

NUTRIGENOMICS EFFECT OF AZOLLA SUPPLEMENTATION ON MILK PRODUCTION AND DGAT1, SCD1 EXPRESSION IN DAIRY CATTLE OF KORAPUT DISTRICT, ODISHA

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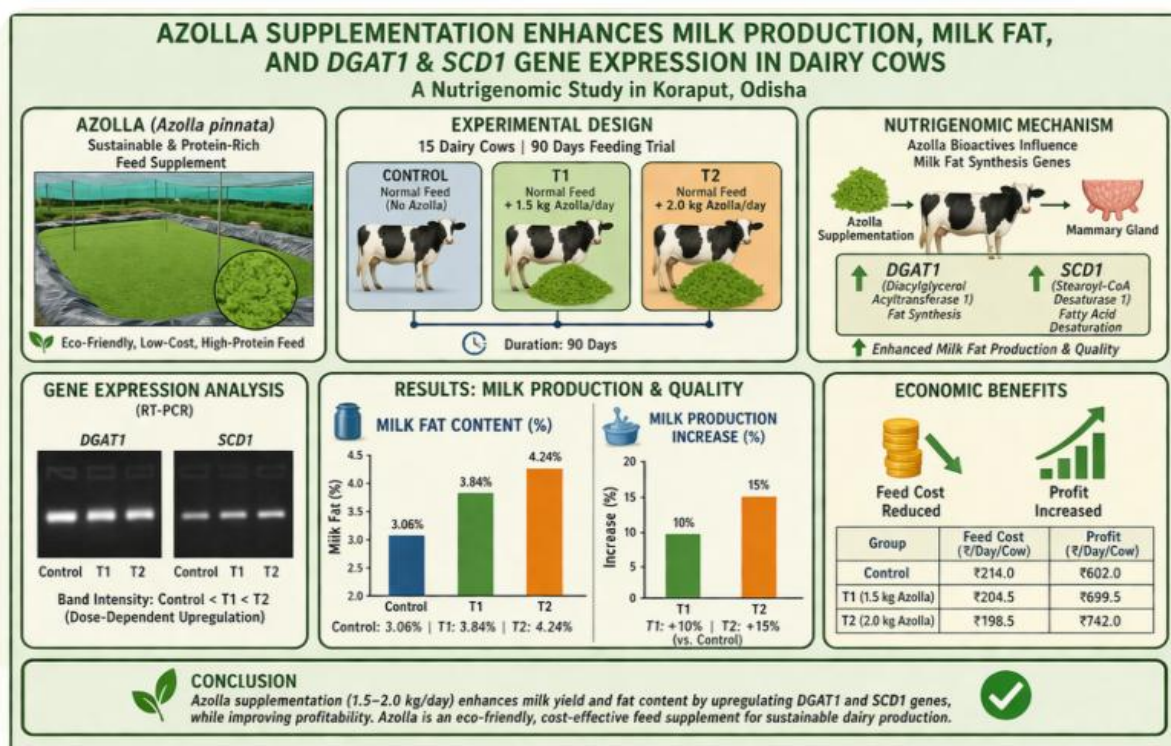
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ABSTRACT

This study examined the impact of Azolla supplement on milk yield, quality, and gene expression in dairy cattle in Koraput, Odisha. Fifteen cattle were distributed into three categories such that a control group and two treatment groups were provided with Azolla supplementation in doses of 1.5 kg/day (T1) and 2.0 kg/day (T2) for 90 days. It was found that there was a marked increase in efficiency and milk content. Milk fat percent increased significantly from 3.04% to 3.84% in T1 and from 3.06% to 4.24% in T2, whereas there was no change in the control group. Likewise, milk yield was increased by 10% in T1 and 15% in T2. Economically, there was profit increase from ₹699.5 in T1 and ₹742 in T2 to ₹602 in the control. Genetically, there was an increase in DGAT1 and SCD1 gene expression.

