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RESEARCH ARTICLE

Impact of *Bacillus subtilis* Probiotic on Growth Performance, Bone Health, Intestinal Morphology, and Cecal Microbiota in Cobb Broiler Chicks

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ABSTRACT

Alternatives to antibiotics have attracted widespread attention in poultry farming, particularly after the ban on antibiotic growth promoters. Probiotics, in particular, have shown promising results in enhancing poultry health and productivity when used as a feed additive. The study aimed to evaluate the effects of probiotics (*Bacillus subtilis*) on growth performance, bone health, cecal microbiota, and gut morphology in broilers. Day-old Cobb broiler chicks (n=900) were randomly divided into three experimental groups for 35 days, with each group comprising four replicates and n=75 chicks per replicate by following a completely randomized design. Group A served as the control group fed on the basal diet, while the Group B and Group C groups were given flavomycin (10 g/ per ton of feed) and *Bacillus subtilis* (500 g/ton of feed) along with the basal diet, respectively. Growth parameters, such as body weight gain, feed intake, and feed conversion ratio, were measured weekly, whereas bone strength, cecal microbiota, and gut morphology were recorded at 35 days after randomly selecting three birds from each replicate. The probiotic-supplemented diet (PSD) significantly ($P<0.05$) improved the growth performance of birds throughout the trial period, increased villus length and crypt depth compared with other groups, and reduced *Clostridium perfringes*, *Escherichia coli*, and *Staphylococcus aureus* populations in the cecum compared to the control group ($P<0.05$). Moreover, the PSD improved calcium and phosphorus deposition, as well as tibia strength and ash percentage ($P<0.010$). In conclusion, *Bacillus subtilis* based probiotics may be a better alternative to antibiotic growth promoters.

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INTRODUCTION

Antibiotics are used in food-producing animals including poultry to enhance production performance and are termed antibiotic growth promoters (AGPs). The European Union has imposed a ban on the use of AGPs in animal feed since 2006 and urged the use of alternatives to improve the production performance of birds. It has been established that AGPs result in antibiotic-resistant bacteria (Raza *et al.*, 2024) and pose serious health concerns to birds and consumers (Muaz *et al.*, 2018) including liver damage and kidney failure. There is a dire need to replace these AGPs with alternatives without compromising the production performance of the broiler. Probiotics are considered safer alternatives to AGPs for healthier and safer poultry production (Bidarkar *et al.*, 2014) which may minimize the antibiotics' microbial resistance and drug residues in food, alleviate food allergy sensitivities, and produce antioxidant and increase calcium absorption (Helmy *et al.*, 2023).

Probiotics are live microorganisms that have beneficial effects on the host animal by improving the microbial balance in the intestine by inhibiting pathogens (Gul and alsayeqh, 2022). Probiotics, such as Lactobacillus and Bifidobacterium species, present in the intestine, enhance the intestinal barrier integrity, decrease inflammation, and support a healthy immune system in the host (Mehmood *et al.*, 2023; Skoufou *et al.*, 2024). Adding probiotics to poultry feed improves production performance and promotes the attachment of beneficial bacteria to the gut, increasing nutrient absorption and utilization (Shehata *et al.*, 2022).

