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Effect of protein supplements on total free amino acid content of honey and worker bees of *Apis cerana indica* F.

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Abstract
Free amino acids (FAA) in honey and bee tissue are sensitive indicators of colony nutrition, yet they have not been examined in *Apis cerana indica* fed regionally available protein supplements. Twenty-one colonies, standardised to four frames, were allocated in a completely randomised design to six plant-protein patties (T_1–T_6) or a 50% sucrose control (T_7) and fed weekly for 120 days (1 January–30 April 2026) at Gangavathi, in the Northern Dry Zone of Karnataka, India. Total FAA of stored honey (Cd-ninhydrin method; mg leucine equivalents 100 g^{-1}) and of worker bees (ninhydrin, pH 5.2; µg leucine equivalents g^{-1} fresh weight) was determined at Days 0, 30, 60, 90 and 120 and analysed by two-way ANOVA (treatment × time) with Tukey's HSD. Treatment, time and their interaction were highly significant for both matrices ($P \leq 0.001$). The chickpea flour + wheat germ diet (T_6) gave the highest mean honey FAA (62.15 mg LE 100 g^{-1}; 1.85 times the control), which rose 2.35-fold from baseline to 87.73 mg LE 100 g^{-1} by Day 120, and the highest worker FAA (17,427 µg LE g^{-1}; 1.82-fold increase over baseline by Day 120). Defatted soybean-based diets (T_1, T_2) ranked next. All supplements raised mean honey FAA above the control, whereas the maize + pea diet (T_5) did not raise worker FAA. Across the seven treatment means, FAA of both matrices was strongly correlated with diet consumption and with six colony growth parameters ($r = +0.923$ to $+0.977$). Total FAA therefore tracked supplement quality and colony performance, and may serve as a rapid biochemical indicator of colony nutritional status. The chickpea–wheat germ formulation is recommended for pre-summer supplementation of *A. cerana indica* in semi-arid Karnataka.

Keywords: *Apis cerana indica*, protein supplement, free amino acids, honey, worker bees, colony nutrition

Introduction
The Indian honey bee, *Apis cerana indica* F., is a managed pollinator and honey producer across southern Asia, but its populations are reported to be in decline (Theisen-Jones and Bienefeld, 2016) [22], and managed pollination remains far below the level needed for India's cropped area. Honey bees influence a large share of global crop production (Klein *et al*., 2007) [10], so maintaining colony health through periods of floral dearth is both an agricultural and an ecological concern. Pollen is the sole natural source of protein, lipids, sterols and minerals, and its scarcity reduces brood rearing, glandular development and immune function (Brodschneider and Crailsheim, 2010; Wright *et al*., 2018) [4, 25]. Beekeepers therefore feed protein-rich pollen substitutes, and many studies have shown that ingredient choice strongly affects consumption, brood area, colony strength and honey yield (Younis, 2019; Painkra *et al*., 2022; Taredahalli *et al*., 2025; Noordyke and Ellis, 2021) [13, 14, 21, 26].

Most evaluations rely on colony-level growth measures alone. Protein quality, however, depends on amino acid supply: bees cannot synthesise ten essential amino acids (De Groot, 1953) [6], and the physiological value of a protein source is determined more by its amino acid composition than by crude protein (Standifer, 1967) [19]. Haemolymph and tissue free amino acids reflect recent dietary intake and the age-related role of the worker (Crailsheim, 1990; Paoli *et al*., 2014; Tafi *et al*., 2024) [5, 16, 20], and infection or malnutrition depletes the pool (Wang and Moeller, 1970) [23]. In honey, the FAA fraction carries information about botanical and geographical origin and about processing by the bee, with total contents of about 23–98 mg 100 g^{-1} reported across honeys of different origins (Dimins *et al*., 2022; White, 1975; Iglesias *et al*., 2004) [7, 9, 24]. FAA in honey and bees could therefore provide a chemical index of supplement adequacy that complements growth parameters.
To our knowledge, no study has examined how regionally available plant-protein supplements alter total FAA in honey and worker bees of *A. cerana indica*. The present study evaluated six locally formulated supplements against an unsupplemented control over a 120-day pre-summer feeding trial, and tested whether FAA of honey and worker bees tracks diet consumption and colony growth.

