# Research Note: Oxidative stress and immune response following the administration of live attenuated *Mycoplasma gallisepticum* vaccination in backyard chicken

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**ABSTRACT** In the present study, we investigated a possible relationship between the immune response and the oxidative stress (**OS**) state trend in a group of 12 chickens after intraocular administration of an attenuated *Mycoplasma gallisepticum* (**MG**) vaccine. Blood samples were collected at the vaccination time (T0), after 14 (T1) and 21 d (T2). White blood cell count (**WBC**), differential leucocyte count, and anti-MG antibodies titer (**S/P**) were studied as immune response indexes. As plasmatic OS biomarkers levels, we considered malondialdehyde (**MDA**), nitric oxide (**NO**), superoxide dismutase (**SOD**), reactive oxygen metabolites derived compounds (**d-ROMs**), the ferric reducing ability of plasma (**FRAP**), and superoxide anion ($\mathbf{O_2}^-$). After antigenic stimulation, it was observed a significant decrease in monocythemia and a significant increase in thrombocythemia, S/P, MDA, and SOD. Furthermore, subjects with high d-ROMs levels at T0 tended to develop higher cellular mobilization with increases in WBC and lymphocytes accompanied by lower antibody release. It was also observed that the antioxidant components FRAP and SOD were moderately positively correlated to the entity of antibody response.

**Key words:** backyard chicken, *Mycoplasma gallisepticum*, vaccination, immunity response, oxidative stress

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## INTRODUCTION

*Mycoplasma gallisepticum* (**MG**), a bacterium belonging to the *Mollicutes* class, is a major agent of mycoplasmosis in avian species, including *Gallus gallus*, where it causes relevant economic losses in both intensive and rural/amateur farming (Riazuddin et al., 2017). The clinical containment strategy of MG infections in the field based on the application of tight vaccinal protocols (Shoaib, 2019), aims both to optimize biohazard management, and minimize the use of antibiotics, thus avoiding drug resistance. Moreover, vaccination results as one of the main weapons for the disease containment, active in the limitation of the pathological effects as well as in the reduction of reinfection risk (Nascimento et al., 2005). Nevertheless, its main criticism is the efficacy that is strictly dependent on the animal's immune response, in fact several exogenous and endogens factors can interfere with the immune response and therefore with its effectiveness (Muneer et al., 1988). Among these factors, a relevant role is played by the oxidative state (**OS**), that is determined by the balance between pro-oxidant and antioxidant pressure (Sorci and Faivre, 2009).

On these bases, we investigated in a group of Australorp breed chickens, the parallel evolution of both the immune response and OS status following the intraocular administration of a MG vaccine. The immune response was monitored by measuring the total white blood cell global counts (**WBC**) and their differential distribution in lymphocytes (**Lym**), monocytes (**Mon**), heterophils (**Het**), eosinophils (**Eos**), basophils (**Bas**), and thrombocytes (**Thro**), in correlation with the trend of plasmatic anti-MG antibody titer. Whereas the systemic oxidative status of the animals was investigated by measuring the levels of several plasma OS biomarkers, including malondialdehyde (**MDA**), nitric oxide (**NO**), superoxide dismutase (**SOD**), reactive oxygen metabolites derived compounds (**d-ROMs**), the ferric reducing ability of plasma (**FRAP**), and superoxide anion ($\mathbf{O_2}^-$).

## MATERIALS AND METHODS

The blood examined was taken from animals that had been sampled according to a plan to monitor the health



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condition of the poultry farm. Therefore, blood sampling must be considered as a medical operation falling within normal veterinary medical practices. The protocol of this study was approved by the Committee for Animal Ethics of the University of Parma (approval number PROT. N. 23/CESA /2021).

The study involved a group of 12 Australorp chickens (*Gallus gallus domesticus*), 23 wk old, including 2 males and 10 spawning females, bred extensively on a small amateur farm in northern Italy, in the province of Parma. In their eighth week of life, the owner accidentally introduced into the group 2 subjects asymptomatically infected by MG. After 10 d, although the other 12 animals showed respiratory symptoms due to MG infection, as subsequently diagnosed by PCR test on a tracheal swab (Mg-Ms PCR from BioChek BV - Fokkerstraat 14 - 2811 ER Reeuwijk - ND), the 2 newly introduced hens were removed from the group. The respiratory symptoms resolved after the treatment with doxycycline-HCl in drinking water at a dosage of 500 mg/L for 8 d, in association with an accurate environment sanitization. At the age of 21 wk, 14 d before the vaccination, the 12 hens were subjected again to a tracheal swab that was negative for MG at the PCR test. Aiming to control of the pathogen, the animals at 23 wk of age were then vaccinated by individual intraocular administration of live attenuated vaccine Nobilis MG 6/85 fl 500d (MSD Animal Health), following the instructions recommended by the manufacturer.

