

Oxygenated Drinking Water Enhances Immune Activity in Broiler Chicks and Increases Survivability against *Salmonella Gallinarum* in Experimentally Infected Broiler Chicks

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ABSTRACT. It has been suggested that drinking oxygenated water may improve oxygen availability, which may increase vitality and improving immune activity. The present study evaluated the immune enhancing effects of oxygenated drinking water in broiler chicks and demonstrated the protective efficacy of oxygenated drinking water against *Salmonella Gallinarum* in experimentally infected broiler chicks. Continuous drinking of oxygenated water markedly increased serum lysozyme activity, peripheral blood mononuclear cell proliferation and the CD4⁺/CD8⁺ splenocyte ratio in broiler chicks. In the chicks experimentally infected with *S. Gallinarum*, oxygenated drinking water alleviated symptoms and increased survival. These findings suggest that oxygenated drinking water enhances immune activity in broiler chicks, and increases survivability against *S. Gallinarum* in experimentally infected broiler chicks.

KEY WORDS: immune enhancement, oxygenated drinking water, *Salmonella Gallinarum*.

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Veterinary drugs, especially antimicrobial agents, were discovered over 50 years ago and have since been widely used as feed additives or additives to drinking water to improve growth performance and to prevent subclinical disease challenge in poultry industries [4, 25]. However, there is worldwide concern about the overuse of antimicrobials including the development and spread of antimicrobial resistant strains of bacteria and resistance genes from animals to humans through the food chain [13]. Moreover, residues of these drugs could lead to allergic reactions in sensitive individuals [12]. For these reasons, the restriction of antimicrobials has become a worldwide trend. Especially, under Regulation 1831/2003/EEC, the European Union initiated a progressive ban on the use of antimicrobials in animal feed and drinking water beginning January 1, 2006 [8]. This political decision has focused increasing attention on the development of eco-friendly alternatives to reduce the use of antimicrobials. One of the most promising methods of reducing antimicrobials in poultry industries is improving the immune statuses of birds through the prophylactic administration of natural immunostimulants such as herbs, probiotics and clay minerals, and improvement of raising conditions (e.g., well-ventilated, availability of fresh water and freedom from overcrowding) [16–18].

Since the early 1990s, oxygenated water or equipment for the oxygenation of tap water has been commercially available by various companies, especially in Europe and the

United States. Normal tap water contains approximately 5–7 mg/l dissolved oxygen and fresh fountain water contains 10–12 mg/l dissolved oxygen. Oxygenation of tap water can increase the concentration of dissolved oxygen from 30 to 120 mg/l [10, 26]. It has been considered that drinking oxygenated water improves oxygen availability, which may increase the vitality and improve immune functions. Hyvärinen *et al.* [15] reported that infusion of oxygenated water into the stomach of dogs increased the oxygen tension in the portal blood. Forth and Adam [9] also reported that oxygenated water applied intragastrically to rabbits increased the oxygen content in the abdominal cavity and portal vein. Accumulating evidence has also indicated that oxygenated water has beneficial biological activities. Some clinical reports described therapeutic effects of oxygenated water for a variety of diseases such as obesity and liver dystrophy [5, 23, 28]. In addition, oxygenated drinking water accelerated alcohol detoxification in monkeys [15]. However, a paucity of data is available concerning the effect of oxygenated drinking water on immunological parameters in animals.

Salmonella enterica serovar Gallinarum (*S. Gallinarum*) causes fowl typhoid, a severe systemic infection affecting galliform birds of all ages. The infection is typified by anemia, leukocytosis, hepatosplenomegaly and intestinal tract hemorrhage [27]. Although fowl typhoid has been eradicated from Australia, North America and most European countries, it is still a significant problem in some countries of Asia, Africa, and Central and South America [21, 27]. In Korea, outbreaks of fowl typhoid have been reported since 1992 and this disease has become the most serious problem in the poultry industry [19, 21].

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The aims of the present study were to evaluate the immune enhancing effects of oxygenated drinking water in broiler chicks and to demonstrate the protective efficacy of oxygenated drinking water against *S. Gallinarum* in experimentally infected broiler chicks as an initial step towards the development of eco-friendly alternatives to reduce the use of antimicrobials.

