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

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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). However, 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. 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.

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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 (T₀) was a control group and was fed as per ICAR (2013) for calf. Second group (T₁) was fed as per control by replacing the 15% protein content of the concentrate with the Azolla pinnata and third group (T₂) 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

| 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.
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.
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. MBS455080). 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
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% H2SO4 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:

\[
\text{Average daily dry matter intake} = A - B
\]

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

where

\begin{align*}
A &= \text{average dry matter offered daily}, \\
B &= \text{average dry matter refused daily}, \\
C &= \text{average dry matter voided in faeces daily.}
\end{align*}

The digestibility of other nutrients were calculated as follows:

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

where

\begin{align*}
A &= \text{total intake of the particular nutrient through feeds and fodder}, \\
B &= \text{out go of the particular nutrient through faeces.}
\end{align*}

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 (T0, T1, and T2) 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 ± 0.18, 3.33 ± 0.18, and 3.33 ± 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 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 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) between T0 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 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).
### 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.
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 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.
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. T3 and T4, 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, also Azolla 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 and similar results were obtained in crossbreed calves through Azolla feeding [24]. It was observed that there was no adverse effect of Azolla pinnata feeding maintained the total plasma protein under the normal range. The glucose level (mg/dL) was nonsignificant and similar results were obtained by Dudi and Datt [19]. 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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.

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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.

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