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Chitin digestibility and intestinal exochitinase activity in Nile tilapia and rainbow trout fed different black soldier fly larvae meal size fractions

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Abstract
Black soldier fly (Hermetia illucens) larvae meal (BSFLM) is a novel fish feed ingredient with a high crude protein content (~40% dry matter, DM). However, BSFLM also contains chitin, which might impair nutrient digestibility. To investigate the effect of chitin on nutrient digestibility and chitinase activity in fish, BSFLM with different chitin contents were tested as feed ingredients for Nile tilapia (Oreochromis niloticus) and rainbow trout (Oncorhynchus mykiss). BSFLM was sieved into three size fractions obtaining different chitin contents: 0–200 μm (fine), 200–400 μm (medium), >400 μm (coarse), 1.8, 2.7 and 15.4% chitin on a DM basis, respectively. Four isoenergetic and isonitrogenous diets were formulated for each species including an experimental reference diet and three diets replacing 25% of the experimental reference diet matrix with one of the three BSFLM size fractions. Dietary inclusion of the coarse BSFLM fraction resulted in significantly lower apparent digestibility of DM, crude protein, nitrogen-free extract and chitin when compared with the other treatment groups in both fish species, which supports the hypothesis that chitin might act as an anti-nutrient. However, it was also found that both species could digest chitin but its digestibility decreased with a higher dietary chitin inclusion level. Furthermore, β-N-acetylglucosaminidase activity (exochitinase) increased with higher dietary chitin inclusion in the proximal and distal intestine of both fish species suggesting that exochitinase production can be upregulated depending on the dietary chitin intake. Overall, the results corroborate that chitin could act as a nutrient source as well as an anti-nutrient in both Nile tilapia and rainbow trout.

KEYWORDS
chitin digestibility, chitin fractionation, chitinase, Hermetia illucens, Oncorhynchus mykiss, Oreochromis niloticus
1 | INTRODUCTION

As the aquaculture sector is growing tremendously due to a worldwide increase in fish consumption (FAO, 2020), higher volumes of fish feed are required to sustain aquaculture production. Fed-based aquaculture is largely dependent on high-quality feed ingredients ensuring optimal feed utilization and high fish performance. Fishmeal is generally considered the optimal protein source for fish due to, among others, its high digestibility, high palatability, high crude protein content, low carbohydrate content, and balanced amino acid profile (Renna et al., 2017; Zhou et al., 2004). Despite the nutritional benefits of fishmeal, its low availability and high price have driven the search for alternative protein sources. Most research has focused on ingredients of plant origin; however, these have only proven to be moderately successful due to their generally imbalanced amino acid profiles, the presence of anti-nutritional factors and low palatability (Glencross et al., 2020). Recent investigations have looked at the use of insects as a fish feed ingredient given that insects are part of the natural diet of several fish species (Henry et al., 2015). Additionally, insects generally contain a high protein content, although this varies depending on the insect’s diet, life stage and species (Nogales-Mérida et al., 2019). One insect species that seems particularly promising is the black soldier fly (BSF, Hermetia illucens) whose larvae can convert a wide variety of low-quality organic matter into larval biomass (Jucker et al., 2017; Nguyen et al., 2015; St-Hilaire, Cranfill, et al., 2007). Furthermore, BSF larvae are relatively high in crude protein (31%–59% dry matter, DM) and crude lipid (11%–49% DM) and their meal has an amino acid profile more comparable with fishmeal than most plant-based ingredients (Barroso et al., 2014; Makkar et al., 2014; Nogales-Mérida et al., 2019). The high nutritional value makes black soldier fly larvae meal (BSFLM) of interest as an alternative fish feed ingredient.

For several fish species, BSFLM has been successfully incorporated as a feed ingredient, including Atlantic salmon (Salmo salar) (10%–20% dietary inclusion), Nile tilapia (Oreochromis niloticus) (1%–10% dietary inclusion) and rainbow trout (Oncorhynchus mykiss) (20%–40% dietary inclusion) (Belghit et al., 2019; Fisher et al., 2020; Tippayadara et al., 2021). However, other studies found that growth performance was repressed with dietary inclusion of BSFLM, including turbot (Psetta maxima) (16.5%–75.6% dietary inclusion) and rainbow trout (6.6%–26.4% dietary inclusion) (Dumas et al., 2018; Kroeckel et al., 2012). These seemingly contradictory results could be due to differences in BSFLM inclusion levels, replaced protein sources and BSFLM quality (Hua, 2021; Liland et al., 2021; Wetthasinghe, Hansen, Myldand, & Øverland, 2021). The quality of BSFLM depends, among others, on processing procedures, protein content, amino acid profile and chitin content. Chitin has been hypothesized by some studies to have a potential negative impact on fish performance and nutrient digestibility (Kroeckel et al., 2012; St-Hilaire, Sheppard, et al., 2007).

