



***In-vivo* anthelmintic activity assessment to dietary incorporation of natural feed additives in sheep: A comparative study**

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ABSTRACT

Anthelmintic activity to dietary incorporation of exogenous fibrolytic enzymes (EFE) and wormwood (*Artemisia absinthium* L.) herb as feed additives was evaluated. Twenty crossbred lambs (11.58±0.01 kg body weight) were distributed into four dietary treatments in completely randomized design and fed for a period of 90 days followed by 6 day digestibility trial. Animals in all the groups were offered oats straw based total mixed ration added with EFE's cocktail at 0.60% substrate dry matter (DM) level (T1) or wormwood herb at 4.50% substrate DM level (T2) either alone, and in-combination of the two feed additives (T3), whereas the TMR without addition of any additive served as control (T0). *In vivo* anthelmintic assays and selected haemato-biochemical parameters of animals were evaluated at start (0d) and subsequently at monthly intervals (30, 60 and 90d). The feed additives in-combination improved nutrient digestibility and digestible nutrient intakes per day. Wormwood herb inclusion had significant positive effect on overall treatment means as well as at each feeding period of per cent faecal egg count reduction and at 90 day period of faecal parasitic eggs per gram assay. Blood profile revealed better physiological health status of lambs fed feed additives compared to those of control group. It is concluded that incorporation of wormwood had better anthelmintic activity and the two feed additives act synergistically in sheep to improve nutrient utilisation and gastrointestinal infested host's health.

Keywords: Anthelmintics, Blood, Exogenous enzymes, Health, Wormwood

Gastrointestinal (GI) parasitism continues to be a major problem in small ruminant production worldwide, due to its impact on animal health and productivity and the associated costs of control measures. The usual strategy of GI nematodes control based on the repeated use of commercial anthelmintics is nowadays under question which has stimulated the search for alternative approaches to be used for the treatment and/or control of parasites (Sindhu *et al.* 2010). One of the alternative strategies to conventional anthelmintics is the manipulation of host nutrition through use of natural feed additives that are intended to enhance nutrient utilization, improve physiological health and host resistance and/or resilience

against parasitic infections (Hoste *et al.* 2005).

Exogenous enzymes as well as phytobiotics (herbs) have been documented as safe additives to improve the animal's health through different mode of actions. Exogenous enzyme applications improve nutrient digestion leading to enhancement of nutritional status (El-Kady *et al.* 2006), that influences positively the body's immune functioning. Moreover, the immune system is generally benefited from the herbs rich in phtochemicals possessing immuno-stimulatory properties (Valenzuela-Grijalva *et al.* 2017). One potential herb of interest is *Artemisia absinthium* L. commonly called wormwood and locally known as *Tethwen* - a medicinal sub-shrub, distributed mainly in the temperate zones. In India, it is naturally distributed in the Himalayan region across Jammu and Kashmir. It is considered to be an effective natural alternative remedy for parasite control both in humans as well as in animals. This provided a suggestion that the two feed additives, viz. exogenous fibrolytic enzymes (EFE) and wormwood herb may affect the nutrient utilization and physiological health, thus could be used for ruminants to control GI helminthiasis. We hypothesized that EFE's cocktail and wormwood herb will act individually or synergistically to affect nutrient

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metabolism resulting in better animal responses. Thus, the present study was undertaken with the aim to assess the effect of EFE cocktail and wormwood herb incorporated either alone and in-combination on the changes of GI parasitic load in lambs with natural sub-clinical nematodosis.

MATERIALS AND METHODS

Experimental feed additives and basal diet: The EFE cocktail preparation (ALLENZIMIX-EP) contained cellulase (800,000 IU/g), phytase (400,000 IU/g), α -glucanase (450,000 IU/g), xylanase (400,000 IU/g) and pectinase (350,000 IU/g). The wormwood herb collected from the southern area of Kashmir valley, was used as whole aerial part in dried form for *in vivo* assay. The total mixed ration (TMR) was based on oats straw - 40 parts, mixed grass hay - 20 parts and concentrate mixture - 40 parts formulated to meet nutrient requirements recommended for growing sheep (ICAR 2013).

