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Encapsulated *Cosmos caudatus* K. Leaf Extract Improved Feed Conversion and Intestinal Bacterial Population of Broilers Stocked at Different Density-Induced Stress

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Abstract

The aim of the present study was to investigate the effect of dietary supplementation of encapsulated *Cosmos caudatus* leaf extract on growth, organ weight, and intestinal bacterial population and morphology of broilers at high-density pens. From day 15 onward, 370 broiler chicks were allocated to 5 treatment groups including LSD (10 chicks/m^2 and fed on basal diet), HSD (16 chicks/m^2 and fed on basal diet), HSD5 (16 chicks/m^2 and supplemented with 0.5 g/kg of encapsulated *C. caudatus* leaf extract), HSD10 (16 chicks/m^2 and supplemented with 1.0 g/kg of encapsulated *C. caudatus* leaf extract), and HSD15 (16 chicks/m^2 and supplemented with 1.5 g/kg of encapsulated *C. caudatus* leaf extract). Study was terminated on day 35. Results showed that the treatments did not affect ($p > .05$) slaughtered body weight, weight gain, internal organ weight, and gut morphology of broilers. Feed intake was highest ($p < .05$) in HSD than that in other groups. Encapsulated *C. caudatus* leaf extract improved ($p < .05$) feed conversion ratio of high-stocked broilers, as compared to those of normal-stocked broilers. Encapsulated *C. caudatus* leaf extract reduced ($p < .05$) coliform and *Enterobacteriaceae*, while increasing lactic acid bacteria counts in ileum and caecum. In conclusion, dietary incorporation of encapsulated *C. caudatus* leaf extract improved feed conversion and bacterial population of broilers under stress condition due to high stocking density.

Keywords: Broilers, feed efficiency, gut bacteria, herbs, stocking density

Introduction

Farmers often increase the number of chicks per square meter kept in broiler house to maximize the use of available resources. Apart from the advantages, keeping broilers in a high-density house can induce stress, which can eventually lead to a reduction in growth rate. Several studies reported that rearing broiler chickens in high-density cages can cause the development and ecology of chicken intestines to be disrupted, and it can interfere with intestinal digestive function (Goo et al., 2019; Shakeri et al., 2015). These conditions can ultimately have an impact on the slow growth of broiler chickens. In general, the negative impact of stress can be minimized by giving antioxidants to broilers (Li et al., 2019). In this regard, synthetic antioxidants have been routinely applied to broilers under stress. However, the prolonged use of synthetic antioxidants can cause carcinogenic effects on consumers of broiler meat (Sugiharto et al., 2019). Surai (2016) reported that plant extracts have a high antioxidant content, hence, they are very effective if used as an alternative antioxidant for broiler chickens. Saracila et al. (2021) further documented that the use of herbal-based antioxidants could improve bacterial balance in the intestine and growth performance of broiler chickens under stress conditions. In line with this, Sugiharto (2022) documented that with antibacterial properties, plant-derived products are effective in limiting the growth of bacterial pathogen in the intestine of broilers raised at high-density pens.

Cosmos caudatus K. is one of the most potential herbal plants used as an alternative source of antioxidants for broiler chickens. *C. caudatus* leaves are rich in phenolic compounds that can act as antioxidants (Chan et al., 2016). It is generally known that phenolic compounds are very sensitive to environmental factors such as light, oxygen, humidity, and temperature, so they need to be protected to ensure the effectiveness of phenolic compounds as a source of antioxidants. To protect phenolic components from the harmful effects of environmental conditions, the encapsulation method has been applied (Sobel et al., 2014). The latter method has the potential to not only

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protect bioactive substances but also to maintain or increase their stability. Recently, encapsulation method has been applied to stabilize biological compounds and essential oils and maintain taste and odor of plat-derived products (Vinceković et al., 2017). One of the encapsulation methods is freeze drying. This method sublimes ice to steam under vacuum after rapid freezing (Yang et al., 2020). In most cases, freeze drying is used to dry materials whose bioactive compounds are sensitive to high temperatures, such as antioxidant compounds (Papoutsis et al., 2018).