KEYWORDS: Azolla, Dairy cattle, DGAT1 and SCD1 genes, Feed cost, Milk fat, Milk yield, Nutrigenomic



1. INTRODUCTION

Dairy farming is an important contributor to the rural economy in India. It provides employment and income to millions of people. It also provides nutrients to the population. However, one of the major problems faced by dairy farmers in India is the high cost of cattle feed. Feed costs contribute to almost 60-70% of the total cost of milk production. This reduces the profit for the farmers. Thus, finding alternative and cost-effective options to traditional cattle feed is important. *Azolla*, a small aquatic floating fern, has been recognized as an alternative to traditional cattle feed. It grows quickly in aquatic environments (Yadav *et al.*, 2023). These aquatic floating ferns grow easily in small pits and tanks with limited resources. Henceforth, it is highly suitable for rural zones. *Azolla* is rich in

numerous nutrients like protein, amino acids, vitamins A and B12 and certain minerals like calcium, phosphorous and iron. The amount of protein content of these floating micro plants varies between 20-35%. The major advantage of *Azolla* is that it contains very low amount of lignin the organic polymer which makes easy to digest by cattle which leads to an in nutrient uptake of the cattle. Earlier research shows an increment in the quality of milk yield and cost of cattle feed has been significantly changed by using *Azolla* based treatment. Considering its advantages, *Azolla* is being advocated as a rural dairy feed option (Singh *et al.*, 2023; Kour and Manpreet, 2020). This feed option is eco-friendly, cost-effective, and sustainable. By adopting *Azolla*, not only will rural farmers benefit, but it will also promote environmentally sustainable agriculture (Pillai *et al.*, 2002). With respect to the benefits to the beneficiaries, the farmers who have taken up *Azolla* cultivation have benefited in terms of reduced costs for feed, increased milk production, and better profitability (Mathur *et al.*, 2013; Kerketta *et al.*, 2020; Lumpkin and Plucknett, 1980). Moreover, *Azolla* cultivation is also an environmentally friendly practice, as it requires lesser land and water and promotes sustainable farming practices (Tamizhkumaran and Rao, 2012). It also provides empowerment to rural households by helping them reduce the cost of feed production. Hence, to study the impact of *Azolla* as a substitute for feed in dairy farming in terms of cost. To study the impact on the quantity and quality of milk produced by the cows (Kumar 2025; Gouri *et al.*, 2012). To study the economic benefits and profitability to rural dairy farmers. To study the level of awareness and adoption of *Azolla* technology by the beneficiaries. To study the environmental benefits and sustainability of *Azolla* cultivation. Overall, *Azolla* seems to be a very promising and sustainable practice that provides benefits to rural dairy farmers while also being an environmentally friendly practice.

METHODOLOGY

The impact of the supplementation of *Azolla* on the quality and quantity of milk in bovines was assessed in the present study. The experiment was conducted in Tuna Dairy Farm, Semiliguda block, Koraput, for a period of 90 days from September to November 2024. The milk production parameters were monitored to assess the impact of the supplementation of *Azolla*. A total of 15 hybrid cows, with an average weight of 500 ± 10 kg and average milk yield of 13 ± 0.55 kg/day, were selected from a dairy farm (Chatterjee *et al.*, 2013, Gowda *et al.*, 2015). *Azolla* cultivation setup demonstrating stepwise methodology for *Azolla* cultivation in artificial ponds for the present experiment with polythene-lined pond and shade net protection has been shown in Figure 1. The selected cows in the present study were randomly divided into three groups, i.e., five cows in each group ($N = 5$), to ensure uniformity in terms of weight and lactation performance. There were three groups: one was a comparison group (C), and two were experimental groups (T1 and T2). The experiment was conducted for a period of 90 days to obtain appropriate results. The cows in group C were given 5.0 kg of commercial concentrate feed along with 3.0 kg of dry roughage. On the other hand, cows in group T1 were given 3.5 kg of commercial concentrate feed, 3.0 kg of dry roughage, and 1.5 kg of fresh *Azolla*. Similarly, cows in group T2 were given 3.0 kg of commercial concentrate feed, 3.0 kg of dry roughage, and 2.0 kg of fresh *Azolla* as mentioned in Table 1 and 2. The chemical composition of both commercial concentrate and *Azolla* was analysed and presented in Table 1, while the ingredient composition of the concentrate feed, as per Singh *et al.* (2023), was shown in Table 2. Milk samples were collected at regular intervals to monitor the quality parameters, such as fat percentage, which was recorded every five days, as shown in Table 3, according to Kour & Manpreet (2020). Furthermore, an economic analysis was presented in Table 4, comparing feed costs and income from milk production before and after the addition of *Azolla*.