Materials and Methods
1. Study site and period
The trial was conducted at the Centre of Excellence in Apiculture, College of Agriculture, Gangavathi (15.45° N, 76.52° E; 406 m a.s.l.), in the Northern Dry Zone (Zone 3) of Karnataka, a semi-arid tract with a normal annual rainfall of about 583 mm. Feeding and sampling ran from 1 January to 30 April 2026, a pre-summer window in which ambient temperature rises from its winter minimum to its summer maximum and relative humidity declines.

2. Colonies and experimental design

Twenty-one disease-free colonies of *A. cerana indica* with age-matched, actively laying queens and comparable brood area and adult population were housed in standard Indian-pattern hives. None had received supplementary feed for at least two weeks before the trial, and all were equalised to four filled frames (of eight) on 1 January 2026. Colonies were randomly allocated, using a random-number sequence, to seven treatments with three replicate colonies each in a completely randomised design (n = 21). Treatments T_1–T_6 were six plant-protein formulations (F_1–F_6) and T_7 was the control, which received 50% sucrose solution only.

3. Formulations and feeding

Each formulation was a semi-solid patty containing, per kilogram, 308 g of a formulation-specific pair of plant-protein flours, 62 g dried brewer's yeast, 315 g sugar powder, 251 ml bee honey and 64 ml water; formulations therefore differed only in the identity and proportion of the two protein flours (Table 1). Feed was prepared fresh each week. Each colony in T_1–T_6 received a 100 g portion on wax paper on the top bars of the brood chamber once a week (17 feeding events); residues were removed and weighed, and consumption was corrected for evaporative loss using muslin-wrapped control packets.

Table 1: Composition of the six plant-protein formulations per kilogram of prepared feed

Treatment	Protein flours (g)	Brewer's yeast (g)	Sugar powder (g)	Honey (ml)	Water (ml)
T_1 (F_1)	Defatted soybean 62 + wheat germ 246	62	315	251	64
T_2 (F_2)	Defatted soybean 62 + chickpea 246	62	315	251	64
T_3 (F_3)	Chickpea 154 + maize 154	62	315	251	64
T_4 (F_4)	Maize 154 + wheat germ 154	62	315	251	64
T_5 (F_5)	Maize 154 + pea 154	62	315	251	64
T_6 (F_6)	Chickpea 154 + wheat germ 154	62	315	251	64
T_7	50% sucrose solution only (control)	–	–	–	–

4. Colony growth parameters

Sealed and unsealed brood area (wire grid), drone cell number, honey production, colony weight and colony strength (frames covered by bees) were recorded monthly (details in the companion analysis); treatment-mean values were used here for correlation with FAA.

5. Sampling

Honey and worker bees were sampled from every colony on Day 0 (1 January), Day 30 (31 January), Day 60 (2 March), Day 90 (1 April) and Day 120 (30 April 2026), giving 105 honey and 105 worker samples. Honey (≥2 ml) was aspirated with a sterile syringe through the capping of sealed honey in the super chamber and stored at −20 °C. Two to three workers per colony (pooled fresh weight ≈ 0.086 g) were transported at 4 °C, killed by brief exposure to −20 °C and stored at −20 °C until analysis.

6. Total free amino acid assay

Total FAA was determined by the ninhydrin colorimetric method (absorbance at 570 nm) against an L-leucine standard curve (20, 50, 100, 150 and 200 µg ml^{-1}) prepared in each session; every sample was analysed in triplicate. For honey, the cadmium–ninhydrin method of Palmer-Jones and Line (1962) [15], as modified by Iglesias *et al.* (2004) [9], was used: 1.0 g honey was diluted in 10 ml distilled water, and 0.5 ml aliquots were reacted with 1.0 ml Cd–ninhydrin reagent at 100 °C for 15 min, cooled, stabilised for 10 min and read. Results were expressed as mg leucine equivalents (LE) 100 g^{-1} honey. For worker bees, tissue was homogenised in 5 ml ice-cold 80% ethanol, centrifuged (8,000 × g, 15 min, 4 °C) and the pellet re-extracted with a further 5 ml; 0.5 ml of the combined supernatant was reacted with 1.0 ml standard ninhydrin reagent at pH 5.2 (Moore and Stein, 1948) [11], heated at 100 °C for 15 min and read at 570 nm. Results were expressed as µg LE g^{-1} fresh weight.

