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Research Paper

Bokashi fermentation of brewery's spent grains positively affects larval performance of the black soldier fly *Hermetia illucens* while reducing gaseous nitrogen losses

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ABSTRACT

Digestion of waste feedstocks by larvae of the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) (BSF) results in proteins for animal feed and organic fertilizer with a reduced environmental footprint, but it can still have negative environmental effects through greenhouse gas (GHG) and ammonia (NH_3) emissions. Both biomass conversion by BSF larvae and associated GHG and NH_3 emissions can depend on substrate properties that may be optimized through microbial inoculation pre-treatments, such as bokashi fermentation. Here, we quantified the effects of bokashi fermentation of brewery's spent grains on BSF rearing metrics and associated GHG and NH_3 emissions at benchtop scale. We found that bokashi fermentation increased larval biomass by 40% and shortened development time by over two days on average, compared with unfermented spent grains. In line with increased larval growth, CO_2 emissions in BSF larvae treatments were 31.0 and 79.0% higher in the bokashi fermented spent grains and Gainesville substrates, respectively, compared to the unfermented spent grains. Adding BSF larvae to the spent grains increased cumulative N_2O emissions up to 64.0 mg N_2O kg substrate$^{-1}_{dry}$ but there were essentially no N_2O emissions when larvae were added to fermented spent grains. Bokashi fermentation also reduced NH_3 fluxes from the volatilization of substrate nitrogen in the BSF larvae treatment by 83.7-85.8% during days 7 and 9, possibly by increasing N assimilation by larvae or by reducing the transformation of substrate NH_4^+ to NH_3. Therefore, bokashi fermentation may be applied to improve performance of BSF larvae on a common industrial waste stream and reduce associated emissions.

1. Introduction

The larvae of the black soldier fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae) are important for global efforts to move toward a circular economy model for organic waste management. Unlike other insect livestock, such as crickets and mealworms, BSF can digest a wide variety of feedstocks. As a result, these insects are now reared on an industrial scale as upcyclers of food and agricultural wastes, which they convert into usable products such as protein, fat, and fertilizer (insect manure) (van Huis, 2022). The outputs and negative environmental consequences of BSF production are quantified using life cycle assessments (LCAs), which are tools to make decisions about rearing practices based on a complete evaluation of emissions and identification of possible points for improvements (Smetana et al., 2021). LCAs to date suggest that BSF-based waste upcycling can contribute to a more sustainable circular economy framework, but also demonstrate that BSF production is not energy efficient and generates emissions of nuisance and greenhouse gases (GHG) (Salomone et al., 2017; Smetana et al., 2019; Ites et al., 2020; Guo et al., 2021; Beyers et al., 2023).

GHG emissions trap heat in the atmosphere, and include carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). CO_2, the dominant GHG, is primarily the product of aerobic respiration of carbon sequestered in photosynthesis. Photosynthetic uptake and respiration release occur in roughly equal proportions. The recent atmospheric rise

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in CO_2 levels is mostly due to combustion of fossil fuels (Global Carbon Project, 2022). N_2O is a potent GHG with a global warming potential (GWP) 298 times higher than CO_2 (Myhre et al., 2014), and is a stratospheric ozone depleting substance. Globally, anthropogenic N_2O emissions primarily come from agricultural sources via application of synthetic and organic fertilizers and subsequent microbially-driven nitrification and denitrification processes (Tian et al., 2020). Landfills, composting, and wastewater treatment are also sources of N_2O, emitting 0.2-0.5 Tg N yr-1 globally (Tian et al., 2020). CH_4 is the second most important GHG after CO_2 and has a 100-year GWP 34 times higher than CO_2. CH_4 is produced during decomposition of organic matter and by ruminant livestock, both of which contribute a substantial fraction of global CH_4 emissions (Steinfeld and Wassenaar, 2007; Saunois et al., 2020). GHG emissions occur during industrial-scale conversion of food wastes by BSF, alongside the nuisance gas, ammonia (NH_3) (Chen et al., 2019; Ermolaev et al., 2019; Mertenat et al., 2019; Pang et al., 2020a, 2020b; Parodi et al., 2020). NH_3 is not a GHG, but is hazardous for animals to inhale (Čičková et al., 2015; Lalander et al., 2015) and a precursor for generation of other pollutants, such as 2.5 micron particulate matter ($PM_{2.5}$) and, via nitrogen deposition to ecosystems, production of N_2O (Mosier, 2001; Pleim et al., 2019). Given that BSF production is predicted to grow substantially in the coming decade, quantifying GHG and NH_3 emissions, and the control of rearing practices on these emissions, are essential to measure the negative environmental effects and devise mitigation measures.

At present, there is limited research on GHG and NH_3 emissions from various insect feedstocks. This lack of data makes it difficult to determine whether waste upcycling using insects is more environmentally sustainable than allowing organic wastes to decompose in landfills, or using them for traditional composting (Oonincx et al., 2010; Nordahl et al., 2020). Available research indicates that emissions vary depending on the feedstock C and N composition (Pang et al., 2020b), moisture level (Chen et al., 2019), and pH (Pang et al., 2020a). This variation means there is potential to mitigate GHG and NH_3 emissions by controlling or modifying feedstock characteristics. A promising strategy for feedstock modification is the addition of microbes that participate in substrate breakdown and conversion. Prior work in this area has documented positive effects of microbe inoculations on feedstock quality, as measured by decreasing BSF larval development time, and increasing conversion efficiency and final biomass (reviewed by Jordan and Tomberlin, 2021; Peguero et al., 2021; Gorrens et al., 2023). These studies mostly focused on the addition of a single microbe species as an inoculant and did not document effectiveness of microbes to decrease GHG or NH_3 emissions, with few exceptions. Ermolaev et al. (2019) pretreated or seeded canteen food waste with bacteria isolated from the gut of BSF larvae, but neither inoculation strategy had a significant effect on GHG emissions. Lindberg et al. (2022) treated orange peels and a mixture of broccoli and cauliflower trimmings with *Trichoderma resei* fungi, observing a significant reduction of cumulative CH_4 and N_2O emissions from the vegetable trimmings, but high CO_2 emissions, whereas emissions from treated orange peels were similar to those from the untreated control. Li et al. (2023) showed that addition of lactic acid bacteria or fruit fermentation broth to kitchen waste increased the body weight of BSF larvae by about 8-10%, and, by lowering the pH of the substrate, also reduced NH_3 emissions. This study suggests that microbial modification of feedstocks may be a rapid and cost-effective way to improve rearing metrics and reduce emissions of harmful gases in industrial BSF operations.