Figure 1: Scientifically managed *Azolla* culture units with polythene-lined pond & shade net protection

Azolla Cultivation Techniques and Its Nutritional Significance in Dairy Farming

Azolla was cultivated in small water-filled pits lined with polythene sheets and provided with partial shade to enhance their growth rate. Cow dung slurry and super phosphate were added to enhance their growth rate. Fresh *Azolla* was harvested daily and fed to the cattle directly after washing them to remove impurities from their bodies. Similarly, the proximate composition of *Azolla* indicating its nutritional value has also consider in the present study. It has moderate moisture content (15%) and high crude protein content (24.46%) (Anitha *et al.*, 2016) that indicates *Azolla* is a good source of protein. However, report also shows that the extract has low content of either extract (2.25%) showing less fat content (Yadav *et al.*, 2023) as mentioned in Table 3 and 4. The crude fibre content (8.34%) is also helpful for digestion. The total ash content (3.82%) shows the mineral content.

Table:1 *Azolla* Feeding Schedule

Group	Treatment details
Control (n=5)	5.0 Kg Concentrate + 3.0 Kg Roughage
T 1 (n=5)	1.5 Kg <i>Azolla</i> + 3.5 Kg Concentrate + 3.0 Kg Roughage
T 2 (n=5)	2.0 Kg <i>Azolla</i> + 3.0 Kg Concentrate + 3.0 Kg Roughage

The culture of *Azolla* was carried out using a simple and low-cost method, which is suitable for a rural environment. The culture was done in shallow pits filled with water and lined with polythene sheets to prevent seepage of water, thus providing a favourable environment for the culture of *Azolla*. The culture units were also provided with partial shading to protect *Azolla* from the effects of direct sunlight, which can inhibit the growth of *Azolla* and reduce its productivity (Meena *et al.*, 2017).

Table: 2 Experimental Design and Feeding Regimen

Gr	T	No.A	BW	AMY	CF	DR	AZ
Control	NA	5	500± 10	13±0.55	5	3	Nil
T1	PRA	5	500± 10	13±0.55	3.5	3	1.5
T2	HRA	5	500± 10	13±0.55	3.0	3	2

T: Treatments; No. A: No of Animals; BW: Body Weight; AMY: Amylase Activity; CF: Crude Fiber Digestibility; DR: Digestibility Rate; AZ: *Azolla* Level

To promote the growth and nutritional content of *Azolla*, a nutrient medium was provided by adding cow dung slurry and a small amount of superphosphate to the water. However, a layout of the *Azolla* cultivation setup demonstrating stepwise methodology for *Azolla* cultivation in artificial ponds has been illustrated in Figure 2. These nutrients are essential to provide a favourable environment for the rapid multiplication of *Azolla*, which grows rapidly under favourable conditions to form a thick mat on the water surface (Regar and Jat 2020). Fresh *Azolla* was collected every day to provide a constant source and to avoid overcrowding, which may hinder the growth of the plants. After the collection of the *Azolla* plants, they were cleaned by washing with fresh water to eliminate impurities such as dirt, extra nutrients, and odor from the culture medium.

Table 3: Composition of Concentrate Mixture (Yadav *et al.*, 2023)

Ingredient Type	Ingredients	Approximate %
Energy Feeds	Maize, Barley, Cereals	75%
Protein Feeds	Soyabean meal, Cottonseed cake	30%
By products	Wheat bran, Molasses, Soy hulls	25%
Minerals/Vitamins	Salt, Dicalcium phosphate mix	5%

Finally, the cleaned *Azolla* plants were fed directly to the cattle in their fresh form. This approach was effective in ensuring that the cattle were fed a nutrient-rich and easily digestible feedstuff that was also cost-effective. The cultivation of *Azolla* was done by utilizing available materials at a low cost. This makes the project suitable for implementation in rural areas. This project started with the preparation of a suitable cultivation structure. A shallow pit or a tank was selected for *Azolla* cultivation. A hard polythene sheet or a silpaulin was placed inside the pit to avoid seepage of water. This helps in maintaining a constant aquatic environment. This polythene sheet *also* aids in easy maintenance of *Azolla* cultivation.

