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Histophysiological Changes in Broilers Fed on Diet Supplemented with Mannanoligosaccharide and Organic Acid Blend

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MUS, SM, HZ, AZD, HR and MR designed the experiment. MUS, KA, AA and MSS executed the research. MUS and SM analyzed the data and wrote the manuscript.

Key words
Villus surface area, Yeast, Crypt depth, Goblet cells, Serum biochemistry.

ABSTRACT

The present study aims at analyzing effects of mannanoligosaccharide (MOS) and organic acid blend (OAB) supplementation individually and in combination on carcass yield, small intestinal microarchitecture and serum biochemistry in Hubbard chicks. A total of 128 one day-old chicks were divided equally in four different groups with each group having 4 replicates. The number of birds per group were 32 whereas the number of birds per replicate were 8.The first group was kept as control (CONT) whereas, the second (MOSG) and third (OABG) groups were given MOS (2g / kg of feed) and OAB (3g / kg of feed), respectively. The fourth group (MOS+OAB) was given combination of MOS (2g / kg of feed) and OAB (3g / kg of feed). Birds were given ad-libitum feed and water during an experiment of 35 days. At the end of trial, two birds from each replicate were slaughtered for analysis of the selected parameters. The results showed that carcass weight and carcass yield were significantly high ($p < 0.05$) in MOS+OAB compared to MOSG, OABG and CONT. The results of small intestine microarchitecture showed that villus length, villus width, villus to crypt ratio, villus surface area, acidic, mixed and total goblet cells increased significantly ($p < 0.05$) in birds that were fed MOS and OAB alone and in combination whereas crypt depth decreased significantly ($p < 0.05$) after the selected supplementation in all small intestinal segments compared to control group. Level of thyroid stimulating hormone (TSH) was increased significantly ($p < 0.05$) by the dietary treatments under study individually and in combination. It is concluded that supplementation of MOS and OAB in combination is beneficial compared to their individual effects in broiler chicks.

INTRODUCTION

For more than half a century antibiotic growth promoters (AGP) were used for reduction of pathogenic bacteria and improving growth potential of birds (Hernandez *et al*., 2006) but now, the use of AGP has been banned due to human health concerns and risks regarding development of antibiotic resistant bacteria (Patterson and Burkholder, 2003; Yakhkeshi *et al*., 2011). The avian gastrointestinal tract (GIT) harbors a diverse population of microorganisms living in a symbiotic relationship which influences metabolism, nutrition and immunity of host (Sohail *et al*., 2012). Due to the short life span of broilers colonization of useful bacteria in their early life is important so they can become more immune to the diseases that can be caused by intestinal pathogenic bacteria (Samanta *et al*., 2010). Maturation of small intestine in broilers plays a crucial role in nutrient assimilation, digestion and absorption (Cheled-Shoval *et al*., 2011). Balance between the number of commensal bacteria, mucous production and intestinal epithelial integrity largely influence the efficiency of intestinal barrier (Faure *et al*., 2006; Torrecillas *et al*., 2011) which is the natural barrier against toxic substances and pathogens that are present in the intestinal lumen.

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These stressors can cause disturbance in the normal microflora of intestine which leads to alterations in this barrier (Podolsky, 1993). These alterations facilitate the absorption of undesirable substances that reduce the digestive and absorptive activities of GIT (Pelicano *et al*., 2005). Since the morphology of intestine plays a crucial role in absorption of nutrients certain feed additives are being used for enhancing the absorptive efficacy of intestine (Hofacre *et al*., 2003; Pelicano *et al*., 2005).

Prebiotics are non-digestible food particles that increase the number of useful bacteria in avian intestine (Awad *et al*., 2011) resulting in enhanced growth potential (Pelicano *et al*., 2005). Yeast products are being fed to birds for increasing their growth performance however, the in-vivo and in-vitro modes of actions of these feed additives are not fully understood (Reisinger *et al*., 2012). Mannanoligosaccharide (MOS) is a mannan-based carbohydrate derived from the cell wall of yeast *Saccharomyces cerevisiae* (Cheled-Shoval *et al*., 2014; Iqbal *et al*., 2015). It is considered that MOS exerts its effect on growth by accelerating the maturation of GIT microflora (Solis de los Santos *et al*., 2007; Yang *et al*., 2007). Dietary MOS also modulates mucin synthesis by increasing the expression of different goblet cell types and up-regulating MUC2 and mRNA expression (Chee *et al*., 2010).