We recently showed that the quality of marginal BSF feedstocks can be improved by a brief anaerobic fermentation period using a formulation of lactic acid bacteria, yeasts, and fungi (Gebiola et al., 2023). This process, known as bokashi fermentation, led to increased growth rate and larval weights relative to unfermented marginal feedstocks. Little is known about the impact of bokashi fermentation on GHG or NH_3 emissions. However, Hillberg (2020) recorded significantly reduced CH_4 emissions and a trend toward reduced N_2O emissions when kitchen waste underwent bokashi fermentation as compared to unfermented waste. The author hypothesized that a pre-fermentation of kitchen waste by the bokashi method may lead to reduced GHG emissions during the final stage of aerobic composting under soil, prior to use as fertilizer. Based on these results, and our findings that bokashi fermentation improves substrate nitrogen conversion into protein biomass, we hypothesized that bokashi fermentation will reduce GHG and NH_3 emissions during the BSF rearing process. Working at a benchtop scale, we evaluated the effects of bokashi fermentation of brewery's spent grains – a common industrial feedstock for BSF – on rearing metrics, GHG emissions, and NH_3 production. Brewery's spent grains were chosen as feedstock as a number of studies have shown that it is a nutritious substrate for BSF larvae, often on par with standard diets such as Gainesville or chicken feed (Chia et al., 2018; Liu et al., 2018; Meneguz et al., 2018b; Shumo et al., 2019; Scala et al., 2020; Adebayo et al., 2021; Ceccotti et al., 2022). We used a factorial design, including fermented and unfermented treatments, each with and without BSF larvae. We cannot fully disentangle the contribution of substrate microbial processes, larval metabolism, and their potential synergistic or antagonistic interactions. However, this factorial experimental design allowed us to determine the overall relative contribution of the BSF larval-rearing process as a factor on GHG and NH_3 emissions across different substrates. We predicted that bokashi fermentation would: 1. benefit BSF larvae development and growth rate as already observed at the industrial scale; 2. lead to increased bioconversion efficiency and substrate utilization by larvae; 3. reduce the amount of nitrogen volatilized as NH_3 and possibly also as N_2O relative to unfermented spent grains and/or conventional diet.

2. Materials and methods

A diagram of the experimental design including the substrates used, experimental conditions, factors, summary of methods, and response variables is given in Figure 1.

2.1. Substrate preparation

Brewery's spent grains, donated by a local brewery, were fermented by mixing them with a bokashi starter (premium bokashi bran, Bokashi Living, Vancouver, Canada) that, according to the manufacturer, is made of "enhanced EM-1+ bacterial cultures", where EM stands for Effective Microorganisms. The exact microbial composition of EM-bokashi products is not disclosed. However, it has been reported that it includes predominantly population of lactic acid bacteria (e.g., *Lactobacillus* spp., *Streptococcus* sp.), yeast (e.g., *Saccharomyces* sp., *Candida* sp.), a small proportion of photosynthetic bacteria (e.g., *Rhodopseudomonas* sp., *Rhodobacter* sp., *Rhodospirillum* sp.), actinomycetes (e.g., *Streptomyces* spp.) and fermenting fungi (e.g., *Aspergillus* sp.) (Khaliq et al., 2006; Mayer et al., 2010; Quiroz and Céspedes, 2019; Hillberg 2020). Brewery's spent grains and the bokashi bran were mixed in a 20-liter bokashi composter bin (Bokashi Living) by adding 15 mg of starter to each 2.5 cm thick layer of substrate as per manufacturer's recommendation, until filling the composter bin. Spent grains fermented for four weeks at room temperature. The leachate that accumulated during fermentation below a grate holding up the substrate above a reservoir at the bottom of the bin was removed through a valve at the base of the bin. Unfermented spent grains were collected the day before the experiment, to prevent spoilage associated with long-term storage. In all experiments, we included as a control the Gainesville substrate (Hogsette, 1992), a standard fly diet made by mixing 50% wheat bran (Baker's Authority, Maspeth, NY, USA), 30% alfalfa meal (Walt's Organic Fertilizer Co., Seattle, WA, USA), 20% corn meal (Yummmico, Inc., Hialeah, FL, USA) and hydrating the mixture to 70%. The Gainesville substrate was prepared on the same day the experiment was set up. Fifty grams of each substrate were placed in a 135-mm diameter plastic petri dish (Genesee Scientific, El Cajon, CA, USA) and dried in a mini-

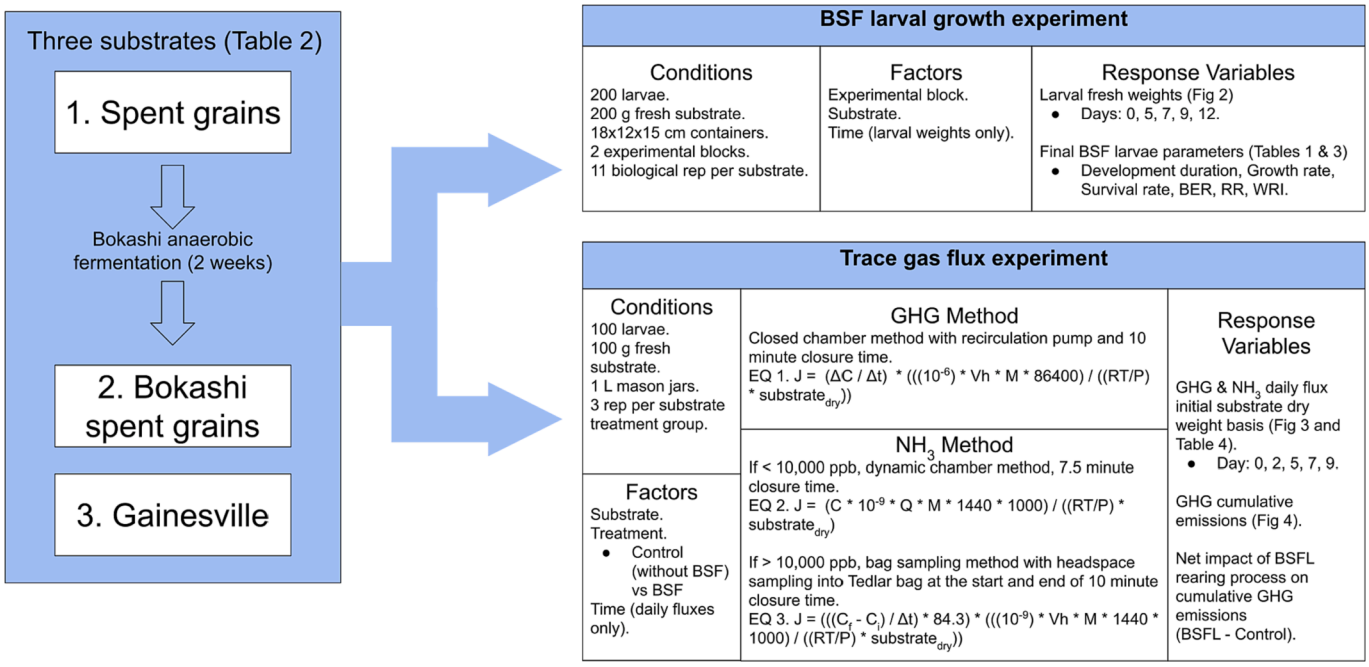

Figure 1. Flow diagram of experimental design. Additional information about the equations used to calculate trace gas flux can be found in the supplemental material.

