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Clostridium perfringens suppressing activity in black soldier fly protein preparations

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ABSTRACT

Clostridium perfringens is a commensal, but also an opportunistic pathogen that can lead to lethal diseases as a result of overgrowth when homeostasis is disrupted. The current course of treatment is antibiotics. However, with increasing antibiotic resistance alternatives are required. We investigated the antimicrobial capacity of digest from different black soldier fly- and mealworm-derived fractions towards *C. perfringens* by using *in vitro* models. Culturing *C. perfringens* with digest of insect-derived fractions showed that fractions containing black soldier fly larvae protein significantly ($p < 0.05$) inhibited the growth of *C. perfringens*. In relation to this effect, many small (<5 amino acids) anti-microbial peptides were identified. The impact on healthy microbiota was also investigated through 16S rRNA sequencing and SCFA secretion following exposure of healthy faecal-derived microbiota to digests. This revealed a small but significant ($p < 0.05$) reduction in abundance and diversity of microbiota, mainly a result of a strong reduction in *Firmicutes* (*e.g. Enterobacter*) and increased abundance of *Proteobacteria* (*e.g. Klebsiella*). These changes coincided with increased levels of acetic, propionic, and butyric acid secretion. The combined impact of black soldier fly larvae protein on these *in vitro* assays suggest it can be a promising additional tool to combat *C. perfringens* infection.

1. Introduction

The human intestinal microbiome is a complex microbial ecosystem (Biedermann & Rogler, 2015). This ecosystem, when in homeostasis, acts in a symbiotic relationship with their host in healthy humans, and supports intestinal health via supporting food digestion and fermentation, providing key nutrients, and preventing pathogenic infection (Kabat, Srinivasan, & Maloy, 2014). However, a disbalance in the intestinal microbiota can lead to disease as a result of overgrowth of pathogenic or opportunistic bacteria (Gagnière et al., 2016). *Clostridium* is such a genus containing many opportunistic and pathogenic species with significant societal impact through a burden on healthcare and economy (Sklenickova et al., 2010). In homeostasis, *Clostridium perfringens,* a Gram-positive, anaerobic, spore-forming pathogenic bacterium of the genus *Clostridium* naturally reside at low frequency in the human and animal intestinal tract. *C. perfringens* can, however, demonstrate dramatic opportunistic growth following the use of antibiotics. Overgrowth of *C. perfringens* can induce diarrhoea and intestinal necrotic enteritis (Sklenickova et al., 2010).

This bacterium can be responsible for lethal diseases such as food borne illness and necrotic enteritis through the production of a number of toxins (Yao & Annamaraju, 2020). In a clinical study including elderly Irish subjects, it was shown that the presence of *C. perfringens* in faeces correlated with a reduced presence of commensals *Bifidobacterium* and *Lactobacillus* (Lakshminarayanan et al., 2013). Lowered levels of these commensals are also observed in intestinal bowel disease and irritable bowel syndrome patients (Gueimonde, Ouwehand, Huhtinen, Salminen, & Salminen, 2007; Kerckhoffs et al., 2009), indicating the potential impact of such a dysbiosis. Pets, such as dogs and cats, are also susceptible to *C. perfringens* infections. A study indicated that up to 28%

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cases of diarrhoea in dogs may occur due to *C. perfringens* poisoning (Weese et al., 2001). In livestock, such as chicken and pigs, *C. perfringens* infection is associated with significant economic losses in livestock industries (Mwangi, Timmons, Fitz-Coy, & Parveen, 2019). Over the years, antibiotics have been used to control *C. perfringens* infection. The ban of antibiotic usage in livestock industry, however, spurred a search for effective alternatives to target *C. perfringens* (Mwangi et al., 2019). Many alternatives to antibiotics are being developed through novel biochemical and genetic technologies, including bacteriophages, antibodies, and probiotics. However, several alternatives that can be directly incorporated into feed already exist in nature, such as insects (Ghosh, Sarkar, Issa, & Haldar, 2019).

Insects have been characterized for their antimicrobial properties as they contain antimicrobial peptides (AMPs), fatty acids, and chitin (Dong, Wichers, & Govers, 2019; Yi, Chowdhury, Huang, & Yu, 2014). Currently, around 300 AMPs have been identified in insects. These AMPs can exert their antimicrobial activity through disruption of the bacterial membrane, interference with metabolism, and/or targeting of cytoplasmic components (Corrêa, Evangelista, de Melo Nazareth, & Luciano, 2019; Jhong et al., 2019). Insect fatty acids are also known for their antimicrobial properties. Medium chain fatty acid derivatives, that are found in high concentration in larvae of the black soldier fly (*Hermetia illucens*), were reported to show activity against Gram-positive bacteria such as *Staphylococcus aureus* (Batovska, Todorova, Tsvetkova, & Najdenski, 2009; Belghit et al., 2019). The antimicrobial activity of fatty acids is linked to the reduction of cellular pH, and the ability to dissociate (Ricke, 2003). Furthermore, different isolates from insects, such as AMPs, fatty acids, and chitin also exert their bactericidal effects via modulating the host immune response. For instance, chitin was shown to prevent *Leishmania major* infection in mice, and the mechanism was putatively associated with the activation of macrophages (Dong et al., 2019; Hoseini et al., 2016). Because insects provide multiple antimicrobial compounds, which limits escape, and poses a critical factor in controlling pathogenic growth (Veldkamp et al., in press) and insects can easily be implemented in feed and food, they constitute an interesting alternative to current antibiotics.

Even though insects contain these three major antimicrobial components, the antimicrobial properties of insects vary between species and life stage. In this study, we explored the bactericidal effects on *C. perfringens* of five different insect-derived products, being mealworm larvae protein meal, black soldier fly larvae protein meal, chitin rich black soldier fly larvae protein meal, black soldier fly cocoon meal, and black soldier fly larvae trilaurin fraction using *in vitro* models.

2. Materials and methods

2.1. Nutritional composition of insect derived products

Mealworm larvae protein meal (MP), black soldier fly larvae protein meal (BP), chitin rich black soldier fly larvae protein meal (BchP), black soldier fly cocoon meal (BC), and black soldier fly larvae trilaurin fraction (BT) were provided by Protix (Dongen, The Netherlands). The details of the nutritional composition, production process, and commercial availability are measured and indicated by the supplier (Protix), and mentioned in Table 1. Specifically, the protein and fat content in insects were measured by using Dumas and Soxhlet method, respectively. The chitin level in insects was evaluated according to the study from Hahn T (Hahn et al., 2018).

