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Abstract

This study evaluated the impact of incorporating varying levels of dried *Azolla pinnata* as a partial or full substitute for soybean meal protein on growth performance, productivity, and selected biochemical, immunological, hormonal, and antioxidant parameters in New Zealand White (NZW) female rabbits. Conducted at the South Sinai Research Station of the Desert Research Center, Egypt, from May to November 2023, the trial involved 45 eight-week-old NZW female rabbits. These were randomly assigned to three dietary groups: a control group fed a standard concentrate diet, a group where 50% of soybean protein was replaced with Azolla (GI), and a group where soybean protein was completely replaced with Azolla (GII). Findings revealed that the GI group (50% Azolla replacement) achieved superior growth performance throughout the study period. Conversely, the GII group (100% Azolla replacement) experienced a significant reduction in body weight during later growth stages. Regarding productivity, no marked differences among the groups were observed in birth numbers. However, birth mortality rates were notably lower in the GII group, despite a significant reduction in kit weights during the first four weeks postpartum. Biochemical analyses showed that Azolla inclusion affected blood parameters based on supplementation level and duration. In the first month, the GII group exhibited elevated total protein and globulin concentrations alongside a reduced albumin-to-globulin ratio, while the trend reversed in the second month. During pregnancy, total protein and globulin levels increased in both supplemented groups. Additionally, Azolla influenced kidney and liver function markers, lipid profiles, immunoglobulin concentrations, thyroid hormones, and reproductive hormones, with variations noted across groups and stages of the experiment. Therefore, partial replacement of soybean meal protein with 50% dried *A. pinnata* improved growth performance without adverse effects on reproductive outcomes or biochemical balance in NZW female rabbits. However, complete replacement (100%) negatively impacted growth and certain productivity parameters, indicating that while Azolla has beneficial properties, its optimal use lies in partial substitution rather than total replacement to maintain rabbit health and performance.

Keywords: *Azolla pinnata*, New Zealand White rabbits, Growth performance, Productivity, Soybean meal protein, Biochemical parameters, Immunity, Hormones.

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Introduction

The world is witnessing a steady increase in food demand due to population growth and rising human consumption rates (Anitha et al., 2016). This puts immense pressure on the agricultural sector to boost food production to meet the needs of an estimated 9 billion people by 2050 (Ramadan, 2019). Projections indicate that global agricultural output will need a 70% increase by 2050 to keep pace with the anticipated 40% population growth. Furthermore, the COVID-19 pandemic in 2019 led to shifts in marketing strategies, with a growing emphasis on local markets (Love et al., 2021).

The demand for crops used in animal feed is particularly escalating, primarily driven by increased human consumption of animal products (Godfray et al., 2010). The scarcity and high prices of concentrated and green fodder in animal nutrition have necessitated the search for readily available and economically viable alternative sources to reduce animal production costs (El Naggar and El-Mesery, 2022).

One of the most significant challenges in this context is securing easily accessible protein sources capable of meeting the nutritional requirements of non-ruminant animals at a low cost. Among potential alternatives, aquatic macrophytes like Azolla have shown promising outcomes (Méndez-Martínez et al., 2019). Several factors contribute to the unsustainability of current feed imports in some countries, where key ingredients such as soybean meal, fishmeal, and cornmeal are imported instead of being domestically produced as raw materials for feed (Nasir et al., 2022).

The widespread reach of media has increased public awareness regarding the importance of high-quality, complete protein foods derived from animals, characterized by high nutritional and biological value. This heightened awareness has subsequently boosted the demand for such foods (Anitha et al., 2016). The need for meat with high nutritional value, specifically low fat, high protein, and low sodium content, coupled with rapid human population expansion, are the primary drivers behind the increased production of rabbit meat (Mottet and Tempio, 2017).

Rabbit and poultry diets rely on feed ingredients derived from important crops also used for human consumption, such as soybean, corn, barley, and wheat (Ogbuewu et al., 2017). To sustain rabbit meat production without competing with humans for these vital crops, the provision of

alternative dietary protein sources becomes essential (Abdelatty et al., 2021). Aquatic macrophytes, such as Azolla, have demonstrated promising potential as such alternatives (Narayan, 2011).

