Effects of partial or complete replacement of soybean meal with commercial black soldier fly larvae (*Hermetia illucens*) meal on growth performance, cecal short chain fatty acids, and excreta metabolome of broiler chickens

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ABSTRACT Black soldier fly larvae meal (**BSFLM**) is receiving great attention as a rich source of protein and antimicrobials for poultry. Therefore, we evaluated the effects of partially or completely replacing soybean meal (**SBM**) with commercial BSFLM on growth performance, tibia traits, cecal short chain fatty acid (**SCFA**) concentrations, and excreta metabolomes in broiler chickens (*Gallus gallus domesticus*). A total of 480 day-old male Ross × Ross 708 chicks were assigned to 6 diets (8 replicates/diet): a basal corn-SBM diet with in-feed bacitracin methylene disalicylate (**BMD**), a corn-SBM diet without BMD (0% BSFLM), and four diets in which the SBM was substituted with 12.5, 25, 50, and 100% BSFLM. Body weight (**BW**), feed intake (**FI**) and cumulative feed conversion ratio (**cFCR**) were monitored on days 14, 28, and 35. Cecal SCFA levels were determined on days 14, 28, and 35. Tibia traits and excreta metabolomes were determined on day (d) 35. On d14, birds fed 12.5 and 25% BSFLM had a similar BW, FI, and cFCR as birds fed BMD ($P > 0.05$). On d 35, birds fed 12.5% BSFLM had a similar BW, FI and cFCR as birds fed BMD or 0% BSFLM ($P > 0.05$). For each phase, birds fed 100% BSFLM had a lower BW, FI and higher cFCR than birds fed BMD or 0% BSFLM ($P < 0.05$). On d 35, BW decreased linearly, quadratically, and cubically with increasing levels of BSFLM ($P < 0.01$). Overall (d 0-35), BSFLM linearly, quadratically, and cubically decreased FI and quadratically and cubically increased cFCR ($P < 0.01$). Quadratic responses were observed for tibia fresh weight ($P = 0.049$) and ash content ($P = 0.022$). BSFLM did not impact cecal SCFAs levels. The excreta metabolome of birds fed 100% BSFLM clustered independently from all other groups and exhibited greater levels of putatively identified methionine, lysine, valine, glutamine, histidine and lower levels of arginine as compared to all diets. Taken together, substitution of SBM with ≤25% of BSFLM in the starter phase may be used as an alternative to BMD.

Key words: black soldier fly larvae meal, antibiotic growth promoter, broiler chicken, bacitracin

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INTRODUCTION

The use of antimicrobials to promote growth, as well as to reduce the amount of feed required to bring broiler chickens (*Gallus gallus domesticus*) to market weight, dates back to the mid-1940s (Moore and Evenson, 1946; Stokstad, 1950). Seventy years later, it was estimated that 73% of all antimicrobials sold globally were used in animals raised for food (Van Boeckel et al., 2017). It is therefore not surprising that the intensive use of antibiotics in livestock production has accelerated the evolution and dissemination of antibiotic resistant genes and bacteria that threaten modern medicine (Endtz et al., 1991; Smith et al., 1999; Dutil et al., 2010; Ward et al., 2014; Liu et al., 2016; de Vries et al., 2018; Carson et al., 2019; Wang et al., 2020b).

Globally, bacitracin is the most commonly used antibiotic growth promoting agent and is available as zinc bacitracin or bacitracin methylene disalicylate (**BMD**)

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(Office of Institutional Equity, 2018). Bacitracin is a mixture of high-molecular weight, non-ribosomal, cyclic polypeptides produced by the bacterium, *Bacillus licheniformis* (Stone and Strominger, 1971), and is mainly active against Gram-positive bacteria and has little effect on Gram-negative bacteria, except for *Neisseria* spp. (Johnson et al., 1945). Depending on the concentration, bacitracin exerts both bacteriostatic and bactericidal activity and acts by inhibiting the enzyme, isoprenyl pyrophosphate, which is involved in bacterial cell wall formation (Siewert and Strominger, 1967; Stone and Strominger, 1971; Storm, 1974). In addition to inhibiting cell wall synthesis, bacitracin has also been shown to suppress protein synthesis (Smith and Weinberg, 1962). Owing to its nephrotoxicity in humans, bacitracin is mainly used as a topical antibiotic ointment to treat minor skin injures and can be used alone or in combination with other antibiotics, including neomycin and polymyxin B (Nguyen R et al., 2022). Nonetheless, bacitracin is considered medically important by the World Health Organization (WHO, 2019).

Restrictions and bans on the use of bacitracin as well as other antibiotic growth promoters (**AGPs**) have been implemented in various countries over the last 36 years; although, regulations on antibiotic growth promoter use vary from one country to another (Rahman et al., 2022). In 1986, Sweden was the first country to ban the use of AGPs (Bengtsson and Wierup, 2006). Several years later in 1999, the EU banned bacitracin and 3 other growth promoting antibiotics (Casewell et al., 2003). In 2006, the EU imposed a complete ban on the use of AGPs, and as of 2022, the EU, excluding the United Kingdom, banned the importation of meat and dairy products using AGPs (Anadón, 2006; Shingler and Nunan, 2022). In 2017, the United States banned the use of medically important antibiotics for growth promotion (Patel et al., 2020). In 2018, Canada removed growth promotion claims on medically important antibiotics, including bacitracin, and veterinary prescriptions are now mandatory to administer antibiotics to animals (Government of Canada, 2021). Still, there are countries that have no policies or legislation governing the use of AGPs (Van et al., 2020). With the removal of AGPs, including bacitracin, in poultry and other livestock, sustainable alternatives are desperately needed in order to meet the increasing demand for animal-origin food products.

One potential alternative to AGPs is to use black soldier fly larvae (*Hermetia illucens*) meal (**BSFLM**) as a functional feed for poultry. BSFLM is an attractive food source due to its ability to convert organic waste such as food waste (Dzepe et al., 2021; Liu et al., 2018) and manure (Lalander et al., 2015), into a rich source of crude protein and fat, essential amino acids (**AAs**), vitamins, and minerals (Tschirner and Simon, 2015; Józefiak et al., 2018; Chia et al., 2020; Dzepe et al., 2021). In addition to its nutritional profile, BSFLM is touted as possessing functional benefits (de Souza Vilela et al., 2021) as it is a known source of both antimicrobial and immunomodulatory compounds including lauric acid (Borrelli et al., 2021), chitin (or its derivatives) (Lagat et al., 2021; Kemboi et al., 2022), and a plethora of antimicrobial peptides (Choi et al., 2012; Park et al., 2014; Park et al., 2015; Vogel et al., 2018; Moretta et al., 2020; Xu et al., 2020; Zhang et al., 2022). In vitro, lauric acid has been shown to inhibit the growth of *Clostridium perfringens,* the etiological agent of necrotic enteritis in poultry (Skřivanová et al., 2005; Timbermont et al., 2010; Matsue et al., 2019). Moreover, lauric acid has been shown to prevent necrotic enteritis lesions in broilers, likely due to its direct effects on *C. perfringens* growth (Timbermont et al., 2010). Chitin, which is the main exoskeleton component of insects and crustaceans, is bacteriostatic and is able to inhibit the growth of Gram-negative bacteria including *Escherichia coli, Vibrio cholera, Shigella dysenteriae*, and *Bacteroides fragilis* (Benhabiles et al., 2012). Recently, chitin and chitosan extracted from black soldier fly pupal shell waste has been shown to inhibit the growth of both human and plant bacterial pathogens (Lagat et al., 2021; Kemboi et al., 2022). In the last decade, several studies have identified a myriad of black soldier fly antimicrobial peptides that are capable of killing both Gram-positive (Park et al., 2014) and Gram-negative bacteria (Choi et al., 2012; Park et al., 2015; Vogel et al., 2018; Moretta et al., 2020; Xu et al., 2020; Zhang et al., 2022). While lauric acid and chitin are known to be present in BSFLM, it is unknown if antimicrobial peptides are present and active in commercially prepared BSFLM.

Several studies have examined the beneficial functional effects of supplementing or substituting poultry diets with BSFLM, black soldier fly prepupae or viable black soldier fly larvae on growth performance, digestibility, gut microbiota composition/function, and immune system status (Dabbou et al., 2018; de Souza Vilela et al., 2021; Heuel et al., 2021). Inclusion of low levels of black soldier fly meal (10−20%) in broiler diets has been shown to improve growth performance when compared to broilers fed a conventional diet without an antibiotic growth promoter (Dabbou et al., 2018; de Souza Vilela et al., 2021). In laying hens, complete replacement of soybean meal (**SBM**) with BSFLM altered the composition of the cecal microbiota in a manner that led to the increased production of microbiota-derived short chain fatty acids (**SCFAs**; e.g., butyrate, acetate, and propionate) (Borrelli et al., 2017), which are known to have numerous beneficial effects on the host as well as antimicrobial activity (Roe et al., 1998, 2002; Chaveerach et al., 2003; Namkung et al., 2011; Yonezawa et al., 2012; Jacobson et al., 2018). Additionally, the beneficial effects of feeding black soldier fly products or oil have been noted in other animals. For example, feeding piglets black soldier fly prepupae or larvae meal led to a reduction in intestinal group D streptococci (Spranghers et al., 2018) and *E. coli* (Yu et al., 2020). Feeding finisher pigs black soldier fly larvae led to an increase in beneficial bacteria and SCFAs and a decrease in the abundance of *Streptococcus* spp. (Spranghers et al., 2018; Yu, et al.,

2019). Substitution of soybean oil with black soldier fly oil was also shown to impair growth of the pathogen *Yersinia enterocolitica* in rabbits (Dabbou et al., 2020) and reduce growth of potentially pathogenic bacteria, *Enterobacteriaceae* spp., in turkey (Sypniewski et al., 2020). Despite these observations, it is currently unclear if black soldier fly products including BSFLM, can serve as an alternative to the antibiotic growth promoter, BMD.