2.2. In vitro digestion

In vitro digestion was performed based on the consensus INFOGEST standardized protocol that is a digestion method that uses constant ratios of meal to digestive fluids and a constant pH for each step of digestion (Minekus et al., 2014). We apply a standard 10-fold reduction in enzyme concentrations, not affecting hydrolysis levels (data not shown), to allow broad usage of digests (*e.g.* ensuring cell viability in cell culture experiments) and inter-assay comparisons and relations (detailed description in Appendix A). Digest of empty control (MilliQ control) (ED), containing all digestive enzymes, and thus constituting a control to digested insect fractions, MP, BP, BchP, BC, and BT were aliquoted and stored in −80 °C immediately after *in vitro* digestion until further analysis.

Table 1
Details of five insect-derived products used in this study (as indicated by supplier). MP: mealworm larvae protein meal, BP: black soldier fly larvae protein meal, BchP: chitin rich black soldier fly larvae protein meal, BC: black soldier fly cocoon meal, BT: black soldier fly larvae trilaurin fraction.

Product	Nutritional composition			Production process	Commercial availability
	Protein (%)	Chitin (%)	Fat (%)		
MP	56	8	29	Produced by pasteurization, drying, and grinding of mealworm larvae	Yes
BP	56	10	15	Produced by mincing, pasteurization, partial defatting, and drying of black soldier fly larvae	Yes
BchP	56	15	18	Produced by mincing, pasteurization, partial defatting, standardization, and drying of black soldier fly larvae	No
BC	63	20	7	Produced by drying and grinding of black soldier fly pupal exoskeleton	No
BT	0	0	100	Fat fraction obtained as co-product of BP during processing	Yes

2.3. Faecal sample preparation

Faecal samples were donated by a healthy adult male donor. Fresh faecal samples were collected in a box containing an anaerobic sponge (AnaeroGenTM 2.5 L, Thermo Scientific, Ochten, Netherlands) to keep an anaerobic atmosphere. Immediately after collection, the faecal sample was transferred to a stomacher bag (Voor't Labo, The Netherlands), and 20% (w/v) autoclaved phosphate buffer was added containing per liter: 8.8 g K_2HPO_4 (Merck), 6.8 g KH_2PO_4 (Merck), 0.1 g sodium thioglycolate (Sigma-Aldrich), and 15 mg sodium thionate (Merck). The sample was homogenized for 5 min at a speed of 230 rpm in a Stomacher® 400 Circulator (Seward, Hampshire, UK) after which it was poured into a 50 ml falcon tube. The tube was centrifuged at 500 g for 5 min, and the supernatant was collected. Part of the supernatant was aliquoted to prepare a glycerol stock and stored at −80 °C, and another part was immediately used for *in vitro* fermentation.

2.4. Proliferation of C. perfringens and faecal derived microbiota

A glycerol stock of *C. perfringens* NCTC8238 was kindly provided by Prof. dr. T. Abee (Food Microbiology department, Wageningen University and Research). A glycerol stock of faecal derived microbiota was prepared as described above. All manipulations were performed under anaerobic conditions. *C. perfringens* was recovered from the glycerol stock by incubating on a tryptose sulphite cycloserine (TSC) (Merck) agar plate at 37 °C for 24h. One *C. perfringens* colony or 0.2 ml microbiota glycerol stock solution were emulsified with 10 ml fluid thioglycolate (FTG) broth (Oxioid, Wesel, Germany) or brain heart infusion (BHI) broth (BD Bioscience, Vianen, The Netherlands), respectively, at 37 °C O/N. Next, 0.2 ml of each inocula was incubated with 10 ml broth

at 37 °C for 2h to reach the exponential growth phase after which 0.2 ml was transferred to tubes containing 10 ml FTG (*C. perfringens*) or BHI (healthy microbiota) broth and 1 ml of digest (*i.e.* empty digest as control and digest of MP, BP, BchP, BC, or BT). Immediately after mixing (t = 0), and after 3, 6, and 24h, 1 ml of the solution was diluted with peptone physical salt (pfz) solution (Tritium Microbiologie, Eindhoven, The Netherlands) to a 10, 1, 0.1, 0.01, and 0.001% mixture of which 100 μl from each dilution was plated on a TSC (*C. perfringens*) or BHI (healthy microbiota) agar plate for O/N incubation. Colonies were counted after 24h and calculated as a log CFU/ml value. The remaining solution of microbiota that were exposed to digests for 24h was centrifuged at 9391 g for 3 min at 4 °C. The pellet was mixed with 1 ml DNA/RNA shield (Zymo Research, Freiburg im Breisgau, Germany), and sent to Baseclear B.V. (Leiden, The Netherlands) for microbiota composition analysis.

2.5. Microbiota composition analysis

The bacterial RNA was extracted for each pellet, and the 16S rRNA gene sequencing was performed by Baseclear B.V. (L457; NEN-EN-ISO/IEC 17025). The genomic RNA was extracted, and a PCR amplification of the V3- V4 region was conducted on an Illumina MiSeq System (Illumina, San Diego, CA, USA). After amplification, the raw paired ends FASTQ files were trimmed, and converted by bcl2fastq2 Conversion Software (version 2.18, Illumina). The resulting data was analysed by using CLC Genomics Workbench (Microbial Genomics toolbox version 20.0, Qiagen). The operational taxonomic units (OTUs) table was prepared based on a 99% sequencing similarity and the taxonomy was assigned using Silva database (version 132). A phylogenetic tree was created by using MUSCLE (version 3.8.425) to evaluate the alpha and beta diversity of each microbiota community. To exclude the influence of dead cells on the microbiota composition analysis, the analysis was based on RNA level.