At the time of vaccination (T0), a blood sample (2 mL) was drawn from the right ulnar vein of each subject, put in a heparinized vial, immediately placed in a refrigerated container, and stored at 5°C. Each animal was then placed in a cardboard box for 30 min, and the feces were collected for individual parasitological investigation, by Mini-FLOTAC technique (Iemmi et al., 2021a). The blood sampling was repeated after 14 (T1) and 21 d (T2) from T0.

The hematological assessment was performed in a 200 $\mu$L aliquot of each blood sample, according to a previously described protocol (Iemmi et al., 2021a). The considered parameters were microhematocrit packed cell volume (**PCV**), red blood cell counts (**RBC**), WBC counts and differential white blood cell count. The remaining 1.8 mL of blood were centrifuged at 1,400 × *g* for 15 min, and the obtained plasma was employed both for antibody-MG titration and for the measurement of the OS biomarkers. The antibody titration was performed with the commercial ID Screen Mycoplasma gallisepticum Indirect ELISA kit (ID Innovative-Diagnostics, 310 rue Louis Pasteur − 34790 Grabels, France) following the manufacturer's instruction. Titers were expressed as S/P ratio, namely the ratio between the optical density at 450 nm of the tested sample (**S**), and that of the positive control (**P**), with a positivity cut-off ≥0.5 (Feberwee et al., 2005). Plasmatic OS biomarkers, including MDA, NO, SOD, d-ROMs, FRAP, and $O_2^-$, were quantitatively determined according to protocols described in a previous study (Iemmi et al., 2021b).

The statistical elaboration of the data was performed with GraphPad Prism ver.7 software (GraphPad Software Inc., La Jolla, CA). Data distribution type was evaluated employing the Kolmogorov−Smirnov test; data with normal distribution were expressed as means ± standard deviations (**SD**), whereas nonparametric data were treated as medians and total ranges. The evaluation of the significance of the differences between data groups were assessed by ordinary 1-way ANOVA, unpaired *t*- test, or multiple Mann−Whitney U tests and Kruskal−Wallis H test, respectively for parametric and nonparametric data. Linear regression analysis and Pearson correlation coefficient analysis were used to assess the correlation between leukocyte levels, S/P ratios, and the levels of the different OS biomarkers. Correlation (r) was considered strong for $r \geq 0.700$ or $r \leq -0.700$, moderate for $0.700 > r \geq 0.300$ or $-0.700 < r \leq -0.300$ and absent for $0.300 > r > -0.300$; the "+" and "-" signs were indicative of either positively or negatively correlated data.

## RESULTS AND DISCUSSION

The data of the hematological and serological parameters at the different sampling times are reported in Table 1, whereas those of the levels of the OS biomarker are reported in Table 2.

The data show that antigen stimulation due to the anti-MG vaccine didn't cause any significant variation in the total and % counts of Lyn, Het, Eos, and Bas. A significant variation was observed for the numbers of Mon ($P = 0.0242$) and Mon% ($P = 0.0438$), highlighting a decrease in Mon (n° of cells) and Mon % levels between T0 and T1, with $P = 0.0124$ and $P = 0.0162$ respectively, followed by an increase between T1 and T2, that restored the count nearly to the values of T0.

Considering the Thro cell population, although ANOVA didn't show significant differences between mean levels at the 3 sampling times, a significant

**Table 1.** Hematological and serological parameters at the different sampling times are reported as means and SD.

| | T0 | T1 | T2 |
|---|---|---|---|
| PCV (%) | 36.61 ± 7.70 | 37.77 ± 7.95 | 37.75 ± 6.62 |
| RBC ($10^6/\mu$l) | 2.78 ± 0.38 | 2.87 ± 0.78 | 2.84 ± 0.79 |
| WBC ($10^3/\mu$l) | 12.21 ± 10.07 | 13.40 ± 5.25 | 18.93 ± 10.09 |
| Het (%) | 38.58 ± 16.54 | 32.75 ± 13.61 | 31.83 ± 9.04 |
| Het ($10^3/\mu$l) | 4.18 ± 3.16 | 3.47 ± 2.01 | 4.32 ± 3.52 |
| Eos (%) | 0.84 ± 1.59 | 2.20 ± 3.31 | 3.06 ± 2.98 |
| Eos ($10^3/\mu$l) | 0.21 ± 0.50 | 0.38 ± 0.68 | 0.59 ± 0.81 |
| Bas (%) | 0.64 ± 1.43 | 0.18 ± 0.40 | 0.59 ± 1.10 |
| Bas ($10^3/\mu$l) | 0.04 ± 0.10 | 0.02 ± 0.06 | 0.17 ± 0.40 |
| Lym (%) | 57.45 ± 14.49 | 64.79 ± 14.41 | 62.86 ± 10.22 |
| Lym ($10^3/\mu$l) | 7.61 ± 6.65 | 8.72 ± 3.85 | 12.00 ± 6.90 |
| Mon (%) | 2.53 ± 2.75 | 0.18 ± 0.60 | 1.66 ± 2.34 |
| Mon ($10^3/\mu$l) | 1.03 ± 1.22 | 0.03 ± 0.10 | 0.25 ± 0.29 |
| Thro ($10^3/\mu$l) | 11.97 ± 9.08 | 18.29 ± 11.09 | 17.42 ± 9.18 |
| S/P ratio | 1.96 ± 1.03 | 2.95 ± 0.65 | 2.79 ± 0.67 |

