South African Journal of Animal Science 2026, vol. 56(11)

The effects of phytogenic supplementation on growth performance, blood metabolites, and diarrhoea occurrence in pre-weaned Holstein calves

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(Submitted 04 March 2026; Accepted 13 October 2026; Published 10 November 2026)

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Abstract

This study evaluated the effects of thymol and cinnamaldehyde (T+C) supplementation on growth performance, selected blood metabolites, and diarrhoea occurrence in pre-weaned Holstein calves. Forty Holstein calves (20 females and 20 males) were initially enrolled, with 10 calves assigned to each treatment × sex combination. Three calves died during the experimental period, leaving 37 calves for the final growth-performance analyses (control: 9 females and 9 males; T+C: 9 females and 10 males). Growth performance (average daily gain, solid-feed dry matter intake, solid-feed conversion ratio, and weaning body weight) was not affected by treatment, sex, or their interaction. Serum phosphorus at birth and blood urea nitrogen at weaning showed nominal sex-associated differences, but neither remained significant after Benjamini–Hochberg false discovery rate correction. The proportion of calves with diarrhoea was numerically lower in the T+C group than in the control group (8/19 versus 11/18), but the difference was not statistically significant. Thus, under the conditions of this study, T+C supplementation did not significantly affect growth performance, measured biochemical variables, or diarrhoea occurrence.

Keywords: calf, cinnamaldehyde, diarrhoea, phytogenic additive, serum biochemistry, thymol

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Introduction

Pre-weaning is a time in the livestock management of a dairy calf when the optimisation of nutrition will benefit both long-term survival and health. Early growth is closely related to the well-coordinated growth of both the gastrointestinal tract and immune system (Edwards & Renaud, 2025). This phase is marked by large physiological changes, especially movement from a milk-based dietary pattern to functional rumen development. As such, proper nutritional approaches have to be taken to ensure that rumen maturation and immune competence are addressed, while also ensuring proper growth performance (Donovan *et al.*, 1998; Khan *et al.*, 2011). While calf management has improved, enteric disorders, including neonatal diarrhoea, continue to be some of the main causes of morbidity and mortality in pre-weaned calves.

URL: www.sajas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
https://doi.org/10.17159/sajas.v56i11.01

Antimicrobials have historically been employed to control these diseases and augment performance, but increasing concerns about antimicrobial resistance have prompted the development of more sustainable alternatives (Cho & Yoon, 2014; World Health Organization, 2014). One such alternative is the addition of phytogenic feed additives, including plant-derived essential oils such as thymol and cinnamaldehyde, to feeds. These products have received increasing attention as bioactive nutritional interventions. Phytogenic compounds have been investigated for their potential effects on microbial activity, rumen fermentation, and nutrient utilisation (Greathead, 2003; Calsamiglia *et al.*, 2007).

However, responses to phytogenic supplementation in pre-weaned calves remain inconsistent. Some reports have indicated improved feed efficiency and a decreased frequency of diarrhoea, while others have reported little or no effect (Jahani-Azizabadi *et al.*, 2022; Nora *et al.*, 2024). Recent studies suggest that liquid diets containing essential oil blends could modulate immune and antioxidant parameters while having only slight effects on body weight gain (Nora *et al.*, 2024; Velázquez-Cruz *et al.*, 2025). There is also limited information on sex-related differences in metabolic responses during early development.

Although phytogenic supplementation has previously been investigated in dairy calves, comparatively little information is available on whether responses differ between female and male calves. Therefore, the objective of this study was to determine whether supplementary thymol and cinnamaldehyde administered through milk affects growth performance, selected serum biochemical variables, and diarrhoea occurrence in pre-weaned Holstein calves. Treatment × sex interactions were also evaluated to determine whether responses differed between female and male calves.

Materials and methods

Ethical approval and experimental location

This study was conducted at the Dairy Research and Application Farm of Çukurova University (Adana, Türkiye; 37.00° N, 35.32° E) between October and December 2024. All animals were managed in accordance with institutional guidelines for the care and use of laboratory animals (reference number: 2, dated 20 May 2024).