2.6. In vitro fermentation

The in *vitro* fermentation was performed in a biological quadruplicate using microbiota from a healthy adult donor. Each fermentation vessel contained 21.5 ml basal medium, with either 10 ml PBS (control) or 10 ml of empty digest or insect digest (*i.e.* MP, BP, BchP, or BC). The basal medium was prepared, and contained per liter: 2 g peptone (Duchefa Biochemie, Haarlem, The Netherlands), 2 g yeast (Sigma-Aldrich), 0.5 g L-cysteine (Sigma-Aldrich), 5.22 g K_2HPO_4 (Merck), 16.32 g KH_2PO_4 (Merck), 2 g $NaHCO_3$ (Merck), 1 g mucin (Sigma-Aldrich), and 2 ml Tween 80 (Sigma-Aldrich). Anaerobic processing of the fermentation vessels was achieved by consistently sparging with oxygen free nitrogen followed by transferring 3.5 ml of freshly prepared faecal-derived microbiota. Subsequently, the fermentation vessels were incubated at 37 °C whilst shaking (200 rpm), and samples (2 ml) were taken after 0, 3, 6, and 24h for analysis. The samples were centrifuged at 20800 g at 4 °C, and the supernatant was collected and stored at −80 °C.

2.7. SCFA analysis

The collected supernatants from fermentation were diluted with 16.6 mM sulphuric acid (Sigma-Aldrich) at a ratio of 1:1 (v/v), and subjected to a SCFA analysis by using high performance liquid chromatography (HPLC) (model Acquity Arc™, Waters, Eschborn, Germany) equipped with a refractive index detector (model R2414, Waters) and an AMINEX HPX-87H column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad Laboratories, Richmond, VA, USA). The column was maintained at 35 °C using an integral column heater (Waters), and sulphuric acid (8.3 mM) was used as eluent for analysis. The standard samples were prepared at a concentration of 13.83 mM for lactic acid (Sigma-Aldrich), 18.15 mM for acetic acid (Merck), 13.82 mM for propionic acid (Sigma-Aldrich), 4.14 mM for isobutyric acid (Fluka, Cheniou, Gmblt, Germany), 12.12 mM for butyrate acid (Sigma-Aldrich), 9.75 mM for isovaleric acid (Fluka), 9.73 mM for valeric acid (Acros Organic™, Geel, Belgium), and 27.07 mM for formic acid (Merck) in 1 L of 8.3 mM sulphuric acid. The standard was injected after every 5 samples, repeatedly, and the calibration curve was constructed by plotting the peak area against the molarity of standard solutions.

2.8. LC-MS/MS analysis

In vitro protein digests ED, MPD, BPD, and BchPD were analysed in duplicate on a UPLC-MS system (Dionex Ultimate 3000 online connected to QexactivePLUS (Thermofisher, Waltham, U.S.A)). Samples were injected (10 ml) both on a pentafluorophenyl F5 core-shell column (Kinetex F5, 15 cm × 2.1 mm, 2.6 mm particles, Phenomenex, Torrance, USA) and separately also on a HSS T3 column (15 cm × 2.1 mm, 1.8 mm particles, Waters, Millford, USA), operated at 40 °C, or respectively 60 °C, and flow rate of 0.2 ml (0.15 ml for T3) per minute. Total run time was developed over a 40 min time window: Starting with buffer A (0.1% formic acid in water) for 5 min, and separated with a gradient of 0–30% buffer B (0.1% formic acid in 100% Acetonitrile) during 20 min, increasing to 80% B in 5 min, stable at 80% B for 3 min, and back to 0% B during 2 min, and stable at 0% B for 5 min. Separated peptides were online injected into the Q ExactivePLUS using the standard ESI source in positive mode, with 3.5 kV spray voltage, 290 °C capillary temperature, nitrogen sheath gas flow 40, and auxiliary gas flow 10 heated at 60 °C. MS spectra were collected with two alternative methods; first within a m/z range of 220–1400, alternatively with a m/z range of 380–1200. MS scans were at 70000 resolution (profile) and AGC target of 3×10^6 ions maxIT for 100 ms; followed with data-dependent switch to MS/MS mode at 17500 resolution (centroid) at AGC target 10^5 ions, NCE 27, minimum AGC 10^4, maxIT 110 millliseconds, and 4 m/z isolation window, loop count 5, a dynamic exclusion of 10 s without further charge exclusion.

2.9. Sequence data analysis

Several approaches were followed to match sequence information to the acquired LC-MS/MS data. Data were searched with MaxQuant software (V1.6.5) versus a protein sequence database of Hermetia (UniProt-tax_343581), Tenebrio (UniProt-tax_7066), without or complemented with Musca_domestica Uniprot proteome_UP000095301 and Drosophila UNiprot proteome_UP000000803. Peptide tables from both searches were filtered to remove contaminants (*e.g.* collagen, trypsin), low scoring matches (Score< 20, PEPscore> 1%), and filtered peptide tables were merged together. Peptides with a Maximum Score> 20 or PEPscore< 1% were considered as a good score, and peptides with a Maximum Score of 100 were considered as perfect score.

Alternatively, LC-MS/MS data were converted to.mgf format using MSconvert (ProteoWizard V3.0.20178). Mgf converted data were interpreted by pNOVO3 *de-novo* search algorithm separately per sample group (ED, MPD, BPD, and BchPD). The resulting peptide sequence tables per sample were matched to the sequence database of LAMP2 containing 22534 peptide sequences of antimicrobial peptides (per July 3rd, 2020). Only matches with complete and identical sequence fit between pNOVO3 output and LAMP2 db were retained. Subsequently, only peptides with a maximum of peptide spectrum matching (PSM) score > 50 were considered.

2.10. Statistic analysis

Data is presented as mean or mean +SD and statistically significant differences between parameters were analysed by one-way ANOVA (Graphpad Prism 8, La Jolla, CA, USA) or Kruskal-Wallist test (Fig. 5).

3. Results

3.1. C. perfringens growth inhibition activity of digested insect-derived fractions

To investigate whether digest of insect-derived fractions exhibit anti-*C. perfringens* activity, *C. perfringens* growth, and viability was studied upon exposure to these digests. Exponentially growing *C. perfringens* were exposed to medium (PBS) as control for no treatment, empty digest (ED) as control for enzymatic effects, or digest of MP, BP, BchP, BC, or BT. The CFU of *C. perfringens* were measured after 0, 3, 6, and 24h of exposure (Fig. 1A). Growth of *C. perfringens* was significantly ($p < 0.05$) inhibited after 3h incubation with a digest of MP (MPD), and after 6h incubation with a digest of BP (BPD), digest of BchP (BchPD), and MPD. After 24h, BPD and BchPD significantly ($p < 0.05$) inhibited viability of *C. perfringens* by 68.5% and 84.6%, respectively (Fig. 1B). Moreover, a digest of BC (BCD) and BT (BTD), although not significantly, showed a lowered growth of *C. perfringens* over time as well.