Azolla pinnata is a free-floating aquatic fern belonging to the Azollaceae family, growing in symbiosis with the nitrogen-fixing blue-green alga *Anabaena Azollae* (Mathur et al., 2013). Azolla is considered a potentially valuable feed component that can aid farmers in achieving sustainability, defined as both environmentally and economically responsible (Kollah et al., 2015). Despite being an uncommon feed source, Azolla can be used as a feed ingredient for growing lambs. It has a relatively low oil content but boasts a high protein content ranging from 25-30%, along with minerals constituting 10-15% and a mixture of amino acids, bioactive compounds, biopolymers, and carbohydrates making up 7-10% (Kathirvelan et al., 2015).

Azolla, a free-floating water fern, is rich in protein and essential amino acids, growth-promoting intermediates, vitamins (A, B12, and beta-carotene), and minerals such as calcium, phosphorus, potassium, iron, copper, and manganese. Furthermore, its high fiber content and low lignin level make it easily digestible for livestock. Azolla is considered one of the most promising alternatives due to its ease of cultivation, low water requirement for growth, high yield, and high nutritional content (Kumar and Chander, 2017).

Based on the aforementioned context, the present study aimed to investigate the effect of graded nutritional levels of sun-dried Azolla as an alternative source of soybean protein on the growth performance and productivity of rabbits.

2. Materials and Methods

This study investigated the impact of supplementary Azolla feeding at varying levels on the growth performance and productivity of New Zealand White (NZW) rabbits. The experiment was conducted at the South Sinai Research Station of the Desert Research Center, Ministry of Agriculture, Egypt, between May and November 2023. All procedures adhered to the guidelines approved by the Research Ethical Committee of the Faculty of Science, Suez Canal University, Ismailia, Egypt.

2.1 Azolla Cultivation and Meal Production

Azolla pinnata was cultivated in six excavated holes (3 × 4 × 0.3 m) within an agricultural greenhouse. Each hole was prepared with fertile soil, a slurry of rabbit manure and superphosphate, and then inoculated with approximately 5 kg of fresh *Azolla* culture. After 10-15 days, approximately 10 kg of *Azolla* was harvested daily (as per Adzman et al., 2022), sun-dried, and analyzed for chemical composition according to AOAC (2005) methods (Table 1).

2.2 Experimental Design

Forty-five 8-week-old NZW female rabbits (initial body weight 1.535±.025 g) were randomly divided into three equal dietary groups:

- **Control:** Basal concentrate diet.
- **GI:** Basal diet with 50% of soybean replaced by *Azolla pinnata*.
- **GII:** Basal diet with 100% of soybean replaced by *Azolla pinnata*.

Rabbits were housed individually in grower cages (50 x 50 x 35 cm) under similar environmental conditions and fed their respective experimental diets with ad libitum water after a 7-day pre-feeding period. Diet formulations, based on NRC (1977) recommendations (Table 2), were prepared at Ras Sudr Research Station, and their chemical analyses are presented in Table 2. After three months of feeding, natural mating with treated bucks (1 buck: 5 does) was conducted. Pregnancy was diagnosed by abdominal palpation 15 days post-mating as shown in the Fig. 1.

2.3 Growth Performance Evaluation

Rabbits were weighed bi-weekly before feeding for 12 weeks. Kits were weighed immediately after birth and weekly until weaning.

2.4 Serum Biochemical Parameters Determination

Blood samples were collected from the auricular vein after one and two months of feeding the experimental diets, and after 21 days of pregnancy, following an overnight fasting period. The serum was separated by centrifugation and stored at -20 °C. Biochemical analyses included total protein (Henry, 1964), albumin (Doumas et al., 1971), globulin (calculated), urea (Patton & Grouch, 1977), creatinine (Bartels, 1972), alanine aminotransferase (ALT), aspartate

aminotransferase (AST) (Reitman & Frankel, 1957), triglycerides (TG) (Fassati & Prencipe, 1982), total cholesterol (Allen, 1974), high-density lipoprotein (HDL-C) (Lopez, 1977), low-density lipoprotein (LDL-C), and very low-density lipoprotein (VLDL-C) (Lee & Nieman, 1996), with VLDL calculated as TG/5 and LDL as (Total cholesterol – HDL-c) – VLDL-c. Commercial kits were used for all colorimetric titrations.

