TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2020) 44: 534-541
© TÜBİTAK
doi:10.3906/vet-1907-95

Humoral immunity provided by a novel infectious bronchitis vaccine supplemented by bacterium-like particles (BLPs)

Emadodin TOHIDI[1], Abolfazl GHANIEI[2,*], Hamidreza FARZIN[3], Alireza HAGHPARAST[4]

[1]Faulty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[3]Mashhad Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran
[4]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Received: 22.07.2019 • **Accepted/Published Online:** 22.02.2020 • **Final Version:** 02.06.2020

Abstract: Infectious bronchitis (IB) is a notable disease of poultry flocks that results in economic loss. As a consequence of the presence of various IB virus (IBV) serotypes, control strategies, such as vaccination, should be replaced by provide broad protective immunity against the disease to date. Gram-positive enhancer matrix particles, or so-called bacterium like particles (BLPs), obtained from the bacterium *Lactococcus lactis* (*L. lactis*), have demonstrated an adjuvancy effect by providing demanding mucosal and humoral immune responses, as well as a protective cellular immunity whenever delivered admixed with a vaccine via intranasal or intraocular administration. In this study, for the first time, attempts were made to investigate the impact of an IBV vaccine supplemented by various doses of BLPs on induced levels of humoral immunity against IB. For this purpose, increasing doses of derived BLPs (0, 0.15, 0.3, and 0.6 mg dry weight per bird) were admixed with IBV live attenuated H120 serotype vaccine, and were delivered via ocular administration to 4 equal groups of 10 specific pathogen-free (SPF) chickens in 4 groups: control, BLP1, BLP2, and BLP3, respectively. In addition, 10 SPF chickens that were not immunized comprised the Unvaccinated group. Blood was collected from 5 members of each group weekly for 35 days. Levels of IgG antibodies in the sera were then assayed using ELISA. Weight gain and the feed conversion ratio of each group were also recorded weekly. Finally, 3 birds from each group were necropsied to evaluate probable lesions. The best results were obtained in the BLP1 group, with IBV vaccination at a low dose of admixed BLPs boosted immediate anti-IBV humoral responses; however, the results were not significantly different from those of the Control group, but were still feasible enough for application in the field. In conclusion, BLPs could be a desirable adjuvant for IBV vaccines to increase immunity in chickens.

Key words: Bacterium like particles, IgG, adjuvant, infectious bronchitis, chicken

1. Introduction

Infectious bronchitis (IB) is a highly prevalent respiratory disease in chickens. The IB virus (IBV) enters through mucosal surfaces of the respiratory system, where virus replication also occurs. Subsequent to a viremia, the virus infects the kidneys, and gastrointestinal and urogenital tracts. Infection of the mentioned organs causes symptoms such as conjunctivitis, respiratory distress, and pathological alterations in the kidneys and trachea, as well as poor weight gain and a low number of eggs in broilers and layers, respectively [1]. In the case of existing accompanied secondary bacterial infection, high mortality is expected from the disease. Thus, IB has a significant economic influence on the poultry industry [2]. The causing agent is a gamma-coronavirus possessing 4 structural proteins, and among them, the S protein (S1 and S2 subunits) is responsible for attachment and entry through the cells [1]. These subunits are exploited in virus identification and antigenic neutralization, and along with a nucleocapsid protein, which contains CTL-inducing epitopes, are capable of triggering host immune responses. Differences in the amino acid sequence of the S1 subunit gene, in addition to recombination between IBV strains, result in the emergence of multiple new variants that are not cross-protected. Apparently, the use of live attenuated vaccine types or the presence of multiple concurrent infections of different IBV serotypes also contributes to this recombination process. Thus, some countries only allow vaccination using one or a few vaccine types, which makes strategies for preventing IB even more complicated. As a consequence, controlling IB has not been completely achieved thus far [3–6].

* Correspondence: ghaniei@um.ac.ir

It has been revealed that while no association exists between circulating antibodies and IB protection [6], both the local antibody response and cell-mediated immunity correlate greatly with protection. Nevertheless, monitoring IgG antibodies still remains as an important method for evaluating the immune response to an IBV challenge [7].

IB could initially be prevented by considering vaccination and biosecurity methods. Immunization by either live or inactivated vaccines of various serotypes has been recognized as the main control strategy against IB. Due to the requirements of multiple immunizations, such as large doses of adjuvants as well as live priming, the application of inactivated vaccines is costly and even less efficient when compared to live types. The existence of so many IBV strains, along with their antigenic alterations, is a huge burden for highly protective immunization. To develop an efficient anti-IBV vaccine, factors such as age, maternally-derived antibodies (MDAs), and the immune competence of the bird, along with immunogenicity of the vaccine, the virulence, and type of the challenge strain, as well as the interval between vaccination and the challenge, should be considered. Correspondingly, various novel promising IB vaccines have been developed; however, their application in the field has been challenged by existing drawbacks, such as the low efficiency and immunogenicity of DNA and peptide vaccines, respectively, as well as safety concerns about the cytotoxicity of the components of nanoparticle vaccine delivery, along with difficulty in maintaining stability and displaying antigens on nanoparticles [1,3,8–10].