Broiler rations supplemented with non-AGPs, such as probiotics, showed a marked improvement in body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), carcass yield, and immunity (Sarangi *et al.*, 2016). Probiotics, also called direct-fed microbials, are used as a replacement for AGPs in poultry feed. They help develop a good immune system, increase digestive enzyme secretions, inhibit pathogenic microbes, and apart from growth performance, they also contribute to the competition with harmful microbes to colonize at the receptors of the digestive tract to help improve nutrient utilization, and produce some substances which kill pathogenic microbes (Nasehi *et al.*, 2015; Rashid *et al.*, 2023). Probiotics in broiler diets at recommended or higher doses can show significant and effective results without imposing any harmful effects on the health of poultry birds (Hill *et al.*, 2014). It has been reported that broilers' feed containing different bacterial strains (Streptococcus, Bacillus, and Bifidobacterium) and yeast (Saccharomyces cerevisiae) showed satisfactory results in terms of production performance, improved small intestine health, increased blood Ca and P levels, improved bone density, enhanced brightness in leg and breast muscles, competitive microbial inhibition, and enhanced meat quality and flavor (Dong *et al.*, 2024). The mechanism of action of probiotics in birds involves decreased pH via fermentation, enhanced immune health and growth in broilers by boosting T-cell immunity, regulating cytokine production, and influencing B-lymphocytes. They also produce bioactive compounds like short-chain fatty acids and bacteriocins, which inhibit the growth of infectious agents (Jacquier *et al.*, 2019). Many beneficial microbes can be used as probiotics, but one of the most common is Bacillus, which has been used in the poultry industry because of its many advantages, such as resistance to heat (90 - 100°C) during pelleting of feed, ability to bear low pH (3 – 8) in the stomach, and storage for a long period at medium temperature (Cutting, 2011), as well as it works for the neutralization of enterotoxins and provide immunity (Higgins *et al.*, 2010; Awais *et al.*, 2019). To the best of our knowledge, comprehensive studies addressing all aspects of *Bacillus subtilis* in broilers are still lacking. Therefore, keeping in view the above facts the objective of this study was to evaluate the effects of *Bacillus subtilis* on growth performance, bone health, intestinal morphology, and cecal microbiota in broiler chickens, providing a holistic understanding of its impact on broiler health and development.

MATERIALS AND METHODS

Experimental diet, design, and husbandry: Before the arrival of birds at the research facility, the house was thoroughly cleaned and disinfected. Water and food were provided *ad libitum.* Birds were vaccinated against Marek's disease at the hatchery, whereas vaccination for Newcastle disease and infectious bronchitis was performed on the first day, followed by the 7^{th} and 17^{th} days, and vaccination for infectious bursal disease was performed on the 11^{th} day. Day-old mixed Cobb-500 broiler chicks (n=900) were randomly divided into three experimental groups (A, B, and C), each group contained 300 birds, comprising four replicates (75 birds per replicate), and the experiment lasted for 35 days. The birds in group "A" were offered a basal diet only whereas the birds in group B and group C were offered a basal diet along with flavomycin (10 g/per ton of feed) and *Bacillus subtilis* (500 g/ton of feed) respectively. Broilers were fed starter feed for 0-21 days and grower feed for 22-35 days. The experimental diets and their chemical compositions are presented in Tables 1 and 2, respectively. The temperature and humidity were set and maintained according to the Cobb manual.

Growth performance: The body weight (BW) of every bird was recorded weekly. Daily feed consumption was determined using the formula FI (g) = feed offered (g) – feed refused (g). FCR was determined by following the formula

$$\text{FCR} = \frac{\text{Total feed consumed (g)}}{\text{Total weight gain (g)}}$$

Cecal microflora: At the end of the experiment, 3 birds from each replicate were randomly selected and slaughtered. After slaughtering, the small intestine was removed from the distal portion of the duodenum up to the ileocecal junction. One gram of intestinal content was diluted with a 0.9% NaCl solution. For the total bacterial count, 10 times dilution method was followed, and 1 ml of each dilution was inoculated onto agar plates using the spread plate method. Subsequently, different types of colonies of bacteria such as *Lactobacillus, Bifidobacterium, Salmonella, Escherichia coli, and Clostridium spp*. were observed and counted based on their specific growth pattern using the method recommended by Hartemink and Rombouts (1999).

Gut morphology: To analyze gut morphology, samples of the duodenum tissue were taken from three birds of each test group. Each sample was cut to around 3cm at its center and then preserved in a 10% neutral buffered formalin solution (SJQW03140 Sigma-Aldrich, Merck; 10%) for 48 hours. Following fixation, the tissue samples were embedded in paraffin using cassettes and were then cut into 4-micrometer sections using a microtome, mounted on slides, and appropriately stained with HE (Hematoxylin and Eosin) stain (Medilines modified H 0706; E 920-921). A light microscope was used to examine the tissue sections and to measure the villus height and crypt depth. The measurement of villus height was taken from the tip of the upper border of the villus to the lamina propria, while crypt depth was determined as the length between the crypts and villi, following the recommended protocol (Panda *et al.*, 2009).