2.5 Determination of Immunoglobulins and Hormones

Serum levels of Immunoglobulin G (IgG) and M (IgM) were determined using specific kits (Bio-diagnostic, Egypt). Serum triiodothyronine (T3) and tetraiodothyronine (T4) were measured using radioimmunoassay kits. Prolactin and progesterone were assayed using ELISA kits.

2.6 Oxidative and Antioxidant Status Evaluation

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) levels using a competitive binding ELISA method. Superoxide dismutase (SOD) activity was assessed using a rat SOD ELISA Kit (Cusabio, USA). Catalase (CAT) activity was determined using a double-sandwich ELISA method (MyBioSource, USA). Glutathione (GSH) levels were measured using an ELISA Kit (Cusabio, USA). Total antioxidant capacity (TAC) was assessed using the OxiSelectTM kit (Cell Biolabs, USA). All procedures followed the manufacturers' instructions.

2.7 Statistical Analysis

Data were presented as means ± standard error (S.E.). One-way ANOVA was used to determine significant differences between experimental and control groups, with significance set at $P \leq 0.05$. Statistical analysis was performed using Minitab software (SAS, 2000).

3. Results

3.1 Growth Performance

Fig. 2 illustrates the effect of replacing soybean meal protein with dried *A. pinnata* on the weekly growth performance of young rabbits across three dietary groups. These groups differ based on the percentage of soybean protein substituted with Azolla. The control group, which received no Azolla, exhibited an increase in body weight from 1531.53 ± 60.54 g at week 8 to

2950.00 ± 62.67 g at week 20. The GI group, with 50% soybean protein replaced, consistently showed higher body weights compared to the GII group, starting at 1556.66 ± 60.00 g at week 8 and reaching 2860.66 ± 50.65 g by week 20, reflecting better growth performance. In contrast, the GII group, with 100% replacement, recorded the lowest body weights throughout the study period. Statistical analysis revealed significant differences in body weights between the control, GI, and GII groups at various time points. A marked decline ($P < 0.001$) was observed in the GII group's weights during weeks 12 to 20, compared to both the control and GI groups. These results suggest that the complete replacement of soybean protein with Azolla negatively affects rabbit growth performance.

The quantity and mortality rate of births in each group with their respective weights. There was no noteworthy distinction observed between the experimental groups when compared to the control rabbits. However, a notable reduction in the mortality rate of births was evident in G II compared to the other groups, as illustrated in Table 3. Furthermore, there was a significant ($P<0.05$) decline in their weights from the first week to the fourth week within G II in comparison to G I and the control group. Additionally, a significant decrease in weights over four weeks was observed between GI and the control group, as depicted in Table 3.

3.2 Effect of Azolla on Serum Biochemical Parameters.

The impact of substituting soybean meal protein with dried Azolla in female rabbit diets on various parameters, including total protein (TP), serum albumin, globulin, and the albumin: globulin ratio (AL: GL ratio) at different sampling intervals. After one month of the study, G II exhibited a significant increase in TP and GL compared to G I and the control group, as depicted in Table 4. Additionally, G II demonstrated a significant decrease in the AL:GL ratio compared to G I and the control group during this period. However, after two months, G II showed a notable reduction in TP and GL in comparison to G I and the control group. Furthermore, the AL: GL ratio was significantly higher in G II compared to G I and the control group, as shown in Table 4. During pregnancy, specifically after 21 days, there was a significant increase in TP and GL of the experimental group compared to the control rabbits. However, the AL: GL ratio in the substitution groups significantly decreased in comparison to the control group during this phase, as illustrated in Table 4

In Tables 4 and 10, G II had significantly decreased ALT and AST after a month than the G I and control rabbits. However, replacement of the diet with different percentages of Azolla for two months and after 21 days of pregnancy, significantly increased the AST of G I than the G II and control group. Also, after 21 days of pregnancy, G I had significantly increased ALT than the G II and control group. After two months, G I had a significantly decreased ALT than the control group.