In this study, encapsulation method was applied for the *C. caudatus* leaf extract. Stress condition in broiler chickens was induced by housing the birds in high-density pens. The aim of the present study was to investigate the effect of dietary supplementation of encapsulated *C. caudatus* leaf extract on growth performance, internal organ weight, and intestinal bacterial population and morphology of broilers stocked at high-density pens. It was hypothesized that encapsulated *C. caudatus* leaf extract would improve growth, maintain internal organ development, and improve intestinal bacterial population and morphology of broilers raised at high-density pens.

Methods

Preparation of Encapsulated *C. caudatus* Leaf Extract

C. caudatus leaves were obtained from a local market in Semarang, Central Java Province, Indonesia. The leaves were weighed, washed, and dried at room temperature. The dried leaves were ground into powder and then macerated using 70% ethanol (1:6, g:mL) for 72 h at room temperature. The filtrate was obtained by filtering the macerated substance with a filter paper. The filtrate was then dried in a vacuum rotary evaporator at a maximum temperature of 60°C until a paste was obtained. Maltodextrin was used as an encapsulation coating material. It was dissolved in distilled water in a ratio of 1:3 (g:mL). The maltodextrin solution was then mixed with the filtrate of *C. caudatus* leaf extract with a ratio of 1:5 (mL:mL) filtrate to maltodextrin. The *C. caudatus* leaf extract was then freeze-dried to produce a powder product. The antioxidant activity of the encapsulated and unencapsulated *C. caudatus* leaf extract was then determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) technique (Seyedreihani et al., 2017). In brief, The UV–Vis spectrophotometer is used to measure the DPPH free radical scavenging activity. The initial absorbance of the DPPH solution (.1 mM) was measured at 515 nm, and the absorbance was monitored at this wavelength throughout the assay. In 1.5 mL of methanolic DPPH solution, an aliquot (20 µL) of sample was added. At 30-minute intervals, the change in absorbance at 515 nm was measured until the reaction curve reached a plateau. The results were averaged after the samples were examined in triplicate. The DPPH inhibition (%) was calculated using the equation as follows: $((OD_{515control} - OD_{515sample})/OD_{515control}) \times 100\%$.

In Vivo Study

The study employed 370 male Lohmann MB-202 broiler chicks, which weighed 41.2 ± 0.76 g on average. The chicks were fed commercial crumble feed with 21%–23% crude protein, 5%–8% crude fat, 3%–5% crude fiber, and 4%–7% ash (based on the feed label) when they were 0–14 days old. Throughout the trial, drinking water was available at all times. At 4 days old, a single dose of active Newcastle disease-infectious bronchitis (ND-IB) vaccine was given via eye drops, and 0.15 mL of inactivated Newcastle disease-avian influenza (ND-AI) vaccine was given subcutaneously. The Gumboro vaccination was also delivered via drinking water on day 14. From day 15 onward, the chicks (447 ± 5.23 g) were allocated to 5 treatment groups including LSD (10 chicks/m^2 and fed on basal diet), HSD (16 chicks/m^2 and fed on basal diet), HSD5 (16 chicks/m^2 and supplemented with 0.5 g/kg of encapsulated *C. caudatus* leaf extract), HSD10 (16 chicks/m^2 and supplemented with 1.0 g/kg of encapsulated *C. caudatus* leaf extract), and HSD15 (16 chicks/m^2 and supplemented with 1.5 g/kg of encapsulated *C. caudatus* leaf extract). The encapsulated *C. caudatus* leaf extract was added into the feed at the end of the mixing process. The mixing process was carried out manually, and feeds were prepared in mash form. The doses of encapsulated *C. caudatus* leaf extract supplemented in the experimental feed were inspired by the study of Abdelli et al. (2021) who supplemented the microencapsulated fumaric acid and thymol to broiler chicken feeds. In this study, broilers were given encapsulated *C. caudatus* leaf extract to compensate for the unfavorable impact of high stocking density, which is usually experienced by broilers from 15 days onward. The formulated basal feed is presented in Table 1.