Normal tap water was aerated with pure oxygen using a model NOW-1000 apparatus (Korea Nature Oxygen Water, Gwangju, Korea) for 3 hr at room temperature. The oxygenated water and the normal tap water were provided in water supply system, which was replaced three times daily. Oxygen partial pressure in the water was measured using a model HI 9146 apparatus (Hanna Instruments, Woonsocket, RI, USA) before filling the water supply system, and after removing the water supply system from the cages. Freshly oxygenated water contained up to 45 mg/l oxygen, 6.4 times more than that of non-oxygenated fresh tap water (7 mg/l), while stale oxygenated water still contained 6.3 times more oxygen than stale non-oxygenated water (38 vs. 6 mg/l).

Twenty-day-old Ross broiler chicks were subjected to immunological assay (n=11 in each group) and evaluation of protective efficacy against *S. Gallinarum* infection (n=21 in each group). In each independent study, chicks were randomized into two groups. One group received only normal tap water (tap water-drinking group) and the other group received only oxygenated drinking water (oxygenated water-drinking group). All chicks were housed in separate air-controlled rooms and allowed free access to a nutritionally complete antibiotic-free chicks feed and their particular drinking water. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Approval number: CNU IACUC-YB-2010-1).

In the immunological assay, all chicks were supplied with the particular drinking water for 2 weeks, and body weight was monitored every 2 days. No significant differences in body weight change were found between the groups for 2 weeks (data not shown). Blood was collected from the wing vein of each chick on day 15. Serum was obtained by centrifugation at $2,000 \times g$ for 10 min at 4°C and used for the determination of lysozyme activity [20]. PBMC was isolated using Lymphoprep™ (Axis-shield, Oslo, Norway) according to the manufacturer's instructions. PBMCs were coincubated with or without 25 µg/ml lipopolysaccharide (LPS; Sigma-Aldrich) and PBMC proliferation was determined as an OD_{540nm} value of colored material after incubation with MTT [30]. Spleen was obtained from each chick, and single cell suspensions were prepared by pushing the tissue through a 40 µm nylon mesh (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Isolated cells were analyzed to determine the component ratio of CD3⁺CD4⁺ cells and CD3⁺CD8⁺ cells using anti-CD3, CD4 and CD8 cell markers (fluorescein isothiocyanate and phycoerythrin-conjugated monoclonal antibodies, outhern Biotech, Birmingham, AL, U.S.A.) [17].

In the evaluation of protective efficacy against *S. Galli-*

narum infection, all chicks were acclimatized to their particular drinking water for 2 weeks before experimental bacterial infection. Prior to the experiment, the chicks were confirmed to be *Salmonella*-free by bacteriological culture of fecal samples obtained by cloacal swabs [14] and a serum plate agglutination test using *S. Gallinarum* antigen [22]. In addition, they were also confirmed to harbor only a low level ($1 \times 10^5 - 1 \times 10^6$ colony forming units (cfu)/g of feces) of *Escherichia coli* by bacteriological culture of fecal samples obtained by cloacal swabs [24]. *S. Gallinarum* (SG3001) was prepared as previously described [29]. Each chick was orally challenged with 5×10^{10} cfu (the optimal dose as determined in our previous study) [17]. The chicks were monitored daily for clinical signs, whereby each chick was observed and examined separately. Clinical signs were scored as + for drowsiness, ++ for drowsiness, ruffled feathers/respiratory distress and +++ for emaciation and whitish creamy diarrhea from the vents. The number of chicks affected in each group was noted and the mortality in each group also was recorded for 7 days after the bacterial challenge. The scoring method was similar to that used by Christensen *et al.* [3]. All the remaining chicks were euthanized on 7 days post-infection (DPI). The liver and spleen from the sacrificed chicks were collected and weighted. Post-mortem examination on all dead and remaining chicks was carried out and any pathological changes associated with fowl typhoid were observed. Viable bacteria cell count was carried out in liver and spleen collected from all remaining chicks as previously described [29].

The data are expressed as mean \pm standard deviation (SD). Student's *t*-test was performed for statistical analysis of the data. All statistical analysis of data was performed using SigmaPlot® version 10.0 software (Systat Software, San Jose, CA, U.S.A.). $P < 0.05$ was considered as the level of significance.

