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Studies on the novel effects of electron beam treated pollen on colony reproductive output in commercially-reared bumblebees (*Bombus terrestris*) for mass pollination applications

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ABSTRACT

Commercially-reared bumblebees provide an important pollinator service that helps support food production and security. The deployment of an appropriate non-thermal disinfection technology for the bulk treatment of pollen collected from honeybees for the feeding of commercial bumblebees is important in order to mitigate against complex diseases and unwanted pathogen spillover to native bees. High level disinfection of pollen was achieved using an electron (e)-beam dose of 100 kGy that corresponded to 78 % loss of cellular viability of bee pathogens before feeding to bumblebees as measured by the novel in vitro use of flow cytometry (FCM). Novel findings showed that e-beam treated-pollen that was fed to bumblebees produced fewer females, gynes and exhibited an absence of males when compared to control bumblebee colonies that were fed untreated commercial pollen. A similar trend emerged in bumblebee colony reproductive outputs when using membrane filtered washed pollen. Proteomic analysis of bumblebees from individual colonies fed with treated-pollen revealed a differential abundance of proteins associated with stress, immunity and metabolism when compared to the untreated pollen control group. Microbiome analysis of the bumblebee gut content revealed differences in microbiota between treated and untreated pollen in bumblebee colony studies. This novel study evaluated the impact of industrial e-beam treated-pollen on complex bee disease mitigation where physically treated-pollen fed to bumblebees was shown to substantially affect colony reproductive outputs.

1. Introduction

Pollination is a vital resource provided by many insects including bees with 87.5 % of angiosperm species benefiting from insect pollination (Theodorou et al., 2020). Animal pollination is required for up to 40 % of global crop production (Krams et al., 2022), and bee pollinators, such as managed honeybee (*Apis mellifera*) and various bumblebees (*Bombus* species) and solitary bees, play a critical role. The western honeybee alone is estimated to be worth $15–$20 billion to the US economy, where it pollinates >90 % of almond, blueberry and apple crops. The honeybee is extremely important on a global scale for crop production; however, there is evidence of regional declines in both Europe and the USA (Murphy and Stout, 2019). Consequently, the commercial production of bumblebees has increased substantially (Hidalgo et al., 2020). Several bumblebee species have also been adapted to commercial pollination with over 2 million commercial bumblebee colonies used on an annual basis to pollinate over 20 crop types worldwide (Trillo et al., 2021). Bumblebees are efficient pollinators and have the ability to forage at low temperatures and in adverse weather conditions. In addition, some species of bumblebee have the ability to buzz pollinate that is necessary for pollination and to increase the yield of many fruit crops such as tomatoes, and peppers (De Luca and Vallejo-Marin, 2013). Due to their size, *Bombus terrestris* has the ability to carry more pollen on their body surfaces relative to honeybees (Knapp et al., 2019) (Stern et al., 2021). Previous Irish surveys reported that commercial bumblebees pollinate strawberries (60 %), apples (25 %) and tomatoes (20 %) (Biodiversity and Series, 2009).

However, bees are one of many pollinators that are under treat in the world today (Peso et al., 2018). The decline in honey bees was first reported in 2006 that was referred to as colony collapse disorder (CCD) (VanEngelsdorp et al., 2017; Tong et al., 2018). Since then, losses have been observed in bee colonies in several countries (Staveley et al.,

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2014), and have raised conservation concerns (Murray et al., 2012). Several factors, such as pesticides, parasites, viruses, climate change, habitat loss and a lack of quality nutrition have been linked with poor colony survivorship (Runckel et al., 2011; Tong et al., 2018; Reeves et al., 2018; Peso et al., 2018). The decline of bee pollinators is an alarming development given their critical role in food security and economic prosperity. Hidalgo et al. (2020) noted that a "concern related to the commercial rearing of bumblebees is the possibility of microbial contamination of food used in the rearing, including pollen obtained from domesticated honeybees". These commercial bumblebees have a high parasite prevalence (41 % of individuals infected) of trypanosomatids, microsporidians, and neogregarines, posing a potential risk to wild pollinators (Trillo et al., 2021).

A key factor influencing bee decline is their susceptibility to diseases caused by parasites and viruses (Walderdorff et al., 2018). Bumblebees tend to be predominantly infected by the parasite *Crithidia bombi* while *Nosema ceranae* and *Varroa* mites tend to infect honeybees. There is evidence to suggest that these pathogens can cross bee species to cause infection (Graystock et al., 2015). *Nosema ceranae* is a significant fungal parasite that shortens the life span of the bee by causing energetic stress (Youngsteadt et al., 2015). This fungal bee pathogen also suppresses bee immunity, disrupts foraging behaviour, and negatively impacts on the synthesis of vital molecules and cellular signalling. *Nosema* infections have been traditionally treated therapeutically such as using fumagillin; however, this has since been banned by the European Union due to its toxicity. It is appreciated that there is currently no technology or intervention that has been proven to effectively disinfect the broad array of complex bee pathogens, such as those contamination wax combs or pollen (Burnham, 2019; Simone-Fintrom et al., 2018). This is due to multiple factors including the lack of interdisciplinary research to inform testing and development of appropriate industrial-scale disinfection technologies commensurate with using bee infectivity colony studies (Goblirsch et al., 2021); the lack of a relevant in vitro bioassay for real-time determination of complex pathogen destruction that will inform appropriate treatment dosage (Simone-Fintrom et al., 2018); the variability in pathogen resistance to applied treatment technologies (Hidalgo et al., 2020); lack of simultaneous or sequential cellular and molecular information on the response of complex bee pathogens to sublethal and lethal treatment doses appropriate for killing pathogens in bee host (that maybe significantly lower dose when required for artificial in vitro disinfection studies (Naughton et al., 2017; Simone-Fintrom et al., 2018); and the lack of consensus on appropriate standard methods to reporting disinfection efficacy that includes future use of potential indicator reference strain(s) representative of different bee pathogen types informing comparative and repeatable studies enabling the relevant testing and development of appropriate breakthrough treatment technologies.

Pollen is an important natural source of protein and lipids for honeybees, where the amount of pollen collected is related to the number of larval and adult bees present in the hive (Ghosh et al., 2020). Commercial pollen is sourced from honey bees and this is then used to feed bumblebees (Pereira et al., 2019a). In natural environments, pollen is exposed to a broad range of microorganisms including pathogens to the extent that pollen collected by honeybees harbour complex microbial bee pathogens that maybe transmitted to the bee colony (Graystock et al., 2013; Graystock et al., 2015). More than 200 tonnes of honeybee-collected pollen is used annually (Velthuis and van Doorn, 2006). Commercial pollen has been found to contain a variety of bee pathogens that are economic importance in that they contaminate bumblebee colonies produced by mass rearing for export to other countries. These bee pathogens include inter alia *N. ceranae*, and *Crithidia mellificae*, *Apicystis bombi* and viruses *Deformed wing virus* (DWV), *Israeli acute paralysis virus* (IAPV), Chronic bee paralysis virus and *Sacbrood virus* (SBV) (Pereira et al., 2019b).

Determining an appropriate and effective method for the non-thermal treatment of natural pollen gathered by honeybees for the mass rearing of commercial bumblebees is a complex challenge (Hidalgo et al., 2020; Goblirsch et al., 2021). There are limited number of industrial-scale technologies that can potentially treated large quantities (tonnes) of pollen, such as on pallets, without affecting its nutritional constituents for this important pollination application (Goblisrsch et al., 2021). However, limited studies thus far have pursed use of irradiation technologies (Hidalgo et al., 2020) that have been applied and regulated for food applications that includes important knowledge transfer from the adjacent medical device sector (McEvoy et al., 2023). Hidalgo et al. (2020) have reported that gamma irradiation at a dose of 7 kGy effectively killed the majority of aerobic bacteria and the spores of fungi that contaminated commercial pollen when treating small sample quantities; however, aerobic endospore forming bacteria survived treatment at 9 kGy, particularly *Bacillus pumilus*. Meeus et al. (2014) reported that gamma irradiation of pollen is capable of reducing the pathogenicity of honey bee virus IAPV, and two commercial bumblebee produces (Biobest, Waesterloo, Belgium; Koppert B. V, Verkel en Rodenrijs, The Netherlands), have started to use this method for pollen sterilization in Europe. Other researchers have also noted that gamma irradiation reduces viral incidences in bumblebee mass rearing, along with reducing the level of other bee pathogens, such as *C. bombi*, *A. bombi*, *N. bombi*, *Nosema apis*, *N. ceranae*, deformed wing virus (DWV), Kashmir bee virus (KPV), black queen cell virus (BQCV), sacbrood virus (SQB), Ascosphaera fungi, and American foulbrood and European foulbrood bacterial (Graystock et al., 2016). However, Simone-Fintrom et al. (2018) reported that the recalcitrant Chronic bee paralysis virus and the Black queen cell virus can withstand 25 kGy gamma irradiation delivered over 9 h 45 min (standard dose used for sanitation purpose; ISO#13409 2002); however, this dosage was reported to effectively inactivate the fungus *Ascosphaera apis*, the microsporidian gut parasite *Nosema ceranae*, and Deformed wing virus.