Table: 4 Chemical Composition of Concentrate vs *Azolla* (Kour *et al.*, 2020)

Component	Crude Protein	Ether Extract	Total Ash (%)	NDF (%)	ADF (%)
Concentrate Feed	22	4.2	8.00	33.3	17.3
<i>Azolla</i>	21.7	2.8	16.1	63.1	34.8

A frame was prepared to provide a suitable structure. This was done by placing thin bamboo sticks or PVC pipes. A shade net was placed over the frame. This helps in maintaining a partial shade condition. This is necessary since *Azolla* grows well in a moderate light condition. Excessive exposure to sunlight may affect *Azolla* growth. A shade net helps to avoid disturbances from outside factors such as birds, fish, etc. The subsequent phases were to generate a nutrient rich substratum by distributing a slim coat of fertile soil with a complexity of around 10-15 cm lined with polyethylene sheets. Afterward the mixture of fresh cow dung and water was allowed to poured on top of the soil, followed by the addition of Single Super Phosphate which serves as a nutrient rich material which provides ambient condition for the rapid growth of *Azolla*.



Figure 2: *Azolla* cultivation setup demonstrating stepwise methodology for *Azolla* cultivation in artificial ponds. Subsequently, fresh water was transferred into the pits in a depth of 5-10 cm. This ambient condition provides better growth for the floating Fern *Azolla*. After complete preparation of the bed, for maintaining a good *Azolla* culture, some fresh floating samples were poured in a uniform manner to the surface. *Azolla* started multiplying its number rapidly in a matter of 7 days. Regular maintenance was performed for maintaining a constant supply of nutrient to the medium. The prepared setup with its management for *Azolla* Pond for the present experiment has been shown in Figure 3.

The farming of *Azolla* was supported out in a systematically controlled manner to acquire finest results in their progress and production. A total of 10 artificial ponds were formed in a moderately shaded zone, preferably under tree cover, as *Azolla* requires 30% sunlight to carry its optimal growth. Afterward each pond was excavated to a depth of 20 cm and covered with a polythene sheet to prevent seepage of water and to maintain a stable aquatic environment. A layer of fertile soil was placed on the top of the polythene sheet which served as a nutrient base for efficient growth of the plants, for a standard pond size of 2x 2 meters, 5-10 kg of soil was applied and replenished monthly. In order to improve the availability of nutrients, cow dung slurry was added to the ponds. This is a mixture of 2kg of cow dung with water, which is then left to decompose for 2 days. This serves as a nutrient medium for the availability of nutrients to the plants. This is replenished every 5 – 7 days for optimal growth conditions. In addition to that, 10 kg of Single Super Phosphate (SSP) is also added regularly (after every 5-7 days) to serve as a phosphorus source for the efficient growth of *Azolla*, as phosphorus is a key element for the rapid multiplication of *Azolla* and for the nitrogen fixation process mediated by the cyanobacterium *Anabaena Azollae*. The fresh inoculum of *Azolla* (250-300 g) was added to the pond for the growth of the crop. Water depth was maintained at a level of 10-15 cm with a water change of 25% after every 10 days for the maintenance of water quality.

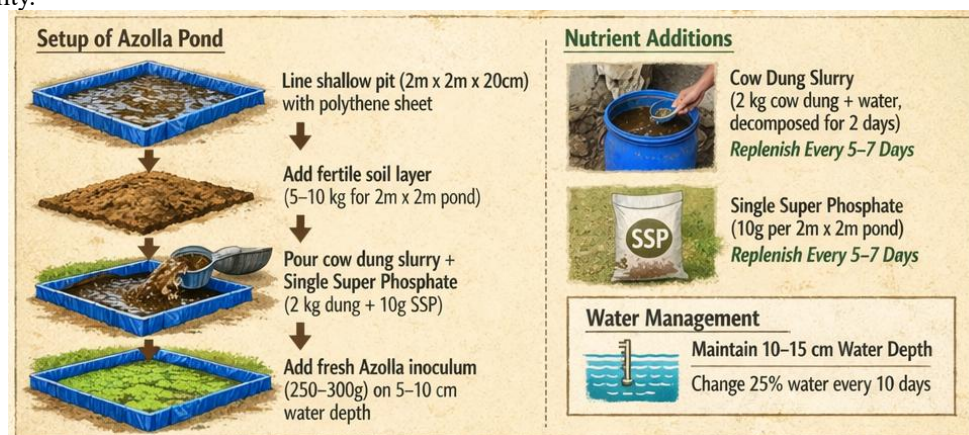


Figure 3: Setup and Management of *Azolla* Pond

Nutrigenomic Effects of *Azolla* Supplementation on Milk Production:

The present study, which showed that milk yield and fat content increase through *Azolla* supplementation, can be further explained at the molecular and genetic levels through the integration of nutrition and gene expression in dairy cattle. *Azolla* is known to be rich in proteins, essential amino acids, vitamins, and minerals that play a significant role as nutritional modulators in gene expression. These nutritional elements play an important role in regulating important metabolic and lactation-related genes in dairy cattle. These include genes that encode for: Milk fat synthesis through DGAT1 (Diacylglycerol O-acyltransferase 1) gene and SCD1 (Stearoyl-CoA desaturase) gene for milk fat synthesis in mammary glands. The increase in milk fat content (from 3.04% to 3.84%, 4.24%) may be due to upregulation of these genes, and also Milk protein synthesis by Casein gene expression (CSN1S1, CSN2) may be positively affected by improved protein intake from *Azolla*. Regulation of lactation:

Hormonal regulation involving PRLR (Prolactin Receptor) and GH (Growth Hormone) gene expression may be indirectly upregulated, thereby increasing milk. To understand the molecular basis of the improved milk fat content in the supplemented groups, an additional molecular approach involving the DGAT1 and SCD1 genes were included in the present study design. Blood samples from all the groups (Control, T1, and T2) and milk samples from all the groups (Control, T1, and T2) were collected during the beginning and end of the 90-day experiment. Genomic DNA samples were isolated from the blood samples of all the groups using standard DNA isolation protocols. The DGAT1 and SCD1 genes' DNA sequences were amplified using PCR and subjected to sequencing reactions. The sequence data were analyzed using bioinformatics tools such as MEGA software to construct phylogenetic trees using the Neighbor-Joining method. Reference gene sequences of different cattle breeds and other related ruminant species, such as buffalo, goat, and sheep, were downloaded from the databases such as GenBank of the National Center for Biotechnology Information (NCBI) and subjected to sequence alignment to identify the regions of conservation and variations in the sequence. Particular attention is given to the functional polymorphisms of the DGAT1 gene, such as the K232A polymorphism.

RESULTS AND DISCUSSION:

Impact of *Azolla* Feeding on Dairy Productivity, Milk Quality, and Profitability in Cattle

The findings of the present study clearly indicate the positive effect of *Azolla* supplementation on the quality and quantity of milk production in dairy cows. The average milk fat percentage before *Azolla* supplementation was almost similar in all the treatment groups, i.e., 2.98% (Control), 3.04% (T1), and 3.06% (T2). However, after supplementation, there was a significant increase in milk fat percentage in both T1 and T2, i.e., 3.84% and 4.24%, respectively, whereas the control group showed no significant increase, i.e., 2.98%. Thus, *Azolla* supplementation improves milk quality. Regarding milk production, all the treatment groups produced an average milk production of 13 liters/day before *Azolla* supplementation. After supplementation, T1 showed an increase in milk production to 14 liters/day (10% increase), whereas T2 showed an increase to 14.5 liters/day (15% increase), whereas the control group did not show any increase in milk production. In terms of economics, the feed cost was reduced from ₹128 to ₹90 in T1 and to ₹78 in T2 due to supplementation, and profit increased from ₹602 to ₹699.5 in T1 and to ₹742 in T2 which has been discussed in Table 5 and 6. Overall, the findings confirm that the supplementation of *Azolla* resulted in increased milk production, higher milk fat content, lower feed costs, and higher profitability, with the highest benefits accruing in the T2 group. As like that, the milk yield (or parameter) for before and after treatment with comparing to Control, T1, and T2 groups were presented has been can be mentioned in the table 5. It has observed that after treatment, T1 and T2 have improved significantly, whereas the control remains unchanged, indicating the positive effect of the treatment, especially on the level of *Azolla* inclusion.

