Turkish Journal of Veterinary & Animal Sciences

Volume 46 | Number 5 Article 7

1-1-2022

Effects of replacing the protein content of Azolla Pinnata with concentrate on physiological and blood profiles changes in Sahiwal calves

NINAD BHATT

RAMESH CHANDRA

Kotresh Prasad CHIKKAGOUDARA

DIKSHA KANDPAL

DEEPESH BHARAT MISRA

See next page for additional authors

Recommended Citation

BHATT, NINAD; CHANDRA, RAMESH; CHIKKAGOUDARA, Kotresh Prasad; KANDPAL, DIKSHA; MISRA, DEEPESH BHARAT; and TYAGI, NITIN (2022) "Effects of replacing the protein content of Azolla Pinnata with concentrate on physiological and blood profiles changes in Sahiwal calves," *Turkish Journal of Veterinary & Animal Sciences*: Vol. 46: No. 5, Article 7. https://doi.org/10.55730/1300-0128.4247
Available at: https://journals.tubitak.gov.tr/veterinary/vol46/iss5/7

Effects of replacing the protein content of Azolla Pinnata with concentrate on physiological and blood profiles changes in Sahiwal calves

Authors

NINAD BHATT, RAMESH CHANDRA, Kotresh Prasad CHIKKAGOUDARA, DIKSHA KANDPAL, DEEPESH BHARAT MISRA, and NITIN TYAGI

TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2022) 46: 724-733
© TÜBİTAK
doi:10.55730/1300-0128.4247

Effects of replacing the protein content of *Azolla Pinnata* with concentrate on physiological and blood profiles changes in Sahiwal calves

Ninad BHATT[1,*], Ramesh CHANDRA[1], Kotresh Prasad CHIKKAGOUDARA[2], Diksha KANDPAL[3], Deepesh Bharat MISHRA[4], Nitin TYAGI[4]

[1]LPM Division, ICAR-NDRI, Karnal, Haryana, India
[2]University of Agricultural Sciences, Raichur, Karnataka, India
[3]Division of Veterinary Pathology, LUVAS, Hisar, Haryana, India
[4]Division of Animal Nutrition, ICAR-NDRI, Karnal, Haryana, India

Received: 19.01.2022 • **Accepted/Published Online:** 13.07.2022 • **Final Version:** 18.10.2022

Abstract: Eighteen female Sahiwal calves of average four month of age and 54 kg body weight were selected for the study. The animals were divided into three groups (T0, T1, and T2) on body weight basis. The animals were fed as per ICAR feeding standard (2013) for calves for 90 days. The animals in T0 group T0 were fed as per feeding standard while in T1 and T2 groups, fresh *Azolla pinnata* was fed by replacing 15% and 30% protein content of concentrate with *Azolla pinnata* on DM basis. The nonsignificant results were obtained for all the physiological parameters. In addition, the nonsignificant results were obtained for the body weight at different intervals among all the three groups. The blood samples were collected at every fortnight from zero days until the end of the study. Plasma concentrations of cortisol, T3 and T4 have shown nonsignificant results throughout the experiments ($p > 0.05$). However numerically cortisol level was lower in the Azolla fed groups. There was a significant difference seen in the levels of growth hormone and IGF-1 level in between T0 and T2 groups ($p < 0.05$). Various biochemical tests such as glucose, protein, NEFA, creatinine, BUN level along with haematological parameters have shown no significant difference ($p > 0.05$). Also, Azolla has influenced the IGF-1 and GH level leading to faster growth rate. It can be concluded that *Azolla pinnata* can act as a novel initiative for protein replacement by maintaining the circulating concentration of various blood profiles up to the basal levels along with better growth.

Key words: *Azolla pinnata* , Sahiwal, hormonal, biochemical, physiological, IGF-1

1. Introduction

Feeding plays a very important part in expression of full genetic ability of the livestock. However, the study conducted by IGFRI scientists have found that India is deficient in concentrates, dry fodder and green fodder and their shortage is found to be 28.9%, 23.4%, and 11.24% respectively [1]. Thus, livestock rely on left out crop residues as their primary feed source which are poor in nutritional quality. Also, the severe deficiency of green fodder during winter season leads to extreme nutritional shortage for livestock [2]. These problems, along with rising demand for animal products justified research into rational use of unconventional feed. Feed is incurring around 60%–70% of the total cost required for raising the livestock and the concentrate feed among them is the most expensive [3]. Thus, if we provide a cheaper alternate source of protein for livestock, it can play an important role in raising income from livestock. To overcome this issue, Azolla can be one of the best alternatives. It requires minimum inputs for growth and can produce green fodder with high nutrients throughout the year [4]. The unit cost of *Azolla Pinnata* production is Rs. 1.36/kg and the cost of the commercially available concentrate is Rs. 23.4/kg [5]. Azolla owing to its higher protein content (21%– 32%) have a definite role in accelerating growth [6] by causing changes in various blood metabolites level. Various essential amino acid such as leucine, tryptophan, methionine, lysine, etc., are present in *Azolla pinnata* as per study. Adequate growth of dairy calves is very crucial for future economics of the herd. The nutrients intake from liquid diet before weaning is very limited to induce fast ruminal functioning [7]. Various factors are responsible for rumen development in young calves [8], but the nature of the feed offered has the most important role [9]. Lee et al. [10] concluded that protein and energy supplied along with the milk replacer have several effects on body weight, health and blood metabolites (i.e. glucose, creatinine, BUN). The varied level of proteins in diet may

* Correspondence: ninadbhatt24@gmail.com

adversely affect the liver and kidney functions of animals [11]. The sudden nutritional change in the diet of animal will affect the concentrations of growth hormone (GH), insulin, T3, T4, and metabolites such as glucose, ammonia nitrogen, etc. [12]. Blood profiling is the most commonly used predictor for disease diagnosis [13]. Changing haematobiochemical parameters are essential indicators of the animal's physiological and pathological states [14]. However, until now, no studies have been conducted to see the effects of *Azolla pinnata* on blood metabolites of indigenous Sahiwal calves. Hence, the present research was conducted with an objective to examine the effect of *Azolla pinnata* feeding on physiological, biochemical and hormonal profile changes in Sahiwal calves during winters.