Yook et al. (1998) previously reported that gamma treatment of pollen at 7.5 kGy dose did not significantly affect the nutritional composition of pollen; thus, intimating that this irradiation method offers potential as a sterilization process for mass rearing of commercial bumblebees. McFadden et al. (2016) reported that electron-beam treatments of heat sensitive infant milk formulae at 10 kGy did not substantially affect nutritional composition. However, there is a marked gap in appropriate experimental studies that focus on bee colony stability, and potential hereditary effects, post consumption of treated pollen by commercial bumblebees.

McEvoy and Rowan (2019) compared the effectiveness of irradiation technologies for large scale medical device applications that would be potentially similar in high through-put treatment of honey-bee gathered pollen destined for mass rearing of commercial bumblebees. E-beam uses high energy electrons emitted via an accelerator or ^{60}Co isotope as its radiation source (McEvoy and Rowan, 2019). It has advantages over gamma-treatments that include short exposure periods (usually minutes), fast cycle times, flexible batch size, good radiation dose distribution, simple validation process, no quarantine period and enables real-time monitoring (McEvoy and Rowan, 2019). E-beam is a non-thermal sterilization method for high-throughput treatment of medical devices and pharmaceutical drugs (McEvoy et al., 2023). E-beam efficiently penetrates bulk densities between 0.05 and 0.3 g per cc (McEvoy and Rowan, 2019). Electron-beam treatment of pollen for rearing commercial bumblebees was investigated in this study due also to the proximity of a large-scale industrial terminal sterilization process that also facilitated fast transportation of samples for analysis and usage in nearby specialist laboratories. In addition, McEvoy et al. (2023b) recently reported that X-ray, gamma and e-beam are comparable in terms of their microbicidal efficacy at equivalent respective dosages for medical device applications where these authors used recalcitrant bacterial endospore bioindicators (*Bacillus* species) that may potentially use in the future as an umbrella in vitro approach representative of the different complex bee pathogens that require an in vivo host (bee) for culture. The determination of bee disease inactivation by treatment

technologies and approaches is challenging given that there is reliance on using bees as host where there is limited information of appropriate in vitro assays. Determining the infectivity of complex pathogens, such as parasites, is generally very difficult as there is a lack of an appropriate in vitro infection models (Gerard et al., 2019; Franssen et al., 2019). Naughton et al. (2017) used a novel combined qPCR and cell culture assay for *Cryptosporidium parvum* oocysts (a waterborne enteroparasite), where survivor plots were used to scope appropriate pulsed UV dose for inactivating the bumblebee trypanosome parasite *Crithidia bombi* prior to using bumblebee infectivity studies. Pulsed UV is not deemed suitable as a technology for bulk pollen treatment due to lack of depth of UV penetration (Rowan, 2019). We report here, an assessment of the effectiveness of industrial electron beam technology for the decontamination of commercial pollen destined for bulk use in feeding commercial bumblebees. The subsequent impact of electron beam-treated and membrane-filtered washed pollen on commercial bumblebee colony reproductive outputs that includes influencing changes in bee types, fatbody proteome and bee gut microbiome composition, is reported. The insect fatbody plays a key role in protection against oxidative stress, accumulation of toxic compounds, nutrient and energy storage and mobilization, which is necessary for biosynthesis of molecules (Strachecka et al., 2021). This makes the fatbody an ideal tissue for the detection of molecular fluctuations associated with dietary changes.

2. Materials and methods

2.1. Industrial electron-beam sterilization

Pollen was obtained from a commercial supplier treated in an industrial electron beam site (STERIS AST, Tullamore Industrial Park, Ireland), and transported to the lab for analysis and use. E-beam treatments of 1 kg pollen samples were conducted at 1.5 kGy, 5 kGy, 10 kGy, 25 kGy, 50 kGy, 75 kGy and 100 kGy. This study was repeated in triplicate. The effectiveness of reducing microbial burden in these e-beam treatment regimes was determined using flow cytometry where propidium iodide (PI) was used to confirm loss of cellular viability as per McEvoy et al. (2021). Noting, there were limited opportunities to investigate treatment doses due to restricted access as it coincided with COVID-19 pandemic where there was increased need for treating medical devices at this industrial e-beam terminal sterilization site.

2.2. Membrane filtration of pollen

Untreated pollen was placed on a 5 μm cut-off filter (Whatman) and washed with sterile water. The water mixes with the pollen, and passes through the membrane filter, while the pollen is retained. It was hypothesised that this process could reduce pathogen load, as small microorganisms can readily pass through the 5 μm pores of the filter.

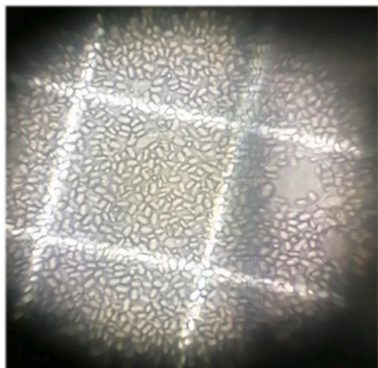

Fig. 1. Microscopic visualization of microbial load suspended in sterile water that was extracted from commercial pollen using membrane filtration.

Microbial contamination present in the filtered water was confirmed using a haemocytometer, where 1×10^6 microbes/mL were counted (Fig. 1). The washed pollen product was blotted dry using Whatman filter paper.

All pollen treatment types were stored in separate containers at 4 °C for the duration of the experiment. All challenged bumblebee colonies were fed on pollen used from the same batch, and fil pollen was made up every three days, using sterile water and 5 μm filter paper.

2.3. Bumblebee colonies

Research grade colonies of *Bombus terrestris audax* were sourced from Agralan with 40–50 bees per colony. These bumblebees had ad libitum access to feeding tubes filled with 40 % (w/v) sucrose. All bumblebee colonies were kept at 24 ± 2 °C and 58 ± 5 % relative humidity in the dark. For the first week, colonies were fed daily with 4 g of pollen, and 8 g of pollen thereafter. Twelve bumblebee colonies were used that were divided them into 3 groups, #1 to 4 were fed untreated pollen (control); Bumblebee colonies #5 to 8 were fed the same commercial pollen that had been treated with a 100 kGy dose of e-beam radiation. Bumblebee colonies #9 to 12 were fed washed pollen. Observations were made on each colony every second day, for a total of 28 days. Colonies were monitored for weight (once a week) and reproductive output (every second day) through visual observations and counting. Reproductive output was determined through counting of eggs, larvae and pupae.

2.4. Tagging callows and dissection for microbiome analysis

Newly emerged bees (callows) can be distinguished from adult bees due to their grey phenotype compared to the adult yellow and black phenotype. Callows were anesthetised under CO_2, tagged and reintroduced into the colony. Tagged callows were removed from the colony after 2 weeks, snap frozen in liquid nitrogen and stored at −80 °C until required.

2.5. DNA extractions for microbiome analysis

Bees were removed from −80 °C storage, pinned to a wax bed through the thorax and stinger prior to thawing. The abdomen was extended slightly and cut along the dorsal and ventral plane to reveal the abdominal contents. The digestive tract was located, transferred toa sterile 1.5 mL tube containing 200 μl of the ATL buffer from the DNeasy Blood and Tissue Kit (Qiagen) and homogenised using a motorised pestle. 100 μl of lysozyme was added and the sample was incubated at 36 °C for 40 min. The remainder of the DNA extraction procedure followed the manufacturer's instructions. Extracted DNA was stored at −20 °C before submission to Novogene Europe (UK) Ltd. for and ITS and 16S amplicon sequencing to determine the bacterial and fungal species present. Five replicate samples per treatment were sequenced. Sequencing data are available from NCBI BioProject PRJNAXXXXXXX. DNA quality control studies exhibited range values between 1.9 and 2.1 for untreated (control), e-beam and filtered-treated samples for Abs 260 and Abs 280 ratios (Supplementary Table 1).

2.6. Proteomic sample preparation

For fatbody collections, bees were prepared and pinned as they were for the digestive tracts. Fat body cells were collected from the inside the dorsal and ventral cuticles using sterilized pins and tweezers and transferred to individual 1.5 ml tubes containing 300 μl of lysis buffer comprising 6 M urea, 2 M thiourea and 1 tablet of Complete™, Mini Protease Inhibitor Cocktail (Roche Diagnostics). Samples were snap frozen in liquid nitrogen and stored at −20 °C. When all dissections were complete the samples were thawed on ice, homogenised for 30 s each using a motorised pestle, and centrifuged at 10000 rpm for 5 min to pellet any remaining cellular debris. Supernatants were aliquoted into

sterile 1.5 mL tubes and stored at −80 °C.