Chitin is a polymer of N-acetyl-D-glucosamine and is found in the exoskeleton of insects (Rathore & Gupta, 2015). The chitin content in BSF larvae is approximately 5.4%–9.0% on a DM basis (Caligiani et al., 2018; Finke, 2013). Despite several studies on chitinolytic activity, it remains unclear which fish species can digest chitin and to what extent. Chitinolytic activity has, for example, been found in the intestinal tract of Atlantic cod (Gadus morhua) and rainbow trout, whereas it was not detected in meagre (Argyrosomus regius) (Danulat & Kausch, 1984; Guerreiro et al., 2021; Lindsay et al., 1984). Chitinolytic enzymes can be categorized either as endochitinases (splitting randomly within the chitin polymer) or exochitinases.

The latter can be further divided into chitobiosidases (catalyzing the release of acetylchitobiose from the non-reducing end of the chitin polymer) and glucosaminidases (catalyzing the release of N-acetylglucosamine from the non-reducing end of the chitin polymer) (Rathore & Gupta, 2015). The role of these chitinolytic enzymes in chitin digestibility in fish, however, remains unknown.

Similar to several other non-starch polysaccharides, chitin seems to possess anti-nutritional properties. In tilapia (Oreochromis niloticus × Oreochromis aureus), feeding diets supplemented with 2%, 5% or 10% chitin for 8 weeks resulted in a dose-dependent reduction in weight gain, lipid digestibility and DM digestibility, and an increase in feed conversion ratio (FCR) (Shiau & Yu, 1999). Along the same line, feeding diets with 1% chitin to common carp (Cyprinus carpio) for 90 days resulted in a significantly lower final body weight compared with fish fed diets without supplementation (Gopalakannan & Arul, 2006). The reduction in growth performance has been associated with chitin impairing the digestibility of certain nutrients. Although evidence in fish is currently lacking, the provision of chitinous material to mammals has been shown to bind lipids in the gastrointestinal tract, making them less available for hydrolysis, reducing lipid absorption and promoting lipid excretion (Muzzarelli, 1996). Furthermore, a monogastric in vitro digestibility study found a negative correlation between chitin content and protein digestibility (Marono et al., 2015).

Despite indications of chitin impairing fish performance, very few studies have examined how to potentially improve the applications of BSFLM in fish feed by reducing the chitin content. Two studies investigated the fractionation of chitin from different BSF stages using chemical extraction methods (Caligiani et al., 2018; Smets et al., 2020), and while these methods were able to fractionate chitin, they produced large volumes of chemical waste and were only applied on a laboratory scale. Aiming at a more sustainable method, the objectives of the current study were to investigate whether sieving activity in Nile tilapia and rainbow trout.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All procedures complied with current Danish and EU legislation (Directive 2010/63/EU) regarding animal experimentation.
2.2 | Experimental diets

A batch of BSF larvae was reared on a mixture of chicken feed and plant-based by-products at ENORM Biofactory A/S (Flemming, Denmark). The larvae were harvested 12 days post-hatching and mechanically separated from their substrate, washed, partially defatted (using mechanical pressing without solvents), dried and ground into a meal. The partially defatted meal was mechanically sieved by a commercial company (Engsko, Randers, Denmark) into three size fractions with particle sizes 0–200μm, 200–400μm, and >400μm. The nutritional composition of the three meal fractions, including their amino acid composition, is shown in Table 1.

An experimental reference diet was formulated for either species of which 25% of the matrix was replaced by one of the three BSFLM fractions (Table 2, amino acid composition described in Table 51), which was the highest inclusion level possible to formulate the diets isoenergetically given the high lipid content in the three BSFLM fractions. Additionally, diets were formulated to be isonitrogenous with estimated amino acid profiles above requirements for both species. The diets were formulated and produced as 3mm extruded pellets by Aller Aqua A/S (Christiansfeld, Denmark).

2.3 | Fish

Juvenile Nile tilapia (~8 g) were obtained from Til-Aqua (Someren, the Netherlands) and transported to the trial location (Technical University of Denmark, Hirtshals, Denmark), where they were maintained on a commercial diet (Inicio Plus, BioMar, Denmark) until reaching a mean body weight of ~40 g. Two weeks before the trial, the fish were fasted for 24 h and weighed using mild sedation (0.04 g/L ethyl-p-amino benzoate). A total of 144 fish with a mean body weight (BW) ± SD of 41.2 ± 1.7 g were distributed in 12 identical tanks (189 L) mounted in a flow-through mass balance system as described by Dalsgaard and Pedersen (2011). The fish were acclimated to the tanks and feeding routine for 2 weeks feeding each diet to three randomly assigned tanks.