Given the growing bans and restrictions on antibiotic growth promoter use in poultry, environmentally sustainable, easily adoptable, non-antibiotic strategies are urgently required that mimic the growth-promoting effects of BMD (Wicker et al., 1977; Kaukas et al., 1988; Guban et al., 2006). In recent years, there has been increasing interest in utilizing insects that can be employed to "upcycle" food wastes and other organic wastes into an alternative food source for poultry and other livestock. As a result, black soldier fly products are being approved for use as feed for aquaculture and in poultry feed in several countries, including Canada (Lähteenmäki-Uutela et al., 2021). In 2016, the Canadian Food Inspection Agency (**CFIA**) authorized the use of black soldier fly products as feed for broiler chickens, ducks, geese, turkey, salmonids, and tilapia (Lähteenmäki-Uutela et al., 2021). In addition to serving as an alternative protein source, it has been suggested that insects and insect-derived products (e. g., oil) may serve as an alternative to in-feed AGPs (Borrelli et al., 2017; Spranghers et al., 2018). Still, there is a dearth of broiler studies directly comparing the functional attributes of different substitution levels of SBM with commercially processed BSFLM to gold-standard performance enhancing feed additives, such as BMD. During the preparation of this manuscript, another study was published demonstrating that substitution of SBM with low amounts of BSFLM fortified with black soldier fly oil could provide some performance enhancing effects in broilers in a manner comparable to broilers fed a conventional corn-SBM diet supplemented with the AGPs, BMD and narasin (narasin, which is mainly used as a coccidiostat also has growth promoting properties in broilers [Plata et al., 2022]) (Facey et al., 2023). Still, it is unclear if BSFLM alone (i.e., no oil) was responsible for the growth enhancing effects observed in that study. Herein**,** we compared the effects of substituting SBM with either low levels (12.5 and 25%) or high levels (50 and 100%) of commercial BSFLM to a conventional diet containing BMD on performance, tibia traits, cecal SCFAs levels, and the excreta metabolome in broiler chickens.

MATERIALS AND METHODS

Animals and Ethics Statement

All experimental procedures performed in this study were approved by the Animal Care Committee of the University of Guelph Animal Ethics Committee (AUP#3521) according to the guidelines of the Canadian Council of Animal Care (Canadian Council on Animal Care, 2009).

Birds, Housing, and Experimental Diets

A total of 480 day (d)-old male Ross × Ross 708 broiler chicks obtained from a commercial hatchery (Maple Leaf Foods, New Hamburg, ON, Canada) were distributed in 48 identical metabolic cages (10 birds/cage) at the Arkell Poultry Research Station (University of Guelph, Guelph, ON, Canada). The chicks were vaccinated with Bronchitis and Marek's vaccines at the hatchery. Cages were allocated to 6 diets (8 replicates/diet) in a completely randomized design. Diets were formulated for a 3-phase feeding program: Starter: d 0−14, Grower: d 15−28, and Finisher: d 29−35 (Tables 1−3) that met or exceeded nutrient specifications for Ross × Ross 708 (Aviagen and Ross, 2014). The composition of the commercially prepared, partially defatted BSFLM (Enterra Feed Inc., Vancouver, BC, Canada, Lot #: L191203M-1) used in this study was determined by A&L Canada Laboratories (London, ON, Canada; Supplementary Table 1). Black soldier fly larvae were reared on a 100% plant-based diet consisting of pre-consumer food wastes. The antibiotic growth promoter, BMD 110 G, was provided by Zoetis Canada Inc. (Kirkland, QC, Canada). Within each phase, diets were formulated to have the same level of nutrients and energy coefficients for standardized ileal digestible (**SID**) amino acids, metabolizable energy (**AME**) values, and nutrient values for BSFLM were obtained from published literature (de Marco et al., 2015; Schiavone et al., 2017; Mwaniki and Kiarie, 2019) and for the other feedstuffs from Evonik Aminodat 5.0 for diet formulation (Evonik Nutrition & Care GmbH., 2016). The dietary treatments consisted of A) a corn- and soybean-meal (**SBM**)-based diet with BMD (BMD group): 110 ppm for starter and 55 ppm for grower and finisher phases, B) a corn-SBM-based diet without an AGP (0% BSFLM) and 4 different diets in which the SBM was replaced by BSFLM at inclusion levels of C) 12.5% (25−32% SBM), D) 25% (20−26% SBM), E) 50% (10−13% SBM), and F) 100% (0% SBM). All diets were prepared in mash form. Diet samples were finely ground and submitted to a commercial lab (SGS Canada Inc, Guelph, ON, Canada) for dry matter, crude protein, crude fat, starch, and minerals analyses (Tables 1−3).

Data Collection

Chicks were weighed at the start of the trial (d 0) and at the end of each growing phase thereafter. Performance parameters including body weight (**BW**), feed intake (**FI**), and cumulative feed conversion ratios (**cFCR**) were measured at d 14, 28, and 35 from each cage using a similar method as previously described (Das et al., 2020). Birds were inspected at least twice daily. Any mortalities or culls were removed. The dates of removal and bird weights were recorded on a data capture sheet. Any birds showing signs of illness or

Table 1. Composition, nutrient content and analyzed provisions of the starter diets, as fed basis.

	Starter, d 0−10					
		BSFLM,%[1]				
Item	BMD	0	12.5	25	50	100
Corn	40.3	40.5	43.1	45.8	51.1	55.6
Wheat	10.0	10.0	10.0	10.0	10.0	10.0
Soybean meal, 46%	39.2	39.2	32.7	26.2	13.1	0.0
L-Lysine HCL	0.26	0.27	0.29	0.31	0.36	0.41
DL-Methionine	0.35	0.35	0.36	0.37	0.40	0.42
L-Threonine	0.14	0.14	0.15	0.16	0.18	0.30
L-Tryptophan	0.00	0.00	0.00	0.00	0.03	0.08
BSFLM	0.0	0.0	4.9	9.8	19.6	29.4
Limestone fine	1.16	1.16	1.05	0.95	0.74	0.53
Monocalcium Phosphate	2.11	2.11	2.09	2.07	2.03	1.99
Salt	0.26	0.26	0.26	0.26	0.26	0.26
Sodium bicarbonate	0.14	0.14	0.12	0.10	0.06	0.03
Vitamin and trace premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Soy oil	4.97	4.89	3.97	3.05	1.19	0.00
BMD® 110[3]	0.10	0.00	0.00	0.00	0.00	0.00
Ingredient total:	100	100	100	100	100	100
Calculated nutrients						
AMEn, Kcal/kg	3,000	2,999	3,000	3,000	3,000	3,033
Crude protein, %	23.0	23.0	23.0	23.0	23.0	23.0
Crude fat, %	6.7	6.6	6.1	5.7	4.7	4.4
SID[4] Lys, %	1.28	1.28	1.28	1.28	1.28	1.28
SID Met, %	0.65	0.65	0.67	0.69	0.72	0.76
SID Met+Cys, %	0.95	0.95	0.95	0.95	0.95	0.95
SID Try, %	0.27	0.27	0.25	0.22	0.20	0.20
SID Thr, %	0.86	0.86	0.86	0.86	0.86	0.95
Calcium, %	0.96	0.96	0.96	0.96	0.96	0.96
Available P, %	0.48	0.48	0.48	0.48	0.48	0.48
Sodium, %	0.16	0.16	0.16	0.16	0.16	0.16
Bacitracin, ppm	110	0	0	0	0	0
Analyzed provisions						
Dry matter, %	90.74	90.85	91.12	90.56	91.63	90.88
Crude protein, %	23.5	20.99	24.09	25.79	24.62	23.27
Crude fat, %	7.30	8.94	8.07	7.40	7.56	5.22
Starch, %	29.48	34.05	31.10	29.62	35.89	42.64
Calcium, %	1.20	0.91	1.08	1.05	1.03	0.96
Phosphorous, %	0.90	0.83	0.91	0.87	0.88	0.78
Potassium, %	1.06	0.93	0.95	0.98	0.74	0.53
Magnesium, %	0.19	0.17	0.18	0.19	0.20	0.18
Sodium, %	0.18	0.19	0.19	0.18	0.19	0.13

[1]Percentage of soybean meal replaced with black soldier fly larvae meal (BSFLM).
[2]Provided per kilogram of diet: vitamin A, 8,800.0 IU; vitamin D3, 3,300.0 IU; vitamin E, 40.0 IU; vitamin B12, 12.0 mg; vitamin K3, 3.3 mg; niacin, 50.0 mg; choline, 1,200.0 mg; folic acid, 1.0 mg; biotin, 0.22 mg; pyridoxine, 3.3 mg; thiamine, 4.0 mg; calcium pantothenic acid, 15.0 mg; riboflavin, 8.0 mg; manganese, 70.0 mg; zinc, 70.0 mg; iron, 60.0 mg; iodine, 1.0 mg; copper, 10 mg; and selenium, 0.3 mg.
[3]BMD, bacitracin methylene disalicylate (Zoetis Canada Inc., Kirkland, Quebec).
[4]SID, standardized ileal digestible.

distress were removed and humanely killed. During flock inspections, birds were observed for activities, and feed and water were checked to assure that each was always available.