Lysozyme, which is secreted by some phagocytes such as macrophages and polymorphonuclear leukocytes, is highly active against bacteria. Lysozyme can destroy glucosidic bonds in the cell walls of *E. coli* and *Staphylococcus* as a result of their phagocytic activity [11]. Therefore, high lysozyme activity in the serum is associated with a high destructive activity of phagocytes [20]. In the present study, the serum lysozyme concentration in the oxygenated water-drinking group (3.803 ± 1.748 µg/ml) was significantly higher than in the tap water-drinking group (2.456 ± 0.825 µg/ml) ($P < 0.05$) (Fig. 1). These results suggest that continuous drinking of oxygenated water may enhance destructive activity of phagocytes, thus enhancing the immune function of the body.

LPS is a constituent of the outer membrane of Gram-negative bacteria. The molecule acts as an endotoxin and elicits a potent immune response in animals [7]. In the present study, LPS-induced lymphocyte proliferation in the oxygenated water-drinking group (1.702 ± 0.248) was significantly enhanced compared with the tap water-drinking group (1.405 ± 0.994) ($P < 0.05$), although proliferation of the unstimulated cells did not differ between the groups (tap

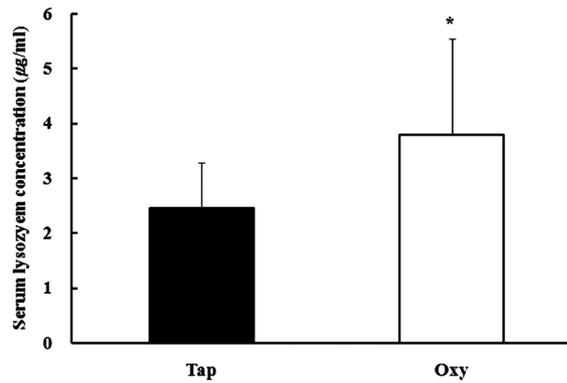


Fig. 1. Effect of oxygenated drinking water on serum lysozyme activity in broiler chicks. Chicks were supplied each particular drinking water for 2 weeks, and blood was collected from the wing vein of each chick on day 15. The serum lysozyme concentration in the oxygenated water-drinking group (Oxy) was significantly higher than in the tap water-drinking group (Tap) (* $P < 0.05$). For each group, data represents the mean \pm SD (n=11).

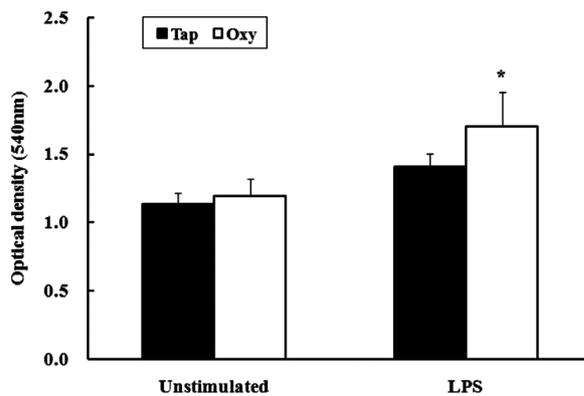


Fig. 2. Effect of oxygenated drinking water on PBMC proliferation in broiler chicks. PBMCs were coincubated with or without mitogen (LPS) and PBMC proliferation was determined as an OD_{540nm} value of colored material after incubation with MTT. When LPS was added into the culture medium, PBMC proliferation in the oxygenated water-drinking group (Oxy) was significantly enhanced compared to the tap water-drinking group (Tap) (* $P < 0.05$). Proliferation of the unstimulated cells did not differ between the groups. For each group, data represents the mean $OD_{540nm} \pm$ SD (n=11).

water-drinking group: 1.137 ± 0.075 ; oxygenated water-drinking group: 1.194 ± 0.123) (Fig. 2). This suggests that the mitogenicity of lymphocytes is enhanced by the drinking of oxygenated water in broiler chicks, especially against Gram-negative bacteria such as *S. Gallinarum*.