Juvenile rainbow trout (~50 g) were obtained from Refsgård Fiskeri (Egtved, Denmark) and transported to the same trial location, where they were quarantined for 2 weeks while feeding a commercial diet (Efico Enviro 920 advance, BioMar, Denmark). After quarantine, the fish were handled as described for the tilapia and 216 fish with a mean body weight of 70.9 ± 1.0 g were distributed in the 12-tank mass balance system at a stocking density of 18 fish per tank, feeding each diet to three randomly assigned tanks during 2 weeks acclimation.

2.4 | Digestibility trials

A 40L/h freshwater flow was applied for both species during the digestibility trials while the dissolved oxygen was maintained between 85% and 100% saturation using an automatic monitoring and dosing system (Oxyguard Pacific System, OxyGuard International A/S, Denmark). The photoperiod during the trial was 14 h light and 10 h dark (06:00–20:00). Nile tilapia were kept at 23±1°C while rainbow trout were kept at 11±1°C, both temperatures were within the optimal temperature range for each species (Huet & Timmermans, 1972; Leonard & Skov, 2022). The water quality was checked daily for pH, NH4+, NO2- and NO3-.

Using mild sedation, the fish were individually weighed before the start of the digestibility trial. Tilapia were fed 2.0% body weight per day (BW/d) for 9 days applying an average FCR of 1.1 as calculated during the acclimatization period. Trout were fed 1.2% BW/d for 9 days based on an average FCR of 0.9 as calculated during the acclimatization period. Approximately 60% of the daily ration was fed during 20 min starting at 10:00 and 40% at 13:00 using automated feeders. Uneaten pellets were collected and counted after each 20 min feeding event and subtracted from the total feed intake. Feces were collected continuously over 24 h for 9 days in sedimentation columns for measuring digestibility directly as originally described by Cho et al. (1982) and refined by Dalsgaard and Pedersen (2011). Total feces samples were pooled per three consecutive days and stored at −20°C while awaiting further analysis. The first pooled samples were used as backup, while apparent digestibility coefficients were calculated for the second and third pooled samples as previously described by Dalsgaard and Pedersen (2011). After the digestibility trial, fish were weighed individually (BWj) and killed using an overdose of benzocaine (0.1 g/L ethyl-p-aminobenzoate). A number of fish (n = 4 for tilapia, n = 3 for trout) were taken from each tank for dissection of the liver and intestinal tract and weights were determined. The intestinal tract in trout was divided into three sections (proximal, middle and distal) as described by Verdille et al. (2020) while in tilapia, the different intestinal sections could not be easily distinguished and intestines were, therefore, cut into three even sections based on the total intestinal length. Crude enzyme extracts were obtained as described by Pfalzgraff et al. (2021) and stored at −80°C until further analysis.

2.5 | Analytical methods

Feed samples were ground with a mill (A10 basic, IKA, Staufen, Germany) and homogenized to collect representative sub-samples. Fecal samples were thawed and homogenized using an Ultra Turrax (T18 basic, IKA, Staufen, Germany). Feed and feces were analyzed for DM by drying samples at 105°C until the weight was constant (Kolar, 1992). Ash was determined by incineration in a muffle furnace at 550°C overnight (Kolar, 1992). Crude protein (N * 6.25) was measured using the Kjeldahl method (Kjeltc 2200, Foss, Hillerød, Denmark) after acid digestion (ISO 2005). Crude lipid was quantified using chloroform-methanol extraction as described by Bligh and Dyer (1959). The amino acid content of the different BSFLM fractions and the diets was measured as previously described by Larsen et al. (2012) with some modifications. Samples were finely ground, and 9 mg (2 replicates per sample) was transferred to a 5 ml vacuum hydrolysis tube by
suspending the sample in 1.8 ml of HCl (6 M, Supelco, USA) containing 0.2% liquefied phenol (Sigma-Aldrich, USA). Oxygen was removed by flushing with nitrogen under vacuum while vortexing at 2000 rpm. The tube was sealed under vacuum conditions and heated at 110°C for 24 h in a reacti-therm heating module (Pierce, Thermo Scientific, Rockford, USA) followed by cooling on ice. After cooling, 100 μl norleucine (30 mM, Sigma-Aldrich) was added as an internal standard and the sample was transferred to an 8-ml glass tube. The hydrolysis tube was rinsed with 1 ml of distilled water, which was added to the sample, followed by vacuum centrifugation until completely dry. The dried sample was suspended in 3 ml of HCl (0.01 M) followed by filter centrifugation at 3000 g at room temperature for 10 min and stored at −80°C until further analysis. Amino acids were derivatized using an AccQ-Tag Ultra Derivatization Kit (Waters, USA) and separated and quantified.