Animal management and experimental feeding: All procedures performed in the study were in accordance to the Institutional Animal Research and Ethical Standards (AU/FVS/Acad/EC-PG/PF/2016/4814-16). Twenty crossbred (Fec-B gene carrying strain $\times$ Hampshire) male lambs (4–6 months age; 11.58 ± 0.01 kg mean body weight) of uniform conformation positive for GI nematodes with parasitic eggs per gram (EPG) of faeces more than 150 counts were procured, fed the same basal TMR for 21 days (adaptation period) without additives. The lambs were then distributed equally into 4 dietary treatments (5 lambs each) in completely randomized design, and fed their experimental diets *ad lib.* for 90 days followed by 6 days duration of digestibility trial.

The experimental diets were control (T0) (basal TMR without any additive), and treatments diets, i.e. TMR added with 0.60% substrate dry matter (DM) of EFE cocktail (T1), 4.50% substrate DM of wormwood herb (T2), and combination of the two feed additives (T3). The doses of the feed additives were chosen on the basis of the results of the orthogonal contrast statements of the pilot *in vitro* nutrient degradability studies. The lambs in all the groups were fed individually the experimental diets once daily in stalls. Clean and fresh drinking water was offered *ad lib.*

Collection and analysis of faecal samples: *In vivo* anthelmintic activity of the feed additives added to TMR's was evaluated by carrying out faecal parasitic egg counts and faecal egg count reduction (FECR) assays in lambs at start (0 d) and subsequently at monthly intervals (30, 60 and 90 d) during the experimental trial. Faecal samples for examination were collected directly from the rectum of lambs and packed in polythene bags. Parasite egg counts were made using the modified McMaster method (Soulsby 1982) and expressed as egg per gram of faeces (EPG). The FECR was calculated in accordance to Coles *et al.* (1992) using the formula:

$$R = 100 \times (1 - X_t/X_c)$$

where, R, per cent reduction in faecal egg count; X_t , average EPG of treatment group; X_c , average EPG of control group.

Haemato-biochemical analysis: The blood samples were taken from jugular vein in the morning before watering and feeding from all the animals at start (0 d) after adaptation period and subsequently on monthly intervals (30 d, 60 d and 90 d) of the experimental feeding trial. Approximately 10 mL of whole blood was collected from each animal, out of which ~2 mL was poured into ethylene diamine-tetra acetic acid (EDTA) vials, agitated for 15–20 seconds to prevent blood clotting and analysed for haematological indices, viz. haemoglobin (Hb), haematocrit (Hct), and eosinophils count immediately after collection using Automatic Haematology Analyser-MS4 (Melet Schloesing Laboratories, France). Another ~8 mL of whole blood samples were poured in dry test tubes allowed for clotting and then centrifuged at $2,000 \times g$ for 10 min to obtain sera for analysis of total serum protein (TSP), serum urea nitrogen (SUN), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in semi-auto biochem analyzer (photometer-5010V5+, Robert Riele INC, Berlin, Germany) using commercial kits.

Digestibility study and chemical analysis: At the end of feeding experiment, six days duration digestibility trial was conducted using cages during which records of feed intake,orts and faeces voided were maintained. Samples of faeces and residue leftover collected during the digestibility trial daily (approximately 10% of total amounts) were pooled for each animal, dried, ground and stored until chemical analysis.

The experimental TMR, refusals and faeces were chemically analysed as per AOAC (2005) for DM, total ash, crude protein (CP) as $6.25 \times N$, and ether extract (EE) to determine digestible nutrient contents of the diets in terms of digestible crude protein (% DCP) and total digestible nutrients (% TDN). Neutral detergent fibre (NDF) content was analyzed according to Van Soest *et al.* (1991).

Statistical analysis: The data of nutrient digestibility and digestible nutrient intakes were analyzed using the General Linear Model procedure of SPSS (2011) based on the statistical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} , dependent variable of the j th lamb on the i th treatment; μ , overall mean; T_i , the fixed effect of i th treatment ($i = 1, 2, 3$); e_{ij} , random residual (error) associated with the dependent variable from the j th lamb on the i th treatment.

Data on haemato-biochemicals and *in vivo* anthelmintic assays were analyzed by repeated measures ANOVA using Proc Mixed procedure of SAS 9.2 (2009) based on the statistical model:

$$Y_{ijk} = \mu + T_i + P_k + e_{ijk}$$

where Y_{ijk} , dependent variable of the k th time on j th lamb and the i th treatment; μ , general mean; T_i , effect of i th treatment; P_k , effect of k th period; e_{ijk} , random error.