The percentage of $CD3^+CD4^+$ T lymphocytes in the spleens of the oxygenated water-drinking group ($14.18 \pm 10.65\%$) were significantly increased compared to spleens of the tap water-drinking group ($6.17 \pm 1.77\%$) ($P < 0.001$). However, no significant difference in spleen $CD3^+CD8^+$ T lymphocytes was observed between the groups (tap water-

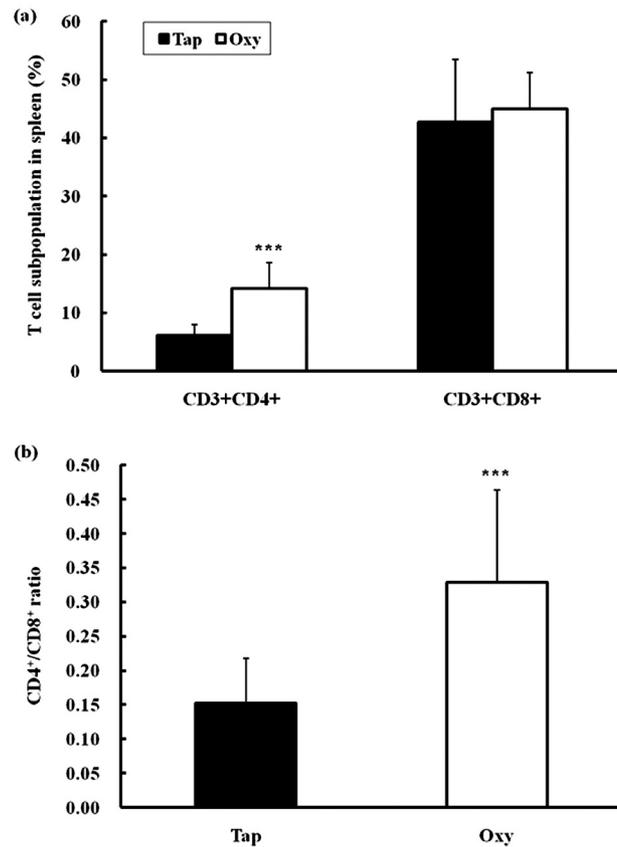


Fig. 3. Effect of oxygenated drinking water on the spleen T lymphocyte subpopulations in broiler chicks. (a) The percentage of $CD3^+CD4^+$ T lymphocyte in spleen of the oxygenated water-drinking group (Oxy) were significantly increased compared to those of the tap water-drinking group (Tap) (***) $P < 0.001$). However, no significant difference in the spleen $CD3^+CD8^+$ T lymphocyte was observed between the groups. (b) The ratio of $CD4^+/CD8^+$ cells in spleens of the oxygenated water-drinking group was significantly increased compared to those of the tap water-drinking group (***) $P < 0.001$). For each group, data represents the mean \pm SD (n=11).

drinking group: $42.77 \pm 4.45\%$; oxygenated water-drinking group: $44.98 \pm 6.28\%$) (Fig. 3a). The ratio of $CD4^+/CD8^+$ cells in spleens of the oxygenated water-drinking group (0.33 ± 0.13) was significantly increased compared to those of the tap water-drinking group (0.15 ± 0.07) ($P < 0.001$) (Fig. 3b). The $CD4^+/CD8^+$ ratio is used as a measure of immune function and response. Low ratios are usually observed in individuals with acute viral diseases and hemophilia [2], whereas high ratios have been associated with an increase in the immunofunctional ability of chicks [1, 6]. Therefore, oxygenated drinking water confers a benefit on the immune function in broiler chicks and may enhance the resistance of broiler chicks to infectious diseases.

The beneficial effects of oxygenated drinking water on immune activity in broiler chicks led us to evaluate the protective efficacy of oxygenated drinking water against *S.*