7. Statistical analysis

FAA data were analysed by two-way factorial ANOVA with treatment (7 levels) and time (5 levels) as fixed factors, each cell replicated across three colonies. Treatment means were compared by Tukey's HSD at $P \leq 0.05$, and critical differences (CD) at 5% were computed for each time point. Pearson correlation coefficients were computed between overall treatment-mean FAA and each growth parameter (n = 7 treatments; n = 6 for diet consumption, the control being unfed). Analyses were performed in SPSS Statistics v22.0 (SPSS Inc., Chicago, IL, USA).

Results

1. Total free amino acids in honey

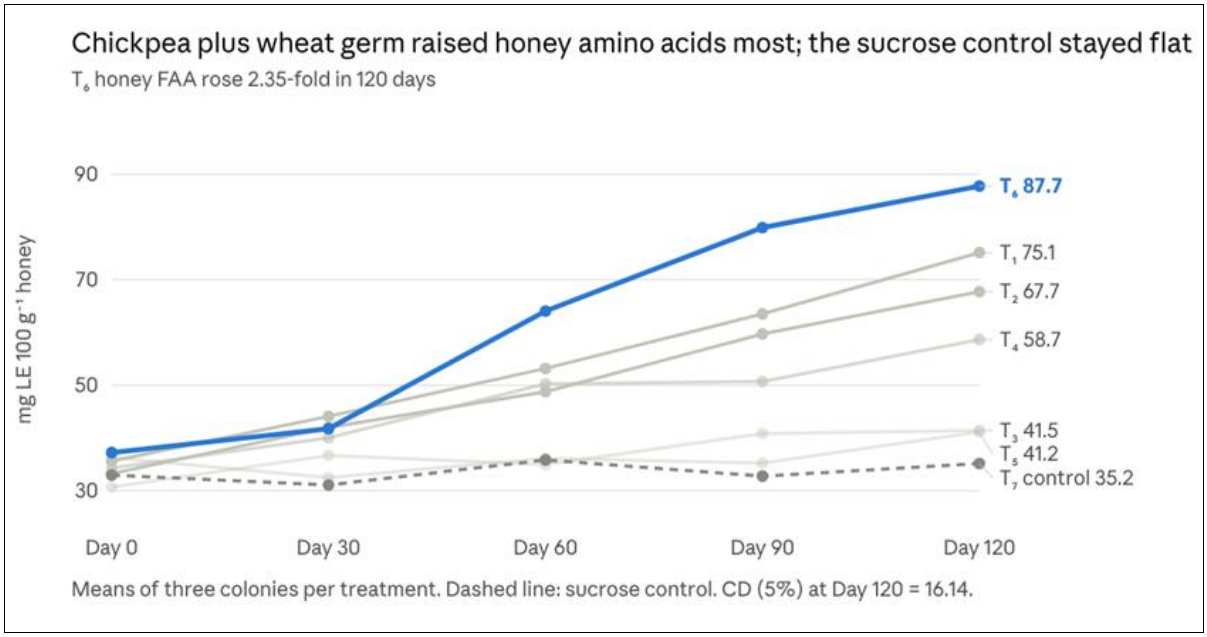
Treatment, time and their interaction significantly affected honey FAA (treatment $F_{6,70} = 64.78$; time $F_{4,70} = 75.30$; treatment × time $F_{24,70} = 7.65$; all $P \leq 0.001$; Table 2, Fig. 1). At Day 0 treatments were statistically similar (30.73–37.27 mg LE 100 g^{-1}; CD = 9.75), confirming a common baseline. From Day 30 the better-supplemented treatments diverged upward at progressively increasing rates, whereas the control remained essentially flat (33.00 at Day 0; 35.20 at Day 120).