Table 1
Description of variables measured for black soldier fly larvae

Variable	Description
Development duration	days from 5-DOL to 5% prepupae
Growth rate	(average larval final body weight − average larval initial body weight [mg]) / development duration (days)
Survival rate	larvae + prepupae alive at the 5% prepupal stage (%)
Bioconversion efficiency (BER)	[(Final larval biomass (g) - Initial larval biomass (g)) / (Initial substrate (g) - Frass (g))] x 100%
Substrate reduction rate (RR)	[(Initial substrate (g) - Frass (g)) / Initial substrate (g)] x 100%
Waste reduction index (WRI)	RR/development duration

incubator (VWR, Radnor, PA, USA) at 60°C until a stable weight was recorded at two consecutive daily weighing to get the dry matter and C:N ratio of the substrates (see below).

2.2. Rearing conditions and BSF larval growth experiment

We conducted the experiment in a room with controlled temperature (27 ± 1°C) and humidity (70 ± 5%), and 12:12 L:D photoperiod. Five-day-old larvae (5-DOL) were purchased from EVO Conversion Systems, LLC (College Station, TX, USA). The same batch of purchased BSF larvae were used to set up also the gas emission experiments described below. Three extra sets of 200 BSF larvae were prepared and oven dried as above to get the initial dry weight and C:N ratio (see below). The experimental unit consisted of 200 5-DOL and 200 g of substrate in 18x12x5 cm plastic black food containers (New Century Plastic Industry Inc., San Leandro, CA, USA) closed by a lid with a meshed opening to allow aeration. The experiment was replicated twice, with each treatment (Gainesville, bokashi-fermented spent grains, unfermented spent grains) having six and five biological replicates, respectively. All 200 BSF larvae were weighed before adding them to the substrates, then 15 BSF larvae/unit were gently rinsed to remove feeding substrates, pat-dried and weighed using a precision balance (U.S. Solid, Cleveland, OH, USA) at days 5, 7, 9 and 12, to get the weight dynamics for the first 12 days of the experiment. After weighing, BSF larvae were returned to the units. When at least 5% of BSF larvae became prepupae, the experiment was terminated, BSF larvae were separated from the substrate (at this point referred to as frass) and three life history traits (development duration, growth rate, survival rate, as defined in Table 1) were measured. Subsets of 5 g of BSF larvae placed in 90-mm diameter plastic petri dishes (Genesee Scientific) were oven dried as described above to get the moisture content to calculate the final dry weight of BSF larvae. Fresh frass was placed in 135-mm diameter plastic petri dishes, weighed and oven dried as well. Three BSF larval performance indices, bioconversion efficiency corrected for the substrate (BER), substrate reduction rate (RR) and waste reduction index (WRI), as defined in Table 1, were calculated based on BSF larvae and frass dry weights. pH of the substrates was measured at the beginning of the experiment using a pH meter (Luster Leaf, Atlanta, GA, USA).

2.3. Greenhouse gas and ammonia emissions experiment

We compared GHG emissions of the three substrates (Gainesville, bokashi-fermented spent grains, unfermented spent grains) with (BSF) and without (Control) BSF larvae feeding on them, for a total of six treatments. Each treatment had three biological replicates, for a total of 18 experimental units. For this experiment we used 100 g of each substrate, keeping the 1:1 ratio of substrate:BSF larvae for the treatments with BSF larvae present. Substrates and BSF larvae were housed in 1-liter wide mouth mason jars (Ball, Atlanta, GA, USA), which were kept alongside the experimental units of the concurrent larval growth experiment, hence under the same abiotic conditions reported above. Jars were left uncovered to avoid buildup of gas. CO_2, CH_4, N_2O, and

Table 2
Initial characteristics of substrates for rearing black soldier fly larvae

Substrate	Total carbon (%)	Total nitrogen (%)	C:N
Spent grains	48.0 ± 0.3[a]	4.67 ± 0.1[a]	10.3 ± 0.2[a]
Bokashi spent grains	46.1 ± 0.3[b]	4.04 ± 0.13[b]	11.4 ± 0.3[a]
Gainesville	41.9 ± 0.1[c]	3.14 ± 0.14[c]	13.4 ± 0.5[b]

Initial total carbon content (%), total nitrogen content (%), and C:N ratio of rearing substrates (mean ± SE). Different letters indicate significant differences ($p<0.05$) between substrate means (n=3).

NH_3 were measured at the beginning of the experiment, and on days 2, 5, 7, 9. Initial rearing substrates were oven dried at 60℃, finely ground to 150 μm, and measured for total carbon (C) and total nitrogen (N) via combustion using a Flash EA112 Automatic Elemental Analyzer (ThermoFisher Scientific, Waltham, MA, USA) (Table 2).

2.3.1. Gas flux measurements

Jar lids were modified by addition of two Swagelok bulkhead fittings to enable laboratory gas flux measurements using Picarro cavity ring-down spectrometers (CRDS). GHG flux (N_2O, CO_2, CH_4) was measured using a closed chamber method with Picarro G2308 CRDS equipped with Picarro A0702 external recirculation pump (Picarro, Santa Clara, CA, USA) for a 10-minute closure time (Christiansen et al., 2015). During GHG measurement the recirculation pump allowed for real-time measurement of the accumulation of gas in the jar headspace. Fluxes were calculated using the change in gas mole fraction over time and converting to mass via the jar volume and ideal gas law.

