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# Effects of single or combined feeding with *Bacillus subtilis* and organic acid mixture on egg production, physiological responses, and cholesterol level in chicken eggs

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**ABSTRACT.** The objective of this study was to evaluate the nutritional effects of single or combined feeding with *Bacillus subtilis* and a mixture of organic acids on egg production, intestinal ammonia concentration, ceacal microflora profile, and individual lipid fractions in chicken egg yolk. Two doses of *B. subtilis* were administered separately or together with an organic acid mixture composed of formic acid, fumaric acid, and sorbic acid. A total of 250 43-week-old Hy-Line Brown layers were divided into 5 groups with 5 replicates per group, and fed one of five experimental diets (control, maize-soybean meal-based diet without *B. subtilis* or organic acids; T1 – *B. subtilis* 1.0 × $10^{10}$ CFU/kg diet; T2 – *B. subtilis* 1.0 × $10^{10}$ CFU/kg diet + 0.15% organic acids; T3 – *B. subtilis* 2.0 × $10^{10}$ CFU/kg diet; and T4 – B. subtilis 2.0 × $10^{10}$ CFU/kg diet + 0.15% organic acids) for 8 weeks. Egg production and daily egg mass showed a raising trend or increased significantly in the group fed *B. subtilis* alone and the *B. subtilis* + organic acids combined groups, respectively. Additionally, the average eggshell strength in the treated groups was significantly higher compared to the control group. The abundance of ceacal lactic acid bacteria in the treated groups was significantly higher compared to the control group. The cholesterol content in egg yolk in all treated groups was significantly decreased compared to the control group. Overall, feeding *B. subtilis* exerted desirable effects on increasing egg production and eggshell quality, as well as modulating the ceacal microbial profile regardless of co-supplementation with organic acids.

## Introduction

Modern commercial laying hens are known for their exceptionally high egg production potential, and ensuring optimal nutrition is essential to maintain high laying performance and the highest egg quality (Hy-Line International, 2018). Feed additives play a crucial role in this regard, as they are incorporated into feed formulations to enhance food utilisation efficiency, promote acceptance, and improve overall performance and health status (Świątkiewicz et al., 2013). With the prohibition of antibiotics in poultry production, alternatives have been sought and incorporated into poultry feeds to regulate the intestinal microbial flora and avoid certain intestinal pathologies (Yaqoob et al., 2022). Probiotics and organic acids have also been applied as alternative feeding strategies to enhance poultry productivity and health (Hag et al., 2017; Carvalho et al., 2022).

Probiotics are live microorganisms added to feeds that exert beneficial effects in the gastrointestinal tract of chickens (Xu et al., 2006; Ahmad et al., 2022). Probiotics have been reported to improve intestinal microflora, immune response, and disease resistance (Rajput et al., 2013; Grant et al., 2018). *Bacillus subtilis* is one of most widely used bacterial strain as a probiotic in poultry. Studies have shown that feeding *B. subtilis* can increase egg production (Neijat et al., 2019) and improve intestinal microbiota profiles in laying hens (Zhang et al., 2021). Organic acids (OM) have long been utilised for feed preservation, as additives enhancing growth and feed efficiency, and in chick protection through competitive exclusion (Muleta, 2021). They play a crucial role in improving the intestinal health of poultry and exhibit antimicrobial properties (Khan and Iqbal, 2016).

Probiotics and organic acids have pivotal functions in promoting intestinal health and immune regulation. Many studies using broiler chickens have demonstrated the effects of these two feed additives on growth performance and physiological responses (Ahmad et al., 2022). However, only limited information is available regarding their effects on egg production and egg quality in laying hens when used alone or in combination. Therefore, the present study was conducted to evaluate the dietary effects of probiotics administered alone or together with an organic acid mixture on laying performance, egg quality, intestinal ammonia concentrations, ceacal microflora profile, and lipid fractions in chicken eggs.

## Material and methods

The laying hens were housed and feeds were prepared at the Konkuk University animal farm, and the experimental protocol was approved by the Institutional Animal Care and Use Committee of the Konkuk University (KU18136).

