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Research article

Earthworm farming for enhanced protein upcycling from spent mushroom substrate

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ABSTRACT

Earthworm farming offers a sustainable method to convert organic residual streams into high-quality edible protein, enhancing nutrient recycling and food systems circularity. This study evaluates the effectiveness of earthworm farming in upcycling protein from maize stover through two pathways: directly feeding earthworms on maize stover or feeding earthworms spent mushroom substrate (SMS) derived from oyster mushroom cultivation on maize stover. Two earthworm species, *Eisenia fetida* and *Eudrilus eugeniae*, were farmed in mesocosms for 37 days and assessed for their biomass gain, protein yield, and essential amino acid composition. Results show significantly enhanced biomass gains when earthworms were fed SMS compared to maize stover alone, attributed to the lower carbon-to-nitrogen (C/N) ratio of SMS. *E. fetida* demonstrated 19.7 % higher total amino acid content, while the amino acid profiles of both species were nutritionally relevant, especially for lysine and tryptophan—critical for regions like Sub-Saharan Africa experiencing protein deficiencies linked to maize-based diets. Using Ugandan maize stover yields, we estimated that consecutive oyster mushroom and earthworm farming could upcycle up to 29 kg of crude protein per hectare annually, enhancing protein upcycling by 115 % compared to mushroom cultivation alone and by 238 % compared to direct stover-to-earthworm conversion. This highlights that, despite practical and logistical challenges, this waste-to-protein pathway offers significant potential for small-scale producers in resource-limited settings to enhance food security and profitability. Further research to optimize feed-specific stocking rates, and develop cost-effective technologies for small-scale production in Sub-Saharan Africa is essential to maximize protein upcycling and scalability.

1. Introduction

Earthworm farming, also known as vermiculture, is a promising approach to produce high-quality protein for food and feed applications from organic residual streams, thereby contributing to food systems circularity (Lowe et al., 2023). Earthworms are protein-rich, low in fat, and contain essential amino acids, important fatty acids, minerals and vitamins (Sonntag et al., 2023). Wild harvested earthworms have long been a traditional food source in diverse cultures worldwide, from New Zealand (Benham, 1904) to China and Japan (Ding et al., 2019) and as far as Venezuela (Paoletti et al., 2003). In the search for new, healthy, and sustainable food sources (Duluins and Baret, 2024), farmed earthworms have been proposed as an alternative protein source for food applications (Sabine, 1983; Sun and Jiang, 2017; Zhenjun et al., 1997). Moreover, litter-dwelling (epigeic) earthworms have been farmed on an industrial scale for decades, primarily to produce vermicompost, a high-quality soil amendment, and earthworms for fishing bait and animal feed (Edwards et al., 2011; Sherman, 2018). A wide range of organic residual streams can be used as feed in earthworm farming (Edwards et al., 2011). However, not all are suitable when producing earthworm

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protein for food applications due to food safety concerns (Conti et al., 2019; Tedesco et al., 2020). Therefore, it is essential to assess the productivity of earthworms when farmed on alternative organic residual streams that are considered food safe.

Spent mushroom substrate (SMS), a by-product from edible mushroom cultivation, is a promising feed material for earthworm farming, but its potential for earthworm protein upcycling remains largely unexplored. The globally expanding mushroom industry generates significant quantities of SMS (Royse et al., 2017), which can pose environmental and potential public health risks due to nutrient leaching if left untreated (Domínguez-Gutiérrez et al., 2022; Yang et al., 2023). SMS primarily consists of mycelium and partially decomposed lignocellulose-rich plant material. Due to its recalcitrant chemical composition and often high salt contents, SMS has limited suitability for direct use as an organic soil amendment (Hřebečková et al., 2020a; Mukhopadhyay, 2023).

Vermicomposting has been shown to improve the fertilizer quality of SMS by enhancing plant-available nutrients, pH, salt content, physical characteristics and microbial diversity (Devi et al., 2020; Patra et al., 2022; Yu et al., 2022). Recent studies have also demonstrated that earthworm farming on SMS can produce considerable earthworm biomass gains (EBG), with increases of up to 399 % relative to the initial earthworm biomass (Bakar et al., 2014; Domínguez-Gutiérrez et al., 2022), with minimal variation between species (Purnawanto et al., 2020). Other studies, in contrast, report more modest EBG of only 86 % on SMS (Yang et al., 2023). Variation in EBG has been linked to the substrate properties, which are influenced by the initial composition of the substrate and the mushroom species cultivated (Hřebečková et al., 2020b; Yue et al., 2019). For example, the high lignocellulose content (0.4 mg g^{-1}) in SMS from shiitake (*Lentinula edodes*) cultivation has been shown to hinder earthworm growth and increase mortality (Shi et al., 2020). Based on comparison of the cited studies, spent oyster mushroom substrate (*Pleurotus* spp.) appears to be the most suitable feed for earthworms. Despite its potential, the extent to which earthworm farming can effectively upcycle protein from SMS remains an under-researched area in the current literature.

The need to increase sustainable protein production for healthy human nutrition is especially urgent in regions with high population growth and limited farmland, such as Sub-Sahara Africa (SSA), where per capita protein intake is low and undernourishment rates are high (Brice and Garnett, 2022). Maize is the most widely cultivated staple crop in SSA (Tesfaye et al., 2015). Utilizing maize stover for oyster mushroom cultivation is being explored as an efficient, low-tech strategy to produce additional protein without expanding agricultural land. Subsequent use of SMS as feed for earthworm farming could further enhance protein upcycling without requiring additional land. Alternatively, maize stover could be fed directly to earthworms, making it essential to determine which pathway yields more edible protein. While some earthworm species may better utilize specific waste streams (Dominguez and Edwards, 2011), the comparative efficiency of different species in upcycling SMS and maize stover has received limited attention. Moreover, investigating the essential amino acid profiles of earthworms fed on these materials is crucial, yet this aspect remains largely unexplored (Sonntag et al., 2023), despite its importance for addressing undernutrition linked to specific amino acids (IPES-Food, 2022).