Organic acids (OA) and organic acid blend (OAB) are among candidate replacement for antibiotics (Smulikowska *et al*., 2010). Dietary OA have shown positive impact on growth performance of broilers (Brown and Southern, 1985; Eidelsburger and Kirchgessner, 1994; Garcia *et al*., 2000). They improve digestibility of minerals like P, Mg, Zn, Ca and also act as substrates in intermediary metabolism (Adil *et al*., 2011). Supplementation with OAB reduces the number of acid intolerant pathogenic bacteria such as *Campylobacter*, *Salmonella* and *E. coli* (Dibner and Buttin, 2002) which is beneficial for birds health. Similar to AGP short chain OA exhibit antibacterial activity but their activity is pH dependent (Kum *et al*., 2010). Undissociated forms of OA have the ability to pass through bacterial cell membrane where alkaline medium of bacterial cytoplasm dissociates OA resulting in production of H^+ ions that decreases pH of cell. Energy of the organism is utilized in restoring normal balance whereas, disruption in synthesis of DNA and protein is caused by $RCOO^-$ anions produced from OA. This results in reducing rapid proliferation of pathogenic bacteria (Paul *et al*., 2007).

Very few experiments have been conducted which divulge about the synergistic effects of organic acid blend (OAB) along with MOS (Ozduven *et al*., 2009), and to the best of our knowledge no data is present regarding the combined effect of MOS and OAB and supplementation on small intestine microarchitecture and selected serum parameters in broilers. Therefore, this study aims at exploring the single and combined effects of MOS and OAB supplementation on carcass yield, small intestine microarchitecture and serum biochemistry in broilers.

Table I.- Composition of experimental diet given to broilers (Hajati *et al*., 2015).

	Starter diet (1-10 days)	Grower diet (11-21 days)	Finisher diet (21-35 days)
Ingredients (%)			
Corn	56.2	59.9	63.34
Soyabean oil	2.26	3.3	3.94
Soyabean meal	37.11	32.55	28.71
Dicalcium phosphate	1.92	1.86	1.74
Oyster shell	1.16	1.12	1.06
Common salt	0.3	0.3	0.3
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25
L-Lysine hydrochloride	0.24	0.21	0.18
DL-Methionine	0.31	0.26	0.23
Nutrient composition			
ME, kcal/kg	3000	3105	3180
CP, %	21.23	19.46	18
AP, %	0.50	0.48	0.45
Ca, %	1	0.96	0.9
Lysine, %	1.32	1.19	1.06
Methionine + cysteine, %	0.98	0.89	0.82

ME, metabolizeable energy; AP, available phosphorus; CP, crude protein. [1]Vitamin premix supplied the following per kg of diet; vitamin A, 18000U; vitamin D_3, 4000U; vitamin E, 36mg; vitamin K_3, 4mg; vitamin B_{12}, 0.03mg; thiamine, 1.8mg; riboflavin, 13.2mg; pyridoxine, 6mg; niacin, 60mg; calcium pantothenate, 20mg; folic acid, 2mg; biotin, 0.2 mg; choline chloride, 5000mg. [2]Mineral premix supplied the following per kg of diet; Cu, 20mg; Fe, 100mg; Mn, 100mg; Se, 0.4mg; Zn, 169.4mg.

MATERIALS AND METHODS

Experimental design and grouping of birds

This study was carried out in environmentally-controlled broiler research shed at University of Veterinary and Animal Sciences (UVAS), Pattoki. The experiment lasted for 35 days and was conducted on a total number of 128 one day-old Hubbard broiler chicks obtained from a commercial hatchery. Upon arrival birds were weighed and randomly assigned to four groups with each group having four replicates. Each group had 32 birds whereas each replicated had 8 birds. The first group (CONT) was kept as