Tibia Bone Traits and Analysis

At the end of the experiment (d 35), 2 birds per cage were randomly selected and sacrificed via cervical dislocation. A total of 96 left tibia (2 tibia/cage, 16 tibia/diet) were sampled from sacrificed birds and frozen at −20°C until analysis using methods outlined by AOAC International (2005; method 932.16; AOAC, 2005) and Kakhki et al. (2019). Bone samples were thawed overnight at room temperature and then, excessive adhering tissues were removed using forceps, the unsharpened side of scissors, and a blade. To further loosen tissues, samples were autoclaved at 121°C for 1 min. Remaining tissues were immediately removed to leave a bare bone that was then placed in a desiccator to cool, and the weight of dried bone recorded after cooling. Tibia bones were then placed in an open tin foil and oven dried (Thermo Laboratory Drying Oven, Thermo Scientific, Canada) at 60°C for 2 d and the weight recorded afterwards to determine the dry matter. After weighing, the dried bones were placed in a muffle furnace (Lindberg/Blue M Moldatherm Box Furnaces, Thermo Scientific) and ashed at 550°C for 8 h for determination of ash content (weight of ash per g of dry bone) and ash concentration (ash weight/bone weight × 100%). To account for differences in BW, tibia fresh weight, and dry weight were expressed relative to the corresponding d 35 BWs.

Excreta Sample Preparation, UHPLC-MS/MS Analysis, and Data Processing

Excreta collection was conducted on d 35 using slide-in trays that were placed underneath each of the 48

Table 2. Composition, nutrient content, and analyzed provisions of the grower diets, as fed basis.

	Grower, d 11−24					
		BSFLM,%[1]				
Item	BMD	0	12.5	25	50	100
Corn	43.2	43.3	45.7	48.1	52.8	57.8
Wheat	10.0	10.0	10.0	10.0	10.0	10.0
Soybean meal, 46%	35.7	35.7	29.7	23.9	12.1	0.0
L-Lysine HCL	0.20	0.21	0.23	0.25	0.29	0.33
DL-Methionine	0.30	0.30	0.31	0.32	0.34	0.37
L-Threonine	0.10	0.10	0.11	0.12	0.14	0.16
L-Tryptophan	0.00	0.00	0.00	0.00	0.02	0.06
BSFLM	0.0	0.0	4.5	8.9	17.8	26.8
Limestone fine	1.03	1.03	0.94	0.84	0.65	0.46
Monocalcium phosphate	1.90	1.90	1.88	1.86	1.82	1.79
Salt	0.26	0.26	0.26	0.26	0.26	0.26
Sodium bicarbonate	0.11	0.11	0.10	0.08	0.05	0.01
Vitamin and trace premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Soy oil	6.14	6.10	5.26	4.43	2.75	1.01
BMD® 110[3]	0.05	0.00	0.00	0.00	0.00	0.00
Ingredient Total:	100	100	100	100	100	100
Calculated nutrients						
AMEn, Kcal/kg	3,100	3,100	3,100	3,100	3,100	3,100
Crude protein, %	21.5	21.5	21.5	21.5	21.5	21.5
Crude fat, %	7.9	7.9	7.5	7.0	6.2	5.3
SID[4] Lys, %	1.15	1.15	1.15	1.15	1.15	1.15
SID Met, %	0.59	0.59	0.60	0.62	0.65	0.68
SID Met+Cys, %	0.87	0.87	0.87	0.87	0.87	0.87
SID Try, %	0.25	0.25	0.23	0.21	0.18	0.18
SID Thr, %	0.77	0.77	0.77	0.77	0.77	0.78
Calcium, %	0.87	0.87	0.87	0.87	0.87	0.87
Available P, %	0.44	0.44	0.44	0.44	0.44	0.44
Sodium, %	0.16	0.16	0.16	0.16	0.16	0.16
Bacitracin, ppm	55	0	0	0	0	0
Analyzed provisions						
Dry matter, %	88.72	88.93	88.99	89.09	89.27	89.67
Crude protein, %	23.0	22.1	21.5	21.9	22.0	23.9
Crude fat, %	7.85	8.25	8.82	7.87	7.17	6.97
Starch, %	32.61	32.66	32.08	33.04	37.95	44.73
Calcium, %	0.86	0.79	0.87	0.85	0.85	0.92
Phosphorous, %	0.80	0.80	0.76	0.72	0.77	0.75
Potassium, %	0.94	0.91	0.81	0.81	0.69	0.63
Magnesium, %	0.17	0.16	0.16	0.17	0.18	0.20
Sodium, %	0.18	0.17	0.18	0.14	0.18	0.17

[1]Percentage of soybean meal replaced with black soldier fly larvae meal (BSFLM).
[2]Provided per kilogram of diet: vitamin A, 8,800.0 IU; vitamin D3, 3,300.0 IU; vitamin E, 40.0 IU; vitamin B12, 12.0 mg; vitamin K3, 3.3 mg; niacin, 50.0 mg; choline, 1,200.0 mg; folic acid, 1.0 mg; biotin, 0.22 mg; pyridoxine, 3.3 mg; thiamine, 4.0 mg; calcium pantothenic acid, 15.0 mg; riboflavin, 8.0 mg; manganese, 70.0 mg; zinc, 70.0 mg; iron, 60.0 mg; iodine, 1.0 mg; copper, 10 mg; and selenium, 0.3 mg.
[3]BMD, bacitracin methylene disalicylate (Zoetis Canada Inc., Kirkland, Quebec).
[4]SID, standardized ileal digestible.

wire-mesh-floor cages. Fresh excreta were aseptically scooped from each tray into sterile 50 mL conical tubes (Thermo Scientific) and placed into an icebox for transportation to the lab for storage and further processing. Excreta samples were sent to the BioZone Mass Spectrometry Facility (Department of Chemical Engineering and Applied Chemistry, University of Toronto, ON, Canada) for metabolomics analysis using an untargeted ultra-high performance liquid chromatography/tandem mass spectrometry (**UHPLC-MS/MS**) approach. A total of 48 samples were used for metabolic profiling and included 8 samples from each experimental group at a single time point (d 35). Briefly, to prepare excreta samples for UHPLC-MS/MS analysis, approximately 300 mg of excreta from each cage (8 replicate cages per dietary treatment) was weighed and placed into a 2 mL microcentrifuge tube. One milliliter of the extraction solvent [40% (v/v) acetonitrile, 40% methanol (v/v), 20% (v/v) water] was added to each sample and vortexed for 1 min. Samples were then incubated at −20°C for 30 min and subsequently centrifuged at maximum speed in a microcentrifuge. The supernatant was collected and transferred to a new 1.5 mL microcentrifuge tube. To enhance the recovery of the metabolites, 200 μL of extraction solvent was added to the pellet, vortexed for 1 min and subsequently incubated at −20°C for 30 min. The resuspended pellet was centrifuged at maximum speed microcentrifuge and supernatant was transferred to the appropriate 1.5 mL microcentrifuge tube. The collected supernatants were then dried by vacuum centrifugation using an Eppendorf SpeedVac and resuspended in 95% water and 5% acetonitrile and standardized to a final concentration of 0.71675 mg/μL, which was based on the lowest amount of starting material recovered following sample preparation.

UHPLC-MS/MS analysis was performed using an UltiMate 3000 UHPLC system (Thermo Scientific, Waltham, MA) equipped with a Thermo Scientific Hypersil

Table 3. Composition, nutrient content, and analyzed provisions of the finisher diets, as fed basis.

