The bio-footprint of a wound debridement device containing *Lucilia sericata* larvae

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

January 2018

Publications

Pritchard, D.I., Čeřovský, V., Nigam, Y., **Pickles, S.F**., *et al.* (2016). TIME management by medicinal larvae. *Int Wound J.*, 13(4): 475-484. [Epub 2015 Jul 15].

Pickles, S.F. and Pritchard, D.I., (2017). Quality control of a medicinal larval (*Lucilia sericata*) debridement device based on released gelatinase activity. *Med Vet Entomol.*, 31(2):200-206. [Epub 2017 Jan 24].

Pickles, S.F. and Pritchard, D.I., (2017). Endotoxin testing of a wound debridement device containing medicinal *Lucilia sericata* larvae. *Wound Rep Regen.,* 25(3): 498-501 [Epub 2017 May 26].

Contents

List of Abbreviations

Agr	Agar solution
AMC	7-Amino-4-methyl coumarin
AMP	Antimicrobial peptide
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BB-50	BioBag-50 debridement device
BLASTp	Basic local alignment search tool (for) proteins
BSE	Bovine spongiform encephalopathy
CD	Carnival Diet
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Expectation value
EC	Enzyme Commission
ECM	Extracellular matrix
Egg	Egg solution
ES	Excretion/secretion
ESI	Electrospray ionisation
EU	Endotoxin unit
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
Gel	Gelatin solution
GTP	Guanosine triphosphate
HILIC	Hydrophilic interaction liquid chromatography
HMDB	Human Metabolome Database
HSP	Heat shock proteins
IDO	Indoleamine 2,3-dioxygenase
JNK	c-Jun N-terminal kinases
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
L. sericata	*Lucilia sericata*
LA	*Lucilia Agar*
LAL	*Limulus* amoebocyte lysate
LAP	Larval alimentary products
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LDS	Lithium dodecyl sulphate
LPS	Lipopolysaccharide
MES-SDS	2-(N-morpholino) ethanesulphonic acid
MHRA	Medicines and Healthcare Products Regulatory Agency
mRNA	Messenger ribonucleic acid
MRSA	Methicillinusa-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MWCO	Molecular weight cut-off

n	Numerical variable
NCBI	National Center for Biotechnology Information (USA)
NHS	National Health Service
ns	No significance
p mol	Picomole
P	Probability value
PBS	Phosphate-buffered saline
PM	Protein marker
PMF	Peptide mass fingerprinting
pNA	*p*-nitroaniline
PTFE	Polytetrafluoroethylene
PVA	Poly-vinyl alcohol
r	Coefficient of determination
R^2	Regression model
RDEA	Radial diffusion enzymatic assay
RNA	Ribonucleic acid
RP-HILIC	Reverse-phase hydrophilic interaction liquid chromatography
RPLC	Reverse-phase liquid chromatography
RSLC	Rapid separation liquid chromatography
SBTI	Soy bean trypsin inhibitor
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serum solution
SOP	Standard operating procedure
Soy	Soy solution
Stch	Potato starch solution
TBS	Tris-buffered saline
tRNA	Transfer ribonucleic acid
Whe	Wholemeal wheat flour solution
$\bar{x}$	Mean
Yea	Yeast solution

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Identification and intensities ($\times 10^3$) of 42 additional larval compounds detected during RP-HILIC metabolomic analysis of LAP collected from BioBag devices (n =6). In total, 56 compounds of interest were identified. Compounds in bold have been identified in comparison with authentic standards (Level 1 metabolites), the remainder are classed as Level 2 putatively annotated compounds having been matched to databases (using mass and retention time).

Abstract

The BB-50 is a wound debridement product which contains live larvae of the common greenbottle blow-fly, *Lucilia sericata* (Diptera: Calliphoridae). It forms part of the 'BioBag'® range of Class I sterile medical devices developed by BioMonde©, a multi-national wound care company, specialising in the manufacture and distribution of larval products. In the United Kingdom, the devices are approved for use by the Medicines and Healthcare Products Regulatory Agency, under the 'Specials' License (reference number MS 25595) to meet the clinical needs of a patient, when a suitable licensed medicine is not available. Despite having been offered by the National Health Service for almost two decades, and supported by clinically-proven efficacy, it is estimated that 'maggot therapy' is administered to only 1% of the 200,000 patients currently suffering with senescent chronic wounds. In order to improve acceptability, sterile maggots have been incorporated within fully-sealed yet porous pouches, to provide ease of application, and to create a physical barrier between the patient and the insects. Globally, larval-assisted debridement is considered as a successful treatment, but one of last resort, after conventional therapies have failed. Furthermore, there are numerous anecdotal reports detailing the disinfecting and healing properties of substances produced by the maggots as they feed upon the necrotic material of the chronic wound, but clinical evidence is lacking.

The original contribution to knowledge provided by this thesis, is the concept of the 'bio-footprint' of the BB-50, which is created upon the initial introduction of the BioBag into the wound environment. The aim of this study was to visualise the impact of this device over 24 hours and to harvest the alimentary products which give rise to the bio-footprint, and ultimately the therapeutic effects of the treatment. From here, the first standard operating procedure for the collection of larval substances was developed, leading to the creation of a Quality Control assay for the assessment of the devices. The project then addressed the possibility that the manufacturing processes were contributing to the activity of the bio-footprint. Once confidence in the quality of the larval substances was achieved, it was possible to undertake the first systematic exploration of a standardised, regulated product, which is in current clinical use.

High and low molecular weight investigations were employed to characterise the content of the alimentary products, with a view to identifying compounds which could be linked to therapeutic effects. It is acknowledged that the mechanisms behind maggot therapy are unclear, but that a range of substances are likely to be responsible for the beneficial outcomes observed. However, only two compounds have been fully characterised from larval material. The enzyme chymotrypsin and the antimicrobial peptide lucifensin, are linked to therapeutic effects and have been detected in the environment of the chronic wound following larval treatment. Contemporary studies have yet to characterise a substance which is associated with the promotion of healing.

This study further contributes to the discipline following the detection of 132 putatively identified compounds, which may be produced by the larvae within the first 24 hours of treatment. Additionally, many of these possess multifactorial actions, which have the potential to positively alter the chronic wound and bring about the onset of healing.

Acknowledgments

Sincere thanks must go to all of those who have assisted in the completion of this study. In particular, Dr Gary Telford and Dr Lina Dahabiyeh, formerly of the University of Nottingham, whose expertise in the laboratory and their presence in the office made all the difference.

Without Professor David Pritchard, this project would not have been possible. His enthusiasm is without bounds, and 'The Prof' has been a joy to work with. I can only hope that the content of this thesis demonstrates my appreciation and admiration.

This project was funded by BioMonde and the University of Nottingham.

Dedication

For Nanny Peg

Chapter One: Introduction

1.0 The origins of maggot therapy for wound treatment

The administration of sterile fly larvae (or maggots) into an existing wound environment to facilitate healing was formally introduced to the medical community as an effective osteomyelitis antiseptic treatment by William Stevenson Baer (1929), the founder of the first orthopaedics clinic at the Johns Hopkins University in Baltimore (Manring & Calhoun, 2011). It was subsequently employed worldwide against a variety of infected wounds in the pre-antibiotic era, (Robinson, 1935b), following Baer's original observations that maggots were feeding on the exposed wound tissues of injured First World War soldiers. Although not an uncommon sight on the battlefield, he noted that the presence of larvae was also associated with unexpected therapeutic effects (Crile & Martin, 1917), which he was later able to replicate in a clinical setting (Baer, 1929). Claims have been made that the origins of the remedy can be traced to 'ancient times' (Church, 1996; Thomas, 2010), however early medical texts from the 15th century (Andrew, 1527) and even 600 BC (Bhishagratna, 1911; Kumar, 2009) refer only to the links between the infirm or the dying, and the attraction of scavenging insects to the bodies of humans and animals. Notably, similar military documentations occurred between the 16th and 19th centuries (Paré, 1557; Millingen, 1809; Larrey, 1832; Keen, 1865; Goldstein, 1931; Chernin, 1986) and despite the implication of a positive relationship, the direct suggestion of the purposeful introduction of larvae into existing wounds is absent. Speaking in 1930, Baer acknowledged the work of Confederate Army surgeon John Forney Zacharias during the Civil War (1861-1865), but could find no evidence of medical or experimental use in times of peace:

> *"I first used maggots to remove the decayed tissue in hospital gangrene and with eminent satisfaction. In a single day they would clean a wound much better than any agents we had at our command. I used them afterwards at various places. I am sure I saved many lives by their use, escaped septicaemia, and had rapid recoveries."*
>
> Zacharias, as quoted by Baer, published posthumously (1931)

Baer is credited with the first scientific study and clinical usage of what became known as 'maggot therapy' which appeared to wane and peak inversely with the development and failure of antibiotics (Pechter & Sherman, 1983), and was most recently revived at the turn of the last millennium, as antibiotic resistance reached critical levels (Sherman *et al.*, 2000; Pritchard *et al.*, 2016). The treatment became widely-known as 'maggot debridement therapy' (Stoddard *et al.*, 1995), as the larvae were also applied for the

removal of decayed wound tissue and associated debris. The necrophagous nature of the maggots, combined with their ability to produce antimicrobial substances, is exploited in wound bed preparation today; actions which are associated with the apparent promotion of healing in otherwise static (or degradative) wounds (Pickles & Pritchard, 2017b).

Following Baer's pioneering work, efforts were made to characterise the compounds responsible for the therapeutic effects, often with a view to replacing the need to use the live organism. This idea was proposed by Baer himself (1929) and one of his earliest colleagues, Stanton Livingston, first attempted to obtain the elusive 'maggot active principle' (Livingston & Prince, 1932). Filth-feeding flies and their larvae appear to conflict with the hygienic practices of medicine, as it is widely known that maggots develop within decomposing organic matter, such as animal waste or corpses (Mackerras & Freney, 1933; Erzinçlioglu, 1996). It is, however, from here that the larvae have developed the survival mechanisms to feed and thrive in the heavy bioburden of these environments, which in turn permits their suitability for use in the chronic wound. Despite the production of larvae under sterile conditions for medical use, as mooted by Baer, the notion of maggot activity tends to generate a repulsion reflex (Matchett & Davey, 1991; Davey, 1994). Larval therapy has a place in modern medicine, but it is generally not considered to be a first-line treatment and a substitution for live larvae is still of interest (Vistnes *et al.*, 1981; Prete, 1997; Britland *et al.*, 2011; Čeřovský *et al.*, 2011; Cazander *et al.*, 2012; Pritchard *et al.*, 2012; Sherman *et al.*, 2013; Valachová *et al.*, 2013).