To address these knowledge gaps, we quantified the biomass gain and nutritional composition of two earthworm species, *Eisenia fetida* (Savigny, 1826) and *Eudrilus eugeniae* (Kinberg, 1866), farmed on SMS derived from oyster mushroom cultivation on maize stover, as well as on maize stover alone. Maize stover yields were quantified and stover samples collected during prior fieldwork in Uganda. This allowed us to calculate the potential annual protein yield per hectare for two pathways: direct stover–to–earthworm conversion versus stover–to–mushroom–to–earthworm, within this specific case study.

2. Material and methods

2.1. Feed materials

Maize stover was sourced from a farm in Kabasekende, Kibaale district, Uganda, dried at 40 °C, and milled to ≤4 mm. It was then either fed directly to earthworms, or used to cultivate grey oyster mushrooms (*Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm.) before being fed to earthworms as SMS.

To cultivate the mushrooms, the milled stover was filled into 14 mushroom grow bags (25 × 50 cm PVC EgBert brand) equipped with micropore filters. The stover was adjusted to 75 % moisture content with water, autoclaved at 121 °C and 15 psi, and inoculated with 2.5 g fresh matter (FM; 42.68 % DM) wheat grain spawn (strain no.: 101,001, Tyroler Glückspilze®, AUT) per 100 g FM maize stover after cooling. The grow bags were then sealed and incubated for 51 days in climate-controlled grow chambers, producing up to two harvests. During the colonization phase (day 0–18), the temperature was maintained at 21–25 °C and relative humidity at 85 %. For primordia and fruit body formation (day 19–51), the temperature was kept at 20–22 °C and relative humidity at 95 %. During mushroom cultivation, 100 g dry matter (DM) of mushroom substrate, composed of 95.91 g DM maize stover and 4.09 g DM oyster mushroom spawn, were converted to 60.37 (±4.29) g DM SMS, resulting in a stover to SMS conversion-rate of 0.63. Only the first flush of oyster mushrooms was harvested, yielding on average 45.02 g FM (13.78 % DM). Table 1 shows the chemical composition of the described materials.

2.2. Earthworm procurement

The earthworms used in this experiment were juvenile *E. fetida* and *E. eugeniae*, representing commonly farmed, litter-dwelling (epigeic) species from temperate and tropical climates, respectively (Dominguez and Edwards, 2011). These species perform well in Sub-Saharan Africa and were selected for their high biomass gains, fecundity, and resilience to handling and variable environmental conditions (Kabi et al., 2020; Reinecke et al., 1992). They were sourced from pure cultures (*E. fetida* from ECT Oekotoxikologie GmbH, DE; *E. eugeniae* from Best Buy Worms, FL, USA) and propagated as stock cultures at Thünen Institute of Organic Farming (Römbke et al., 2016). The stock cultures were maintained at 25 ± 2 °C in plastic containers (568 × 368 × 116 mm) covered with lids ventilated by thirteen 8 mm holes. Each container was filled with 5 kg FM of a base substrate mix consisting of coconut coir, pine bark mulch, mature vermicompost, and mature green waste compost at a 4:2:1:3 FM ratio, with a top-layer of 3 kg FM SMS (*P. ostreatus* on maize stover). Cultures were fed weekly with approximately 500 g of fresh cow manure.

After six weeks, mature earthworms were separated from the composted substrate mix and transferred to new containers with fresh substrate mix. The remaining composted substrate, now containing

Table 1
Total carbon (C) and nitrogen (N) contents as percentage of dry matter, and C/N-ratio for maize stover, oyster mushroom spawn, mushroom substrate (= stover + spawn), spent mushroom substrate (SMS) and harvested oyster mushrooms. Numbers in brackets show standard deviation.

Material	n	C (% DM)	N (% DM)	C/N ratio
Maize stover	6	47.44 (±0.39)	0.58 (±0.10)	84.81 (±16.70)
Spawn	1	43.79	2.87	15.24
Mushroom substrate	calculated	47.29	0.67	70.19
SMS	14	45.23 (±0.25)	0.79 (±0.12)	54.26 (±2.08)
Oyster mushrooms	14	45.76 (±0.22)	2.67 (±0.31)	17.37 (±1.92)

earthworm cocoons, was transferred to separate containers to facilitate the hatching of juvenile earthworms. Juveniles grew for approximately 40 days, depending on the slightly variable hatching time. At the start of the experiment (day 0), juveniles were hand-sorted from the substrate and transferred to aerated 650 mL plastic containers. Remaining substrate was carefully removed, and the juveniles were kept moist and in dark conditions for 60–120 min before being introduced into the mesocosms for the experiment.

2.3. Earthworm farming

The collected juvenile earthworms were farmed in mesocosms, which consisted of clear plastic containers (1800 mL; 192 × 128 × 106 mm) containing the feed material. Six 8 mm holes were drilled into the container lids and covered with micropore tape to reduce evaporation and prevent earthworm escapes while ensuring adequate ventilation. Ten mesocosms were filled with 600 g FM of SMS, while another ten mesocosms were filled with maize stover, after adjusting the moisture content of the feed materials to 80 % with water.

The treatments in this experiment were normalized based on earthworm biomass, with 1 g of FM earthworm added for every 100 g of FM feed material. Due to species size differences, this resulted in varying numbers of earthworms per species. On average, 93 ± 9.91 juveniles of *E. fetida* and 31 ± 1.73 juveniles of *E. eugeniae* were added, with average fresh weights of 66 ± 0.01 mg and 195 ± 0.01 mg FM per individual, respectively, achieving a total of 6 g FM earthworms per mesocosm.