	Finisher, d 25−35					
		BSFLM,%[1]				
Item	BMD	0	12.5	25	50	100
Corn	47.7	47.8	49.8	51.9	56.0	60.2
Wheat	10.0	10.0	10.0	10.0	10.0	10.0
Soybean meal, 46%	30.6	30.6	25.6	20.4	10.3	0.0
L-Lysine HCL	0.20	0.20	0.22	0.24	0.27	0.31
DL-Methionine	0.28	0.28	0.29	0.29	0.31	0.33
L-Threonine	0.09	0.09	0.10	0.11	0.12	0.15
L-Tryptophan	0.00	0.00	0.00	0.00	0.01	0.05
BSFLM	0.0	0.0	3.8	7.7	15.3	23.0
Limestone fine	0.92	0.92	0.84	0.76	0.60	0.43
Monocalcium phosphate	1.72	1.72	1.71	1.69	1.66	1.63
Salt	0.26	0.26	0.26	0.26	0.26	0.26
Sodium bicarbonate	0.12	0.12	0.10	0.09	0.06	0.03
Vitamin and trace premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Soy oil	7.06	7.02	6.31	5.58	4.14	2.66
BMD® 110[3]	0.05	0.00	0.00	0.00	0.00	0.00
Ingredient total:	100	100	100	100	100	100
Calculated nutrients						
AMEn, Kcal/kg	3,200	3,200	3,200	3,200	3,200	3,200
Crude protein, %	19.5	19.5	19.5	19.5	19.5	19.5
Crude fat, %	9.0	9.0	8.6	8.2	7.5	6.7
SID[4] Lys, %	1.03	1.03	1.03	1.03	1.03	1.03
SID Met, %	0.54	0.54	0.55	0.56	0.59	0.62
SID Met+Cys, %	0.80	0.80	0.80	0.80	0.80	0.80
SID Try, %	0.22	0.22	0.21	0.19	0.16	0.16
SID Thr, %	0.69	0.69	0.69	0.69	0.69	0.70
Calcium, %	0.79	0.79	0.79	0.79	0.79	0.79
Available P, %	0.40	0.40	0.40	0.40	0.40	0.40
Sodium, %	0.16	0.16	0.16	0.16	0.16	0.16
Bacitracin, ppm	55	0	0	0	0	0
Analyzed provisions						
Dry matter, %	88.67	88.49	88.92	88.63	89.41	88.53
Crude protein, %	20.6	18.9	19.8	20.1	20.0	20.6
Crude fat, %	8.32	8.00	8.51	8.76	9.57	7.65
Starch, %	33.09	43.63	35.36	35.32	38.56	43.92
Calcium, %	0.86	0.57	0.62	0.65	0.89	0.82
Phosphorous, %	0.71	0.63	0.65	0.71	0.74	0.71
Potassium, %	0.78	0.84	0.81	0.74	0.67	0.54
Magnesium, %	0.15	0.15	0.16	0.16	0.18	0.18
Sodium, %	0.16	0.12	0.14	0.16	0.20	0.17

[1]Percentage of soybean meal replaced with black soldier fly larvae meal (BSFLM).
[2]Provided per kilogram of diet: vitamin A, 8,800.0 IU; vitamin D3, 3,300.0 IU; vitamin E, 40.0 IU; vitamin B12, 12.0 mg; vitamin K3, 3.3 mg; niacin, 50.0 mg; choline, 1,200.0 mg; folic acid, 1.0 mg; biotin, 0.22 mg; pyridoxine, 3.3 mg; thiamine, 4.0 mg; calcium pantothenic acid, 15.0 mg; riboflavin, 8.0 mg; manganese, 70.0 mg; zinc, 70.0 mg; iron, 60.0 mg; iodine, 1.0 mg; copper, 10 mg; and selenium, 0.3 mg.
[3]BMD, bacitracin methylene disalicylate (Zoetis Canada Inc., Kirkland, Quebec).
[4]SID, standardized ileal digestible.

Gold C18 column (1.9 μm, 2.1 mm × 50 mm, equipped with a guard column), coupled to a Q Exactive Mass Spectrometer (Thermo Scientific) equipped with a HESI II source, as described by Liu et al. (2021), at the BioZone Mass Spectrometry Facility (Department of Chemical Engineering and Applied Chemistry, University of Toronto, ON, Canada). The eluents were comprised of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). Gradient elution was conducted at a flow rate of 0.3 mL/min over 15 min with the following parameters: 5% B, 0−1 min; linear gradient to 98% B, 1−7 min; 100% B, 7−10 min; linear gradient to 5% B, 10−10.5 min; followed by 5% B, 10.5 −15 min. Ten microliters of each extract of the d 35 excreta samples were injected using the above method. The Q Exactive HESI II Mass Spectrometer was operated in both positive and negative polarity mode. MS1 spectra were acquired over an m/z range from 70 to 1,000 with the mass resolution set to 70k, AGC target of 3 x 10^6, max injection time 100 ms, spray voltage 3.5 kV, capillary temperature 320°C, sheath gas 15, aux gas 5, sweep gas 2, and S-lens RF level 55. Data dependent MS2 spectra were acquired using a Top5 approach with a mass resolution of 17.5k, AGC Target of 1 x 10^5, maximum injection time of 50 ms, an isolation window of 0.4 m/z and HCD collision energy of 30. Data was processed using Thermo Fisher Scientific Compound Discoverer 3.2 software, and searched against the HMDB, Kegg, BioCyc, mzCloud, and a custom AA database with a maximum error allowance of 5 ppm to putatively identify compounds. Peak abundance was determined by integrating the area under the curve.

SCFA Analysis and Quantification

On days 14, 28, and 35, two birds from each cage were randomly selected, weighed, and euthanized by cervical

dislocation and ceca were aseptically removed. Cecum from one bird were collected and transferred into 2 separate, sterile Whirl-Pack bags and immediately frozen in a cooler containing dry ice for subsequent analysis of SCFAs. Frozen cecal samples were cut open on weighing paper and 200 mg of content was immediately weighed and placed into a 15 mL screw cap plastic tube. Four milliliters of 0.01 M of HCl in water was added to the tube, capped and vortexed to mix, and then shaken for 2 h at room temperature on a Heildoph Multi Reax shaker (Heidolph Corp., Germany). The sample was centrifuged for 10 min at 4,000 × *g* at 4°C and the supernatant was collected. The supernatant was then filtered through a 0.22 μM PVDF syringe filter (Mandel Scientific, Guelph, ON, Canada). After that 200 μL of the filtrate was mixed with 800 μL of milli-Q water in a GC vial for GC analysis. A Thermo Scientific Trace 1310 GC-Flame Ionization Detector (**FID**) with TriPlus RSH auto sampler (Thermo Scientific, Mississauga, ON, Canada) was used for SCFAs analysis. A Phenomenex Zebron capillary GC ZB-FFAP column (30 m × 0.25 mm (I.D.) × 0.25 μm) was used for separation. One microliter of the prepared cecal samples or of a mixed SCFA standard solution was injected into the column at a 10:1 split ratio under split mode at 200°C. Helium at flow rate of 1.0 mL/min was used as the carrier gas. The oven temperature program was initially set at 100°C, then increased to 200°C at 10.0°C/min, and maintained at 200°C for 1 min. The FID condition was set at: temperature 250°C, air flow 350 mL/min, hydrogen 30.0 mL/min, nitrogen makeup gas 28.0 mL/min. The data were acquired and processed with Xcalibur Software Version 4.2.28.14 (Thermo Fisher Scientific, Mississauga, ON, Canada). A certified reference material CRM46975 volatile free acid mix from Sigma-Aldrich Canada (Oakville, ON, Canada) was used for the identification and calculation of the concentration of each SCFA in the diluted extract of chicken cecal samples. The calculation of each SCFA in chicken cecal samples was based on the GC-FID measured concentration in the diluted extract, multiplied by dilution factor and volume of extract, divided by the net weight of the chicken ceca sampled.

Data and Statistical Analysis

Data from the 6 dietary treatments on growth performance (BW, FI, cFCR, tibia bone traits, and mortality), cecal SCFA content, and putatively identified AAs were analyzed as a complete randomized design using the General Linear Mixed Model (**GLMM**) procedure of SAS 9.4 (SAS Institute Inc., 2016). Diets (treatments) were used as sources of variation and the individual cage (8/diet) as experimental units. The Interactive Matrix Language (**IML**) procedure of SAS was used to obtain contrast coefficients (SAS Institute Inc., 2022) to determine linear, quadratic, or cubic effects of diets using contrast statements. The Fisher least significant difference test was used to separate means whenever the F-value was significant. In all instances, a *P*-value of 0.05 was used to declare significance. All metabolites were annotated using the HMDB (https://hmdb.ca/metabolites) database and matched with the mzCloud database (https://www.mzcloud.org/). Principal component analysis (**PCA**) of the metabolomes were performed using Compound Discoverer version 3.2 software (Thermo Scientific). Figures were created using GraphPad Prism Software version 9.2 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Growth Performance

The effects of substituting SBM with different levels of BSFLM (12.5, 25, 50, or 100% BSFLM) on broiler chicken growth performance were assessed and compared to birds fed a conventional corn-SBM diet without (0% BSFLM) or with BMD (antibiotic growth promoter control) (Table 4). On d 14, the BW for birds fed BMD, 12.5% or 25% BSFLM were similar ($P > 0.05$), albeit, higher than the BW of birds in the 0, 50, and 100% BSFLM groups ($P < 0.05$). The BW of birds in the 0% BSFLM or 50% BSFLM groups were similar ($P > 0.05$), while the BW of birds fed 100% BSFLM were lower than the BWs of birds in all other groups ($P < 0.05$). This clearly indicates that complete substitution of SBM with BSFLM has an adverse impact on bird BW. From d 0 to 14, FI was higher for birds fed BMD when compared to the 0% BSFLM group ($P < 0.05$). There were no significant differences in FI for birds fed 12.5, 25, and 50% BSFLM as compared to the 0% BSFLM and BMD groups ($P > 0.05$). Consistent with the low BW observed for birds fed 100% BSFLM, FI for this group was lower as compared to birds in all other treatment groups ($P < 0.05$). From d 0 to 14, there were no significant differences in the cFCRs for birds fed 0, 12.5, 50, and 100% BSFLM as compared to the BMD group ($P > 0.05$).