Protein quantification was conducted using a Qubit® fluorometer version 2.0 following manufacturer guidelines. 100 μg of protein was removed from each sample and processed with a 2-D clean up kit (GE HealthCare) to remove biological impurities. The resulting pellet was resuspended in 50 μl resuspension buffer (6 M urea, 0.1 M Tris HCl, pH 8.0), of which 20 μl was used for protein digestion. 115 μl of 50 mM ammonium bicarbonate was added to each sample. Proteins were reduced and alkylated by adding 0.5 M dithiothreitol (DTT) at 56 °C for 20 min followed by 0.5 M iodoacetamide (IAA) and incubated at RT for 15 min in the dark. 1 μl of 1 % (w/v) Protease Max (Promega) and 1 μl sequence grade trypsin (Promega) were added to each sample and incubated at 37 °C for 16 h. Subsequently, 1 μl of 100 % trifluoroacetic acid (TFA) was added to each sample to terminate digestion and the samples were incubated at room temperature for 5 min and centrifuged at 10,800 rpm for 10 min. The resulting supernatants were purified using Pierce C18 spin columns (Thermo Scientific) following manufacturer guidelines and the eluted purified peptides were dried using a vacuum concentrator (Thermo Scientific Savant DNA 120) and stored at 4 °C. Peptides were resuspended in a volume of loading buffer (2 % (v/v) acetonitrile and 0.05 % (v/v) TFA) to yield a concentration of 0.5 μg/μl, sonicated for 2 min and centrifuged at 13,400 rpm for 5 min.

2.7. Mass spectrometry analysis

1 μg of peptide mix for each sample was eluted onto the Q Exactive (Thermofisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. An increasing acetonitrile gradient was used to separate peptides on a Biobasic C18 Picofrit™ column (200 mm length, 75 mm ID), using a 120-minute reverse phase gradient at a flow rate of 250 nL/min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300–2000 Da) was carried out to select the 15 most intense ions prior to MS/MS. MaxQuant version 1.6.17.0 (www.maxquant.org) was used for protein identification and LFQ normalisation of all MS/MS data. The Andromeda search algorithm in MaxQuant was used to correlate all MS/MS data against protein reference sequences obtained from the National Centre for Biotechnology to correlate the data against the protein reference sequences derived from the *B. terrestris* genome (Saad et al., 2015) obtained from the National Centre for Biotechnology Information (NCBI) repository (17,508 entries, downloaded September 2021). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXDXXXXXX).

2.8. Proteomic data analysis

Perseus version 1.6.1.1 was used for data visualization and statistical analysis. Normalised LFQ intensity values were used as a measure of protein abundance. The data was filtered for the removal of contaminants and peptides identified by site. LFQ intensity values were log_2 transformed and samples were allocated to groups corresponding to treatment. Proteins absent from any samples in at least one group were not used for further analysis. A data imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1 times the mean standard deviation (SD) of all measured values and a width of 0.1 times this SD. Normalised intensity values were used for principal component analysis. A two-sample *t*-test was performed using a cut-off value of $p \leq 0.05$ to identify statistically significant differentially abundant (SSDA) proteins. Volcano plots were produced by plotting –Log *p*-values on the y-axis and Log_2 fold-change values on the x-axis to visualize differences in protein abundance between treatment groups. Hierarchical clustering of SSDA proteins was performed using z-score normalised intensity values to produce a heat map of protein abundance.

2.9. Functional annotation

The Search Tool for the Retrieval of Interacting Genes/proteins (STRING) version 11 (Szklarczyk et al., 2019) (www.string-db.org) was used to map protein-protein interaction networks. Protein sequences were inputted into the STRING database and protein-protein interactions were analysed using the homologous *Apis mellifera* match for each identified *B. terrestris* protein. STRING clusters were inspected for the protein sets of higher or lower abundance in fatbodies from bees fed *E*-beam-treated or washed pollen with respect to the control pollen. The gene ontology categories enriched with each set (Fisher's Exact test; $p < 0.05$) were obtained for molecular function, biological process, cellular compartment, KEGG term and others where appropriate to determine the pathways and processes affected by the different pollen treatments.

3. Results

3.1. Preliminary screening and assessment of treatments for pollen sterilization

E-beam technology was tested for its suitability to decontaminate bulk honeybee gathered pollen for the purpose of feeding commercial bumblebees. E-beam treatment was carried out on pre-determined microbial and parasitic bee pathogens extracted from bulk pollen at Steris Advanced Sterilization Technologies (AST), Tullamore, Ireland. E-beam treatments were not effective at lower treatment doses (≤25 kGy), where there was increased level of inactivation achieved using 50 kGy, 75 kGy and 100 kGy doses that were equivalent to 36.6 %, 51.2 % and 77.9 % loss of cellular activity respectively using flow cytometry (Fig. 2). E-beam samples left in storage at room temperature revealed no mold growth after 8 months compared to untreated pollen where fungal growth was observed after one-month storage (Fig. 3). Due to this study coinciding with the COVID-19 pandemic, it was not possible to access the industrial e-beam facility for additional optimisation. Accordingly, a dose of 100 kGy for e-beam treatment of pollen was selected that would provide a fixed dosage for maximal destruction of pathogens in the pollen based on initial findings.

3.2. Colony reproductive output

Bumblebee colonies were observed every second day for changes in brood development with the changes in eggs clumps, larvae and pupae numbers noted and recorded. A reduction in the number of larvae and pupae being produced in both treated colonies (e-beam treated and washed pollen) compared to untreated colonies was observed after four weeks (Fig. 4). A change in egg, larvae, and pupae colour was also observed. Eggs from colonies fed with treated pollen failed to develop into larvae. Colonies fed untreated pollen continued to produce eggs, larvae and pupae.

Colonies fed on e-beam treated and washed pollen produced significantly more eggs compared to those fed on control pollen (Fig. 4A) ($P \leq 0.05$). However, control colonies produced significantly more larvae than e-beam-treated and washed pollen-fed colonies ($P \leq 0.001$, $P \leq 0.05$) (Fig. 4B). Control colonies also produced significantly more pupae ($P \leq 0.001$) (Fig. 4C).

3.3. Colony numbers and sex typing

Colonies were examined post-experiment and sex typed bees that had died during the course of the experiment were included in the overall count, but were not typed due to the poor condition of their bodies. These were seen mainly in the control colonies (Fig. 5). Colonies fed on treated pollen produced fewer males, while control groups produced males in all 4 colonies. Female bees that were deemed to be larger

50 kGy

PI +
%P:36.58
Cells/ml:49298.16

75 kGy

PI +
%P:51.20
Cells/ml:189804.92

100 kGy

PI +
%P:77.85
Cells/ml:274664.06

SSC-A

Propidium Iodide

Fig. 2. E-beam treatments were administered to microbial samples derived from industrial pollen at intensities of 50, 75 and 100 kGy. Samples were prepared for flow cytometry analysis by staining with viability dye Propidium Iodide (PI). Dot plots show percentages and total cell numbers positive for PI fluorescence, representative of cell damage/death.

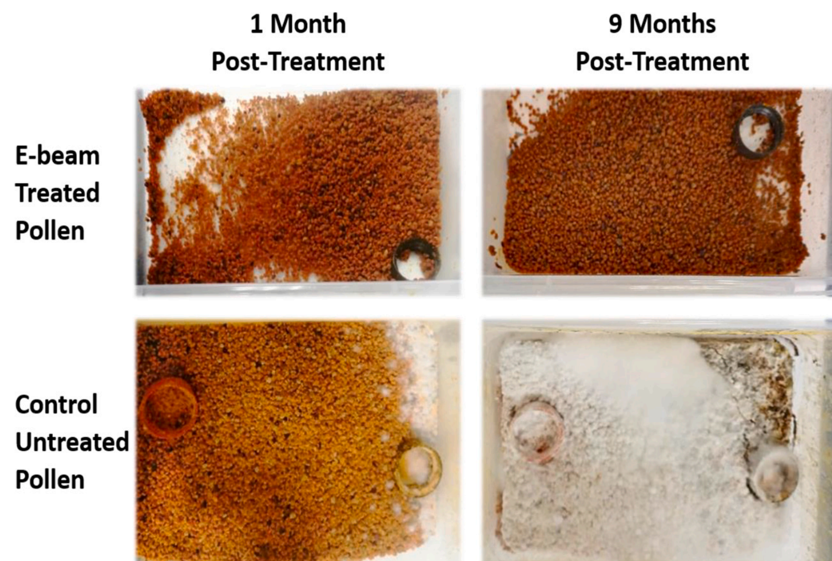

Fig. 3. Pollen treated with E-beam radiation and control untreated pollen was stored in sealed containers for 9 months post-treatment. Pollen treated with E-beam exhibited no fungal growth 9 months post treatment. Untreated pollen exhibited a small amount of fungal growth 1-month post treatment and major growth 9 months post treatment.

