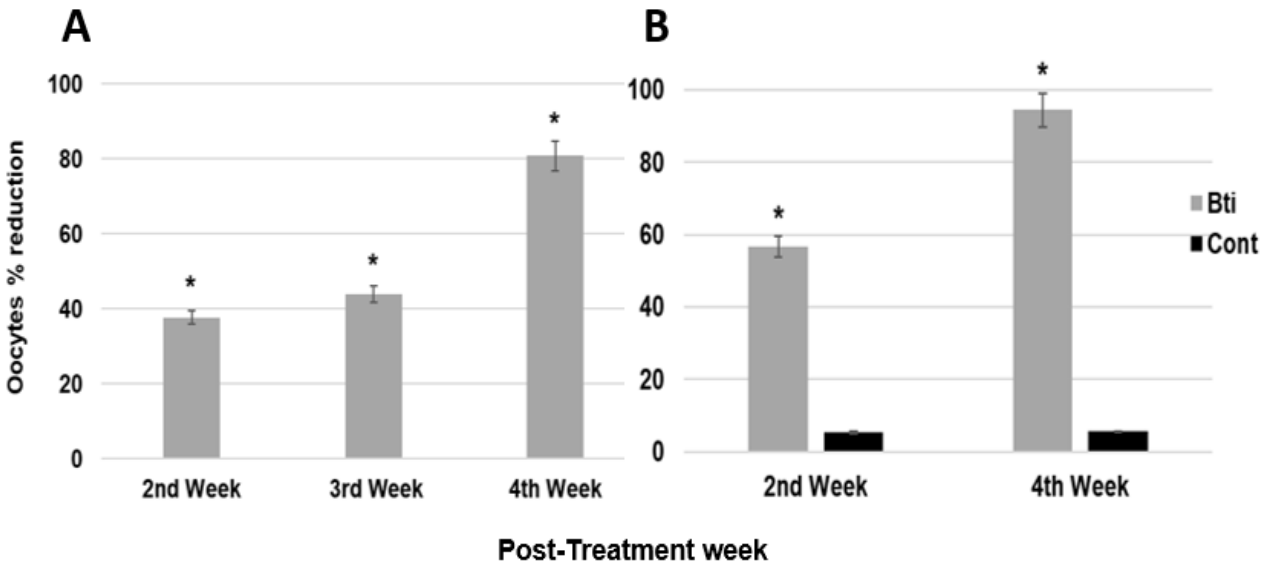


Figure 1. Percentage of oocysts reduction. **A**. The data represent the oocysts reduction in feces of treated *Bti* compared with the control groups oocysts reduction, during the 2nd, 3rd and 4th weeks post-treatment. **B**. The data represent the oocysts reduction in feces of treated *Bti* compared with the control groups oocysts reduction from the first week of experiment. Asterisk (*) represent statistic difference ($P < 0.05$) between supplemented and control group.

Evaluating the weight of the birds at the end of period of study we observed that the *Bti* group had mean weight of 339.68 g representing a gain of 40.32 g (+/- 7.79 S.D), and the control group a mean weight of 327.52 g representing a gain of 31.93 g (+/- 3.43 S.D). However, the weight gain difference was not significant (P=0.236) between the groups.

DISCUSSION

The identification of the species of *Eimeria* that infected the quails was made based on the observed morphological characteristics and comparing with the description of Teixeira and coauthors [22] and Berto and coauthors [3].

In this study, a significant effect on the reduction of *E. bateri* oocysts in the feces of *Bti*-supplemented quails was observed. By the second week of *Bti* administration, a significant ($P < 0.05$) reduction (37.73%) in oocysts was observed and this reduction was more pronounced at the fourth week, amounting to 80.76%. During the same time period, the variation in the oocysts number in the control group was 5.2% and 5.6%, respectively. Sun and coauthors also found a significant reduction of 62.5% of *Eimeria tenella* oocysts in chicken after 4 weeks of treatment with another probiotic microorganism, *Saccharomyces cerevisiae* [23].

In other studies performed by our group, it was possible to demonstrate that *Bti*, administered to cattle and sheep, has a larvicidal effect on the nematode *Haemonchus contortus,* which is an important livestock parasite [13–15]. In protozoa, experimental studies using *Bacillus* spores on the control of *Cryptosporidium*, *Giardia*, and *Eimeria* demonstrated the toxic activity that can inhibit parasite development [24]. However, to the best of our knowledge, no *Bti* active molecule has thus far been reported as acting against *Eimeria* spp. that could reduce oocyte production.