3.2. Overview of peptides in the digest of protein-containing insect-derived fractions

As the protein enriched insect-derived fractions BPD and BchPD demonstrated to significantly ($p < 0.05$) reduce the viability of *C. perfringens*, we performed a proteomics analysis to identify the presence of antimicrobial peptides. First, to identify peptide sequences matching an insect protein sequence database, a standard MS/MS search was performed using MaxQuant software with trypsin as the digestion enzyme (Cox & Mann, 2008; Tyanova, Temu, & Cox, 2016). Using the available proteins from the Uniprot database from genus *Hermetia* (Black Soldier fly, 92 sequences), *Tenebrio* (mealworm, 620 sequences), *Musca domestica* (housefly, 16984 sequences) and *Drosophila* (fruit-fly, 4974 sequences), only 54 peptide sequences were identified with a good score, either the Max of Score> 20 or the Min of PEP < 1% (Table B1). The peptide length distribution was between 6 and 20 amino acids, and none of these peptides had a sequence identical to sequences in the LAMP2 database (data not shown).

As the MaxQuant search algorithm is designed to match peptide sequences longer than 6 amino acids, whereas in our sample the majority of peptides had very short sequences, we applied a separate approach for identifying short peptides. *De novo* interpretation of MS/MS spectra was performed using the pNOVO3 algorithm applied to the multiple LC-MS/MS data per sample group (Chi et al., 2010). From pNOVO3 a large number of peptide sequences were derived, not all of them having a maximum score of 100. In total, 1639, 43368, 51198, and 57426 peptides were identified in the ED, MPD, BPD, and BchPD, respectively. Among these peptides, 23.9%, 19%, 19.4%, 23.1% of peptides in ED, MPD, BPD, and BchPD, respectively, had a sequence smaller than 5 amino acids (Fig. 2A). After obtaining all the peptides in insect digests, we searched for identical matches with peptide sequence in the LAMP2 antimicrobial database. A total of 23 peptides were identified in BPD, 24 peptides in MPD, and 24 peptides were found in BchPD (Fig. 2B). Moreover, 14 identical peptides were found both in the MPD, BPD, and BchPD, 6, 2, and 3 unique antimicrobial peptides were found in MPD, BPD, and BchPD respectively. After quality control of antimicrobial peptides, using a cut-off value of a max peptide spectrum matching (PSM) score > 50 or max average amino acid (Avg AA) score > 50, we found 17, 19, and 16 peptides in MPD, BPD, and BchPD respectively, and 10 peptides were shared among these three digests (Table B.2).

3.3. Impact of digest of insect-derived fractions on healthy microbiota viability

As the digest of insect-derived fractions decreased the viability of *C. perfringens* over time to a different extent, we examined whether this effect also applied to gut microbiota in general. To this end, we harvested microbiota from a faecal sample from a healthy donor and exposed these during their exponential growth phase to the digest of insect-derived fractions, empty digest, and medium control at the same concentration as for the *C. perfringens* assay. Microbiota CFU/ml was measured after 0, 3, 6, and 24h of exposure (Fig. 3A). This revealed that the digests had minimal effect on the growth and viability of the microbiota. However, upon 24h incubation, the viability of microbiota was slightly, but significantly ($p < 0.05$), reduced by BPD, BCD, and BchPD by 8.6%, 6.2%, and 10.2%, respectively, when compared to medium control, and by 9.7%, 7.3%, and 11.3%, respectively, when compared to ED (Fig. 3B).

3.4. Microbiota compositional changes after exposure to digest of insect-derived fractions

To gain insight on the specific impact of BPD and BchPD on the healthy microbiota, we performed 16S rRNA sequencing to analyse the composition of microbiota after 24h exposure to medium, empty digest, or the digested insect-derived fractions. This revealed that ED neither significantly ($p > 0.05$) changed the total operational taxonomic units (OTUs) (Fig. 4A) nor the diversity indicated by Shannon index (Fig. 4B) when compared to medium control. In contrast, BPD and BchPD induced a significant ($p < 0.01$) decrease of 16.7% and 20.3%, respectively, in the total OTUs when compared to medium (Fig. 4A). In addition to OTUs, BPD and BchPD led to a significant ($p < 0.01$) reduction in the Shannon index when compared with medium control (Fig. 4B).

To explain the reduced diversity induced by BPD and BchPD, we evaluated the changes in the relative abundance at phylum (Fig. 4C) and genus (Fig. 4D) level after exposure to ED, BPD, and BchPD. Again, no significant ($p > 0.05$) changes were detected in bacteria phyla and

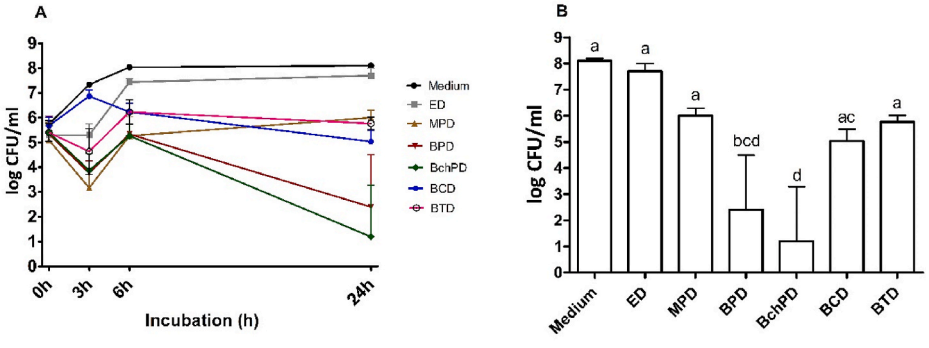

Fig. 1. Impact of digest of insect-derived fractions on *C. perfringens* growth and viability. Insect-derived fractions MP, BP, BchP, BC, BT, and MilliQ (empty control) were digested and added separately, as well as medium as control, to *C. perfringens* during its exponential growth phase. The CFU of *C. perfringens* were measured at t = 0, 3, 6, and 24h of incubation by counting colonies, and reported here as log CFU/ml (A). The log CFU/ml values after 24h were shown as bar charts (B). Both line and bar charts represent the mean of 3 independent experiments +SD. Statistical analysis was performed by one-way ANOVA, and different letters indicate significant ($p < 0.05$) differences; ED: empty digest; MPD: digest of mealworm larvae protein meal; BPD: digest of black soldier fly larvae protein meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BCD: digest of black soldier fly cocoon meal; BTD: digest of black soldier fly larvae trilaurin fraction; CFU: colony-forming units.