Wang [11] has cited that Roozmalen et al. developed an antigen delivery platform.. in his paper.so the number should be placed at the end of statement. developed an antigen delivery platform comprising bacterium-like particles (BLPs) for mucosal vaccines. The BLPs were derived from *Lactococcus lactis* (*L. lactis*), a nonpathogenic gram-positive bacterium that is considered as a generally recognized and safe organism. As BLPs are the size of a bacterium, their uptake by membranous cells is easily facilitated [12]. Their advantages comprise reducing secondary effects, the applicability of either a monovalent or a polyvalent vaccine, along with decreasing bacterial colonization and amplification in the lungs, being easily administered for mass immunization, not requiring booster immunization, triggering both the systemic and mucosal immune systems, possessing a higher binding capacity, as well as less anticarrier response, when compared to to a living lactic acid bacteria (LAB) [13]. Surprisingly, their capacity for being stored without the need for a cold chain over a long period of time was also noticeable [14]. BLPs are able to reduce the bacterial load in the lungs [14]. Additionally, it was found that when a secondary bacterial infection also existed, the mortality of young chickens caused by IBV was higher [15]. Thus, BLPs could probably be a great advantage against mortality from IB, particularly in young chickens.

In the current experiment, the influence of BLPs admixed with an IBV vaccine delivered via ocular administration on levels of anti-IBV serum IgGs of specific pathogen-free (SPF) chickens was investigated and attempts were made to discover the proper dose of BLPs that induced the best humoral immune response against IB. Performance data and necropsy lesions of the birds were also evaluated. To our knowledge, it was the first application of BLPs in favor of poultry health.

2. Materials and methods

2.1. BLP preparation

Lyophilized *L. lactis* was obtained from a microorganism bank. Afterwards, it was transferred into a proper culture medium (M17 broth supplemented with glucose). The inoculated bacterium in liquid culture medium was then centrifuged at 18,000 rpm for 10 min before washing to remove the surface solution and subsequently, 10% trichloroacetic acid was added to the pellet. Following vortexing, the suspension was placed in boiling water for 5 min. The resultant solution was centrifuged and then the pellet was washed with phosphate-buffered saline (PBS). After the last centrifugation, PBS was added to the pellet. Finally, it was stored at –70 °C until use. This method was performed in accordance with a previous study [16] with some modifications.

IBV live attenuated H120 serotype was obtained from the Razi Vaccine and Serum Research Institute, in Iran, and admixed with 3 doses of BLPs at 0.15, 0.3, and 0.6 mg dry weight per bird. These BLP doses, except for the dose administered to the BLP1 group (BLPs at 0.15 mg dry weight per bird) were previously used by Wang et al. [11].

2.2. Experimental design

A total of 50 SPF chicken eggs were purchased from an Indian company (Venky's Poultry Products, Pune, MH, India) and hatched in an incubator. Afterwards, these 50 SPF chickens were divided randomly into 5 equal groups. While one group was not vaccinated (Unvaccinated group), the other 4 groups (Control, BLP1, BLP2, and BLP3 groups) were immunized at 1 day old with a single dose of Razi IBV live attenuated H120 serotype via ocular administration, and in addition, the BLP1, BLP2, and BLP3 groups received the vaccine admixed with BLPs at 0.15, 0.3, and 0.6 mg dry weight per bird, respectively.

2.3. Blood sample collection and ELISA assay

Blood was collected via brachial venipuncture from the wings of 5 day-old SPF chickens on day 1 prevaccination and then from 5 birds of each group (birds of each group were marked with 5 different colored leg bands in advance) weekly, for 35 days, in the same manner as mentioned above. Blood samples on days 7, 14, 21, 28, and 35 postvaccination (p.v.) were obtained and kept in microtubes at 4 °C in the

refrigerator. A total of 130 samples were collected, and the relevant sera were subsequently extracted by centrifugation at 4000 × g for 5 min and stored at −20 °C until evaluation.

Sera samples were measured for titers of the IgG antibodies against IB using the ELISA chicken IgG antibodies kit, commercially available from IDVet (Grabels, France), according to the manufacturer's instructions.

2.4. Recording of the weekly weight gain and feed conversion ratio

All SPF chickens in the 5 groups were weighed weekly and the measured weight values were recorded for later weight gain evaluation. Weekly feed intake of the birds was also recorded to estimate the feed conversion ratio (FCR) of each group at the end of each week.

2.5. Postmortem examinations

From each group, 3 SPF chickens were euthanized at 35 days of age and different specific organs, such as the bursa of fabricius, cecal tonsils, thymus, kidney, spleen, liver, and lung, as well as trachea, were evaluated for probable lesions.

2.6. Statistical analysis

Values of the anti-IBV serum IgG titers and weights of the birds were presented as the mean ± SD. ANOVA coupled with the Tukey post hoc test was used to compare the means of the variables recorded among the groups. Repeated measure ANOVA was used to analyze changes of the variables of each group over time. Statistical analyses were performed using SPSS software v.16 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. IBV-specific IgG titers

Expectedly, over the duration of the experiment, the serum IgG titer levels in the Unvaccinated group (* < 27.6 ± 4.93), and from days 1 to 7 p.v. in all of the groups (* < 102 ± 143.96), were consistently negligible. No significant difference was observed among all groups within the first 7 days of the experiment (Table 1).