### TABLE 1

Nutritional composition of the three black soldier fly larvae meal (BSFLM) fractions with different particle sizes (0–200 μm, 200–400 μm and >400 μm) after mechanical fractionation (values expressed as mean, n = 2) compared with soybean meal and fishmeal.

<table>
<thead>
<tr>
<th>Analyzed composition</th>
<th>BSFLM</th>
<th>Soybean meal (solvent extracted)</th>
<th>Fishmeal (combined species)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fine (0–200 μm)</td>
<td>Medium (200–400 μm)</td>
<td>Coarse (&gt;400 μm)</td>
</tr>
<tr>
<td>Dry matter (DM, g/kg)</td>
<td>965</td>
<td>964</td>
<td>976</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>549</td>
<td>557</td>
<td>559</td>
</tr>
<tr>
<td>Corrected crude protein (g/kg DM)</td>
<td>541</td>
<td>545</td>
<td>493</td>
</tr>
<tr>
<td>Crude lipid (g/kg DM)</td>
<td>215</td>
<td>147</td>
<td>14</td>
</tr>
<tr>
<td>Ash (g/kg DM)</td>
<td>136</td>
<td>113</td>
<td>72</td>
</tr>
<tr>
<td>Chitin (g/kg DM)</td>
<td>18</td>
<td>154</td>
<td>–</td>
</tr>
<tr>
<td>NFE (g/kg DM)</td>
<td>47</td>
<td>3</td>
<td>308</td>
</tr>
<tr>
<td>Calculated GE (MJ/kg DM)</td>
<td>22.4</td>
<td>21.6</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Amino acid (g/kg DM)

<table>
<thead>
<tr>
<th>Essential amino acids (EAA)</th>
<th>BSFLM</th>
<th>Soybean meal</th>
<th>Fishmeal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>30.4</td>
<td>19.8</td>
<td>35.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>14.6</td>
<td>13.8</td>
<td>14.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>25.3</td>
<td>21.1</td>
<td>22.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>39.8</td>
<td>40.0</td>
<td>38.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>36.2</td>
<td>20.4</td>
<td>31.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>8.3</td>
<td>6.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>26.2</td>
<td>13.8</td>
<td>25.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>23.8</td>
<td>18.0</td>
<td>19.8</td>
</tr>
<tr>
<td>Valine</td>
<td>25.9</td>
<td>37.7</td>
<td>21.7</td>
</tr>
<tr>
<td>Sum of EAA</td>
<td>230.5</td>
<td>190.8</td>
<td>215.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-essential amino acids (NEAA)</th>
<th>BSFLM</th>
<th>Soybean meal</th>
<th>Fishmeal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>27.3</td>
<td>31.8</td>
<td>51.6</td>
</tr>
<tr>
<td>Aspartate + asparagine</td>
<td>57.2</td>
<td>52.3</td>
<td>32.5</td>
</tr>
<tr>
<td>Glutamate + glutamine</td>
<td>63.8</td>
<td>60.7</td>
<td>40.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.5</td>
<td>35.6</td>
<td>21.3</td>
</tr>
<tr>
<td>Proline</td>
<td>25.1</td>
<td>46.5</td>
<td>27.4</td>
</tr>
<tr>
<td>Serine</td>
<td>22.4</td>
<td>27.5</td>
<td>24.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>30.6</td>
<td>42.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Sum of NEAA</td>
<td>249.9</td>
<td>276.7</td>
<td>253.5</td>
</tr>
<tr>
<td>Sum of total amino acids</td>
<td>480.4</td>
<td>467.5</td>
<td>469.0</td>
</tr>
</tbody>
</table>

**a**Crude protein calculated using a nitrogen-to-protein conversion factor of 6.25.

**b**Crude protein corrected for 6.9% nitrogen in pure chitin (Liu et al., 2012).

**c**Nitrogen-free extract (g/kg) calculated as: dry matter (g/kg) - (crude protein g/kg + crude lipid g/kg + ash g/kg + chitin g/kg).

**d**Calculated gross energy (GE) used 23.66 MJ/kg protein, 39.57 MJ/kg lipid and 17.17 MJ/kg NFE and chitin.

**e**Obtained from NRC (2012).
using reverse-phase separation and UV detection at 260nm (UPLC, Waters). Amino acid separation was performed on an AccQ-Tag Ultra C18, 2.1 x 100mm column with the mobile phase composed of distilled water (eluent A) and acetonitrile (eluent B), with eluent B increasing from 0%–60% over 10 min following a gradient using a flow rate of 0.7 ml/s. Amino acids were quantified using Waters Empower 3 software. Tryptophan could not be quantified with the applied method as it is decomposed during acid hydrolysis.

