ORIGINAL ARTICLE

Veterinary Research Forum. 2026; 17 (4): 273 - 280
doi: 10.30466/vrf.2025.2053788.4668
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

In vitro synergistic efficacy of postbiotics and specific immunoglobulin Y antibodies against *Mycobacterium avium* subsp. *paratuberculosis*

Mera Sharif[1], Naheed Mojgani[2]*, Nader Mosavari[2], Fattah Sotoodehnejad Nematalahi[1]

[1] Department of Biology, SR.C., Islamic Azad University, Tehran, Iran; [2] Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization, Karaj, Iran.

Article Info

Article history:

Received: 24 February 2025
Accepted: 19 July 2025
Available online: 15 April 2026

Keywords:

Immunoglobulin Y antibody
Indirect ELISA
Mycobacterium avium Subsp. Paratuberculosis
Postbiotics

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease and a potential contributor to Crohn's disease, presents a significant challenge due to its resistance to conventional antibiotics. This necessitates the development of innovative strategies for prevention and treatment. This study aimed to evaluate the anti-bacterial activity of pathogen-specific antibodies derived from chicken egg yolks (immunoglobulin Y [IgY]) and the postbiotics from lactic acid bacteria against MAP. Immunoglobulin Y antibodies were produced by immunizing hens with formalin-killed MAP strain antigens. The IgY was extracted and purified, and the anti-MAP titers were quantified by indirect enzyme-linked immunosorbent assay. The minimum inhibitory concentration of different concentrations of specific anti-MAP IgY and the mixture of postbiotics (from four different probiotic strains, including *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Pediococcus acidilactici*) individually and in combination against MAP was determined at various time intervals. Anti-MAP IgY titers in egg yolks increased within 2 weeks of immunization, reaching peak levels at 6 weeks. Growth inhibition assays revealed that postbiotics concentration as low as 6.25 mg mL^{-1} effectively inhibited MAP growth. Anti-MAP IgY demonstrated anti-bacterial activity with a minimum inhibitory concentration of 50.00 mg mL^{-1}, while the combined IgY-posbiotics treatment achieved MAP growth inhibition at a minimum inhibitory concentration of 3.125 mg mL^{-1}. The findings of the study suggest that combination therapy with specific IgY and postbiotics may be a promising preventive strategy for controlling MAP infections. Further *in vivo* studies are needed to elucidate the underlying mechanisms and optimize the application of this approach for broader use in veterinary and human medicine.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of *paratuberculosis*, commonly known as Johne's disease, affecting cattle, particularly dairy cows. This disease leads to significant economic losses in the livestock industry due to chronic infections, reduced productivity, and animal losses.[1] In addition to its economic impact, MAP is a pathogen of public health importance, as its high resistance to environmental factors facilitates zoonotic transmission between animals and humans.[2] Extensive research has also suggested a potential link between MAP and Crohn's disease in humans, sparking increased concern regarding its role in zoonotic diseases. Extensive research and studies have shown an association between MAP and Crohn's disease in humans.[3-6]

Globally, MAP infection rates have risen, largely due to the asymptomatic nature of the silent phase of infection, which allows for prolonged fecal shedding and hinders early diagnosis. For example, in countries with intensive farming systems, the prevalence of MAP exceeds 50.00% in dairy herds.[2] In Iran, infection rates among cattle range from 2.27 to 35.88%, further emphasizing the need for effective control measures.[7]

Currently, no effective treatments or vaccines for MAP are available, and efforts to develop viable vaccines have yielded limited success. Considering the chronic nature of MAP infections, their economic toll, the potential for food-borne transmission, and increasing prevalence of antibiotic resistance, it is evident that novel preventive and therapeutic strategies are urgently needed.[8]

Alternative approaches, such as passive immuno-therapy, have emerged as adjunctive treatments for

***Correspondence:**

Naheed Mojgani. PhD
Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization, Karaj, Iran
E-mail: n.mojgani@rvsri.ac.ir

pathogens resistant to conventional antibiotics.[8] One promising strategy involves oral (inactive) immunotherapy using pathogen-specific immunoglobulin Y (IgY) antibodies. Using cost-effective methods, these antibodies can be efficiently produced and purified from chicken egg yolks, making them a viable alternative to mammalian serum antibodies. Unlike mammalian antibodies, IgY production is non-invasive and scalable, and avoids immunological cross-reactivity with mammalian IgG. Additionally, IgY antibodies exhibit a broader capacity to bind multiple epitopes, enhancing their effectiveness against pathogens.[9-11]

Probiotics represent another promising strategy for disease prevention and treatment. Probiotics are live microorganisms that confer health benefits when consumed in sufficient quantities.[12] Among probiotics, lactic acid bacteria are widely used in therapeutic and preventive applications.[13] More recently, postbiotic metabolites which refer to substances derived after the microorganisms are no longer alive, have gained attention for their therapeutic potential.[14] These inactivated bacterial fractions with their intra-cellular metabolites have been reported to contain several bioactive compounds, including short-chain fatty acids (acetic, butyric, and propionic acids), lactic acid, anti-bacterial peptides (bacteriocins and bacteriocin-like substances), proteins, hydrogen peroxide, exopolysaccharides, enzymes, and vitamins.[15] Postbiotic metabolites derived from probiotic bacteria have demonstrated anti-microbial, anti-inflammatory, and immunomodulatory properties in medical research.[16]

Given MAP's intra-cellular nature and the chronic persistence of its infection, a multifaceted approach is required for effective control. Strategies that combine anti-microbial activity, immune system activation, and intestinal microbiota modulation are particularly promising.[17] While several studies have investigated the effects of probiotics on MAP, no study to date has evaluated the combined anti-bacterial effects of postbiotics and IgY antibodies against MAP. In this study, we produced anti-MAP-specific IgY antibodies in immunized hens and evaluated their anti-bacterial activity against MAP. Additionally, we assessed the synergistic effects of IgY antibodies in combination with postbiotics derived from four probiotic strains, offering a novel approach for MAP control and infection prevention.