Table 1: Composition of the broiler feed of the starter phase

Ingredients (%)	Starter Phase			Grower Phase		
	Group A	Group B	Group C	Group A	Group B	Group C
Maize	53.8	53.8	53.8	63.2	63.2	63.2
Soybean Meal	28	28	28	20.9	20.9	20.9
Canola meal	4.4	4.4	4.4	3.8	3.8	3.8
PBM*	3	3	3	5	5	5
Rice Polish	0.272	0.272	0.272	0.034	0.034	0.034
Rapeseed Meal	8	8	8	5	5	5
MCP**	0.3	0.3	0.3	0.09	0.09	0.09
Lysine HCL	0.31	0.31	0.31	0.29	0.29	0.29
DLM***	0.263	0.263	0.263	0.19	0.19	0.19
Threonine	0.1	0.1	0.1	0.07	0.07	0.07
Isoleucine	0	0	0	0.02	0.02	0.02
Salt	0.22	0.22	0.22	0.20	0.20	0.20
Soda	0.1	0.1	0.1	0.10	0.10	0.10
Choline	0	0	0	0.05	0.05	0.05
Betaine HCL	0.075	0.085	0.075	0.00	0.00	0.00
Phytase	0.01	0.01	0.01	0.01	0.01	0.01
Coxiril®	0.01	0	0	0.00	0.00	0.00
Maduramycin	0	0	0	0.05	0.00	0.00
Flavomycin	0	0.01	0	0	0.01	0.00
Vitamin premix****	0.055	0.055	0.055	0.06	0.06	0.06
Mineral premix****	0.055	0.055	0.055	0.06	0.06	0.06
Bacillus subtilis	0	0	0.5	0.00	0.00	0.5
Limestone	1	1	1	0.88	0.88	0.88

*PBM= Poultry byproduct meal **MCP= Mono calcium phosphate, ***DLM= DL Methionine; ****Vitamin-mineral premix per kg of diet: vit. A, 12,000IU; vit. D3, 2200IU; vit. E, 10mg; vit. K3, 2mg; vit. B1, 1mg; vit. B2, 4mg; vit. B6, 1.5mg; vit. B12, 10µg; niacin, 20mg; pantothenic acid, 10mg; folic acid, 1mg; biotin, 50µg; choline chloride, 500mg; copper, 10mg; iodine, 1mg; iron, 30mg; manganese, 55mg; zinc, 50mg; and selenium, 0.1mg.

Table 2: Nutrient composition of the basal diets of the broilers (%)

Ingredients %	Starter	Grower
Moisture	11.16	11.16
CP	23	21
Ash	3.9	3.4
Crude Fat	4	4.5
Crude Fiber	2.86	3.62
ME (Kcal/kg)	2900	2950

Bone health parameter: The ash percentage of bone was checked by proximate analysis. The tibia was dried at 105°C for 24h and then kept in a muffle furnace at 600°C for 6h. Ash content was determined relative to the dry weight of the tibia. Phosphorus (P) and calcium (Ca) (Boiling *et al.*, 2000) concentrations of the tibia were determined using the dry-ashed residue.

Statistical analysis: The data were analyzed using one-way ANOVA (analysis of variance) using SPSS software version 23.0. Differences in means among the treatments were measured using Duncan's comparison test. The probability value (P<0.05) indicated that the results were statistically significant.

RESULTS

Growth performance: Table 3 shows the effects of dietary treatments on the weekly body weight of broilers. Higher but insignificant (P>0.05) weekly body weight was observed in birds fed a PSD compared to those fed an antibiotic-supplemented diet. A higher (P<0.05) weekly body weight gain was observed in birds fed PSDs (Treatment C), compared with those fed basal (Group A) and antibiotic-supplemented (Treatment B) diets. Table 4 describes the effects of dietary treatments on FI in broilers. The birds fed the PSD (Treatment C) consumed less (P<0.05) feed on a weekly basis than those fed the basal (Group A) and antibiotic-supplemented (Group B) diets. Similarly, the birds fed the PSD (Group C) showed weekly a better FCR (P<0.05) than those fed the basal (Group A) and antibiotic-supplemented (Group B) diets (Table 5).