**TABLE 2** Ingredient formulation of Nile tilapia and rainbow trout experimental reference diet and three experimental diets containing black soldier fly larvae meal (BSFLM) of different size fractions (fine: 0–200 μm, medium: 200–400 μm and coarse: >400 μm) together with their analyzed nutrient content as g/kg on as is basis (values expressed as mean, n = 2)

<table>
<thead>
<tr>
<th>Ingredient (g/kg)</th>
<th>Nile tilapia</th>
<th>BSFLM diets</th>
<th>Rainbow trout</th>
<th>BSFLM diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental reference diet</td>
<td>Fine</td>
<td>Medium</td>
<td>Coarse</td>
</tr>
<tr>
<td>Wheat flour^a</td>
<td>375.8</td>
<td>281.9</td>
<td>281.9</td>
<td>281.9</td>
</tr>
<tr>
<td>Soybean protein concentrate^b</td>
<td>250.0</td>
<td>187.5</td>
<td>187.5</td>
<td>187.5</td>
</tr>
<tr>
<td>BSFLM 0–200 μm</td>
<td>-</td>
<td>250.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSFLM 200–400 μm</td>
<td>-</td>
<td>-</td>
<td>250.0</td>
<td>-</td>
</tr>
<tr>
<td>BSFLM &gt;400 μm</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>250.0</td>
</tr>
<tr>
<td>Poultry meal^c</td>
<td>80.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Feather meal^d</td>
<td>80.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Haemoglobin^e</td>
<td>80.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Fishmeal LT^f</td>
<td>50.0</td>
<td>37.5</td>
<td>37.5</td>
<td>37.5</td>
</tr>
<tr>
<td>Rapeseed oil^g</td>
<td>30.0</td>
<td>22.5</td>
<td>22.5</td>
<td>22.5</td>
</tr>
<tr>
<td>Monoammonium phosphate</td>
<td>20.2</td>
<td>15.2</td>
<td>15.2</td>
<td>15.2</td>
</tr>
<tr>
<td>Salmon oil^h</td>
<td>20.0</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Vitamin-mineral premix^i</td>
<td>14.0</td>
<td>10.5</td>
<td>10.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Diamol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Analyzed composition (g/kg)**

<table>
<thead>
<tr>
<th></th>
<th>Nile tilapia</th>
<th>BSFLM diets</th>
<th>Rainbow trout</th>
<th>BSFLM diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>940</td>
<td>940</td>
<td>939</td>
<td>941</td>
</tr>
<tr>
<td>Crude protein^j</td>
<td>452</td>
<td>474</td>
<td>479</td>
<td>486</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>69</td>
<td>103</td>
<td>98</td>
<td>87</td>
</tr>
<tr>
<td>Ash</td>
<td>71</td>
<td>79</td>
<td>80</td>
<td>72</td>
</tr>
<tr>
<td>Chitin</td>
<td>-</td>
<td>9</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>NFE^k</td>
<td>348</td>
<td>276</td>
<td>271</td>
<td>254</td>
</tr>
<tr>
<td>Calculated GE (MJ/kg)^l</td>
<td>19.4</td>
<td>20.2</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Digestible protein^m</td>
<td>427</td>
<td>443</td>
<td>451</td>
<td>446</td>
</tr>
<tr>
<td>DE (MJ/kg)^n</td>
<td>17.8</td>
<td>18.1</td>
<td>18.2</td>
<td>17.2</td>
</tr>
<tr>
<td>DP/DE (g/MJ)</td>
<td>24.0</td>
<td>24.5</td>
<td>24.8</td>
<td>25.9</td>
</tr>
</tbody>
</table>