### Animals, diets, and management

The study utilised *B. subtilis* strain BS1010 isolated from traditional soy sauce (BS; $1.0 \times 10^{10}$ CFU/g; BS1010, ACC Inc. Seongnam-si, Gyeonggi-do, Republic of Korea). A mixture of OM used in study consisted of 25% formic acid, 10% fumaric acid, and 10% sorbic acid (ACC Inc. Seongnam-si, Gyeonggi-do, Republic of Korea). A total of 250 43-week-old Hy-Line brown layers were randomly assigned to five groups (50 layers per group) to ensure similar egg production across all groups. The layers were housed in a commercially designed caged layer house with water and feed provided *ad libitum.* The control group was fed a maize-soybean meal-based diet without BS or OM. The four experimental groups were fed diets containing two different doses of BS ($1.0 \times 10^{10}$ CFU/kg or $2.0 \times 10^{10}$ CFU/kg diet) alone or a combination of these doses and 0.15% OM (T1, BS $1.0 \times 10^{10}$ CFU/kg diet; T2, BS $1.0 \times 10^{10}$ CFU/kg diet + 0.15% OM; T3, BS $2.0 \times 10^{10}$ CFU/kg diet; T4, BS $2.0 \times 10^{10}$ CFU/kg diet + 0.15% OM) for eight weeks. The basal diet was formulated to meet and exceed the nutritional requirements provided by the National Research Council (1994). The formulation and chemical compositions of the basal diets are outlined in Table 1. Fresh diets were provided daily, and the feed intake of each replicate was recorded weekly. Room temperature was maintained at 22 ± 3 °C, with artificial lighting provided for 15 h each day throughout the experimental period.

**Table 1.** Ingredients and chemical composition of the experimental diets

| Ingredients, % | |
|---|---|
| Yellow maize | 57.91 |
| Soybean meal | 22.91 |
| Wheat bran | 1.54 |
| Corn gluten meal | 4.73 |
| Tallow | 1.50 |
| DL-methionine (99%) | 0.07 |
| Choline chloride (50%) | 0.13 |
| Dicalcium phosphate | 1.42 |
| Limestone | 9.19 |
| Salt | 0.30 |
| Vitamin mixture[1] | 0.15 |
| Mineral mixture[1] | 0.15 |
| Total | 100.00 |
| Calculated values | |
| crude protein, % | 18.00 |
| MEn, kcal/kg | 2 812 |
| methionine + cysteine, % | 0.72 |
| lysine, % | 0.86 |
| Ca, % | 3.90 |
| available P, % | 0.35 |

[1] provided per kg of diet: IU: vit. A 40 000, vit. $D_3$ 8 000, vit. E 10; mg: vit. $K_3$ 4, vit. $B_1$ 4; vit. $B_2$ 12, vit. $B_6$ 6, vit. $B_{12}$ 0.02, niacin 60, pantothenic acid 20, folic acid 2, biotin 0.02, Fe 30, Zn 25, Mn 20, Cu 5.0, Se 0.1

### Egg production and egg quality

During the experimental period, eggs were collected at a fixed time every day, and their number and weight were measured. Abnormal eggs were excluded from the measurements and recorded on a per-replicate basis. Egg mass was calculated by multiplying the hen-day egg production by the average egg weight.

Egg quality was determined at the end of the experiment. Five eggs from each replicate were collected, weighed and stored overnight at room temperature (20 °C) for subsequent analyses. The shell breaking strength of each collected egg was measured using a DET-6000 digital egg tester (Nabel Co., Ltd., Kyoto, Japan). Eggshell thickness, excluding the shell membrane, was measured using a 547-360 Digimatic micrometer (Mitutoyo, Japan). Egg yolk colour was determined by comparison with a Roche yolk colour fan (Hoffman-La Roche, Basel, Switzerland). Albumen height was measured using the same DET-6000 digital egg tester (Nabel Co., Ltd., Kyoto, Japan), followed by Haugh unit calculation.

### Blood profiles

At the end of the experiment, one or two birds were randomly selected from each replicate, and blood was drawn from the wing vein. Serum was obtained after gentle centrifugation (2 000 × *g* for 15 min). Serum samples were stored at −20 °C until analysis. Serum total cholesterol levels were measured using a Labospect 008AS automatic blood analyser (Hitachi High-Tech Co., Tokyo, Japan). Serum glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) levels were measured using a colorimetric method. Serum immunoglobulin G (IgG) titre was determined by a single radial immune-diffusion assay using the ELISA Chicken IgG Core kit (Komabiotech Co., Seoul, Korea).