Animals and experimental design

The study initially included 40 newborn Holstein calves (20 females and 20 males). On day 4, calves were blocked by birth date and initial body weight and allocated to the two dietary treatments in a 2 × 2 factorial arrangement (treatment × sex), with 10 calves initially assigned to each treatment × sex combination. During the experimental period, three calves died: one control female, one control male, and one thymol and cinnamaldehyde (T+C) female. Consequently, 37 calves completed the study and were available for the final growth-performance analyses (control: n = 18, 9 females and 9 males; T+C: n = 19, 9 females and 10 males). The control group received no phytogenic supplementation, whereas the T+C group received the same feeding regimen supplemented with a thymol and cinnamaldehyde product, as described below.

Calf management

Immediately following parturition, calves were separated from their dams to minimise pathogen transmission and control colostrum intake. Each calf received 2 L of high-quality colostrum (immunoglobulin G >50 g/L) via a nipple bottle within the first 2 hours of life, followed by an additional 2 L within 24 hours. To ensure passive immunity transfer, standard health management protocols were strictly followed, including navel disinfection with a 7% iodine solution and the administration of prophylactic serum according to the established farm veterinary programme. Calves were housed in individual pens (1.1 × 2.3 m) designed to prevent physical contact between animals while maintaining visual social interaction. The pens were bedded with clean wheat straw that was fully replaced twice daily to maintain dry and hygienic conditions.

Feeding management

Calves were routinely fed 4 L of whole milk (3.78% crude protein, 3.71% fat) per day from day 4 until weaning, divided into two equal feedings at 06:00 and 18:00 and delivered in individual buckets. Starter feed was formulated to meet or exceed the nutrient requirements of pre-weaned calves,

according to the National Academies of Sciences, Engineering, and Medicine (NASEM, 2021). Starter feed was provided in pelleted form to ensure uniform intake and prevent selective sorting. Lucerne hay, the sole forage source, was chopped to 2–3 cm to stimulate rumination and support rumen development. Water and lucerne hay were provided *ad libitum* throughout the study.

Phytogenic supplementation consisted of 6 g/day of a commercial product containing thymol and cinnamaldehyde (MenoherbTM, Menon, Shangai, China), administered by dissolving 3 g of the product into each milk-feeding ration. This provided daily intakes of 1089.6 mg cinnamaldehyde and 91.8 mg thymol, which were maintained throughout the experimental period. The stability of the phytogenic blend in milk and its palatability were not formally evaluated. Milk refusals were not systematically recorded during the experimental period.

The chemical composition and metabolisable energy content of the calf starter feed and lucerne hay are provided in Table 1. The calf starter feed and lucerne hay were ground through a 1-mm screen, dried, and analysed in triplicate using Association of Official Analytical Chemists (AOAC, 2005) methodology to determine the dry matter (AOAC 930.15), ash (AOAC 942.05), ether extract (AOAC 920.39), and crude protein (Kjeldahl method, AOAC 984.13) content. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were evaluated according to Van Soest *et al.* (1991), including heat-stable α-amylase and residual ash. Metabolisable energy values were calculated from chemical fractions and total digestible nutrients using NASEM (2021) equations.

Table 1 Ingredients and chemical composition of calf starter feed and lucerne hay provided to pre-weaned Holstein dairy calves, on a dry matter basis

	Calf starter feed	Lucerne hay
Ingredients (%):		
Ground maize	45	
Soybean meal	22	
Ground barley	15	
Wheat bran	10	
Sugar beet molasses	5	
Limestone	Minor inclusion	
Dicalcium phosphate	Minor inclusion	
Salt	Minor inclusion	
Vitamin-mineral premix (tailored)	Minor inclusion	
Proximate composition:		
Dry matter (%)	92.38	92.72
Crude protein (%)	18.12	11.75
Ether extract (%)	4.2	0.82
Crude cellulose (%)	8.78	33.23
Acid detergent fibre (%)	11.32	39.12
Neutral detergent fibre (%)	21.12	44.89
Crude ash (%)	7.38	7.23
Metabolisable energy (Mcal/kg of dry matter)	2.550	1.842