Feed consumption, body weight, and feed conversion ratio (FCR) were recorded weekly from days 15 to 35. On day 35, two chicks per pen (ten chicks per treatment groups) were slaughtered, internal organs were collected and weighed (empty weight), and intestinal segments (duodenum, jejunum and ileum, around 2 cm each) were

Table 1.
Dietary Composition of Broilers (Days 15–35)

Ingredients	Proportion, % (Unless Otherwise Noted)
Yellow corn	57.9
Coconut oil	2.55
Soybean meal	34.8
DL-metionine	0.19
Bentonite	1.00
Limestone	1.34
Monocalcium phosphate	1.51
Premix[1]	0.27
Chlorine chloride	0.07
Natrium chloride	0.40
Nutrient composition	
Metabolizable energy, kcal/kg[2]	3411
Moisture	11.9
Crude protein	23.2
Crude fiber	2.92
Crude fat	1.66
Ash	8.57

[1]Premix contained (per kg of feed) vit A 7750 IU, vit B_1 1.25 mg, vit B_2 3 mg, vit B_6 1.88 mg, vit B_{12} 0.01 mg, vit C 25 mg, vit D_3 1550 IU, vit E 1.88 mg, folic acid 1.50 mg, Ca-d-pantothenate 7.5 mg, niacin 1.88 mg, biotin 0.13 mg, Co 0.20 mg, Cu 4.35 mg, Fe 54 mg, I 0.45 mg, Mn 130 mg, Zn 86.5 mg, Se 0.25 mg, L-lysine 80 mg, choline chloride 500 mg, DL-methionine 900 mg, $CaCO_3$ 641.5 mg, dicalcium phosphate 1500 mg.
[2]Metabolizable energy was calculated according to Bolton formula: 40.81 {0.87 [crude protein + 2.25 crude fat + nitrogen-free extract] + 2.5}

obtained and placed in a tube containing 10% buffered formalin to measure the intestinal morphology. At the same time, the contents of ileum and caecum were collected and placed in sterile sample bottles for the enumeration of selected bacterial population in the intestine. Chickens were harvested at 35 days of age, as is customary in Indonesian commercial broiler farms.

Histological measurements of the small intestinal segments were conducted as detailed by Tunç et al. (2019). Hematoxylin and eosin staining of 5 µm intestinal slices was carried out, and the measurement of villous height (VH) and crypt depth (CD) was done using an optical microscope coupled to a digital camera. Each bird's mean VH and CD were computed using five measurements. Total coliform and lactose-negative *Enterobacteriaceae* were enumerated as red and colorless colonies, respectively, on MacConkey agar (Merck KGaA, Darmstadt, Germany) after 24 hours of aerobic incubation at 38°C. Coliform and lactose-negative *Enterobacteriaceae* were then summed to determine the number of *Enterobacteriaceae*. The number of lactic acid bacteria (LAB) on De Man, Rogosa, and Sharpe (MRS) (Merck KGaA) agar was counted after anaerobic incubation at 38°C for 48 hours.

Statistical Analysis

The power analysis was performed prior to in vivo study to determine the sample size of the study. The data obtained were evaluated using a one-way analysis of variance with a 5% significance threshold to identify the effect of treatment, and if there was a significant effect, Duncan's multiple distance test was used to determine the differences between treatments. Statistical Package for the Social Sciences software version 16.0 was used to analyze the data.

Results

Antioxidant Activity of Encapsulated *C. caudatus* Leaf Extract

The DPPH assay showed that the encapsulated *C. caudatus* leaf extract had percent DPPH inhibition of 74.6%, while the non-encapsulated one had percent DPPH inhibition of 20.52%. This result suggested that encapsulation increased antioxidant activity of the *C. caudatus* leaf extract as compared to that of non-encapsulated one. A previous study revealed that encapsulation was attributed to the preserved phenolic compounds and hence improved antioxidant activity of plant materials. Indeed, encapsulation may protect phenolic compounds against oxidation. In agreement with earlier study, Shaygannia et al. (2021) reported that encapsulation improved antioxidant activity of lemon extract. In this respect, the latter authors suggested that encapsulation improved bioactivity and bioavailability of polyphenols and eventually increased antioxidant activity. In this study, the herbal bioactive compounds were not determined. Therefore, it was difficult to assess which compounds contributed the most to antioxidant activity before and after the encapsulation.