### Caecal microbial content and ammonia concentration

The caecal content was aseptically sampled from each bird for microbial testing at the end of the experiment. Caecal digesta homogenates were prepared in sterile phosphate-buffered saline and serially diluted from $10^{-1}$ to $10^{-7}$. These dilutions were subsequently plated on duplicate selective agar media to determine the abundance of the target bacterial strains. The total count of microbes, coliforms, and *Lactobacillus* spp. was determined using plate count agar, MacConkey agar, and MRS agar (Difco Laboratories, Detroit, MI, USA), respectively. All plates were incubated at 37 °C for 24 to 72 h, and subsequently the grown colonies were counted. The results obtained are presented as base-10 logarithm colony-forming units per gram of caecal digesta. Ammonia concentration in the caecal digesta was measured using the AA0100 ammonia assay kit (Sigma, St. Louis, MO, USA).

### Yolk lipid fraction contents

The contents of each lipid fraction in the yolks were measured following a previously described method by An et al. (1997) with some modifications. Briefly, total lipids in eggs were extracted using the method of Folch et al. (1957). Subsequently, each lipid fraction was separated by a thin-layer chromatography on silica gel chromatorods using a mixture of hexane, diethyl ether, and formic acid (85:15:0.1, *v/v*, Sigma-Aldrich St. Louis, MO, USA) as the developing solvent and quantified using a TH-10 TLC/FID Iatro Scan analyser (Iatron laboratory Inc, Tokyo, Japan).

### Statistical analysis

Data were analysed using the GLM procedure implemented in SAS 9.0 software (SAS Institute, 2002) with the cage lot as the experimental unit to assess egg production, egg quality, and yolk lipid fraction contents. Individual layers were considered as units for other criteria. Multiple comparisons between the groups were performed using Tukey's test, and contrast statements for differences between control and treatments were considered significant at $P < 0.05$.

## Results

The dietary effects of separate or combined feeding with BS and OM on egg production are presented in Table 2. Overall, dietary supplementation with BS significantly increased egg production, with the exception of group T3. There were no significant differences observed in egg weight and feed intake between the treatment and control groups. Additionally, daily egg mass showed a significant increase when BS was fed alone or in combination with OM compared to the control group. Concurrent feeding with BS and OM did not affect egg production or daily egg mass compared to the groups fed BS alone. Moreover, the relative weights of the liver and spleen were not affected by dietary treatments.

The average eggshell strength was significantly higher in the groups fed BS and OM compared to the control group. There were no significant differences in eggshell thickness and colour, yolk colour, or Haugh units between any of the experimental groups and control (Table 3). No differences were also found between egg and eggshell quality in the groups fed BS alone and the groups fed BS and OM.

**Table 2.** Effects of single or combined feeding of probiotics and organic acid mixture on egg production and organ weights in laying hens

| | Control | T1 | T2 | T3 | T4 | SEM |
|---|---|---|---|---|---|---|
| Egg production, % | 88.8$^{b}$ | 91.2$^{a}$ | 92.2$^{a}$ | 90.7$^{ab}$ | 92.0$^{a}$ | 0.326 |
| Egg weight, g/egg | 68.8 | 69.2 | 68.7 | 68.9 | 68.6 | 0.136 |
| Daily egg mass | 61.2$^{b}$ | 63.4$^{a}$ | 63.0$^{a}$ | 62.7$^{a}$ | 62.8$^{a}$ | 0.224 |
| Feed intake, g/bird/day | 120.7 | 120.9 | 118.4 | 123.6 | 118.2 | 1.673 |
| Liver, g/100 g BW | 1.81 | 1.93 | 1.90 | 1.74 | 1.87 | 0.058 |
| Spleen, g/100 g BW | 0.09 | 0.09 | 0.09 | 0.09 | 0.08 | 0.005 |

| | Probability of contrast | | |
|---|---|---|---|
| | Control vs. BS | Control vs. BS+OM | BS vs. BS+OM |
| Egg production | 0.047 | 0.008 | 0.127 |
| Daily egg mass | 0.042 | 0.023 | 0.342 |