The effects of dietary soybean meal protein substitution with dried Azolla on lipid profile. After a month, all lipid profiles showed a significantly greater increase in G II than those of GI and the control, except LDL-c, that depicted in Table 4. All lipid profiles were significantly greater in the G I than in the control. Nevertheless, over two months, all lipid profiles in the G II group significantly increased compared to those in the GI and control groups. Additionally, TC of the GI group significantly increased as compared to the control group, as seen in Table 4. TC, HDL-c, and LDL-c in the control group had significantly decreased after 21 days of pregnancy compared to other experimental groups. However, LDL-c, TG, and VLDL in the G II group were significantly higher relative to other experimental rabbits (Table 4).

The effects of dietary soybean meal protein substitution with dried Azolla on biomarkers of kidney functions (urea and creatinine) and liver function enzymes (AST and ALT) of female rabbits. The G I had significantly decreased urea than the G II and control group after a month and 21 days of pregnancy (Table 4). Throughout the entire experiment, there was no significant change in the creatinine level of all the groups (Table 4).

3.5 Effect of Azolla on immunoglobulin and hormones

The results of immunoglobulin G and M levels concerning dietary soybean meal protein substitution levels with dried Azolla. After one month, there was a significant reduction in IgM in the G II group compared to the G I group control (Table 5); however, after two months, there was a significant reduction in IgG in the G I group compared to the G II group and control (Table 5)

The effects of dietary soybean meal protein substitution with dry Azolla on T3 and T4. A month later of feeding, T3 was significantly lower in G II than G I and the control group (Fig. 3), whereas T4 was significantly higher in G I than G II and the control group (Fig. 4). However, T3 and T4 weren't significantly different between the groups after two months. After 21 days of

pregnancy, T3 and T4 were significantly lower in the control group than in the other experimental animals.

The effects of dietary soybean meal protein substitution levels with dried Azolla on progesterone, prolactin, and estradiol hormones. Prolactin significantly decreased in G I compared to control, while prolactin and progesterone significantly decreased in G II compared to control (Fig. 5). Fig. 6 shows a significant decrease in estradiol in G I compared to G II and control, as well as a significant decrease in prolactin in G II compared to control.

3.6 **Effect of Azolla on oxidative stress/antioxidant status.**

The impact of substituting dietary soybean meal protein with dried Azolla on various parameters including MDA, SOD, CAT, GSH, and TAC. Results indicated that after one and two months of experimentation, the MDA levels were notably higher in group GI compared to other experimental groups and the control. However, after one month, MDA levels significantly increased in the control group compared to group GII. Conversely, after two months, MDA levels significantly decreased in the control rabbits compared to group GII, as illustrated in Table 4.

One month later, the SOD, CAT, GSH, and TAC outcomes displayed a noteworthy rise in group GII compared to both the other experimental groups and the control. Moreover, the levels of SOD, CAT, GSH, and TAC exhibited a significant increase in the control group compared to group GI, as depicted in Table 4.

Two months later, the outcomes for SOD, CAT, GSH, and TAC indicated a notable increase in the control group compared to the other experimental groups. Additionally, the levels of SOD, CAT, GSH, and TAC exhibited a significant increase in group GII compared to group GI, as illustrated in Table 4.

Discussion

The present study aims to evaluate the feasibility of using dried *Azolla pinnata* as an alternative protein source in rabbit diets by partially or completely replacing soybean meal protein. Specifically, the study investigates the effects of different levels of Azolla supplementation on the growth performance, productivity, and selected biochemical, immunological, hormonal, and antioxidant parameters of New Zealand White (NZW) female rabbits. This research addresses the increasing global demand for sustainable and cost-effective animal feed sources, driven by rising

food consumption and competition between human and animal dietary needs. By exploring Azolla's potential as a locally available, nutritionally rich, and environmentally sustainable feed ingredient, the study seeks to offer practical solutions to reduce reliance on expensive imported feed components without compromising animal health or production efficiency.