Growth-performance measurement

Body weight was measured weekly from birth until 8 weeks of age using a digital scale (Ata Scale, Türkiye). Average daily gain (ADG) was calculated as the difference between initial and final body weight divided by the total number of experimental days. Individual feed intake was determined daily before the morning provision of calf starter feed and lucerne hay at 08:00. *Ad libitum* calf starter feed and chopped lucerne hay were available, with the amounts modulated daily for approximately 10% refusals. A digital scale (0.1 g readability) was used to weigh forage and starter feed refusals separately to determine individual intake. Solid-feed dry matter intake (DMI) was calculated as the combined DMI

of calf starter feed and lucerne hay. Milk intake was not included in this calculation. Accordingly, the solid-feed conversion ratio (SFCR) was calculated as the solid-feed DMI (kg dry matter/day) divided by the ADG (kg/day). This ratio therefore represents the efficiency of solid-feed use rather than the total dietary feed conversion rate.

Blood sampling and biochemical analysis

Blood samples were collected via jugular venepuncture at birth and at weaning prior to the morning provision of solid feed at 08:00, to ensure consistency in metabolic state. Samples were allowed to clot at room temperature before being centrifuged at 4000 rpm for 15 min at 4 °C, and serum was aliquoted and stored at −20 °C until analysis. Serum concentrations of aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), glucose, cholesterol, calcium, phosphorus, magnesium, total protein, albumin, and blood urea nitrogen (BUN) were determined using commercial diagnostic kits (Mindray Bio-Medical Electronics Co., Shenzhen, China). Analyses were performed using a Mindray BS-120 automated biochemical analyser following the manufacturer's instructions and standard clinical chemistry procedures (Rifai *et al.*, 2018). Catalogue numbers for the assays were: AST (105-000443-00), GGT (105-000448-00), glucose (105-000450-00), cholesterol (105-000446-00), BUN (105-000463-00), total protein (105-000461-00), albumin (105-000441-00), calcium (105-000444-00), phosphorus (105-000459-00), and magnesium (105-000458-00).

Diarrhoea assessment

Faecal consistency was scored daily for each calf at 08:00 by the same trained observer to reduce inter-observer variation. A standardised four-point system (0–3) adapted from McGuirk (2008) was used: 0 = firm, well-formed; 1 = soft or semi-formed; 2 = loose, non-formed; 3 = watery, potentially containing mucus or blood. A calf was classified as having diarrhoea when a faecal score of ≥2 was recorded during the experimental period. Calves with diarrhoea received supportive therapy according to standard farm veterinary protocols, including oral rehydration therapy and non-steroidal anti-inflammatory drugs if pyrexia (>39.5 °C) or systemic illness occurred. Formal blinding of faecal scoring and laboratory analyses to treatment allocation was not implemented, and no separate allocation-concealment procedure was used.

Statistical analysis

Data were analysed using a two-way analysis of variance (ANOVA) in a 2 × 2 factorial design, with treatment (control versus T+C), sex (female versus male), and their interaction included as fixed effects. The individual calf was considered the experimental unit. Growth-performance endpoints, including ADG, weaning body weight, solid-feed DMI, and SFCR, were analysed using the same factorial structure. Weekly body weights were used to monitor growth during the experimental period; inferential analyses reported in the present manuscript were based on the predefined growth-performance endpoints. Baseline serum biochemical variables measured at birth were compared between sexes using one-way ANOVA because these measurements preceded treatment administration. Serum biochemical variables measured at weaning were analysed using the 2 × 2 factorial model including treatment, sex, and treatment × sex interaction. All statistical analyses were performed using jamovi software (The jamovi project, Sydney, Australia; version 2.6.19.0). Model assumptions were assessed by inspection of residual quartile-quartile plots and residual-versus-fitted plots, together with the Shapiro–Wilk test of residuals. Missing biochemical observations were not imputed; analyses were conducted using the available observations for each endpoint. The proportion of calves classified as having diarrhoea was compared between treatment groups using a two-sided Fisher's exact test. To account for multiple comparisons among biochemical endpoints, *P*-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure. Both nominal *P*-values and FDR-adjusted *P*-values (q-values) were considered, and findings that did not remain significant after FDR correction were interpreted as exploratory. Statistical significance was set at $P < 0.05$. A sensitivity analysis based on the final treatment group sizes (n = 18 and n = 19), a two-sided α of 0.05, and 80% power indicated a minimum detectable standardised between-group difference of approximately Cohen's $d = 0.95$. Thus, the study had limited power to detect small-to-moderate treatment effects.