2. Material and methods

2.1. Animals

The present research was conducted at Livestock Research Centre (LRC) in National Dairy Research Institute (NDRI), Karnal, Haryana. Eighteen Sahiwal female calves, averaging 56 kg live weight and 4 months of age were collected from herd and were randomly assigned to three treatments of six animals each. The animals were housed as per the space and welfare standard set by BIS (Bureau of Indian Standards). The animals were properly vaccinated for FMD, HS, BQ, etc., and dewormed before the start of experiment. The duration of experiment was 90 days (13 weeks) from 1 November 2018 to 29 January 2019. Azolla was grown at the Azolla production unit established at Fodder Research Institute. A total of about 19 pits were selected for propagating *Azolla pinnata* and their size was about 11×4.5 feet and 2 feet depth. The average yield per pit/day was 1.05 kg and the average yield from 19 pits/day was 20.01 kg/day [5].

2.2. Chemical analysis of feed samples

Samples of wheat straw, concentrate mixture, oats and *Azolla pinnata* were analysed as per AOAC 2000 [15]. The proximate analysis of different feeds and fodder fed to Sahiwal calves has been presented in Table 1. The composition of concentrate used has been mentioned in Table 2. During the trial period, samples of feed and fodder offered, residue and faeces were collected daily. Samples of feed, refusal and faeces were analysed for proximate principles [15].

2.3. Amino acid analysis

Amino acids present in sun-dried Azolla were analysed using high performance liquid chromatography (HPLC). The contents of the various amino acids identified were expressed as a percentage of Azolla dry matter and as a percentage of protein content. The amino acid profiling of *Azolla pinnata* is mentioned in Table 3.

2.4. Feeds and feeding

Two weeks adaptation period on the regular diet of *Azolla pinnata*, oats, concentrate were provided for calves. It was followed by the experimental period of 13 weeks (90 days) during which the groups were fed with three different treatments. The animals were fed as per ICAR (2013) feeding standard. First group (T_0) was a control group and was fed as per ICAR (2013) for calf. Second group (T_1) was fed as per control by replacing the 15% protein content of the concentrate with the *Azolla pinnata* and third group (T_2) was fed the same but by replacing the 30% protein content of concentrate with the *Azolla pinnata.* The diets formulated were isonitrogenous in all the groups. All the animals were fed individually. Concentrate mixture mixed with Azolla was provided at 8.30 a.m., roughage mixed with wheat straw was offered at 9.30 a.m. and same feeding was done two times a day. The dry matter (DM) offered per day was around 2.5 kg per 100 kg live body weight. Then these growing calves were fed with DM in the ratio of 60**:**40 as roughage**:**concentrate. Dry matter intake of animals was adjusted fortnightly. First of all, the fortnight body weight of calves were calculated. Based on it, DM was fed at 2.5 kg/100 kg live body weight. The DM offered was

Table 1. Proximate composition of different feed used in trial (% DM basis).

Components	Oats	Concentrate	Wheat straw	*Azolla pinnata*
% DM	14.5 ± 0.16	90 ± 0.02	89.9 ± 0.06	9.95 ± 0.03
OM	92.9 ± 0.24	91.85 ± 0.20	89.8 ± 0.23	79.7 ± 0.18
NDF	56.8 ± 0.47	27.6 ± 0.08	73.1 ± 0.04	44.28 ± 0.18
ADF	28.9 ± 0.08	17.4 ± 0.52	55.88 ± 0.58	39.4 ± 0.06
CP	10.6 ± 0.06	23.4 ± 0.08	3.3 ± 0.11	26.5 ± 0.08
EE	2.9 ± 0.02	4.4 ± 0.05	0.9 ± 0.08	3.9 ± 0.13
TA	7.1 ± 0.25	8.15 ± 0.25	10.2 ± 0.16	20.3 ± 0.281`

DM: dry matter, OM: organic matter, NDF: neutral detergent fibre, ADF: acid detergent fibre, CP: crude protein, EE: ether extract, TA: total ash.

Table 2. Ingredient composition (% on DM basis) of concentrate mixture.

Ingredients	Maize	Ground nut cake GNC	Mustard cake	Wheat bran	Mineral mixture	Salt
Quantity	30	18	24	25	2	1

slightly above the ICAR requirement in each group so as to prevent any deficiency in diet. Along with it, the wheat straw was fed at 200 g/calf/day. This was done in order to check diarrhoea that was seen initially before the start of experiment.

Procedure for replacement of concentrate with *Azolla pinnata* is as follows:

1. First of all, the amount of concentrate to be offered is calculated as per the body weight of animals on DM basis.
2. Then protein content present in that concentrate amount is calculated on DM basis.
3. Afterward 15% and 30% protein content of concentrate is calculated.
4. Then the amount of Azolla protein required to replace this 15% and 30% protein content of concentrate is calculated on DM basis.
5. Afterward the *Azolla pinnata* required on fresh basis is calculated and finally fed to calves in different treatment groups.
6. Remaining concentrate left after replacement was calculated on fresh basis and then fed to each groups as per requirements.