Water vapor corrected NH_3 mole fraction was measured with Picarro 2123 CRDS and all Polytetrafluoroethylene (PTFE, Teflon™) (Chemours, Wilmington, DE, USA) tubing and fittings. Because NH_3 is a sticky molecule, the closed chamber method is not appropriate given surfaces NH_3 could stick to. Instead, NH_3 mole fractions were initially measured using a dynamic chamber system, where the same jar setup described above was connected to the analyzer pumping at 1.77 L per minute from the jar which was connected upstream to a 100 L Tedlar bag filled with NH_3-free zero air, serving as a reservoir to replace chamber air pumped into the analyzer. Using this method, NH_3 flux was calculated by multiplying the measured NH_3 mole fraction at the end of 7.5-minute closure time by the flow rate of air through the chamber system and using the ideal gas law to convert to molar concentration. On day 5, NH_3 mole fractions during dynamic chamber measurement began to exceed the 10,000 ppb operating range of the analyzer in some samples. On day 7 and 9, for treatments where NH_3 mole fractions exceeded the analyzer's operating range, NH_3 was measured using a closed static chamber and bag sampling method. Sample jars were closed with a lid modified with a rubber septa and 60 mL of headspace air was transferred using a plastic syringe and 1.5-inch 20-gauge Luer-Lok™ syringe into a Restek™ Tedlar bag filled with 5L of NH_3-free zero air immediately after lid closing and after 10 minutes closure time. Tedlar bag samples were connected directly to the analyzer for about 2.5 minutes until the bag was emptied. When using the bag sampling method, NH_3 concentration was multiplied by a factor of 84.3 to account for dilution of sampled headspace air injected into a full Tedlar bag. Flux was calculated using the difference in NH_3 mole fraction between the two headspace samples collected over 10 minutes, the headspace volume, and the ideal gas law to convert from volumetric to molar concentration. All daily flux rates were expressed per kg of initial substrate dry weight and cumulative emissions were calculated using trapezoid rule to interpolate between sampling points. Additional details on the equations used to calculate trace gas flux are provided in the Supplementary material. GWP was calculated using a 100-year GWPs of 298 for N_2O and 34 for CH_4. The net contribution of BSF larvae rearing on GHG emissions was calculated from the difference between the BSF and control treatments.

2.4. Statistical analyses

As the experiment was repeated twice, to account for nuisance factors such as batch of brewery's spent grains (obtained from the same brewery but at different times) or temporal changes that might have occurred to the bokashi-fermented spent grains, a blocking effect was considered in the statistical models to increase their power. Development duration, BER, RR and WRI data were rank-transformed, as they did not conform to normality and homoscedasticity. Data on development duration, survival and growth rate were analyzed by a two-way ANOVA model that was fitted using the *lm* function, adding block as factor. We evaluated model residuals (Crawley, 2012) and performed multiple comparisons of the means by the Tukey HSD test using the *HSD.test* function of the R package *agricolae*. To account for the repeated measurements of BSF larvae weight data, these were analyzed by a two-way mixed ANOVA model fitted using the *lmer* function of the R package *lmer4* that included rearing substrates, time and their interaction as explanatory variables, experimental units (the plastic boxes) and block as random factors and daily weights as the response variable. For analysis of BER, RR and WRI data, a mixed model with block as random effect and substrate moisture as random covariate, given an issue that occurred with preparation of the Gainesville substrate at the second experimental block that affected the dry weights, was built. After evaluating model residuals, multiple comparisons of the means by the Tukey HSD test were carried out using the R package *multcomp*. Statistical significance of substrate and treatment factors with time in daily gas fluxes were determined with repeated measures ANOVA. Statistical significance (p value) of substrate and treatment factors in cumulative emissions were determined with a two-way ANOVA and post hoc multiple pairwise comparisons of group means with Bonferroni correction at the 95% confidence level were analyzed using the *rstatix* and *emmeans* packages in R. Due to missing measurements and use of two methods, starting on day 5 statistical analyses for NH_3 were conducted separately for each method on a given day.

3. Results

Initial pH of the rearing substrates before digestion by BSF larvae was 6, 5.5 and 5 for Gainesville, unfermented and bokashi-fermented spent grains, respectively. Total C and N content of the substrates were highest from the unfermented spent grains, followed by the bokashi fermented spent grains, then Gainesville substrate (Table 2). The Gainesville substrate had a significantly higher C:N ratio while the ratio was similar

Table 3
Variables measured for black soldier fly larvae

Variables	Feeding substrates			
	Spent grains	Bokashi spent grains	Gainesville	Unit
Development duration	15.1 ± 0.3[b]	12.5 ± 0.2[a]	12.0 ± 0.0[a]	days
Growth rate	6.2 ± 0.4[b]	9.6 ± 0.4[a]	10.1 ± 0.5[a]	mg/day
Survival rate	94.0 ± 1.0[a]	92.7 ± 1.1[a]	93.6 ± 0.9[a]	%
Bioconversion efficiency (BER)	11.5 ± 0.9[a]	17.8 ± 1.3[b]	9.0 ± 1.0[a]	%
Reduction rate (RR)	62.2 ± 1.6[b]	59.8 ± 1.8[b]	69.1 ± 2.8[a]	%
Waste reduction index (WRI)	4.2 ± 0.2[b]	4.8 ± 0.4[c]	5.8 ± 0.2[a]	

Development duration (mean ± SE) from 5-DOL stage to prepupal stage, growth rate (mean ± SE), survival rate (mean ± SE), bioconversion efficiency corrected for the substrate (BER, mean ± SE), substrate reduction rate (RR, mean ± SE) and waste reduction index (WRI, mean ± SE) of black soldier fly larvae reared on different substrates. Different letters indicate significant differences ($p < 0.01$) at the Tukey HSD test.

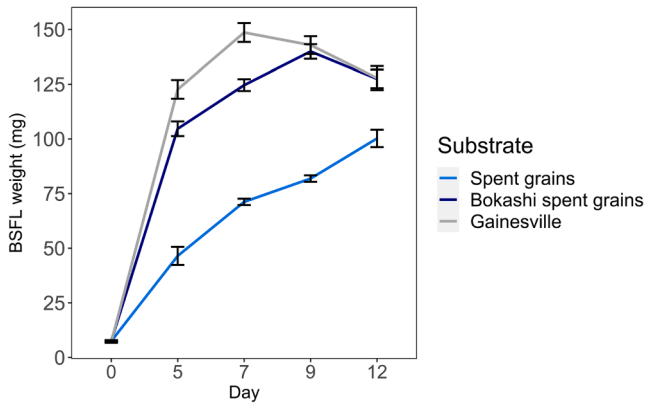

Figure 2. Average weight (mg, mean ± SE) of black soldier fly larvae reared on different substrates.

between the unfermented and bokashi fermented spent grains (Table 2). Rearing substrates had a significant effect on all variables measured, except for survival (summarized in Table 3). For development duration (ANOVA, $F_{2,29}$=42.9, p<0.001), larvae feeding on unfermented spent grains took about three days longer, on average, to complete the larval development than those reared on the Gainesville substrate and on bokashi-fermented grains. The same pattern was observed for growth rate (ANOVA, $F_{2,29}$=33.0, p<0.001). For weight dynamics, both substrate (ANOVA, F=221.5, df=2, p<0.001) and time (ANOVA, F=828.3, df=4, p<0.001) as well as their interaction (ANOVA, F=38.3, df=8, p<0.001) had a significant effect on larval weight (Figure 2). The peak weight was reached at day 7 for larvae on Gainesville substrate, between day 7 and 9 for larvae on bokashi-fermented spent grains, and between day 9 and 12 for larvae on unfermented spent grains (Figure 2). Peak larval weight on fermented grains was 40% higher, on average, than those reared on unfermented grains. Survival was not affected by the feeding substrate (ANOVA, F=0.5, df=2, p=0.6). Substrate had a significant effect on all three indices that assess the efficacy and performance of BSF larvae: BER (ANOVA, F=9.5, df=2, p<0.001), RR (ANOVA, F=17.4, df=2, p<0.001) and WRI (ANOVA, F=67.2, df=2, p<0.001). BSF larvae feeding on the Gainesville diet had the highest RR and WRI and the lowest BER, and BSF larvae feeding on bokashi-fermented grains performed equally (RR) or better (BER and WRI) than those feeding on unfermented grains.