control and was fed basal diet (Table I). The second group (MOSG) and third group (OABG) were supplemented with MOS (Actigen®- Alltech Lexington, UK, 2g/kg of basal diet) and OAB (Acid Lab – Pulse, Wuzburg, Germany, 3g/kg of basal diet) respectively whereas, the forth group (MOS+OAB) was given combination of both MOS and OAB (2g and 3g, respectively per kg of basal diet) (Ozduven *et al*., 2009). The OAB contained formic acid 16.84%, ammonium formate 9.25%, lactic acid 33.60%, propionic acid 5.05% and sodium chloride 35.26 %. Birds were vaccinated intraocularly by live attenuated Newcastle disease virus (Ceva-Phylaxia, Budapest, Hungary) on day 1, with a booster on day 21 in drinking water. Similarly vaccination against infectious bursal disease (Lohman Animal Health GmbH, Cuxhaven, Germany) was done by intraocular route on day 8 and repeated on day 20 in drinking water. The study was conducted according to the guidelines of Animal Care and Use Committee UVAS, Lahore, Pakistan.

Carcass weight and carcass yield

During the whole experiment feed and water to birds of each replicate were provided ad-libitum. At the end of the experiment two birds nearest to the average weight of the same replicate were selected and exsanguinated by cutting carotid arteries and jugular vein. Birds were allowed to bleed for approximately two minutes, viscera were removed immediately and carcass weight was taken using a sensitive digital scale. Carcass yield was calculated as the percentage of live body weight (Dehghani-Tafti and Jahanian, 2016).

Small intestine morphometry

About 3cm long small intestinal segments from midpoints of duodenum (segment encompassing the duodenal loop), jejunum (segment between duodenum and ileum) and ileum (distal segment before the ileo-cecal junction equaling the length of caecum) were taken and fixed in 10% neutral buffered formalin. Segments were then embedded in paraffin, stained by haematoxylin and eosin (Bancroft *et al*., 2013) and observed under microscope (Labomed, USA) at 4×. Measurements were made by using a commercial morphometric program (Prog Res 2.1.1). Villus length (VL) was measured from the tip of the villus to villus crypt junction. The villus width (VW) was measured from the base, middle, tip of villus and the average of the results was considered as VW. The crypt depth (CD) was measured from base of crypt to the transition region between crypt and villus. Villus surface area (VSA) was calculated by the formula $(2\pi)(VW/2)(VL)$ (Solis de los Santos *et al*., 2007). Measurements were made in triplicates on 5 well oriented villi which were selected on the basis of intact lamina propria and average of results was reported.

Histochemistry of goblet cells and intraepithelial lymphocyte count

Slides prepared by paraffin embedding technique were stained by combined alcian blue – periodic acid Schiff (AB-PAS) (Bancroft *et al*., 2013) and observed at 10X for counting goblet cells. Five villi per bird were studied for counting the goblet cells. Goblet cells (GC) were differentiated as acidic and mixed on the basis of staining ability of mucin contents. Acidic goblet cells (AGC) having acidic mucin stained blue whereas, mixed goblet cells (MGC) having both acidic and mixed mucin were stained purple (Leknes, 2010).

Slides used for morphometry were used for counting intra epithelial lymphocytes (IEL) at 40×. The IEL are identified as rounded cells with large central or eccentric nuclei and scant cytoplasm (Ashraf *et al*., 2013). Counts were made in triplicates on 5 well oriented villi which were selected on the basis of intact lamina propria and average of results was reported.

Serum collection and analysis

Blood was collected at the time of slaughter was centrifuged at 1500 × g at 4°C for 20 min. Serum was harvested and stored at -20°C until analysis. Concentration of thyroid stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) were calculated by using commercially available ELISA kits (Autobio Diagnostics Co. Ltd. Brussels, Belgium). Total oxidants status (TOS) was determined on the basis of the oxidation of ferrous to ferric ion in the presence of various oxidant species after being calibrated with hydrogen peroxide using a spectrophotometer (BTS-330; Biosystems, Barcelona, Spain) at 800nm wavelength (Erel, 2005). Total antioxidant status (TAS) was estimated using protocol of Erel (2004) in which *o*- dianisidine dihydrochloride was used as substrate. Enzymatic activity of arylesterase was determined using the protocol described by Juretic *et al*. (2006) using phenyl acetate as substrate. Ceruloplasmin activity was determined by using *o*-dianisidine dihydrochloride as substrate (Schosinsky *et al*., 1974).