BS – *Bacillus subtilis*, OM – organic acid mixture; Control – basal diet without BS or OM, T1 – BS 1.0 × $10^{10}$ CFU/kg diet, T2 – BS 1.0 × $10^{10}$ CFU/kg diet + 0.15% OM, T3 – BS 2.0 × $10^{10}$ CFU/kg diet, T4 – BS 2.0 × $10^{10}$ CFU/kg diet + 0.15% OM; SEM – standard error of the mean, BW – body weight. Data are presented as the least square of the mean of five replicates with ten birds per replicate; $^{ab}$ mean values with different superscripts in the same row are significantly different at $P < 0.05$

**Table 3.** Effects of single or combined feeding of probiotics and organic acid mixture on egg qualities in laying hens

| | Control | T1 | T2 | T3 | T4 | SEM |
|---|---|---|---|---|---|---|
| Eggshell strength, kg/cm$^2$ | 3.11$^{b}$ | 3.29$^{ab}$ | 3.35$^{a}$ | 3.50$^{a}$ | 3.37$^{a}$ | 0.032 |
| Eggshell thickness, mm/100 | 36.34 | 36.20 | 36.16 | 36.73 | 36.58 | 0.296 |
| Eggshell colour | 26.58 | 26.26 | 26.00 | 26.76 | 27.54 | 0.514 |
| Yolk colour, RCF | 7.43 | 7.36 | 7.45 | 7.35 | 7.51 | 0.070 |
| Haugh unit | 92.02 | 94.46 | 92.99 | 92.70 | 92.79 | 0.652 |

| | Probability of contrast | | |
|---|---|---|---|
| | Control vs. BS | Control vs. BS+OM | BS vs. BS+OM |
| Eggshell strength | 0.039 | 0.032 | 0.374 |

BS – *Bacillus subtilis*, OM – organic acid mixture; Control – basal diet without BS or OM, T1 – BS 1.0 × $10^{10}$ CFU/kg diet, T2 – BS 1.0 × $10^{10}$ CFU/kg diet + 0.15% OM, T3 – BS 2.0 × $10^{10}$ CFU/kg diet, T4 – BS 2.0 × $10^{10}$ CFU/kg diet + 0.15% OM; SEM – standard error of the mean, RCF – Roche colour fan. Data are presented as the least square of the mean of five replicates with ten birds per replicate; $^{ab}$ mean values with different superscripts in the same row are significantly different at $P < 0.05$

The abundance of lactic acid bacteria significantly increased ($P < 0.05$) in the intestine of birds fed BS alone or administered a combination of BS and OM (Table 4). In contrast, the count of coliforms decreased ($P < 0.05$) in groups T1 and T3 compared to the control group. Ammonia concentrations were significantly lower in the treated groups compared to the control group. The microbial profiles in the

**Table 4.** Effects of single or combined feeding of probiotics and organic acid mixture on intestinal microflora abundance and ammonia concentrations in laying hens

| | Control | T1 | T2 | T3 | T4 | SEM |
|---|---|---|---|---|---|---|
| Intestinal microflora | | | | | | |
| Total microbes, log CFU/g | 6.11 | 6.66 | 6.96 | 6.97 | 7.00 | 0.150 |
| Lactic acid bacteria, log CFU/g | 7.12$^{b}$ | 7.42$^{a}$ | 7.60$^{a}$ | 7.64$^{a}$ | 7.47$^{a}$ | 0.082 |
| Coliforms, log CFU/g | 5.26$^{a}$ | 4.56$^{b}$ | 4.77$^{ab}$ | 4.36$^{b}$ | 4.79$^{ab}$ | 0.140 |
| Ammonia concentration, µ/ml | 2.24$^{a}$ | 1.82$^{b}$ | 1.64$^{b}$ | 1.71$^{b}$ | 1.69$^{b}$ | 0.106 |

| | Probability of contrast | | |
|---|---|---|---|
| | Control vs. BS | Control vs. BS+OM | BS vs. BS+OM |
| Lactic acid bacteria | 0.018 | 0.027 | 0.540 |
| Coliforms | 0.029 | 0.048 | 0.129 |
| Ammonia concentration | 0.033 | 0.047 | 0.132 |

BS – *Bacillus subtilis*, OM – organic acid mixture; Control – basal diet without BS or OM, T1 – BS 1.0 × $10^{10}$ CFU/kg diet, T2 – BS 1.0 × $10^{10}$ CFU/kg diet + 0.15% OM, T3 – BS 2.0 × $10^{10}$ CFU/kg diet, T4 – BS 2.0 × $10^{10}$ CFU/kg diet + 0.15% OM; SEM – standard error of the mean. Data are presented as the least square of seven birds per treatment; $^{ab}$ mean values with different superscripts in the same row are significantly different at $P < 0.05$

caecum of commercial laying hens improved significantly after the inclusion of dietary BS, whereas the addition of OM did not have any beneficial effects.