3.1. *Trace gas fluxes:*

CO_2 fluxes varied with time (RM ANOVA, F=18.6, df=4, p<0.001), depending on the day, substrate, and treatment (Figure 3a-b). Larval rearing increased cumulative CO_2 emissions (ANOVA, F=42.1, df=1, p<0.001) from the bokashi spent grains and Gainesville substrates but not the unfermented spent grains (Figure 4a). Addition of BSF larvae increased cumulative CO_2 emissions by 31% and 79% in the bokashi spent grains and Gainesville substrates, respectively. Substrate significantly affected CO_2 (ANOVA, F=75.7, df=2, p<0.001) as emissions were greatest from the Gainesville substrate with and without BSF larvae rearing while emissions were significantly greater from the bokashi spent grains compared to the unfermented spent grains in the BSF larvae treatment only (Figure 4b). The net effect of the BSF larvae rearing process on cumulative CO_2 emissions was about 95.2 and 81.5 g CO_2 kg substrate$^{-1}_{dry}$ in bokashi spent grains and Gainesville substrates, respectively, and only about 35.7 gCO_2 kg substrate$^{-1}_{dry}$ with spent grains (Figures 4a,b). CH_4 fluxes were mostly negligible (Figure 3c-d). Generally, CH_4 emissions did significantly differ by substrate (ANOVA, F=15.4, df=2, p<0.001), with the Gainesville substrate being significantly higher than both spent grain substrates, but were not affected by BSF larvae rearing.

N_2O fluxes varied with time (RM ANOVA, F=9.7, df=4, p=0.004) depending on substrate and treatment factors (Figure 3e-f). N_2O was greatest from unfermented spent grains during BSF larvae rearing after 5 days while N_2O fluxes from the bokashi spent grains were very low (Figure 3e-f). Cumulative N_2O emissions were affected by the interaction between substrate and treatment (ANOVA, F=4.2, df=2, p=0.04). BSF larvae treatment increased cumulative N_2O emissions up to 64.0 mg N_2O kg substrate$^{-1}_{dry}$ from the unfermented spent grains only, and this substrate had greater N_2O emissions compared to bokashi spent grains (p = 0.01) during BSF larvae rearing (Figure 4f). BSF larval-rearing had effectively no net contribution on N_2O emissions using bokashi spent grains and Gainesville but had a contribution of about 60.8 mg N_2O kg substrate$^{-1}_{dry}$ from spent grains. N_2O emissions dominated the overall GWP (Figure 4g-h). BSF larvae rearing had essentially no net contribution to GWP using bokashi spent grains and Gainesville substrate but had an average net contribution of up to 18.1 g CO_2eq kg substrate$^{-1}_{dry}$ in the spent grains substrate. NH_3 fluxes were low enough to be measured with the dynamic chamber method during the first two days and generally did not significantly differ by substrate or treatment (Table 4). NH_3 mole fractions from the bokashi spent grains substrate and Gainesville BSF larvae treatment continued to be low through the first five days, with fluxes reaching up to 16.2 mg NH_3 kg substrate$^{-1}_{dry}$ day^{-1} (Table 4). Starting on day 5, NH_3 concentrations quickly exceeded the instrument's operational range for the spent grains substrate with and without BSF larvae and the Gainesville control without BSF larvae, requiring a bag sampling method, which recorded fluxes up to 132.9 mg NH_3 kg substrate$^{-1}_{dry}$ day^{-1} (Table 4). Generally, samples that could be measured using the dynamic chamber method had NH_3 mole fractions under 500 ppb, while samples that required dilution with the bag sampling method had raw NH_3 mole fractions up to 20,000 ppb almost immediately after lid closure. BSF larvae rearing significantly increased NH_3 flux from spent grains compared to the control on day 7 (ANOVA, F=22.9, df=2, p=0.002) (Table 4b). By day 9 NH_3 fluxes were affected by substrate (ANOVA, F=30.5, df=4, p<0.001) with the largest emissions from the spent grains (Table 4). On day 7 and 9 of BSF larvae rearing, NH_3 fluxes using bokashi spent grains were 83.7 to 85.8% lower compared to unfermented spent grains.

4. Discussion

We found that anaerobic fermentation of a common BSF feedstock (brewery's spent grains) improves conversion efficiency and larval output while reducing GHG and NH_3 emissions. BSF larvae fed with bokashi-fermented spent grains had a higher growth rate than those reared on unfermented grains, resulting in a 40% higher average peak weight (i.e., harvesting time of larvae for industry) that was also reached earlier. This would translate into shorter and more numerous industrial rearing cycles. Compared to larvae reared on unfermented feedstock, BSF larvae feeding on the fermented feedstock took almost three days less, on average, to complete development and had a more favorable bioconversion efficiency ratio and waste reduction index. Biological metrics of BSF larvae fed on fermented feedstock were on par with those of larvae reared on the nutritionally complete control substrate, and even superior for one of the three performance metrics evaluated, bioconversion efficiency. We also demonstrated reductions in gaseous emissions, as bokashi fermentation of spent grains greatly reduced gaseous nitrogen losses as N_2O and NH_3. Therefore, our results indicate that bokashi fermentation has the potential to improve both environmental and economic aspects of BSF rearing.