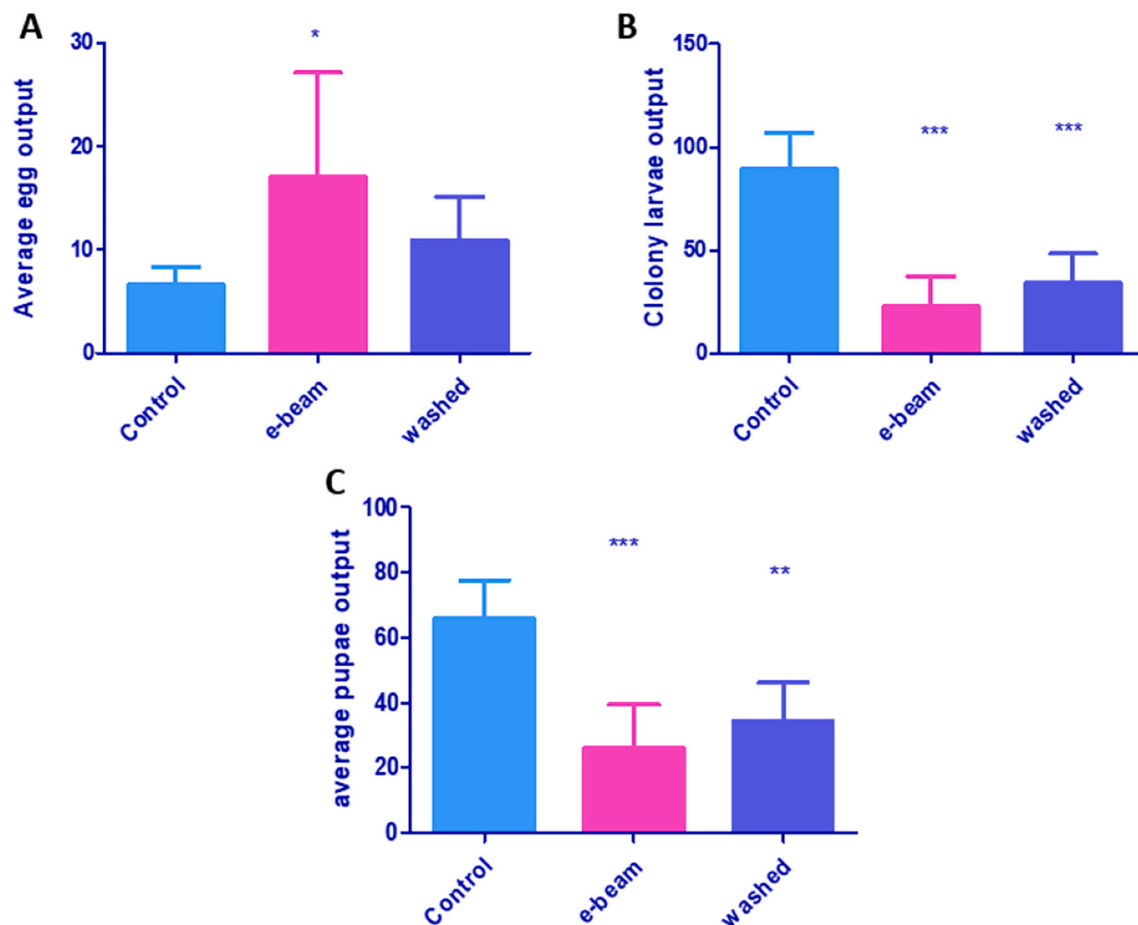

Fig. 4. Colony reproductive output was measured after 28 days of feeding on untreated, e-beam treated, and washed pollen. Both e-beam and washed colonies produced significantly more eggs ($P \leq 0.05$) than the control colony (A). The control colony produced significantly more larvae compared to the e-beam treated pollen fed colony ($P \leq 0.001$), and washed pollen fed colony ($p \leq 0.05$) (B), as well as significantly more pupae ($P \leq 0.001$) (C). Colonies were analysed using a one-way ANOVA repeated measure test.

than average were denoted as Gyne/Queen. Each colony had 1 queen and any other bee of similar size within that colony was labelled as a gyne. In the control group three of the four colonies produced gynes. In the treated colonies, only the colony fed washed pollen produced a single gyne, which was found in colony 11. In total, control groups produced 781 females, 856 males and 39 queens/gynes, compared to e-beam producing 379 females, 11 males and 4 queens/gynes and washed pollen fed colonies producing 661 females, 2 males and 5 queens/gynes (Fig. 5B).

3.4. E-beam pollen vs control pollen proteomic analysis

A total of 177 statistically significant differentially abundant (SSDA) proteins were identified in fatbodies from e-beam treated pollen fed and control bees (relative fold change (RFC) range: +21.7 to −169.2). There were 103 proteins with increased abundance and 74 with decreased abundance. The top 15 proteins that were upregulated were associated with toxicity, immunity, translation and sex hormone production (Table 1). The top 15 proteins that were downregulated in colonies fed on e-beam treated pollen were associated with bee venom, chitin degradation, exoskeleton, peptidoglycan recognition, cellular respiration and heat shock. Cytochrome b5 was detected in high abundance in the e-beam treated pollen fed colonies. Cytochrome enzymes are generally associated with detoxification.

Proteins that were in lower abundance in the e-beam treated pollen fed group included melittin (RFC-168.9), Chitinase 2 (RFC-10.5) and proteins involved in defence and immunity such as the serine proteinase Chymotrypsin (RFC-4.3) and Peptidoglycan recognition protein (RFC-4.6). It is suggestive of an upregulation of detoxification proteins within the e-beam treated pollen fed group coupled with a downregulation of proteins involved in immunity.

3.5. Washed pollen vs control pollen analysis

A total of 523 (SSDA) proteins were identified in fatbodies from washed and control pollen fed bees (RFC range: +47.5 to −394.3). There were 337 proteins with increased abundance and 185 with decreased. The top 15 proteins that were upregulated were associated with toxicity, immunity, translation and sex hormone production, with clusters in proteasomal upregulation and amino acid biosynthesis being identified (Table 2, Fig. 6). The top 15 proteins that were downregulated in colonies fed on e-beam treated pollen were associated with bee venom, chitin degradation, exoskeleton, peptidoglycan recognition, cellular respiration and heat shock.

The most prominent microbiota identifed in individual bumblebee colonies were *gilliamella*, *snodgrassella*, *lactobacillus*, chloroplast and mitochondria. *Gilliamella* was more abundant in e-beam treated pollen fed colonies (0.3992) compared to washed pollen fed colonies (0.3665) and control colonies (0.2568). *Snodgrassella* was more abundant in control colonies (0.3505) compred to e-beam (0.1714) and washed (0.2710). *Lactobacillus* was more abundant in e-beam treated colonies (0.2670) compared to washed (0.2313) and control colonies (0.1245), (Fig. 7A). The mean abundant differences between species from various genera of microbiota found in the digestive tracts of bees fed on either