From days 7 to 14 p.v., a more prolonged serum IgG response was observed in the BLP3 (from 102 ± 143.96 to 758 ± 1257.68) and Control (from 59 ± 16.64 to 866 ± 530.10) groups when compared to the BLP1 (from 39.4 ± 12.97 to 1897.60 ± 1266) and BLP2 (from 62.6 ± 51.97 to 1654.6 ± 1013.27) groups (Figure). On day 14 p.v., the highest serum IgG titer levels were observed in the BLP1 group (1897.60 ± 1266); however, no significant differences were detected between the serum IgG titers of the groups, except in the BLP1 and the Unvaccinated groups (Table 1).

From days 14 to 21 p.v., the lowest among the vaccinated groups surge was observed in the BLP2 group (from 1654.6 ± 1013.27 to 2641.8 ± 1682.71) (Figure). On day 21 p.v., the highest serum IgG titer levels were observed in the BLP1 (3718.2 ± 640.96) group, whereas the serum IgG values in the BLP2 and the BLP3 groups were within the same range, while those of the Control group (2468.4 ± 433.12) were the lowest among the vaccinated groups. No significant difference was observed between the serum IgG titers of the vaccinated groups (Table 1).

From days 21 to 28 p.v., the highest surge in the serum IgG titer levels was observed in the Control group (from 2468.4 ± 433.12 to 3614 ± 1161.82) when compared to the other groups (Figure). On day 28 p.v., while the serum IgG titer levels of the Control group (3614 ± 1161.82) exceed those of the BLP2 group (3399.2 ± 2697.66), the serum IgG values of the BLP2 and BLP3 groups were within the same range. No significant difference was observed between the serum titers of the vaccinated groups (Table 1).

From days 28 to 35 p.v., surprisingly, the serum titer levels of the BLP3 group decreased (from 3405.4 ±

Table 1. Mean ± SD of the weekly recorded IBV-specific serum IgG titer levels in the 5 different groups of birds over the 35 days of the experiment. Mean titer of the day-old chickens was 33.8 ± 16.28.

Groups	Sampling day				
	7	14	21	28	35
Unvaccinated	15.8 ± 3.83^{Aa}	17 ± 3.08^{Aa}	22.4 ± 2.4^{Aab}	27.6 ± 4.93^{Ab}	21.2 ± 3.03^{Aab}
Control	59 ± 16.64^{Aa}	866 ± 530.10^{ABa}	2468.4 ± 433.12^{Bb}	3614 ± 1161.82^{Bbc}	4493.6 ± 934.5^{Bc}
BLP1	39.4 ± 12.97^{Aa}	$1897.60 \pm 1266.19^{Bab}$	3718.2 ± 640.96^{Bbc}	4186.8 ± 1147.55^{Bc}	4964.8 ± 1566.1^{Bc}
BLP2	62.6 ± 51.97^{Aa}	1654.6 ± 1013.27^{ABa}	2641.8 ± 1682.71^{Ba}	3399.2 ± 2697.66^{Ba}	3724.4 ± 2892.68^{Ba}
BLP3	102 ± 143.96^{Aa}	758 ± 1257.68^{ABab}	2617 ± 1034.84^{Bbc}	3405.4 ± 1469.81^{Bc}	3326 ± 1011.07^{Bc}

$^{A, B, C}$ Values with different superscripted uppercase letters indicate statistically significant differences among the groups at each time point ($P < 0.05$).

^{a,b,c} Values with different superscripted lowercase letters indicate statistically significant differences between the data in the same row ($P < 0.05$).

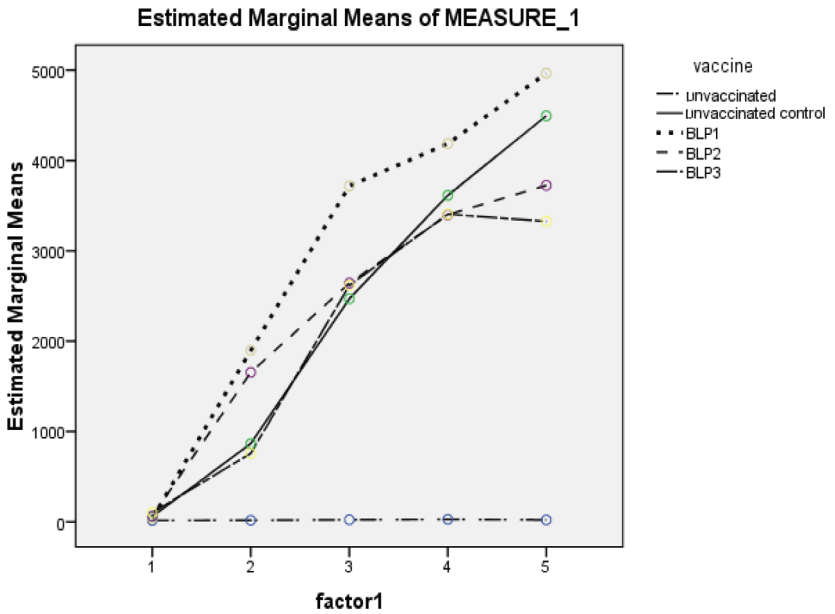

Figure. Trend of the estimated means of the IBV-specific serum IgG titer levels using ELISA among the 5 groups (Unvaccinated, Control, BLP1, BLP2, and BLP3) over the 35 days of the experiment.