Table 5: Average Milk Fat (%) Before and After *Azolla* Feeding

Cow No.	Before Treatment			After Treatment		
	Control	T1	T2	Control	T1	T2
1	3.1	3.2	3.1	3.0	3.8	4.1
2	3.0	3.0	3.1	3.0	3.9	4.2
3	3.1	3.0	2.9	3.2	4.1	4.5
4	2.7	3.2	2.9	2.8	4.2	3.8
5	3.1	2.8	3.2	2.9	4.2	4.6
Avg	2.98	3.04	3.06	2.98	3.84	4.24

The milk fat percentage of the groups the Control, T1, and T2 groups before and after treatment with $\pm$ SD has been mentioned and discussed in Figure 3. The milk fat percentage of the groups before treatment is relatively close to each other at around 3%. However, the milk fat percentage of the groups after treatment is different. The milk fat percentage of the Control group is relatively the same as before, showing no changes. On the other hand, the milk fat percentage of the T1 and T2 groups has greatly improved, especially the latter, which has a milk fat percentage of over 4% as shown in Table 4, 5 and Figure 4. The error bars show minimal variability. The graph clearly shows that the treatment, especially the higher *Azolla* percentage of the T2 group, greatly improves the milk fat percentage compared to the Control group.

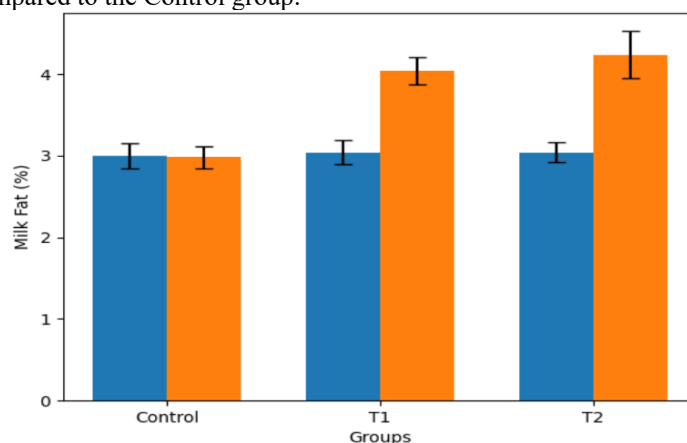


Figure 4: Statistical representation of milk fat % before and after treatment

The dairy performance prior to and after *Azolla* supplementation has been elucidated in Table 6. Before supplementation, all dairy groups had the same feeding pattern, cost (₹128), milk yield (13 L/day), and profit (₹602). No differences were observed. After supplementation, the control group remained the same. However, T1 (1.5 kg *Azolla*) recorded a reduced feed cost of ₹90, increased milk yield of 14 L/day (10%), and increased profit to ₹699.5. T2 (2 kg *Azolla*) recorded a lower feed cost of ₹78, increased milk yield of 15 L/day (15%), and recorded the highest profit of ₹742 as mentioned in Table 6 and Figure 5. *Azolla* supplementation greatly improved dairy performance, particularly T2.

Table 6: Economic Analysis Before and After *Azolla* Feeding

Particulars	Before Treatment			After Treatment		
	Control	T1	T2	Control	T1	T2
Feeding Regime (Kg)	0 + 5 + 3	0 + 5 + 3	0 + 5 + 3	0 + 5 + 3	1.5 + 3.5 + 3	2 + 5 + 3
Feed Cost (₹)	128	128	128	128	90	78
Milk Yield (L/Day)	13	13	13	13	14	15
% IMY	0	0	0	0	10	15
Accessory cost (₹)	50	50	50	50	50	50
Revenue (60/L)	780	780	780	780	840	870
Total Profit (₹)	602	602	602	602	699.5	742

IMY: Increase Milk Yield, T1: Treatment 1; T2: Treatment 2

Similarly, the performance comparison between the Control, T1, and T2 groups before and after the *Azolla* treatment has been mentioned in Figure 4. In the first pie chart, the performance before the treatment is shown. In this case, the performance of the Control, T1, and T2 groups is almost the same, at 33% and 34%, respectively. This shows that the groups were producing the same amount of milk before the treatment. In the second pie chart, the performance after the treatment is shown. In this case, the performance of the T2 group has increased to 42%, while the performance of the Control and T1 groups remains the same at 34%. This shows that the *Azolla* treatment enhances productivity, especially for the T2 group.