^aDLG, Fredericia, DK.
^bNordic Soy, Uusikaupunki, FI.
^cSONAC, Denderleeuw, BE.
^dGepro, DiepHolz, D.
^eBadenhop, Verden, D.
^fFF Skagen, Skagen, DK.
^gEmmelev, Otterup, DK.
^hBioceval, Cuxhaven, D.
^iVilofoss, Gråsten, DK.
^jCrude protein calculated using a nitrogen-to-protein conversion factor of 6.25.
^kNitrogen-free extract (NFE) (g/kg) calculated as: Dry matter (g/kg) – (Crude protein g/kg + crude lipid g/kg + ash g/kg + chitin g/kg).
^lCalculated gross energy (GE) using 23.66 MJ/kg protein, 39.57 MJ/kg lipid and 17.17 MJ/kg nitrogen-free extract and chitin.
^mDigestible protein (g/kg) = (Nitrogen intake g/kg * Nitrogen digestibility %) * 6.25.
^nDigestible energy = Calculated gross energy in feed MJ/kg * Energy digestibility %.
Chitin in BSFLM and fecal samples was determined spectro-photometrically using a modified version of Tsui et al. (1969) and Guerreiro et al. (2020). Approximately 1 g of sample was defatted by incubation in 10 ml of acetone (Supelco) for 1 h with agitation. The sample was centrifuged at 4700rpm at 5°C for 15 min and the supernatant was discarded. The pellet was washed three times with 10 ml of distilled water, freeze-dried until constant weight, and stored in a desiccator at room temperature until further use. Each freeze-dried sample (10 mg) was suspended in 10 ml of 0.5 M NaOH (Merck, Germany) solution for deproteinization, agitated for 2 h, and centrifuged at 4700rpm at 5°C for 15 min. The supernatant was discarded and the pellet was washed three times with distilled water followed by freeze-drying. The deproteinized sample (10 mg) was hydrolyzed in 3 ml of 6 M HCl (Supelco) for 24 h in a heating block at 100°C and cooled to room temperature. The hydrolysate was filtered through a 0.2-μm filter and pH adjusted to 6.0–6.5 with NaOH, adding up to 10 ml with distilled water. Subsequently, 1 ml of sample or 1 ml of distilled water (experimental blank) was mixed with 1 ml of 5% w/v NaNO₂ (Sigma-Aldrich) and 1 ml of 5% w/v KHSO₄ (VWR, USA). After 15 min at room temperature, 1 ml of 12.5% w/v NH₄NO₂ (Sigma-Aldrich) was added followed by constant shaking for 5 min at 210rpm. Freshly prepared 0.5% w/v 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (1 ml) (Merck) was added and after 60min at room temperature, 1 ml of 0.5% w/v freshly prepared FeCl₃ (Merck) was added and the samples were left for minimally 30 min. Subsequently, the absorbance was measured on a plate reader (CLARIOstar, BMG Labtech, Germany) at 650nm against the experimental blank. Chitin nitrogen was calculated as 6.9% of the chitin content as previously described for pure chitin (Liu et al., 2012).

Exochitinase β-N-acetylglucosaminidase (EC 3.2.1.52) activity was measured in crude enzyme extracts obtained from the proximal, middle and distal intestinal sections of Nile tilapia and rainbow trout using a modification of the procedure described by McCreath and Gooday (1992). Crude extracts (5 μl) were diluted in 495 μl ice-cold distilled water and kept on ice to avoid enzyme activity reduction. A fluorogenic substrate was prepared by diluting 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (M2133, Sigma-Aldrich) in phosphate-citrate buffer (pH = 5.2) to a final concentration of 0.2 mg/mL. In a black flat-bottom 96-well plate, 5 μl diluted crude extract was added followed by the addition of 95 μl diluted fluorogenic substrate. The plate was incubated at 37°C for 15 min followed by the addition of 200 μl glycine-NaOH buffer (pH = 10.6), and fluorescence was measured on a plate reader (CLARIOstar, BMG Labtech, Germany) applying an excitation wavelength of 360nm and an emission wavelength of 450nm. Enzyme activity was calculated using a standard curve treated similarly to the diluted crude enzyme extracts with 4-methylumbelliferone (M1381, Sigma-Aldrich) dissolved in glycine-NaOH buffer (pH = 10.6) at different concentrations and replacing the diluted fluorogenic substrate with phosphate-citrate buffer (pH = 5.2). One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 μmole of 4-methylumbelliferone per min, expressed per gram of wet tissue.

2.6 | Calculations and statistical analysis

Apparent digestibility coefficients (ADC) were determined directly as (Jobling, 1994):

\[
ADC_i = \left( C_i - F_i \right) / C_i \times 100
\]

where i represents dietary content of DM, ash, lipid, protein (corrected and non-corrected), energy, chitin, or NFE; C the consumed amount of i; and F the faecal loss of i.

Gross energy (GE) was calculated as described by Jobling (1994) and Gutzowska et al. (2004):

\[
GE \, (MJ/kg) = (23.66 \times \text{Crude protein} \, [g/kg] + 39.57 \times \text{Crude lipid} \, [g/kg] + 17.17 \times \text{NFE} + \text{Chitin} \, [g/kg]) / 1000
\]

Nitrogen-free extract (NFE) was calculated as:

\[
NFE \, (g/kg) = \text{Dry matter} \, (g/kg) - (\text{Crude protein} \, [g/kg] + \text{Crude lipid} \, [g/kg] + \text{Ash} \, [g/kg] + \text{Chitin} \, [g/kg])
\]

Data are presented as mean ± standard deviation (SD), unless otherwise mentioned, with each tank being considered an experimental unit. All data were tested for normality of residuals and homogeneity of variances using the Shapiro–Wilk and Levene’s test respectively. Data were subjected to a one-way ANOVA to compare means between the different treatment groups. When differences between means were significant (p < 0.05), a Tukey’s honest significant post hoc test was performed. All statistical tests were performed using IBM SPSS Statistics 25.0 (IBM Corp., USA). Graphs were generated by GraphPad Prism 9.2.0 software (GraphPad Software, USA).