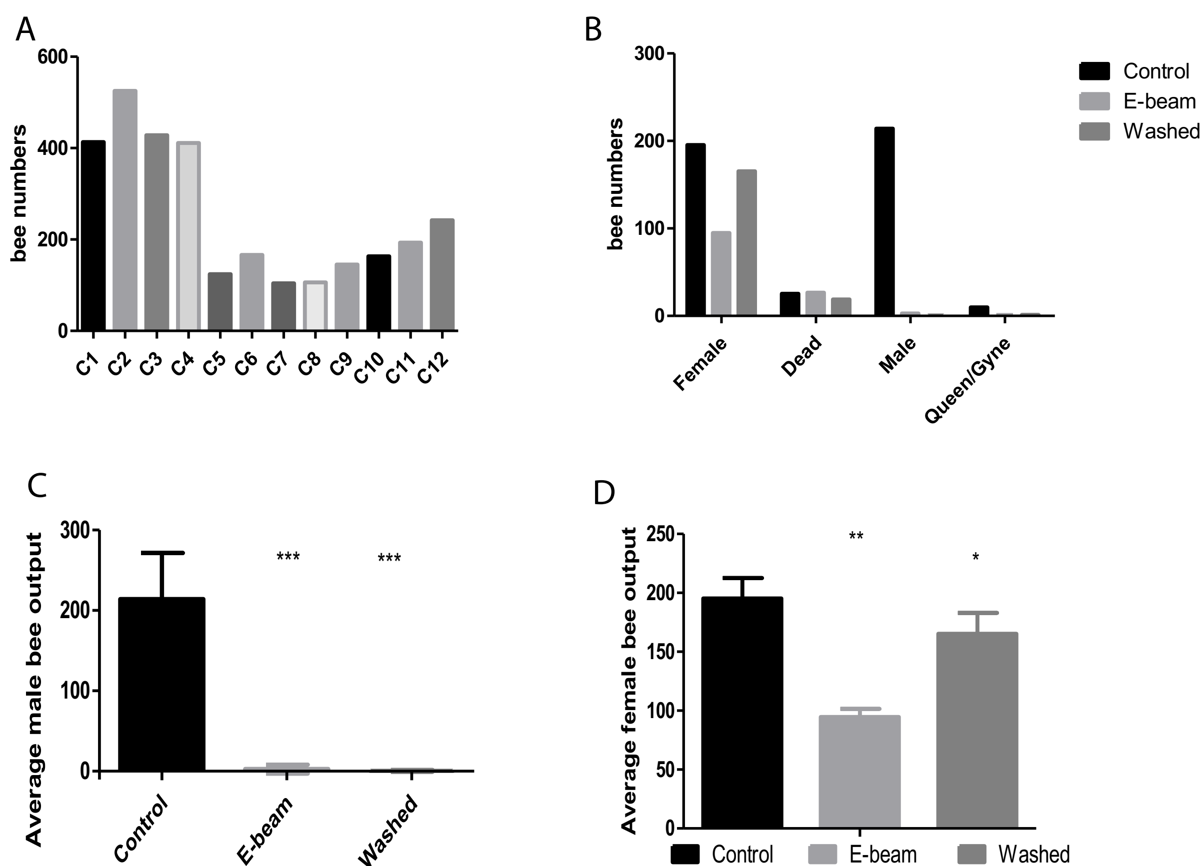

Fig. 5. Bee output from colonies fed on untreated, e-beam treated, or washed pollen. The differences observed for individual colonies total bee counts (A) and the categories they were divided into via treatment (B). Treated colonies as a whole failed to produce male bees (C), whereas control colonies produced more males compared to females. Fewer females were also produced in e-beam and washed colonies (D). Data was analysed using one-way ANOVA, Turkey's method.

untreated, e-beam treated pollen or washed pollen were recorded with confidence intervals between the groups of a upper limit of 95 % (Fig. 8).

The abundance and diversity of fungi present varied per colony and per pollen source. *Zygosaccharomyces* averaged in control colonies (0.2482), e-beam (0.0041) and washed (0.2759), *Rhizopus* ranged in the control (0.0004), e-beam (0) and washed (0.1085), *Wickerhamomyces* ranged in the control (0.0172), e-beam (0.0049) and washed (0.1339), *Kazachstania* ranged in the control (0), e-beam (0.0129) and washed (0), *Candida* ranged in the control (0.0002), e-beam (0.0096) and washed (0.0010). *Bettsia* was not present in the control (0) or washed (0), while small amounts were detected in e-beam (0.0037). *Neoascochyta* was also found in e-beam (0.0074) with a smaller amount found in the control (0.00003) and none detected in washed (0). A total of 10 genus were identified In bees fed on control pollen 8 of the 10 were present, 9 of the 10 in bees fed on e-beam treated pollen and 7 of the 10 in the bees fed on washed pollen. Bees fed on e-beam treated pollen also had the greatest number of unidentified fungi (Fig. 7B).

4. Discussion

4.1. Decontamination efficacy

This study investigated e-beam sterilization as a novel non-thermal technology for the purpose of sterilizing pollen for commercial bumblebee rearing application. Samples treated with 100 kGy exhibited no microbial growth 2 months post treatment when compared to untreated controls, based on light microscopy observations. The microbial burden treated at 100 kGy exhibited a residual vitality at ca. 22 % of treated population as measured by flow cytometry that intimates high level disinfection; however, it is likely that this high dosage possibly killed bee pathogens such as complex parasites through a multi-hit biocidal process. There were no survival of bee parasites when treated bumblebees were monitored over a 30 day period. Naughton et al. (2017) observed lingering viability in the bumblebee trypanosome parasite *Crithidia bombi* when treated with high intensity pulsed UV (PUV). Simone-Fintrom et al. (2018) noted that it is critical to determine appropriate physicochemical and operational parameters that inform an effective dose for gamma irradiation of honey bee fungal agent causing chalkbrood disease, the microsporodium gut parasite Nosema spp. and several viruses in the bee host where studies were conducted at 25 kGy for disinfecting wax combs with reported survival of Black queen cell virus (BQCV) and Chronic bee paralysis virus (CBPV).

There is currently a reliance on use of surrogate in vitro methods to estimate inactivation of broad range of pathogens without properly considering the ability of treated pathogens to elicit an infection process in the bee host. For example, Farrell et al. (2011) reported that lethal action of pulsed light is attributed to a multi-hit biocidal process in treated *Candida albicans* where increased fluence or UV dose enhanced the severity of simultaneous or sequential sub-lethal stresses (namely membrane-lipid peroxidation, membrane permeabilization, reactive oxygen species accumulation, DNA damage, necrosis and apoptosis) to a point of irreversible lethality, and death. This study reported on the novel use of flow cytometry for determining total microbial bioburden lethality; for example, an e-beam treatment dose of 100 kGy achieved 78 % lethality based on DNA and membrane stability staining. However, it is possible that a much lower treatment dose may have been effective at killing various types of bumblebee pathogens present in the contaminated pollen if challenged in bumblebees. Interestingly, the

Table 1
The top 15 SSDA proteins in bees from e-beam treated and control pollen fed colonies. Positive relative fold change (RFC) values indicate proteins with higher abundances in fatbodies from bees fed with the e-beam treated pollen whereas negative RFCs indicate proteins with reduced abundances in bees fed with the e-beam treated pollen in comparison to those fed the non-treated control pollen.

Accession number	Protein name	Relative fold change
XP_003397300.2	Uncharacterized protein	21.1
XP_012176245.1	Cytochrome b5	6.9
XP_012163499.1	Vitellogenin	6.4
XP_020721756.1	Uncharacterized protein	6.1
XP_003396646.1	Uncharacterized protein	5.0
XP_003393261.1	Mitochondrial coenzyme A transporter SLC25A42	4.3
XP_003395337.1	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	4.3
XP_003393699.1	Cytochrome P450 9e2	4.3
XP_003399652.1	PRA1 family protein 3	3.7
XP_003398970.1	long chain fatty acid transport protein 1	3.7
XP_012164498.1	ADP-ribosylation factor 2	3.5
XP_012164498.1	Chaoptin	3.2
XP_020723232.1	60S ribosomal protein L39	3.2
XP_003402576.2	Serine protease inhibitor 3/4 partial	3.2
XP_003401025.1	Ornithine amninotransferase	3.0
XP_020718386.1	Melittin	−168.9
XP_003395447.1	Epididymal secretory protein E1	−17.1
XP_003400637.1	Probable chitinase 2	−10.5
XP_003395094.1	Uncharacterized protein	−9.8
XP_012165909.1	Uncharacterized protein	−9.2
XP_020718419.1	Glycine rich cell wall structural protein 1	−9.2
XP_003397852.1	Nicotinamidase	−9.2
XP_020718442.1	Transmembrane protease serine 9	−8.6
XP_012169980.1	Protein lethal(2) essential for life	−7.0
XP_12172148.1	Uncharacterized protein	−5.3
XP_003400160.1	Peptidoglycan recognition protein	−4.6
XP_003394953.1	Cuticle protein 16.5	−4.6
XP_012169306.1	Uncharacterized protein	−4.6
XP_020718419.1	Glycine rich cell wall structural protein 1	−4.6
XP_003402742.1	Chymotrypsin	−4.3

Table 2
The top 15 SSDA proteins in bees from washed and control pollen fed colonies. Positive relative fold change (RFC) values indicate proteins with higher abundances in fatbodies from bees fed with the washed pollen whereas negative RFCs indicate proteins with reduced abundances in bees fed with the washed pollen in comparison to those fed the non-treated control pollen.

Accession number	Protein name	Relative fold change
XP_003395548.1	Insulin gf binding protein acid labile subunit	48.5
XP_020721715.1	Putative fatty acyl CoA reductase CG8306	17.1
XP_012164498.1	Chaoptin	16
XP_012163499.1	Vitellogenin	13.9
XP_020718283.1	Membrane metallo-endopeptidase 1	12.1
XP_003402924.1	UDP = Gluctonosyltransferase 2B15	11.3
XP_003399880.1	Putative fatty acyl CoA reductase	11.3
XP_012175289.2	Retinol dehydrogenase 14	9.8
XP_003396646.1	Uncharacterized protein	9.1
XP_003402892.1	Glucosylceramidase	8.5
XP_003395752.1	Ribosomal protein S29	8
XP_003397300.2	Uncharacterized protein	7.4
XP_020723232.1	Ribosomal protein	7.4
XP_003398436.1	Pancreatic lipase relasted protein 4	6.9
XP_003395762.1	Omega conotoxin protein 3	6.9
XP_020718386.1	Melittin	−388.0
XP_020718442.1	Transmembrane protease serine 9	−27.8
XP_003400637.1	Probable chitinase 2	−27.8
xp_012170989.1	Phospholipase A2	−18.3
XP_003402742.1	Chymotrypsin 1	−14.9
XP_003400160.1	Peptidolycan recognition protein	−13.9
XP_003393535.1	Chymotrypsin 1	−12.9
XP_003398056.1	DNA-directed RNA polymearse II subunit RPB1	−10.5
XP_003394953.1	Cuticle protein 16.5	−9.8
XP_003394058.1	Probable salivary secreted peptide	−9.8
XP_003401050.2	Serine protease 53	−9.8
XP_003395094.1	Uncharacterized protein	−8
XP_003402225.2	Maltase A1	−8
XP_003399666.1	Collagen alpha (IV) chain	−7.4
XP_012165896.1	Uncharacterized protein	−7.4