1469.81 to 3326 ± 1011.07) (Figure). On day 35 p.v., the serum IgG titer levels of the Control (4493.6 ± 934.5) and BLP1 (4964.8 ± 1566.1) groups were higher than those of the BLP2 (3724.4 ± 2892.68) and BLP3 (3326 ± 1011.07) groups. No significant differences were observed between the serum IgG titers of the vaccinated groups (Table 1).

On days 21, 28, and 35 p.v., a significant difference was evident between the serum IgG titer levels of all the vaccinated groups and the group receiving no vaccine (Table 1).

3.2. Weights and FCR

The mean weights of the birds from the highest to the lowest at each time point were in the BLP3, BLP2, BLP1, Control, and Unvaccinated groups, respectively, except for those in the Control group on day 21, which were higher than those of the other groups, excluding the BLP3 group (Table 2).

The lowest recorded FCR was in the BLP1 group throughout the duration of the experiment, while those of both the BLP3 and BLP2 groups were only lower during week 1 (Table 3).

3.3. Necropsy lesions

While no pathologic alterations were observed in the Unvaccinated, Control, BLP1, and BLP2 groups, 2 chickens in the BLP3 group experienced bleeding in the trachea and larynx, along with hemorrhages in the bursa of fabricius and thymus. Minor pneumonia and nephritis, as well as urate deposits in the kidneys, were other observations in these 2 birds.

4. Discussion

This study evaluated BLPs as a novel adjuvant for IBV vaccines in chickens and yielded promising results. As the experiment was performed on SPF chickens, the serum IgG titers were nonsignificant in the Unvaccinated group over the duration of the study and in all of the other groups over the first 7 days. It was assumed that immune system immaturity at the time of vaccination was responsible for such inadequate and/or delayed anti-IBV immune response. Therefore, a significant rise in antibody levels may take time to become evident [17]. This was in accordance with a previous study where a significant discrepancy was not observed until 28 days p.v. [18].

At the end of week 2, the titer levels in the BLP1 (1897.60 ± 1266.19) and BLP2 (1654.6 ± 1013.27) groups were remarkable; however, they were nonsignificant when compared to those of the Control (866 ± 530.10) and BLP3 (758 ± 1257.68) groups. Surprisingly, the titer levels of the BLP3 group were the lowest among the vaccinated groups (758 ± 1257.68) at the end of week 2. This could mean that in comparison with a nonadjuvanted IBV vaccine, low doses of BLPs (0.15 and 0.3 mg dry weight of BLPs per bird) as a vaccine adjuvant were able to rapidly boost immune response against IBV, particularly in younger birds.

The BLP3 group was the only vaccinated group to exhibit a decrease in titer levels (from 3405.4 ± 1469.81 to 3326 ± 1011.07) in the final week p.v. The insufficient serum IgGs response was observed in the BLP2 group to a lesser extend as well, particularly when compared to the

Table 2. Weekly mean weights of the 5 different groups of birds over the 35 days of the experiment (mean ± SD).

Groups	Sampling day					
	1	7	14	21	28	35
Unvaccinated	32 ± 2.3^{Aa}	47 ± 2.1^{Ab}	83.5 ± 4.7^{Ac}	133.5 ± 9.1^{Ad}	204 ± 16.9^{Ae}	278.5 ± 19^{Af}
Control	33.2 ± 2.7^{Aa}	50.5 ± 4.9^{Aa}	91.5 ± 11.7^{ABb}	174.5 ± 19.9^{Bc}	225 ± 33.4^{ABd}	307.2 ± 39.4^{ABe}
BLP1	33.3 ± 1.6^{Aa}	58.8 ± 4.7^{Bb}	99.6 ± 17.3^{BCc}	164.3 ± 24^{BCd}	252.3 ± 31.3^{Be}	341.3 ± 27.8^{BCf}
BLP2	33.5 ± 2.2^{Aa}	60.3 ± 7^{Ba}	108.1 ± 16.8^{Cb}	173.3 ± 32.4^{BCc}	261.8 ± 45.6^{BCd}	345 ± 49.6^{BCe}
BLP3	34.6 ± 3.3^{Aa}	68.5 ± 5^{Cb}	123 ± 9.9^{Dc}	193.3 ± 18.5^{Cd}	292.1 ± 29.2^{Ce}	367 ± 41.3^{Cf}

^{A,B,C,D} Values with different superscripted uppercase letters indicate statistically significant differences among the groups at each time point ($P < 0.05$).
^{a,b,c,d,e,f} Values with different superscripted lowercase letters indicate statistically significant differences between the data in the same row ($P < 0.05$).