2.5. Body weight parameters

The calves were weighed at each fortnight on a weighing balance before offering feed and water for two days. The weight average for these two days is the actual weight for that fortnight.

2.6. Physiological parameters

The rectal temperature (RT) of calves was recorded with a digital thermometer by touching the calf's rectal mucosa for nearly a minute. The pulse rate (PR) was counted by putting the index finger at the base of the tail to feel the pulsation of the middle coccygeal artery and the results are presented in pulsations per minute. The calves' respiration rate (RR) was recorded by visual observation of flank inward and outward movements. The peripheral skin temperature (ST) was recorded using noncontact tele thermometer (Raytek, Model Raynger ST2L, Surrey, UK) by placing it around 2–3 inches away from the surface of the calculating site.

2.7. Hormonal and biochemical parameters

Blood samples were collected at the beginning (0 day), and then repeated at fortnightly interval until the end of study. The collection was done at 8.30 a.m. before offering feed and water. The blood was immediately transferred into tubes containing EDTA as anticoagulant. Afterwards, the tubes were rotated gently in between the palms for facilitating the mixing the blood with anticoagulant. The test tubes were then sealed and placed in refrigerator immediately. In order to obtain plasma, blood samples were centrifuged at 3000 rpm for 25 min. The plasma thus obtained was transferred to labelled storage vials and stored in deep freezer for estimation of blood metabolites, enzymes and hormones. The fresh blood collected was used for RBC, WBC, and haemoglobin. All the biochemical and hormonal profiles were assessed with the help of the kits purchased from different companies. The analysis was done as per the instructions provided by the manufacturer along with the kits.

Plasma IGF-1 level was estimated using commercially available bovine insulin-like growth factor (IGF1) ELISA Kit manufactured by MyBioSource Incorporation (San Diego, CA, USA) (Product no. MBSS455080). Plasma NEFA was estimated using free fatty acids, FFA ELISA Kit manufactured by BIOTANG Incorporation (Lexington, MA, USA) (Product no. HU9163). Leptin was measured by bovine leptin ELISA Kit manufactured by MyBioSource Incorporation (Product no. MBS2020003). Blood cortisol was estimated through bovine cortisol

Table 3. Amino acid profiling of *Azolla pinnata*.

Amino acids	% DM	% protein content
Threonine	0.96	3.8
Leucine	2.43	9.1
Isoleucine	0.83	3.7
Tryptophan	0.48	1.9
Lysine	1.3	5.1
Phenylalanine	1.1	4.7
Tyrosine	0.68	2.8
Glycine	1.55	6.1
Methionine	0.52	1.6
Arginine	1.36	5.4
Cystine	0.10	1.1
Serine	0.85	3.7
Alanine	1.71	6.8
Arginine	1.53	5.5

ELISA Kit manufactured by MyBioSource Incorporation (Product no. MBS2608983). Blood growth hormone was estimated using GH ELISA Kit manufactured by MyBioSource Incorporation (Product no. MBS703041). Blood triiodothyronine (T3) was estimated using bovine triiodothyronine (T3) ELISA Kit manufactured by MyBioSource Incorporation (Product no. MBS2609823). Blood thyroxine T4 was estimated using bovine thyroxine, T4 ELISA Kit manufactured by MyBioSource Incorporation (Product no. MBS702370).

Blood haemoglobin of calves was estimated using Sahli's hemoglobinometer. The fresh blood collected was used for estimating of RBC, WBC, and haemoglobin. All the biochemical and hormonal profiles were assessed with the help of the kits purchased from different companies as per the instructions provided by the manufacturer along with the kits. The plasma glucose level was estimated using ABSbio glucose calorimetric detection kit manufactured by Advanced BioReagent Systems (Hayward, CA, USA) (Product no. K189-100). Total plasma protein was estimated using ABSbio protein detection kit manufactured by Advanced BioReagent Systems (Product no. K138-200). Blood urea was estimated using ABSbio urea detection kit (Product no. K358-200). Total plasma creatinine was estimated using ABSbio creatinine detection kit by Advanced BioReagent Systems. Blood albumin was estimated through commercially available albumin test kit (bromocresol green, end point assay methods), Span Cogent Diagnostics Ltd. (Surat, GJ, India) (Product no. 84LS100-60).

2.8. Data collection and sampling

A digestibility trial of seven days was conducted to determine the digestibility of different nutrients by conventional total collection method. The dung voided by each animal during the digestibility trial was collected daily, weighed, and dried in a hot air oven overnight at 100 °C, then cooled in desiccators and weighed to determine the dry matter content. The representative sample was ground and stored in an airtight container and further used for chemical analysis. About 1/300th part of fresh faeces voided by each animal daily was preserved in 25% H_2SO_4 for nitrogen estimation.

2.8.1 Calculation

Voluntary dry matter intake and apparent dry matter digestibility of the feed are calculated for each animal as follows:

Average daily dry matter intake = A – B

$$\text{Dry matter digestibility} = \frac{(A - B - C) \times 100}{(A - B)}$$

where

A = average dry matter offered daily,

B = average dry matter refused daily,

C = average dry matter voided in faeces daily.

The digestibility of other nutrients were calculated as follows:

$$\text{Digestibility \% of a particular Nutrient} = \frac{(A - B) \times 100}{(A)}$$

where

A = total intake of the particular nutrient through feeds and fodder,

B = out go of the particular nutrient through faeces.