detection of a bioactive to represent occurrence of an irreversible death phase in treated pathogens (such as apoptosis) may also indirectly inform future process efficacy that may negate the need for using complex surrogate in vitro assays. A dose of 100 kGy was selected in this study as it produced the greatest level of microbial lethality and there was limited opportunities to test less severe dosages due to the COVID-19 pandemic where the industrial e-beam facility was prioritised for medical device sterilization. Albeit limited, studies using gamma irradiation of pollen (Hildago et al., 2020), or wax combs (Simone-Fintrom et al., 2018), using ca 10 kGy (a standard dose employed by commercial bumblebee producers) maybe of a sufficient intensity for achieving the desired high level disinfection or sterilization of treated pathogens in the bee host itself; however, it is likely that higher treatment doses are potentially required given the resistance profile of recalcitrant viruses as exhibited by BQCV and CPBV in related honey bee inactivation studies (Simone-Fintrom et al., 2018). Hildago et al., (2018) reported the survival of *Bacillus pumilus* endospores in wax comb studies at 9 kGy; thus, inferring the potential use of this particular bacterial species as a bioindicator of sterilization efficacy for bee disease migitation where these particular spore-forming bacteria are used to confirm sterility assurance levels for the adjacent medical device sector (McEvoy et al., 2023; Rowan et al., 2023).

It is recognised that the doses of e-beam ranging from 7 kGy to 44 kGy have been applied for treating foods (Lee et al., 2017); however, the higher dose of 100 kGy was also selected in this study to assure sterilization (or high-level disinfection) so as to avoid any unnecessary use of test bumblebees. Use of flow cytometry was used to indirectly measure or gauge disinfection of complex parasites as there is no specific in vitro infection model for studying bee parasites. Interestingly, use of flow cytometry has also been used as a rapid in vitro enumeration method for medical device sterilization (McEvoy et al., 2021). Naughton et al. (2017) also used a surrogate approach for determining efficacy of destroying *C. bombi* parasites where these researchers used a waterborne protozoan parasite as the challenge bioindicator where survivors were determined using in vitro combined cell culture-qPCR infectivity assay. Recent studies have reported on the development of honey bee and bumblebee cell lines that may inform future in vitro infectivity studies that would inform scoping in vitro studies to limit use of actual bumblebees (Goblirsch and Adamczyk, 2023).

Bumblebee colonies fed on either e-beam treated or washed pollen, produced significantly fewer females, gynes and males compared to bees fed on non-treated pollen. They also produced significantly fewer larvae and pupae, despite producing more eggs. This is likely due to the eggs failing to develop into larvae, meaning a constant egg count was observed in treated colonies. The failure to produce adult bees in these colonies presents the risk of having fewer workers and nurses to tend to the brood, and a lack of gynes means there is less chance of new colonies being founded. The lack of males will also heavily impact on reproduction as the primary role of males in a bumblebee colony is to mate with the queen (Amin et al., 2010). There was a significant difference in thorax width and intertegular distance between colonies fed on treated and control pollen. As sucrose was readily available across all colonies, the differences noted in size and reproductive output could be due to nutritional alterations in the pollen, with key components either being washed out or denatured. It is possible that the microbial community that inhabits commercial pollen plays a key role in nutrition, which is required for reproductive output too. Changes in the proteome were observed, likely due to microbial and nutritional alterations in the polled due to treatment. Several pathways involved in stress and metabolism, growth and development and immunity were altered in bees fed on

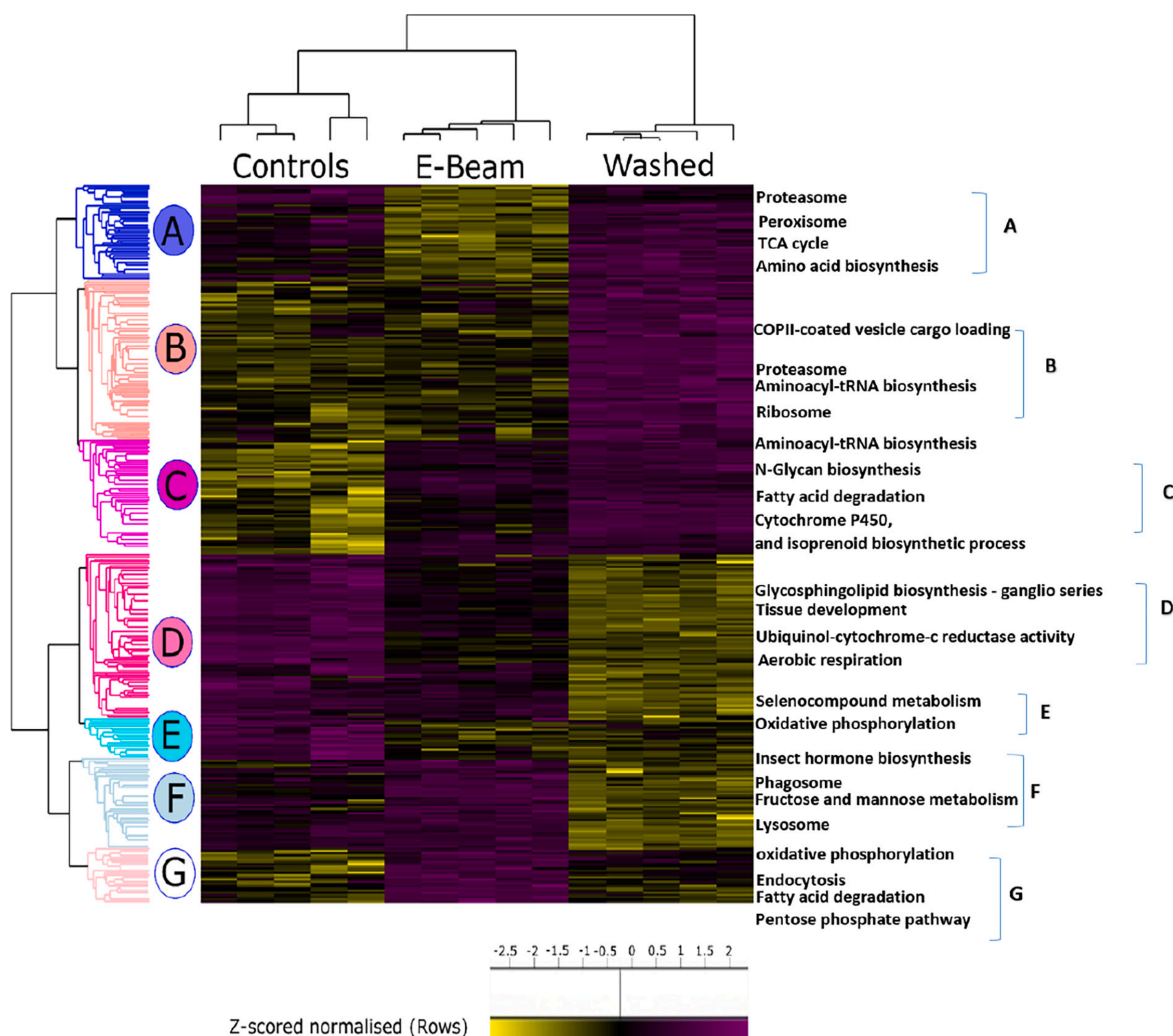

Fig. 6. Heat map of treated clusters from fat body analysis showing proteins divided into clusters sorted by abundance. Clusters in black/dark purple are normalised, with yellow denoting proteins and pathways of low abundance and bright purple denoting proteins and pathways of high abundance. Pathways involved in immunity, stress and metabolism were upregulated or downregulated depending on pollen type fed to the commercial bumble bee colonies.

treated pollen compared to those fed on control pollen.

4.2. Increased abundance in E-beam treated pollen fed colonies

4.2.1. Metabolism

Colonies fed on e-beam treated pollen exhibited upregulation in oxidative phosphorylation, endocytosis, fatty acid degradation and the pentose phosphate pathway. The pentose phosphate pathway is a key pathway in the formation of molecules for nucleic acid and amino acid biosynthesis while the non-oxidative pathway can supply glycolysis with the necessary metabolites required for normal function. Fructose and mannose metabolism was upregulated in the colonies fed on e-beam treated pollen, perhaps due to an excess of both sugars in the diet, or a dependency on both sugars in the diet in order to maintain energy levels for normal function. Mannose and fructose metabolism was down-regulated in the washed group and normalised in the control, suggesting e-beam treated pollen is altered and affecting the metabolism of the colonies. An upregulation of cytochrome *b*5 in e-beam treated pollen fed colonies could be a direct result of fatty acid degradation. If bees in these colonies were facing a nutrient deficit, then it is possible that fatty acids are being broken down to generate ATP, and a remodelling of degraded fatty acids is occurring to maintain lipid metabolism. Mitochondrial coenzyme A transporter SLC25A42 was upregulated. This mitochondrial inner membrane protein transports both cytosolic dephosphoCoA (dPCoA) and coenzyme A (CoA), with CoA playing a role in fatty acid and cholesterol biosynthesis, fatty acid oxidation and amino acid metabolism (Philip et al., 2012). This suggests that there is a breakdown and restructuring of various fatty acids in order to maintain homeostasis.