Table 3. Weekly FCR records of the 5 different groups of birds during the experiment.

	Week 1	Week 2	Week 3	Week 4	Week 5
Unvaccinated	2.12	2.45	2.65	2.96	2.97
Control	1.73	2.21	1.77	2.52	2.85
BLP1	1.27	1.68	1.73	1.91	2.19
BLP2	1.24	2.01	2.36	2.63	2.84
BLP3	1.09	1.77	2.12	2.38	2.70

Control group, this results in further investigation in the necropsy findings, such as hemorrhage in the lymphoid organs including the bursa of fabricius and thymus of 2 individuals in the BLP3 group.

It was supposed that single-dose vaccine administration could have been a reason for not observing a significant discrepancy between the titers of the groups. As it was expected to observe antibody alterations over a short period such as 35 days, a booster vaccination was excluded, since it could excessively complicate the results. As a consequence, the low-dose BLP-adjuvanted IBV booster on days 7 or 14 p.v. may have resulted in significantly different possible IgG titer levels in the BLP-treated groups. Due to a TLR expression deficiency in chickens at early ages [17] and considering that BLPs codelivered at 1 day old are a toll-like receptor 2 (TLR2) agonist [19], it was speculated that the ultimate potential of the codelivered BLPs in inducing expectative highly significant titer levels in this period could not be attained. Nevertheless, further investigation is required to support this hypothesis.

In addition to providing protective inflammation, TLR agonists are also capable of amplifying exacerbated inflammation, leading to pathological lesions [20]. It has been shown that TLR2, TLR4, and TLR7 are involved in inflammation and renal problems, such as lupus nephritis [21]. Moreover, it has been recognized that TLR7 signaling, leading to interleukin-6 production, takes part in the development of nephritis or tissue injuries in chickens challenged with an IBV strain [22]. Therefore, it was opined that an excess amount of BLPs, as was applied as a high dose in the BLP3 group (0.6 mg dry weight of BLPs per bird), may have caused unwanted inflammatory responses, since renal lesions were observed in 2 chickens in this group.

The best weight records were observed in the BLP3 group (Table 2), even though the best FCR was recorded in the BLP1 group (Table 3). As the mean weight records showed, the codelivered BLPs were highly significantly correlated with weight gain. Interpretation of the performance results was difficult, and it seemed that there existed a need for further investigation.

In summary, it was supposed that in developing a BLP-adjuvanted IBV vaccine, the proper suggested dose of BLPs for providing boosted humoral immune response could be less than 0.3 mg dry weight of BLPs per bird, as was administered in the BLP1 group (0.15 mg dry weight of BLPs per bird) in the current study.

An argument exists regarding whether antibodies are involved in preventing IB or not. In a study by Feng et al.

[7], it was revealed that local and cellular immunity, but not humoral immunity, were involved in protection against IBV. Moreover, it was shown by Bru et al. [15] that anti-IBV antibodies were not always associated with protection. Nevertheless, it was reported that locally-produced anti-IBV IgGs induced by day old vaccination probably play a role in protection against IBV, specifically in controlling upper respiratory tract infection [18]. In another study by Orr-Burks et al. [1], it was demonstrated that anti-IBV IgA titers could be more correlated with protection when compared to anti-IBV IgG titers; however, it was indicated that tear antibody levels were not considered as an accurate parameter of protection against IB. Furthermore, Lopes et al. [23] stated that elicited mucosal IgG and IgA antibodies in the respiratory tract, as well as cell-mediated immunity responses, were important factors in preventing IBV replication and protection against ciliostasis and lesions of the trachea. It was assumed that IgGs are involved in the protection of oviducts in layers by neutralizing IBV serum antigens, resulting in prevention against the persistency of IBV infection in chickens [24]. In a recent study, there existed evidence that the ovaries were considerably less degenerated in a group that received an inactivated booster after a live vaccine than in a group receiving only a live vaccine [24,25]. Feng et al. [7] stated that monitoring IgGs was of crucial importance in evaluating the immune response to an IBV challenge. In another study, the evaluation of serum antibodies for predicting the potency of live IBV vaccination was mentioned as improper since the antibody titer levels induced by vaccination at an early age were low; however, this method was preferred by some regulatory authorities over performing in vivo protection studies [26]. Despite all of the mentioned results, because the immune status of poultry flocks is commonly assessed by serological assays, the measurement of serum antibodies (IgGs) was therefore considered in the design of this study.