1.1 The rationale behind maggot usage in contemporary medicine

Reflecting the concerns of modern healthcare, larval treatment focus has shifted from osteomyelitis to chronic wounds arising from more generalised skin and cardiovascular conditions. This includes lower-limb ulcers associated with diabetes, or pressure sores arising from reduced mobility, as well as burns or traumatic injuries, with accompanying persistent infections (Mustoe *et al.*, 2006; Werdin *et al.*, 2009). In 2012/2013, the National Health Service (NHS; UK) managed 2.2 million wound sufferers and each year, approximately 10% present with chronicity (Posnett & Franks, 2008; Guest *et al.*, 2017). It is estimated that maggot therapy is applied to 1% of these patients in the UK (BioMonde UK, pers. comm). Globally, it is generally considered as

an effective treatment, but one of last resort, ahead of amputation and after other conventional therapies have failed (Sherman, 2003; Steenvoorde *et al.*, 2007; Peck *et al.*, 2015).

An acute wound, such as an abrasion or cut to the skin, is expected to progress through four physiological stages (homeostasis, inflammation, repair and remodelling), leading to healing within four weeks (Harding *et al.*, 2002; Falanga, 2005; Cowan *et al.*, 2012). Those that have stagnated or worsened are likely to exhibit chronicity by three months, which is marked by inappropriate inflammation at this phase, limited blood and oxygen circulation, leading to the formation of necrotic tissue (Martin & Nunan, 2015; Harries *et al.*, 2016). This material creates a physical barrier to healing and is also open to infection. Although the patient status and individual metabolic factors will contribute to delayed healing, infection is considered to be the likeliest cause of chronic wounds (Leaper *et al.*, 2015). Where the body is unable to remove the dead and/or contaminated material naturally, medical intervention is required. A combination of debridement methods may be employed (**Table 1**), the selection of which will depend upon the type and location of the wound, patient status, practitioner expertise and associated costs (Enoch & Harding, 2003; Gray *et al.*, 2010).

Table 1: Summary of recommended debridement methods which may be used in the management of chronic wounds. Larval therapy is referred to here as a form of 'biosurgery'. Modified from Vowden & Vowden (2011).

Debridement	Method	Outcome
Autolytic	Dressings and topical treatments.	Slow softening of hardened tissues, facilitated by enzymes of the body and moisturisation.
Biosurgical	Live blow-fly larvae.	Rapid removal of various types of necrotic tissue and associated micro-organisms.
Hydrosurgical	High energy saline beam.	Wound lavage, quick removal of the majority of necrotic tissues from the wound bed.
Mechanical	Wet and dry gauze wipes.	Slow non-selective removal of material from the top layer of the wound bed.
Sharp	Scalpel or scissors and forceps.	Quick removal of necrotic material above the viable tissue level.
Surgical	As sharp and/or other instruments.	As sharp method (quick removal of large areas), but also requires analgesia.
Ultrasonic	Ultrasound plus irrigation system.	Agitation of the wound bed, rapid removal of tissues for excision or maintenance.

Sharp debridement is considered as the gold standard (Herberger *et al.*, 2011), which involves cutting away the tissue with a scalpel or scissors, but this carries the risk of additional injury. For some patients, this may need to be performed under general anaesthetic, as in the case of surgical debridement, and may not suited to those receiving

immunosuppressant or anticoagulant medications (Harries *et al.*, 2016). The removal of devitalised material prepares the wound bed and surrounding tissues for spontaneous healing or further treatment (Strohal *et al.*, 2013), to force the wound onto the next phase. Debridement concomitantly reduces the bacterial bioburden, by removing the tissues which harbour microbial populations, to permit the use of topical antiseptics or systemic antibiotics. With chronic wounds, regular debridement is often necessary for maintenance and to manage progressive deterioration. By applying several hundreds of juvenile maggots to the wound, approximately 10-15 g of necrotic tissue could be selectively removed within 24 hours and larvae may remain in the wound for up to 3-5 days (Mumcuoglu, 2001; Fleischmann *et al.*, 2004a). With the exception of larval therapy, none of the other debridement methods contain integrated antimicrobials or additional therapeutic actions. Baer may have recognised the three therapeutic outcomes of larval treatment: debridement, disinfection and the promotion of healing (Baer, 1931), but contemporary acceptance has been slow.

The first prospective controlled clinical study began in California in 1990, and although maggot therapy was shown to debride and heal pressure ulcers more rapidly than conventional treatments ($P = 0.01$), regulatory approval did not follow for over a decade (Sherman *et al.*, 1995; Thomas, 2010). Further studies have reported similar significant ($P = 0.01$ to < 0.001) findings (Wayman *et al.*, 2000; Sherman, 2002b, 2003; Dumville *et al.*, 2009; Wang *et al.*, 2010; Mudge *et al.*, 2014) but clinical evidence is lacking, particularly beyond debridement (Sherman, 2014), and this deficit is likely to have played a part in the delayed recognition of the treatment. Researchers in South Wales (Thomas *et al.*, 1999) published the first preliminary laboratory study of maggot antibacterial properties with activity against methicillin-resistant *Staphylococcus aureus* (MRSA), which had been emerging as a substantial healthcare problem since 1980 (NNIS, 2001; Sydnor & Perl, 2011). It is this finding which is acknowledged as having a pivotal effect on the acceptance of larval treatment by the modern medical community (Beasley & Hirst, 2004), and was featured in a special report by The Lancet (Bonn, 2000). It was not until 2005 that the first preliminary clinical observational study detailing MRSA elimination following larval treatment was initiated. Clinicians from Manchester Royal Infirmary reported a success rate of 92% ($n = 13$ patients), and a reduction in treatment time to 19 days, compared to 28 weeks for standard antibiotic therapy (Bowling *et al.*, 2007). The complexity of wounds, the variety of patient status

and a duty of care create a problematic landscape for trial design. The measurement of healing (and comparatively), is challenging and its definition is subjective between studies (Gottrup & Apelqvist, 2010; Frykberg & Banks, 2015). The reduction of necrotic tissue alone does not necessarily equate to healing, or the complete eradication of infection. The complexity of the healing process, following the removal of these barriers is also unlikely to be completely underway when the maggot treatment is withdrawn, which usually occurs following the debridement phase. Due to efficacy, this tends to be much shorter in the larval-treated groups (Sherman, 2002b; Dumville *et al.*, 2009; Mudge *et al.*, 2014). Continuing the use of the maggots beyond initial debridement would increase the therapeutic benefits (Pritchard & Nigam, 2013) but this appears to be absent in existing studies. Clinical cases and reports demonstrate the success of the treatment outside of the remit of formal trials and larval treatment is acknowledged as possessing therapeutic properties, leading to a decrease in amputations and antibiotic administration (Jukema *et al.*, 2002; Armstrong *et al.*, 2005; Sherman, 2009, 2014; Baillie *et al.*, 2017).

1.2 The development of modern maggot therapy

To date, there is only one fly species approved by the Medicines and Healthcare Products Regulatory Agency (MHRA, UK) and the Food and Drug Administration (FDA, USA). Colonies of the common 'greenbottle blow-fly' *Lucilia* (formerly *Phaenicia*) *sericata* (Diptera: Calliphoridae) are maintained to mass-produce sterile larvae for use as a prescription-only debridement treatment (FDA, 2007; MHRA, 2015). This was the main blow-fly species utilised therapeutically by Baer (1931) and his successors, as he reported that *"maggots of this type destroy only dead tissue, or tissue which is pathological"*. Furthermore, it has a cosmopolitan distribution, great seasonable abundance, and is suited to culturing in the laboratory (Wall *et al.*, 1992; Stevens & Wall, 1996; Zheng *et al.*, 2017). Many members of the Calliphoridae fly family are readily-obtainable in the warmer months of the natural environment, by means of a meat-based lure, such as animal carcasses or offal, in which the female flies deposit their eggs (**Appendix**). Following hatching, the resultant larvae continue to feed within the substrate (usually no more than 5 days), until the onset of metamorphosis, leading to the development of the adult fly (Lowne, 1892b; Smith, 1986; Grassberger & Reiter, 2001). Following species identification and colony establishment, maggots

destined for patient usage must be produced under sterile conditions. This is achieved by liquid chemical sterilisation of the outside of the eggs, and the rearing of larvae on a sterile food substrate (Simmons, 1934; Sherman & Wyle, 1996; Mohd-Masri *et al.*, 2005). Maggots from regulated sources, such as BioMonde® or Monarch Labs (active predominantly in Europe and America, respectively), have methodologies approved to avoid the possibility of pathogenic or transmissible agents which may arise during larval rearing and production.

Recently, larval therapy was proposed for inclusion in the TIME framework (Pritchard *et al.*, 2016), an acronym (**Table 2**) developed to enable medical practitioners to identify and manage the dynamic barriers to wound healing using a structured approach (Schultz *et al.*, 2003; 2004). Almost a decade after the establishment of the framework, the use of maggots for debridement was incorporated under the 'tissue' component (Leaper *et al.*, 2012). However, as shown in **Table 2**, larval activity is relevant to each aspect, something which no other debridement method can achieve.

Table 2: The 'TIME' acronym framework for wound bed preparation, essential in the treatment of chronic wounds. The action of blow-fly larvae is applicable to each component, unlike other traditional debridement methods. Modified from Pritchard *et al*. (2016).

Acronym	Component	Larval Action
T	Tissue (non-viable)	Enzymatic impact. Production of enzymes to digest tissue and bioburden. Physical act of feeding.
I	Infection and Inflammation	Multifactorial impact. Increase in alkalinity, production of antimicrobial and antifungal peptides.
M	Moisture (imbalance)	Impacts on T, I and restores balance. Liquefaction of hardened tissues, reduction of wound exudate.
E	Edge (epithelial, non-advancing)	Alters cell activity conducive to healing. Promotion and stimulation of various cells types, angiogenesis. Production of granulation tissue.

Many of these actions were observed and recorded anecdotally by Baer and others, but the molecular mechanisms behind these actions are of interest in modern research. The characterisation of enzymes (Chambers *et al.*, 2003), antimicrobial (Čeřovský *et al.*, 2010) and antifungal (Pöppel *et al.*, 2014) peptides has provided scientific evidence in support of treatment efficacy. The investigations have utilised live larvae and tissue homogenates, but the collection of products excreted by *Lucilia sericata* larvae was shown to be a suitable and simple alternative to the whole organism (Hobson, 1931b). Similar techniques were employed in agricultural research (Bowles *et al.*, 1988), but 15

years elapsed before a method associated with clinical studies was published (Chambers *et al.*, 2003). Following this, the use of larval 'excretion/secretion' (ES) material became commonplace, and such methods are now used routinely (Bexfield *et al.*, 2004; van der Plas *et al.*, 2008; Cazander *et al.*, 2012).