Performance of Broilers

In most cases, housing at a high-density pen is associated with the growth reduction in broilers (Shakeri et al., 2015; Goo et al., 2019). In contrast, our current experiment showed no substantial effect ($p > .05$) of high density on slaughtered weight and body weight gain of broilers (Table 2). In line with this, Aziz and Al-Hawezy (2021) reported an absent effect of different stocking density on the body weight of broilers at day 35 of age. The competition between birds at 16 chicks/m² was most likely insufficient to generate severe stress, which could have harmed broiler growth. In this study, the total live body weight of high-stocked broilers was about 30 kg/m². Indeed, the European Union (Council Directive 2007/43/EC) stated that the stocking density for modern broiler chickens is still permissible at 33 kg/m². In this regard, the absent effect of high stocking density on body weight of broilers could be understood. However, this inference should be interpreted carefully, as feed efficiency (FCR) was worse ($p < .05$) in HSD than that in LSD chicks in the present study (Table 2). Results in the present study showed that feed consumption was highest ($p < .05$) in HSD than that in other treatment groups (Table 2). This finding was in contrast to Goo et al. (2019) but in agreement with Aziz and Al-Hawezy (2021). At week 5, the latter researchers pointed out that broilers reared in high-density pens consumed more feed than those reared in normal-density pens. The exact reason for the enhanced feed consumption in high-stocked broilers is yet to be determined. However, Rohmadi et al. (2021) suggested that the high density of chickens leads to more noise, so the chickens stay awake and hence eat more feed. Indeed, the sight and sound of a chicken eating have been shown to cause other chickens to eat. Compared to HSD, the high-stocked chickens receiving *C. caudatus* leaf extract consumed less feed in this study. This could indicate that supplementing with *C. caudatus* leaf extract led to more efficient nutrient utilization for growth. Our inference was actually supported by the fact that *C. caudatus* leaf extract was capable of improving ($p < .05$) FCR of broiler housed in high-density pens, as compared to those reared at normal-density

Table 2.
Performance of Broilers (Days 15–35)

Items	LSD	HSD	HSD5	HSD10	HSD15	SEM	*p*
Total live BW, g/m²	19,020[b]	29,568[a]	30,528[a]	29,152[a]	30,432[a]	922	<.01
Slaughtered BW, g/bird	1902	1848	1908	1822	1902	16.4	.36
BWG, g/bird	1447	1402	1462	1377	1457	16.1	.37
FI, g/bird	2385[d]	2794[a]	2531[b]	2464[c]	2552[b]	29.3	<.01
FCR	1.65[b]	1.99[a]	1.73[b]	1.79[b]	1.76[b]	0.03	<.01

[a,b,c,d]Means with different superscripts within similar row differ significantly ($p < .05$)
Note: LSD = 10 chicks/m² and fed on basal diet; HSD = 16 chicks/m² and fed on basal diet; HSD5 = 16 chicks/m² and supplemented with 0.5 g/kg of encapsulated *C. caudatus* leaf extract; HSD10 = 16 chicks/m² and supplemented with 1.0 g/kg of encapsulated *C. caudatus*; HSD15 = 16 chicks/m² and supplemented with 1.5 g/kg of encapsulated *C. caudatus*; BW = body weight; BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio; SEM = standard error of mean.

Table 3.
Internal Organs Weight of Broilers

Items, g/100 BW	LSD	HSD	HSD5	HSD10	HSD15	SEM	*p*
Heart	0.36	0.40	0.40	0.38	0.40	0.01	.27
Liver	2.02	2.05	2.03	2.07	2.08	0.05	.99
Proventriculus	0.40	0.43	0.40	0.38	0.44	0.01	.31
Gizzard	1.17	1.14	1.10	1.19	1.13	0.03	.89
Pancreas	0.22	0.20	0.21	0.18	0.20	0.01	.37
Duodenum	0.40	0.43	0.40	0.39	0.39	0.01	.73
Jejunum	1.01	0.94	0.84	0.85	0.88	0.03	.32
Ileum	0.65	0.67	0.58	0.66	0.65	0.02	.37
Caeca	0.47	0.52	0.42	0.42	0.47	0.02	.29
Abdominal fat	1.42	1.15	1.23	1.22	1.23	0.05	.63
Spleen	0.16	0.12	0.13	0.15	0.11	0.02	.90
Thymus	0.16	0.14	0.15	0.14	0.10	0.04	.15
Bursa of fabricius	0.08	0.08	0.06	0.08	0.06	<0.01	.57