2.9. Statistical analysis

The data collected was analysed by multiple ANOVA techniques using PROC ANOVA procedure of SAS 9.3. Homogenous subsets were separated using the Duncan's multiple range test and the level of significance was declared at $p < 0.05$. The level of significance was declared at $p < 0.05$.

3. Results

The average DMI, CP intake along with nutritive value of ration has been mentioned in Table 4. The average feeding of different components (fresh *Azolla pinnata* and green fodder) in different groups has been mentioned in Table 5.

3.1. Growth performance

The overall increase in the body weight and average daily weight gain (ADG) of different groups has been mentioned in Table 6. The ADG was significantly ($p < 0.05$) higher in T2 group in comparison to T0 groups as seen in Table 6. The results obtained from the digestibility trial revealed that there were no significant effects ($p > 0.05$) in the digestibility of DM, OM, NDF, ADF, EE, and NFE among all the three (T_0, T_1, and T_2) groups.

3.2. Physiological parameter variations

All the values of physiological parameters (rectal temperature, skin temperature, respiration and pulse rate) were nonsignificant among all the groups and have been mentioned in Table 7.

3.3. Serum hormonal indicators

The average plasma cortisol levels (ng/mL) in T0, T1, and T2 groups were 3.07 ± 0.13, 2.54 ± 0.21, and 2.66 ± 0.18, respectively (Table 8). The results of the average plasma leptin level (mg/dL) in T0, T1, and T2 groups are presented in Table 8. The levels were nonsignificant ($p > 0.05$) between T0 and T1 but significant difference is obtained between T0 and T2 groups ($p < 0.05$). The overall average of the GH (ng/mL) level in T0 group was 9.06 ± 0.34, and in T1 it was 10.77 ± 0.58 and in T2 group, the average value was 11.83 ± 0.65. The significant results ($p < 0.05$) were found between T0 and T2 groups (Table 8). The results of the average plasma T3 level (ng/mL) in T0, T1, and T2 groups are presented in Table 8. The average plasma T4 levels (ng/mL) in T0, T1, and T2 groups were 48.38 ± 2.56, 47.3 ± 3.56, and 49.32 ± 1.28, respectively, and were nonsignificantly ($p > 0.05$) among all the groups (Table 8).

Table 4. Mean DMI, CP intake in different groups (kg/day).

Parameters	T0 (control)	T1 (treatment 1)	T2 (treatment 2)
DMI (kg/day)	$1.88^{a} \pm 0.07$	$1.94^{a} \pm 0.07$	$1.98^{a} \pm 0.09$
CPI (kg/day)	$0.335^{a} \pm 0.09$	$0.346^{a} \pm 1.1$	$0.354^{a} \pm 0.08$
CP % (ration)	15.66 ± 0.12	15.75 ± 0.14	15.82 ± 0.12
TDN % (ration)	67.29 ± 0.60	67.56 ± 0.73	68.12 ± 0.89

DMI: dry matter intake, CPI: crude protein intake, CP: crude protein, TDN: total digestible nutrients.

Table 5. Average feeding of different components in treatment groups (90 days).

Groups	Roughage offered (kg)	Conc. before replacement (g)	Conc. CP	CP to be replaced (15%)	Conc. after replacement	Azolla req. for replacement (DM basis)	Azolla fresh (kg)	Wheat Straw (g)
T0	7.78	835.56	-	-	-	-	-	0.200
T1	8.03	862.22	0.18	27.2	732.89	102.8	1.03	0.200
T2	8.19	880.00	0.19	55.6	616.00	209.8	2.11	0.200

Table 6. Average body weight and ADG of different groups.

Parameters	Control (T_0)	Treatment 1 (T_1)	Treatment (T_2)
Initial body weight (kg)	56.76 ± 2.67	56.87 ± 2.26	56.92 ± 3.09
Final body weight (kg)	93.36 ± 3.76	95.62 ±.59	97.97 ± 3.33
Body weight gain (kg)	36.6 ± 2.39	38.75 ± 2.17	41.05 ± 2.93
Average daily weight gain (ADG) (kg)	$0.411^{a} \pm 0.02$	$0.431^{ab} \pm 0.01$	$0.456^{b} \pm 0.01$

Mean ± SD (n = 6). ^{abc}: different superscript implies significant difference ($p < 0.05$) among treatments.

Table 7. Various physiological parameters in the treatment groups.

Parameters	T0 (control)	T1 (treatment 1)	T2 (treatment 2)
Rectal temperature (°C)	$38.1^{a} \pm 0.11$	$37.8^{a} \pm 0.19$	$37.5^{a} \pm 0.14$
Respiration rate (per minute)	$16.88^{a} \pm 0.47$	$18.08^{a} \pm 0.87$	$17.34^{a} \pm 0.75$
Pulse rate (per minute)	$64.81^{a} \pm 0.93$	$65.12^{a} \pm 1.22$	$64.38^{a} \pm 1.13$
Skin temperature (°C)	$23.8^{a} \pm 0.68$	$24.8^{a} \pm 0.55$	$23.46^{a} \pm 0.37$

Mean ± SD (n = 6). ^{abc}: different superscript implies significant difference ($p < 0.05$) among treatments.

3.4. IGF-1 level at various fortnights

The significant difference ($p < 0.05$) was obtained for IGF-1 among T0, T1, and T2 groups (Table 8). All the values of IGF-1 at fortnight level were nonsignificant ($p > 0.05$) to each other statistically except at the 5th, 6th and 7th fortnight where T0, T1, and T2 are statistically different ($p < 0.05$) to each other (Table 9).