One might suggest that a possible role mediated by *Bti* is to compete with *Eimeria* for nutrients and/or the ecological environment in the intestinal tract [25]. *Eimeria* need to invade the cell to replicate, however, first it needs to adhere to the cell surface, so if the *Bacillus* dispute for the same site less *Eimeria* will adhere, penetrate and replicate, and as a result less oocysts shedding may occur. Another mechanism used by *Bacillus* against pathogens is the capacity of stimulating both innate and adaptive immune responses by activating intestinal epithelial cells and immune cells, providing protection in the intestinal mucosa of the host [26]. Dalloul and coauthors demonstrated that chickens supplemented with probiotics had more intestinal intraperitoneal lymphocytes expressing surface-marked CD4+, CD8+, and αβTCR and a reduced number of *Eimeria* oocysts in the feces [27]. We recently demonstrated that *Bacillus toyonensis* have the ability to stimulate cytokine production, which drives the development of the local and systemic immune responses [28, 29]. Therefore, cytokines expressed during *Bti* treatment may have a major role by leading a more rapid immune response to *Eimeria*. Lillehoj and Choi showed the role of interferon gamma (IFN-g) in the development of resistance to *Eimeria*, demonstrating that IFN-g inhibits *E. tenella* development *in vitro* and reduces oocysts production [30].

Even not observing statistical difference in weight gain for the Bti group, one may suggest that the difference of 9 grams in the mean weight in favor of the Bti group might be relevant, considering the weeks and the number of birds evaluated.

Nevertheless, proposing the use of *Bti* as an alternative to conventional treatments, such as drugs or vaccines to control *Eimeria* spp. appears unreasonable. However, it is suggested that *Bti* may be used as a complementary method to reduce oocyst infestation to improve *Eimeria* control. A better understanding of molecular mechanisms underlying the beneficial effects of *Bti* on *Eimeria* infection control is essential to validate the approach.

Conflicts of Interest: The authors have no conflicts of interest to declare. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

There was no unnecessary cruelty in animal experimentation.

REFERENCES

1. Chapman HD. Milestones in avian coccidiosis research: a review. Poult Sci. 2014 Mar;93(3):501–11.
2. Lillehoj HS. Role of T lymphocytes and cytokines in coccidiosis. Int J Parasitol. 1998 Jul;28(7):1071–81.
3. Berto BP, Borba HR, Lima VM, Flausino W, Teixeira-Filho WL, Lopes CWG. Eimeria spp. from Japanese quails (Coturnix japonica): new characteristic features and diagnostic tools [Internet]. Vol. 33, Pesquisa Veterinária Brasileira. 2013. p. 1441–7. Available from: http://dx.doi.org/10.1590/s0100-736x2013001200008
4. Shah HL, Johnson CA. Eimeria bateri Bhatia, Pandey and Pande, 1965 from the Hungarian quail Coturnix c. coturnix in the United States and its attempted transmission to the chicken. J Protozool. 1971 May;18(2):219–20.
5. Norton CC, Peirce MA. The Life Cycle of Eimeria bateri(Protozoa, Eimeriidae) in the Japanese Quail Coturnix coturnix japonicum [Internet]. Vol. 18, J. Parasitol. 1971.p. 57–62. Available from: http://dx.doi.org/10.1111/j.1550-7408.1971.tb03280.x
6. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev vaccines, 2006,5.1:143-63.
7. Abbas RZ, Iqbal Z, Blake D, Khan MN, Saleemi MK. Anticoccidial drug resistance in fowl coccidia: the state of play revisited. Worlds Poult Sci J, 2011, 67.2:337-50.
8. Conway DP, Mckenzie, ME. Poultry coccidiosis: diagnostic and testing procedures. John Wiley & Sons, 2007
9. Vermeulen AN, Schaap DC, Schetters, ThPM. Control of coccidiosis in chickens by vaccination. Vet. Parasitol, 2001,100.1-2:13-20.
10. Shah MAA. DNA vaccines as sustainable Coccidiosis control strategies in chickens. Sci Lett. 2013,1:1–4.
11. Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C, Panwar H. Bacillus As Potential Probiotics: Status, Concerns, and Future Perspectives [Internet]. Vol. 8, Frontiers in Microbiology. 2017. Available from: http://dx.doi.org/10.3389/fmicb.2017.01490
12. Bravo A, Likitvivatanavong S, Gill SS, Soberón M. Bacillus thuringiensis: A story of a successful bioinsecticide. Insect. Biochem Mol Biol. 2011 Jul;41(7):423–31.
13. Sinott MC, Cunha Filho NA, Castro LLD, Lorenzon LB, Pinto NB, Capella GA, et al. Bacillus spp. toxicity against Haemonchus contortus larvae in sheep fecal cultures [Internet]. Vol. 132, Exp Parasitol. 2012. p. 103–8. Available from: http://dx.doi.org/10.1016/j.exppara.2012.05.015