Note: LSD = 10 chicks/m^2 and fed on basal diet; HSD = 16 chicks/m^2 and fed on basal diet; HSD5 = 16 chicks/m^2 and supplemented with 0.5 g/kg of encapsulated *C. caudatus* leaf extract; HSD10 = 16 chicks/m^2 and supplemented with 1.0 g/kg of encapsulated *C. caudatus;* HSD15 = 16 chicks/m^2 and supplemented with 1.5 g/kg of encapsulated *C. caudatus;* BW = body weight; SEM = standard error of mean.

pens. Perhaps, the encapsulated *C. caudatus* leaf extract was able to alleviate stress in high-stocked broilers, allowing more energy to be allocated to growth rather than stress recovery. In general, stress is associated with the lower protein digestibility and energy retention (Souza et al., 2016). This condition may therefore result in lower feed efficiency, as indicated by the higher FCR in HSD than in LSD and encapsulated *C. caudatus* leaf extract-supplemented HSD birds.

Internal Organ of Broilers

It was apparent in this current investigation that relative weight of the internal organs of broilers did not differ ($p > .05$) among the treatment groups (Table 3). In respect particularly to density effect, Sapsuha et al. (2021) also did not find any substantial effect of stocking density on the relative weight of internal organs of broilers. In this study, treatment with encapsulated *C. caudatus* leaf extract had no effect on broiler internal organs relative weight. This was consistent with Sapsuha et al. (2021) showing no impact of nutmeg flesh (*Myristica fragrans* Houtt) extract on the internal organs weight of broilers housed at different stocking density conditions. In most circumstances, the relative weight of internal organs is proportional to the live body weight of broilers as the organs were stated based on the live weight percent of the birds (Heidari & Toghyani, 2018). In this study, the slaughtered body weight of broilers did not vary among treatment groups, and hence the relative weight of internal organs was not different as well.

Intestinal Morphology of Broilers

On broiler chicks, measurements of villi height and crypt depth were taken, and the results are listed in Table 4. According to Goo et al. (2019), stress caused by high stocking density was associated with disturbed intestinal morphology, as seen by reduced villi height and deeper crypt depth, hence reducing broiler absorption capacity. Broiler intestine morphology was not affected by housing broilers in varied stocking densities ($p > .05$) in this study. Abudabos et al. (2013) found no significant variation in villi height of the duodenum and jejunum between broilers stocked at low (28 kg/m^2) and high (40 kg/m^2) density conditions, which is consistent with our findings. The specific reason for the lack of effect of stocking density was unknown until recently, although the variation in stress intensity caused by stocking density could be attributable to broilers' varied responses to intestinal cell growth. As previously observed by Yakhkeshi et al. (2011), encapsulated *C. caudatus* leaf extract had no significant influence on the intestinal morphology of broilers. The latter researcher found no influence of a commercial herbal product (Sangrovit) on the villi height and crypt depth of broiler villi in the duodenum, jejunum, and ileum. The various responses of broilers

Table 4.
Intestinal Morphology of Broilers

Items	LSD	HSD	HSD5	HSD10	HSD15	SEM	*p*
Duodenum							
Villi height, µm	1075	995	1065	1131	927	37.8	.50
Crypt depth, µm	137	121	109	112	128	5.15	.40
Jejunum							
Villi height, µm	741	920	676	704	780	32.4	.13
Crypt depth, µm	109	101	102	113	98.2	2.71	.36
Ileum							
Villi height, µm	608	623	586	641	590	21.6	.93
Crypt depth, µm	108	112	111	105	92.2	4.61	.67

Note: SEM = standard error of mean; LSD = 10 chicks/m^2 and fed on basal diet; HSD = 16 chicks/m^2 and fed on basal diet; HSD5 = 16 chicks/m^2 and supplemented with 0.5 g/kg of encapsulated *C. caudatus* leaf extract; HSD10 = 16 chicks/m^2 and supplemented with 1.0 g/kg of encapsulated *C. caudatus*; HSD15 = 16 chicks/m^2 and supplemented with 1.5 g/kg of encapsulated *C. caudatus*.