On d 28, no significant difference in BW were observed between the 0% BSFLM and BMD groups ($P > 0.05$). While the BW observed for birds fed 12.5 and 25% BSFLM were similar to the BW of the 0% BSFLM group ($P > 0.05$), they were lower than the BW of the BMD treated group ($P < 0.05$). Once again, the BW of birds fed 100% BSFLM were lower than the BWs of birds in all other groups ($P < 0.05$). The BW of birds fed 50% BSFLM was lower than the BMD, 0, 12.5, and 25% BSFLM groups ($P < 0.05$). From d 15 to 28, birds fed 12.5, 25, or 50% BSFLM exhibited a similar FI compared to the 0% BSFLM and BMD groups ($P > 0.05$). In contrast, FI was lowest for birds fed 100% BSFLM when compared to the FI of birds in all other groups ($P < 0.05$), and again, this is consistent with the low BW exhibited for this group. From d 15 to 28, the cFCRs was lowest for birds fed BMD ($P < 0.05$), and greatest for birds fed 100% BSFLM ($P < 0.05$), while all other diets were intermediate.

Table 4. Effect of partially or completely substituting SBM with BSFLM on growth performance, tibia traits, and mortality of broiler chickens.

	Diet[1]										
		BSFLM, %[2]							Responses to BSFLM[5]		
Parameter[3]	BMD[4]	0	12.5	25	50	100	SEM	*Overall P*-value	Linear	Quadratic	Cubic
BW (g/bird)											
d 0	41.9	42.1	41.9	42.0	42.0	42.0	0.09	0.458	-[6]	-	-
d 14	230.4[a]	215.6[b]	235.2[a]	237.9[a]	216.2[b]	190.2[c]	4.27	<0.01	0.276	0.014	<0.01
d 28	1,112.5[a]	1,082.9[ab]	1,067.2[b]	1,065.4[b]	987.3[c]	793.4[d]	15.16	<0.01	<0.01	<0.01	<0.01
d 35	1,736.3[a]	1,648.3[ab]	1,655.5[ab]	1,625.6[b]	1,527.9[c]	1,241.7[d]	32.03	<0.01	<0.01	<0.01	<0.01
FI (g/bird)											
d 0–14	309.1[a]	283.1[b]	296.1[ab]	297.0[ab]	296.2[ab]	261.1[c]	6.88	<0.01	0.347	0.865	<0.01
d 15–28	1142.9[a]	1157.5[a]	1139.5[a]	1158.8[a]	1144.7[a]	913.8[b]	23.96	<0.01	<0.01	0.008	<0.01
d 29–35	842.0[a]	856.9[a]	821.4[a]	819.1[a]	808.0[a]	697.2[b]	20.90	<0.01	0.004	0.010	0.002
d 0–35	2294.1[a]	2297.6[a]	2257.0[a]	2275.0[a]	2249.0[a]	1872.1[b]	36.51	<0.01	<0.01	0.002	<0.001
cFCR[4]											
d 0–14	1.651[ab]	1.640[ab]	1.534[b]	1.519[b]	1.705[a]	1.780[a]	0.06	0.016	0.679	0.067	0.011
d 15–28	1.288[d]	1.328[cd]	1.349[bcd]	1.383[bc]	1.423[b]	1.513[a]	0.03	<0.01	0.052	0.010	0.008
d 29–35	1.289	1.435	1.356	1.379	1.395	1.477	0.04	0.08	0.826	0.775	0.053
d 0–35	1.351[d]	1.421[bcd]	1.399[cd]	1.434[bc]	1.491[ab]	1.557[a]	0.03	<0.01	0.336	0.004	0.004
Tibia attributes, d 35											
Fresh weight, g/kg BW	8.13	8.01	8.00	8.14	8.24	8.41	0.203	0.714	0.694	0.196	0.362
Dry weight, g/kg BW	3.31	3.31	3.31	3.38	3.45	3.53	0.074	0.205	0.527	0.049	0.206
Ash content, %	35.0	36.9	35.4	35.5	33.0	35.2	0.953	0.141	0.480	0.022	0.508
Total mortality (%)											
d 0–35	0	0.42	0	0	1.74	0	0.506	0.117	-	-	-

[1]Data represents least square means±SEM of 8 replicates/treatment arranged in a completely randomized block design. *P*-value was obtained by ANOVA.
[2]BSFLM, %, percentage of soybean meal replaced with black soldier fly larvae meal (BSFLM).
[3]BW, body weight; FI, feed intake; cFCR, cumulative feed conversion ratio.
[4]BMD, bacitracin methylene disalicylate control (corn-SBM diet with antibiotic growth promoter).
[5]Contrasts in LS Means tested among diets 0, 12.5, 25, 50, and 100% BSFLM.
[6]Not determined.
[abcd]Means within a row lacking a common superscript differ ($P < 0.05$).

On d 35, no significant differences in BW were observed between birds in the 0%, 12.5% BSFLM, and BMD groups ($P > 0.05$). The BW for birds fed 25% BSFLM was lower when compared to the BW for BMD-fed birds ($P < 0.05$), but not the 0% BSFLM group ($P > 0.05$). Again, BW was lowest for birds fed 100% BSFLM as compared to all other groups ($P < 0.05$). The BW of birds fed 50% BSFLM was lower compared to the BMD, 0, 12.5, and 25% BSFLM control groups ($P < 0.05$).

From d 29 to 35 and d 0 to 35, no significant differences in FI were observed between the BMD, 0, 12.5, 25%, and 50% BSFLM groups ($P > 0.05$), while FI was lower for birds fed 100% BSFLM as compared to all treatment groups ($P < 0.05$). From d 29 to 35, no significant differences in the cFCRs were observed between all groups ($P = 0.08$). From d 0 to 35, no significant difference in the cFCR values was observed between the BMD, 0%, and 12.5% BSFLM groups ($P > 0.05$), while an increase in cFCR was observed for the 25, 50, and 100% BSFLM groups as compared to the BMD group ($P < 0.05$). Notably, across all groups, birds fed 100% BSFLM demonstrated the highest cFCR ($P < 0.05$). No significant differences were observed for the cumulative mortality rate for all diets tested ($P > 0.05$) (Table 4).

To assess the dose-response effects of increasing inclusion levels of BSFLM on growth performance, linear, quadratic, and cubic contrasts analyses was employed for the 0, 12.5, 25, 50, and 100% BSFLM groups (Table 4). On d 14, BW demonstrated quadratic ($P = 0.014$) and cubic ($P < 0.01$) responses to increasing BSFLM, with a maximum observed for the 25% BSFLM group. On d 28 and 35, BW decreased linearly ($P < 0.01$), quadratically ($P < 0.01$), and cubically ($P < 0.01$) with increasing levels of BSFLM. From d 0 to 14, cubic responses to increasing inclusion levels of BSFLM were noted for FI ($P < 0.01$) and cFCR ($P = 0.011$), with a maximum FI and cFCR observed for 25% BSFLM and 100% BSFLM, respectively. From d 15 to 28, FI decreased linearly ($P < 0.01$), quadratically ($P < 0.01$), and cubically ($P < 0.01$) as BSFLM inclusion levels increased. From d 15 to 28, quadratic ($P = 0.010$) and cubic ($P = 0.008$) responses were observed for cFCR between groups, with a maximum corresponding to the 100% BSFLM group. From d 29 to 35, FI decreased linearly ($P < 0.004$), quadratically ($P < 0.010$), and cubically ($P = 0.002$) with increasing levels of BSFLM, with a maximum FI corresponding to the 0% BSFLM group. A similar response was observed from d 0 to 35 for FI. From d 29 to 35, no linear, quadratic, or cubic effects were noted for cFCR ($P > 0.05$). From d 0 to 35, quadratic ($P = 0.004$) and cubic ($P = 0.004$) responses were noted for cFCR, with a maximum observed for the 100% BSFLM group.

Tibia Traits

The effect of the experimental diets on d 35 tibia bone traits are shown in Table 4. No differences or dose-response effects in in tibia fresh weight were observed

between all groups ($P > 0.05$). A quadratic response ($P = 0.049$) was observed for tibia dry weight adjusted for bird BW, with a maximum corresponding to the 100% BSFLM group. This increase in tibia dry weight is likely due to the low BW exhibited by the 100% BSFLM group. Surprisingly, a quadratic response was also observed for ash content ($P = 0.022$), with 50% BSFLM exhibiting the lowest ash content.

Cecal SCFA Profile

To assess the impact of various levels of BSFLM on cecal SCFA production, SCFA concentrations were measured at the end of each growth phase. As seen in Table 5, no significant differences in total and specific SCFA levels were observed for any of the diets tested at each phase of growth ($P > 0.05$). Similarly, no linear, quadratic, or cubic effects were noted for any of the SCFAs measured ($P > 0.05$).

Excreta Metabolite Profiles

To assess if the observed effects of BSFLM on bird performance were associated with additional metabolic changes, we investigated the impact of diets containing BSFLM on the metabolome of d 35 excreta samples (mixture of both urine and feces). Altogether, 1,548 putative metabolites were present across all treatment groups. As seen in Figure 1, PCA of the excreta metabolite profiles showed independent clustering of birds fed 50 or 100% BSFLM from one another and from all other groups. The excreta metabolome of birds fed 25% BSFLM showed some independent clustering from the 0% BSFLM group, but not the BMD and 12.5% BSFLM groups. The excreta metabolomes of birds in the BMD, 0% and 12.5% BSFLM appeared to mostly overlap with one another (Figure 1). Taken together, these data suggest that high levels, but not low levels of BSFLM inclusion induces major metabolic changes in birds.