Statistical analysis

Analysis of data was conducted with Statistical Package for Social Science (SPSS for windows version 20.0 SPSS, Chicago, USA). Data was found to be normally distributed after checking with Kolmogrov Simirnov test. Data for groups was analyzed with one way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$ and were calculated by applying Duncan's multiple-range test (Duncan, 1955).

Table II.- Effect of dietary supplementations on carcass weight and carcass yield of broilers.

Parameters	Treatment groups			
	CONT	MOSG	OABG	MOS+OAB
Carcass weight (g)	1383.88 ± 2.49[a]	1431.19 ± 2.14[b]	1443.18 ± 2.61[c]	1505.01 ± 1.57[d]
Carcass yield (%)	61.46 ± 0.11[a]	62.61 ± 0.09[b]	62.46 ± 0.11[b]	63.41 ± 0.06[c]

Results are presented as mean ± standard error. Superscripts [a-d] within a row indicates significant difference between groups ($p < 0.05$). CONT, control; MOS, mannanoligosaccharide; OAB, organic acid blend; MOSG, group supplemented with MOS; OABG, group supplemented with OAB.

Table III.- Effect of dietary supplementations on small intestine microarchitecture in broilers.

Intestinal segments	Parameters	Treatment groups			
		CONT	MOSG	OABG	MOS+OAB
Duodenum	Villus length (mm)	0.93 ± 0.01[a]	1.14 ± 0.01[b]	1.22 ± 0.01[c]	1.31 ± 0.01[d]
	Villus width (mm)	0.22 ± 0.01[a]	0.31 ± 0.01[b]	0.31 ± 0.01[b]	0.32 ± 0.01[b]
	Crypt depth (mm)	0.33 ± 0.01[b]	0.32 ± 0.01[b]	0.32 ± 0.01[b]	0.3 ± 0.01[a]
	Villus : Crypt (mm)	2.78 ± 0.02[a]	3.55 ± 0.03[b]	3.78 ± 0.02[c]	4.36 ± 0.02[d]
	Villus surface area (mm^2)	0.67 ± 0.01[a]	1.11 ± 0.01[b]	1.20 ± 0.01[c]	1.32 ± 0.01[d]
	Intraepithelial lymphocytes (per villus)	80.25 ± 1.70[b]	73.25 ± 1.53[a]	72.25 ± 1.53[a]	70.75 ± 1.43[a]
	Acidic goblet cells (per villus)	39.25 ± 1.66[a]	51.75 ± 1.69[b]	59.75 ± 1.69[c]	62.75 ± 1.82[c]
	Mixed goblet cells (per villus)	17.75 ± 1.04[a]	22.75 ± 1.04[b]	24.75 ± 1.04[bc]	27.75 ± 1.04[c]
	Total goblet cells (per villus)	57.00 ± 2.68[a]	74.50 ± 2.73[b]	84.50 ± 2.73[c]	90.50 ± 2.85[c]
Jejunum	Villus length (mm)	1.27 ± 0.01[a]	1.37 ± 0.01[b]	1.37 ± 0.01[b]	1.40 ± 0.01[c]
	Villus width (mm)	0.28 ± 0.01[a]	0.34 ± 0.01[b]	0.33 ± 0.01[b]	0.34 ± 0.01[b]
	Crypt depth (mm)	0.41 ± 0.01[c]	0.40 ± 0.01[bc]	0.412 ± 0.01[c]	0.39 ± 0.01[a]
	Villus : Crypt (mm)	3.05 ± 0.03[a]	3.38 ± 0.04[b]	3.36 ± 0.05[b]	3.55 ± 0.01[c]
	Villus surface area (mm^2)	1.12 ± 0.02[a]	1.46 ± 0.02[b]	1.46 ± 0.02[b]	1.50 ± 0.02[b]
	Intraepithelial lymphocytes (per villus)	28.25 ± 1.53	25.25 ± 1.53	24.25 ± 1.43	24.25 ± 1.43
	Acidic goblet cells (per villus)	82.25 ± 1.75[a]	97.75 ± 1.69[b]	117.25 ± 1.90[c]	121.75 ± 2.00[c]
	Mixed goblet cells (per villus)	30.25 ± 0.99[a]	35.25 ± 0.99[b]	37.25 ± 0.99[b]	40.25 ± 0.99[c]
	Total goblet cells (per villus)	112.50 ± 2.73[a]	133.00 ± 2.68[b]	154.00 ± 2.85[c]	162.00 ± 2.93[c]
Ileum	Villus length (mm)	0.97 ± 0.01[a]	1.22 ± 0.01[b]	1.23 ± 0.01[b]	1.32 ± 0.01[c]
	Villus width (mm)	0.18 ± 0.01[a]	0.24 ± 0.01[b]	0.25 ± 0.01[b]	0.26 ± 0.01[c]
	Crypt depth (mm)	0.25 ± 0.01[a]	0.22 ± 0.01[b]	0.22 ± 0.01[b]	0.21 ± 0.01[b]
	Villus : Crypt (mm)	3.80 ± 0.04[a]	5.50 ± 0.06[b]	5.49 ± 0.05[b]	6.14 ± 0.06[c]
	Villus surface area (mm^2)	0.57 ± 0.01[a]	0.95 ± 0.01[b]	0.97 ± 0.02[b]	1.08 ± 0.02[c]
	Intraepithelial lymphocytes (per villus)	27.75 ± 1.82	25.75 ± 1.82	25.75 ± 1.47	24.25 ± 1.53
	Acidic goblet cells (per villus)	45.75 ± 1.82[a]	58.75 ± 1.82[b]	66.25 ± 1.75[c]	69.25 ± 1.75[c]
	Mixed goblet cells (per villus)	18.75 ± 1.04[a]	23.75 ± 1.04[b]	25.75 ± 1.04[bc]	28.75 ± 1.04[c]
	Total goblet cells (per villus)	64.50 ± 2.85[a]	82.50 ± 2.85[b]	92.00 ± 2.63[c]	98.00 ± 2.63[c]