Results and discussion

Growth performance

Of the 40 calves initially enrolled, 37 completed the experimental period. Three calves died during the study (two in the control group and one in the T+C group), resulting in final group sizes of 18 and 19 calves, respectively. Table 2 presents the descriptive statistics for the effects of thymol and cinnamaldehyde supplementation during the pre-weaning period on growth performance in Holstein calves.

Table 2 Effects of thymol and cinnamaldehyde supplementation (T+C) on the growth performance of pre-weaned Holstein calves (mean ± standard deviation)

Treatments	Control		T+C		*P*-value		
	Female	Male	Female	Male	Treatment	Sex	T × S
Number	9	9	9	10			
ADG (kg/day)	0.39 ± 0.12	0.35 ± 0.14	0.41 ± 0.11	0.39 ± 0.10	0.469	0.400	0.787
SFCR	1.08 ± 0.64	1.33 ± 0.74	1.03 ± 0.51	0.98 ± 0.37	0.292	0.613	0.427
Birthweight (kg)	36.93 ± 4.35	38.33 ± 5.06	37.24 ± 4.98	38.54 ± 4.55	0.869	0.394	0.973
Solid-feed DMI (kg/day)	0.36 ± 0.08	0.38 ± 0.06	0.38 ± 0.10	0.35 ± 0.08	0.797	0.869	0.445
Weaning weight (kg)	63.49 ± 7.13	65.36 ± 7.79	67.10 ± 5.40	65.49 ± 5.72	0.392	0.953	0.427

ADG: average daily gain, SFCR: solid-feed conversion ratio, DMI: dry matter intake, T × S: treatment by sex interaction. Growth-performance data are presented for the 37 calves that completed the experimental period; three calves that died during the study were not included in final endpoint calculations.

No statistically significant differences were observed between the control and treatment groups (Table 2). The observed ADG values were below the expected growth targets of 600–800 g/day for Holstein calves (Van Amburgh & Drackley, 2005). This relatively low ADG should be interpreted in the context of the fixed milk allowance of 4 L/day used under the farm management protocol. This feeding level also limits the direct extrapolation of the present findings to calves managed under higher milk-feeding programmes. Drackley (2008) reported that ADG values of 0.30–0.45 kg/day may result from limited liquid feeding regimes relative to the calves' maintenance requirements. Furthermore, Khan *et al.* (2011) suggested that an ADG below 0.40 kg/day in the pre-weaning period could arise from early transitions to starter feed or the use of low-quality milk replacers. For instance, Göncü & Gökçe (2004) reported pre-weaning ADG values ranging from 253.83 to 367.55 g/day for Holstein calves raised in different hutch types, without significant differences between the groups. Moreover, Göncü & Özkütük (1998) stressed that the growth rate in the region could range from 511 to 613 g/day, depending on the hutch material and environmental stressors. These results are consistent with those of Vakili *et al*. (2013), who reported that phytogenic compounds are often unable to affect growth performance under conditions of reduced rations. These results are also in line with previous research indicating that essential oils do not enhance growth performance under controlled feeding conditions (Benchaar *et al*., 2008; Santos *et al.*, 2015). Minimal effects of thyme oil or cinnamaldehyde supplementation on nutrient utilisation and production responses in dairy cattle have also been reported in the literature (Benchaar *et al*., 2008). Similarly, Vakili *et al.* (2013) found no improvement in performance parameters in calves treated with thyme and cinnamon essential oils. Nonetheless, the influence of phytogenic additives may vary by dosage, diet, and animal health status (Greathead, 2003; Calsamiglia *et al*., 2007).

Diarrhoea occurrence

Diarrhoea cases are summarised in Table 3. Diarrhoea was recorded in 11 of 18 calves (61.1%) in the control group and 8 of 19 calves (42.1%) in the T+C group. Although the proportion was numerically lower in the supplemented group, the difference was not statistically significant (two-sided Fisher's exact test, $P = 0.330$). Therefore, the present study does not provide sufficient evidence to

conclude that T+C supplementation reduced diarrhoea occurrence. Diarrhoea was evaluated as a calf-level occurrence endpoint in the present study. Pathogen identification, dehydration scoring, faecal microbiology, and other detailed clinical measures were not evaluated; therefore, the findings should not be interpreted as a comprehensive assessment of gastrointestinal health. The numerical difference should be interpreted cautiously, particularly given the limited sample size. The absence of formal blinding during faecal scoring should also be considered when interpreting the health-related outcomes.