Table 5.
Intestinal Bacterial Populations of Broilers

Items (log cfu/g)	LSD	HSD	HSD5	HSD10	HSD15	SEM	*p*
Ileum							
Coliform	7.42^{ab}	8.35^{a}	6.74^{b}	6.48^{b}	6.31^{b}	0.21	.01
LNE	6.17	7.00	6.32	6.60	6.44	0.19	.69
Enterobacteriaceae	7.86^{ab}	8.42^{a}	7.01^{b}	6.99^{b}	6.70^{b}	0.20	.04
LAB	9.50	9.62	10.3	10.4	10.5	0.14	.07
Caecum							
Coliform	8.68^{ab}	9.15^{a}	8.08^{b}	8.41^{b}	8.38^{b}	0.10	<.01
LNE	7.69	8.36	7.75	7.21	7.58	0.16	.27
Enterobacteriaceae	8.77^{ab}	9.22^{a}	8.60^{b}	8.50^{b}	8.61^{b}	0.08	.03
LAB	11.5^{a}	10.3^{b}	11.7^{a}	11.6^{a}	11.5^{a}	0.10	<.01

^{a,b}Means with different superscripts within similar row differ significantly ($p < .05$)
Note: cfu = colony forming unit; LNE = lactose-negative *Enterobacteriaceae*; LAB = lactic acid bacteria; LSD = 10 chicks/m^2 and fed on basal diet; HSD = 16 chicks/m^2 and fed on basal diet; HSD5 = 16 chicks/m^2 and supplemented with 0.5 g/kg of encapsulated *C. caudatus* leaf extract; HSD10 = 16 chicks/m^2 and supplemented with 1.0 g/kg of encapsulated *C. caudatus*; HSD15 = 16 chicks/m^2 and supplemented with 1.5 g/kg of encapsulated *C. caudatus*; SEM = standard error of mean.

can be attributed to the nature of the herbal products, levels, delivery routes, and experimental conditions.

Intestinal Bacterial Populations of Broilers

It was observed in this present study that stocking the chicks at different densities had no substantial effect ($p > .05$) on the numbers of coliform, lactose-negative *Enterobacteriaceae*, and *Enterobacteriaceae* in the ileum and caecum. However, high stocking density resulted in lower ($p < .05$) LAB counts especially in caecum of broilers (Table 5). This finding was in agreement with Kridtayopas et al. (2019) showing that high stocking density negatively influences bacterial population in the intestine of broilers. In this case, the stress condition was attributed to the changes in normal microbial population leading to the decrease in commensal/beneficial bacteria and increase in pathogenic bacteria in the intestine of broilers (Abudabos et al., 2013). At high-stocking density pens, dietary incorporation of encapsulated *C. caudatus* leaf extract reduced ($p < .05$) populations of coliform and *Enterobacteriaceae* both in the ileum and caecum of broilers, when compared with those that received no supplement. The antibacterial properties of *C. caudatus* leaf extract (Cheng et al., 2015) was most likely responsible for lowering the numbers of potential pathogenic bacteria in this present study. Our present finding also showed that treatment with encapsulated *C. caudatus* leaf extract increased ($p < .05$) the count of LAB in the intestine of broilers. In the intestines of broilers, LAB has a competitive exclusion activity against harmful microorganisms (Sugiharto, 2016). In this regard, the greater LAB counts may be linked to the lower harmful bacteria adhering to the intestinal mucosa of broilers as a result of this feature. Overall, the improvement in intestinal microbial balance could be attributed to the improved intestinal digestive function, and hence feed utilization and efficiency of broilers.

Conclusion and Recommendations

Dietary incorporation of encapsulated *C. caudatus* leaf extract improved feed conversion and bacterial population of broilers under stress condition due to high stocking density.

Ethics Committee Approval: The *in vivo* study was approved by the Animal Ethics Committee of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro (Date: September 5, 2020, No. 57-09/A3/KEP/FPP).

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Author Contributions: Concept and design – S.S.; In vivo trial – D.M.N., T.A.S., E.W.; Writing manuscript – D.M.N.; Revision – S.S., T.Y,. Hl.W., E.W.

Declaration of Interests: The authors declare that they have no competing interest.

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