Differences among treatments were considered significant at $P < 0.05$, whereas $0.05 < P < 0.10$ presented a tendency using Tukey's procedure for multiple comparisons among means.

RESULTS AND DISCUSSION

Nutrient digestibility and plane of nutrition: The feed additives incorporated in-combination had a significant ($P < 0.05$) positive effect on digestibility of nutrients (OM, CP and NDF) (Table 1). Improvement in digestibility of nutrients represents the efficacy of the feed additives incorporation in augmenting the nutrient utilization. Higher dietary nutrient supply improves ability of the infected host to develop immunity during challenge and repair mucosal damage (Stear *et al.* 2007). This beneficial effect of nutritional alteration over the parasite-host relationship is an alternative to chemoprophylaxis.

Higher protein supplementation improves host resistance to GI nematodes, observed as reduced faecal egg counts and worm burdens, especially during the phase of expression of immunity. Resistance to parasitism in lambs is likely to get compromised when CP intake falls below $14 \text{ g/kg}^{0.75}$ BW (Downey *et al.* 1972). In the present study,

intake of digestible nutrients was improved by feed additives when incorporated singly; however, developed statistical significances by inclusion in-combination, which could have hastened the development of immunity to GI parasites in the experimental lambs. The nutrient intake per unit BW by lambs on high nutritional plan was more than infected (challenged with *Haemonchus contortus*) animals resulting in their better performance (Khan *et al.* 2017).

In vivo anthelmintic assay: GI parasitic load assessment includes counting of parasitic eggs in faecal sample of the host animals or calculating their per cent reduction with time. No significant effect of any feed additive inclusion was evident on overall mean as well as at all periods except 90d for EPG count assay; however, there was significant ($P < 0.01$) effect of period in wormwood herb incorporated groups (T2 and T3) although consistent reduction in EPG from 0 d to 90 d was noticed in all the groups (Table 2, Fig. 1). For % FECR assay, significantly lower overall mean ($P < 0.05$) as well as mean values at each feeding period ($P < 0.01$) were noticed in wormwood herb incorporated groups (Table 2, Fig. 2). Also, there was significant ($P < 0.01$) effect of period on means of % FECR in wormwood herb incorporated groups only. In the present study, reduction

Table 1. Nutritional profile of lambs fed diets incorporated with feed additives (exogenous fibrolytic enzymes cocktail and wormwood herb) alone or in-combination

Attribute	Treatment groups			
	T0	T1	T2	T3
<i>Nutrient digestibility (%)</i>				
Organic matter*	60.35 ^a ±2.01	67.34 ^{ab} ±1.94	67.65 ^{ab} ±2.79	69.79 ^b ±1.88
Crude protein*	60.01 ^a ±2.94	64.38 ^{ab} ±2.90	68.60 ^{ab} ±2.11	71.26 ^b ±1.72
Neutral detergent fibre*	47.48 ^a ±3.25	58.39 ^b ±3.89	50.66 ^a ±2.70	59.52 ^b ±2.04
<i>Digestible nutrient intakes</i>				
DCPI				
g/d**	87.38 ^a ±6.78	101.08 ^{ab} ±5.70	113.36 ^{ab} ±6.20	119.82 ^b ±6.13
g/kgW ^{0.75} *	10.06 ^a ±0.19	10.24 ^a ±0.39	11.06 ^b ±0.30	11.16 ^c ±0.25
TDNI				
g/d**	515.51 ^a ±28.26	616.22 ^{ab} ±24.56	657.52 ^{ab} ±40.86	687.95 ^b ±38.36
g/kgW ^{0.75}	59.60±0.79	62.52±1.71	64.07±2.08	64.04±1.83

The means across rows with different superscripts differ significantly (* $P < 0.05$; ** $P < 0.01$) among the groups. DCPI, digestible crude protein intake; TDNI, total digestible nutrients intake

Table 2. *In vivo* anthelmintic assays in lambs fed diets incorporated with feed additives (exogenous fibrolytic enzymes cocktail and wormwood herb) alone or in-combination