Clinical evidence in support of the excretion/secretion model has been provided by the development of contained debridement devices, in which the live larvae are enclosed within a sealed, porous dressing for administration. Usually, loose larvae are applied to the wound, but in order to improve acceptability of the treatment, the first 'biobag' was developed by the University of Vienna and Heidelberg University Hospital (Grassberger & Fleischmann, 2002). The design has been modified over time and the patent for the BioBag® is held by BioMonde, although bagged larvae are produced by BIOLLAB in Poland and MEDALT in Slovakia (Čičková *et al.*, 2013). The devices may be preferred by medical staff and patients (Gottrup & Jorgensen, 2011; Čičková *et al.*, 2015) but they have been shown to require more larvae per treatment and twice the number of treatments for debridement ($P = 0.028$ and $P < 0.001$), as shown by clinical (Steenvoorde *et al.*, 2005; Dumville *et al.*, 2009; Čičková *et al.*, 2013) and laboratory studies (Thomas *et al.*, 2002b). However, the same authors reported that contained larval therapy reduced the time to debridement compared to traditional methods (Dumville *et al.*, 2009) and was essential for difficult or unusual wound positions (Thomas *et al.*, 2002b; Steenvoorde *et al.*, 2005). Opletalová *et al.* (2012) confirmed that contained larvae were faster during the first week of treatment, when compared with sharp debridement ($P = 0.04$), but that the two methods were equal by the end of the study (15 days). In general, larval treatment is accepted to be as good as, or better than standard debridement, and is associated with additional therapeutic outcomes. Loose larvae, which are confined by structured dressings but have unrestricted access to the entire wound, are acknowledged as being more effective than those which are contained within sealed pouches. Although BioMonde offer both loose and contained larvae, the smallest BioBag (the 'BB-50') is the product most frequently requested by practitioners (BioMonde UK, pers. comm).

1.2.1 The BB-50 debridement device

The BioBags are available in five sizes, the construction of each is the same, but scaled up accordingly. The BB-50, as the name suggests, contains a minimum of 50 recently-

hatched larvae and comprises a heat-sealed polyester yarn pouch (2.5 × 4 cm) and a poly-vinyl alcohol foam 'spacer' (1 cm^3), which prevents the bag from collapsing on the maggots. The largest device at 10 cm^2 is the BB-400. Each bag is contained within an individual polypropylene delivery tube, with integrated polytetrafluoroethylene (PTFE) filter-membrane (0.22 μm) to ensure sterility from production until opening for use (**Figure 1**).

Figure 1: Examples of BioBag debridement devices, as produced by BioMonde. A 50 mL delivery tube containing a BioBag is shown, alongside the BB-100 (centre) and the BB-50 (lower right). Each BioBag is wetted with a small amount of sterile saline for transportation. Image courtesy of BioMonde. (Scale bar = 10 mm.)

The maggots destined for patients are reared and handled under sterile conditions. Eggs are collected and pooled from fly colonies, having been deposited on a non-meat substance. To remove the possibility of the transmission of infectious agents from animal products, as dictated by the European Commission (2011), BioMonde developed the 'Carnival Diet' as a substitution for bovine and porcine liver. The Carnival Diet is utilised to support the maintenance and mass-rearing of the stock colonies, the resultant eggs are chemically-treated and hatched on an alternative, sterile media **(Section 1.3)**. The '*Lucilia* Agar' formulation was also created as a non-meat substrate, and is used to support the incubation and initial growth of the disinfected larvae (≤ 24 hours), prior to microbial testing and inclusion into the BioBags.

Although the BioBags are in use clinically, research involving the devices is limited, and studies are concerned primarily with the feeding efficacy and survival of the contained maggots (Thomas *et al.*, 2002b; Blake *et al.*, 2007; Čičková *et al.*, 2015; Wilson *et al.*, 2016). The studies employ meat-based models (cuts of animal meat, or hand-made 'burgers' of minced meat) which are used in traditional entomological

studies for rearing and behavioural or developmental investigations (Saunders & Bee, 1995; Donovan *et al.*, 2006; Boatright & Tomberlin, 2010). The BioBags are stitched on to the meat and a reduction in substrate mass against the growth of the larvae is monitored (both visual and measurable). Although the models are suited for such purposes, they cannot be used to obtain larval products, as these are lost to the surroundings. Furthermore, the collection methodologies available to obtain larval ES products use loose maggots (Chambers *et al.*, 2003; Kerridge *et al.*, 2005; van der Plas *et al.*, 2008; Bexfield *et al.*, 2010; Cazander *et al.*, 2012; Bohova *et al.*, 2014), and no studies detailing otherwise appear to have been published. To investigate the impact of the BioBag upon administration to the patient, and to determine the content of the larval products, alternative methods were developed in this study.

1.3 Larval alimentary products

The maggot 'ES' terminology is used to encapsulate all substances that may be produced by the larvae to facilitate feeding. Conflicting findings in modern literature will claim that certain enzymes or peptides are expressed in specific organs, but not in others, and that the compounds are utilised internally, or released only from the anterior or posterior portion of the larvae. The maggots possess a variety of innate and inducible mechanisms to facilitate feeding, the activity and content of the substances produced, will vary depending upon the age of the larvae and the substrate in which they feed. In the absence of standardised methodologies, meaningful conclusions regarding consistent larval content are difficult to make. The term 'larval alimentary products' (LAP) will be used henceforth, to encompass all substances formed and utilised by maggots. Feeding is enzymatically driven, a process that occurs both internally and extracorporeally, and by a variety of substances that are produced anteriorly and posteriorly by the larvae, which may be secreted, excreted, recycled, shed, or retained (Lowne, 1892a; Hobson, 1931b; Waterhouse, 1954; Wigglesworth, 1987; Casu *et al.*, 1996; Tellam *et al.*, 2000). The LAP terminology was proposed, alongside the publication of a standard operating procedure (SOP) for the collection of larval products from regulated larvae contained within BioBag debridement devices, as a result of this study (Pickles & Pritchard, 2017b).

1.3.1 Enzymes responsible for debridement

Baer's work (1929), was concerned with the clinical results of larval therapy, and although he postulated as to *"whether we can get the active principle, whether this is an enzyme, whether we can get out that enzyme"* he did not have the chance to perform any such research. He died of a stroke in 1931 (Manring & Calhoun, 2011), although his colleague Livingston was attempting compound extractions shortly after Baer's pivotal publications (**Section 1.1**). The initial removal of necrotic tissue in cases of osteomyelitis was performed by surgeons prior to the introduction of larvae, and the focus of the treatment was combatting sepsis and ensuring healing; the subsequent publications that followed in the early 1930s reflect this (Livingston & Prince, 1932; Weil *et al.*, 1933; Stewart, 1934b; Robinson, 1935c; Simmons, 1935). The discovery of debridement agents was led by entomological researchers, studying the natural history of necrophagous flies. By 1932, the works of Ralph Percival Hobson (London School of Hygiene and Tropical Medicine, **Section 1.3** and **Section 1.4**) were being cited in clinical publications (Maseritz, 1932; Robinson & Norwood, 1933; Stewart, 1934c), detailing the presence of proteolytic enzymes in the maggot gut and excreta (Hobson, 1931b; 1931a; 1932b). Hobson was investigating the digestive system of *Lucilia sericata* larvae, not due to an interest in maggot therapy, but because the species was (and still is) a major pest of livestock (Froggat, 1914; Davies, 1929; James, 1947; Tongue *et al.*, 2017).

It may be that the enzymatic efficacy of *L. sericata* larvae is related to this evolved ectoparasitic habit (Stevens & Wall, 1997), alongside its natural role in recycling the nutrients of decomposing organic matter, such as animal carcasses (Smith & Wall, 1997). In 'sheep-strike', the fly is capable of initiating traumatic myiases in livestock, following the deposition of eggs into damp and soiled fleece (Hall & Farkas, 2000); the larvae then feed on the tissues of the live 'host'. However, Baer (1931) claimed that the maggots fed only dead tissues (**Section 1.3**) and Weil *et al.* (1933) stated that *"we have found that the larvae of* Lucilia sericata *will starve on clean granulation tissue"*. Additionally, Dominique-Jean Larrey (1832), a French surgeon in Napoleon's army, reported that "*these larvae are, indeed, greedy only after putrefying substances, and never touch those parts which are endowed with life"* following his observations of the maggot-infested wounds of soldiers (**Section 1.1**). Of particular interest, is the reference by Stewart (1934c) of the William Marsh Rice University (Texas) whereby his intact

skin was penetrated by feeding *L. sericata* larvae after 48 hours of contact on his arm. In modern therapy, the introduction and removal of maggots is controlled, and the insects need only be *in-situ* for approximately 4 days, by which time they have finished feeding (**Section 1.3**). It is therefore proposed that the action of the maggots is to be considered as *preferential* or *selective*. This may be under the control of external stimuli (such as odour, or availability of a suitable substrate) or the specificity of the enzymes produced (d'Almeida & Salviano, 1996; Cardoso *et al.*, 2016).

Various enzymes may mediate the removal of necrotic wound tissues, with proteolytical classes likely to dominate, based on the proteinaceous diet of the larvae. Hobson's pivotal work refers to early observations by French entomologist Jean-Henri Fabre, who claimed blow-fly larvae emitted a pepsin anteriorly, following the observation of the degradation of cooked egg white (Fabre, 1894), although its identity was not experimentally confirmed. It was also reported that the work of Weinland in 1906 detected the presence of tryptase in maggot excreta. Hobson confirmed this finding, and with sterile larvae, in addition to determining the presence of other peptidase and lipase activities (Hobson, 1931b), and that collagen digestion was aided by the alkaline conditions created by the maggots (Hobson, 1931a). He also went on to describe the general feeding behaviour of the larvae (Hobson, 1932b), and his studies are so frequently cited in larval therapy literature, it is often overlooked that the work was of agricultural, not medical, concern.