Materials and Methods

Bacterial strains and culture conditions. The MAP strains III and V were obtained from the Razi Vaccine and Serum Research Institute (RVSRI; Karaj, Iran), and cultured on Herrold's egg-yolk medium (HEYM) without mycobactin (Becton, Dickinson, USA) at 37.00 ˚C for 4 weeks. Probiotic bacteria, including *Lactobacillus reuteri* (strain LRE-B16), *Lactobacillus rhamnosus* (strain LRH-B2), *Lactococcus acidophilus* (starin LA-A18), and *Pediococcus acidilactici* (starin PAC-153) obtained from a local probiotic manufacturer (Nature Biotechnology Co., Karaj, Iran) were grown in DeMan Rogosa and Sharpe (HiMedia, Thane, India) media at 37.00 ˚ C for 24 hr under anaerobic conditions.[18,19]

Preparation of postbiotic. Overnight-grown cultures of *L. reuteri*, *L. rhamnosus, L. acidophilus*, and *P. acidilactici* were centrifuged (8,000 *g* for 15 min at 4.00 ˚C), and the collected pellets were washed with sterile 0.85% (w/v) NaCl (Merck, Darmstadt, Germany) solution. The obtained pellets were mixed in equal volumes and the final concentrations of cell suspension were adjusted to 1.00×10^9 CFU mL^{-1}. The cells were then treated with 1.00 mg mL^{-1} of lysozyme (Merck) for 30 min at 37.00 ˚ C and further sonicated at 5 min intervals in an ice bath. The cell debris was discarded *via* centrifugation (8,000 *g* for 15 min at 4.00 ˚ C), and the obtained suspension was filtered and sterilized using 0.22 µM Millipore membranes (Sartorius Minisart, Gottingen, Germany). Finally, the filter-sterilized suspension was freeze-dried as described earlier.[18,19] The sterile postbiotic powders were stored at –20.00 ˚ C for future use.

Immunization of hens with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Bacterial cells of MAP harvested from the freshly grown cultures by centrifugation (1,500 *g* for 15 min) were inactivated in 10.00% (v/v) formalin overnight at 37.00 ˚C. The collected cellular debris was washed in phosphate-buffered saline (PBS; pH: 7.40) and adjusted to a 1.00 McFarland standard. An emulsion consisting of 500 µL of prepared bacterial suspension, serving as the MAP antigen, was mixed with an equal volume of Freund's incomplete Adjuvant (FIA; Sigma-Aldrich, St. Louis, USA) for immunization. A total of 20 white leghorn chickens (Bovans strain) aged 90 days were obtained from the Department of Animal Sciences of the Campus of Agriculture and Natural Resources of Tarbiat Modares University, Tehran, Iran. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee's guidelines at Islamic Azad University, Science and Research Branch, Tehran, Iran (Ethical Code: IR.IAU.SRB.REC.1398.198). The chickens were randomly divided into two groups (n = 10). The first group received four intra-muscular injections into the breast at 2-week intervals (0.50 mL *per* site), using inoculation containing the two inactivated strains with FIA. The second group was administered PBS combined with FIA without MAP in the same manner as a control.[20]

Immunoglobulin Y extraction and purification. Eggs were collected daily after the first immunization and stored at 4.00 ˚C for extraction and purification of IgY from the yolks. To extract the antibody from the water-soluble part of the egg yolk, the polyethylene glycol-6000 (Merck)

method described by Fishman and Berg was employed. The final product was dialyzed overnight against a 0.10% NaCl solution,[21] and freeze-dried according to the process described by Wang *et al*.[9]

Protein estimation and molecular weight determination. The protein concentration of the purified IgY was determined using the Kjeldahl method.[22] The approximate molecular weight of the extracted and purified IgY was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses under reducing condition as described by Amro *et al*.[23]