It has been supposed that the presence of MDAs impaired active immunization following day-old vaccination nevertheless, some studies have shown that MDAs do not may impair IBV vaccination, since local immunity is probably of far more importance and serum antibodies are not involved in this response [4]. In another experiment, the transfer of B and T cell subpopulations to the upper respiratory tract was not observed in SPF chickens. Thus, despite minor neutralizing vaccine antigens, MDA appeared to have no significant influence over responses induced by an IBV vaccine unless it was administered on the day of hatching [27]. In a study by van Ginkel et al. [17], it was purposed that early immunization did not result in the expected innate and adaptive immune responses. Chickens vaccinated on days 1, 7, or 14 after hatching displayed delayed serum antibody response. Furthermore, a lower antibody production and IgG avidity index, along with increased tracheal inflammation and subsequently, lower protection, were all found in the group vaccinated at 1 day old when compared to the other groups. Thus, immunization at 1 week after hatching was proposed as an alternative for the day-old vaccination. As the lack of TLR expression in day-old chickens was probably the cause of lower antibody affinity maturation and subsequently lower protection, exploiting a TLR-activating adjuvant was suggested to boost antibody production and elevate affinity maturation in day-old chickens, which could consequently help to avoid the challenges of vaccination at an early age [17]. In this study, it was decided to administer day-old vaccination for the birds since it is routinely performed in poultry flocks in Iran as current practice.

BLPs are able to act as an adjuvant because they mediate TLR2 signaling through their cell-wall components. Neonatal DCs, CD4, and $CD8^+$ cell stimulation, as well as proinflammatory and Th1 polarizing cytokine secretion in favor of biased Th1 type T cell response [28-31], along with their role in Th17 activation in a TLR2-dependent manner make their activity potent, even when simply mixed with a subunit antigen. On the whole, TLR agonists, because of their immune stimulation potency, have been noted as efficient future adjuvants [32]. Thus, the TLR2 mediating activity of BLPs, in addition to their safety properties, makes them an eligible candidate as a promising alternative adjuvant for an IBV vaccine.

Other successful applications of BLPs in previous studies have included: the prevention of bacterial infections in early life by *L. lactis* germ particles carrying vaccine antigens [33]; germ particles acting as a potent adjuvant for intranasal influenza immunization [19]; BLP-adjuvanted influenza vaccine, delivered via intranasal administration, enhancing systemic and local antibody responses against influenza, leading to protective resistance against homologous or heterologous infection when compared to conventional intramuscular immunization [31]; intranasal vaccination of mice or cotton rats with BLPs resulting in potent induction of recombinant fusion protein-specific immunoglobulins and a significant decrease of virus titers in lungs with respiratory syncytial virus challenge [34]; nasal immunization by BLP mixed with split influenza vaccine inducing influenza A virus-specific T cell and B cell responses, both systemically and at the site of virus entry in a TLR2 dependent manner [29]; Shigella invasion plasmid antigen B and D, displayed on the BLPs of *L. lactis*, inducing protective immunity in adult and infant mice [35]; pneumococcal surface protein, bound with protein anchors of BLPs (BLPs/PspA-PA), efficiently inducing IgG and IgA levels and providing complete protection in mice challenged with pneumococci of different PspA families [11]; intranasal pertussis vaccine with BLP adjuvant enhanced immune responses and protected mice from an intranasal challenge and pathological injury [30]; and recently, BLP based pneumococcal vaccine, displaying PspA2 and PspA4 fragments, inducing broad protective immune responses [36]. To the best of our knowledge, this

experiment was the first attempt toward the application of BLPs as a novel adjuvant for vaccines in the poultry industry. Evidently, the results of the present study were in agreement with those of previous studies, wherein BLPs are a robust adjuvant for vaccines.

This study was a novel and primary attempt to understand the efficiency of BLPs supplemented with a vaccine against IB. Evaluation of mucosal and cellular immunity influenced by the BLP-adjuvanted IBV vaccine was not considered in this experiment despite its substantial duty in protection [23]. Challenges were also not performed herein, although they are essential to estimate the real potency of the applied BLP-adjuvanted vaccine against a field strain [8,9,37]. Thus, to further understand the immune system stimulating potency of the BLP-adjuvanted vaccine, studies consisting of challenges along with evaluating levels of immunity other than humoral, in addition to viral load detection, as well as ciliostasis and histopathology investigations are suggested.

In summary, it could be concluded that admixing low doses of BLPs, such as 0.15 mg dry weight per bird, with a live H120 IBV vaccine is capable of rapidly boosting higher anti-IBV serum IgG titer levels than a nonadjuvanted live IBV vaccine, even at 2 weeks after hatching.

Acknowledgments
The authors would like to thank Mr Ali Kargar, a poultry science technician at the School of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. This project was funded by the Ferdowsi University of Mashhad, Iran (grant no. 48602). This experiment was approved by the Ethics Committee of Ferdowsi University of Mashhad, Iran and performed in accordance with the relevant guidelines and regulations.

Conflict of Interest
The authors declare that there is no conflict of interest.