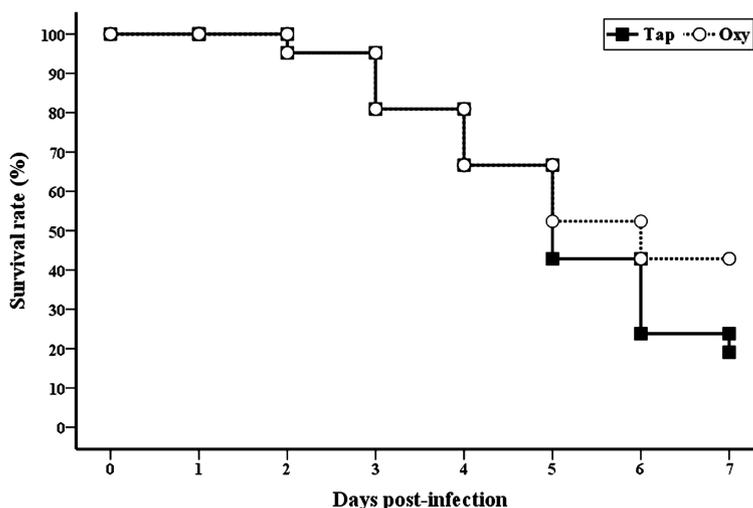


Fig. 4. Trends in survival rates of experimentally *Salmonella Gallinarum* infected broiler chicks. Mortality was first observed on 2 DPI in both groups. The survival rates progressively increased in the oxygenated water-drinking group (Oxy) as compared to the tap water-drinking group (Tap) from 5 DPI, although the survival rates in the both groups were the same by 4 DPI. By 7 DPI, the survival rates were 19.05% (4/21) in the tap water-drinking group and 42.86% (9/21) in the oxygenated water-drinking group.

Table 1. Scoring of the clinical signs noted in the present study

DPI	Tap water-drinking group (n=21)				Oxygenated water-drinking group (n=21)			
	-	+	++	+++	-	+	++	+++
0	21	0	0	0	21	0	0	0
1	19	1	1	0	19	1	1	0
2	13	4	2	1	14	3	2	1
3	3	3	5	6	11	3	2	1
4	3	3	4	4	7	3	4	0
5	2	1	1	5	4	4	2	1
6	1	2	1	1	4	3	2	0
7	1	1	1	1	4	3	2	0

Arabic numerals under each clinical sign score indicate number of chicks identified with the specific signs. Scoring procedures were according to Christensen *et al.* [4]. The abbreviation DPI denotes days post-infection. Symbols: +, drowsiness; ++, ruffled feathers and/or respiratory distress; +++, ruffled feathers, emaciation, white creamy diarrhea; -, no clinical symptoms.

Gallinarum, which is the most serious problem in the Korean poultry industry [22]. Daily monitoring of clinical signs in chicks infected with *S. Gallinarum* showed that the severity of symptoms associated with fowl typhoid were gradually reduced in the oxygenated water-drinking group as compared to the tap water-drinking group from 3 DPI, although onset of clinical symptoms was not delayed (Table 1). Mild and moderate clinical signs including drowsiness and ruffled feathers and/or respiratory distress were observed from 1 DPI until the end of the experiment in both groups. However, severe clinical signs including emaciation and creamy whitish diarrhea were observed in the tap water-drinking group from 2 DPI until the end of the experiment, while severe clinical signs were only observed in the

oxygenated water-drinking group on 2, 3 and 5 DPI. Mortality was first observed on 2 DPI in both groups. Although the survival rates in the both groups were the same (66.67%; 14/21) by 4 DPI, it progressively increased in the oxygenated water-drinking group as compared to the tap water-drinking group from 5 DPI. By 7 DPI, the survival rates were 19.05% (4/21) in the tap water-drinking group and 42.86% (9/21) in the oxygenated water-drinking group (Fig. 4). The number of viable bacteria in the liver and spleen of the oxygenated water-drinking group showed a tendency to decrease compared with the tap water-drinking group at the end of the experiment, although the difference was not significant (liver, $P=0.080$; spleen, $P=0.156$) (Fig. 5). Those protective effects of oxygenated drinking water against *S.*

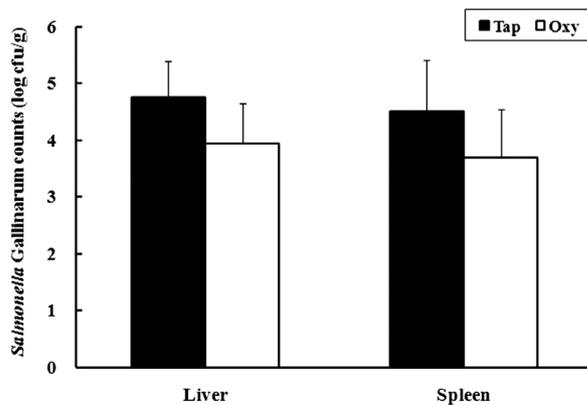


Fig. 5. Viable bacteria cell count in the liver and spleen. The number of the viable bacteria in the liver and spleen of the oxygenated water-drinking group (Oxy) showed a tendency to decrease compared with the tap water-drinking group (Tap) at the end of the experiment, although the difference was not significant (liver, $P=0.080$; spleen, $P=0.156$). For each group, data represents the mean \pm SD.