4.2.2. Stress-related protein upregulation

Vitellogenin (Vg), which is vital for egg maturation and embryonic development (Wu et al., 2021), was upregulated in e-beam treated pollen fed colonies. It not only plays a role in egg maturation, but also in protecting against oxidative stress, and can act as a pathogen recognition receptor (PRR) in *Apis mellifera* (Wu et al., 2021). It is possible that Vg upregulation was in response to stress conditions, as an upregulation in phagosome, lysosome and oxidative phosphorylation was observed in the heat maps. This could be a result of poor nutrition and Vg could be acting as sensor for sugar.

Other proteins upregulated included members of the cytochrome *p*450 family cytochrome p4509e2, which metabolise toxins and promote tolerance. In *Apis mellifera*, these enzymes play a role in detoxification of pesticides (Mao et al., 2011). A change in the molecular makeup of the pollen due to e-beam treatment may be inducing toxic effects on the

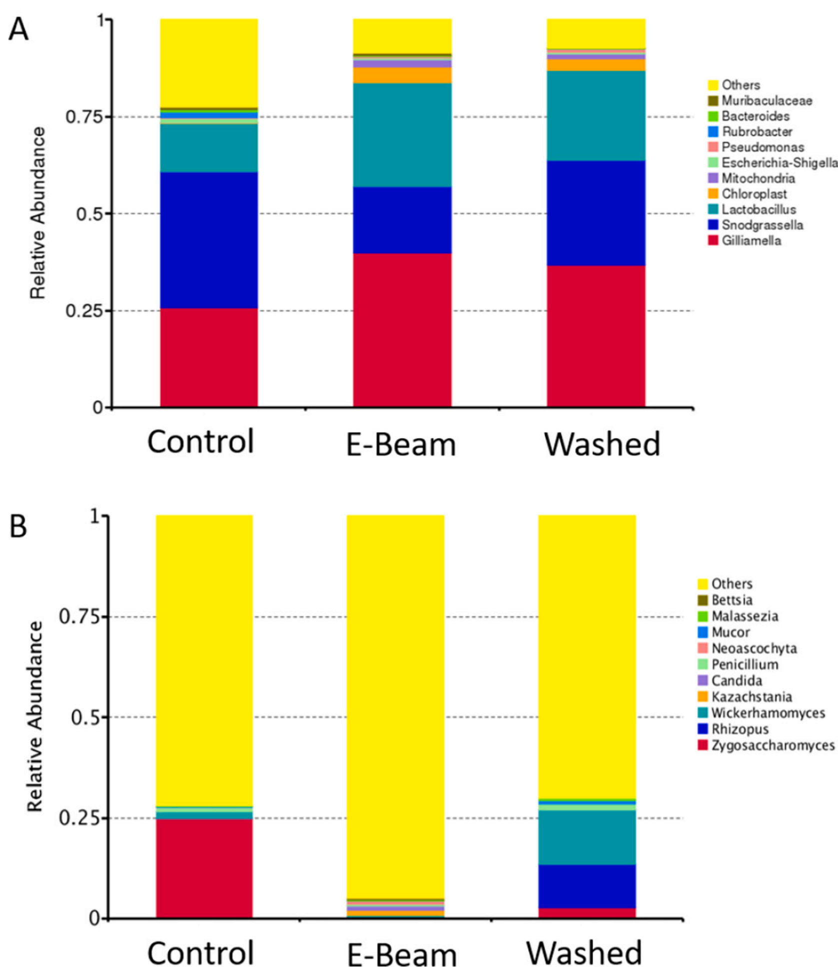

Fig. 7. The relative abundance of the microbiome (A) and mycobiome (B) from the gut of bees fed on either control, e-beam treated or washed pollen. Genus distribution varied depending on the type of pollen the colonies were fed. The three core bacteria *Gilliamella*, *Snodgrassella* and *Lactobacillus* made up the majority of the microbiota present, with differences being observed in the numbers present depending on the type of pollen the bees were fed. Bees fed on e-beam treated pollen had the greatest diversity of fungi in their mycobiome.

colonies. Indeed, e-beam treated pollen visually differed from non-treated commercial pollen, it had a waxy glow, resembling plasticine and took on a darker colour.

4.3. Increased abundance in washed pollen fed colonies

4.3.1. Unsaturated fats and fatty acids biosynthesis

Biosynthesis of fatty acids and unsaturated fats were upregulated in bumblebees fed on washed pollen, they had a less diverse gut microbiome, and a greater abundance of *Firmicutes* and *Bacteroidota* compared to control colonies. High *Firmicutes* and *Bacteroidota* levels are an indicator of a high fat diet (HFD). An increase in saturated fatty acids (SFA) in the diet decreases microbiota diversity (Wang et al., 2021). When considered together, this suggests that bees fed on washed pollen are potentially living on a HFD/SFA diet. The upregulation in the biosynthesis of unsaturated fats is possibly a direct result of nutrition defects within the pollen. Upregulation of fatty acid biosynthesis along with fatty acid degradation may suggest that the pollen diet is driving a change in metabolism and a reliance on fat stores in order to maintain homeostasis.

4.3.2. Metabolism

Proteins associated with the TCA cycle, amino acid biosynthesis, the peroxisome and proteasome were upregulated in washed pollen fed colonies. Pollen has been shown to affect amino acid levels, with non-essential amino acid production being increased in pollen fed honeybees (Gage et al., 2020). While additional research is required to determine amino acid profiles, altered pollen may not provide enough nutrition for sufficient the same level of amino acids as good quality pollen. Previous studies have shown altered amino acid profiles in the brains of bees deprived of pollen.

4.3.3. Growth and development

Insulin like growth factor (IGF) plays a role in growth development, reproduction, stress resistance and lifespan, and was upregulated in colonies fed on washed pollen. This could be why larger bees were observed in these colonies compared to control colonies. IGF works to promote body and tissue growth during development, in response to the nutritional status (Naoki Okamoto, 2018). Glucosylceramidase breaks down glucosylceramide (GlcCer) to glucose and ceramide, and play roles in cell adhesion, recognition, growth, development and inflammation (Reza et al., 2021). Deficiencies in glucosylceramidase can impair memory, and movement via accumulation of glucosylceramide (Reza et al., 2021). Glycosphingolipid biosynthesis was downregulated in colonies fed on washed pollen and upregulated in control colonies, suggesting further negative impacts on colonies fed washed pollen.

4.4. Decreased abundance in E-beam treated pollen fed colonies

4.4.1. Immunity

Proteins associated with inflammatory responses and immunity were down regulated in e-beam treated pollen fed colonies. Chymotrypsins are a family of proteins that have developed in response to defence mechanisms of plants against insects that feed on them (Kim et al., 2022). It is possible that mechanisms produced by pollen such as proteinase inhibitors and defensive enzymes (Kim et al., 2022) have been deactivated by e-beam sterilization process. Peptidoglycan recognition proteins were downregulated, potentially because the nutrient quality is