References

1. Orr-Burks N, Gulley SL, Toro H, van Ginkel FW. Immunoglobulin A as an early humoral responder after mucosal avian coronavirus vaccination. Avian Diseases 2014; 58 (2): 279-286. doi: 10.1637/10740-120313-Reg.1
2. Jordan B. Vaccination against infectious bronchitis virus: a continuous challenge. Veterinary Microbiology 2017; 206: 137-143. doi: 10.1016/j.vetmic.2017.01.002
3. Sjaak de Wit JJ, Cook JK, van der Heijden HM. Infectious bronchitis virus variants: A review of the history, current situation and control measures. Avian Pathology 2011; 40 (3): 323-335. doi: 10.1080/03079457.2011.566260
4. Jackwood MW, Jordan BJ, Roh HJ, Hilt DA, Williams SM. Evaluating protection against infectious bronchitis virus by clinical signs, ciliostasis, challenge virus detection, and histopathology. Avian Diseases 2015; 59 (3): 368-374. doi: 10.1637/11026-012415-reg.1
5. Bande F, Arshad SS, Bejo MH, Moeini H, Omar AR. Progress and challenges toward the development of vaccines against avian infectious bronchitis. Journal of Immunology Research 2015; 2015: 424860. doi: 10.1155/2015/424860
6. Cook JK, Jackwood M, Jones RC. The long view: 40 years of infectious bronchitis research. Avian Pathology 2012; 41 (3): 239-250. doi: 10.1080/03079457.2012.680432
7. Feng K, Xue Y, Wang J, Chen W, Chen F et al. Development and efficacy of a novel live-attenuated QX-like nephropathogenic infectious bronchitis virus vaccine in China. Vaccine 2015; 33 (9): 1113-1120. doi: 10.1016/j.vaccine.2015.01.036
8. Zhao Y, Cheng JL, Liu XY, Zhao J, Hu YX et al. Safety and efficacy of an attenuated Chinese QX-like infectious bronchitis virus strain as a candidate vaccine. Veterinary Microbiology 2015; 180: 49-58. doi: 10.1016/j.vetmic.2015.07.036
9. Yan S, Zhao J, Xie D, Huang X, Cheng J et al. Attenuation, safety, and efficacy of a QX-like infectious bronchitis virus serotype vaccine. Vaccine 2018; 36 (14): 1880-1886. doi: 10.1016/j.vaccine.2018.02.053
10. Li J, Helal ZH, Karch CP, Mishra N, Girshick T et al. A self-adjuvanted nanoparticle based vaccine against infectious bronchitis virus. PLoS One 2018; 13 (9): 1-17. doi: 10.1371/journal.pone.0203771
11. Wang D, Lu J, Yu J, Hou H, Leenhouts K et al. A novel PspA protein vaccine intranasal delivered by bacterium-like particles provides broad protection against pneumococcal pneumonia in mice. Immunology Investigation 2018; 47 (4): 403-415. doi: 10.1080/08820139.2018.1439505
12. Lu J, Hou H, Wang D, Leenhouts K, Roosmalen MLV et al. Systemic and mucosal immune responses elicited by intranasal immunization with a pneumococcal bacterium-like particle-based vaccine displaying pneumolysin mutant plym2. Immunology Letters 2017; 187: 41-46. doi: 10.1016/j.imlet.2017.05.003
13. Mao R, Wu D, Wang Y. Surface display on lactic acid bacteria without genetic modification: strategies and applications. Applied Microbiology and Biotechnology 2016; 100 (22): 9407-9421. doi: 10.1007/s00253-016-7842-8
14. Yu J, Li B, Chen X, Lu J, Wang D et al. Comparison of immunogenicity and protection of two pneumococcal protein vaccines based on PsaA and PspA. Infection and Immunology 2018; 86 (6): e00916-17. doi: 10.1128/IAI.00916-17
15. Bru T, Vila R, Cabana M, Geerligs HJ. Protection of chickens vaccinated with combinations of commercial live infectious bronchitis vaccines containing Massachusetts, Dutch and QX-like serotypes against challenge with virulent infectious bronchitis viruses 793B and IS/1494/06 Israel variant 2. Avian Pathology 2017; 46 (1): 52-58. doi: 10.1080/03079457.2016.1203393

16. Bosma T, Kanninga R, Neef J, Audouy SA, van Roosmalen ML et al. Novel surface display system for proteins on non-genetically modified gram-positive bacteria. Applied Environmental Microbiology. 2006; 72 (1): 880-889. doi: 10.1128/AEM.72.1.880-889.2006

17. van Ginkel FW, Padgett J, Martinez-Romero G, Miller MS, Joiner KS et al. Age-dependent immune responses and immune protection after avian coronavirus vaccination. Vaccine 2015; 33 (23): 2655-2661. doi: 10.1016/j.vaccine.2015.04.026

18. Okino CH, Alessi AC, Montassier Mde F, Rosa AJ, Wang X et al. Humoral and cell-mediated immune responses to different doses of attenuated vaccine against avian infectious bronchitis virus. Viral Immunology 2013; 26 (4): 259-267. doi: 10.1089/vim.2013.0015

19. Saluja V, Amorij JP, van Roosmalen ML, Leenhouts K, Huckriede A et al. Intranasal delivery of influenza subunit vaccine formulated with GEM particles as an adjuvant. The AAPS Journal 2010; 12 (2): 109-116. doi: 10.1208/s12248-009-9168-2

20. Sabroe I, Parker LC, Dower SK, Whyte MKB. The role of TLR activation in inflammation. Journal of Pathology 2008; 214 (2): 126-135. doi: 10.1002/path.2264

21. Smith KD. Toll-like receptors in kidney disease. Current Opinions in Nephrology and Hypertension 2009; 18 (3): 189-196. doi: 10.1097/MNH.0b013e32832a1d5f