Enzyme-linked immunosorbent assay (ELISA). To measure IgY antibody titers in serum, blood samples were taken from the wing vein on day 0 and at 2, 4, 6, and 8 weeks, and sera were separated and stored at -20.00 ˚C.[20] Indirect ELISA was performed to measure specific IgY antibody in sera and egg yolk samples.[24] Briefly, 100 µL of MAP suspension was prepared in bicarbonate coating buffer (0.10 M; pH: 9.60; Merck) and added in each of the ELISA micro-titer plate well (PolySorp; Thermofischer Scientific Inc., Roskilde, Denmark) in triplicates. In the last column, coating buffer without MAP was added as a negative control. After 16 - 18 hr of incubation at 4.00 ˚ C, the wells were washed three times with 10.00 mM PBS with Tween 20 (PBST; Merck) solution, then a 150:1 solution of 2.50% casein (Sigma-Aldrich) was added to each well as a blocker and incubated for 1 hr at room temperature. One-hundred µL of a serially diluted serum, egg yolk, and sera's IgY fractions in PBST were added to each well for a 45 min incubation at room temperature. The plate was then washed three times with PBST. Subsequently, horseradish peroxidase-conjugated mouse anti-chicken IgY antibody (Abcam, Cambridge, UK) in 10.00 mM PBS (pH: 7.20) was added to each well, and the plate was incubated again for 30 min at room temperature. After incubation, the wells were washed with PBST, 100 µL of tetra-methyl benzidine substrate (Merck) was added to the wells, and the plate was incubated for 15 min in darkness at room temperature. In the last step, 100 µL of HCl 1.00 N was added as a stop solution, and the plate was read at 450 nm. Crude IgY from non-immunized hens was used as a control.[24]

Minimum inhibitory concentration (MIC). The MIC of the extracted IgY antibodies and postbiotics against MAP was determined by the tube dilution assay described earlier. Initially, log phase cultures of MAP in HEYM were centrifuged (38,000 *g* for 20 min), and the pellet was washed in Middlebrook 7H9 broth (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) supplemented with 0.20% glycerol and 10.00% oleic acid albumin dextrose catalase (Unitech, Dublin, Ireland). The treated cells were suspended in Middlebrook 7H9 broth, and their density was adjusted to 3.00×10^8 CFU mL^{-1} using 1.00 McFarland standard.[25] Different concentrations of the postbiotics and specific IgY antibodies (100, 50.00, 25.00, 12.50, 6.25, 3.125, and 1.50 mg mL^{-1}) prepared in Middlebrook 7H9 broth were treated with the respective pathogen suspension (approximately 1.00×10^8 CFU mL^{-1}). The tubes were incubated at 37.00 ˚C, and the optical density at 600 nm was monitored for 42 days at regular periods. A growth curve was plotted for the treatment group and compared with positive and negative controls that included MAP in HEYM media without any treatments and HEYM medium without MAP, respectively. To investigate the anti-bacterial effects of the combination of postbiotics and IgY against MAP, equal amounts of each were combined at the same concentrations mentioned above (100, 50.00, 25.00, 12.50, 6.25, 3.125, and 1.50 mg mL^{-1}). The lowest concentration of IgY, postbiotics, and the mixture of IgY and postbiotics in the test tubes with no visible or detectable bacterial growth or turbidity was considered to represent the MIC. All experiments were repeated thrice for reproducibility before statistical analysis.[26]

Statistical analysis. Statistical analysis was carried out using GraphPad Prism (version 9.0; GraphPad Software Inc., San Diego, USA) program. To analyze and compare the anti-MAP effect of extracted IgY, postbiotics, and the mixture of postbiotics and IgY at various concentrations and over 6 weeks, a two-way analysis of variance followed by Tukey's multiple comparison test was performed. All experiments were performed in triplicate and given as mean ± standard deviation. The *p*-values less than 0.05 were considered statistically significant.

Results

Specific anti-MAP IgY production. In this study, we evaluated the titers of specific anti-MAP IgY antibodies produced in immunized chicken blood serum and egg yolk over a 6-week period using indirect ELISA. The results revealed a marked difference in IgY levels between the treated and control groups following immunization. In the control group (non-immunized chicken), serum IgY levels remained relatively constant throughout the experiment, with no significant changes. However, in the immunized chickens, serum IgY levels revealed a significant rise after the first immunization, with the highest concentrations recorded at week six (Fig. 1), indicating a robust and sustained immune response in the treated hens. The IgY titers displayed significant variation at different time intervals in immunized and control animals. In the immunized group, IgY concentrations in egg yolk increased substantially as early as two weeks post-immunization, highlighting the rapid onset of antibody production. In contrast, the control group exhibited no notable changes in the IgY levels at any injection time point during the study (Table 1). This indicates the efficiency of the immunization protocol in stimulating specific IgY production.

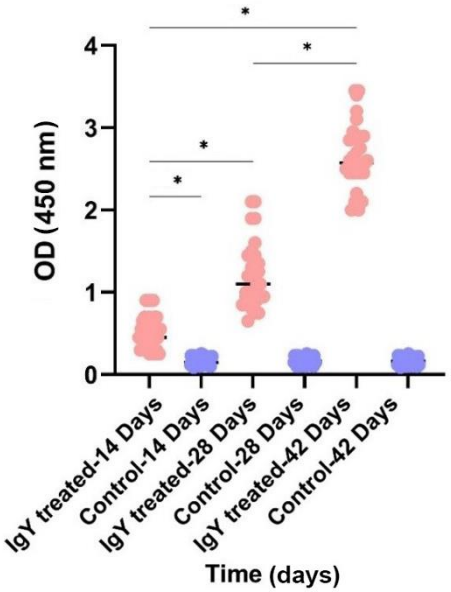

Fig. 1. Assessment of serum immunoglobulin Y (IgY) levels over a 6-week period using indirect enzyme-linked immunosorbent assay. OD: Optical density.
* indicates statistically significant differences compared to the baseline ($p < 0.0001$).

Table 1. Specific immunoglobulin Y (IgY) concentration in egg yolk before (Control group) and after immunization at different time intervals as determined by indirect enzyme-linked immunosorbent assay (n = 12).