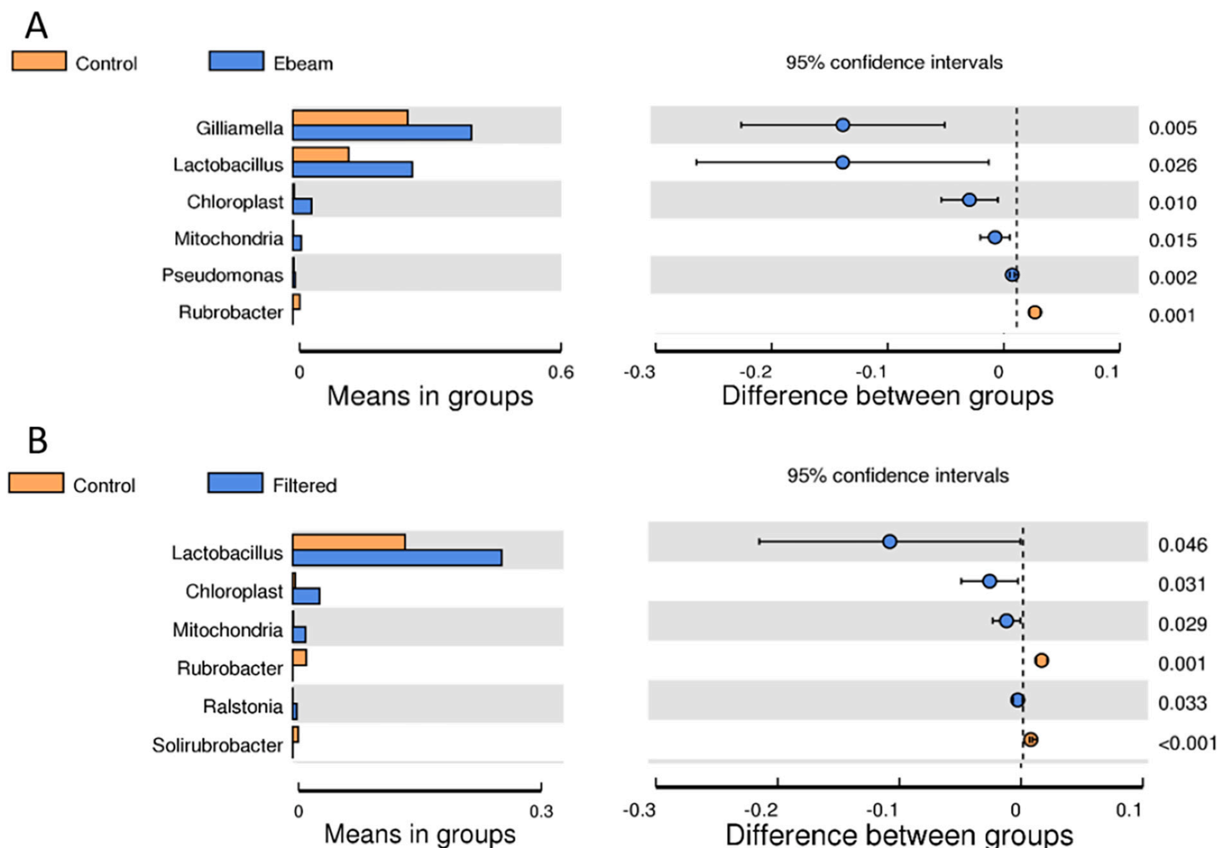

Fig. 8. The mean abundance of microbiota species from the digestive tracts of bees fed on e-beam treated pollen compared to control pollen (A) and fed on washed pollen (B). The confidence interval of between group variation demonstrates the lower and upper confidence limits of the 95 % confidence interval, with the centre representing the difference of the mean value.

poor, and the necessary precursors for maintaining homeostasis are not available. Fatty acid degradation was upregulated, suggesting that starvation is playing a role in protein regulation and the immune pathways are being downregulated in favour of maintaining pathways for fatty acid degradation for ATP generation.

4.5. Decreased abundance in washed pollen fed colonies

4.5.1. Immunity studies

The fatbody proteomic profile is similar to what was observed in the bees fed on e-beam treated pollen, with a downregulation of proteins that play a role in innate immunity. These included serine proteases, chymotrypsin, peptidoglycan recognition, phospholipase and chitinase. There is upregulation of proteins involved with ATP generation and lipid degradation suggests that bees also generate energy through alternative pathways under stressful conditions.

4.6. Microbiomes studies

Bacteria belonging to the Genus *Gilliamella*, *Snodgrassella* and *Lactobacillus* were all present in each treatment group, but varied in relative abundance. In each group, several fungi were identified with the greatest diversity of fungal species evident in bees that were fed on e-beam treated-pollen. Bumblebee colonies fed with untreated control pollen exhibited the greatest abundance of *Snodgrassella* in their digestive tracts, whereas bees fed on treated pollen had a greater abundance of *Gilliamella*. This organism has been previously reported to degrade polysaccharides and can trigger host immune responses (Zhang et al., 2022). There was no clear dominant fungal genus present in the e-beam treated group; whereas *Rhizopus* and *Wickerhamomyces* were found at elevated levels in the washed group compared to the e-beam and untreated control group. Adding sterile water to the pollen during the filtration step increases the water activity value, and would have supported greater growth of fungal and other microbial organisms retained on the pollen.

The food spoiling yeast *Zygosaccharomyces* was the dominant fungal species present in bees fed with untreated pollen. *Zygosaccharomyces* has been detected in bee bread and offers a possible transmission route to newly emerging bees; it has the ability to grow in pollen due to its high osmotolerance (Detry et al., 2020). These fungal pathogens appear to have been fully inactivated in e-beam treated pollen. However, the presence of colonizing microorganisms (such as yeast and bacteria) in the gut microbiome of bees is beneficial as this aids digestion such as fermentation of complex sugars (Tauber et al., 2019).

5. Conclusion

Identifying an appropriate and effective non-thermal decontamination technology for pollination industry remains a complex but critical challenge. This constitutes the first study to provide an initial insight into the impact of decontaminating honeybee pollen, and subsequent effects on colony reproductive outputs in commercial-reared bumblebees. A range of reproductive and colony level changes were observed in bumblebees fed e-beam treated pollen. This coincided with changes in the bumblebee proteome and microbiome, which appear to impact negatively on bee health. A similarity between test bumblebees fed e-beam treated and washed pollen was observed where no males were produced. A failure to produce males is most possibly evident of stunt in colony stage development. Fewer females were also recorded within treated colonies and this failure to produce offspring would most likely affect colony productivity with less workers to attend the brood and then less foragers to gather food from the developing brood. Also, the failure

to produce gyne (virgin queens) would most likely mean that new colonies would not be established. While this might not necessarily be a negative factor for commercial colony establishment or production, as they are only required for a single growing season; however, it does post the question that if these effects are being observed at the colony and individual organism level, then what is potentially happening at the molecular level. It also poses the question as to whether or not a change in the chemical or nutritional makeup of the pollen was a driver for changes at the colony level, or perhaps the removal of other microorganisms that be beneficial to bee nutrition. Currently, there is a strategic need to identify appropriate decontamination technology for appropriately treating bulk pollen so as to mitigate potential transfer of complex parasites and viruses to native pollinators (bees). It appears that a high-intensity e-beam sterilization dosage (100 kGy) is not appropriate for treating pollen that was informed by using an artificial in vitro flow cytometry enumeration approach; thus inferring that additional research is required focused on studying lower doses of industrial scale e-beam or gamma irradiation.

5.1. Future related studies should address

Conduct extensive comparative testing and development of industrial-scale gamma irradiation and electron-beam treatment of pollen over a range of (appropriate) dosages that targets and measures the inactivation performance of different types of problematical bumblebee pathogens. There is also a possible future role for the use of X-ray technology for bulk pollen treatment as demonstrated by McEvoy et al., 2023b in the adjacent medical device area.

Commensurate physicochemical characterization and visualization of pollen post treatments to confirm no structural or nutritional changes in treated pollen for rearing commercial bumblebees, such as combined novel use of scanning electron microscopy and image analysis. Studies revealed that physically treating pollen affects colony outputs; thus to co-monitoring of pollen structure over treatment regimes is important.

Identification of an appropriate non-thermal technology and treatment dosage that will support and enable bee disease mitigation without affecting colony reproductive outputs, possibly the use of combinational industrial treatments such as X-ray or vaporized hydrogen peroxide (such as McEvoy et al., 2021).

Co-creation and development of an appropriate in vitro diagnostic method for studying pathology and physiology of complex bee pathogens post treatments, such as possibly the future use of cell lines derived from bumblebees similar to approaches adopted from honey bee (such as Goblirsch and Adamczyk, 2023).

Investigate simultaneous and sequential occurrence of lethal stresses in treated bee pathogens to inform appropriate dosage for killing similar problematical pathogens in the bee host.

Investigate the potential relationship between producing super clean or sterilized pollen on subsequent bee immunity that may affect critical housekeeping activities such as foraging in bumblebee colonies fed these artificially-treated resources.

Investigate the potential relationship between floral types and bee parasite and virus contamination of gathered pollen with implications for effective disease mitigation for the pollinator industry.

Establish stakeholder consensus on the use and adoption of appropriate decontamination technologies for the pollinator industry, such as for treating bulk pollen, hive equipment and wax combs.

Investigate the development of digital (twin) tools for end-to-end sterility assurance of pollen and commercially reared bumblebees including monitoring for decision-making.

Investigate the sustainable development of appropriate sterilization technologies for pollination and ecosystem service management including applying life cycle assessment and other key performance indicator tools (such as intimated in adjacent areas by Rowan and Pogue, 2021; Garvey et al., 2022; McEvoy et al., 2023; McEvoy et al., 2023b; Rowan, 2023).

CRediT authorship contribution statement

Jack Eakins (JE), Mark Lynch (ML), James Carolan (JC), Neil J Rowan (NR).

Conceptualization (NR, JC); Research methods (JE, ML, JC, NR); Data generation (JE), Data analysis (JE, ML, JC, NR); research funding (NR), research supervision (NR, JC, ML), draft research paper writing, review (JE, ML, JC, NR).

Declaration of competing interest

The authors declare no competing conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165614.

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