Table 3 Effects of thymol and cinnamaldehyde (T+C) supplementation on the proportion of pre-weaned Holstein calves with diarrhoea

	Control (n = 18)	T+C (n = 19)	*P*-value
With diarrhoea (n)	11	8	0.330
Without diarrhoea (n)	7	11	
Total	18	19	

Diarrhoea was defined as a faecal score ≥2, according to the scoring system described in the Materials and methods. *P*-value was obtained using the two-sided Fisher's exact test.

In past studies, the incidence of diarrhoea was reduced after the supplementation of essential oils in calves (Windisch *et al*., 2008), and in other related studies, modest or varying effects were found (Vakili *et al*., 2013). Due to the limited sample size, the statistical power to detect significant differences may have been limited. The lower numerical proportion of diarrhoea occurrence in the supplemented group should be interpreted with caution because the between-group difference was not statistically significant and the study had limited power to detect small-to-moderate effects. Therefore, the present results do not demonstrate a protective effect of T+C against diarrhoea.

Blood metabolites

Table 4 presents the comparative analysis of blood metabolites between female and male calves at birth, while Table 5 provides the comparative analysis of blood metabolites between the four experimental groups at weaning.

Table 4 Serum biochemical metabolites and mineral concentrations of Holstein calves at birth (mean ± standard deviation)

Parameters	Female (n = 16)	Male (n = 18)	*P*-value	FDR q-value
Aspartate aminotransferase (U/L)	83.75 ± 32.80	96.11 ± 53.34	0.423	0.826
Gamma-glutamyl transferase (U/L)	34.90 ± 11.05	31.08 ± 20.49	0.512	0.826
Cholesterol (mg/dL)	165.00 ± 29.27	150.00 ± 33.50	0.191	0.637
Glucose (mg/dL)	55.10 ± 19.82	53.68 ± 20.73	0.843	0.856
Calcium (mg/dL)	11.35 ± 1.25	11.04 ± 0.80	0.368	0.826
Phosphorus (mg/dL)	9.79 ± 1.75	8.37 ± 1.48	0.015	0.150
Magnesium (mg/dL)	2.56 ± 0.35	2.74 ± 1.47	0.628	0.826
Total protein (g/dL)	8.11 ± 1.15	8.26 ± 3.10	0.856	0.856
Albumin (g/dL)	3.51 ± 0.08	3.45 ± 0.12	0.113	0.565
Blood urea nitrogen (mg/dL)	10.88 ± 2.26	10.52 ± 2.45	0.661	0.826

Benjamini–Hochberg false discovery rate (FDR) correction was applied across the baseline biochemical sex comparisons. Statistical significance after correction was considered at q <0.05. No baseline biochemical variable remained statistically significant after FDR correction. Nominal *P*-values are shown. The baseline biochemical analyses were performed using the available observations from 16 female and 18 male calves (n = 34). Missing observations were excluded from the statistical analyses without imputation.

Table 5 Effects of dietary thymol and cinnamaldehyde (T+C) supplementation and calf sex on serum biochemical variables in Holstein calves at weaning (mean ± standard deviation)