The effects of single or combined supplementation with BS and OM on blood profiles and yolk lipid fractions are shown in Table 5. GOT and GPT activities and total serum cholesterol levels were not affected by dietary treatments. Although serum IgG concentrations in the treated groups tended to be higher than those of the controls, the difference was not significant. Yolk cholesterol levels were significantly lower in the groups with BS supplementation, nevertheless, no significant differences in triacylglycerol and phospholipid levels were detected. There was no difference in yolk cholesterol levels between the groups fed BS alone and animals fed BS and OM in combination.

et al. (2019) reported that diets containing single-strain or multi-strain probiotics did not significantly increase average egg weight. In contrast, administration of BS at 1 g/kg diet was found to increase egg weight (Abdelqader et al., 2013). Probiotics have a beneficial impact on egg production and egg quality, which is attributed to several mechanisms, including increased nutrient absorption, improved immune function, and the promotion of intestinal health. These effects may help explain the results obtained in the present study (Yaqoob et al., 2022).

Eggshell strength in the treated groups differed significantly compared to the control birds, except for group BS1. However, no differences were found in eggshell quality between the group administered BS alone and those fed BS and OM in combination. Previous studies on probiotic supplementation

**Table 5.** Effects of single or combined feeding of probiotics and organic acid mixture on blood profiles and yolk lipid fraction contents in laying hen

| | Control | T1 | T2 | T3 | T4 | SEM |
|---|---|---|---|---|---|---|
| Blood profiles | | | | | | |
| total cholesterol, mg/dl | 127.5 | 114.6 | 126.6 | 114.5 | 120.4 | 5.268 |
| GOT, U/l | 104.9 | 102.5 | 106.9 | 106.6 | 102.3 | 4.824 |
| GPT, U/l | 7.6 | 8.1 | 8.4 | 8.1 | 8.3 | 0.806 |
| IgG, mg/dl | 4.7 | 5.7 | 5.6 | 5.6 | 5.1 | 0.340 |
| Egg yolk | | | | | | |
| cholesterol, mg/g | 12.93[a] | 10.77[b] | 11.42[b] | 10.97[b] | 11.46[b] | 0.167 |
| triacylglycerol, mg/g | 234.1 | 230.6 | 241.7 | 211.5 | 225.6 | 12.036 |
| phospholipid, mg/g | 143.9 | 128.4 | 134.8 | 129.6 | 129.5 | 10.082 |

| | Probability of contrast | | |
|---|---|---|---|
| | Control vs. BS | Control vs. BS+OM | BS vs. BS+OM |
| Yolk cholesterol | 0.028 | 0.043 | 0.097 |

BS – *Bacillus subtilis*, OM – organic acid mixture; Control – basal diet without BS or OM, T1 – BS 1.0 × $10^{10}$ CFU/kg diet, T2 – BS 1.0 × $10^{10}$ CFU/kg diet + 0.15% OM, T3 – BS 2.0 × $10^{10}$ CFU/kg diet, T4 – BS 2.0 × $10^{10}$ CFU/kg diet + 0.15% OM; GOT – glutamic oxaloacetic transaminase, GPT – glutamic pyruvic transaminase, IgG – immunoglobulin G, SEM – standard error of the mean SEM – standard error of the mean. Data are presented as the least square of seven birds per treatment; [ab] mean values with different superscripts in the same row are significantly different at $P < 0.05$