3.5. Serum biochemical indicators

The results of the average plasma glucose level (mg/dL) in T0, T1 and T2 groups are presented in Table 10 and no significant results ($p > 0.05$) obtained among all the groups. The plasma BUN levels (mg/dL) in T0, T1, and T2 groups are presented in Table 10 and the non significant ($p > 0.05$) results were found among all the three groups.

Table 8. Different hormonal parameters estimated in the treatment groups.

Parameters	T0 (control)	T1 (treatment 1)	T2 (treatment 2)
Cortisol (ng/mL)	$3.07^{a} \pm 0.13$	$2.54^{a} \pm 0.21$	$2.66^{a} \pm 0.18$
Leptin (ng/mL)	$5.33^{a} \pm 0.05$	$5.89^{a} \pm 0.13$	$6.97^{b} \pm 0.22$
GH (ng/mL)	$9.06^{a} \pm 0.34$	$10.77^{ab} \pm 0.58$	$11.83^{b} \pm 0.65$
T3 (ng/mL)	$0.95^{a} \pm 0.08$	$1.02^{a} \pm 0.14$	$0.98^{a} \pm 0.10$
T4 (ng/mL)	$48.38^{a} \pm 2.56$	$47.3^{a} \pm 3.56$	$49.32^{a} \pm 1.28$
IGF 1 (g/dL)	$129.73^{a} \pm 1.53$	$133.64^{b} \pm 2.58$	$137.80^{c} \pm 2.77$

(IGF 1: insulin like growth factors, GH: growth hormone, T3: triiodothyronine, T4: thyroxine. Mean ± SD (n = 6). ^{abc}: different superscript implies significant difference ($p < 0.05$) among treatments**.**

Table 9. IGF-1 values at different intervals.

Fortnight	T0 (control)	T1 (treatment 1)	T2 (treatment 2)
1.	$116.61^{aF} \pm 0.91$	$120.79^{Fa} \pm 1.54$	$120.44^{Da} \pm 1.61$
2.	$123.9^{Ea} \pm 1.34$	$124.24^{Ea} \pm 0.65$	$126.41^{Ca} \pm 0.69$
3.	$127.66^{Da} \pm 0.77$	$128.68^{Ea} \pm 0.80$	$130.9^{Ca} \pm 1.46$
4.	$130.77^{Ca} \pm 0.62$	$134.14^{Db} \pm 0.87$	$137.44^{Bb} \pm 0.23$
5.	$132.1B^{Ca} \pm 0.74$	$137.11^{Cb} \pm 0.70$	$141.97^{Bc} \pm 1.27$
6.	$133.1^{Ba} \pm 0.482$	$143.14^{Bb} \pm 0.64$	$149.94^{Ac} \pm 0.32$
7.	$140.0^{Aa} \pm 0.50$	$148.39^{Ab} \pm 0.27$	$150.41^{Ab} \pm 0.51$
Average	$129.7^{a} \pm 1.15$	$133.64^{b} \pm 1.44$	$137.80^{c} \pm 1.66$

Mean ± SD (n = 6). ^{abc}: different superscript implies significant difference ($p < 0.05$) among treatments. ^{ABC}: different superscript implies significant difference ($p < 0.05$) among fortnights.

Table 10. Different biochemical parameters in the three treatment groups.

Parameters	T0 (control)	T1 (treatment 1)	T2 (treatment 2)
Glucose (mg/dL)	$56.12^{a} \pm 2.14$	$55.06^{a} \pm 1.75$	$56.93^{a} \pm 1.68$
BUN (mg/dL)	$18.46^{a} \pm 0.77$	$17.52^{a} \pm 0.78$	$16.80^{a} \pm 0.83$
Creatinine (mg/dL)	$1.25^{a} \pm 0.10$	$1.29^{a} \pm 0.12$	$1.27^{a} \pm 0.14$
Protein (g/dL)	$7.31^{a} \pm 0.54$	$7.60^{a} \pm 0.61$	$7.62^{a} \pm 0.72$
Albumin (mg/mL)	$32.7^{a} \pm 0.53$	$33.8^{a} \pm 0.38$	$34.6^{a} \pm 0.81$
NEFA (µmol/L)	$44.31^{a} \pm 1.12$	$43.73^{a} \pm 1.36$	$42.22^{a} \pm 1.57$
Alpha-amino nitrogen (µg/mL)	$62.55^{a} \pm 6.32$	$63.43^{a} \pm 8.27$	$64.11^{a} \pm 5.88$
RBC (million/mm³)	$6.67^{a} \pm 0.07$	$7.33^{a} \pm 0.19$	$7.11^{a} \pm 0.13$
WBCs (cells/µL)	$14482^{a} \pm 549$	$12743^{a} \pm 189$	$13478^{a} \pm 345$
Haemoglobin (g%)	$12.56^{a} \pm 0.24$	$13.13^{a} \pm 0.18$	$11.88^{a} \pm 0.26$

BUN: blood urea nitrogen, NEFA: nonesterified fatty acid, WBC: white blood cells, RBC: red blood cells.
Mean ± SD (n = 6). ^{abc} different superscript implies significant difference ($p < 0.05$) among treatments.