	Control		T+C		*P*-value		
Parameter	**Female**	**Male**	**Female**	**Male**	**Treatment**	**Sex**	**T × S**
Aspartate aminotransferase (U/L)	86.70 ± 31.60	94.30 ± 20.50	105.00 ± 50.50	86.30 ± 19.90	0.536	0.784	0.641
Gamma-glutamyl transferase (U/L)	33.30 ± 22.90	22.30 ± 11.50	19.30 ± 4.68	24.80 ± 14.20	0.421	0.783	0.381
Cholesterol (mg/dL)	165.00 ± 43.70	162.00 ± 38.70	148.00 ± 39.90	140.00 ± 25.40	0.084	0.441	0.361
Glucose (mg/dL)	36.70 ± 21.30	37.30 ± 16.20	39.70 ± 12.00	38.30 ± 22.90	0.948	0.779	0.304
Calcium (mg/dL)	11.30 ± 0.37	10.70 ± 0.57	10.90 ± 0.51	10.90 ± 0.89	0.943	0.944	0.085
Phosphorus (mg/dL)	7.96 ± 1.30	8.33 ± 2.33	7.46 ± 1.96	9.20 ± 1.29	0.330	0.175	0.280
Magnesium (mg/dL)	2.27 ± 0.60	2.51 ± 0.36	2.30 ± 0.64	2.32 ± 0.39	0.794	0.106	0.466
Total protein (g/dL)	7.73 ± 1.15	7.29 ± 1.05	6.66 ± 1.43	6.95 ± 1.40	0.193	0.554	0.958
Albumin (g/dL)	3.48 ± 0.06	3.48 ± 0.11	3.38 ± 0.19	3.51 ± 0.08	0.500	0.651	0.770
Blood urea nitrogen (mg/dL)	11.90 ± 3.55	9.19 ± 3.96	13.10 ± 5.53	8.98 ± 3.08	0.565	0.038	0.482

T × S: treatment by sex interaction. Nominal *P*-values are shown. Benjamini–Hochberg adjustment was applied separately to the treatment, sex, and treatment × sex significance tests, with statistical significance after correction considered at $q < 0.05$. No biochemical effect remained significant after FDR correction.

Serum phosphorus concentrations were higher in female than in male calves (9.79 ± 1.75 versus 8.37 ± 1.48 mg/dL), reaching nominal statistical significance ($P = 0.015$). However, this difference did not remain significant after Benjamini–Hochberg correction for multiple comparisons (q = 0.150) and was therefore considered exploratory. No other baseline biochemical variable showed a significant sex-associated difference. Similarly, Gökçe *et al.* (2021) reported that differences in total protein, albumin, urea, BUN, beta-hydroxybutyrate, non-esterified fatty acids, total cholesterol, alanine aminotransferase, AST, GGT, calcium, phosphorus, and magnesium concentrations in Holstein calves were not statistically significant. In contrast, Göncü *et al.* (2023) noted that while blood parameter differences between milk replacer groups were not statistically significant, this was with the exception of GGT. They also indicated that the interaction effects between average values of total protein and cholesterol were significant. Nonetheless, all measured values were within physiological reference ranges for healthy Holstein calves (Mohri *et al*., 2007).

The biochemical data recorded at weaning indicate that the majority of the parameters investigated remained stable across the groups, with no statistically significant differences observed between the T+C treatment group and the control group (Table 5). The biochemical profile observed in this study suggests that most parameters remained within physiological limits, consistent with the findings of Gökçe *et al.* (2021), who observed that blood parameters in Holstein calves undergo specific fluctuations during early developmental stages.

At weaning, BUN values showed a nominal sex-associated effect ($P = 0.038$); however, this association did not remain significant after Benjamini–Hochberg FDR correction (q = 0.380) and was therefore considered exploratory. None of the treatment, sex, or treatment × sex effects on the measured biochemical variables remained statistically significant after FDR adjustment.

Conclusions

Under the conditions of this study, thymol and cinnamaldehyde supplementation did not significantly improve growth performance or alter the measured serum biochemical variables in pre-weaned Holstein calves. Nominal sex-associated differences observed for selected biochemical variables did not remain significant after correction for multiple comparisons and should therefore be interpreted with caution. The proportion of calves with diarrhoea was numerically lower in the T+C group, but the difference was not statistically significant. Thus, the present data do not provide sufficient evidence for a growth-promoting or protective health effect of T+C supplementation at the dose evaluated. Larger studies are needed to determine whether treatment effects occur under different feeding and management conditions.

Acknowledgements

The authors thank the staff of the Çukurova University Dairy Research Farm for animal care and technical assistance. The authors also acknowledge Makrovit (Konya, Türkiye) for providing the phytogenic product used in this study.

Authors' contributions

Conceptualisation: Ş.E., methodology: A.E., investigation: A.E. and S.G., data curation: A.E. and C.A.O., writing (original draft preparation): A.E., writing (review and editing): Ş.E., supervision: Ş.E. All authors have read and agreed to the published version of the manuscript.

Conflict of interest declaration

The authors have no conflicts of interest to declare.

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