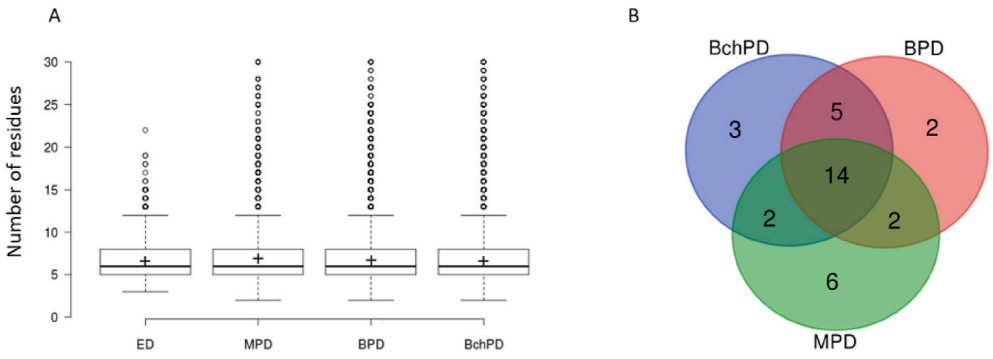

Fig. 2. Peptide distribution in digest of protein-containing insect-derived fractions. Insect-derived fractions MP, BP, BchP, and Milli-Q (empty control; ED) were digested, and the supernatant was used to analyse the presence of antimicrobial peptides. LC-MS/MS was performed. All peptide sequences were *de-novo* derived (using pNOVO3), and the distribution of all peptides based on their length. In total, 1639, 43368, 51198, and 57426 peptides were identified in the ED, MPD, BPD, and BchPD, respectively (A). *De-novo* derived peptides were matched on identity with sequences in LAMP2 database, and a venn diagram indicates the overlap, and differences of the antimicrobial peptides in MPD, BPD or BchPD (B). Box plots show the number of residues. Boxes represents the second and third quartiles, and whiskers represent 1.58x interquartile range (IQR). ED: empty digest; MPD: digest of mealworm larvae protein meal; BPD: digest of black soldier fly larvae protein meal; BchPD: digest of chitin rich black soldier fly larvae protein meal.

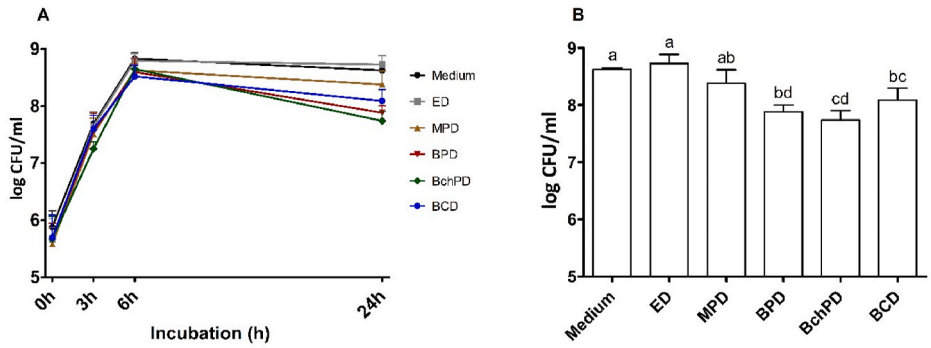

Fig. 3. Impact of digest of insect-derived fractions on growth of faecal-derived microbiota. Insect-derived fractions MP, BP, BchP, BC, and MilliQ (empty digest) were digested and added, as well as medium control, to faecal-derived microbiota during its exponential growth- and plateau phase. The growth of microbiota was measured at t = 0, 3, 6, and 24h by counting colonies, and reported here as log CFU/ml after overnight incubation (A). The log CFU/ml values after 24h are shown as a bar chart (B). Both line and bar charts represented the mean of 3 independent experiments +SD. Statistical analysis was performed by one-way ANOVA, and different letters indicate significant ($p < 0.05$) differences; ED: empty digest; MPD: digest of mealworm larvae protein meal; BPD: digest of black soldier fly larvae protein meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BCD: digest of black soldier fly cocoon meal; CFU: colony-forming units.

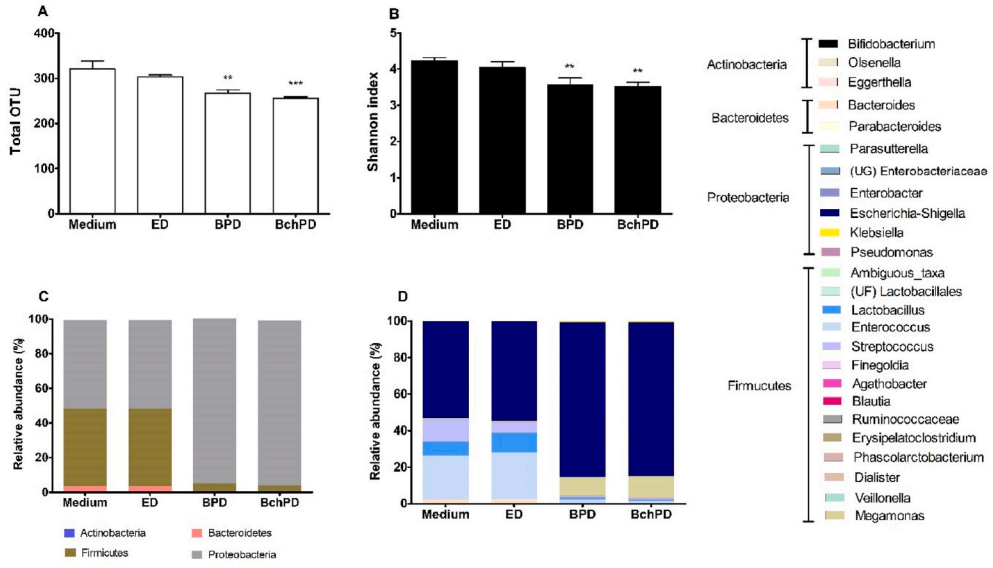

Fig. 4. Microbiota compositional changes after 24h exposure to BPD or BchPD. Insect-derived fractions BP, BChP, and MilliQ (empty digest) were digested and added, as well as medium control (BHI broth), to faecal-derived microbiota during its exponential growth phrase. After 24h exposure, the microbiota was collected and subjected to 16S rRNA sequencing to analyse the composition. The observed species were indicated as total OTUs (A), and the evenness of microbiota distribution was indicated as a Shannon index (B). The relative abundance of microbiota was showed at phylum (C) and genus (D) level. Bar charts represented the mean of 3 independent experiments +SD. Statistical analysis was performed by one-way ANOVA with **: $p < 0.01$; ***: $p < 0.001$. ED: empty digest; BPD: digest of black soldier fly larvae protein meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; OTU: operational taxonomic unit; UF: unknown family; UG: unknown genus.