## Discussion

Probiotic supplementation of laying hens' diets improved production performance either by increasing egg number or sustaining egg production. Yörük et al. (2004) showed that the addition of multi-strain probiotics composed of *Lactobacillus*, *Bifidobacterium,* and *Enterococcus* spp. at 0.1% or 0.05% resulted in significantly better egg production compared to controls, but did not affect feed intake. Similarly, Xu et al. (2006) found that feeding dried BS caused significant positive effects on egg production and feed efficiency. Conflicting results have been previously reported regarding the nutritional influence of probiotics on egg weight and egg mass. Studies by Haddadin et al. (1996) and Xiang

that used different strains and inclusion levels, and potentially other factors, have also reported varied results. For instance, feeding dried BS was shown to significantly improve internal and external egg qualities (Xu et al., 2006),while dietary probiotic addition at 0.5 g/kg resulted in a significant reduction in the number of damaged eggs (Balevi et al., 2001). However, despite many positive findings concerning eggshell quality, Haddadin et al. (1996) found that dietary *Lactobacillus acidophilus* exerted no significant effect on eggshell thickness. Organic acids can be used as an important additive improving mineral absorption and calcium availability in the gut, thereby contributing to improved eggshell quality (Soltan, 2008). However, contrary to expectations, some studies on the use of organic acids in layers' diets have reported results that do not align with this hypothesis (Światkiewicz et al., 2013). In the present study, OM did not show any additional effects when fed together with BS. Further studies are needed to clarify the effects of separately applied OM on laying performance and egg quality.

Feeding probiotics has been consistently associated with positive changes in intestinal microflora profiles. Mountzouris et al. (2007) observed that broiler chickens fed multi-strain probiotics showed higher abundance of *Bifidobacterium* and *Lactobacillus* spp. in their caecal microflora. Similarly, Watkins and Kratzer (1983) reported a significant decrease in caecal coliform bacterial counts in chicks fed a diet containing *Lactobacillus*. Moreover, supplementation with BS alone or in combination with *Saccharomyces boulardii* exerted beneficial effects on intestinal microflora and gut histology in broiler chickens (Rajput et al., 2013). Consistent improvements in gut microflora modulation and resulting metabolite production in chicks fed *Bacillus* spp. were also reported by Abdelqader et al. (2013) and Grant et al. (2018). The significant reduction in intestinal ammonia levels as a result of BS supplementation might be partly attributed to improved nitrogen availability and protein digestibility (Table 4). Our findings are consistent with those obtained in another study utilising BS cultures (Santoso et al., 1999).

In the current study, yolk cholesterol levels were significantly lower in groups fed diets with BS, although no significant differences in triacylglycerol and phospholipid concentrations were recorded. This finding is consistent with previous reports demonstrating significant decreases in yolk cholesterol levels as a result of inclusion of dietary probiotics (Mátéová et al., 2009). Haddadin et al. (1996) reported a decrease of 18.8% in yolk cholesterol concentrations when laying hens were fed *Lactobacillus* for 48 weeks. Similarly, Mikulski et al. (2012) observed a reduction of more than 10% in egg yolk cholesterol level in birds fed probiotics. In the present study, feeding BS alone or in combination with OM reduced yolk cholesterol levels from 12.8 to 17.9%. Egg yolk cholesterol is mainly derived from cholesterol synthesised in the liver. Both hepatic cholesterol production and endogenous triacylglycerol-rich lipoprotein levels have important functions in yolk cholesterol deposition (Huang et al., 2019). Dietary lactic acid bacteria have been previously shown to significantly reduce yolk cholesterol levels, which was associated with lower hepatic cholesterol synthesis through the regulation of key enzyme pathways (Deng et al., 2020). The reduction in cholesterol levels induced by probiotics may be due to the modulation of lipid metabolism, cholesterol assembly into lipoproteins, and its incorporation into developing oocytes. These possibilities need to be elucidated through further research.

## Conclusions

Overall, the inclusion of *Bacillus subtilis* obtained from traditional soy sauce, either alone or in combination with an organic acid mixture (OM), yielded favorable outcomes, including increased egg production and eggshell quality, along with a reduction in yolk cholesterol levels. In addition, the incorporation of BS at levels up to $2.0 \times 10^{10}$ CFU/kg diet did not negatively affect target physiological parameters and positively modulated caecal microbial profiles, indicating its safe application in commercial laying hen breeding. However, OM did not exert any additional effects when administered in combination with *B. subtilis*.

## Conflict of interest

The Authors declare that there is no conflict of interest.

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