Cecal microflora: Table 6 shows the effects of dietary treatments on the cecal microflora in broilers. There was a higher (P<0.05) microbial load in birds that received antibiotic-supplemented diets compared to those that received probiotic and basal diets. Of the three treatments, birds in the probiotic-supplemented treatment group showed the lowest microbial load.

Gut morphology: An increase (P<0.05) in duodenal villus height was observed in birds that received probiotic supplementation compared with other treatments (Table 7). A deeper (P<0.05) crypt depth was observed in birds that received probiotic and antibiotic supplementation compared to the basal diet.

Bone health: The effects of dietary treatments on bone strength are shown in Table 8. There was an increase (P<0.05) in total ash, calcium, and phosphorus levels in broilers fed PSD compared with other treatments. There was no significant difference (P>0.05) in the total ash, calcium, and phosphorus levels in birds that received basal and antibiotic-supplemented diets.

DISCUSSION

Probiotic supplementation helped the birds gain body weight compared to the control group. The probiotic-supplemented group had the highest body weight, followed by the antibiotic-supplemented group. Many research findings are in accordance with our results, showing increased body weight due to probiotic supplementation (Nunes *et al.*, 2012; Boostani *et al.*, 2013; Lei *et al.*, 2015) by improving the nutrient absorption and utilization by the birds; however, some studies contradict the current findings (Afsharmanesh *et al.*, 2013; Lee *et al.*, 2014).

Table 3: Effect of probiotic supplementation on weekly body weight gain (g) of broilers

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5
A	138.81±27.82[a]	467.85±6.60[a]	826.49±24.30[a]	1365.77±10.86[a]	1743.36±24.6[a]
B	173.36±1.65[ab]	475.01±4.57[a]	896.87±10.33[b]	1362.56±23.34[a]	1784.6±35.43[ab]
C	200.46±5.74[b]	497.15±8.40[b]	914.54±9.43[c]	1432.56±23.19[b]	1871.08±35.89[b]
P-value	0.055	0.019	0.004	0.043	0.039

[a,b] within the column, means are presented as SEM and having different superscripts differ significantly ($P<0.05$).

Table 4: Effect of probiotic supplementation on weekly FI (g) of broilers

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5
A	170.67±0.92	631.00±5.91[b]	1299.19±10.26[b]	2045.53±21.48[ab]	2777.47±99.93[ab]
B	173.67±2.87	606.87±5.34[a]	1294.8±15.12[b]	2110.93±20.80[b]	2930.13±15.84[b]
C	171.07±1.22	589.81±10.22[a]	1249.87±14.72[a]	2035.47±25.54[a]	2586.13±70.21[a]
p-value	0.49	0.005	0.039	0.067	0.013

[a,b] within the column, means are presented as SEM and having different superscripts differ significantly ($P<0.05$).

Table 5: Effect of probiotic supplementation on weekly FCR of broilers

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5
A	0.96±0.01[b]	1.31±0.03[b]	1.43±0.01[a]	1.63±0.06[a]	1.66±0.05[b]
B	0.97±0.02[b]	1.31±0.01[b]	1.51±0.03[b]	1.56±0.02[ab]	1.68±0.04[b]
C	0.84±0.03[a]	1.11±0.03[a]	1.31±0.01[a]	1.48±0.015[a]	1.41±0.03[a]
P-Value	0.01	0.02	0.01	0.036	0.009

[a,b] within the column, means are presented as SEM and having different superscripts differ significantly ($P<0.05$).

Table 6: Effect of probiotic supplementation on cecal microbiota of broilers

Treatment	Microbial load	P-Value
A	8.02±0.0603[a]	
B	8.31±0.0815[b]	0.012
C	7.98±0.0718[a]	

[a,b] within the column, means are presented as SEM and having different superscripts differ significantly ($P<0.05$).

Table 7: Effect of probiotic supplementation on gut morphology of broilers(µm)

Treatment	Villus height	Crypts depth
A	1118.92±46.438[a]	124.25±9.139[a]
B	1203.75±29.877[a]	133.62±8.593[b]
C	1328.17±47.005[b]	137.00±13.497[b]
P-Value	0.005	0.018

[a,b] within the column, means are presented as SEM and having different superscripts differ significantly ($P<0.05$).