genera after exposure to ED. At phylum level, *Proteobacteria* and *Firmicutes* were dominant in microbiota after exposure to control and ED. However, the relative abundance of *Proteobacteria* elevated from 51% to 95%, and *Firmicutes* reduced from 45% to 5% after exposure to BPD or BchPD when compared with ED. At genus level, *Escherichia-Shigella*, *Enterococcus*, and *Lactobacillus* were the dominant genus after incubation with medium or ED. When comparing BPD and BchPD to ED, the genus of *Escherichia-Shigella* elevated from 54.5% to 84.5% and 83.9%,

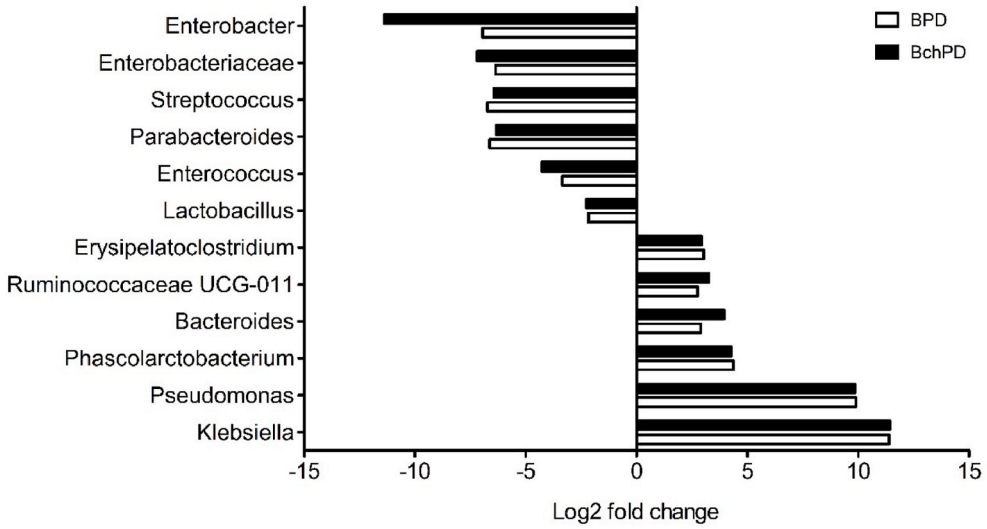

Fig. 5. Changes in the microbiota at a genus level after 24h incubation with BPD or BchPD. Only the bacteria significantly (FDR $p < 0.05$) changed after incubation with BPD and BchPD when compared with ED were showed. Statistical analysis was performed using Kruskal-Wallis test. Bar charts show the mean of 3 independent experiments. BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal.

respectively, *Megamonas* elevated from 0.5% to 9.7% and 11.3%, respectively, *Enterococcus* reduced from 25.6% to 9.7% and 11.3%, respectively, and *Lactobacillus* reduced from 10% to 1% and 0.9%, respectively (Fig. 4D). Statistical analysis revealed that among the 26 genera detected in all microbiota samples, 12 genera were significantly (FDR $p < 0.05$) changed after incubation with BPD or BchPD when compared with ED (Fig. 5). Moreover, it showed that BPD and BchPD induced a 2704- and 2760- fold increase of *Klebsiella*, respectively, although only reaching a 2% relative abundance, and 123- and 2678-fold decrease of *Enterobacter*, respectively, when compared with ED. Of note, we did not observe any changes in the abundance of the *Clostridium* genus upon incubation with BPD or BchPD, which might be due to the absence of *Clostridium* in the medium control (data not shown).

3.5. Fermentation of digest of insect-derived fractions increased SCFA release by faecal-derived microbiota

Short chain fatty acids (SCFAs) are a group of metabolites secreted by the microbiota that support a healthy gut (LeBlanc et al., 2017). A change in SCFA levels, regardless of a change in microbiota composition, can therefore also impact health. To this end, the levels of acetate, butyrate, propionate, valerate, formic acid, iso-butyrate, and iso-valerate were measured after 24h incubation of the microbiota with MPD, BPD, BchPD, BCD, and ED (Fig. 6). Analysis revealed that MPD, BPD, and BchPD significantly ($p < 0.05$) elevated the total SCFA secretion by microbiota when compared with ED. In particular, incubation with MPD induced a significant ($p < 0.001$) increase in acetate, propionate, iso-butyrate, and iso-valerate release by microbiota when