Assay	Period (days)	Treatment groups			
		T0	T1	T2	T3
Faecal egg counts (eggs/gram of faeces)	0	626.00 ^{aA} ±62.63	647.40 ^{aA} ±77.70	672.80 ^{aB} ±92.58	681.20 ^{aB} ±58.21
	30	560.50 ^{aA} ±63.57	559.60 ^{aA} ±85.66	460.00 ^{aAB} ±79.06	448.40 ^{aAB} ±50.57
	60	518.25 ^{aA} ±65.73	499.40 ^{aA} ±86.44	347.20 ^{aAB} ±101.56	329.20 ^{aA} ±59.78
	90*	497.50 ^{cA} ±69.77	464.40 ^{bcA} ±92.68	245.60 ^{abA} ±84.67	224.80 ^{aA} ±64.88
	Mean±SE	550.56±28.36	542.70±40.06	431.40±91.61	420.90±98.05
Percentage reduction in faecal egg counts	30**	10.85 ^{aA} ±1.70	15.12 ^{aA} ±3.53	32.94 ^{bA} ±2.88	34.71 ^{bA} ±2.65
	60**	17.92 ^{aA} ±2.78	25.08 ^{aA} ±4.98	52.71 ^{bAB} ±8.00	53.11 ^{bAB} ±4.73
	90**	21.48 ^{aA} ±3.85	31.43 ^{aA} ±6.85	67.46 ^{bB} ±7.40	69.01 ^{bB} ±6.56
	Mean*±SE	16.75 ^a ±3.12	23.88 ^a ±4.75	51.04 ^b ±10.00	52.28 ^b ±9.91

The means across the rows with different lower case superscript differ significantly (* $P < 0.05$; ** $P < 0.01$) among the groups; The means within the columns across the rows for each attribute with different uppercase superscripts differ significantly ($P < 0.01$) among the periods.

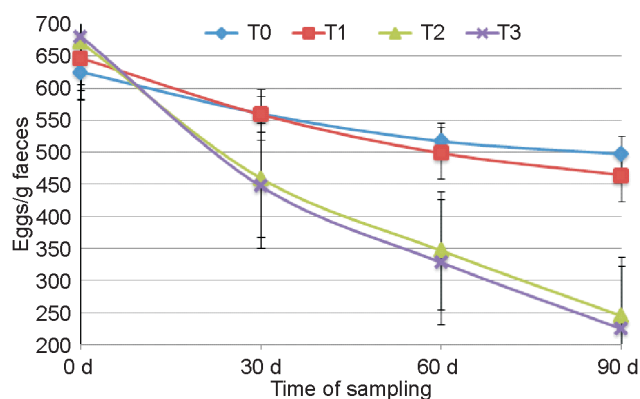


Fig.1. Faecal egg counts in lambs fed diets incorporated with feed additives (exogenous fibrolytic enzymes cocktail and wormwood herb) alone or in-combination.

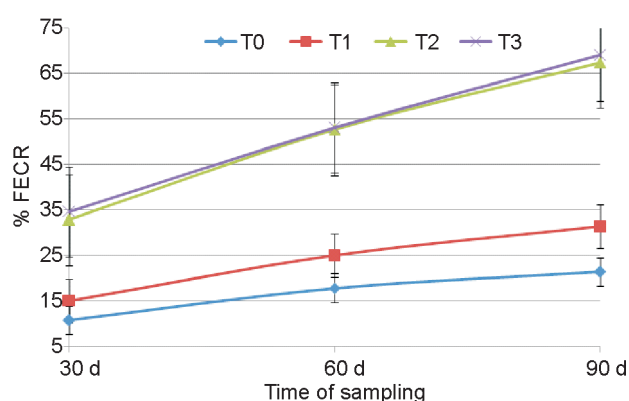


Fig.2. Percentage reduction in faecal egg counts (%FECR) of lambs fed diets incorporated with feed additives (exogenous fibrolytic enzymes cocktail and wormwood herb) alone or in-combination. T₀, Control (basal TMR without feed additives) ; T₁, TMR incorporated with 0.60% substrate DM of EFE cocktail alone; T₂, TMR incorporated with 4.50% substrate DM of wormwood herb alone; T₃, TMR incorporated with 0.60% substrate DM of EFE cocktail and 4.50% substrate DM of wormwood herb in-combination.

in EPG count while increase in % FECR values across the feeding periods was possibly due to stall feeding of animals with no provision to infection through grazing and/or improvement in nutriture of animals with time. Increased availability of essential nutrients are expected to improve host resistant to GI helminthes (Houdijk 2012).