The results demonstrated that partial substitution of soybean meal protein with 50% Azolla (GI group) enhanced growth performance, as reflected by higher body weights compared to the GII group (100% Azolla replacement), which showed significant growth retardation from weeks 12 to 20. This trend aligns with previous studies reporting that Azolla can be a valuable protein source when used moderately in animal diets, thanks to its high protein content, essential amino acids, and bioavailability (Kumar & Chander, 2017; Samad et al., 2020). However, complete replacement appears detrimental, possibly due to the presence of anti-nutritional factors like tannins and phytic acid in Azolla, which may interfere with nutrient absorption and digestion (Hassan et al., 2020; Abdelatty et al., 2021).

Interestingly, although GII exhibited reduced body weights, it showed a lower birth mortality rate, indicating that Azolla may contribute positively to reproductive outcomes despite impairing growth at high inclusion levels. These results suggest a need to balance Azolla inclusion to maximize benefits while minimizing adverse effects.

In terms of serum proteins, the elevated total protein and globulin levels observed in GII after one month may be attributed to Azolla's rich amino acid profile. However, the subsequent decline in these parameters after two months suggests potential long-term limitations in the bioavailability of Azolla's nutrients when used as the sole protein source, as supported by previous findings (Méndez-Martínez et al., 2019; Abdelatty et al., 2021). The reduced albumin-to-globulin ratio in GII further underscores the need to avoid excessive Azolla inclusion to maintain protein balance.

Liver enzyme (AST, ALT) fluctuations, particularly the decrease in GII after one month and the increase in GI after two months, may reflect the liver's adaptive response to Azolla's bioactive compounds. This is consistent with studies indicating that moderate Azolla supplementation supports liver function, while higher levels may pose metabolic stress (Nasir et al., 2022). Similarly, the stable creatinine levels across all groups suggest that kidney function remained largely unaffected, while urea levels were lower in GI, indicating efficient protein metabolism at moderate Azolla levels (Ismail et al., 2023).

Azolla inclusion led to significant increases in total cholesterol, triglycerides, and LDL-c, particularly in the GII group. While Azolla is rich in bioactive compounds, high fiber, and anti-nutritional components may have interfered with lipid metabolism, aligning with previous observations in rabbits and poultry (Kathirvelan et al., 2015; Ogbuewu et al., 2017). These findings suggest that excessive Azolla inclusion could disrupt lipid homeostasis.

Reduced IgM levels in GII and decreased IgG in GI reflect Azolla's immunomodulatory effects, which are dosage-dependent. Previous studies noted that moderate levels of Azolla enhance immune responses, while higher levels may suppress immunity due to potential anti-nutritional factors (Mishra et al., 2016; Ismail et al., 2023).

Regarding hormonal profiles, the altered T3, T4, prolactin, progesterone, and estradiol levels suggest that Azolla inclusion influences thyroid and reproductive hormones. While partial replacement improved hormonal balance, complete replacement negatively affected these parameters, indicating stress or endocrine disruption, a finding supported by prior studies (Mottet & Tempio, 2017; Abdelatty et al., 2021).

The improved antioxidant status (SOD, CAT, GSH, TAC) observed in GII after one month may be linked to Azolla's rich antioxidant content, including vitamins and bioactive compounds (Chichilichi et al., 2015). However, prolonged inclusion resulted in fluctuating MDA levels, indicating oxidative stress at higher Azolla levels. This pattern is consistent with other studies emphasizing the dose-dependent antioxidant benefits of Azolla (Kamel and Hamed 2021; Punyatong et al., 2024).

In Conclusion

Azolla is a protein-rich floating fern with essential nutrients but low carbohydrates and lipids. It is valuable for animal feed, biofertilization, and bioremediation. Overall, the study highlights the potential of *A. pinnata* as a sustainable, cost-effective partial replacement for soybean meal protein in rabbit diets. While 50% replacement supports growth, productivity, biochemical balance, and antioxidant status without adverse effects, complete replacement negatively impacts growth, lipid metabolism, and certain hormonal parameters. These findings suggest that moderate inclusion levels of Azolla optimize its nutritional benefits while avoiding the drawbacks associated with high inclusion rates. Future research should focus on processing

techniques to reduce anti-nutritional factors in Azolla, improving its viability as a complete protein substitute.

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