Gallinarum may relate to general immune enhancing effects of oxygenated drinking water, consistent with the results of the immunological assays in the present study. Our previous report has also shown that the survivability of broiler chicks against *S. Gallinarum* is increased by the enhancement of general immunity such as increments of lysozyme activity, LPS-induced lymphocyte proliferation and CD4⁺/CD8⁺ ratio [17].

On post-mortem examination of experimentally *S. Gallinarum* infected chicks, pathological changes characteristic of fowl typhoid were evident. Features observed were septicemic carcass with congested subcutaneous blood vessels and skeletal muscles that were also congested and dark brown in appearance. The liver and spleen were enlarged; the liver displayed a cooked appearance, while petechial hemorrhages and necrotic foci were common features in liver, spleen and myocardium. In addition, hydropericardium, catarrhal enteritis and nephropathy were also observed. Although there was no significant difference in the overall severity of lesions between both groups, the oxygenated water-drinking group displayed less severe petechial hemorrhages and necrotic foci in the liver compared with the tap water-drinking group (Fig. 6a). Moreover, enlargement of the liver in the oxygenated water-drinking group (46.27 ± 7.24 gram) was significantly milder compared with in the tap water-drinking group (61.03 ± 16.57 gram) at the end of the experiment ($P<0.05$) (Fig. 6b). This finding suggests that continuous drinking of oxygenated water might be beneficial for recovery from liver damage by *S. Gallinarum* infection. This is consistent with previous reports that oxygenated drinking water increases oxygen tension in portal blood and has therapeutic effects in liver dystrophy and alcohol detoxification [9, 15, 28].

Taken together, these findings suggest that oxygenated drinking water enhances immune activity in broiler chicks,

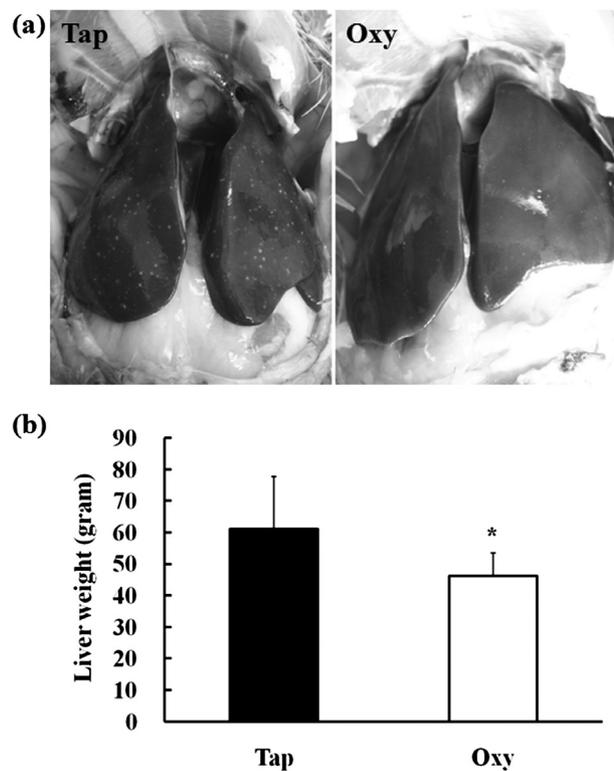


Fig. 6. Gross findings and weight of liver in experimentally *Salmonella Gallinarum* infected broiler chicks. (a) The oxygenated water-drinking group (Oxy) displayed less severe petechial hemorrhages and necrotic foci in liver compared with the tap water-drinking group (Tap). (b) Enlargement of liver in the oxygenated water-drinking group was significantly milder compared with the tap water-drinking group at the end of the experiment (* $P<0.05$). For each group, data represents the mean \pm SD.

and alleviate symptoms and increases survivability against *S. Gallinarum* in experimentally infected broiler chicks. Hence, oxygenated drinking water may provide an alternative way to reduce use of antimicrobials through the promotion of immune activity and prevention of diseases, especially in the Korean poultry industry that remains susceptible to outbreaks of fowl typhoid. Before this potential is realized, however, much work remains to be done. The present study did not investigate the exact mechanisms of oxygenated drinking water against *S. Gallinarum* in broiler chicks. Therefore, precise knowledge of mechanisms of oxygenated drinking water against *S. Gallinarum* is required. In addition, confirmation of the protective efficacy of oxygenated drinking water is required in chicks naturally occurring fowl typhoid.

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