Injection time	Concentration (mg mL^{-1})
Before immunization	1.01 ± 0.31
First dose	3.45 ± 0.18*
Second dose	6.09 ± 0.15*
Third dose	8.97 ± 0.21*
Fourth dose	9.40 ± 0.22*

* The difference of values in each row were significant compared to the control group ($p < 0.05$).

The SDS-PAGE analysis under reducing condition (with β-mercaptoethanol which breaks the IgY's inter-chain disulfides, yielding two sub-unit bands) indicated the purity of produced IgY antibodies. Two distinct protein bands were observed, corresponding to the heavy (65.00 kDa) and light (27.00 kDa) chains of IgY (Fig. 2). This result verifies the structural integrity of the extracted IgY and highlights the reliability of the isolation method employed.

Anti-bacterial effects of specific anti-MAP IgY and the postbiotics. The MIC of specific IgY antibodies and postbiotics against MAP was assessed by adding varying concentrations of IgY and postbiotics to MAP cultures. The results revealed that all tested concentrations of IgY exhibited significant inhibition of MAP growth. The MIC of IgY was determined to be 50.00 mg mL^{-1}, which effectively suppressed bacterial growth over the 6-week experimental period (Fig. 3A). During the first weeks, all concentrations of IgY (1.50 - 100 mg mL^{-1}) inhibited bacterial growth

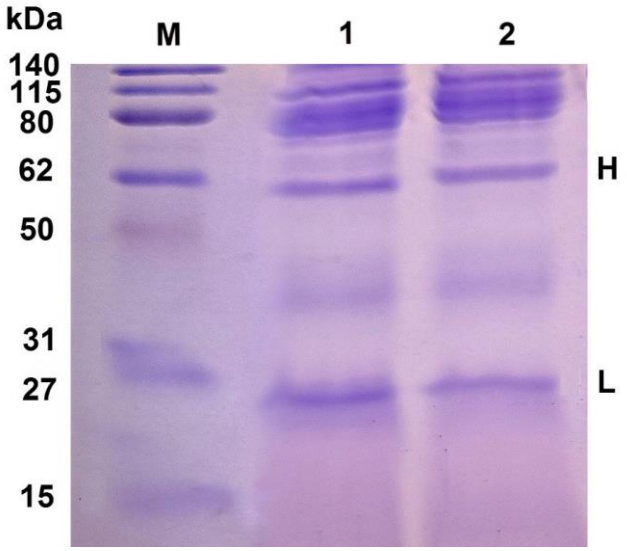

Fig. 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of extracted immunoglobulin Y (IgY). Lane M: Pre-stained protein ladder (BioLegend, San Diego, USA); Lane 1: 0.50 mg mL^{-1} specific IgY at week two; Lane 2: 0.50 mg mL^{-1} specific IgY at week four; H: Heavy chain 65.00 kDa; L: Light chain 27.00 kDa.

effectively. This is likely due to the slow growth rate of MAP during the early stages of culture, which may amplify the observed inhibitory effects. However, over time, as the bacterial growth resumed and began to proliferate more robustly, the lower concentrations (1.50, 3.125, and 6.25 mg mL^{-1}) of IgY had diminishing inhibitory effects. This indicates that higher concentrations of IgY are particularly effective at maintaining long-term inhibition of MAP growth. Interestingly, no significant differences in the inhibitory effects among these lower concentrations were observed at week six, indicating that they may have a similar threshold of efficacy over longer durations. In contrast, higher concentrations (≥ 12.50 mg mL^{-1}) consistently maintained significant bacterial inhibition throughout the study period. These findings suggest a concentration-dependent effect, where IgY at higher doses can sustain bacterial suppression over extended periods, while lower doses may lose efficacy as MAP adapts to growth conditions.

The anti-bacterial effects of the varying concentrations of postbiotics derived from probiotics were evaluated. The results demonstrated that the postbiotics effectively inhibited MAP growth across all concentrations tested, with the MIC determined to be 6.25 mg mL^{-1} (Fig. 3B). Higher concentrations of postbiotics (≥ 6.25 mg mL^{-1}) displayed strong inhibitory activity, maintaining bacterial suppression throughout the 6-week period. At lower concentrations, particularly 1.50 mg mL^{-1}, MAP growth was inhibited during the 1st week; however, as observed with IgY, as the pathogen culture began to recover and grow over time, higher postbiotic concentrations were

required to inhibit the growth. This suggests that while low concentrations of postbiotics have some initial anti-microbial effects, they are insufficient to sustain long-term inhibition, possibly due to an insufficient dose of active anti-microbial metabolites in the particular concentrations of the postbiotic. Comparatively, with increasing doses of the postbiotic, enhanced anti-bacterial effects were observed, indicating the dose-dependent effectiveness of the postbiotics. This might be dependent on the higher ratio of the anti-microbial compounds present in the higher concentrations of the postbiotics that could actively suppress MAP growth.