The plasma creatinine levels (mg/dL) in T0, T1, and T2 groups are presented in Table 10 and they also did not differ significantly ($p > 0.05$) (Figure 1). The results of the average of plasma protein level (g/dL) in T0, T1, and T2 groups are presented in Table 10 and it was found that the there was no significant difference ($p > 0.05$) found among all the groups

both fortnightly as well as overall. The results of the average plasma albumin level (mg/dL) in T0, T1, and T2 groups are presented in Table 10. All the values at different fortnights were nonsignificant ($p > 0.05$) to each other among all the three groups. However numerically higher values were reported in the treatment groups. The results of the average of NEFA (µmol/L) in T0, T1, and T2 groups are presented in Table 10 and they also did not differ significantly ($p > 0.05$) (Figure 2). The results of the average alpha-amino nitrogen (µg/mL) level in T0, T1, and T2 groups are presented in Table 10. The nonsignificant results ($p > 0.05$) were obtained for different fortnight as well as overall average. The results of RBC, WBC, and haemoglobin content in different groups are given in Table 10 and these values were nonsignificant ($p > 0.05$) to each other.

4. Discussion

4.1. Effect of Azolla supplementation on growth performance

The highest ADG was observed in T2 followed by T1 and least in T0. The reason is due to higher CP digestibility of *Azolla pinnata* along with the higher metabolisable protein that is around 84% that implies the capability of Azolla as a protein supplement [16]. Essential amino acids such as leucine, tryptophan, methionine, lysine, etc., are present in *Azolla pinnata* as per the study. These amino acids play an important role in activating protein synthesis and retarding the proteolysis. Thus, they act as an ideal protein source and contribute positively to body growth and hence better growth was seen in Azolla fed groups.

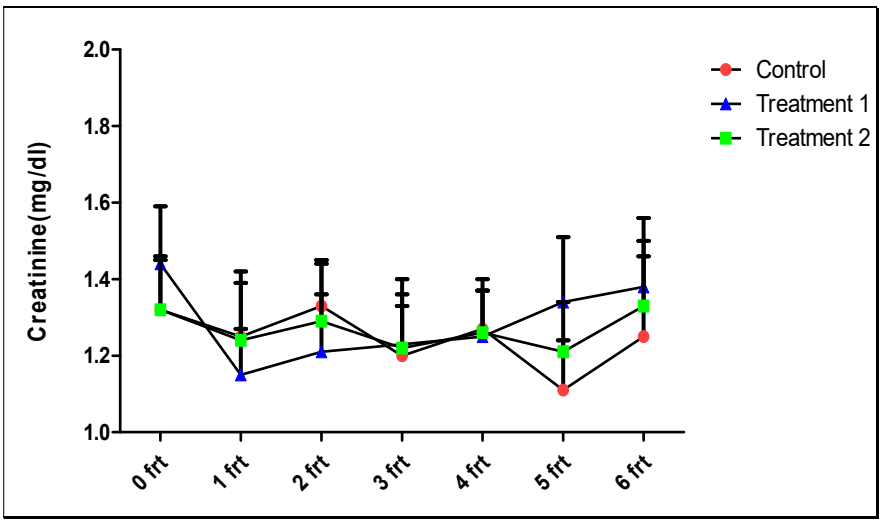

Figure 1. Creatine level at different fortnights ($p < 0.05$). **X axis:** fortnights (frt); **Yaxis:** creatinine level at different fortnights.

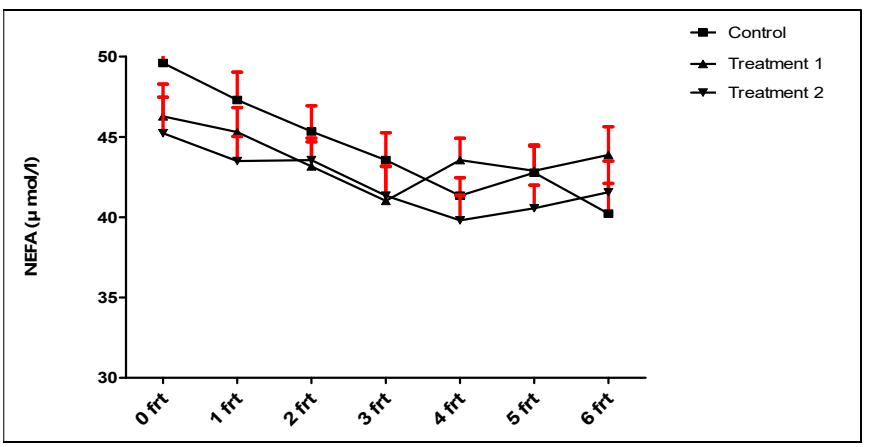

Figure 2. NEFA levels at different fortnights ($p < 0.05$). **X axis:** fortnights (frt); **Y axis:** NEFA level at different fortnights.

4.2. Effect of Azolla supplementation on physiological parameters

Due to the presence of such an excellent mineral profiling (Ca, P, K, Mg as 1.16%, 1.29%, 1.25%, 0.35% in DM %, respectively), the essential amino acids and minerals might be responsible for keeping the physiological parameters under normal range which in turn are represented in Azolla fed groups.