14. Sinott MC, de Castro LLD, Leite FLL, Gallina T, De-Souza MT, Santos DFL, et al. Larvicidal activity of Bacillus circulans against the gastrointestinal nematode Haemonchus contortus in sheep [Internet]. Vol. 90, J. Helminthol. 2016. p.68–73. Available from: http://dx.doi.org/10.1017/s0022149x14000844
15. De Lara APDESS, Lorenzon LB, Vianna AM, Santos FDS, Pinto LS, et al. Larvicidal activity of Bacillus thuringiensis var. israelensis Cry11Aa toxin against Haemonchus contortus [Internet]. Vol. 143, Parasitol. 2016. p. 1665–71. Available from: http://dx.doi.org/10.1017/s0031182016001451
16. Parker MW, Feil SC. Pore-forming protein toxins: from structure to function. Progress in biophysics and molecular biology, 2005. 88(1), 91-142.
17. Dalloul RA, Lillehoj HS, Tamim NM, Shellem TA, Doerr JA. Induction of local protective immunity to Eimeria acervulina by a Lactobacillus-based probiotic. Comp Immunol Microbiol Infect Dis. 2005 Sep;28(5-6):351–61.
18. Lee S, Lillehoj HS, Park DW, Hong YH, Lin JJ. Effects of Pediococcus - and Saccharomyces-based probiotic (MitoMax®) on coccidiosis in broiler chickens [Internet]. Vol. 30, Comparative Immunology, Microbiology and Infectious Diseases. 2007.p.261–8. Available from: http://dx.doi.org/10.1016/j.cimid.2007.02.002
19. Yousten AA. Bacillus sphaericus: microbiological factors related to its potential as a mosquito larvicide. Adv Biotechnol Processes. 1984;3:315–43.
20. Duszynski DW, Wilber PG. A guideline for the preparation of species descriptions in the Eimeriidae. J Parasitol. 1997 Apr;83(2):333–6.
21. Menezes RCA, Lopes CWG. [Epizootiology of Eimeria arloingi in goats in the Serrana Fluminense microregion, Rio de Janeiro, Brazil]. Journal of the Federal Rural University of Rio de Janeiro. 1995. 5:12–2. Portuguese.
22. Teixeira Filho WL, Lopes CWG. Coccidiosis in japanese quails (Coturnix japonica): characterization of a naturally occurring infection in a commercial rearing farm [Internet]. Vol. 6, Revista Brasileira de Ciência Avícola. 2004. p. 129–34. Available from: http://dx.doi.org/10.1590/s1516-635x2004000200010
23. Sun H, Wang L, Wang T, Zhang J, Liu Q, Chen P, et al. Display of Eimeria tenella EtMic2 protein on the surface of *Saccharomyces cerevisiae* as a potential oral vaccine against chicken coccidiosis. Vaccine. 2014 Apr 1;32(16):1869–76.
24. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J Gastroenterol. 2009 Jan 22;44(1):26–46.
25. Pinchuk IV, Bressollier P, Verneuil B, Fenet B, Sorokulova IB, Mégraud F, et al. In vitro anti-Helicobacter pylori activity of the probiotic strain Bacillus subtilis 3 is due to secretion of antibiotics. Antimicrob Agents Chemother. 2001 Nov;45(11):3156–61.
26. Forsythe P, Bienenstock J. Immunomodulation by commensal and probiotic bacteria. Immunol Invest. 2010;39(4-5):429–48.
27. Dalloul RA, Lillehoj HS, Shellem TA, Doerr JA. Enhanced mucosal immunity against Eimeria acervulina in broilers fed a Lactobacillus-based probiotic. Poult Sci. 2003 Jan;82(1):62–6.
28. Santos FDS, Menegon YA, Piraine REA, Rodrigues PRC, Cunha RC, Leite FPL. Bacillus toyonensis improves immune response in the mice vaccinated with recombinant antigen of bovine herpesvirus type 5. Benef Microbes. 2018 Jan 29;9(1):133–42.
29. Habil N, Al-Murrani W, Beal J, Foey A. Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain-dependent and cell subset-specific manner [Internet]. Vol. 2, Benef Microbes. 2011. p. 283–93. Available from: http://dx.doi.org/10.3920/bm2011.0027
30. Lillehoj HS, Choi KD. Recombinant chicken interferon-gamma-mediated inhibition of Eimeria tenella development in vitro and reduction of oocyst production and body weight loss following Eimeria acervulina challenge infection. Avian Dis. 1998 Apr;42(2):307–14.