Interest in enzymatic LAP continued in trends (**Section 1.1**), with varying degrees of purpose and results (Ziffren *et al.*, 1953; Waterhouse & Irzykiewicz, 1957; Vistnes *et al.*, 1981; Bowles *et al.*, 1988), until Chambers *et al.* (2003) described the presence of serine, aspartyl and metalloproteinases from the LAP of sterile *L. sericata* larvae. These findings were supported by the detection of trypsin and chymotrypsin in the LAP of *Lucilia cuprina* (Sandeman *et al.*, 1990), the warm temperate/sub-tropical counterpart to *L. sericata*, and the detection of larval enzymes in the wound environment following treatment (Schmidtchen *et al.*, 2003). The group created a recombinant form of *L. sericata* chymotrypsin, which was shown to degrade venous leg ulcer slough and eschar *ex vivo*, that it differed from endogenous human chymotrypsins and was not restrained by endogenous inhibitors found in the wound (Telford *et al.*, 2010; Britland *et al.*, 2011; Telford *et al.*, 2011; Pritchard & Brown, 2015). Furthermore, they showed that the serine proteases produced migratory effects on keratinocytes and fibroblasts

(Horobin *et al.*, 2006; Smith *et al.*, 2006). Variations of these enzymes have been reported, indicating that the larvae produce a wide range of these substances, and their activities concomitantly reduce the bioburden of the wound (Andersen *et al.*, 2010; Brown *et al.*, 2012; Telford *et al.*, 2012; Harris *et al.*, 2013; Valachova *et al.*, 2014; Pöppel *et al.*, 2016).

1.3.2 Peptides responsible for disinfection

The removal of necrotic tissue will reduce the bacterial populations present in such material and although the link with healing failure is well documented (Edwards & Harding, 2004; Wolcott *et al.*, 2009; Nusbaum *et al.*, 2012) debridement is not a treatment *per se*, as the bioburden may still be present and continue to thrive (**Section 1.2**). Wound infection represents a physical and cellular barrier to healing, particularly in the case of biofilm formation, whereby the sessile organisms adhere to a surface (as opposed to free-floating planktonic forms), which present increased difficulties in eradication (Davis *et al.*, 2008; Metcalf & Bowler, 2013). Extracellular polymeric substances produced by the bacteria surround the colonies, creating a matrix of sugars, proteins, and DNA from the host and the microbes, which may extend below the surface of the wound bed, be protected from host defences and require higher concentrations of antibiotic administration (Wolcott *et al.*, 2010; Cowan *et al.*, 2013; Cooper *et al.*, 2014). Based on studies by James *et al.* (2007) and Malone *et al.* (2017), it is likely that at least 70% of chronic wounds comprise biofilm, whereas less than 10% are present in acute wounds. Larval treatment, which is enzymatically driven, will not only remove the physical barrier, but has the added benefits of broad-spectrum disinfection and biofilm eradication, by means of bacterial DNAse and the removal of bacterial docking sites and adhesins (Brown *et al.*, 2012; Harris *et al.*, 2013; Pritchard & Brown, 2015). Hobson (1931b) described an acidic region of the midgut of *L. sericata*, which he believed was responsible for reducing the microbial bioburden likely to be present in the normal diet of the larvae. He also reported the presence of a natural intestinal flora of Gram-negative bacilli beyond this region, (Hobson, 1932a) whose purpose was to destroy toxic excretion products (Hobson, 1932c). Baer (1931) had believed that it was the feeding action of the maggots which caused wounds to become alkaline (subsequently confirmed by Hobson), which in turn diminished the growth of bacteria (Parnés & Lagan, 2007).

The work of Livingston and Prince (1932) failed to characterise the 'active principle' they were seeking (**Section 1.1**), despite reporting they were attempting to do so by unspecified 'ongoing chemical analyses'. Their spurious results were soon criticised by other contemporaries (Maseritz, 1932; Robinson & Norwood, 1933) and notwithstanding the clinical literature citing 'antiseptic properties', very few studies appear to be concerned with determining what may be the cause. This became more of a pressing concern following the emergence of antibiotic resistance (**Section 1.1.** and **Section 1.2**). Entomologist Bernard Greenberg (University of Illinois at Chicago) had been studying the transmission of pathogens by various filth-feeding adult flies, which led him to explore larval feeding. He determined that prior to metamorphosis (**Section 1.3**), maggots almost completely eradicated an administered bacterial load, and that the larval gut must contain forms of bactericides (Greenberg, 1959; 1965). The studies of juvenile maggot digestion and the construction of the gut were similar to that of Hobson's, but Greenberg confirmed that the acidic region resulted in considerable microbial reduction. He was unable to determine the composition of the acid (suggested by Hobson as a phosphate) by comparison with phosphoric and hydrochloric acid. Greenberg did, however, report that the natural fly bacterium *Proteus mirabilis* (Gram-negative) was also a part of the larval flora, and was shown to be resistant in the acid region; he concluded that its presence was necessary for the destruction of other bacteria (Greenberg, 1968; 1969).

Complementing these mechanisms, alongside the disruption of bacteria by enzymes (both internally and extracorporeally to the maggot) and the reports of antibacterial action (Thomas *et al.*, 1999; Mumcuoglu *et al.*, 2001; Kerridge *et al.*, 2005; Huberman, *et al.*, 2007a) additional substances within LAP have been reported to possess antimicrobial properties. Innate and inducible mechanisms have been described in several insects species, and many antibacterial peptides (AMPs) are synthesised by the fat body (Keppi *et al.*, 1986). The 'defensins' (anti-Gram positive) are of particular interest (Dimarcq *et al.*, 1990) as they have been shown to be non-homologous to human counterparts (Otvos, 2000) and of particular relevance, have been recently characterised in *L. sericata* (Čeřovský *et al.*, 2010). The peptide known as 'lucifensin' was shown to be active against *Micrococcus luteus*, *Bacillus subtilis* and *Staphylococcus* and *Streptococcus* species, alongside possessing a slight antifungal action against *Candida albicans*. It has since been characterised (Čeřovský *et al.*, 2011),

produced in recombinant form (Andersen *et al.*, 2010), detected in wound exudate following maggot therapy (Čeřovský & Bem, 2014), and therefore the confidence level associated with its presence and clinical effects are equivalent to that of chymotrypsin. Studies have reported similar activities of compounds with homology to other insect antimicrobial or antifungal peptides, but full characterisations have not been completed (Pöppel *et al.*, 2014; Tellez & Castano-Osorio, 2014; Valachová *et al.*, 2014). It has been suggested that to verify the existence and effect of any larval-derived products, the substance must be a) characterised molecularly, b) linked pre-clinically to a beneficial effect, c) detected in the chronic wound environment following larval treatment and d) be active at realistic concentrations. Currently, this maxim cannot be applied to any of the substances which have been linked to healing outcomes.

1.3.3 Compounds responsible for healing

Aside from the lack of sepsis, one of the most striking initial observations made by Baer (1929) was *"on washing out those maggots I found the most beautiful pink granulation tissue that you can imagine"*. This is a stroma comprised of new connective tissue, blood vessels, macrophages and fibroblasts, which restore functionality and integrity to the injured site (Häkkinen *et al.*, 2012), and the granulation response was often used as an indicator of treatment success (Galbraith, 1931; Martin & Heeks, 1932). As granulation material fills the wound bed, new layers of epithelial tissue form to cover, contract and ultimately, close the wound. Only Robinson & Norwood (1933) doubted the speed of the development of granulation material, remarking that following cleaning by maggots, the wound tissues grew spontaneously at a normal rate and only appeared faster because of the previous delays. In subsequent studies by Robinson, his own work refutes this suggestion (to be discussed as follows), and the response is still used as a marker/end-point in clinical trials and treatment observations (Courtenay *et al.*, 2000; Mumcuoglu, 2001; Graninger *et al.*, 2002; Sherman, 2003; Bowling *et al.*, 2007; Wilasrusmee *et al.*, 2014).

In 1934, Stewart (**Section 1.4.1**) claimed that larvae exuded calcium carbonate through the body walls which was a significant finding as calcium ions were known to stimulate phagocytosis. He combined the compound with a solution of picric acid (to mediate the action of bacterial exotoxin on recruited phagocytes), to create calcium picrate, an analgesic substance. Stewart reported that the type of granulation tissue which followed

after applying this treatment, was macroscopically identical to that produced after larval treatment *"a smooth type and possesses a peculiar sheen - such tissue appears to be specifically characteristic of maggot therapy*" (Stewart, 1934a). The presence of calcium carbonate was detected (in a LAP-style solution) by a colourimetric method used to estimate the amount of calcium present in blood samples (Roe & Kahn, 1926), it does not appear to have been confirmed since, despite numerous references throughout the literature that the compound is produced by the larvae (Wollina *et al.*, 2000; Mumcuoglu, 2001; Fleischmann *et al.*, 2004b; Choudhary *et al.*, 2016).

Baer (1931) also hypothesised that *"maggots seem to have other more subtle biochemical effects within the wound itself"* and the most likely candidate for this appears to be allantoin, as originally suggested by William Robinson (U.S. Department of Agriculture). Robinson (1935c) was convinced by the digestive and disinfecting properties of larvae, but felt that the rapidity of healing in otherwise non-healing wounds and development of granulation tissue following maggot therapy suggested the production of a *"definite substance which stimulates the growth"*. He stated that *"no organism but man does anything primarily to benefit another unrelated organism"* and therefore the results observed must be due to a secondary, or involuntary act, such as the excretion of waste products, but that extensive experimentation would be required to investigate such material. Instead, by referring to medical and entomological literature, and correlating the findings to larval-treated wounds, he arrived at the conclusion that maggots produced allantoin, a compound already known to medicine to possess 'healing properties' (Macalister, 1912), and confirmed its identity in LAP by crystallographic methods. As a commercially available substance, produced from uric acid, Robinson (1935a) obtained allantoin and created a solution which he and others (Greenbaum, 1936; Gordon, 1937; Kaplan, 1937; Posner, 1958) began using successfully in wound care. Despite this, Robinson stated that larvae were invaluable and that his solution was not intended to completely replace the live organism.

Ammonia production has long been associated with maggots, liberated as a pungent gas during their feeding. Although regarded as highly toxic, ammonia may be used to treat unconsciousness as 'smelling salts', which trigger an inhalation and stimulation reflex (McCrory, 2006). Maggot-derived odours were reportedly used as a cure for consumption, according to the *San Francisco Call* newspaper (1911), but Robinson (1940) reported that this reactive waste product (Hobson, 1931b) occurred as the

unstable ammonium carbonate compound, prior to forming ammonium bicarbonate, and was responsible for stimulating wound healing, the production of granulation tissue and a rise in alkalinity (Messer & McClellan, 1935). This discovery, and subsequent medical usage, followed a similar process as with his work on allantoin, and its related compound urea (Robinson & Baker, 1939; Sloan, 1941), although in the larvae, this minor component appears as uric acid, prior to the excretion of allantoin (Brown, 1938a; Dow, 2013). Allantoin was known to be a product of purine metabolism (Gordon, 1937), and although the mechanisms leading to ammonia formation were not clear (Brown, 1935; Brown & Farber, 1936; Brown, 1938b; Lennox, 1940), it was acknowledged that maggot excretory waste was the source of a potent mixture of substances with extraordinary wound healing effects.