Abbreviations: Bas, basophils; Eos, eosinophils; Het, heterophils; Lym, lymphocytes; Mon, monocytes; PCV, packed cell volume; RBC, red blood cell; Thro, thrombocytes; WBC, white blood cell.

**Table 2.** OS biomarkers at the different sampling times; d-ROMs, NO, and SOD values are represented as means and SD (parametric), whereas MDA, $O_2^-$ and FRAP values are indicated as medians and ranges (nonparametric). In some cases, the result of the measurement was under the limit of detection (LOD).

| | T0 | T1 | T2 |
|---|---|---|---|
| d-ROMs (UCAR) | 50.88 ± 18.19 | 57.20 ± 28.21 | 61.03 ± 25.16 |
| NO ($\mu$mol/L) | 24.69 ± 16.71 | 31.20 ± 26.73 | 14.74 ± 22.34 |
| SOD (U/mL) | 2.25 ± 0.72 | 2.33 ± 1.23 | 3.06 ± 1.28 |
| MDA (mmol/L) | <LOD (<LOD−4.00) | 2.10 (<LOD−172.60) | 35.27 (<LOD−90.20) |
| $O_2^-$ (mABS) | 0.27 (0.22−0.64) | 0.27 (0.17−0.65) | 0.23 (0.18−0.64) |
| FRAP ($\mu$mol/L) | 175.99 (122.29−350.87) | 169.07 (102.65−274.40) | 146.58 (97.14−231.71) |

Abbreviations: d-ROMs, reactive oxygen metabolites derived compounds; FRAP, ferric reducing ability of plasma; MDa, malondialdehyde; NO, nitric oxide; $O_2^-$, superoxide anion; SOD, superoxide dismutase.

increase could be observed by the *t* test between T0 and T1($P = 0.0251$).

Regarding the serological examination, as expected, the chickens were already positive at T0, as a consequence of the field MG infection determined by the introduction of 2 MG subclinical subjects 15 wk before the time of the vaccination. Consequently, the vaccination should be considered as a booster for the reinforcement of the immune response previously acquired against the pathogen.

As expected, following the vaccination, there was a significant increase in the average anti-MG antibody (**Ab**) titers ($P = 0.0155$) for the whole duration of the study, with $P = 0.0139$ between T0 and T1, followed by a moderate decrease in the interval T1−T2. However, the average levels of the Ab titers between T0 and T2 remained statistically significant with $P = 0.0376$.

Examining the OS biomarkers, as regards MDA, consequently to vaccinal stimulation a significant difference was observed between the means measured at the 3 sampling sessions ($P = 0.0030$). The extremely low levels found at T0 underwent a significant increase in most of the vaccinated chickens (64% of subjects) at T1 ($P$=0.0258), which continued until T2, with a $P$ value for the whole T0−T2 interval of 0.0004. This phenomenon could have been determined, in general, to peroxidation of cell membrane phospholipids due to the production of oxidative compounds by active leucocytes, as suggested by a previous investigation (Zeb and Ullah, 2016).

After moderate growth in the T0−T1 interval, the mean levels of SOD exhibited a notable increase after T1, with a statistical significance considering the whole interval T0−T2 ($P = 0.0472$). Regarding the other OS biomarkers examined, no significant variations were observed.

For the following correlation study, the values of the different parameters were examined with WBC and S/P levels, being these the 2 main indicators of the immune response evaluated in the present study.