Synergistic anti-bacterial actions of postbiotics and IgY. To evaluate the combined anti-bacterial effects of IgY and postbiotics, equal concentrations of both components were mixed and tested against MAP. The results demonstrated that the mixture exhibited significantly stronger anti-MAP activity compared to the either IgY or postbiotic alone, with the MIC of the mixture determined to be as low as 3.125 mg mL^{-1} (Fig. 3C). This highlights the synergistic interaction between IgY and postbiotic, where

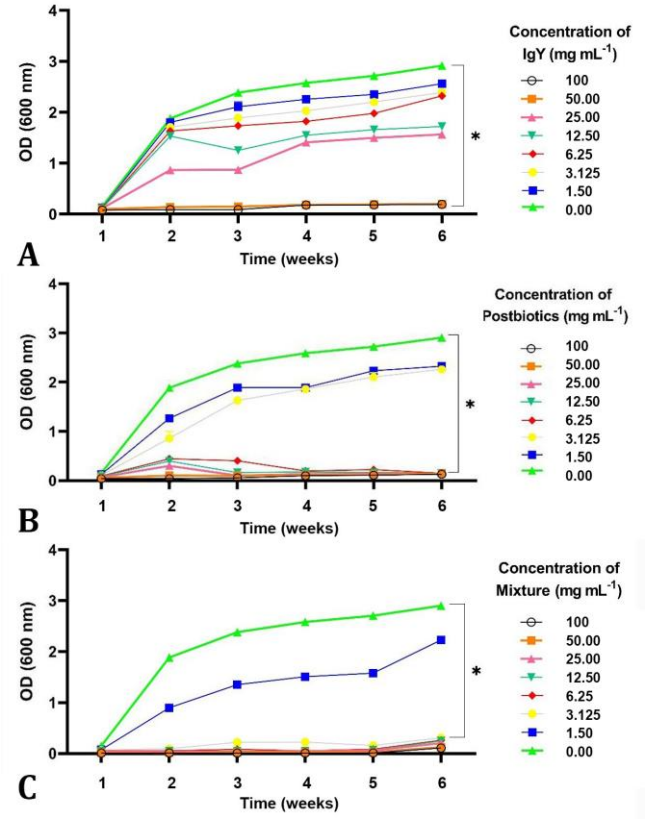

Fig. 3. Anti-*Mycobacterium avium* subsp. *paratuberculosis* (MAP) activity of different concentrations of **A)** purified anti-MAP immunoglobulin Y (IgY) antibodies, **B)** postbiotics, and **C)** combination of IgY and postbiotics. Anti-microbial activity was assessed by measuring the optical density (OD) of MAP cultures after treatment.
* indicates a statistically significant reduction in OD compared to the untreated control group ($p < 0.0001$).

the combination enhances the overall inhibitory effect against MAP. The superior efficacy of the IgY-postbiotic mixture was particularly evident at week six, where bacterial growth inhibition was sustained even at lower concentrations (*e.g.*, 3.125 mg mL^{-1}). In contrast, IgY and postbiotics individually at the same concentration exhibited reduced inhibitory effects by week six. This suggests that the combined action of IgY and postbiotics amplifies their individual anti-bacterial properties, possibly through complementary mechanisms.

Figure 4 provides a direct comparison of the anti-MAP effects of IgY, postbiotics, and the IgY-postbiotic mixture at various concentrations during the examined 6-week period. The mixture consistently demonstrated the strongest inhibitory effect, with the lowest bacterial growth observed at the concentrations of 50.00, 6.25, and 3.125 mg mL^{-1}. Among the three treatment groups, IgY alone exhibited the lowest inhibitory effects, particularly at lower concentrations. Postbiotics performed better than IgY, maintaining suppression at intermediate concentrations. The mixture of IgY and postbiotics showed the highest efficacy, effectively inhibiting bacterial growth even at low concentrations, indicating a synergistic interaction.

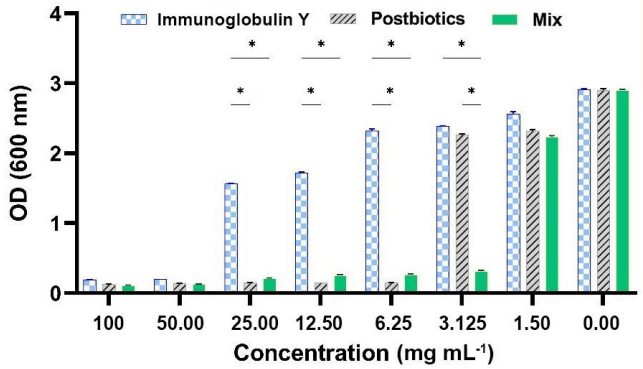

Fig. 4. Comparison of anti-*Mycobacterium avium* subsp. *paratuberculosis* (MAP) activity among three treatment groups. The activity was evaluated based on optical density (OD) measurements of MAP cultures.
* indicates statistically significant differences compared to the control group at the same concentration ($p < 0.0001$).