3 | RESULTS

3.1 | Larvae meal size fractions and experimental diets

All BSFLM fractions had a similar gross energy content (21.6–22.4 MJ/kg) and crude protein content (53.0%–54.6%) (Table 1). The chitin content was affected by fractionation, increasing from 1.8% and 2.7% in the fine and medium fractions, respectively, and 15.4% in the coarse fraction. In contrast, the crude lipid content decreased from 20.7% in the fine fraction to 14.3% in the coarse fraction. The coarse fraction was lower in several essential amino acids (EAA, including especially arginine, lysine, phenylalanine, and threonine) compared with the fine and medium fractions, while it was higher in valine and total non-essential amino acids (NEAA) (Table S1).

Inclusion of 25% of the different BSFLM fractions in the feed for Nile tilapia and rainbow trout resulted in 0.9% and 1.1% chitin, respectively, in diets including the fine and medium fractions, and...
4.4% for diets including the coarse fraction (Table 2). The DP/DE ratio was slightly higher in diets including the coarse fraction than in the other diets, while the sum of EAA was lower.

3.2 | Apparent nutrient digestibility

There was no mortality in the Nile tilapia trial, whereas in total three fish died in the rainbow trout trial but no effect of diet was observed (Table S2). Significant differences were observed for apparent digestibility in Nile tilapia of all dietary parameters except for crude lipid (Table 3). Dry matter, crude protein (non-corrected as well as corrected for chitin), NFE, and energy digestibility were all significantly lower for BSFLM diets compared with the experimental reference diet (p < 0.001). Furthermore, the digestibility of these dietary parameters, along with chitin, was significantly lower in the coarse BSFLM diet compared with the fine and medium BSFLM diets. When correcting crude protein digestibility for chitin nitrogen, significant differences between the treatments remained. Ash digestibility was significantly lower in the experimental reference diet and coarse BSFLM diet compared with the fine BSFLM diet (p = 0.013).

For rainbow trout, apparent digestibility was significantly different between treatment groups for all investigated nutrients (Table 3). A significantly lower digestibility was found for the coarse BSFLM diet for dry matter, crude protein (non-corrected and corrected), NFE, and chitin compared with the other diets including the experimental reference diet (p < 0.05). Crude lipid digestibility was significantly lower in fish fed the experimental reference diet compared with the BSFLM diets (p < 0.001). Ash digestibility was lower for BSFLM diets compared with the experimental reference diet, while energy digestibility was lowest for the experimental reference and coarse BSFLM compared with the other diets.

3.3 | Chitinase activity

The β-N-acetylglucosaminidase activity was significantly higher in the distal sections compared with the proximal and middle sections for both Nile tilapia (Figure 1a) and rainbow trout (Figure 1b) (p < 0.001). Furthermore, β-N-acetylglucosaminidase activity was significantly higher in the proximal intestine in both fish species fed the medium and coarse diet compared with the experimental reference diet (p < 0.001). Lastly, the exochitinase activity in the distal intestine was significantly higher in fish fed the coarse BSFLM fraction compared with the experimental reference in both tilapia (p = 0.002) and trout (p = 0.049) and the activity in the distal intestine generally appeared to increase with higher dietary chitin inclusion.

TABLE 3 Apparent digestibility in Nile tilapia and rainbow trout fed the experimental reference diet or one of the three experimental diets containing black soldier fly larvae meal (BSFLM) with different size fractions (fine, medium and coarse) (mean ± standard deviation, n = 3)

<table>
<thead>
<tr>
<th>Dietary parameter (%)</th>
<th>Nile tilapia</th>
<th>BSFLM diets</th>
<th>Rainbow trout</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental reference diet</td>
<td>Fine (0–200 μm)</td>
<td>Medium (200–400 μm)</td>
</tr>
<tr>
<td></td>
<td>Dry matter</td>
<td>87.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.1 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Crude protein</td>
<td>94.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.0 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Corrected crude protein&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-</td>
<td>93.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Crude lipid</td>
<td>92.8 ± 0.5</td>
<td>94.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Ash</td>
<td>44.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.8 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>NFE</td>
<td>87.1 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.8 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Energy</td>
<td>92.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Chitin</td>
<td>-</td>
<td>58.8 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: Dissimilar superscript letters indicate differences in means within the same row (p < 0.05).

Abbreviation: NFE, nitrogen-free extract.

*Corrected crude protein corrects for chitin nitrogen, with pure chitin having 6.9% nitrogen (Liu et al., 2012).
et al., 2017). Pure chitin contains approximately 6.9% nitrogen (Liu et al., 2017). Due to the presence of non-protein nitrogen, including chitin (Janssen et al., 1995), embedding in a matrix of other components including proteins, lipids and minerals (Kramer et al., 1995), and this constellation could impair absorption may also be impaired due to chitin, which was suggested by Abro et al. (2014) in a study with Arctic char (Salvelinus alpinus) that showed that L-lysine uptake in the proximal intestine was reduced when fish were fed a diet containing chitin. In addition, insect chitin is embedded in a matrix of other components including proteins, lipids and minerals (Kramer et al., 1995), and this constellation could impair the accessibility of digestive enzymes to these nutrients. However, these suggested mechanisms require further investigation in fish to identify the role of chitin as an anti-nutrient.