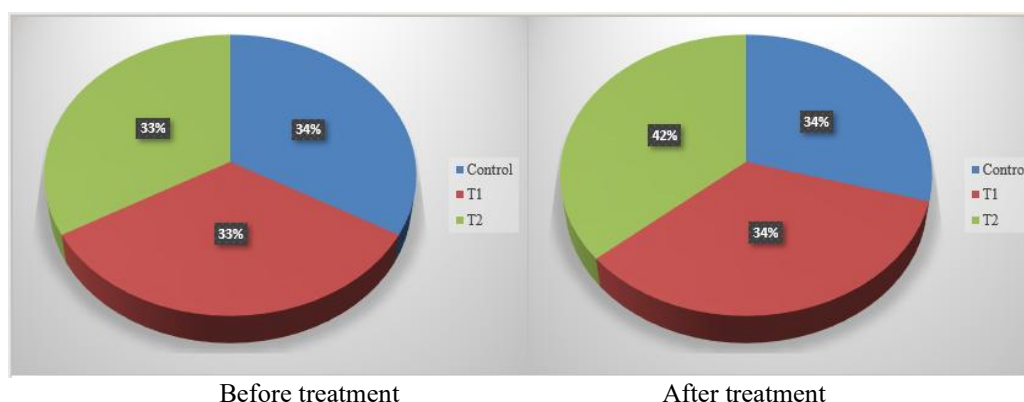


Figure 5: Comparative analysis of Dairy performance before and after *Azolla* supplementation

The economic benefits of *Azolla* feed are shown in the Table 7 and Figure 5. The control group, which was not fed *Azolla*, had a feed cost of Rs. 128, milk production of 13 L/day, and a profit of Rs. 602. The T1 group, which was fed 1.5 kg of *Azolla*, had a feed cost of Rs. 90, milk production of 14 L/day (10%), and a profit of Rs. 699.5. The T2 group, which was fed 2 kg of *Azolla*, had a feed cost of Rs. 78, milk production of 14.5 L/day (15%), and a profit of Rs. 742. The use of *Azolla* as feed is profitable because it decreases feed cost and increases milk production.

Table 7: Economic Analysis After 90 Days *Azolla* Feeding

Particulars	Control	T1	T2
Feeding Regime	0 + 5 + 3 Kg	1.5 + 3.5 + 3 Kg	2 + 3 + 3 Kg
Feed cost (₹)	128	90	78
Milk Yield (L/Day)	13	14	14.5
% Increase	0%	10%	15%
Accessory cost (₹)	50	50	50
Revenue (₹60/L)	780	840	870
Profit (₹)	602	699.5	742

Unlikely, a summary of the comparative results of the supplementation of *Azolla* on dairy performance has been mentioned in Table 8. In the control group, the fat content of the milk was constant at 2.98%, as were the levels of milk yield, which remained constant at 13 L/day. In T1, the fat content increased from 3.04% to 3.84%, and the levels of milk yield increased from 13 L/day to 14 L/day, showing a moderate improvement with a decrease in feed cost and an increase in profit by about Rs 97.5. In T2, the fat content increased from 3.06% to 4.24%, and the levels of milk yield increased from 13 L/day to 14.5 L/day, showing the best improvement in the performance of the dairy cows. The reduction in feed cost was also the maximum in T2, showing an increase in profit by about Rs 140.

Table: 8 Overall Impact of *Azolla* Supplementation

Parameter	Control	T1	T2
Milk Fat % (Before to After)	2.98 → 2.98	3.04 → 3.84	3.06 → 4.24
Milk Yield (L/Day)	13 → 13	13 → 14	13 → 14.5
Feed cost Reduction	No change	Moderate	High
Profit Increase (₹)	No change	±97.5	± 140
Overall Effect	No improvement	Good	Excellent