In total, 20 mesocosms were prepared, representing four combinations of earthworm species and feed materials, with five replicates per combination. The mesocosms were incubated in dark conditions at 25 ± 2 °C for 37 days, at which biomass had peaked in pre-studies. At the end of the incubation period, earthworms in each mesocosm were counted, their total biomass (FM) measured, and all earthworms were frozen at −20 °C without prior gut voiding.

2.4. Compositional analyses

Earthworms were freeze-dried and ground into a homogeneous powder using mortar and pestle. The DM content was determined during the lyophilisation process (Christ Alpha 1–4 LSC plus with LyoCube 4–8, Martin Christ Gefriertrocknungsanlagen GmbH, DE) by weighing the samples before and after drying. The drying conditions were: −20 °C and a vacuum of 1 mbar until a sample temperature of 24 °C was reached; drying was completed after 24 h with a vacuum of 0.1 mbar. Feed materials and oyster mushrooms were oven-dried at 40 °C for 48 h, and DM content was determined by weighing the samples before and after drying. The dried samples were then homogenized using a colloid grinder (StarBeater, VWR®).

Total carbon and nitrogen were determined by total combustion (Flash*Smart*™ Elemental Analyzer, Thermo Scientific™), and the carbon-to-nitrogen (C/N) ratio was calculated. Amino acids were quantified using high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Samples were prepared for HPLC analysis by oxidation and hydrolysis according to Commission Regulation (EC) No 152/2009, Appendix III, Method F for all amino acids except tryptophan, where method G was used (EC, 2009). The subsequent derivatization and quantification were performed according to Cohen and Michaud (1993), with 2-aminobutyric acid (Carl Roth GmbH & Co KG, DE) used as the internal standard. The entire adapted analytical procedure is detailed in Witten et al. (2020). Quality control for each analytical run included the use of the internal standard 2-aminobutyric acid and an in-house control sample (feed sample from the annual official VDLUFA feed survey in Germany, in which the laboratory regularly participates) from preparation to quantification.

2.5. Statistical analyses

Statistical analyses were performed using R (R Core Team, 2023) and RStudio (Posit team, 2024). To validate normal distribution and homogeneity of variance, Shapiro-Wilk and Levene's tests were applied, respectively. Due to violations of normality in several cases and the small sample size ($n = 5$), we used Aligned Rank Transformation (ART) consistently for all response variables. This was followed by a two-way analysis of variance (ANOVA) to examine the effects of earthworm species, feed materials and their interaction on the response variables. A significance threshold of $p < 0.05$ was adopted. P-values for statistical tests are presented in supplementary Table A1.

2.6. Calculations

To explore protein upcycling through earthworm farming at the farm level in Uganda, we calculated the potential protein yield obtainable from utilizing all maize stover available per hectare per year for two different pathways. The first pathway involved cultivating oyster mushrooms on maize stover, followed by earthworm farming on the resulting SMS. The second pathway directly used maize stover as earthworm feed. Protein yields from mushrooms and two earthworm species were calculated based on biomass gains and nitrogen contents (N x 6.25) quantified in this study (see Section 2.4). The mushroom protein yield was adjusted to account for the nitrogen content of the mushroom spawn, as this external nitrogen source was not added when earthworms were fed directly on maize stover. The calculated mushroom and earthworm protein yields were then multiplied by the average annual maize stover yield of 4.69 Mg/ha from the Ugandan farm where the stover was sourced. The calculations also account for material reduction based on the measured stover-to-SMS conversion rate of 0.63 (see Section 2.1).

Nitrogen conversion efficiency (NCE) was determined by dividing the nitrogen content (g) in the harvested mushroom or earthworm biomass by the nitrogen content (g) of the corresponding feedstock (e.g., maize stover) and multiplying the result by 100.

3. Results

3.1. Productivity of earthworm farming

Farming earthworms on SMS produced significantly higher total EBG compared to maize stover in both earthworm species (Fig. 1, A.; $p_{E.eug.} < 1e^{-4}$, $p_{E.fet.} = 1.7e^{-3}$). In *E. fetida*, EBG on SMS was 3.15 times higher than on maize stover, while in *E. eugeniae*, it was 2.00 times higher. Regardless of feed material, *E. fetida* showed higher EBG than *E. eugeniae*, but this difference was statistically significant only on SMS ($p_{SMS} < 1e^{-4}$), where EBG was 2.02 times higher.

Individual EBG (Fig. 1, B.), calculated to account for differences in the number of earthworms introduced (see Section 2.3), revealed a consistent substrate effect but an opposite trend in species performance compared to total EBG. Specifically, *E. eugeniae* achieved significantly higher individual EBG than *E. fetida*, being 1.48 times higher on SMS and 2.21 times higher on maize stover ($p_{SMS} = 1.87e^{-2}$, $p_{stover} = 3.75\ e^{-2}$).

Overall, earthworm farming was more productive on SMS, yielding higher total and individual EBG for both species. However, due to differences in the initial number of earthworms introduced, the relative productivity of the two species remains inconclusive.

3.2. Nutritional composition of farmed earthworms

The DM content of the harvested earthworm biomass was consistent across treatments, with an average of 13.5 % FM (Table 2). Nitrogen content was significantly higher in *E. fetida* compared to *E. eugeniae*, being 1.15 times higher on SMS ($p_{SMS} < 1e^{-4}$) and 1.21 times higher on maize stover ($p_{stover} < 1e^{-4}$). The feed materials had a minor effect on

Fig. 1. Mean total (A.) and individual (B.) earthworm biomass gain for two earthworm species farmed for 37 days on maize stover alone or spent mushroom substrate (SMS) from oyster mushroom cultivation on maize stover. Letters, where they differ, denote significant differences between treatments ($p < 0.05$). Error bars indicate standard error ($n = 5$). Note that y-axes differ in scale by factor ten.