There is some overlap (or perhaps synergy) between the enzymes, antimicrobials and the lower-molecular weight substances produced by the maggots, in terms of their role in debridement, disinfection and healing promotion. Larval enzymes possess antimicrobial and cell-stimulatory properties (**Section 1.4.1.**), these are optimum at alkaline conditions, which appear to be caused by the products of nitrogen metabolism and excretion. In turn, these products have also been linked with debriding and disinfecting properties, as well as the production of growth factors (van der Plas *et al.*, 2009; Honda *et al.*, 2011). Contemporary interest in smaller compounds discovered further antimicrobial properties (Bexfield *et al.*, 2004; Huberman, *et al.*, 2007b; Bexfield *et al.*, 2008), including microRNAs (Wang & Zhang, 2011), alongside amino acid (Bexfield *et al.*, 2010) and fatty acid (Zhang *et al.*, 2010) derivatives. These demonstrated pro-angiogenic effects, which may also play a role in the profound vascularisation and improved circulation to the wound, following larval therapy (Wilson *et al.*, 1932; Sherman, 2002a; Wollina *et al.*, 2002; Maeda *et al.*, 2014). Unlike chymotrypsin or lucifensin, no modern studies have characterised a substance from *Lucilia sericata* larvae responsible for the healing effects observed.

1.4 Justification of the current study

Despite the volume of evidence detailing the numerous compounds which may be found in LAP, the normalisation of these findings cannot be assumed. Blow-flies are suited to laboratory rearing, and as demonstrated by their natural plasticity, the larvae may thrive in many substrates. However, the variation of insect diets and general

husbandry used to culture a biologically dynamic organism, produces variations in the results. Many laboratories study *L. sericata*, on account of its medical, agricultural, veterinary, ecological and forensic relevance, and although such results add to the existing knowledge of the insect, not all are applicable to maggot therapy. Even within those studies that do focus on wound care, there is disparity in the methodologies, which conflicts with the strict regulations of medical device production. It is known that the larvae can adapt to differing food sources, and that their immune strategies may be innate or inducible, depending upon the nature of the challenge. It is no wonder that such filth-feeding organisms can produce a myriad of substances, a strategy which is essential to thrive in an environment of decomposition, such as a decaying carcass. The use of animal offal for larval feeding (**Section 1.3.1**) may be readily obtained and affordable, but it is not homogenous. Crucially, the flies and the larvae are not reared on animal tissues, but synthetic diets (created specifically for the product of 'medicinal maggots'). Furthermore, the 'maggot' is not a single state, but represents three developmental stages in the life-cycle of the fly (**Appendix**). Many of the studies conducted employed meat-based diets and investigate the properties of the largest maggot stage (the third instar larva), which on account of their size, are easier to handle. However, each instar follows and precedes another biologically dynamic state, and it cannot be assumed that all pressures and responses on these stages are equal. Likewise, it is acknowledged that extreme variation also exists within the type of chronic wound (and the individual patient status) to which larvae may be administered. The purpose of this study was to determine the likely impact of the most commonly utilised debridement device, the BB-50, upon its initial introduction to a patient. To this end, it is essential to study those insects which have been produced specifically for wound care. The maggots dispatched to practitioners by BioMonde are reared using consistent, approved methods, conforming to the medical regulations stipulated by the FDA and the MHRA. All BioBag devices used in this study were received as supplied to practitioners.

Beyond their usage in trial, or basic debridement laboratory studies (**Section 1.3.1**) no research concerning larvae within contained debridement devices appears to have been conducted. Prior to this study, there were no published methods pertaining to the standardised collection of LAP from larvae within regulated containment devices or any investigations into the initial impact of LAP upon administration. The research

conducted herein relates to a standardised, regulated product, and represents the largest study of such material.

1.4.1 Aims and objectives

The primary aim of this work was to characterise the bio-footprint produced by the most frequently used debridement device, the BB-50. Acknowledging that the bio-footprint occurs due to the action of alimentary products, the components of the bio-footprint were collecting into a working solution. By investigating LAP collected under standardised conditions (**Section 2.2.2**), using larvae produced from a consistent and regulated source, the true composition of medical-grade LAP was determined.

Larval alimentary products were then assessed following the development of a Quality Control assay (**Section 2.3**), which is now the current pinnacle of standardisation for LAP gelatinase activity (Pickles & Pritchard, 2017b). This culminated in the application of the assay for the interrogation of the Carnival Diet (**Section 3.1.1**) and *Lucilia* Agar (**Section 3.1.2**) formulations, and the elimination of false positives in an endotoxin assay (**Section 3.1.3**). These steps were undertaken to determine the contribution of insect diet and gut microflora to the LAP profile (Pickles & Pritchard, 2017a).

A series of initial objectives were defined in preparation of achieving this level of characterisation (**Figure 2**):

1) Visualisation of the bio-footprint of the debridement device: to demonstrate the physical impact of the BB-50 device during the first 24 hours of application.
2) Creation of a standard operating procedure for the collection of LAP from live larvae contained within the BB-50 device for 24 hours: to obtain the bio-footprint in solution form and of sufficient quality, to enable the investigation of its composition.
3) Development of an alternative Quality Control assay: to assess the enzymatic activity of LAP without the use of meat-based models, in a homogenous system which can be applied and modified according to the investigational need.
4) Assessment of the insect-rearing substrates: to determine the contribution of dietary components to composition of LAP and its enzymatic activity.

Once confidence in the quality of the LAP solution was achieved, it was possible to employ analytical methods to explore the composition of the bio-footprint. By investigating standardised material, it was possible to identify larval substances which have the potential for impact within the wound environment, following the first 24 hours of BioBag administration. This was to be accomplished by the secondary objectives:

5) High molecular weight characterisation: for total protein profiling of the bio-footprint.
6) Low molecular weight characterisation: for total metabolomic profiling of the bio-footprint.

By investigating the complete solution, this study represents the first systematic approach to LAP exploration, leading to full understanding of the bio-footprint of a well-used device. The study also identified larval compounds which hold biomarker potential, and may be linked to clinical effects. If these candidates could be detected in the wound environment post-treatment, as in the manner of chymotrypsin (**Section 1.4.1**) and lucifensin (**Section 1.4.2**), they could be accepted by the medical community as key healing compounds, something which has not yet been achieved beyond debridement and disinfection.

This study not only represents a significant contribution of knowledge to the existing discipline of maggot therapy (**Figure 2**), but also raises awareness of the need to ensure that future studies are conducted using regulated material, if the findings are to be applied to a clinical setting and patient experience.

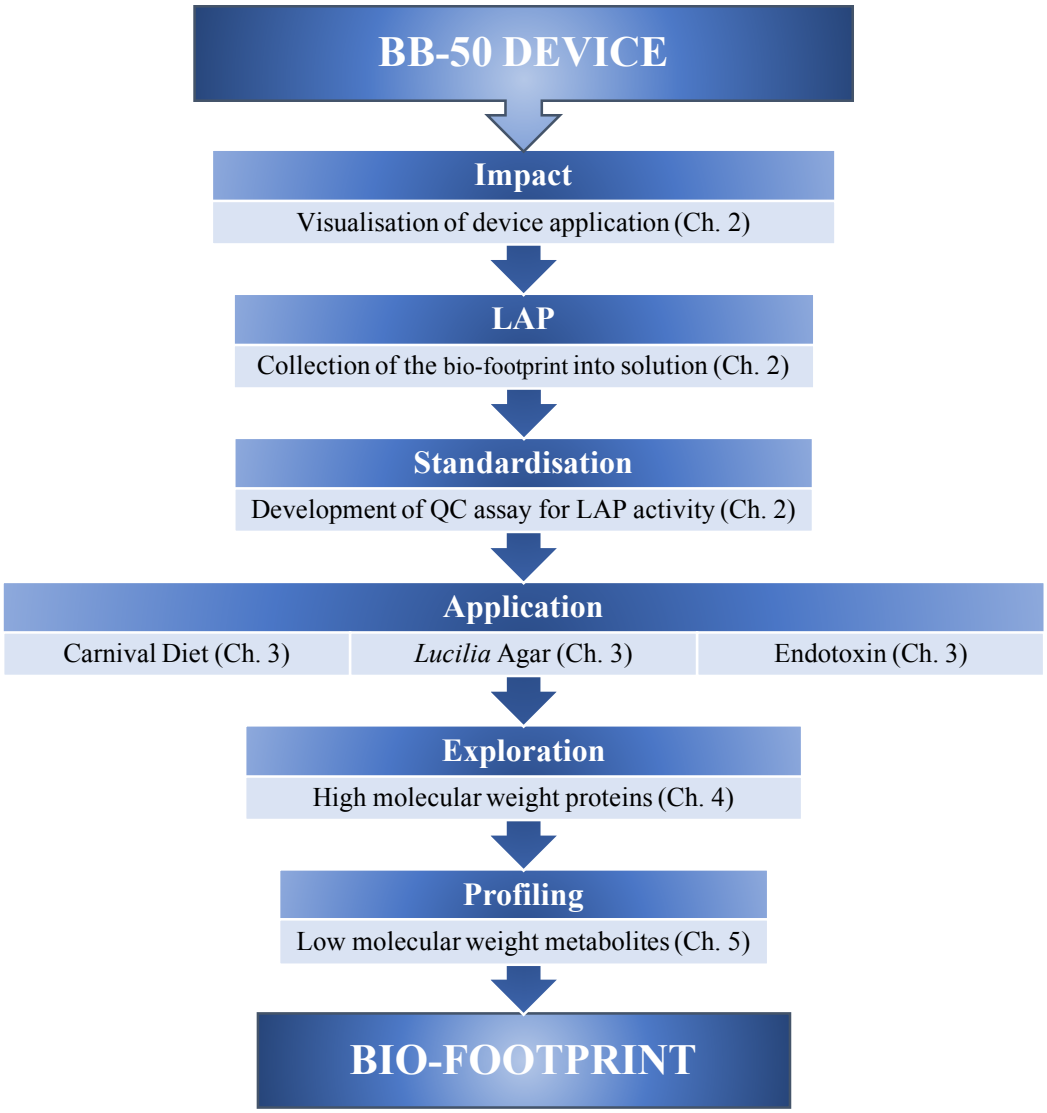

Figure 2: Study overview, commencing with the application of the BB-50 debridement device and the visualisation of the impact of the bio-footprint. Larval alimentary products (LAP) were collected into phosphate-buffered saline, thereby capturing the bio-footprint in a working solution, suitable for analysis. An alternative Quality Control (QC) assay for the enzymatic assessment of LAP, without the use of meat-based models, was developed and subsequently applied to interrogate the rearing substrates used in colony maintenance (Carnival Diet) and BioBag production (*Lucilia* Agar). The assay also supported the exploration of possible endotoxin presence in LAP. Once confidence in the quality of the solution was achieved, high and low molecular weight exploration was undertaken to characterise the content of the total bio-footprint. Chapter (Ch.) numbers are shown in parentheses.