Provision of diet incorporated with EFE alone improved the nutritional status of animals compared to control, although could not develop significance in statistical terms which were reflected by non-significant enhancement of *in vivo* anthelmintic assays in the respective group. Perusal of the results revealed significant anthelmintic activity of the wormwood herb, confirming the earlier findings of Urban *et al.* (2008) who reported significant reduction in egg development and juvenile (L3) larval motility of *Ascaris suum* by ethanolic extracts of *A. absinthium* under *in vitro* study. The present findings also scientifically validate the traditional usage of wormwood as an anthelmintic, being used in indigenous systems of medicine as a vermifuge.

The anthelmintic properties of wormwood herb are attributed to the presence of volatile oil rich in thujone (α and β) (Meschler and Howlett 1999).

Haemato-biochemistry: Blood metabolic profiling is considered to be one of the important diagnostic tool for assessing inflammatory reaction, along with malnutrition and debility caused by the parasitic infestation. Incorporation of the feed additives had no effect on overall means of Hb levels, while significantly lower ($P < 0.05$) Hct and higher ($P < 0.05$) eosinophil counts were noticed in control and EFE alone incorporated groups compared to EFE-herb in-combination fed group (Table 3). The haematological indices in animals of all the groups were within the normal physiological ranges for sheep (Jain 1986), suggesting general health of experimental animals remained normal throughout the investigation period. The periodic increase of Hb and Hct ($P < 0.05$) in animals fed wormwood herb incorporated diets might be due to improved nutriture of animals that improved the erythropoiesis and/or reduced the establishment of adult helminths, as immature parasites (larval stages) cause seepage of blood from injured tissues due to migration leading to lower haemogram levels. The observed higher values for eosinophil counts in control and EFE alone incorporated groups may be attributed to the immune response to comparatively higher parasitic load as depicted by EPG counts in the respective groups.

Though the mean values of all the serum biochemicals were within normal ranges for sheep (Kaneko *et al.* 1997), no significant effect of the feed additives were observed on overall mean values of TSP and AST; however, SUN ($P < 0.01$) and ALT activity ($P < 0.05$) were lower in the wormwood herb incorporated groups (Table 3). The comparatively lower ($P > 0.05$) TSP level in the control group might be due to haemodilution, a compensatory mechanism for intestinal haemorrhage caused by parasitic larva and later on due to loss of large quantities of serum protein in the gut through exudation. Non-significant increase in TSP was also reported by Gupta *et al.* (2006) in heifers fed mixture of different herb (*Bacopa monnieri*, *Urtica dioica* and *Eclipta alba*), and Peters *et al.* (2015) in lactating dairy cows fed diets incorporated with exogenous enzymes. Wormwood herb incorporation lowered ($P < 0.01$) SUN levels probably due to efficient utilisation of dietary proteins as also depicted by higher CP digestibility.

In light of the results of present study, use of *Artemisia absinthium* L. herb alone or in synergism with EFE's cocktail as feed additive seems valid for inclusion as natural anthelmintic in diet of sheep with improvement of the infested host's health. With the rapid development of resistance against most chemical anthelmintics along with accumulation of their residues in animal products and environment and the unaffordable costs for small livestock farmers, inclusion of the wormwood herb in diets of ruminants may be recommended as alternative control strategy to treat cases of parasitism for organic livestock farming.

Table 3. Haemato-biochemical profile of lambs fed diets incorporated with feed additives (exogenous fibrolytic enzymes cocktail and wormwood herb) alone or in-combination