4.3. Effect of Azolla supplementation on serum hormonal indices

Leptin hormone is the major regulator for the amount of feed intake and energy balance. Leptin is the result of the obesity (ob) gene [17] that controls feeding behaviour and energy consumption. Leptin works directly on the bovine pituitary and induces GH and PRL secretion [18]. In our study, also leptin might have induced GH secretion and hence significant growth rate in T2 groups. The average IGF-1 level showed significant difference between T0, T1, and T2 groups [19]. The source of growth hormone is anterior pituitary gland, from where it is released into the bloodstream and stimulated to produce IGF-1 in the liver [20]. IGF-1 then stimulates systemic body development and has growth promoting impacts on nearly every body cell, particularly skeletal muscle, cartilage, bone, skin, and haematopoietic cells. The dietary increase in protein intake will stimulate IGF-1 level production, and this is not dependent on calorie consumption [21]. Thus, in T1 and T2 groups, there was a higher growth rate due to better IGF-1 production. IGF-1 is a vital growth hormone, facilitating the protein anabolism and linear growth [22]. Thus, it exhibits that *Azolla pinnata* have some anabolic effects. The growth hormone level (GH) was nonsignificant among all the three groups except at the 4th fortnight. Chandra et al. [23] have seen a significant difference with Vitamin E supplementation in GH level. However, Khare et al. [24] have obtained nonsignificant results in between Azolla and control groups**.** GH plays an important role in body growth of cattle [25]. Thus, Azolla feeding might have triggered GH release and provided better growth in treatment groups. T3 and T4 levels were nonsignificant among all the three groups throughout the experiment. T_3 and T_4 are known thermoregulators in animals, involved in nutritional and environmental metabolic activities of animals. These hormones regulate the energy balance and protein metabolism, thermoregulation [26]. Thus, the normal range of these hormones is very important for maintaining the proper basal metabolic rate and as well proper functioning of various vital functions of body. In our study, alsoAzolla have maintained them within the normal range and hence contributing to homeostasis and positive energy balance. Similarly, Khare et al. [24] and Chandra et al. [23] also reported a normal range of T3 and T4 levels in their experiments.

4.4. Effect of Azolla supplementation on serum biochemical indices

It was observed that there was no adverse effect of Azolla supplementation on the haematological parameters of the Sahiwal calves [5,24]. Since Azolla does not have any antinutritional factors with better mineral profiling and antioxidative property [27], all these haematological values were under the normal range. The glucose level (mg/dL) was nonsignificant among all the groups. Similar results were obtained [5,24]. Glucose is the main indicator of energy. There is an increase in the glucose level with the time due to the fact that there is higher growth rate of animals as the experiment proceeds. Higher energy demand due to faster growth rate with time may had caused the potential triggering for release of hepatic glucose, which in turn is transformed into energy-intensive acetyl coenzyme A and is used by the calves as the energy source for growth [28]. The albumin level was nonsignificant and similar results were obtained by Bhatt et al. [5]. There is a reduction in NEFA level as the age of animal progressed but not a significant one [29]. The average plasma protein level (g/dL) was nonsignificant and similar results were obtained in crossbreed calves through Azolla feeding [24]. It was found that *Azolla pinnata* feeding maintained the total plasma protein under the normal range [3]. The level of BUN was nonsignificant among all the groups. Similar nonsignificant results were obtained by Dudi and Datt [19]. The overall average of plasma creatinine level (mg/dL) was nonsignificant to each other. Similar results were obtained by Roy et al. [30]. Creatinine is the key indicator of kidney function and also represents the glomerular filtration rate [31]. As the values are under normal state, it does not contribute to any harmful effects to kidney. Therefore, incorporation of *Azolla pinnata* is safe with regard to kidney functions and does not hold any adverse effects.

5. Conclusion

From the present research, it may be concluded that crude protein content of concentrate can be replaced by *Azolla pinnata* at 15% and 30% level without any adverse effects by maintaining the concentration of various critical physiological, biochemical, and hormonal profiles up to the basal levels. Azolla was also seen to influence IGF-1 and GH hormone activity, leading to better growth of calves. Hence *Azolla pinnata* can be used as a novel feed for protein replacement in nearby future to bridge the demand and supply gap for nutrients and for providing better productivity.

Animal care

All animal experiments were carried out in accordance with the existing standard of the Institutional Animal Ethics Committee, established by the Ministry of Animal

Husbandry, Dairying and Fisheries, GOI, pursuant to article number 13 of the CPCSEA rules. All persons gave their informed consent prior to their inclusion in the study.

Acknowledgements
The authors are highly grateful to ICAR – National Dairy Research Institute, Karnal (Haryana), India for providing financial assistance.

Conflict of interest
The authors declare no conflict of interest and certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