2.0 Abstract

This chapter, 'Standardisation' introduces the concept of the BB-50 bio-footprint, by visualising the initial impact of the debridement device in a wound model and tracking its progress over a period of 24 hours. The methodology led on to the creation of the first standard operating procedure for the collection of alimentary products from live *Lucilia sericata* larvae, whilst still contained within the BioBags, as supplied to practitioners. By capturing the components of the bio-footprint into solution, it was possible to design an alternative assay to the meat-based model for the enzymatic assessment of larval products. The theme of standardisation is continued throughout this section, as the substrate for the developed radial-diffusion enzymatic assay was the same as that used for the visualisation of the bio-footprint, and the collection procedure incorporated the passive harvesting of alimentary products, also over a period of 24 hours.

2.1 Visualising the bio-footprint

The rearing of blow-fly larvae in the laboratory for experimental purposes has been traditionally achieved using relatively fresh animal tissues (**Section 1.2** and **Section 1.3**), to simulate the natural conditions required for necrophagous carrion-breeders, which supports oviposition and larval feeding (Lowne, 1892a; Atkins, 1908; Baer, 1931). The substrate may vary, depending upon the requirements of the study, cost and general availability, although bovine or porcine liver offal are routinely used, the latter particularly as a model for human tissues. (Clark *et al*., 2006; Niederegger *et al*., 2013; Bernhardt *et al*., 2017). A reduction in the mass of the substrate, the growth of the maggots (instar, length, weight) and the progression to adulthood (pupariation, adult emergence, reproduction) are monitored and measured where possible (Moore *et al*., 1985; Chaudhury, 2009b). Meat-based assays were designed predominantly for behavioural or developmental investigations (Saunders & Bee, 1995; Ireland & Turner, 2006) and are usually conducted until the larvae have completed feeding, marking the onset of pupariation, in preparation for adult metamorphosis (**Appendix**). Although such methodologies are suited to studies relating to maggot debridement, and are employed currently by industrial and academic institutions, including BioMonde (Thomas *et al*., 2002a; Wilson *et al*., 2016), they are not suitable for studying larval enzymes *in vitro*. Alimentary products are released into the surroundings in order to

exert their digestive effects, thereby facilitating feeding by liquifying tissues and mounting a response to bacterial challenge (Wall & Shearer, 1997). Larval development is visible over time, with each day physical changes may be observed, but hourly progression is negligible. To determine the initial impact of the BioBag upon administration to a patient, an alternative relevant substrate was developed to visualise the bio-footprint.

To ensure comparability and to maintain relevance to wound studies, gelatin was selected as the substrate for incorporation into an inert and robust agar medium. Gelatin is readily digested by LAP (Hobson, 1931a) and its degradation can be visualised using enzymological gel electrophoresis and protein staining methodologies (Pritchard & Brown, 2015). In this context, it acts a cost-effective model for collagen, which is the most abundant component of the dermal skin layer and a structural protein found in the extracellular matrix (Rangaraj *et al*., 2011; Byron *et al*., 2013). Building upon these principles, a substrate plate for direct BioBag contact was formulated, whereby the digestive output of the maggots was visible within 60 minutes of application, and up to a period of 24 hours.

2.1.1 Materials and methods

All reagents were obtained from Sigma-Aldrich Ltd (Missouri, USA), unless otherwise stated. The substrate base was prepared as 2% agar and 5 mg/mL porcine skin gelatin in Tris-buffered saline (TBS, pH 8.2) and microwave-heated until clear of particulates. Forty millilitres of molten solution were added to individual 10 cm^3 Petri plates (Sterilin; Gwent, UK) and allowed to solidify prior to overnight refrigeration. Various arrangements were trialled, and the final formulation was proven to be suitable for handling, visualisation of enzymatic activity and to support four rotations of a single BB-50 per gel-plate.

Six BB-50 debridement devices as shipped to practitioners were kindly supplied by BioMonde Ltd (Bridgend, UK). Upon immediate receipt, BioBags were removed from the sealed delivery tubes and agitated gently to remove excess saline (**Figure 1**, **Section 1.3.1**), before positioning upon the surface of an individual gel-plate within a Class II microbiological safety cabinet. A single gel-plate was utilised as an environmental control (no BioBag), alongside the test plates. After one hour in the dark, due to the phototactic behaviour of the larvae (Yadav, 2003), each BioBag was rotated

clockwise and positioned upon a fresh area of the gel-plate. The process was repeated after the following time periods had elapsed: 3, 8 and 12 hours (total 24 hours in the dark).

Upon completion, gels were fixed within the plates using glacial acetic acid, methanol and water (10:25:65) for one hour, prior to overnight staining (20 hours, ambient temperature) with 0.05% Coomassie Brilliant Blue R-250 to confirm protein digestion. All gel-plates were photographed with a calibrated scale at a fixed height, on a transmitted light box. All BioBags were immersed in just-boiled hot water for 60 seconds and transferred to 80% ethanol for the preservation and storage of the larvae, in accordance with standard entomological practice (Smith, 1989; Amendt *et al*., 2007).

2.1.2 Results

Prior to fixation, the apparent digestion of the gelatin substrate of the test plates was visible with the naked eye, with discrete areas being observed at each position. The control plate was devoid of any such changes. Following staining, digestion was confirmed and enabled the visualisation of the bio-footprint (**Figure 3**). Gelatinolytic activity was predominantly concentrated around the spacer and corners of the BioBag pouch which is to be expected with Calliphoridae larvae, which are known to feed in aggregates to concentrate enzymatic output (to facilitate feeding and to maintain moisture levels) and by means of thigomatatic behaviour; movement in response to a touch stimulus (Boulay *et al*., 2013).

For each gel-plate, the impact of the enzymatic action of LAP could be seen with the naked eye, following the first rotation (after 60 minutes), and prior to staining. With each successive position the action could still be observed, but visualisation was improved following Coomassie staining (**Figure 3**). The control gel-plate (no BioBag) remained uniformly blue, indicating that in the absence of larvae, degradation of the substrate did not occur (not shown).

Two of the gel-plates (**Figure 3a** and **3b**) showed a greater distribution of LAP, which was linked to the observation that the associated BioBags were received within delivery tubes containing an excess of saline, and were therefore more moist upon receipt. The spread reduced slightly with each rotation and by the final position, the degree of digestion in the final position (after 12 hours of contact) appeared consistent between

the gel-plates. The spacer appears to have created a focal point of the larvae, and digestion was localised to its position (**Figure 3f**), and despite the dampness of the BioBag from **Figure 3a**, the site of the spacer could still be resolved. This was also the case as shown in **Figure 3c**, although a secondary zone of digestion can be seen, within the area of the same BioBag, which is associated with the edge/corner of the pouch. Although the study was not intended for behavioural study, the findings have provided an insight into the location and action of the larvae within the device over time, which is not possible with meat-based assays.

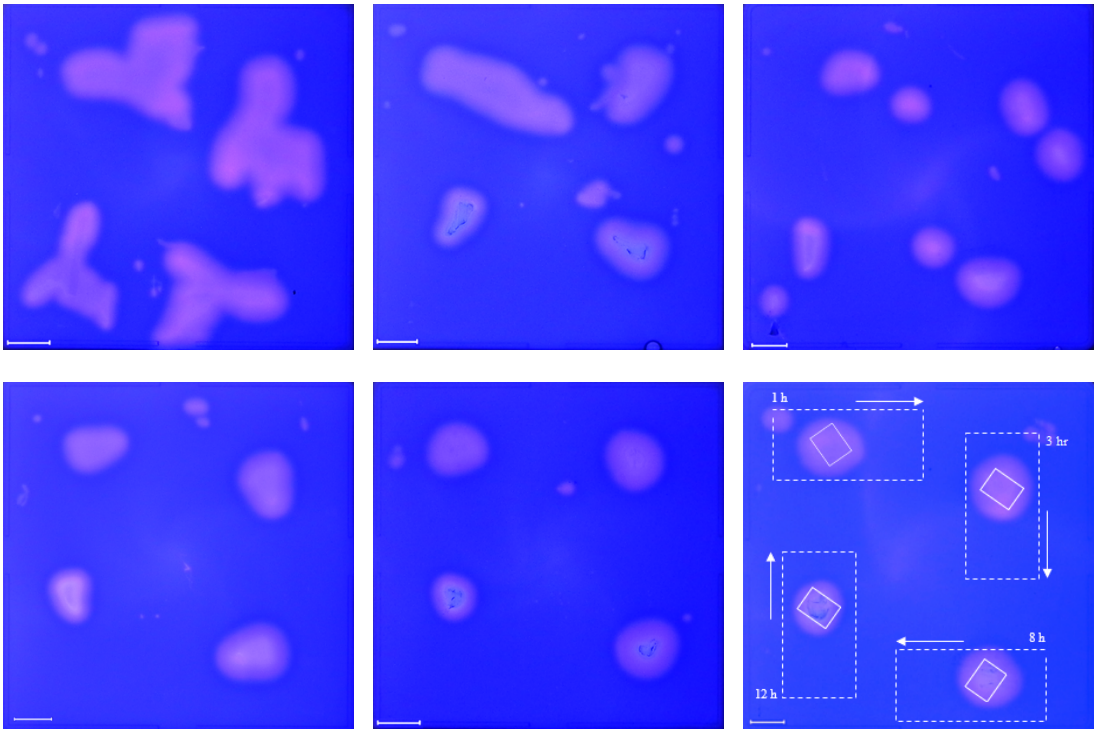

Figure 3: Visualisation of the bio-footprints produced by live larvae contained within six BioBag-50 devices (**a** to **f**) following direct contact with individual gel-plates (2% agar and 5 mg/mL gelatin) after 1, 3, 8 and 12 hours (total elapsed time, 24 hours). Devices were rotated clockwise within each plate, starting from the top left corner (overlay, image **f**) and protein digestion was confirmed following Coomassie Brilliant Blue staining. A corresponding control plate without BioBag contact produced a completely blue, and therefore undigested plate, following staining (not shown).