T_6 recorded the highest overall mean (62.15 mg LE 100 g^{-1}), 1.85 times the control, followed by T_1 and T_2 (statistically similar), then T_4, T_3 and T_5 ($T_3 \approx T_5$), with the control lowest. Over 120 days T_6 rose 2.35-fold from its own baseline (37.27 to 87.73), T_1 2.11-fold, T_2 2.04-fold and T_4 1.71-fold, against 1.35-fold for T_3, 1.13-fold for T_5 and 1.07-fold for the control. At Day 120, T_6, T_1, T_2 and T_4 exceeded the control by more than the CD (16.14), whereas T_3 and T_5 did not. Small decreases between consecutive sampling dates in T_3 (Day 60) and T_5 (Day 30) fell within the CD and were not significant.

Table 2: Total free amino acid content of honey (mg leucine equivalents 100 g⁻¹) of *A. cerana indica* colonies under protein supplement diets

Treatment	Day 0	Day 30	Day 60	Day 90	Day 120	Mean
T_1	35.67	44.13	53.20	63.53	75.13	54.33[b]
T_2	33.27	42.00	48.80	59.73	67.73	50.31[b]
T_3	30.73	36.73	35.00	40.87	41.47	36.96[d]
T_4	34.33	40.07	50.27	50.73	58.67	46.81[c]
T_5	36.33	32.53	36.20	35.27	41.20	36.31[d]
T_6	37.27	41.80	64.07	79.87	87.73	62.15[a]
T_7 (control)	33.00	31.13	35.87	32.80	35.20	33.60[e]
SEm (±)	2.02	2.77	2.95	3.46	3.34	
CD (5%)	9.75	13.40	14.25	16.72	16.14	
CV (%)	10.18	12.53	11.06	11.57	9.95	

Means of three replicate colonies. Means followed by a common letter in the final column do not differ significantly at $P \leq 0.05$ (Tukey's HSD).



Treatment means of three colonies · Days 0–120

Fig 1: Total free amino acid content of honey (mg leucine equivalents 100 g⁻¹) in *Apis cerana indica* colonies fed six protein supplements (T_1–T_6) or sucrose only (T_7) over 120 days.

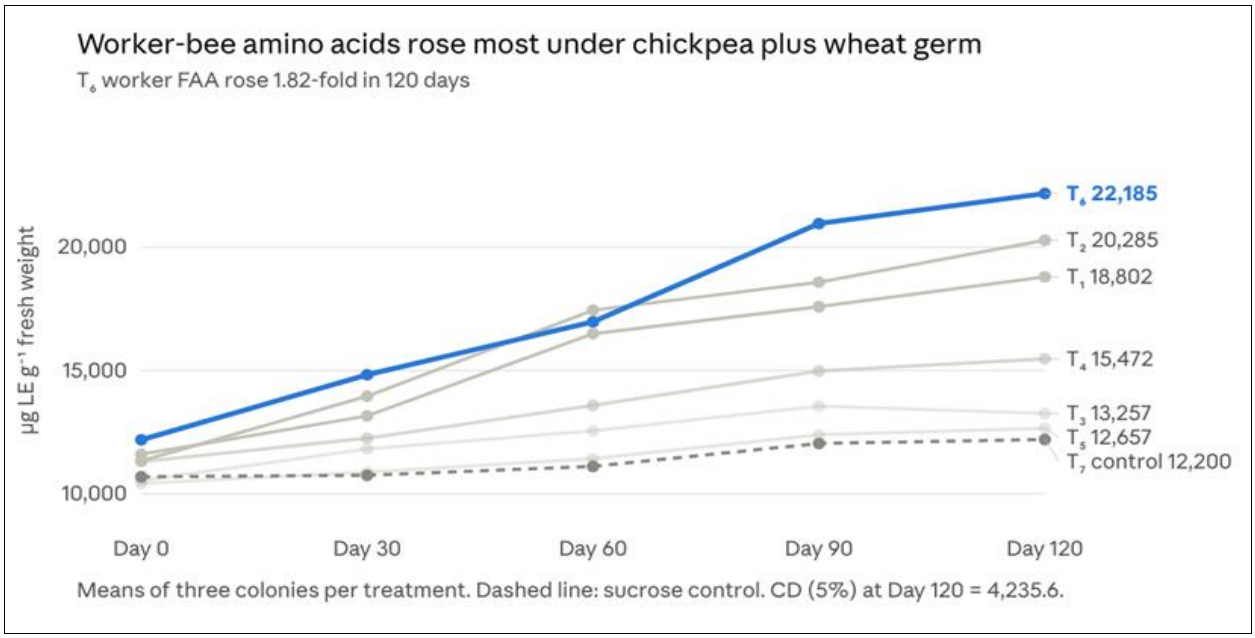
2. Total free amino acids in worker bees