Table 2
Means and standard deviation (StD) per treatment (species x feed; $n = 5$) for contents of dry matter (DM), nitrogen (N), total amino acids (TAA), and amino acids of two earthworm species farmed on maize stover and spent mushroom substrate (SMS). Letters, where they differ, denote significant differences between treatments ($p < 0.05$).

Earthworm species	*Eisenia fetida*				*Eudrilus eugeniae*			
Feed material	Maize stover		SMS		Maize stover		SMS	
	mean	StD	mean	StD	mean	StD	mean	StD
DM (% FM)	14.00^{a}	0.26	13.35^{a}	0.19	13.58^{a}	0.49	13.10^{a}	0.86
N (% DM)	10.28^{a}	0.15	10.24^{a}	0.16	8.95^{b}	0.16	8.50^{c}	0.08
TAA (% DM)	58.14^{a}	0.81	58.49^{a}	0.48	49.23^{b}	0.15	48.19^{c}	0.40
Essential amino acids (g/kg DM)								
Histidine	19.72^{a}	0.20	19.78^{a}	0.30	16.29^{b}	0.25	16.34^{b}	0.51
Isoleucine	27.31^{a}	0.45	27.40^{a}	0.31	23.18^{b}	0.45	22.35^{b}	0.70
Leucine	48.45^{a}	0.60	48.55^{a}	0.31	41.39^{b}	0.26	40.23^{c}	0.45
Lysine	43.72^{a}	0.74	43.71^{a}	0.60	38.60^{b}	0.48	37.59^{b}	1.37
Methionine	11.25^{a}	0.22	10.97^{a}	0.28	8.89^{b}	0.18	8.72^{b}	0.05
Phenylalanine	25.60^{a}	0.37	26.03^{a}	0.18	22.60^{b}	0.16	22.56^{b}	0.61
Threonine	30.53^{a}	0.44	31.16^{a}	0.18	25.64^{b}	0.22	25.24^{b}	0.58
Tryptophan	8.98^{a}	0.07	9.30^{a}	0.18	7.14^{b}	0.30	6.69^{c}	0.26
Valine	29.55^{a}	0.48	29.99^{a}	0.44	25.63^{b}	0.60	24.92^{b}	0.76
Semi-essential amino acids (g/kg DM)								
Cysteine	9.21^{a}	0.28	9.12^{a}	0.22	7.63^{b}	0.13	7.29^{b}	0.08
Tyrosine	20.24^{a}	0.29	20.80^{a}	0.19	17.04^{b}	0.25	16.94^{b}	0.61

nitrogen content, which was only significant in *E. eugeniae*, containing 1.05 times more nitrogen when fed maize stover compared to SMS ($p_{E.eug.} = 5e^{-4}$).

We further observed significant differences in the total amino acid (TAA) content per kg DM between the two earthworm species, with *E. fetida* exceeding *E. eugeniae* by 9.6 % DM on average across feed materials (Table 2; $p_{SMS} < 1e^{-4}$, $p_{stover} < 1e^{-4}$). Feed had a small effect on TAA that was only significant in *E. eugeniae* ($p_{E.eug.} = 2.75e^{-2}$), being 1.02 time higher when fed stover compared to SMS. Individual essential and semi-essential amino acids varied significantly between species. The largest relative differences were observed in Tryptophan, Methionine and Cysteine contents, with *E. fetida* showing 1.32, 1.26, and 1.23 times higher concentrations, respectively, compared to *E. eugeniae* across feed materials. In absolute terms, the largest differences were found in Leucine, Lysine and Threonine contents, with 7.69, 5.62, and 5.41 g/kg DM, respectively. Absolute differences in some non-essential amino acids were even more pronounced (supplementary Table A2). Feed material had a slight but significant effect on the DM content of Leucine and Tryptophan, though this effect was only evident in *E. eugeniae*.

To account for potential variation introduced by differences in gut content between the two earthworm species, we compared amino acid profiles as a percentage of TAA (Fig. 2). This analysis revealed only minor, yet in eight cases significant, qualitative differences in amino acid composition between species. *E. fetida* had higher levels of Methionine ($p < 1e^{-4}$), Threonine ($p = 4.1e^{-2}$), Tryptophan ($p < 1e^{-4}$), and Cysteine ($p = 1.08e^{-2}$), while *E. eugeniae* contained more Leucine ($p = 3.3e^{-3}$), Lysine ($p = 1e^{-3}$), Phenylalanine ($p < 1e^{-4}$), and Valine ($p = 4.93e^{-2}$). The effect of feed materials on amino acid profiles was limited, affecting Cysteine ($p = 4.8e^{-2}$), Leucine ($p = 1.93e^{-2}$), Phenylalanine ($p = 2.4e^{-2}$) and Tyrosine ($p = 4.85e^{-2}$; supplementary Table A3).