Discussion

Passive immunity through the use of specific antibodies, such as IgY, has emerged as an attractive approach for combating gastrointestinal pathogens in humans and animals. Laying hens have gained significant attention as a sustainable and efficient source of antibodies for the prevention and treatment of infectious diseases. Compared to the mammalian antibodies, IgY is remarkably robust in terms of shelf-life stability, and the production is cost-effective and scalable. Hens can produce significant amounts of IgY (30.00 - 45.00 g) annually, and dried egg powder containing IgY can be used directly as a

low-cost supplement in livestock feed.[27] The IgY antibodies do not cross-react with rheumatoid factors or human anti-mouse antibodies and do not activate the mammalian complement system.[28] Furthermore, IgY lacks hetero-agglutinins and often provides stronger immunogenic responses to conserved mammalian peptides than antibodies produced in rabbits.[28] This makes poultry a valuable and low-cost source for producing antibodies with specific and efficient immunological characteristics.[28,29]

In the present study, anti-MAP IgY was produced by immunizing hens with formalin-inactivated MAP mixed with FIA. The IgY levels in the egg yolk rose significantly within 2 weeks, peaking at 6 weeks post-immunization. After the fourth injection, antibody levels began to decline gradually, but specific antibodies were produced for over 168 days. This demonstrates that chickens require approximately 2 weeks to initiate antibody production, making them efficient hosts for generating large quantities of specific IgY non-invasively. Lohmann hens, in particular, proved to be excellent candidates for anti-MAP IgY production.[27]

For IgY isolation, polyethylene glycol precipitation was employed, which is a low-toxic and widely used method in pharmaceutical research. This technique offers several advantages, including simplicity, efficiency, and safety, making it ideal for large-scale IgY extraction compared to other methods, such as sodium sulfate precipitation, chloroform extraction, and gel-filtration chromatography. Although the presence of concomitant protein bands in SDS-PAGE is expected with polyethylene glycol-6000-based purification, which typically yields ~70.00 - 80.00% purity, these additional bands may result from lipoproteins and livetins being not fully removed by this method.[23]

The SDS-PAGE results of the purified IgY in this study were consistent with those of Sudjarwo *et al*., who used IgY immunotherapy for *Mycobacterium tuberculosis* complex infection.[30] Both studies demonstrated distinct protein bands corresponding to the two sub-units of IgY (27.00 and 65.00 kDa).

The anti-bacterial properties of IgY, as highlighted in this study, are an important focus in the literature. For example, Van Nguyen *et al*. showed that IgY exhibited immunogenic activity against microbial infections,[31] while Lee *et al*. reported that chicken IgY antibodies effectively inhibited *Salmonella enteritidis* and *Salmonella Typhimurium* growth under laboratory conditions. Also, it was demonstrated that immunized chickens suppressed *Salmonella* colonization in organs and reduced bacterial shedding in feces.[32]

Previous studies have shown that IgY is generally more effective against Gram-negative bacteria than Gram-positive bacteria. This is likely due to the complex structure of Gram-positive bacterial cell walls, which may limit IgY binding. Furthermore, unlike mammalian IgG, IgY does not fully participate in phagocytosis as an opsonin. As a result, monotherapy with IgY may be less effective against certain bacteria, prompting growing interest in its combined use with other compounds, such as probiotics, postbiotics, propolis, and phages.[33]

The combined effect of IgY and probiotics has been stated in previous studies. Rahimi *et al.* demonstrated that the combination of IgY and probiotics significantly reduced *Salmonella* infection in broilers, with reductions in bacterial growth in the liver, spleen, and ileum.[34] Similarly, Xie *et al*. evaluated probiotics and Igs as adjunctive treatments for pediatric rotavirus enteritis. Their findings showed that probiotics reduced the frequency of diarrhea and secondary bacterial infections, while Igs significantly increased fecal secretory IgA levels and reduced disease duration.[35] However, in the present study, for the very first time, we compared the anti-bacterial effects of IgY and postbiotics against MAP.

In 2019, Ali *et al*. investigated the inhibitory effect of nisin (postbiotic) on MAP and evaluated its effect on the mycobacterial cell wall.[26] Nisin is extensively utilized as a bio-preservative, making it the most notable postbiotic-derived compound sourced from *Lactococcus lactis* that is available in the market. These researchers showed that nisin had a higher MIC against the laboratory strain (MAP K10), at 500 U mL^{-1}, compared to the field isolates (*e.g.*, MAP 4B and JTC 1281), at 15.00 U mL^{-1}. In milk, growth of MAP was inhibited after treatment with levels of nisin that are permissible in human food at 4.00 and 37.00 ˚C. These researchers indicated that postbiotics reduced membrane integrity by forming pores in the mycobacterial cell wall, thereby decreasing survival of paratuberculosis; hence, recommended that nisin treatment could be implemented as a control measure to reduce MAP from infected herds.[26]

In the current study, we used a mixture of postbiotics by combining the extracts from four probiotic bacteria in equal proportions. As seen from our results, the mixture of postbiotics was highly effective in inhibiting the pathogen and revealed enhanced anti-bacterial actions compared to the IgY alone. However, it was interesting to note that the combination of IgY and postbiotics had further superior anti-bacterial effects against MAP compared to either IgY or the postbiotics alone.

The enhancement in the anti-bacterial effects of the combination of specific antibodies and probiotics has been shown earlier by Karamzadeh-Dehaghani *et al*. These researchers reported improved immune function and reduced *Escherichia coli* infections in calves treated with IgY and multi-strain probiotics.[36] This synergy emphasizes the potential for combined therapies to enhance immunity and suppress bacterial infections more effectively. Although the exact mechanism involved in the synergistic effects is not yet clear, but the mechanism underlying this synergistic effect might highlight the complementary

actions of IgY and the postbiotics. The IgY can neutralize MAP before it adheres to or invades intestinal epithelial cells, while postbiotics strengthen gut-epithelial junctions and modulate immune responses, thus preventing chronic inflammation. This combination offers immediate and long-term protection in the gut.[37] Furthermore, IgY likely targets specific antigens on the pathogen's surface, while the anti-microbial metabolites in the postbiotics, such as organic acids, bacteriocins, hydrogen peroxide, and anti-microbial peptides, might disrupt bacterial cell structures or metabolic pathways. Together, these agents provide a multifaceted approach to bacterial inhibition.[37]