Our finding that the addition of a microbial mixture improved production metrics is in line with other studies (Jordan and Tomberlin, 2021; Peguero et al., 2021; Gorrens et al., 2023), including a preliminary study we carried out at the industrial scale (Gebiola et al., 2023). In the present study, we found similar trends, such as higher growth rate and peak weight of BSF larvae fed with bokashi-fermented spent grains, although the average weight gain associated with feeding on bokashi-

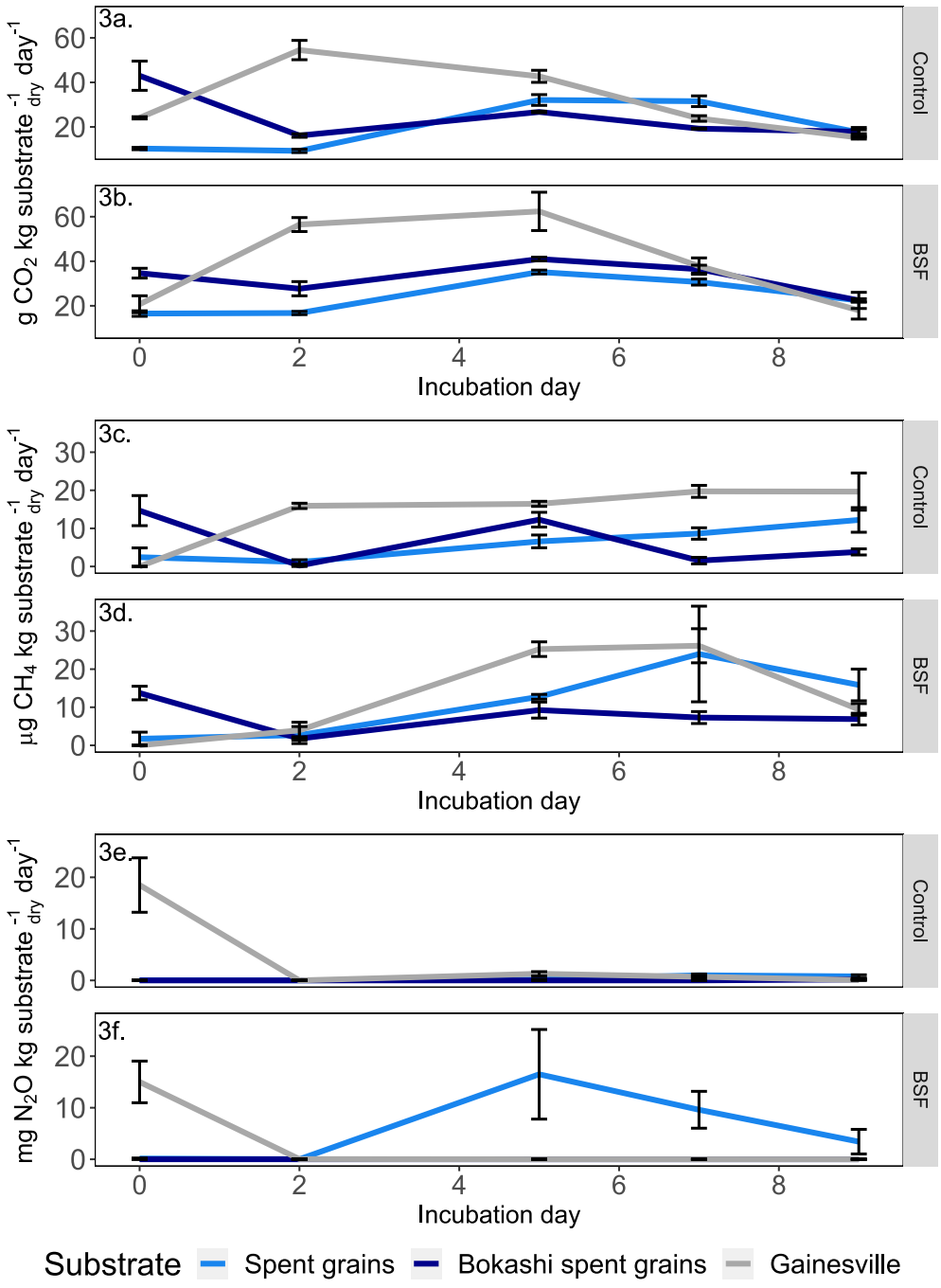

Figure 3. Average CO_2 (a-b), CH_4 (c-d), and N_2O (e-f) daily flux per kg of initial substrate dry weight measured during incubation experiment. Error bars are one standard error of the mean (n=3). For each gas the top panel represents the control treatment, and the bottom panel represents the BSF treatment.

fermented spent grains compared with unfermented spent grains in our benchtop scale experiments was double that observed in our industrial scale experiments (40% vs 21%) (Gebiola et al., 2023). Also, while no difference in developmental duration was observed at an industrial scale between larvae fed fermented and unfermented spent grains (Gebiola et al., 2023), here BSF larvae fed on unfermented spent grains developed significantly faster (over two days on average). Given the large agreement between the two studies for larvae fed on fermented spent grains and the control substrate, these differences are likely due to different sources of spent grains, whose physical and chemical properties can indeed vary among breweries (Naibaho and Korzeniowska, 2021) and to a lesser extent even within a brewery (Santos et al., 2003), and differences in BSF rearing scale, which are known to affect measurements (Yang and Tomberlin, 2020). That our overall conclusions are similar at different scales suggests that bokashi fermentation may be widely applicable as a tool for improving feedstock conversion metrics and increasing larval output.

Using a factorial approach, we were able to separate the effects of bokashi fermentation and feedstock digestion by BSF larvae on the dynamics of GHG and ammonia emissions over time. For CO_2, cumulative emissions tracked with larval metabolism and feedstock quality, as the substrates that supported the greatest larval growth, Gainesville and bokashi spent grains, led to the highest CO_2 emissions, with a significant effect of BSF larvae addition. This result is also indirectly supported by similar cumulative CO_2 emissions between the unfermented spent grains with and without BSF larvae, suggesting that little of the unfermented