Overall, while *E. fetida* contained higher levels of nitrogen, as well as total and individual essential and semi-essental amino acids, the

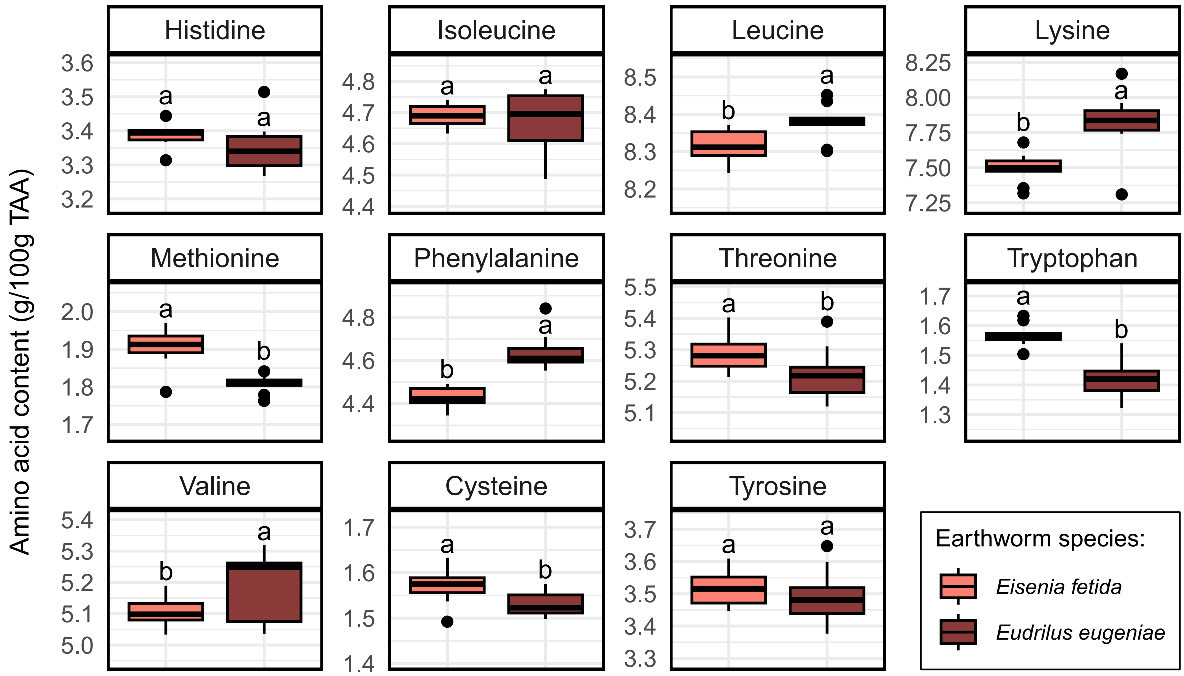

Fig. 2. Content of essential and semi-essential amino acids in g/100 g total amino acids (TAA) for two farmed earthworm species as means of both feed materials, maize stover and spent mushroom substrate ($n = 10$). Note that y-axes were adjusted to the data range of individual amino acids. Letters, where they differ, denote significant differences between species ($p < 0.05$). The whiskers indicate the data range within 1.5 times the interquartile range from the first and third quartiles, with dots showing outliers.

essential amino acid profiles were similar for both species.

3.3. Estimated protein upcycling potential

The cultivation of oyster mushrooms produced an average of 6.46 (±2.06) g DM mushrooms, with a nitrogen content of 2.67 (±0.31)% (Table 1), per 100 g DM of maize stover. Utilizing annual maize stover yields from 1 ha at an Ugandan case study location for consecutive oyster mushroom cultivation and earthworm farming, we estimate that, on average, 13.69 kg of crude protein (N x 6.25) can be obtained from

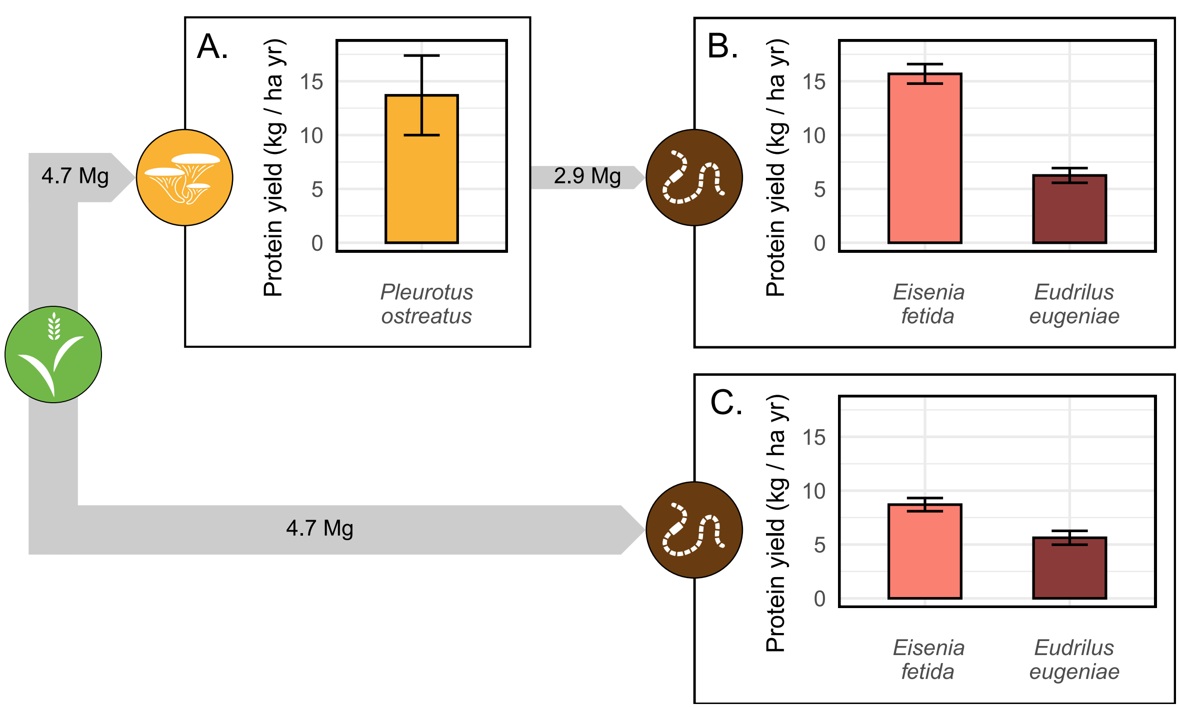

Fig. 3. Protein upcycling from 4.7 Mg ha^{-1} yr^{-1} Ugandan maize stover was assessed through two pathways: (A.) stover used for oyster mushroom cultivation, (B.) followed by earthworm farming on the resulting 2.9 Mg ha^{-1} yr^{-1} spent mushroom substrate, versus (C.) stover fed directly to the earthworms. Potential annual protein yield per hectare was calculated based on biomass gain and crude protein content ($N \times 6.25$) of the earthworm species and oyster mushrooms, with nitrogen added through mushroom spawn subtracted.