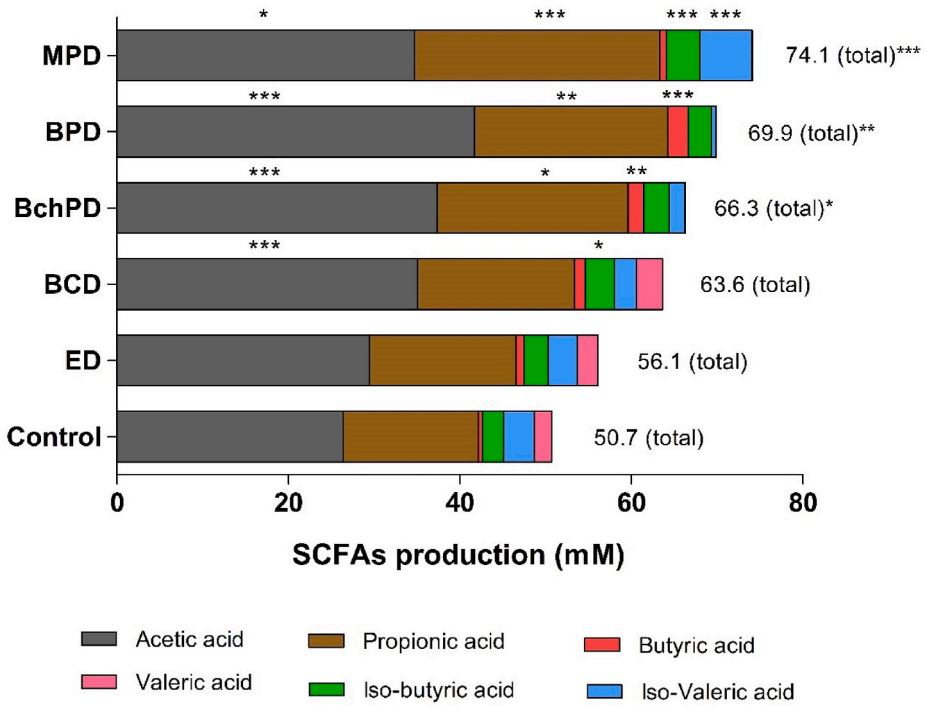

Fig. 6. SCFAs secretion by faecal-derived microbiota following exposure to digest of insect-derived fractions. Insect-derived fractions MP, BP, BchP, BC, and MilliQ (empty digest) were digested and added, as well as medium control (PBS), to faecal-derived microbiota. The secretion of acetate, propionate, butyrate, iso-butyrate, iso-valerate, and valerate was measured after 24h of incubation. Stacked bar shows the mean of 4 independent experiments. Statistical analysis was performed by one-way ANOVA with *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. MPD: digest of mealworm larvae protein meal; BPD: digest of black soldier fly larvae protein meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BCD: digest of black soldier fly cocoon meal; ED: empty digest.

compared with ED. Incubation of microbiota with BPD or BchPD also led to a significant ($p < 0.05$) increase of acetate, propionate, and butyrate secretion, and BCD induced a significant ($p < 0.05$) elevation of acetate and iso-butyrate secretion.

4. Discussion

C. perfringens is a commensal bacterium, but also an opportunistic pathogen that can cause severe health issues in humans, pets (*e.g.* dogs and cats), and livestock (*e.g.* chickens) (Silva & Lobato, 2015; Sklenickova et al., 2010). Here, we set out to identify whether specific insect-isolates contain anti-*C. perfringens* activity. Insects are already applied as human food, pet food, and livestock feed due to their high nutritional value (Anankware, Fening, Osekre, & Obeng-Ofori, 2015). After the ban on using antibiotic growth promoter in livestock industries, the antimicrobial property of insects such as mealworm and black soldier fly acquired increasing attention (Mwangi et al., 2019). Insects have also gained popularity as sustainable and health promoting pet food ingredients (Mouithys-Mickalad et al., 2020; Smetana, Schmitt, & Mathys, 2019) with this sector being now the biggest outlet for insect proteins (IPIFF, 2020). Even though the bactericidal effects of insect as a whole have been extensively researched, studies on the antimicrobial property of insect-derived fractions are still scarce. In this study, we tested five insect-derived fractions, and digested them according to the adapted INFOGEST consensus protocol (Minekus et al., 2014).

The *in vitro* digestion method used in this study aims to mimic the oral, gastric, and intestinal digestion in human by simulating parameters such as electrolytes, enzymes, bile, pH, and digestion time based on available physiological data. The digestive system in humans contains stomach, small intestine, and large intestine, and some physiological similarities were found between human and animals (Kararli, 1995). For instance, the colon morphology appears similar in humans and pigs, the stomach morphology and empty characteristics are similar in humans and dogs, and the pH of gastric fluid is similar in humans, dogs, and pigs (Kararli, 1995). Hence, we need to be aware of that, in addition to human, our *in vitro* data might also indicate the effect of insect-derived fractions in certain species of pets or livestock.

When incubating *C. perfringens* with digested insect-isolates, we observed that growth was strongly inhibited by BPD and BchPD with 69% and 85%, respectively. The antimicrobial effects of insects as a whole result from different bioactive components, being antimicrobial peptides (AMPs), chitin, and fatty acids (Mari et al., 2014; Wu, Patočka, & Kuča, 2018). Black soldier flies are naturally rich in lauric acid, known to exhibit activity against both Gram-positive bacteria (*e.g. Staphylococcus aureus*) and Gram-negative bacteria (*e.g. Fusobacterium nucleatum*) (Fischer et al., 2012; Matsue et al., 2019). Lauric acid can be released after lipase digestion of trilaurin, a triglyceride that contains a significant amount of lauric acid (Kam, Woo, & Ong, 2017). However, the results showed that BTD did not significantly ($p < 0.05$) inhibit the growth of *C. perfringens* (Fig. 1). This could result from insufficient or ineffective hydrolysation during *in vitro* digestion as the INFOGEST digestion model has yet to be optimized for digestion of fat. Next, the antimicrobial activity of chitin depends on its physiochemical property. The deacetylated form of chitin, chitosan, was demonstrated to be effective against a broad range of microbes by permeating their membrane (Jeon, Oh, Yeo, Galvao, & Jeong, 2014). Unlike chitosan, the antimicrobial effects of chitin with a low deacetylation level (<50%), the form found in black soldier fly (D'Hondt et al., 2020), was limited, and increasingly associated with immunomodulatory activity (Dong et al., 2019). The results revealed that digest of BC, which has a relatively high amount of chitin (Table 1), did not significantly ($p > 0.05$) decrease the viability of *C. perfringens*. Taken together, the bactericidal effects on *C. perfringens* of BPD and BchPD were most likely due to the presence of AMPs, rather than lauric acid or chitin.