2.1.3 Significance of findings

Larval feeding is usually assessed by monitoring the growth of the insect and the reduction of the tissues in which they develop. In the laboratory, meat-based assays are usually conducted until the onset of pupariation (**Section 1.3** and **Appendix**) when the maggots have finished feeding and are preparing for metamorphosis (Donovan *et al*.,

2006; Martín-Vega *et al*., 2017). *Lucilia sericata* larvae feeding on liver require approximately 6 days to attain this stage at 30ºC, and although a difference in the size of the larvae will be apparent after 24 hours, the initial impact of the insects on the substrate will be negligible (Grassberger & Reiter, 2001). The relevance of such feeding assays to wound debridement is clear (Blake *et al*., 2007), but the procedure is not suitable for the exploration of the BB-50 bio-footprint.

The agar/gelatin substrate permitted visualisation of enzymatic activity after 60 minutes. Utilising basic laboratory reagents and methodologies, a simple, homogenous system was developed, which produced consistent outcomes across the replicates (n= 6). Gelatinolytic activity was predominantly concentrated around the spacer and corners of the BioBag pouch, which suggest the maggots are behaving normally within the confines of the BioBag. Calliphoridae larvae facilitate feeding and survival by aggregation (and by means of thigomatatic behaviour; movement in response to a touch stimulus (Hobson, 1932b; Charabidze, 2011; Boulay *et al*., 2013). Although the gel-plates were not developed to assess larval behaviour, the arrangement could be utilised further for such studies.

The observation that two of the devices were received with a larger volume of saline, was not considered to be a negative outcome, despite the differences in the spread of LAP (**Figure 3**). Indeed, it suggests that the enzymes may be further distributed beyond the area occupied by the BioBag, to access further parts of the wound bed. Although the degree of digestion is likely to be greater around (and within) the spacer, the saline used to keep the larvae moistened, is also enzymatically-active. This finding suggested that the bio-footprint may be captured within an excess volume of saline, and that harvesting LAP into this solution, whilst larvae remained within the BioBag, would be possible. A summary of the key findings is shown in **Figure 4**.

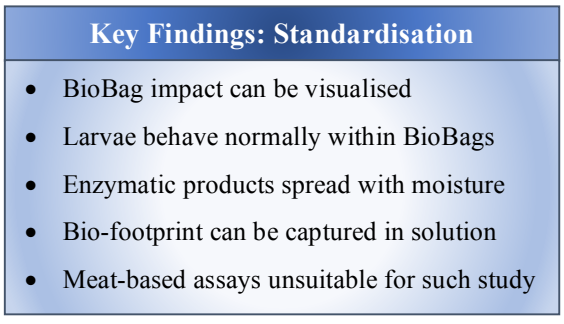

Figure 4: Summary of the key findings associated with the visualisation of the bio-footprint and the behaviour of live larvae within the BioBag device.

2.2 Collection of larval alimentary products

Prior to this study, no methodologies for the collection of LAP from BioBags had been published. An existing protocol developed at the University of Nottingham was trialled for its suitability and was subsequently modified to develop a standard operating procedure (SOP) for the collection of LAP from live larvae contained within BB-50 devices (Pickles & Pritchard, 2017b).

2.2.1 Initial protocol

Six BioBag-50 devices were received from BioMonde as shipped to practitioners. A corresponding set of six control devices (devoid of larvae) was requested for comparative purposes. All devices were identical, with the exception of the presence or absence of larvae. Therefore, all BB-50 devices were comprised of a sealed 50 mL delivery tube, containing one heat-sealed polyester yarn pouch (2.5 × 4 cm), which enclosed one PVA foam spacer (1 cm^3). Larval life was verified upon receipt by observing the appearance and movement of larvae through the delivery tube.

2.2.1.1 Materials and methods

Collection of LAP

All reagents were obtained from Sigma-Aldrich Ltd, unless otherwise stated. All actions were conducted at ambient temperature inside a Class II microbiological safety cabinet, unless otherwise specified.

An aliquot of sterile phosphate-buffered saline (5 mL) was added to each delivery tube and the screw-cap replaced, prior to vertical-rotation for 30 minutes (HulaMixer, Invitrogen; Oslo, Norway) on the slowest setting. Solutions were decanted, and all tubes were stored within a dark-room for 45 minutes, to allow the larvae to acclimatise before the next step. The process was repeated twice more, pooling the subsequent solutions per individual device. All tubes were lightly centrifuged to collect extraneous liquid, and additional liquid retained within the bag and spacer was removed by direct pipetting. Final solutions (15 mL) were stored at -20ºC until use. At this stage of the existing method, complete BioBags would also be frozen, but this action aggregates, distorts and discolours the maggots as they expire. Alternatively, all devices were preserved as aforementioned (**Section 2.1.1**) to maintain the morphology and

appearance of the larvae at the time of killing by hot water and to preserve the larvae for identification. Control devices were treated in the same manner.

Evaluation parameters

Confirmation of existing enzymatic activity of each collection solution was achieved by means of the specific chymotrypsin assay as described by Chambers *et al*. (2003), and as recommended in the existing University of Nottingham protocol (**Section 2.2.1**). Following the cleavage of the substrate (succinyl-alanyl-prolyl-phenylalanyl-AMC*) by chymotrypsin, fluorescent molecules of 7-amino-4-methylcoumarin (AMC*) are released per minute and the optical density is measured using a micro-plate fluorometer (Tecan Spark 10M microplate-reader; Grödig, Austria). The activity is expressed per microgram of total protein, estimated by means of Coomassie quantitation kit (Pierce Biotechnology; Illinois, USA).

As an addendum to the protocol, the maggots contained within each BioBag were removed and counted by hand, as each BB-50 is reported to contain at least 50 larvae (where larvae were noted within the spacer, the figure was rounded up to the nearest five). Furthermore, *Lucilia sericata* species identification was confirmed by slide-preparation and microscopy (Niederegger *et al*., 2011) and the rearing of live larvae to the fly stage, obtained from additional BioBags supplied for this purpose. Identifications were supported by published keys (Smith, 1989; Szpila *et al*., 2013) and comparison with voucher specimens, (including Hobson's original examples from 1932) as obtained from the Medical Entomology Department of the London School of Hygiene and Tropical Medicine (kindly supplied by Mrs Cheryl Whitehorn).

Data analysis

All statistical analyses were performed using GraphPad Prism v.7.0b (GraphPad Software Inc; San Diego, USA). Values are presented as ranges, or the mean ($\bar{x}$) with standard deviation (± SD). Data normality was determined using Shapiro-Wilk test for small sample sizes. Specific tests are listed and where relevant, a probability value (P) of < 0.05 was considered significant.

2.2.1.2 Results

The protocol previously devised at the University of Nottingham was utilised for the collection of LAP from one batch of BioBags and a set of control devices (both $n= 6$).

Protein content, chymotryptic activity and larval number parameters yielded normally-distributed data. No values were obtained for any of the control devices.

By preserving the larvae appropriately, it was possible to count the number contained within each device. Bags were loaded with an average of 215 (± 72) larvae, and this was shown to be significantly different than the theoretical mean of 50 maggots per BB-50, by means of one sample *t*-test (**Chart 1**).

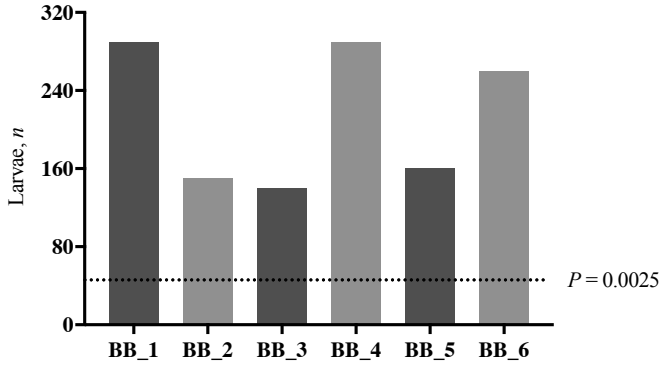

Chart 1: Number of larvae (hand-counted) contained within BioBag-50 debridement devices (n= 6). Dotted line represents 50 larvae, the minimum number reported to be contained within each device. Actual mean (215 ± 72) is significantly different from the theoretical mean (50; one sample *t*-test, P= 0.0025).

As LAP is known to contain a variety of highly active enzymes (**Section 1.4**) elevated protein levels may be expected. However, of the BioBag samples, the total protein content ranged from only 5.9 to 9 μg/mL and the chymotryptic activity liberated 15.3 to 25.2 pmol of AMC* per minute, for every microgram of protein. These values suggest that the potency of this key debridement enzyme may not be directly related to the protein parameter. A weak, but non-significant relationship emerged when tested for linearity (R^2= 0.3270, P= 0.2357, **Chart 2**).

Potential relationships with larval number were observed with this parameter when tested with the protein content and chymotryptic activity (**Chart 3a** and **3b**). Linearity was observed in both cases, but was deemed significant (P= 0.0392) and non-significant (P= 0.0950) respectively.

Although normally distributed, the dataset was treated with caution, due to the small sample size (n= 6). Furthermore, LAP was harvested for only 90 minutes in total, and collected in a large volume of PBS (15 mL). The initial protocol was modified to reflect

the conditions of the bio-footprint (24 hours, **Section 2.1.1**) and to reduce the volume of PBS for LAP collection.

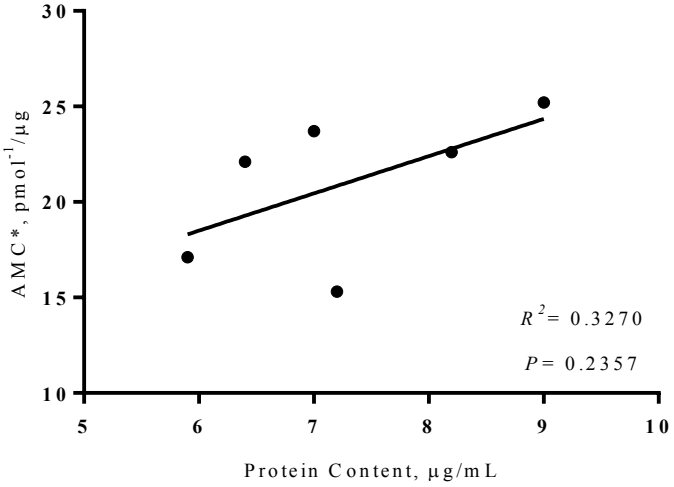

Chart 2: Exploration of the potential relationship between the total protein content and the specific chymotryptic activity (release of fluorescent AMC*) of LAP collected from BB-50 devices (*n*= 6). The weak linear relationship observed was not significant.