mushrooms, 15.69 kg from *E. fetida*, and 6.25 kg from *E. eugeniae*, assuming that all SMS is fed to the respective earthworm species (Fig. 3, A. & B.). These outputs correspond to NCEs of 8.1 % for oyster mushrooms, 11.3 % for *E. fetida,* and 4.5 % for *E. eugeniae*. Total annual protein yield per hectare could reach 29.38 kg through the combined mushroom and *E. fetida* pathway, and 19.94 kg through the mushroom and *E. eugeniae* pathway, reflecting NCEs of 19.4 % and 12.6 %, respectively. In contrast, direct earthworm farming on maize stover without prior mushroom cultivation would result in significantly lower protein upcycling, yielding 8.70 kg of protein for *E. fetida* and 5.62 kg for *E. eugeniae*, with NCEs of 5.2 % and 3.3 %, respectively (Fig. 3, C). Overall, earthworm farming on SMS considerably enhanced potential protein upcycling, compared to both mushroom cultivation and earthworm farming on maize stover alone.

4. Discussion

4.1. Productivity of earthworm farming

This study is the first to directly compare the productivity and nutritional composition between earthworms fed maize stover alone versus SMS derived from oyster mushroom cultivation on maize stover. We observed substantial biomass gains in earthworms fed SMS, consistent with previous studies (Ahmad et al., 2020; Bakar et al., 2014; Hřebečková et al., 2020a; Purnawanto et al., 2020; Yang et al., 2023), and expand existing knowledge by showing that these gains exceed those of earthworms fed the initial substrate, maize stover (Fig. 1). This difference is likely due to the contrasting C/N ratios of the two feed materials (Table 1), which may enhance nitrogen use efficiency in SMS (Aira et al., 2006; Cappellozza et al., 2019; Curry and Schmidt, 2007). The lower C/N ratio in SMS compared to maize stover can be attributed to two factors: first, the addition of nitrogen-rich mushroom spawn reduced the C/N ratio from 85 in maize stover to 70 in the mushroom substrate; second, mycelial respiration during mushroom cultivation further decreased the C/N ratio of SMS to 54 (Table 1). Additionally, the activity of extracellular fungal enzymes present in SMS after mushroom cultivation (Ko et al., 2005; Lim et al., 2013) may facilitate nutrient release and promote biomass gain during earthworm farming.

Despite achieving significant EBG of up to 129 % on SMS, productivity was 2.6–3.1 fold lower compared to previous studies using the same feed material and earthworm species (Domínguez-Gutiérrez et al., 2022; Purnawanto et al., 2020). This discrepancy may be attributable to various factors, including feed availability, particle size, initial earthworm weight and maturity, cultivation cycles, total duration of mushroom cultivation, and the type and composition of the initial mushroom substrate.

A critical factor in earthworm farming is the C/N ratio of the feed material, which influences microbial activity and consequently the availability of nutrients for earthworm growth (Aira et al., 2006; Biruntha et al., 2020; Cappellozza et al., 2019). The SMS used in our study has a relatively high C/N ratio of 54.26, which falls outside the optimal range of 25–30 for earthworm farming (Sherman, 2018), and was 1.1–5.4 fold higher than the feed used in aforementioned studies (Domínguez-Gutiérrez et al., 2022; Purnawanto et al., 2020). Prior research has shown that decreasing the C/N ratio of SMS with nitrogen-rich additives, such as ruminant manure or sewage sludge, can enhance EBG compared to pure SMS (Bakar et al., 2011; Patra et al., 2022; Yang et al., 2023). Conversely, amendments with chicken manure negatively affected EBG (Ahmad et al., 2020), indicating that factors beyond the C/N ratio, such as salt levels, also influence productivity. Given potential food safety concerns related to animal manures, further investigation into nitrogen-rich organic residual streams, such as kitchen or green wastes, as additives to SMS could provide advantages for producing edible earthworm protein (Gong et al., 2019;Ruangjanda et al., 2022).

Another critical factor influencing EBG is farming duration (Domínguez et al., 2017). The extended cultivation periods of 75 or 90 days in the aforementioned studies, which are more than twice the duration of our study (Domínguez-Gutiérrez et al., 2022; Purnawanto et al., 2020), likely contributed to their higher EBG. These longer durations enabled earthworms to complete one to two life cycles, thereby increasing EBG through reproduction (Dominguez and Edwards, 2011).