Considering the direct comparison of the individual levels observed for all the chickens at any singular sampling sessions, only moderate and/or discordant correlations were detected referring both to WBC and S/P; exceptions were represented by d-ROMs and $O_2^-$. In particular, d-ROMs levels changed with a positive correlation with respect to those of S/P both at T0 (r = 0.721) and T2 (r = 0.339), that was absent at T1; the same pattern of variations was observed for $O_2^-$, with r values of 0.464 and 0.348 at T1 and T2, respectively. Although at T0 the subjects had not yet undergone the vaccine infection, the S/P initial values depended exclusively on the stimulation of a previous field infection. Therefore, the variability of the S/P values at T0 could be associated either with a more pronounced and protracted individual immune response, linked to the subject's metabolic single characteristics, or to a depletion of the most recent field infection. Interestingly both scenarios justify higher levels of oxidizing factors (indicated by d-ROMs and $O_2^-$) derived by immune activation (Costantini and Møller, 2009). The positive correlation observed in T2 for these parameters however reinforces the hypothesis that pronounced and prolonged immune activation of its humoral component leads to higher releases and a progressive accumulation of oxidizing factors.

In addition to the correlations between the levels highlighted for single sampling sessions, we evaluated those between the variations (Δ) of the different parameters (interval T0−T1, T1−T2, and T0−T2) and those of WBC and S/P values at the 3 sampling times.

Values of ΔWBC are positively strongly correlated, in all the 3 intervals (r > 0.956), with those of ΔLym, whereas a moderate positive correlation (r = 0.639) could be observed with the values of ΔHet, but only for the T0−T1 interval, thus suggesting an indication of the cell types involved in the antigenic response.

About the correlations between ΔWBC and ΔS/P, a slightly positive value could be observed only for the T0−T1 interval, whereas a negative value was detected in the whole T0−T2 interval (r = -0.362), foreshadowing that high humoral responses are not necessarily connected with high increases of leukocytes.

Examining the relation between ΔWBC and the OS biomarkers variations, the low and discordant levels of correlation observed were of difficult interpretation.

As predictable, the same relation already observed between ΔS/P and ΔWBC appears also between ΔS/P and ΔLym; although the WBC numerosity is strongly determined by the entity of Lym, this result supports the hypothesis that the circulating leukocyte and lymphocyte increase does not lead to higher antibody production.

A moderate negative correlation of ΔS/P between ΔHet (r = -0.356) for the T0−T1 interval could probably be explained by the prevalence of the nonspecific immune cellular response with respect to the humoral one in its early phase (T0−T1).

Considering the ΔFRAP, only an extremely moderate negative correlation (r = -0.363) in relation to ΔS/P in the T0−T1 interval was observed; this finding could be eventually linked to the consumption of non-enzymatic antioxidant factors derived from immune activation, as already observed, with higher evidence, for the correlation with respect to ΔWBC. No correlation has been found between ΔMDA and ΔS/P.

Moreover, referring to ΔS/P, a negative moderate correlation could be observed with both ΔNO (r = -0.329) and $\Delta O_2^-$ (r = -0.526) for the whole T0−T2 interval. This last evidence could be eventually explained by the increased oxidative pressure possibly caused by the immune activation that leads to an inhibition of the antibody production. In support of the above-mentioned hypothesis, the positive correlation found between ΔS/P and ΔSOD (r = 0.659) for the T0−T2 interval, could indicate that the increase of the enzymatic antioxidant protection may be effective to decrease the levels of the prooxidant compounds that seem to obstacle the antibody synthesis.

After comparing both the values of the various parameters at each sampling session and their variations considering the single intervals, we finally examined the variations of WBC and S/P levels for the intervals T0−T1, T1−T2, and T0−T2, in comparison with those of the OS biomarkers detected at the beginning and at the end of each interval.

Starting from FRAP levels, as regards those of ΔWBC for the different intervals, no significant correlation was highlighted, on the contrary, a moderate positive correlation (r = 0.396) was detected between $FRAP_{T0}$ and $\Delta S/P_{T0T1}$ values, which could indicate that, following an antigenic stimulus, higher levels of antioxidants in the plasma could favor a higher antibody response, at least in the first 14 d from the stimulus.

Except for the levels of d-ROMs at T1 with respect to that of ΔWBC in the T0−T1 interval, which resulted not correlated, the other d-ROMs values were positively correlated with those of ΔWBC at the T0−T2 ($r_{T0}$ = 0.386 and $r_{T2}$ = 0.604) and T1−T2 ($r_{T1}$ = 0.643 and $r_{T2}$ = 0.446) intervals, highlighting that an oxidative environment seems to promote the increase of WBC number and, at the same time, high leukocyte proliferation could be associated with final high levels of d-ROMs, and therefore of those of free radicals. About the correlations between the levels of the other OS markers and ΔS/P or ΔWBC, the discordant data found, do not allow to draw any interpretation on the measurements detected here.

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## DISCLOSURES

The authors declare no conflicts of interest.

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