22. Okino CH, Mores MA, Trevisol IM, Coldebella A, Montassier HJ et al. Early immune responses and development of pathogenesis of avian infectious bronchitis viruses with different virulence profiles. PLoS One 2017; 12 (2): 1-17. doi: 10.1371/journal.pone.0172275

23. Lopes PD, Okino CH, Fernando FS, Pavani C, Casagrande VM et al. Inactivated infectious bronchitis virus vaccine encapsulated in chitosan nanoparticles induces mucosal immune responses and effective protection against challenge. Vaccine 2018; 36 (19): 2630-2636. doi: 10.1016/j.vaccine.2018.03.065

24. Tan L, Liao Y, Fan J, Zhang Y, Mao X et al. Prediction and identification of novel IBV S1 protein derived CTL epitopes in chicken. Vaccine 2016; 34 (3): 380-386. doi: 10.1016/j.vaccine.2015.11.042

25. de Wit JJS, Malo A, Cook JKA. Induction of IBV strain-specific neutralizing antibodies and broad spectrum protection in layer pullets primed with IBV Massachusetts (Mass) and 793B vaccines prior to injection of inactivated vaccine containing Mass antigen. Avian Pathology 2019; 48 (2): 135-147. doi: 10.1080/03079457.2018.1556778

26. de Wit JJ, Cook JK. Factors influencing the outcome of infectious bronchitis vaccination and challenge experiments. Avian Pathology 2014; 43 (6): 485-497. doi: 10.1080/03079457.2014.974504.

27. Smialek M, Tykalowski B, Dziewulska D, Stenzel T, Koncicki A. Immunological aspects of the efficiency of protectotype vaccination strategy against chicken infectious bronchitis. BMC Veterinary Research 2017; 13 (1): 1-7. doi: 10.1186/s12917-017-0963-1

28. Leenhouts K. MimopathTM-based vaccine delivery. In: Singh M (editor). Novel immune potentiators and delivery technologies for next generation vaccines. Cambridge, MA, USA: Springer; 2013. pp. 245-265

29. Keijzer C, Haijema BJ, Meijerhof T, Voorn P, de Haan A et al. Inactivated influenza vaccine adjuvanted with bacterium-like particles induce systemic and mucosal influenza. A virus specific T-Cell and B-Cell responses after nasal administration in a TLR2 dependent fashion. Vaccine 2014; 32 (24): 2904-2910. doi: 10.1016/j.vaccine.2014.02.019

30. Shi W, Kou Y, Jiang H, Gao F, Kong W et al. Novel intranasal pertussis vaccine based on bacterium-like particles as a mucosal adjuvant. Immunology Letters 2018; 198: 26-32. doi: 10.1016/j.imlet.2018.03.012

31. de Haan A, Haijema BJ, Voorn P, Meijerhof T, van Roosmalen ML et al. Bacterium-like particles supplemented with inactivated influenza antigen induce cross-protective influenza-specific antibody responses through intranasal administration. Vaccine 2012; 30 (32): 4884-4891. doi: 10.1016/j.vaccine.2012.04.032

32. Matoo JJ, Bashir K, Kumar A, Krishnaswamy N, Dey S et al. Resiquimod enhances mucosal and systemic immunity against avian infectious bronchitis virus vaccine in the chicken. Microbial Pathogenesis 2018; 119: 119-124. doi: 10.1016/j.micpath.2018.04.012

33. Ramirez K, Ditamo Y, Rodriguez L, Picking WL, van Roosmalen ML et al. Neonatal mucosal immunization with a non-living, non-genetically modified lactococcus lactis vaccine carrier induces systemic and local Th1-type immunity and protects against lethal bacterial infection. Mucosal Immunology 2010; 3 (2): 159-171. doi: 10.1038/mi.2009.131

34. Rigter A, Widjaja I, Versantvoort H, Coenjaerts FE, van Roosmalen M et al. A protective and safe intranasal RSV vaccine based on a recombinant prefusion-like form of the F protein bound to bacterium-like particles. PLoS One 2013; 8 (8): e71072. doi: 10.1371/journal.pone.0071072

35. Heine SJ, Franco-Mahecha OL, Chen X, Choudhari S, Blackwelder WC et al. Shigella IpaB and IpaD displayed on L. lactis bacterium-like particles induce protective immunity in adult and infant mice. Immunology and Cell Biology 2015; 93 (7): 641-652. doi: 10.1038/icb.2015.24

36. Lu J, Guo J, Wang D, Yu J, Gu T et al. Broad protective immune responses elicited by bacterium-like particle-based intranasal pneumococcal particle vaccine displaying PspA2 and PspA4 fragments. Human Vaccine Immunotherapy 2019; 15 (2): 371-380. doi: 10.1080/21645515.2018.1526556

37. de Wit JJ, Swart WA, Fabri TH. Efficacy of infectious bronchitis virus vaccinations in the field: Association between the α-IBV IgM response, protection and vaccine application parameters. Avian Pathology 2010; 39 (2): 123-131. 10.1080/03079451003604639