For abbreviations and statistical details, see Table II.

Table IV.- Effect of dietary supplementations on serum biochemistry of broilers.

Parameters	Treatment groups			
	CONT	MOSG	OABG	MOS+OAB
Total oxidants (µm of H_2O_2 equivalent/L)	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Total antioxidants (m*M* Eq. of vitamin C/L)	7.08 ± 0.01	7.79 ± 0.01	7.86 ± 0.01	8.13 ± 0.01
Ceruloplasmin (U/L)	43.26 ± 0.26	43.20 ± 0.30	43.11 ± 0.30	43.25 ± 0.34
Arylesterase (U/L)	86.69 ± 0.46	86.80 ± 0.54	86.87 ± 0.54	86.82 ± 0.49
TSH (µg/dl)	1.50 ± 0.01[a]	1.76 ± 0.01[b]	1.76 ± 0.01[b]	1.85 ± 0.01[c]
T_3 (ng/ml)	3.16 ± 0.03	3.21 ± 0.02	3.16 ± 0.03	3.20 ± 0.03
T_4 (µg/dl)	3.22 ± 0.01	3.21± 0.01	3.21 ± 0.02	3.22 ± 0.02

For abbreviations and statistical details, see Table II.

RESULTS

Carcass weight and carcass yield increased significantly ($p < 0.05$) by supplementation of dietary treatments with MOS+OAB birds having highest carcass weight and carcass yield followed by OABG and MOSG as shown in Table II.

Table III shows that in all the segments of small intestine of broilers VL, VW, villus to crypt ratio, VSA, AGC, MGC and GC increased significantly ($p < 0.05$) by supplementation of MOS and OAB alone and in combination with birds from group MOS+OAB having the highest values for the above mentioned parameters. However, CD decreased significantly ($p < 0.05$) after supplementing bird with MOS and OAB and only in duodenum the number of IEL was significantly decreased ($p < 0.05$) in birds from group MOS+OAB.

The dietary supplementations used in our study had no influence on the concentrations of T3, T4, TOS, TAS, ceruloplasmin and arylesterase however, TSH concentration of was significantly high ($p < 0.05$) in birds of group MOS+OAB, MOSG and OABG compared to the birds of CONT as shown in Table IV.