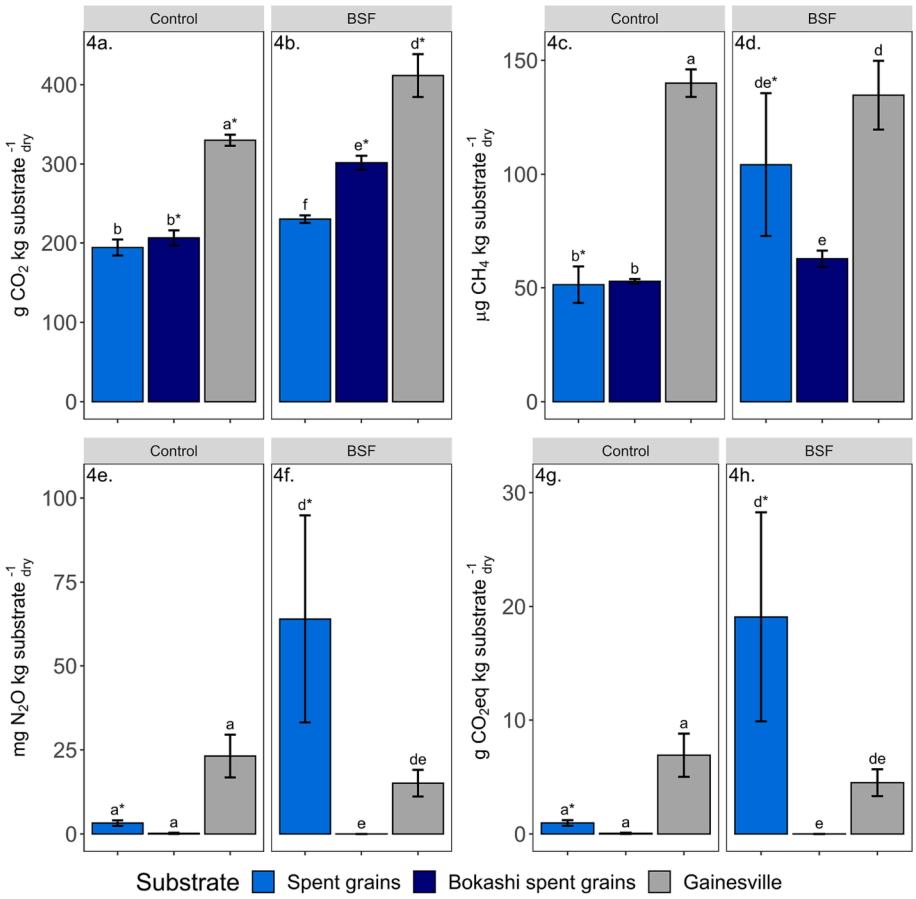

Figure 4. Average cumulative CO_2 (a-b), CH_4 (c-d), and N_2O (e-f) emissions and calculated GWP in CO_2eq (g-h) per kg of initial substrate dry weight over the 9-day incubation. Error bars are one standard error of the mean (n=3). For each gas, the left panel represents the control treatment, and the right panel represents the BSF treatment. CO_2eq was calculated using a 100-year GWP of 298 for N_2O and 34 for CH_4. Letters a-c indicate significant differences between substrates in the control treatment and letters d-f indicate significant differences between substrates in the BSF treatment ($p < 0.05$). An asterisk indicates a significant difference between control and BSF treatments within the same substrate ($p < 0.05$).

Table 4
Daily NH_3 flux rates measured during incubation of substrates without BSF larvae (control treatment) (a) and with BSF larvae (BSF treatment) (b).

a.

IncubationDay	Spent grains		Bokashi spent grains		Gainesville	
	Dynamic chamber	Bag sampling	Dynamic chamber	Bag sampling	Dynamic chamber	Bag sampling
Day 0	1.1 ± 0.08^{a}	-	1.0 ± 0.04^{a}	-	$1.1 \pm 0.1^{a*}$	-
Day 2	3.1 ± 0.2^{a}	-	4.8 ± 1.1^{a}	-	2.3 ± 0.6^{a}	-
Day 5	NA		3.3 ± 0.2	-	NA	
Day 7	-	$28.5 \pm 7.6^{a*}$	4.4 ± 0.7*	-	-	11.4 ± 6.1^{a}
Day 9	-	116.2 ± 15.14^{a}	14.0 ± 2.4	-	-	43.0 ± 12.8^{b}

b.

IncubationDay	Spent grains		Bokashi spent grains		Gainesville	
	Dynamic chamber	Bag sampling	Dynamic chamber	Bag sampling	Dynamic chamber	Bag sampling
Day 0	1.1 ± 0.3^{e}	-	1.2 ± 0.1^{e}	-	$2.0 \pm 0.3^{d*}$	-
Day 2	4.3 ± 1.3^{d}	-	4.5 ± 0.5^{d}	-	10.9 ± 7.6^{d}	-
Day 5	NA		3.0 ± 0.2^{e}	-	16.2 ± 2.4^{d}	-
Day 7	-	100.1 ± 14.0*	14.2 ± 2.6*	-	5.9 ± 3.4	3.35
Day 9	-	132.9 ± 15.6^{d}	-	21.7 ± 3.2^{e}	-	31.2 ± 19.1^{e}

Daily NH_3 flux (mean ± SE) per kg of initial substrate dry weight (mg NH_3 kg $substrate_{dry\text{-}1}$ day^{-1}) during the incubation experiment. Table 4a shows the NH_3 flux from the control treatment and table 4b shows the NH_3 flux from BSF larvae treatment. Statistical analyses on a given day were conducted separately for each method. Letters a-c indicate significant differences between substrates in the control treatment and letters d-f indicate significant differences between substrates in the BSF larvae treatment ($p < 0.05$). An asterisk indicates a significant difference between control and BSF larvae treatments within the same substrate ($p < 0.05$). A - symbol indicates that method was not used to measure samples from that group. NA indicates these groups were not measured with the dynamic chamber method on day 5 due to elevated NH_3 mole fractions. During gas measurements on day 7 one replicate from the Gainesville substrate with BSF larvae treatment had high NH_3 flux measured via bag sampling method while remaining replicates could be measured with a dynamic chamber method (n=2).

spent grains were metabolized by BSF larvae. The temporal pattern of CO_2 emissions was overall similar to previous studies, with a low initial flux, a peak on day 5 and a gradual decrease, as in Ermolaev et al. (2019), Chen et al. (2019) and Pang et al. (2020b). Consistent with other studies (Perednia et al., 2017; Chen et al., 2019; Ermolaev et al., 2019; Mertenat et al., 2019; Parodi et al., 2020), with the notable exception of (Pang et al., 2020a), CH_4 was barely detected, indicating that metabolic CH_4 production from BSF larvae is low or absent in absolute terms, but was still responsive to bokashi fermentation and BSF addition.

Both BSF presence and bokashi fermentation influenced emissions of N_2O and NH_3. BSF larvae increased emissions of N_2O from unfermented spent grains, which spiked at day 5 and remained higher than emissions from bokashi fermented spent grains. Emissions from fermented spent grains were negligible regardless of BSF presence. Therefore, bokashi fermentation counteracted the tendency for N_2O producing microbial activity during rearing. This treatment led to reduced GWP due to lower N_2O emissions, since CH_4 emissions were negligible and CO_2 emissions of biogenic origin are not included in the GWP calculations. NH_3 emissions were similarly reduced, as BSF addition to spent grains increased NH_3 emissions on day 7, but this increase did not occur for bokashi-fermented spent grains. Emissions from the Gainesville substrate were largely unresponsive to BSF larvae addition, with low but steady emission of N_2O and possibly reduced NH_3 fluxes during most of the rearing process. This indicates that substrate composition will affect whether BSF addition leads to increased emissions. Similar temporal patterns in fluxes of nitrogen-containing gases may indicate that addition of BSF larvae promotes nitrogen mineralization in spent grains, but the larvae are unable to efficiently assimilate mineralized nitrogen (Pang et al., 2020b). Nitrogen assimilation into larval biomass may be more efficient from the Gainesville substrate, due to its higher C:N ratio that may have led to less NH_3 volatilization, allowing for more N to be available for BSF larvae uptake and a significant increase in larval biomass weight compared to the unfermented spent grains substrate. Higher substrate C:N ratio and C availability have been shown to reduce NH_3 emissions from BSF rearing (Pang et al., 2020b) and from food waste composting by increasing the ammonia assimilating bacteria populations and enzymatic activity (Meng et al., 2016; Wang and Zeng, 2018). Differences in NH_3 emissions between substrates may also be affected by chemical transformations of nitrogen to NO_3^- or retention as NH_4^+ due to differences in substrate pH. Future studies should measure substrate NH_4^+ concentration and its transformation to NO_3^- throughout the rearing process to determine how BSF larvae affect nitrogen cycling and the contribution of substrate NH_4^+ content to GHG and NH_3 emissions.