The impact of earthworm species on productivity in this study was inconclusive. While *E. fetida* exhibited higher total EBG, *E. eugeniae* demonstrated greater individual EBG. Previous research indicates that higher stocking densities increase total EBG but reduce individual EBG (Domínguez, 2018; Sun, 2003). This pattern has been observed in *E. eugeniae*, *E. fetida*, and *Lumbricus rubellus* when farmed on SMS, where EBG did not differ between species (Purnawanto et al., 2020). Consequently, the differences in EBG observed in our study may partly stem from the varying initial earthworm numbers used to compensate for species-specific weight differences (see Section 2.3), rather than productivity alone. From a practical perspective, farming smaller earthworms at higher stocking densities is likely to result in higher protein yields, similar to findings with commonly farmed black soldier fly larvae (Barragan-Fonseca et al., 2018). To further optimize earthworm growth efficiency, future research should examine the relationship between stocking density and feed C/N ratio (Mnkeni and Mupambwa, 2023) across locally sourced SMS to develop feed-specific stocking strategies.

4.2. Nutritional quality of farmed earthworms

Our analyses confirmed the high nutritional quality of earthworms regarding total amino acid content and composition, consistent with previous reports (Sonntag et al., 2023; Sun and Jiang, 2017). *E. fetida* exhibited significantly higher levels of both total and individual amino acids compared to *E. eugeniae* (Table 2). However, when amino acid profiles were expressed as a percentage of total amino acids, these differences were notably reduced and, in some cases, reversed (Fig. 2). The observed variations were within the range reported for different earthworm species fed identical diets in previous studies (Graff, 1981; Reinecke et al., 1991), and also comparable to intra-species variation due to dietary differences (Alcívar-Cedeño et al., 2016). This raises the question of whether the observed variation reflects a genuine nutritional difference between species or is partially an artifact of the differing number and size of earthworm individuals used (see Section 2.3). The higher number of relatively small E. *fetida* may have increased the tissue-to-gut content ratio in the harvested earthworm biomass, potentially contributing to the higher amino acid contents observed in comparison to the larger *E. eugeniae*. To minimize such potential distortions and better replicate real world earthworm farming conditions, future studies should utilize larger-scale rearing containers with mixed age populations, periodical sub-sampling, and extended cultivation periods.

While the total amount of protein is nutritionally important, amino acid composition is crucial for determining protein quality (Ghosh et al., 2012; Semba, 2016). Common amino acid deficiencies in humans include lysine, threonine, cysteine, methionine and tryptophan (Hambræus, 2014). Diets centred on maize, prevalent in SSA, often lack sufficient tryptophan and lysine (Nuss and Tanumihardjo, 2011). Our findings indicate that farmed earthworms can supply these essential and semi-essential amino acids at nutritionally relevant levels. When compared to whey protein, a typical reference for protein quality, earthworm protein contained 104–109 % of Lysine, 92–93 % of Threonine, 113–119 % of Methionine, 81–83 % of Cysteine and 95–105 % of Tryptophan (Babault et al., 2015), although whey protein composition can vary (Almeida et al., 2016; Naidoo et al., 2018). All of these, except Lysine, are found at higher concentrations in *E. fetida* than in *E. eugeniae*, potentially indicating slightly greater nutritional value of the former species. However, factors such as odour-active compounds may limit the direct suitability of *E. fetida* as food without further processing, such as delipidation (Bou-Maroun and Cayot, 2011).

In summary, these findings highlight the high protein content and quality of farmed earthworms, with *E. fetida* upcycling greater quantities of nutritionally important amino acids than *E. eugeniae*, although protein quality, based on amino acid profiles, was similar for both species.

4.3. Potential and limitations for protein production

We demonstrated significant potential for protein upcycling from maize stover through successive oyster mushroom cultivation and earthworm farming on the farm level in a specific Ugandan study area (Fig. 3). Earthworm farming on SMS enhanced protein upcycling by up to 115 % compared to mushroom cultivation alone. The combined approach of mushroom and earthworm farming yielded 238 % more protein than direct earthworm farming on maize stover. Using all annually available maize stover for this combined pathway could yield up to 29.4 kg of crude protein per hectare. Assuming severely limited farmland availability of 607 m^2 per person in 2100 (Rahmann et al., 2020), this would provide 1.8 kg of protein annually per person. This amount would cover 8.7 % of the annual protein requirement of 20.4 kg for an average 70 kg adult, based on the recommended daily allowance of 0.8 g protein per kg body weight (Institute of Medicine, 2005). In contrast, direct earthworm farming on maize stover upcycled approximately 8.7 kg of protein per hectare per year, covering 2.6 % of protein needs under the same assumptions.

These estimates should be interpreted cautiously due to several methodological and practical limitations. The assumption of 607 m^2 of land per person, while admittedly pessimistic, was used to highlight the potential for protein upcycling under extreme conditions. Protein production was calculated using crude protein (N x 6.25) rather than TAAs, as TAA data were unavailable for oyster mushrooms. Nitrogen added via mushroom spawn was subtracted from the nitrogen harvested with mushrooms, but not all of the nitrogen may have been absorbed by the mushrooms. Some nitrogen likely remained in the SMS, contributing to earthworm growth. As a result, mushroom protein yields may be underestimated, and earthworm protein yields overestimated in Fig. 3. Further research utilizing stable nitrogen (^{15}N) and carbon (^{13}C) isotopes could help disentangle material flows in this upcycling process.

Maize and maize stover yields vary widely across regions and farming systems, even within Uganda (Uganda Bureau of Statistics, 2020), and maize stover availability fluctuates seasonally with maize harvest periods. The dry storage and transportation of large volumes of maize stover present logistical and economic challenges. Additionally, competition from traditional uses, such as livestock feed and soil improvement (Duncan et al., 2016), along with emerging applications (Lwasa et al., 2023; Roobroeck et al., 2019), may further limit its availability for mushroom and earthworm production.

Additionally, mushroom cultivation requires substrate pasteurization, associated with technical, energy and water requirements that could constrain production in certain areas (Grimm et al., 2024). Economic and technical challenges related to harvesting earthworm biomass (Domínguez et al., 2017) and sociocultural acceptance issues, similar to those faced by edible insects in Western markets (Hartmann et al., 2015; Ruby et al., 2015; van Huis, 2020), may hinder the adoption of earthworms as a viable protein source.