DISCUSSION

Effects of MOS have been studied extensively in broilers however, its exact mode of action for expressing beneficial effects in broilers remain unclear. Mechanisms like partial absorption of oligosaccharides and there direct interaction with the carbohydrate receptors on immune and epithelial cells are suggested to be involved (Seifert and Watzl, 2007; Cheled-Shoval *et al*., 2011). Other mechanisms like alterations in gut microflora by pathogenic exclusion through competitive binding to mannose-specific type 1 fimbriae of pathogens like *Campylobacter* and *Escherichia coli* have also been suggested for beneficial effects of MOS (Spring *et al*., 2000; Baurhoo *et al*., 2007; Yang *et al*., 2008). Similarly it has been reported that the increase in weight gain after use of OAB is due the antimicrobial property of OA (Cengiz *et al*., 2012). Comparable results have been reported by Vogt *et al*. (1982) and Skinner *et al*. (1991) who reported an increase in body weight gain after use of OAB. Increase in body weight is positively correlated to the carcass yield (de Jong *et al*., 2014) and carcass weight (Correa *et al*., 2006) which explains our results of a higher carcass weight and carcass yield after inclusion of MOS and OAB in broilers' diet.

Growth and development of birds are dependent on the microarchitecture of intestine. A better intestinal microarchitecture safeguards better growth performance and higher profitability (Mitchell and Carlisle, 1992). Greater VSA offers augmented digestion and absorption of nutrients and is dependent on increased VH and VW (Amat *et al*., 1996). Results similar to our findings have been reported by Zikic *et al*. (2011) and Adil *et al*. (2011) after supplementation of MOS and OA to birds respectively. A higher villus to crypt ratio is indicative of a lower rate of enterocyte cell migration from crypt to villus. It can be suggested that MOS and OAB supplementation reduces enterocytic damage and need for cell renewal in small intestine (m5). Lower number of IEL in duodenum is due to the fact that OA diminish the growth of many pathogenic bacteria naturally present in the intestine thereby reducing their colonization and infectious processes thus, reducing inflammatory reactions in the intestinal mucosa (Pelicano *et al*., 2005). The mucous layer secreted by GC preset in the epithelium of GIT is vital for protection of brush border area of intestinal epithelium and also provides assistance in digestion and absorption (Cheled-Shoval *et al*., 2014). Our results regarding GC count after feeding MOS confirms findings from previous studies (Smirnov *et al*., 2005; Baurhoo *et al*., 2007; Solis de los Santos *et al*., 2007; Chee *et al*., 2010). Greater CD results in increased cell turnover resulting in rapid renewal of villus which is demanding during an increased pathogenic load (Awad *et al*., 2009). Both epithelial and GC are derived from the same progenitor cells that are residing in crypts. Mucin composition from neutral to acidic changes during the maturation process of GC. This maturation and differentiation process implies that an increase number of GC after supplementation of MOS and OAB reflects their enhanced activity and metaplasia (Ashraf *et al*., 2013).

Long-term stress is induced by hypothalamic-pituitary-adrenal (HPA) axis which results in modified immune response, cardiovascular diseases and gastrointestinal lesions (Siegel, 1960). Enteric and central nervous systems are bidirectionally linked forming the brain gut axis via autonomic nervous system. Enhanced HPA activity increases levels of certain hormones in serum (Eutamene and Bueno, 2007). At present it is unclear that hoe MOS and OAB increased TSH levels in serum however, Aluwong *et al*. (2013) in a study demonstrated that TSH levels in broilers were increased by supplementation of live yeast *Saccharomyces cerevisiae*. As OA are having a eubiotic effect (Abu-Elala and Ragaa, 2015) and MOS used in our study is derived from the cell wall of *Saccharomyces cerevisiae* therefore an elevated level of TSH can be expected as seen in our study.

CONCLUSION

It is concluded that compared to individual

supplementation mannanoligosaccharide (MOS) and organic acid blend (OAB) in combination increased carcass weight, carcass yield and small intestinal microarchitecture in broilers. Therefore, both MOS and OAB should be used in combination which might aid in improved performance of birds that will ultimately result in improved profitability.

Statement of conflict of interest

The authors have no conflicts of interest to declare regarding the publication of this manuscript.

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