While the solo effects of Igs and probiotics in treating infectious and inflammatory diseases, such as inflammatory bowel disease, have been widely studied, the combined use of these agents remains a relatively new field requiring further exploration. The findings of the present study highlight the synergistic anti-bacterial actions of the IgY and postbiotics, underscoring the potential of combining IgY and postbiotics as a viable and targeted preventive strategy for the control of MAP in calves. Future research should focus on elucidating the exact mechanisms of this synergy and assessing its efficacy *in vivo* to expand its application to both human and veterinary medicine.

In conclusion, this study is the first to demonstrate the synergistic anti-bacterial effects of IgY and postbiotics against MAP, offering a promising way to prevent infections in both animals and humans. As seen during our results, the addition of postbiotics to IgY significantly enhanced its inhibitory activity, offering a safe, effective, and cost-efficient approach for controlling MAP, especially asymptomatic MAP infections. The combination works through different mechanisms; IgY targets specific antigens on MAP, while postbiotics contribute beneficial compounds, like bacteriocins and peptides that enhance anti-bacterial effects. Since this approach is scalable and sustainable, and works in multiple ways, it could be a valuable tool for preventing MAP infections. Further, *in vivo* studies are needed to be conducted to evaluate the efficacy in laboratory animals. Based on our results, it is highly presumed that a combination of IgY and postbiotics could be considered an effective bio-preventive strategy for controlling diseases, like Johne's disease in animals and inflammatory bowel disease in humans.

Acknowledgments

We express our sincere appreciation to Biorun Nature Biotechnology Company, Karaj, Iran, for supplying the probiotic strains and lyophilization of the postbiotic fractions. Additionally, we extend our gratitude to Dr. Rouhollah Keshavarz for his support in conducting the ELISA, as well Dr. Mohammad Babaie for his contributions to the writing and completion of the data analysis. This work is supported by Islamic Azad University and RVSRI, Karaj, Iran.

Conflict of interest

The authors declared no conflicting interest regarding the publication of the article.

References

1. Malvisi M, Palazzo F, Morandi N, et al. Responses of bovine innate immunity to *Mycobacterium avium* subsp. *paratuberculosis* infection revealed by changes in gene expression and levels of microRNA. PloS One 2016; 11 (10): e0164461. doi: 10.1371/journal.pone.0164461.
2. Grant IR. Zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: the current position. J Appl Microbiol 2005; 98(6): 1282-1293.
3. Richter E, Wessling J, Lügering N, et al. *Mycobacterium avium* subsp. *paratuberculosis* infection in a patient with HIV, Germany. Emerg Infect Dis 2002; 8(7): 729-731.
4. Waddell LA, Rajić A, Stärk K, et al. The zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: a systematic review and meta-analyses of the evidence. Epidemiol Infect 2015; 143(15): 3135-3157.
5. Chaubey KK, Singh SV, Gupta S, et al. *Mycobacterium avium* subspecies *paratuberculosis* - an important food borne pathogen of high public health significance with special reference to India: an update. Vet Q 2017; 37(1): 282-299.
6. Garvey M. *Mycobacterium avium* subspecies *paratuberculosis*: a possible causative agent in human morbidity and risk to public health safety. Open Vet J 2018; 8(2): 172-181.
7. Abdolmohammadi Khiav L, Khangahi Abyaneh H, Fallah Mehrabadi MH, et al. Meta-analysis of Johne's disease in Iranian animals' population (1999 - 2020). Arch Razi Inst 2024; 79(1): 168-179.
8. Wong SY, Grant IR, Friedman M, et al. Antibacterial activities of naturally occurring compounds against *Mycobacterium avium* subsp. *paratuberculosis*. Appl Environ Microbiol 2008; 74(19): 5986-5990.
9. Wang Z, Liu W, Duan X, et al. Effects of freezing and drying programs on IgY aggregation and activity during microwave freeze-drying: protective effects and interactions of trehalose and mannitol. Int J Biol Macromol 2024; 260(Pt 1): 129448. doi: 10.1016/j.ijbiomac.2024.129448.
10. Kumaran T, Citarasu T. IgY-technology: production of antibodies in chickens and use in therapy of infectious diseases. IJSRME 2016; 1(1): 29-35.
11. Spillner E, Braren I, Greunke K, et al. Avian IgY antibodies and their recombinant equivalents in research, diagnostics and therapy. Biologicals 2012;