The temporal dynamics of N_2O and NH_3 can further illuminate factors that affect emissions. When BSF larvae are used for organic waste recycling, substantial NH_3 emissions can be expected from the increasingly aerobic process due to larval movement in the substrate, from the material drying out and warming up, and from the pH of the residues becoming alkaline (Čičková et al., 2015; Meneguz et al., 2018a). On the other hand, NH_3 emissions may be limited by larval assimilation of part of the available nitrogen in the feeding substrate, transformation by microorganisms to be emitted as N_2O, or a delay in the release of NH_3 produced by microbial metabolism due to pH changes in the substrate (Green and Popa, 2012; Parodi et al., 2020). In this study, N_2O and NH_3 were produced mostly from day 5 onwards, which confirms that emissions are the result of changes in the substrates driven by BSF larvae metabolism and inefficient assimilation of NH_4^+ by larvae, which likely lead to more opportunities for gaseous nitrogen loss. N_2O emissions from BSF treatment with unfermented spent grains substrate were highly variable, suggesting that N_2O losses may be sensitive to small differences in moisture, aeration, and microbial activity across replicates within the same substrate.

It is not yet known how bokashi fermentation of feedstocks could improve larval metrics and reduce GHG emissions. One possible mechanism is that the bokashi lactic acid fermentation may have increased nitrogen mineralization and digestibility of the substrate (Quiroz and Céspedes 2019; Wang et al. 2021), which allowed for an increase in nitrogen assimilation into BSF larval tissues. The higher growth rate and biomass of BSF larvae fed on spent grains after bokashi fermentation does suggest increased nitrogen transformation and assimilation as a mechanism for reduced gaseous nitrogen losses. This proposed mechanism is further supported by our observation that N_2O emissions are reduced during aerobic decomposition with and without BSF larvae rearing. The unfermented and bokashi fermented spent grains have a similar initial C:N ratio but differ in both trace gas emissions and larval growth variables. This suggests that differences in emissions and larval performance may not be due to C:N ratio alone but rather the effects of bokashi fermentation on other substrate characteristics. Bokashi fermentation also lowers the pH of the substrate, as it is mostly a lactic acid fermentation (Hillberg, 2020). This shift helps mitigate NH_3 emissions that result from higher pH, preventing a rapid shift of the equilibrium between NH_4^+ and NH_3 towards NH_3 (Ermolaev et al., 2019; Pang et al., 2020a; Parodi et al., 2020). The lower pH of the fermented grains before larval digestion compared with the other two substrates and the associated lower NH_3 emissions observed in our study confirms the role played by pH in mitigating NH_3 emissions. Taken together, this information suggests several avenues for future work to reduce GHG and NH_3 emissions from BSF farming. While feedstock composition may not be possible to manipulate, as it depends on waste that is available, fermentation could be applied broadly across feedstocks.

Use of Picarro CRDS allowed us to measure low N_2O emissions from bokashi fermented substrate, as well as the very large N_2O emissions from unfermented spent grains. It is notable that emissions from this substrate were much higher than those reported in any other BSF study thus far (e.g., Pang et al., 2020b). N_2O emissions vary considerably among studies due to differences in key experimental conditions (Boakye-Yiadom et al., 2022). Large emissions in our study may be due to the high nitrogen content and low C:N ratio of the substrates used in this study (10.3-13.4) which are lower than C:N ratios for substrates used in most prior work. Substrates with lower C:N ratios experience accelerated nitrogen mineralization and subsequent nitrification, denitrification, and volatilization processes. As a result, nitrogen could be lost before being assimilated as microbial or larval biomass.

Our sampling methods for N_2O revealed important effects of bokashi fermentation on the flux of this GHG, but we had more difficulty capturing the full range of NH_3 emissions. Using standards, we found that the bag sampling method only captured about 63.5% of possible NH_3 flux due to NH_3 sticking to chamber, syringe, and Tedlar bag surfaces. Similarly, the dynamic chamber method may only capture about 45% of possible NH_3 flux due to the apparatus not reaching a steady state in the short measurement time. While both methods were necessary due to the large dynamic range of emissions from our treatments, the methods are not directly comparable due to different flux calculations and contact surfaces. As a result, it is difficult to accurately construct a complete flux time series and calculate cumulative NH_3 emissions. However, relative differences between substrates and treatments on individual sampling events are still apparent, showing clear beneficial effects of bokashi fermentation on NH_3 emissions.

Another aspect that should be investigated is the effect of bokashi fermentation on downstream emissions associated with the post-BSF composting process (i.e., further composting of BSF frass), which has been flagged as an environmental burden not only in terms of energy use but also of GWP (Guo et al., 2021; Song et al., 2021). This should be investigated because BSF frass is an excellent biofertilizer and soil amendment (Barragán-Fonseca et al., 2022), but this application may be associated with high soil N_2O emissions (Rummel et al., 2021). More research is needed to determine if frass derived from bokashi-fermented substrates produces lower N_2O emissions, making this product more environmentally sustainable in the context of the BSF-based circular economy model for waste recycling.

5. Conclusions

Using a factorial approach, we explored how bokashi fermentation and the BSF larvae rearing process affects trace gas emissions from different feedstocks and the relationship between rearing performance and gaseous emissions. We showed that bokashi fermentation of spent grains significantly increased BSF larvae growth and performance, while reducing N_2O and NH_3 emissions. In the context of prior studies, our results suggest that larval performance and increases in trace gas emissions as a result of the BSF rearing process may be dependent on substrate properties as well as rearing conditions. Results presented here should be validated at the industrial scale, along with a complete mass balance of carbon and nitrogen and including emissions from the bokashi fermentation itself. Future studies should also characterize the changes in microbial composition during the bokashi fermentation and possibly link the microbial taxa to their function and potential effect on the reduction of trace gas emissions.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.09.033.

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