1. Roy A, Agarwal R, Bhardwaj N, Mishra A, Mahanta SK. Revisiting National Forage Demand and Availability Scenario 2019.
2. Beigh YA, Ganai A, Rather MA. Livestock feed resources availability, feeding practices, and nutrient balances in high Himalaya (Gurez) valley of Kashmir. Tropical Animal Health Production 2020; doi: 10.1007/s11250-020-02273
3. Shukla M, Bhattacharyya A, Shukla PK, Roy D, Yadav B, Sirohi R. Effect of Azolla feeding on the growth, feed conversion ratio, blood biochemical attributes and immune competence traits of growing turkeys, Veterinary World 2018; 11 (4): 459-463.
4. Chatterjee A, Sharma P, Ghosh MK, Mandal M, Roy PK. Utilization of Azolla Michrophylla as feed supplement for crossbred cattle. International Journal of Agriculture and Food Science and Technology 2013; 4 (3): 207-214.
5. Bhatt N, Chandra, R, Chikkagoudara, KP, Tyagi N, Mishra, DB. Effect of Azolla pinnata Feeding on Linear Type Traits and its Relation to Economic Feasibility in Sahiwal Calves. Asian Journal of Dairy and Food Research 2022; doi:: 10.18805/ajdfr. DR-1853
6. Bhatt N, Chandra R, Kumar S, Singh K, Singh NP, Rajneesh. Nutritive Analysis of Azolla pinnata and its Cultivation during Winter Season. International Journal of Current Microbiology and Applied Science 2020; 9 (3): 2012-2018.
7. Bush RS, Nicholson JWG. The effects of weaning schedule, duration of milk feeding and fishmeal on calf performance. Canadian Journal of Animal Sciences 1986; 66: 691-698.
8. Baldwin RL, McLeod VI, KR Klotz, JL, Heitmann RN. Rumen development, intestinal growth and hepatic metabolism in the pre- and post-weaning ruminant. Journal of Dairy Science 2004; 87 (E Suppl.): E55-E65.
9. Khan MA, Weary DM, Keyserlingk MAG. Hay intake improves performance and rumen development of calves fed higher quantities of milk. Journal of Dairy Science 2011; 94: 3547-3553.
10. Lee H, Khan Aj, Lee W, Yang S, Kim S et al. Influence of equalizing the gross composition of milk replacer to that of whole milk on the performance of Holstein calves. Journal of Animal Science 2008; 87: 1129-37. doi: 10.2527/jas. 2008-1110
11. Khan MA. Starch source evaluation in calf starter: I. Feed consumption, body weight gain, structural growth, and blood metabolites in Holstein calves. Journal of Dairy Science 2007; 90 (11): 5259-5268.
12. Acuti G, Todini L, Malfatti A, Antonini M, Barbato O et al. Effects of field bean (Vicia faba L. var. minor) dietary supplementation on plasma thyroid hormones, insulin, insulin-like growth factor-1 concentrations and mohair characteristics in growing Angora goat kids. Journal of Animal Physiology and Animal Nutrition 2008; 93. 456-66. doi: 10.1111/j.1439-0396.2008. 00827.x
13. Ndlovu T, Chimonyo M, Okoh AI, Muchenje V, Dzama K et al. Assessing the nutritional status of beef cattle: current practices and future prospects. African Journal of Biotechnology 2007; 6: 27-34.
14. Hassan MM, Hoque MA, Islam SA, Khan SA, Hossain MB et al. Efficiency of anthelmintic against parasitic infections and their treatment effect on production and blood indices in Black Bengal goats in Bangladesh. Turkish Journal of Veterinary & Animal Sciences 2012; 30: 400-408.
15. AOAC. Association of Official Analytical Chemists, Official Methods of Analysis. 18th Edition., Maryland, USA 2005.
16. Parashuramulu S, Swain PS, Nagalakshmi D. Protein fractionation and in vitro digestibility of Azolla in ruminants. Online Journal of Animal and Feed Research 2013; 3 (3): 129-132 .
17. Schwartz MW, Woods SC, Porte DJ, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature 2000; 404: 661-671.
18. Accorsi PA, Munno A, Gamberoni M, Viggiani R De, Ambrogi, M et al. Role of Leptin on Growth Hormone and Prolactin Secretion by Bovine Pituitary Explants. Journal of Dairy Science 2007; 90 (4): 1683-1691. doi:10.3168/jds. 2006-611
19. Dudi K, Datt C. Relationship of residual feed intake with blood metabolites and hormones in Sahiwal female calves. Forage Reserach 2015; 41: 40-45.
20. Laron Z. The somatostatin-GHRH-GH-IGF-I axis. *Growth hormone, IGF-I and growth: new views of old concepts. Modern endocrinology and diabetes* 1996; Vol. 4. London-Tel Aviv: Freund Publishing House Ltd, 5-10.
21. Levine ME, Suarez JA, Brandhorst S, Balasubramanian P, Cheng CW et al. Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. Cell Metabolism 2014; 19 (3): 407-417.

22. Laron Z. Insulin-like growth factor 1 (IGF-1): a growth hormone. Molecular Pathology 2001; 54 (53): 11-16.

23. Chandra A. Prevalence of hypothyroidism in patients with chronic kidney disease: A cross-sectional study from North India. Kidney Research Clinical Practice 2016; 35: 165-168.

24. Khare A, Chatterjee A, Mondal M, Karunakaran M, Ghosh MK et al. Effect of Supplementing Azolla microphylla on Feed Intake and Blood Parameters in Growing Crossbred Female Calves. Indian Journal of Animal Nutrition 2016; 33. 224. doi: 10.5958/2231-6744.2016.00039.6

25. Etherton TD, Bauman DE. Biology of somatotropin in growth and lactation of domestic animals. Physiological Reviews1998; 78: 745-761.

26. Kim B. Thyroid Hormone as a Determinant of Energy Expenditure and the Basal Metabolic Rate. Thyroid 2008; 18: 141-144.

27. Katayamaa N, Yamashitab Mi, Kishidac Yo, Chung-Chu L, Watanabee I et al. Azolla as a component of the space diet during habitation on Mars. Acta Astronautica 2008; 63: 1093-1099.

28. Trefts, E. Exercise and the Regulation of Hepatic Metabolism. Progress in Molecular Biology and Translational Science 2015; 135: 203-25.

29. Mondal M, Prakash B. Changes in plasma non-esterified fatty acids, glucose and α-amino nitrogen and their relationship with body weight and plasma growth hormone in growing buffaloes (Bubalus bubalis). Journal of Animal Physiology and Animal Nutrition 2004; 88: 223-228. doi: 10.1111/j.1439-0396.2004.00476.x

30. Roy D, Kumar V, Kumar M, Sirohi R, Singh Y et al. Effect of feeding Azolla pinnata on growth performance, feed intake, nutrient digestibility and blood biochemical's of Hariana heifers fed on roughage-based diet. Indian Journal of Dairy Science 2015; 69.

31. Gowda S, Desai PB, Kulkarni SS, Hull VV, Math AA et al. Markers of renal function tests. North American Journal of Medical Sciences 2010; 2 (4): 170-173.