40(5): 313-322.
12. Fijan S. Microorganisms with claimed probiotic properties: an overview of recent literature. Int J Environ Res Public Health 2014; 11(5): 4745-4767.
13. El Far MS, Zakaria AS, Kassem MA, et al. Promising biotherapeutic prospects of different probiotics and their derived postbiotic metabolites: *in-vitro* and histopathological investigation. BMC Microbiol 2023; 23(1): 122.
14. Vinderola G, Sanders ME, Salminen S. The concept of postbiotics. Foods 2022; 11(8): 1077. doi: 10.3390/foods11081077.
15. Prajapati N, Patel J, Singh S, et al. Postbiotic production: harnessing the power of microbial metabolites for health applications. Front Microbiol 2023; 14: 1306192. doi: 10.3389/fmicb.2023.1306192.
16. Mani-López E, Arrioja-Bretón D, López-Malo A. The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria *in vitro* and foods. Compr Rev Food Sci Food Saf 2022; 21(1): 604-641.
17. Kuenstner JT, Naser S, Chamberlin W, et al. The consensus from the *Mycobacterium avium* ssp. *paratuberculosis* (MAP) conference 2017. Front Public Health 2017; 5: 208. doi: 10.3389/fpubh.2017.00208.
18. Moradi M, Molaei R, Guimarães JT. A review on preparation and chemical analysis of postbiotics from lactic acid bacteria. Enzyme Microb Technol 2021; 143: 109722. doi: 10.1016/j.enzmictec.2020.109722.
19. Jalali S, Mojgani N, Sanjabi MR, et al. Functional properties and safety traits of *L. rhamnosus* and *L. reuteri* postbiotic extracts. AMB Express 2024; 14(1): 114. doi: 10.1186/s13568-024-01768-3.
20. Shin SJ, Lee SS, Manning EJ, et al. Production of and applications for a polyclonal IgY diagnostic reagent specific for *Mycobacterium avium* subsp. *paratuberculosis*. J Microbiol 2009; 47(5): 600-609.
21. Fishman JB, Berg EA. Isolation of IgY from chicken eggs. Cold Spring Harb Protoc 2018; 2018(6). doi: 10.1101/pdb.prot099150.
22. AOAC. Official methods of analysis. 20th ed. Gaithersburg, USA: Association of Official Analytical Chemists; 2019.
23. Amro WA, Al-Qaisi W, Al-Razem F. Production and purification of IgY antibodies from chicken egg yolk. J Genet Eng Biotechnol 2018; 16(1): 99-103.
24. Keshavarz R, Mirjalili A, Mosavari N, et al. Development and optimization a high sensitive and specific ELISA system for rapid detection of paratuberculosis in cattle. Int J Adv Biotechnol Res 2016; 7(1): 1-9.
25. Carroll J, Draper LA, O'Connor PM, et al. Comparison of the activities of the lantibiotics nisin and lacticin 3147 against clinically significant mycobacteria. Int J Antimicrob Agents 2012; 36(2): 132-136.
26. Ali ZI, Saudi AM, Albrecht R, et al. The inhibitory effect of nisin on *Mycobacterium avium* ssp. *paratuberculosis* and its effect on mycobacterial cell wall. J Dairy Sci 2019; 102(6): 4935-4944.
27. Schade R, Calzado EG, Sarmiento R, et al. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. Altern Lab Anim 2005; 33(2): 129-154.
28. Tabll AA, Shahein YE, Omran MM, et al. Monoclonal IgY antibodies: advancements and limitations for immunodiagnosis and immunotherapy applications. Ther Adv Vaccines Immunother 2024; 12: 25151355 241264520. doi: 10.1177/25151355241264520.
29. Zeng X, Wang H, Huang C, et al. Evaluation of the immunogenic response of a novel enterobactin conjugate vaccine in chickens for the production of enterobactin-specific egg yolk antibodies. Front Immunol 2021; 12: 629480. doi: 10.3389/fimmu. 2021.629480.
30. Sudjarwo SA, Eraiko K, Sudjarwo GW, et al. The potency of chicken egg yolk immunoglobulin (IgY) specific as immunotherapy to *Mycobacterium tuberculosis* infection. J Adv Pharm Technol Res 2017; 8(3): 91-96.
31. Van Nguyen S, Umeda K, Yokoyama H, et al. Passive protection of dogs against clinical disease due to canine parvovirus-2 by specific antibody from chicken egg yolk. Can J Vet Res 2006; 70(1): 62-64.
32. Lee EN, Sunwoo HH, Menninen K, et al. *In vitro* studies of chicken egg yolk antibody (IgY) against *Salmonella enteritidis* and *Salmonella typhimurium*. Poult Sci 2002; 81(5): 632-641.
33. Xu Y, Li X, Jin L, et al. Application of chicken egg yolk immunoglobulins in the control of terrestrial and aquatic animal diseases: a review. Biotechnol Adv 2011; 29(6): 860-868.
34. Rahimi S, Moghadam Shiraz Z, Zahraei Salehi T, et al. Prevention of *Salmonella* infection in poultry by specific egg-derived antibody. Int J Poultry Sci 2007; 6(4): 230-235.
35. Xie YM, Gao S, Wang LY, et al. Therapeutic effect of probiotics and oral IgY as supplementary drugs in the treatment of pediatric rotavirus enteritis: a comparative study [Chinese]. Zhongguo Dang Dai Er Ke Za Zhi 2013; 15(11): 1000-1005.
36. Karamzadeh-Dehaghani A, Towhidi A, Zhandi M, et al. Combined effect of probiotics and specific immunoglobulin Y directed against *Escherichia coli* on growth performance, diarrhea incidence, and immune system in calves. Animal 2021; 15(2): 100124. doi: 10.1016/j.animal.2020.100124.
37. El-Kafrawy SA, Abbas AT, Oelkrug C, et al. IgY antibodies: The promising potential to overcome antibiotic resistance. Front Immunol 2023; 14: 1065353. doi: 10.3389/fimmu.2023.1065353.