As our samples were derived by *in vitro* digestion simulating the gastric and ileum conditions, the majority of proteins were digested into very small peptides and single amino acids. Most proteomics database search algorithms are designed to identify peptides with a length of minimally 6 amino acids. Using a *de-novo* approach, large number of potential sequence match, with very short sequences, was generated. Following the application of a stringent filter (PSM score>50), sequences were matched on identical sequences with the LAMP2 database. We identified 23 and 24 AMPs in BPD and BchPD, respectively, and 19 AMPs that were common in both BPD and BchPD (Fig. 2B). So far, studies that identified AMPs in black soldier fly larvae were performed through bioinformatic analysis based on gene transcription or chromatography analysis of insect extracts (Moretta et al., 2020; S.-I. Park, Kim, & Yoe, 2015; S. I. Park, Chang, & Yoe, 2014). Up until recently, more than 3000 proteins with antimicrobial property have been found in different organisms, among which 300 AMPs have been identified in insects (Jhong et al., 2019). Most active AMPs are small peptides of 20–50 residues (Yi et al., 2014). However, AMPs found in our study are smaller than 5 amino acids as a result of the *in vitro* digestion procedure. To interpret these small peptides in the digest of insect-derived fractions and control, they were annotated based on an identical sequence match in the database of antimicrobial activity and structure of peptides (DBAASP) (data now shown). Even though the AMPs in the digests were determined by its identical sequence, we need to note that the antimicrobial properties of AMPs are also dependent on their structure (Huang, Huang, & Chen, 2010; Leon et al., 2020). For instance, a peptide (LPLP) identified in both BPD and BchPD possess the same sequence of a peptide cyclo-[LPLP] extracted from Thai sponge *Halisarca ectofibrosa* but their structure is different (Rungprom et al., 2008), which might lead to different functionality. Therefore, further studies are needed to verify the antimicrobial activity of individual peptides or peptides present in major concentrations found in BPD and BchPD and to identify their structure-dependency.

Next to *C. perfringens*, the microbiota community consists of healthy microbiota which we investigated using faecal-derived microbiota from a healthy donor. Generally, human microbiota composition shares some similarity with that of some animals (*e.g.* dog and pigs), and differences mainly relate to variation in the relative abundance of microbiota (Coelho et al., 2018; Ellis et al., 2013; Pilla & Suchodolski, 2020). Hence, results obtained from this *in vitro* study with human microbiota could also be predicable for monogastric animals. Our results revealed that BPD and BchPD significantly ($p < 0.05$) reduced diversity, richness, and viability of faecal-derived microbiota (Figs. 3 and 4). We used a single donor, therefore representing only a single enterotype (Arumugam et al., 2011), but this does not take away from the general finding that insect-fractions impacted the diversity. These general effects on the microbiome were not a consequence of decreased *Clostridium* species abundance or presence, as *Clostridium* species were absent from both control and insect digests treated groups. Our results showed that the incubation of faecal-derived microbiota with BPD reduced *Firmicutes* abundance from 45% to 5%, and elevated *Proteobacteria* abundance from 51% to 95%, and same was observed for BchPD (Fig. 4C). A bloom of *Proteobacteria* in the gut microbiota reflects an unstable microbiota community as can be observed in the neonatal period, in patient after gastric bypass surgery, and in patients with intestinal diseases (Shin, Whon, & Bae, 2015). At genus level, the relative abundance of dominant genus *Escherichia-Shigella* was elevated 30%, and *Enterococcus* reduced around 10% after exposure to BPD or BchPD when compared with ED (Fig. 4D). Increased opportunistic bacteria such as *Escherichia-Shigella* has been found in patients with inflammatory bowel disease, and higher abundance of this genera is recognized as the signature of intestinal microbiota dysbiosis of patients with Crohns diseases (Pascal et al., 2017; Zhang et al., 2020). In contrast to these findings, a study performed with rainbow trout reported that oral administration of black soldier fly larvae elevated the diversity of gut microbiota as evidenced with an increased total OTU, richness, and Shannon diversity (Huyben, Vidaković, Hallgren, & Langeland, 2019). Furthermore, a broiler chicken study also showed that replacement of soybean diet by black

soldier fly diet increased the total OTUs as well as the Shannon diversity (Borrelli et al., 2017). The difference between our study and these animal experiments might be explained by the use of a single donor versus groups of 160 fishes or 24 laying hens, or the presence of AMPs in the digested insect-derived fractions. Generally, most proteins will be digested and absorbed in the small intestine, and only a fraction may reach the proximal colon *in vivo* (Davila et al., 2013). Therefore, the observed impact on OTU and diversity by BPD and BchPD might be less pronounced *in vivo*. Furthermore, the observed reduction in OTU and diversity when incubating faecal-derived microbiota with BPD or BchPD is less than that resulted from antibiotics usage. A clinical study has demonstrated that usage of broad-spectrum antibiotics fluoroquinolones and β-lactams reduced the diversity of faecal-derived microbiota with 25%, and the core phylogenix taxa by more than 50% (Panda et al., 2014). So, although a strong decrease in *Firmicutes* and increase in *Proteobacteria* is undesirable, the total impact on healthy microbiota of BPD or BchPD is less invasive than currently applied antibiotics in humans.

Next to the changes in microbiota composition, SCFAs secretion increased after faecal-derived microbiota was exposed to the digested insect-derived fractions. SCFAs are associated with many beneficial effects, and our findings were consistent with animal studies (Borrelli et al., 2017; El-Hack et al., 2020). Most notably, we found an increase in butyrate secretion by microbiota after BPD or BchPD exposure (Fig. 6). Butyrate is the major energy source for colonocytes, and it can promote the differentiation and proliferation of intestinal epithelial cells. Hence, butyrate has been demonstrated to be beneficial for intestinal health, ameliorate mucosal inflammation, reinforce intestinal barrier function, and modulate intestinal motility (Canani et al., 2011). Even though peptides are not the major substrate for gut microbiota, they can be fermented by a range of microbes such as *Bacteroides* and *Fusobacterium* (Davila et al., 2013). Therefore, animal studies are required to further characterize the impact of insect-derived fractions on SCFAs production and gut microbiota metabolism.

C. perfringens infections significantly impact health of pets, livestock, and humans directly and indirectly via ever rising antibiotic resistance. Increasing antibiotic resistance in animal husbandry is of major concern as there are well established negative implications of their usage (Llor & Bjerrum, 2014; Panda et al., 2014; Pilla et al., 2020; Qin, Pilot, Thompson, & Maskell, 1986). Taken together, the results of these *in vitro* studies on the inhibition of *C. perfringens* growth, alteration of the healthy microbiota composition, and increase in SCFAs secretion indicated that black soldier fly-derived protein can be an effective anti-*C. perfringens* product. Further *in vivo* studies with insect-fractions are required to investigate the optimal dosage and settings at whether this additional tool might contribute to a reduction in antibiotics usage.

Credit author contribution statement

L.D, C.G, and J.J.M conceived and designed the experiments. L.D, R. A and A.A performed the experiments. L.D, R.A and A.A performed data analysis and interpretation. L.D, A.P, C.G, A.A wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

A.P. is employed by Protix B.V.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111806.

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