Worker FAA showed the same direction of response with a smaller relative amplitude (treatment $F_{6,70} = 63.00$; time $F_{4,70} = 71.55$; interaction $F_{24,70} = 4.37$; all $P \leq 0.001$; Table 3, Fig. 2). Baseline values were similar (10,415–12,187 µg LE g⁻¹; CD = 2,512). T_6 again gave the highest mean (17,427 µg LE g⁻¹; 1.53 times the control), reaching 22,185 µg LE g⁻¹ at Day 120 (1.82-fold over baseline). T_2 (16,321) and T_1 (15,528) formed the second group, followed by T_4 and T_3. The maize + pea diet T_5 (11,553) did not differ from the control (11,357). At Day 120, T_6, T_2 and T_1 exceeded the control by more than the CD (4,235.6), whereas T_4 (difference 3,272), T_3 and T_5 did not.

Table 3: Total free amino acid content of worker bees (µg leucine equivalents g⁻¹ fresh weight) of *A. cerana indica* colonies under protein supplement diets

Treatment	Day 0	Day 30	Day 60	Day 90	Day 120	Mean
T_1	11,600	13,157	16,493	17,591	18,802	15,528[b]
T_2	11,330	13,957	17,449	18,586	20,285	16,321[b]
T_3	10,533	11,818	12,554	13,556	13,257	12,344[d]
T_4	11,301	12,255	13,584	14,981	15,472	13,518[c]
T_5	10,415	10,877	11,421	12,394	12,657	11,553[e]
T_6	12,187	14,827	16,976	20,961	22,185	17,427[a]
T_7 (control)	10,688	10,748	11,111	12,040	12,200	11,357[e]
SEm (±)	520.2	546.4	648.3	764.0	877.1	
CD (5%)	2,511.9	2,638.6	3,130.5	3,689.3	4,235.6	
CV (%)	8.08	7.56	7.89	8.41	9.26	

Means of three replicate colonies. Means followed by a common letter in the final column do not differ significantly at $P \leq 0.05$ (Tukey's HSD).



Treatment means of three colonies · Days 0–120

Fig 2: Total free amino acid content of worker bees (µg leucine equivalents g^{-1} fresh weight) under the same treatments over 120 days.

Discussion

Supplement quality is imprinted on honey and worker FAA: Colonies fed the chickpea + wheat germ patty (T_6) accumulated the most FAA in both honey and workers, and the defatted soybean-based patties (T_1, T_2) followed. The ranking $T_6 > T_1 \approx T_2 > T_4 > T_3 \geq T_5$ matched the ranking of diet consumption (66.42, 57.00, 56.25, 50.17, 44.21 and 28.92 g $colony^{-1}$ for T_6, T_1, T_2, T_4, T_3 and T_5), and the same diet ordering is seen in brood, drone, honey, weight and strength responses. This is consistent with reports that chickpea flour, wheat germ and soybean-based diets perform well (Younis, 2019; Ghramh and Khan, 2023; Painkra *et al*., 2022) [8, 14, 26] and with the view that palatability and amino acid balance, rather than protein quantity alone, govern supplement value (Standifer, 1967; Noordyke and Ellis, 2021; Paray *et al*., 2021) [13, 17, 19]. Our design cannot separate the contribution of higher intake from that of amino acid composition, because the two covaried; crude protein and amino acid profiles of the formulations were not determined.