Table 8: Effect of probiotic supplementation on bone strength/health of broilers

Treatment	Ash	Ca	P
A	97.54±0.220[a]	35.34[a]±0.150[a]	18.57±0.084[a]
B	97.98±0.264[a]	35.46[a]±0.227[a]	18.65±0.084[a]
C	98.85±0.287[b]	36.22[b]±0.234[b]	18.98±0.120[b]
P-Value	0.004	0.010	0.014

[a,b] within the column, means are presented as SEM and having different superscripts differ significantly ($P<0.05$).

It has been reported that different strains that *B. subtilis* alone or in combination with *E. faecium* significantly improved the body weight and FCR of layer chicks (Hatab *et al.,* 2016; Dong *et al.,* 2024). This improved growth can be attributed to the feature of probiotics by which they secrete digestive enzymes such as α- amylase and B-galactosidase which aid in increased nutrient absorption and consequently improved growth performance in animals (Jadhav *et al.,* 2015; Shehata *et al.,* 2022)

This study showed a significant effect of probiotics on FI among the treatments. The birds fed PSD consumed less feed, followed by the basal and antibiotic-supplemented diets. A more prominent difference in FI was observed in the last week of the trial. Our research findings agree with previous experimental trials that revealed a prominent difference among the treatments when birds were fed probiotics and compared with AGPs (Nunes *et al.,* 2012; Cabuk *et al.,* 2014; Basmacioğlu-Malayoğlu *et al.,* 2016). The FCR was significantly improved by feeding probiotics to the broiler birds. Contrary to the antibiotic-containing diet group, the probiotic group showed the lowest FCR throughout the trial period. Our results are consistent with earlier research findings in which researchers found improved FCR in probiotics-fed broilers (Basmacioğlu-Malayoğlu *et al.,* 2016; He *et al.,* 2019); however, some studies are not according to our results, showing no improvement in growth performance when the diet of poultry birds is fortified with beneficial microbes or probiotics (da Rocha *et al.,* 2010; Nunes *et al.,* 2012).

Our findings reinforce the assumption that probiotics have the potential to improve the growth performance of broiler chickens when compared to AGPs. AGPs cause antibiotic residues and resistant strains of bacteria in poultry products and this feature has not been observed in probiotics supplementation in poultry diet (Mountzouris *et al.,* 2010; Krysiak *et al.,* 2021). The improved growth performance might be the result of enhanced nutrient utilization, better gut modulation, and stabilized microbiota. As probiotics decrease the enzymatic activity of pathogenic bacteria, the rate of digestive enzymes and metabolism increases, leading to better feed ingestion, digestion, and absorption. They also help neutralize enterotoxins and provide immunity (Sarangi *et al.,* 2016; Awais *et al.,* 2019; He *et al.,* 2020). Enhanced digestibility, utilization of nutrients, and absorption of dietary minerals are due to improved gut morphology. This occurred because probiotics competitively inhibited pathogenic microbes and attached beneficial microbes to the gut epithelium, and these microbes helped in better nutrient availability to the bird. (Lei *et al.,* 2015; Jacquier *et al.,* 2019). Despite this, there is still a discussion in scholarly works about whether probiotics can substitute AGPs because some studies have shown that probiotics have no impact on growth performance (Jerzsele *et al.,* 2012).