***Azolla*-Induced Upregulation of DGAT1 and SCD1 Genes in Dairy Cattle:**

Present study also confirms that *Azolla* supplementation positively influences the gene expression related to fat metabolism, leading to improved milk quality and overall dairy performance. The phylogenetic conservation of DGAT1 and SCD1 genes and their functional significance in the metabolism of milk fat has been well established (Grisart *et al.*, 2002; Schennink *et al.*, 2008; Kgwatalala *et al.*, 2009). Nutrigenomic regulation of the genes due to dietary interventions such as *Azolla* supplementation has been supported by earlier literature (Bauman & Griinari, 2003; Yadav *et al.*, 2023). As shown in the gel electrophoresis image, the expression of DGAT1 (~650 bp) and SCD1 (~550 bp) genes in Control, T1, and T2 groups was examined. Both gel electrophoresis images, distinct bands are observed for both genes, indicating successful PCR amplification of DGAT1 and SCD1 genes as mentioned in gel in Figure 6. The control group shows a lighter band, indicating lower gene expression. The T1 group, where the plants are subjected to 1.5 kg of *Azolla*, shows a moderate increase in band intensity, indicating upregulation of DGAT1 and SCD1 genes. The T2 group, where plants are subjected to 2.0 kg of *Azolla*, shows the highest intensity of bands, indicating the highest expression of DGAT1 and SCD1 genes. The DNA ladder, designated as "M," confirms the size of the bands. The negative control, designated as "NC," does not show any bands, confirming that there is no contamination. As shown in the gel electrophoresis images, the increase in band intensity from the control to T1 and then to T2 indicates that DGAT1 and SCD1 genes are upregulated in a dose-dependent manner, correlating well with the increase in milk fat percentage.

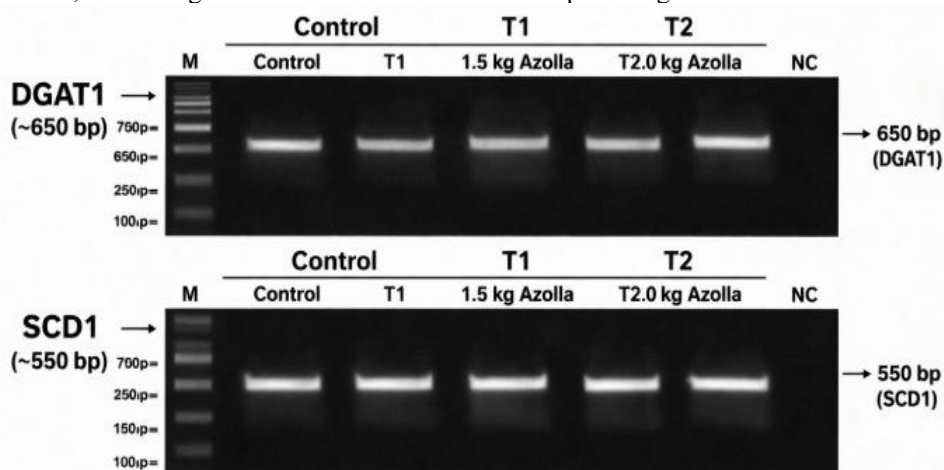


Figure:6 Agarose gel Electrophoresis of GGAT1 and SCD1 Gene Expression in Control and *Azolla* treated Dairy Cows

CONCLUSION

The current study has clearly shown that *Azolla* supplementation has a positive effect on dairy farming in terms of enhancing milk quality and economic benefits. Milk fat percentage has shown significant improvement in the treatment groups. Milk fat percentage has increased from 3.04 to 3.84 in T1 and from 3.06 to 4.24 in T2, whereas it has remained constant in the control group. Milk yield has also shown improvement of 10% in T1 and 15% in T2. These findings are in agreement with those of earlier researchers, which have shown *Azolla*'s role in enhancing dairy farming. At the molecular level, it has been found that *Azolla* improves milk fat percentage due to the expression of DGAT1 and SCD1 genes. *Azolla* is found to be an efficient and sustainable option for dairy farmers. *Azolla* is found to be cost-effective, and its higher concentration is found to be beneficial to rural dairy farmers.

Author contributions: CRediT

AP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Resources; **NP:** Formal analysis, Investigation, Software, Supervision, Validation; **PM:** Conceptualization, Data curation, Formal analysis,

Investigation, Software, Supervision, Validation; **PCP**: Formal analysis, Methodology, Supervision; **SP**: Data curation, Investigation, Methodology, Supervision, Validation, Resources.

Declaration of competing interest

The writers assert that they have no possible conflicts of interest.

Funding sources

This work is not supported by any external funding.

Data availability

The data supporting the outcome of this research work has been reported in this manuscript.

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