Why honey FAA rises: During nectar ripening, workers add salivary and glandular secretions to the maturing honey, so FAA is partly a bee-derived signal (White, 1975; Iglesias *et al*., 2004) [9, 24]. Better-nourished nurse and house bees, with larger hypopharyngeal-gland and amino acid reserves (Brodschneider and Crailsheim, 2010; Alaux *et al*., 2010) [1, 4], may therefore transfer more nitrogenous compounds to honey. The near-flat control and the monotonic divergence from Day 30 are compatible with this explanation. Total FAA values in our honeys (about 30–88 mg LE 100 g^{-1}) lie within the range reported for honeys from other regions by chromatographic methods (Dimins *et al*., 2022) [7], although values are not directly comparable because ninhydrin yields leucine equivalents and not individual amino acids. A direct contribution from honey in the patties (251 ml kg^{-1}) or from supplement material stored by bees cannot be excluded, but these inputs were common to all six supplemented treatments and so do not explain the differences among them.

Why worker FAA responds less: The relative amplitude was smaller in workers (T_6 1.82-fold) than in honey (2.35-fold). Tissue amino acid pools are under homeostatic control, with surplus amino acids directed to vitellogenin and other proteins instead of being accumulated indefinitely (Amdam and Omholt, 2002; Nelson *et al*., 2007; Amdam *et al*., 2003) [2, 3, 12], and honey is an integrating store that continues to accumulate. Free amino acid status also depends on worker age and role (Crailsheim, 1990; Paoli *et al*., 2014; Tafi *et al*., 2024) [5, 16, 20]. Because workers here were sampled without age standardisation, within-treatment variation (CV 7.6–9.3%) may partly reflect age composition. The increase in worker FAA under adequate diets is in the direction expected if dietary protein elevates the amino acid pool, as reported for protein-fed *A. cerana indica* (Pote *et al*., 2025) [18], but we did not measure haemolymph, hypopharyngeal glands or vitellogenin directly.

FAA as an indicator of colony nutrition: The correlations of +0.92 to +0.98 indicate that FAA in either matrix ranks supplements in the same order as colony growth parameters. Honey FAA, which can be sampled without harming bees, is the more practical indicator. These coefficients rest on seven treatment means, one of which is the control, and are not independent colony-level observations; they show agreement in ranking but do not establish causation, and the common seasonal trend (brood and colony size also rose over time) may inflate associations. Confirmation at colony level and with chromatographic profiles of individual amino acids (notably proline and the essential amino acids) is needed.

Limitations: The trial covered a single pre-summer season at one apiary with three colonies per treatment; honey FAA may also be influenced by nectar sources, which varied through the trial but were common to all colonies; and the ninhydrin assay measures total α-amino compounds, not amino acid composition. The proportion of protein in each formulation was not standardised, so protein level (Zheng *et al*., 2014) [27] cannot be separated from ingredient identity.

Conclusions

1. Protein supplementation significantly increased total FAA in honey and worker bees of *A. cerana indica*, with the response developing from Day 30 and widening to Day 120; the unsupplemented control showed little change.
2. The chickpea flour + wheat germ formulation (T_6) was most effective (honey 62.15 mg LE 100 g^{-1}, 1.85 times control; workers 17,427 µg LE g^{-1}), followed by the defatted soybean-based diets (T_1, T_2); the maize + pea diet (T_5) was least effective and did not raise worker FAA.
3. Honey and worker FAA were strongly correlated with diet consumption and all measured colony growth parameters (r = +0.923 to +0.977), supporting FAA, particularly of honey, as a convenient biochemical indicator of supplement quality and colony nutritional status.
4. The chickpea–wheat germ formulation can be recommended as a locally available supplement for *A. cerana indica* during the pre-summer dearth in the Northern Dry Zone of Karnataka. Multi-season trials with amino acid profiling and haemolymph measures are warranted.

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Conflict of interest

The authors declare no conflict of interest.

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