Attribute	Period (days)	Treatment groups			
		T0	T1	T2	T3
Haemoglobin (g/dL)	0	9.85±0.22	9.98±0.26	9.64 ^A ±0.23	9.52 ^A ±0.18
	30	10.45±0.27	10.78±0.43	10.94 ^B ±0.37	10.90 ^B ±0.09
	60**	10.25 ^a ±0.27	10.98 ^{ab} ±0.31	11.44 ^{abB} ±0.41	11.94 ^{bC} ±0.14
	90**	10.17 ^a ±0.31	10.62 ^a ±0.38	10.86 ^{aB} ±0.34	12.24 ^{bC} ±0.22
	Mean±SE	10.18±0.12	10.59±0.22	10.72±0.38	11.15±0.61
Haematocrit (%)	0	31.48±1.90	31.68±1.27	30.20 ^A ±0.54	29.94 ^A ±0.80
	30	32.10±1.87	32.50±1.05	34.74 ^B ±0.83	35.10 ^B ±0.91
	60**	30.78 ^a ±1.36	32.76 ^{ab} ±0.88	36.06 ^{bcB} ±0.88	37.06 ^{bcC} ±0.60
	90**	29.20 ^a ±0.94	29.90 ^a ±0.86	35.70 ^{bB} ±0.99	38.24 ^{bC} ±0.68
	Mean*±SE	30.89±0.62	31.71 ^{ab} ±0.65	34.17 ^{ab} ±1.35	35.08 ^b ±1.83
Eosinophil count (×10 ³ /μL)	0	0.29 ^A ±0.08	0.31 ^A ±0.04	0.39 ^B ±0.04	0.42 ^C ±0.04
	30**	0.56 ^{aB} ±0.11	0.64 ^{bB} ±0.06	0.31 ^{aB} ±0.04	0.32 ^{aB} ±0.03
	60**	0.71 ^b ±0.15	0.60 ^{bB} ±0.09	0.24 ^{aA} ±0.04	0.18 ^{aA} ±0.01
	90**	0.63 ^{cAB} ±0.14	0.57 ^{bcB} ±0.12	0.23 ^{abA} ±0.04	0.13 ^{aA} ±0.01
	Mean*±SE	0.55 ^b ±0.09	0.53 ^b ±0.07	0.29 ^a ±0.04	0.26 ^a ±0.07
Total serum protein (g/dL)	0	6.57±0.21	6.34±0.09	6.50 ^A ±0.09	6.52 ^A ±0.10
	30	6.50±0.24	6.40±0.17	6.68 ^{AB} ±0.15	6.66 ^{AB} ±0.14
	60	6.50±0.29	6.68±0.19	6.80 ^{AB} ±0.16	6.96 ^{AB} ±0.17
	90	6.55±0.27	6.80±0.19	6.94 ^B ±0.16	7.12 ^B ±0.18
	Mean±SE	6.53±0.02	6.55±0.09	6.73±0.07	6.81±0.09
Serum urea nitrogen (mg/dL)	0	34.47 ^A ±0.90	34.01±0.62	32.54±0.74	33.13±0.83
	30	35.84 ^{AB} ±1.26	34.75±0.98	33.42±0.86	33.61±0.92
	60	37.08 ^{AB} ±1.36	34.91±1.21	33.36±1.57	32.92±1.15
	90	38.46 ^B ±1.47	36.19±1.37	35.50±1.08	34.85±1.05
	Mean**±SE	36.46 ^b ±0.85	34.97 ^{ab} ±0.45	33.71 ^a ±0.63	33.63 ^a ±0.43
Alanine amino-transferase (IU/L)	0	24.03±0.75	24.06±0.54	24.60 ^B ±0.62	25.32 ^B ±0.90
	30	23.65±0.86	23.46±0.59	23.30 ^{AB} ±0.76	21.22 ^A ±1.06
	60*	24.70 ^b ±1.73	23.06 ^{ab} ±0.58	20.98 ^{aA} ±1.04	20.44 ^{aA} ±1.02
	90*	25.00 ^b ±1.66	24.26 ^b ±1.33	22.54 ^{abAB} ±1.14	20.14 ^{aA} ±0.87
	Mean*±SE	24.34 ^b ±0.31	23.71 ^{ab} ±0.40	22.85 ^{ab} ±0.52	21.78 ^a ±0.65
Aspartate amino-transferase (IU/L)	0*	73.85 ^{ab} ±1.93	72.48 ^{ab} ±1.13	75.06 ^{abD} ±0.88	75.94 ^{bd} ±0.70
	30	72.02±2.53	71.06 ^{AB} ±1.21	71.16 ^C ±0.68	70.78 ^C ±1.04
	60	70.90±2.76	69.38 ^{AB} ±1.58	67.22 ^B ±1.13	67.16 ^B ±1.33
	90*	69.67 ^b ±3.06	67.58 ^{abA} ±1.96	63.42 ^{aA} ±1.40	61.90 ^{aA} ±1.41
	Mean±SE	71.61±0.89	70.12±0.81	69.21±1.11	68.94±1.29

The means across the rows with different lower case superscript differ significantly (* $P<0.05$; ** $P<0.01$) among the groups. The means within the columns across the rows for each parameter with different uppercase superscripts differ significantly ($P<0.05$).

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