Excreta Amino Acid Analysis

Since AA metabolism is a critical nutritional process in birds and changes in AA content in excreta may reflect the nutritional status of the birds, we further mined the excreta metabolomes for changes in AA levels to better gauge AA digestibility among BSFLM-fed birds compared to BMD-fed birds. A total of 16 AAs were putatively identified in the excreta of all treatment groups (Table 6). No significant differences in the abundance of these AAs were observed for birds fed 25% BSFLM, 12.5% BSFLM when compared to the BMD or 0% BSFLM control groups. There were, however, several putative AAs that were differentially abundant in birds fed 50% or 100% BSFLM compared to BMD or 0% BSFLM fed birds (Table 6). Specifically, the excreta of

Table 5. Effect of partially or completely substituting SBM with BSFLM on cecal SCFA concentrations (μmol/g) for each phase of growth.

		Diet[1]									
			BSFLM[5]							Responses to BSFLM[2]	
SCFA[3]	Day	BMD[4]	0	12.5	25	50	100	SEM	Overall *P*-value	Linear	Quadratic	Cubic
	14											
Acetate		39.55	32.43	41.32	44.65	41.07	39.02	4.788	0.6131	0.519	0.311	0.217
Propionate		5.77	5.35	6.72	8.44	7.34	6.62	1.092	0.4133	0.918	0.197	0.205
Isobutyrate		3.45	4.58	3.32	4.11	4.49	3.69	0.789	0.8047	0.223	0.769	0.871
Butyrate		8.75	8.57	9.61	8.27	8.55	6.75	1.436	0.8274	0.967	0.410	0.218
Isovalerate		3.43	3.22	3.05	3.85	4.45	3.92	0.814	0.8418	0.703	0.164	0.868
Valerate		3.50	3.26	3.22	4.19	4.62	4.15	0.842	0.7928	0.763	0.131	0.802
Total		64.44	57.41	67.23	73.51	70.52	67.69	8.160	0.8026	0.714	0.265	0.325
	28											
Acetate		61.75	58.09	42.94	76.53	57.94	68.49	13.311	0.6088	0.360	0.293	0.720
Propionate		5.38	5.52	4.34	7.54	5.78	5.44	0.969	0.3452	0.092	0.213	0.438
Isobutyrate		1.62	2.01	1.82	2.03	1.35	2.08	0.275	0.3783	0.912	0.345	0.406
Butyrate		11.60	10.60	9.50	13.74	9.72	10.38	2.388	0.8245	0.378	0.766	0.584
Isovalerate		2.48	2.31	2.42	3.81	1.92	3.10	0.997	0.8076	0.930	0.815	0.898
Valerate		2.17	1.82	1.87	2.21	1.78	2.46	0.279	0.457	0.772	0.453	0.540
Total		80.49	82.46	72.25	117.04	80.50	88.76	16.250	0.4712	0.536	0.397	0.883
	35											
Acetate		61.07	58.66	61.46	54.26	62.28	63.86	7.395	0.9558	0.418	0.784	0.508
Propionate		7.13	8.54	7.91	7.56	7.34	7.27	0.736	0.7691	0.533	0.203	0.989
Isobutyrate		3.19	4.08	4.22	3.26	3.78	2.11	0.537	0.0871	0.480	0.062	0.055
Butyrate		13.31	14.22	16.80	14.14	14.20	11.82	1.415	0.2774	0.605	0.213	0.049
Isovalerate		2.78	3.24	3.21	3.09	2.76	2.48	0.501	0.8677	0.670	0.257	0.455
Valerate		3.78	3.61	3.60	3.36	3.22	2.82	0.534	0.8307	0.724	0.312	0.488
Total		87.90	96.28	85.33	87.77	93.22	90.28	9.603	0.9702	0.534	0.785	0.699

[1]Data represents least square means±SEM of 8 replicates/treatment arranged in a completely randomized block design. *P*-value was obtained by ANOVA. Means within a row lacking a common superscript differ ($P < 0.05$).
[2]Contrasts in LS Means tested among diets 0, 12.5, 25, 50, and 100% BSFLM.
[3]SCFA, short chain fatty acids.
[4]BMD, bacitracin methylene disalicylate.
[5]BSFLM, %, percentage of soybean meal replaced with black soldier fly larvae meal (BSFLM).

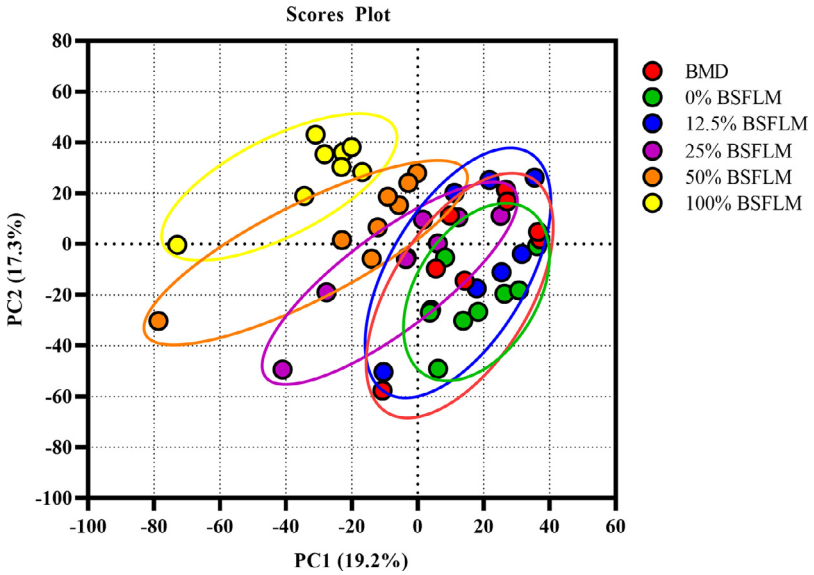

Figure 1. Principal component analysis (PCA) plot built from 1548 putative metabolites identified in excreta taken from d 35 birds fed BMD, 0, 12.5, 25, 50, and 100% BSFLM diets. The plot was generated from metabolite abundances. Each dot in the figure represents one replicate, and different colors indicate experimental diets. The 95% confidence regions are displayed for each diet. Data for the figure was generated using Compound Discoverer 3.2 software (Thermo Fisher Scientific).

birds fed 100% BSFLM contained higher levels of methionine, lysine, valine, glutamine, and histidine and lower levels of arginine as compared to the BMD group ($P < 0.05$; Table 6). The excreta of birds fed 50% BSFLM also contained higher levels of histidine and valine as compared to the BMD group ($P < 0.05$). These data suggest that substitution of SBM with high levels of BSFLM may impact AA metabolism in poultry.

DISCUSSION

This study investigated the effects of substituting SBM with various levels of BSFLM as a potential alternative to the widely used antibiotic growth promoter, BMD, in broilers. Overall, inclusion of 12.5% BSFLM had a similar effect on broiler performance including BW, FI, cFCR, tibia traits, excreta metabolome, and excreta AA content as broilers fed a corn-SBM diet with in-feed BMD. During the starter phase, both 12.5 and 25% BSFLM promoted growth similar to BMD-fed broilers. Indeed, this was the conclusion of a recent study, published while this manuscript was being prepared, demonstrating that substitution of SBM with BSFLM at 12.5 or 25% fortified with black soldier fly oil could provide some growth-promoting effects in broilers in a similar manner as broilers fed BMD and narasin (Facey et al., 2023). Importantly, black soldier fly oil

Table 6. Effect of partially or completely substituting SBM with BSFLM on d 35 excreta amino acid levels[1].

	Diet							
		BSFLM, %[3]						
Amino acid	BMD[2]	0	12.5	25	50	100	SEM	*P*-value
Alanine	4.27E+07	3.85E+07	5.57E+07	4.37E+07	4.87E+07	4.11E+07	4.84E+06	0.1726
Arginine	1.24E+08^{ab}	1.36E+08^{b}	1.26E+08^{ab}	7.67E+07^{ac}	1.11E+08^{ab}	3.38E+07^{c}	1.95E+07	0.005
Asparagine	5.12E+06	4.53E+06	7.58E+06	6.73E+06	8.22E+06	6.44E+06	1.12E+06	0.1872
Aspartic acid	6.91E+06	5.91E+06	7.15E+06	8.52E+06	7.24E+06	6.23E+06	8.27E+05	0.3141
Glutamine	3.14E+07^{a}	3.78E+07^{a}	3.89E+07^{ab}	3.85E+07^{ab}	4.97E+07^{bc}	5.24E+07^{c}	3.95E+06	0.0043
Glutamic acid	1.63E+08	1.53E+08	1.79E+08	1.80E+08	1.84E+08	1.95E+08	1.91E+07	0.69
Histidine	2.03E+07^{a}	2.93E+07^{abc}	3.03E+07^{bc}	2.47E+07^{ab}	3.81E+07^{c}	3.55E+07^{c}	3.39E+06	0.0063
Isoleucine	1.87E+09	1.86E+09	2.31E+09	1.84E+09	2.68E+09	2.01E+09	2.18E+08	0.0532
Lysine	9.84E+07^{ab}	9.05E+07^{a}	1.09E+08^{ab}	1.14E+08^{bc}	1.33E+08^{bc}	1.49E+08^{c}	1.23E+07	0.0163
Methionine	1.12E+06^{a}	1.58E+06^{a}	1.63E+06^{a}	2.88E+06^{a}	4.10E+06^{ab}	6.01E+06^{b}	1.09E+06	0.0226
Phenylalanine	1.44E+09	1.63E+09	2.07E+09	1.59E+09	2.10E+09	1.47E+09	1.90E+08	0.0523
Proline	3.94E+08	4.60E+08	4.98E+08	4.05E+08	4.72E+08	3.69E+08	4.64E+07	0.3321
Threonine	5.87E+07	6.50E+07	5.99E+07	6.77E+07	5.99E+07	7.30E+07	7.06E+06	0.6766
Tryptophan	2.96E+07	3.38E+07	4.00E+07	2.77E+07	3.93E+07	3.23E+07	5.12E+06	0.4523
Tyrosine	2.00E+08	1.81E+08	2.89E+08	2.01E+08	1.83E+08	2.61E+08	3.48E+07	0.1647
Valine	5.38E+08^{a}	6.31E+08^{a}	5.74E+08^{a}	7.03E+08^{a}	9.48E+08^{b}	1.47E+09^{c}	8.55E+07	<0.0001