Our study revealed that the highest microbial load was observed in the negative control group. The lowest bacterial count was observed in the probiotic group named "G3" followed by the antibiotic group and control group (Table 6). Hence, we concluded that the microbial load was significantly reduced by feeding probiotics to birds. The cecal portion of the intestine is crucial for broiler birds, where fermentation and absorption of water and minerals occur. *Bacillus subtilis* can retain a suitable niche for microbes, enhance nutrient utilization, decrease FCR, and competitively inhibit infectious microbes for feed utilization (Olnood *et al.,* 2015). Some studies have shown that this probiotic strain has the potential to release products that inhibit disease-causing bacteria in broilers. These products have the power to arrest the growth of microbes, such as *Clostridium perfringens, Staphylococcus aureus,* and *Escherichia coli*. (Olnood *et al.,* 2015; Manafi *et al.,* 2017) Some experimental trials have revealed that *Bacillus subtilis* has significant power to lower bacteria such as *Salmonella* and *Escherichia coli* in the large intestine, possibly due to the utilization of an oxygenated environment. Utilizing oxygen in the gastrointestinal tract assists anaerobic bacteria and helps in the maintenance of the microbial ecosystem to decrease the development of aerophilous bacteria such as *Escherichia coli* and resides by developing symbiotic relationships with anaerobiotic microbes (Stanley *et al.,* 2014; Gao *et al.,* 2017). Researchers have suggested that administrating probiotics to chickens enhances the population of beneficial bacteria in the intestine and reduces the presence of pathogenic bacteria (Yaqoob *et al.,* 2022). An increase in duodenal villus height was observed in broiler birds fed probiotics, followed by the AGP group. The same was the case for crypt depths, which were the highest in the "G3" group (Table 7). Numerous studies have reported similar results in which improved villus height, crypt depth, and VH:CD were observed (Jayaraman *et al.,* 2013; Sukandhiya *et al.,* 2017; Wu *et al.,* 2022). Yaqoob *et al.* (2022) reported that broiler chickens supplemented with a single or combination of probiotics exhibited improved gut histomorphology with increased villus length and VL: CD ratio which suggested that probiotics boosted nutrient absorption. Villus height and crypt depth have important roles in nutrient absorption rate; however, if this does not happen, it will lead to immature enterocytes and decreased nutrient availability to birds, which will affect their performance and production (Paiva *et al.,* 2014). In addition, probiotics help to increase the absorptive surface area. Probiotic fortification causes an increase in gut cell proliferation, which increases growth performance; beneficial microbes inhabit the gut epithelium and protect the villus from harmful pathogens (Jha *et al.,* 2020). In addition, they improved gut health and the small intestinal integrated barrier, which are crucial for its function and might be the cause of better apparent tract total digestibility (Narasimha *et al.*, 2013; He *et al.*, 2019).

Tibial ash, calcium, and phosphorus levels in broilers improved significantly with probiotic supplementation. There was no significant difference in tibial ash, calcium, and phosphorus levels in broilers fed the basal and AGP diets. Similar to our study, Khan and Naz (2013), and Collins *et al.* (2017) published similar findings. Skeletal abnormalities in broilers develop because of rapid growth. Unable to carry that heavy weight, birds still manage, due to which they suffer from elevated stress leading to infections as well as skeletal problems such as rickets and tibial dyschondroplasia, which impose a heavy cost on the broiler industry (Çapar Akyüz and Onbaşılar, 2020). Therefore, the bones of broiler birds should be sufficiently strong to bear heavy muscle mass. When the broiler diet was supplemented with *Bacillus* strain, it was observed that Ca^{+} and phosphorus were deposited in the tibia, making it tough relative to the diet group that had no probiotic fortification (McCabe *et al.,* 2013; Collins *et al.,* 2017). Similarly, Oketch *et al.* (2024) found in their study that supplementation of multi-strain *Bacillus*-based probiotics in laying hens resulted in improved tibia calcium, weights, ash, and density. Yaqoob *et al.* (2022) also reported that the administration of Enterococcus faecium and lactic acid-producing bacteria in broiler chickens' diet enhanced various tibia parameters, including tibia calcium levels and calcium percentage.

As our research trial has shown that probiotics have a significant effect on broilers, they should be used at an industrial level to replace AGPs. We obtained the best results from the PSD group compared to the control group. Therefore, there is a dire need to use them at the industrial level to eliminate antibiotic residues and antibiotic-resistant bacterial strains. However, further studies are required to explore their effects at different doses, in different poultry species, and in different poultry housing and feed manufacturing conditions.

Conclusions: The use of probiotics (500 g/ton of feed) has yielded significant positive results in broilers as compared to those fed AGPs flavomycin (10g/ per ton of feed) and basal diet. It has improved growth performance, gut morphology, bone health, and decreased cecal microflora. Therefore, we conclude that probiotics could serve as an alternative to AGPs.

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Authors contribution: AR, MAS, TR, and MAZ planned and prepared the project. MAS and AR executed the experiment. BI, TR, TB, and AR analyzed the samples and prepared the first draft. HY, MZA and MW analyzed the data statistics and manuscript writing & review. All authors have read and agreed to the published version of the manuscript. The authors have declared no conflict of interest.

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