In addition to acting as an anti-nutritional factor, chitin has a caloric value of ~17.17 MJ/kg and could potentially be used as a nutrient source.
carbon and nitrogen source (Gutowska et al., 2004). For fish to be able to utilize chitin, chitinases need to be present along the intestinal tract. Previous studies have demonstrated the presence of chitinolytic activity in a wide range of fish species including Atlantic cod and rainbow trout (Danulat & Kausch, 1984; Lindsay et al., 1984). In the current study, exochitinase activity was present in both tilapia and trout and its activity seemed to increase with higher dietary chitin inclusion. Furthermore, the largest differences between treatments were observed in the distal intestine, where exochitinase activity was highest. Higher activity of exochitinase in the distal intestine was also previously observed in Arctic char, while exochitinase activity was approximately the same in the proximal and middle intestine (Abro et al., 2014). A higher exochitinase activity in the distal intestine could be a mechanism to maximize chitin exposure to endochitinases prior to the activity of exochitinases. Chitinases in the digestive tract of vertebrates have been suggested to originate from endogenous production, exogenous production or ingested food (Goody, 1990). In the current study, the presence of chitinases in the feed is unlikely while it could be possible that the exochitinase activity derived from chitinolytic bacteria in the distal intestine. However, a previous study in rainbow trout showed that antibiotic treatment did not affect chitinolytic activity, suggesting that the bacterial contribution to the chitinolytic activity is minimal and that most of the activity is produced endogenously (Lindsay et al., 1984).

It needs to be mentioned that the chitinolytic enzyme activity measured in this study likely overestimated the in vivo activity because of the high assay incubation temperature of 37°C. This is the commonly used temperature for chitinase activity determination but does not represent the temperature at which Nile tilapia (23°C) and rainbow trout (11°C) were reared. This hypothesis is supported by Lindsay et al. (1984), who expected four to five times lower enzyme activity in rainbow trout tissue samples incubated at 15°C compared with 37°C.

Despite the increase in exochitinase activity with a higher dietary chitin inclusion, chitin digestibility in both fish species was significantly higher in fish fed diets containing the fine and medium fractions (49–59%) compared with the coarse fraction (30% in tilapia and 12% in trout). A similar trend was found in a study with penaeid shrimp, showing that chitin digestibility was lower in shrimp fed 4% dietary chitin compared with those fed 1% or 2% dietary chitin (Clark et al., 1993). Additionally, a previous study found that chitin digestibility in rainbow trout was 1.2%–4.9% with dietary chitin inclusion levels of 4%, 10%, and 25% (Lindsay et al., 1984). These findings may suggest that the chitin uptake capacity in the current study was exceeded at the highest dietary chitin inclusion level despite an increase in intestinal exochitinase activity.

5 | CONCLUSIONS AND FUTURE WORK

The current study found that sieving could be used as a simple mechanical method to obtain BSFLM fractions with different chitin contents. However, the applied sieve sizes did not allow complete separation of the chitin fraction. It is, therefore, that more work is needed on further reducing (or removing) chitin in BSFLM in a sustainable manner, focusing, for example, on using other sieve sizes, mechanical exoskeleton removal or enzymatic extraction. It was found that both Nile tilapia and rainbow trout can digest chitin and could potentially use chitin as a nutrient source. However, their capacity to digest chitin decreased with higher dietary inclusion of chitin despite an upregulated exochitinase activity along the intestinal tract. Further research is needed to identify the role of different chitinases in chitin digestion in different fish as well as their overall capacity to digest chitin. Furthermore, this study showed that the dietary inclusion of the coarse BSFLM size fraction resulted in reduced digestibility of certain nutrients presumably due to the anti-nutritional properties of chitin. However, modes of action in which chitin acts as an anti-nutrient remain to be investigated.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

Kyllan Manon Eggink: conceptualization, methodology, formal analysis, writing - original draft, writing - review and editing. Per Bovbjerg Pedersen: conceptualization, writing - review and editing, funding acquisition. Ivar Lund: conceptualization, writing - review and editing. Johanne Dalsgaard: conceptualization, writing - original draft, writing - review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
ETHICS APPROVAL
All procedures complied with current Danish and EU legislation (Directive 2010/63/EU) regarding animal experimentation.

REFERENCES


**Supporting Information**

Additional supporting information can be found online in the Supporting Information section at the end of this article.


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