Moreover, the food safety of earthworms farmed on maize stover-derived spent oyster mushroom substrate requires thorough assessment. Although the contamination risk from SMS, as a by-product from food production, appears low (Conti et al., 2019; Tedesco et al., 2020), the carry-over of mycotoxins, pesticides, and microbial contaminants from crop residues should be further evaluated in additional studies. In this context, immediate processing of earthworms should occur right after harvest to extend shelf-life (Bou-Maroun et al., 2013). To achieve this in Sub-Saharan Africa, energy-efficient drying methods such as solar drying or tunnel drying, are particularly promising as they preserve protein quality and require minimal equipment (Suárez Hernández et al., 2016).

Previous studies typically used nitrogen-rich SMS from industrial mushroom cultivation as earthworm feed (Domínguez-Gutiérrez et al., 2022; Purnawanto et al., 2020), often without specifying the substrate composition, mushroom species, and cultivation period (Hřebečková et al., 2020b). In contrast, our study highlights the innovative potential for protein upcycling from nitrogen-poor maize stover via successive cultivation of oyster mushrooms and earthworms. This context is important when comparing the NCE of up to 19.4 % achieved here with other seemingly more effective protein upcycling pathways, such as black soldier fly larvae (8–80 %) or yellow mealworm (22–58 %) (Javourez et al., 2021, 2024), which are commonly fed high-quality feeds.

4.4. Profitability for small-scale enterprises

While the practical feasibility of consecutive mushroom and earthworm farming across different geographical locations and scales still requires validation, evidence supports the technical and economic viability of both activities for small-scale producers in Uganda (Jjagwe et al., 2019, 2020; Naome, 2018). Fresh oyster mushrooms are sold for 1.18 to 2.94 USD/kg, generating between 2,420 and 55,520 USD, with an average annual profit of 9,541 USD for small-scale producers in the Kampala metropolitan area, Uganda (Mayanja and Tipi, 2018; Pavlik et al., 2023). Another study found annual profits ranging from 1,760 to 170,400 USD in the same area (Pavlik et al., 2023). The profitability of mushroom farming could be enhanced by replacing the currently used substrate, cotton seed hull—which is becoming increasingly expensive (Serunjogi et al., 2005)—with maize stover, a readily available by-product of Uganda's most widely cultivated staple crop (Uganda Bureau of Statistics, 2020).

For small-scale earthworm farms in Kampala, Lalander et al. (2015) reported a 170–280 % return on investment within five years, when feeding *E. eugeniae* with cow manure. In the same area, feeding *E. eugeniae* with pineapple waste or cow manure generated annual profits of 4,073 to 5,241 USD, with 56–64 % from selling earthworms as animal feed at 19 USD/kg and 4.7–5.8 % from vermicompost at 0.08 USD/kg (Zziwa et al., 2021). Combining mushroom and earthworm farming could diversify the income of small-scale producers while enhancing protein upcycling and profitability of maize stover utilization, ultimately contributing to improved food security in the local community.

Scalability of this combined approach to other regions depends on feedstock availability, market demand, and adaptation of production technology. Mushroom cultivation using crop residues offers economic potential in Sub-Saharan Africa if challenges related to spawn production and storage are addressed (Boukary et al., 2024; Kazige et al., 2022). Although interest in earthworm farming is growing, its potential remains underutilized in Africa due to limited policy support (Chianu et al., 2012; Dada and Balogun, 2023). Both systems offer scalability from low-cost, small-scale setups to capital-intensive, high-tech operations (Sherman, 2018; Stamets, 2011). While low-cost systems are easier to adopt and enhance local food security and circularity, high-tech systems provide controlled environments, reducing reliance on the local climate conditions.

5. Conclusion

This study highlights the potential of earthworm farming as a viable strategy for upcycling edible protein, particularly using spent oyster mushroom substrate derived from maize stover. The higher biomass gains in earthworms fed SMS underscore the importance of substrate quality, with its lower C/N ratio contributing to enhanced productivity. The inconclusive productivity differences between *E. fetida* and *E. eugeniae* suggest that research into optimizing feed-specific stocking densities may be more effective for maximizing protein yields than species selection. While *E. fetida* showed superior amino acid content, both species demonstrated high nutritional quality, with potential to address amino acid deficiencies in maize-based diets, particularly in Sub-Saharan Africa. Combining oyster mushroom and earthworm farming could upcycle up to 29 kg of crude protein per hectare annually, significantly enhancing protein upcycling from maize stover over either approach alone. This strategy aligns with global efforts to enhance food system circularity and tackle protein undernutrition in food-insecure regions. Overcoming challenges such as resource competition, technical barriers, and sociocultural acceptance could transform this waste-to-protein pathway from a promising concept into a practical solution for sustainable protein upcycling.

CRediT authorship contribution statement

Enno Sonntag: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alix Vidal:** Writing – review & editing, Supervision, Conceptualization. **Karen Aulrich:** Writing – review & editing, Data curation. **Daniel Grimm:** Data curation. **Gerold Rahmann:** Supervision, Conceptualization. **Jan Willem Van Groenigen:** Writing – review & editing, Supervision. **Hannah van Zanten:** Writing – review & editing, Supervision. **Alejandro Parodi:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Chat GPT in order to improve wording in several sentences and to help with coding in R. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.125325.

Data availability

Sonntag, E., Vidal, A., Aulrich, K., Grimm, D., Rahmann, G., Parodi, A., 2024. Dataset for Earthworm Farming on Spent Mushroom Substrate and Maize Stover. https://doi.org/10.3220/DATA20241007083224-0.

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