[1]Each value represents the least squares means of the peak area under the curve of putatively annotated amino acids in day 35 broiler chicken excreta of 8 replicates/treatment arranged in a completely randomized block design. *P*-value was obtained by ANOVA.
[2]BMD, bacitracin methylene disalicylate control.
[3]BSFLM, %, percentage of soybean meal replaced with black soldier fly larvae meal (BSFLM).
[abc]Means within a row lacking a common superscript differ ($P < 0.05$).

has been shown to harbor antimicrobial properties (Dabboo et al., 2020; Sypniewski et al., 2020) and thus, it is unclear if the oil or meal alone or a combination of both were responsible for the observed growth enhancing effects in that study (Facey et al., 2023). In the present study, we demonstrate for the first time that BSFLM alone, at a substitution level of ≤25%, is sufficient to confer growth enhancing effects in broilers during the starter phase. Similar positive effects on performance have also been observed in broilers fed diets containing partially defatted BSFLM at 15% or full-fat BSFLM at 20% for 35 d and 42 d, respectively, including improving FCR, live weight, and daily FI (Dabbou et al., 2018; de Souza Vilela et al., 2021). However, in these instances, the impact of BSFLM on broiler performance was not directly compared to broilers fed a conventional diet (e. g., corn-SBM) containing an antibiotic growth promoter. Nevertheless, in agreement with these studies, our data suggests that low levels of BSFLM in broiler diets may not only serve as a substitute for SBM, but may also possess functional benefits.

Mechanistically, the positive effects of low inclusion levels of BSFLM on broiler performance is currently unclear, but like AGPs, may involve bacteria-centric (i. e., modulate enteric microbiota) and/or host-centric (i. e., immunomodulatory) modes of action (Brown et al., 2017). One mechanism by which AGPs are hypothesized to enhance growth is by modulating the levels of microbiota-derived SCFAs (Cho et al., 2012). In mice, subtherapeutic antibiotic therapy has been shown to increase the concentration of microbiota-derived SCFAs in the gut, which in turn, are delivered to the liver and promote lipogenesis and weight gain (Cho et al., 2012). Consistent with this model, in broilers, the AGPs, virginiamycin or a combination of neomycin and oxytetracycline have been shown to increase cecal SCFA production (Pourabedin et al., 2015; Kareem et al., 2017). In the present study, neither BMD or inclusion of BSFLM in the diets of broilers impacted cecal SCFAs concentrations, clearly indicating that the growth promoting effects of BMD or low levels of BSFLM occur in a SCFA-independent manner. Consistent with this, Kawasaki et al., (2019) demonstrated that inclusion of 10% black soldier fly larvae or prepupae in the diets of hens had no effect on cecal acetate, propionate, and butyric acid levels. Similarly, previous studies reported no effect of in-feed zinc bacitracin (Akbaryan et al., 2019) or BMD (Oladokun et al., 2021) on cecal SCFA levels in broilers. Another bacteria-centric mode of action of AGP involves the modulation of certain intestinal bacterial populations. BMD is thought to promote broiler growth by directly inhibiting the growth of intestinal *Lactobacillus* spp. (Guban et al., 2006; Zhou et al., 2007). *Lactobacillus* spp. produce an enzyme known as bile-salt hydrolase that negatively effects host fat digestion and utilization (Guban et al., 2006; Zhou et al., 2007). As a result, BMD-mediated depletion of *Lactobacillus* spp. has been suggested to reverse the negative effects of the bile-salt hydrolase enzyme on fat metabolism. (Guban et al., 2006; Zhou et al., 2007). To this end, cecal *Lactobacillus* spp. levels were reduced in laying hens fed diets containing 10% black soldier fly larvae or prepupae, although no effect of the larvae or prepupae on hen BW or FI were noted when compared to the control group (i. e., no dietary inclusion of black soldier fly larvae or prepupae (Kawasaki et al., 2019). In contrast, broilers fed 10% partially defatted BSFLM diets exhibited an increase in the cecal levels of the genus *Lactobacillus* (Biasato et al., 2020). At present, the impact of low levels of BSFLM on the intestinal microbiota composition and its relationship to performance is currently unclear.

Consistent with the immunomodulation hypothesis of antibiotic growth promoter action, it was recently shown that inclusion of full-fat black soldier fly larvae at 20% in broiler diets improved the feed conversion ratio and reduced the number of lymphocytes, white blood cells, and intestinal cytotoxic lymphocytes as compared to the control group (i. e., no dietary inclusion of black soldier fly larvae) (de Souza Vilela et al., 2021). One possibility is that low levels of BSFLM may dampen the intestinal immune response resulting in a reduction in the catabolic costs of maintaining an immune response thus allowing more resources to be used for anabolic processes (i. e., tissue development) (Niewold, 2007; Costa et al., 2011). Nevertheless, to better understand the growth promoting effects of low levels of BSFLM, future studies aimed at elucidating the impact of BSFLM on the intestinal immune system are warranted.

In this study, the growth promoting effects of BMD on broiler performance were only observed during the starter phase. While bacitracin is generally regarded as a growth promoting agent (Wicker et al., 1977; Kaukas et al., 1988; Guban et al., 2006), conflicting results exist in the literature with respect to its growth-promoting properties. For example, Engberg et al. (2000) demonstrated that zinc-bacitracin (20 mg) did not significantly impact pen-raised broilers BW and FI at all phases of growth compared to the no antibiotic control group (Engberg et al., 2000). Similarly, feeding bacitracin (55 ppm) did not impact the BW of d 18 pen-raised broilers fed a corn-SBM-based diet; however, it did promote the growth of broilers fed a sucrose-based diet (Stutz et al., 1983). More recently, no significant differences in growth performance was observed in cage-raised broilers fed BMD at subtherapeutic (55 ppm) and therapeutic (275 ppm) levels (Robinson et al., 2020). Similarly, in 1 of 3 trials, 50 ppm of bacitracin fed to broilers maintained in floor pens did not demonstrate a significant increase in BW compared to the untreated control group for all periods of growth, although, in the other 2 trials, bacitracin enhanced bird BW of d 10 birds when compared to the untreated control group (Guban et al., 2006). In a recent study, BMD at 55 ppm was shown to significantly enhance growth and performance parameters of floor-cage raised birds during the starter and grower phases (Li et al., 2022). BMD at 55 ppm was also shown to improve the overall body weight gain and the feed conversion ratio of pen-raised broilers (Facey et al., 2023). High concentrations of in-

feed BMD (500 ppm) have been shown to enhance BW in cage-raised birds during the grower and finisher phases, but not during the starter phase (Rivera-Pérez et al., 2021). A lack of reproducibility demonstrating the positive growth responses to dietary bacitracin in broilers may be attributed to many factors including, concentration of bacitracin used, environmental conditions, rearing conditions (pen vs. cage), feed composition, broiler breed, genetics, age, gender, and disease status of the birds (e. g., prevalence of *C. perfringens*). Verification and standardization of the conditions in which bacitracin reproducibly exhibits a significant positive growth response is necessary to not only understand the mechanism of action of this agent on growth, but also to identify alternatives. Still, in this study, both BMD and ≤25% BSFLM improved broiler performance as compared to the 0% BSFLM control during the starter phase, thus providing a precedence for the use BSFLM as a potential alternative to BMD.

In addition to being used as an antibiotic growth promoter, bacitracin is effective at reducing the morbidity and mortality and improving growth parameters in *C. perfringens*-challenged broilers (Brennan et al., 2003; Krueger et al., 2017). To this end, black soldier fly protein preparations have been shown to suppress growth of *C. perfringens* in vitro (Dong et al., 2021). However, supplementing broiler diets with 10% BSFLM was shown to increase the levels of *C. perfringens* in the ileum and cecum, but this did not result in intestinal lesions or necrotic enteritis (Van der Heide et al., 2021). Thus, to truly serve as an AGP alternative to BMD, future studies assessing the effects of low levels of BSFLM on the prevalence and management of *C. perfringens*-induced necrotic enteritis in broilers are warranted.

Consistent with previous studies, and reconfirmed here, a dose-response effect of including BSFLM in poultry diets was observed, with high levels of BSFLM negatively impacting poultry performance (Marono et al., 2017; Dabbou et al., 2018; Cutrignelli et al., 2018; Murawska et al., 2021; Facey et al., 2023). The cause(s) of the growth depressive effects of high levels of BSFLM (≥50%) have yet-to-be elucidated (Borrelli et al., 2017; Marono et al., 2017; Cutrignelli et al., 2018), although several theories have been postulated. Marono et al. (2017), suggested that the lower FI of hens fed high levels of BSFLM may be due to the undesirable darker color and different flavor compared to SBM and, therefore, birds are less willing to consume the meal (Marono et al., 2017). Alternatively, it has been suggested that the high chitin content of the feed interferes with protein digestibility, in turn resulting in poor growth performance (Cutrignelli et al., 2018). In agreement with this, in vitro crude protein digestibility was shown to negatively correlate to the chitin content of black soldier fly meal (Marono et al., 2015). Moreover, feeding broilers a diet containing 3.23% shrimp chitin has been shown to have negative effects on growth performance and digestibility, while lower levels of chitin (1.36% and 2.42%) were shown to have beneficial effects on growth performance and no adverse effect on digestibility (Khempaka et al., 2006a,b). The chitin content of BSFLM appears to vary and has been estimated to range from 2.8 to 7.29% (Marono et al., 2015; Cutrignelli et al., 2018; Mwaniki and Kiarie, 2019). Using a previously established protocol for chitin isolation (Wang et al., 2020a), the chitin content of the BSFLM sourced from Enterra Feed Inc. was determined to be 8.0% ± 0.29 (mean ± SD) of the meal (M. Diarra, unpublished data) and is in excellent agreement with previously experimentally determined levels of chitin extracted from black soldier fly larvae (∼7.5-10.1%) (Soetemans et al., 2020). Assuming an average chitin content of 8.0%, then diets containing 100% BSFLM contained approximately 2.3% (starter), 2.1% (grower), and 1.8% (finisher) of chitin. Taking into account the average FI and a chitin content of 8%, then broilers in the 100% BSFLM group consumed approximately 6.1 g, 19.6 g, and 12.8 g of chitin per bird during the starter, grower, and finisher phases, respectively. Black soldier fly larvae chitin ingestion at these levels may contribute to the growth depressive effects of high levels of BSFLM in broilers. Still, the percentage of chitin estimated in the diet of broilers fed 100% BSFLM are within the range of shrimp chitin levels shown to have a positive effect on growth performance in broilers (Khempaka et al., 2006a,b). Consequently, a detailed study of various levels of chitin purified from BSFLM on broiler performance should be performed to provide insight into whether or not chitin is responsible for the observed growth depressive effects of BSFLM.

Untargeted metabolomics of d 35 excreta revealed an increase in the levels of several putatively identified AAs from broilers fed high levels of BSFLM. More specifically, the essential AAs methionine, lysine, valine, histidine, and the nonessential AA, glutamine were found to be higher in the excreta of broilers fed 100% BSFLM. These data suggest that diets containing 100% BSFLM interferes with the absorption of these AAs, which in turn affects protein synthesis and ultimately, broiler growth. In contrast, arginine levels decreased in the excreta of broilers fed 100% BSFLM and this may be due to a deficiency in the diet and/or enhanced catabolism. It is well-established that AA imbalance in poultry diets can affect FI, weight gain, and carcass composition (Bunchasak, 2009). Methionine, lysine, and valine are limiting AAs in corn-SBM-based diets and consumption of diets deficient in any one of these AAs have been shown to depress broiler growth performance parameters including weight gain, FI, FCR (Bunchasak and Keawarun, 2006; Farran and Thomas, 1992; Tian et al., 2019). Consumption of diets deficient in histidine have also been shown to negatively impact broiler body weight gain and FI (Han et al., 1991). Glutamine is a nonessential AA and plays an important role in intestinal development, structure and function, and nutrient absorption (Nassiri Moghaddam and Alizadeh-Ghamsari, 2013). Therefore, reduced absorption of these AAs may contribute to the growth depressive effects of high levels of BSFLM. Arginine is considered the fifth

limiting AA for broiler chickens and also plays an important role in broiler growth (Kidd et al., 2001; Portocarero and Braun, 2021) and immunity (Lee et al., 2002). Intriguingly, Facey et al. (2023) demonstrated that diets containing 25, 50, and 100% BSFLM were deficient in the AA, arginine, and this deficiency increased as the levels of BSFLM increased. Importantly, this negatively impacted the arginine: lysine ratios to levels that were below the NRC recommendations of 0.9 to 1.18 in diets containing 100% BSFLM for all three phases, and in diets containing 25 and 50% BSFLM for the starter and finisher phases (Facey et al., 2023). It is well-established that changes in the optimum value of the arginine: lysine ratio adversely affects the performance of growing poultry (Balnave and Barke, 2002). Therefore, an imbalance in the arginine: lysine ratio in diets containing high levels of BSFLM may, in part, explain the poor performance of the broilers observed in this and other studies. In line with this, substitution of SBM with 50% BSFLM in diets fortified with the AAs lysine, methionine, arginine, and valine was shown to improve the performance of broiler chickens compared to diets containing 50% BSFLM and a basic level of AA supplementation (Velten et al., 2018), indicating that broilers fed high levels of BSFLM were not receiving sufficient AAs including arginine, in their diets.

In this study, tibia dry weight standardized to BW was higher for birds fed high levels of BSFLM, further indicating poor/imbalanced body tissue growth. For the most part, the diets used in this study did not impact tibia ash content with the exception of broilers fed 50% BSFLM, in which tibia ash content was reduced. It is well-established that tibia ash content is impacted by the ratios of calcium and available phosphorous in the feed (Nguyen et al., 2021). At present, the reduced tibia ash content of the 50% BSFLM group appears to be an anomaly, as no major differences in the levels of calcium and phosphorous levels between the diets were noted. Nevertheless, low levels of BSFLM had a similar effect on tibia bone traits as BMD-fed birds and thus, can be incorporated into broiler diets without adversely impacting bone development.

There is growing concern that intensive use of antibiotics in livestock production contributes to environmental release of antibiotics and antibiotic resistant bacteria. Worryingly is the observation that the bacterial *mcr-1* gene, which confers resistance to last-resort antibiotic, colistin, has been found in bacterial isolates from poultry and has been shown to confer cross-resistance to bacitracin (Xu et al., 2018; Song et al., 2020). One possibility then, is that the continued use of bacitracin in poultry production may facilitate the persistence and transmission of colistin-resistant bacteria in livestock and eventually in humans (Xu et al., 2018). While the link between bacitracin use and colistin resistance has yet-to-be elucidated in poultry production and in humans, substitution of BMD with BSFLM is one strategy to minimizing the release of antibiotic residues into the broader environment. However, chitin and its derivatives have been shown to increase DNA uptake in the bacterial pathogen, *Vibrio cholera* (Meibom et al., 2005). The implication being that chitin present in BSFLM may facilitate the horizontal transfer of antibiotic resistance genes in poultry. More recently, in vitro exposure of the human pathogen, *S. aureus* to chitosan, a derivative of chitin co-selected for antibiotic resistant isolates of *S. aureus* (Raafat et al., 2017).Therefore, future research elucidating the impact of BSFLM on the abundance and diversity of clinically relevant antibiotic resistant genes and bacteria in broiler production is required.

There are several limitations in the present study. One limitation of this study is the use of excreta for untargeted metabolomics analysis and subsequent determination of putative AA levels. While the present study sheds some insight into the potential negative effects of BSFLM on AA levels, it is well-established that using ileal digesta for AA level determination is far more accurate than using excreta (Ravindran and Bryden, 1999a; Ravindran et al., 1999b). The differences in AA levels in excreta of the birds fed BSFLM may not only be the result of non-absorbed dietary AAs, (Ravindran and Bryden, 1999a; Ravindran et al., 1999b), but of changes in AA catabolism, hindgut microflora AA metabolism (Ravindran and Bryden, 1999a; Ravindran et al., 1999b; Xu et al., 2016) and to a lesser extent, urinary contribution of AAs to excreta (Ravindran and Bryden, 1999a; Ravindran et al., 1999b). Furthermore, the AAs in the excreta were putatively identified and therefore a targeted metabolomics approach to confirming the identity and concentration of the AAs present in the excreta by using reference standards is required (Schymanski et al., 2014). Future studies evaluating the impact of high levels of partially defatted BSFLM on AA apparent ileal digestibility coefficients is necessary to better understand the effects of this novel feed ingredient on AA utilization. Moreover, while high levels of BSFLM induced major metabolic shifts in the broiler excreta, the identity of the metabolites contributing to this shift have yet-to-be determined. Using a targeted metabolomics approach, confirmation of the identities of the metabolites that are either enriched or depleted may provide mechanistic insight into how high levels of BSFLM negatively broiler health and performance.

In summary, our data suggests that 12.5% or 25% BSFLM can be incorporated into broiler diets during the starter phase to enhance broiler growth, thus minimizing the need for medically important antibiotics in poultry diets. Future studies investigating the growth enhancing properties BSFLM compared to other AGPs are warranted. Finally, despite being touted as a sustainable protein alternative to SBM, research focused on identifying the factor(s) contributing to the performance depressive effects of high levels of BSFLM are required if BSFLM is going to truly serve as a complete replacement to SBM and its use as a feed ingredient be adopted by poultry producers worldwide.

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MF, MD, EK, ET conceived and designed the study. MK performed the experiments. MK, MF, MD, and EK analyzed the data. MF wrote the paper. MF, MD, EK, and ET edited the paper.

DISCLOSURES

The authors declare that they have no conflicts of interest or competing interests.

SUPPLEMENTARY MATERIALS

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