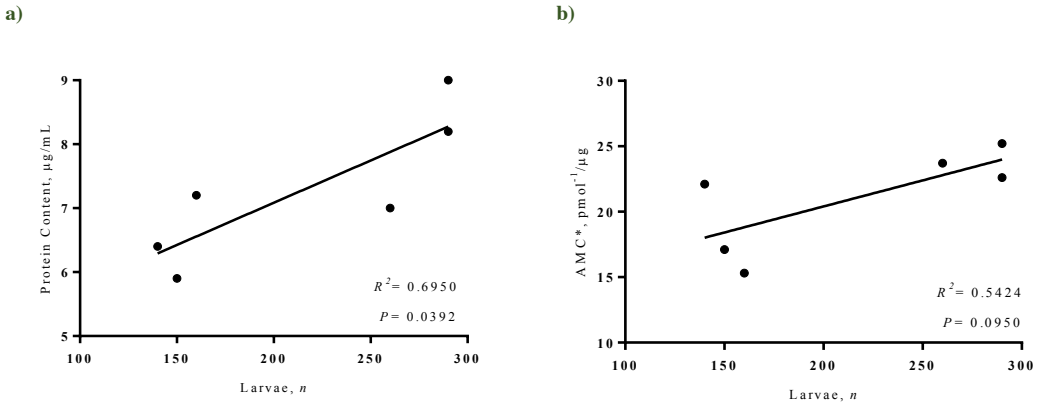

Chart 3a and **3b:** Exploration of the potential relationships between larval number and **a)** protein content and **b)** specific chymotryptic activity (release of fluorescent AMC*) revealed a significant and non-significant relationship, respectively (*n*= 6).

2.2.2 Developed standard procedure

A simplified procedure with a focus on insect husbandry, sterility and the bio-footprint conditions, was developed from the initial protocol. BioBag-50 devices were received from BioMonde as shipped to practitioners, alongside corresponding control devices. Larval viability was verified upon receipt by observing the appearance and movement of larvae through the delivery tube. In total, LAP was collected from 60 larval-loaded BioBags, across a period of two years.

2.2.2.1 Materials and methods

The volume of PBS was reduced to 2.5 mL per BioBag, and the caps of the delivery tubes were replaced but not tightened. The passive collection of LAP was undertaken by permitting the larvae to circulate freely within the BioBag (which was not folded or distorted) whilst the tubes remained static, positioned along their length, with the cap-end raised slightly. This allowed the larvae to remain moistened, but without full immersion. Control devices were treated in the same manner, and all tubes remained within the cabinet, in the dark for 24 hours. Upon completion, solutions were collected by centrifugation and direct pipetting as previously stated, but were transferred directly to individual sterile 1.5 mL micro-centrifuge tubes, prior to storage at -20ºC. All BioBags and control devices were preserved as aforementioned, and all evaluation parameters (larval counts, speciation, protein content and chymotryptic activity) were conducted as described previously (**Section 2.2.1.1**).

2.2.2.2 Results

Larval number

Sixty BioBag-50 debridement devices were used for the collection of LAP under standardised conditions and this dataset demonstrated that bags were loaded with an average of 124.1 (± 34.05) larvae. This was again shown to be significantly different from the theoretical mean of 50 maggots, by means of one sample *t*-test ($P = < 0.0001$). The values for the larval counts were not normally-distributed; however, a frequency histogram was utilised (**Chart 4**), and this produced a Gaussian distribution with the removal of outliers (selected range 80 to 145: $\bar{x} = 110.7 \pm 16.52$, $n = 49$). As before, no devices contained exactly 50 larvae, and BioBags with 100 and 105 maggots were most commonly encountered ($n = 8$ and $n = 9$, respectively). No larvae were recovered from any of the control devices.

(Continued after **Chart 4**)

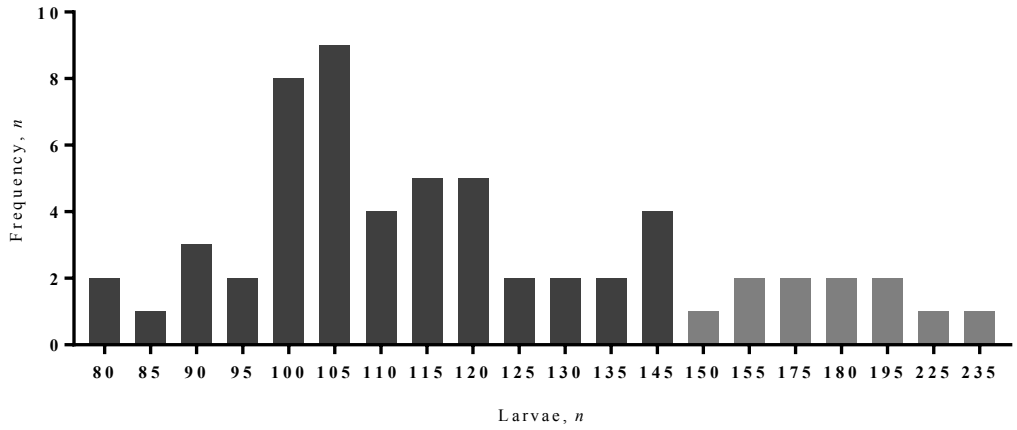

Chart 4: Frequency histogram of the number of larvae (hand-counted) contained within BioBag-50 debridement devices (*n*= 60). With the removal of outliers (150 to 235 larvae; light grey shading) the dataset becomes normally-distributed (*n*= 49).

Protein content

Total protein content ranged from 7.5 to 98.57 μg/mL ($\bar{x}$= 26.48 ± 15.49, *n*= 60). Frequency distribution (bin centres, due to the range) demonstrated that the most commonly encountered protein concentrations were within the range of 15 to 30 μg/mL. The data was found to be skewed, and remained as such with the removal of the greatest value as an outlier (**Chart 5**).

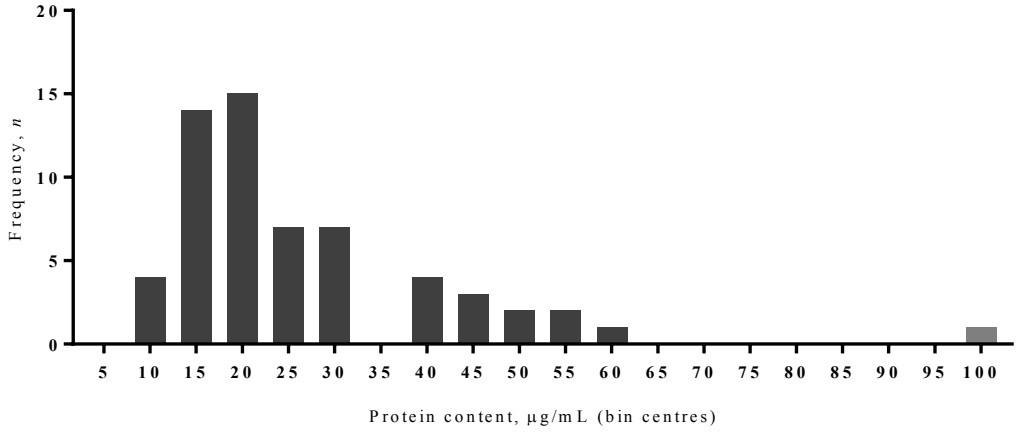

Chart 5: Frequency histogram (bin centres) of the total protein concentration of LAP collected from larvae contained within BioBag-50 debridement devices (*n*= 60). With the removal of the predominant outlier (100; light grey shading) the dataset remains skewed.

Chymotryptic activity

Chymotryptic activity ranged from 9.9 to 81.5 pmol of AMC* released per minute, for every microgram of protein ($\bar{x}$= 33.08 ± 14.34, *n*= 50). Frequency distribution (bin centres, due to the range) demonstrated that the most commonly encountered values for

chymotryptic activity were within the range 15 to 45 $pmol^{-1}/\mu g$. Data was found to be normally-distributed, and remained as such with the removal of the greatest value as an outlier (**Chart 6**).

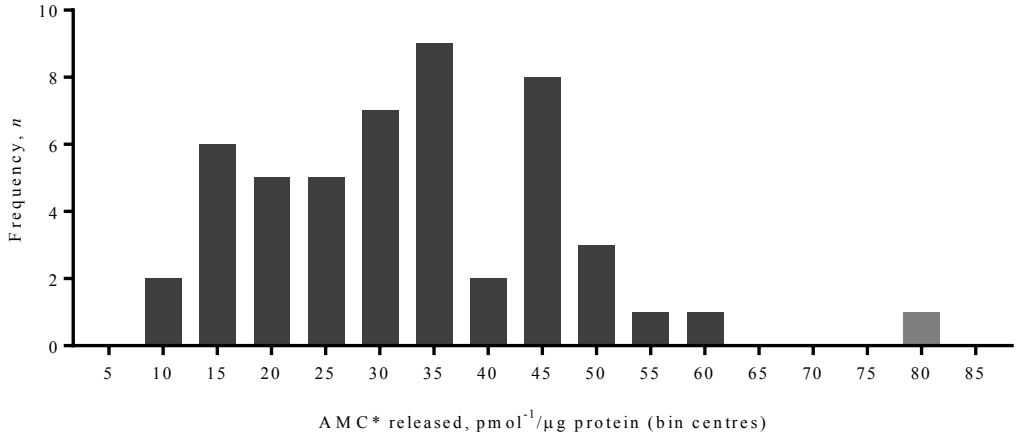

Chart 6: Frequency histogram (bin centres) of specific chymotryptic activity (release of fluorescent AMC*, pmol/minute/µg of protein) of LAP collected from larvae contained within BioBag-50 debridement devices (n= 50). With the removal of the predominant outlier (80; light grey shading) the dataset remains normally-distributed.

Relationship: protein content and chymotryptic activity

The relationship between protein content and chymotryptic activity for the larger sample size was again tested for linearity (**Section 2.2.1.2**), despite the protein parameter being skewed. Although a poor regression value was obtained (R^2= 0.1752), the relationship was deemed significant (P= 0.0025) however, caution should be exercised due to the inclusion of non-parametric data. Therefore, the relationship was tested using the Spearman correlation (**Chart 7**), and although this yielded weak but significant inverse (r= -0.3659, P= 0.0090) results, it should be borne in mind that chymotryptic activity is expressed per microgram of protein, and this may have an impact on the significance.

(Continued after **Chart 7**)

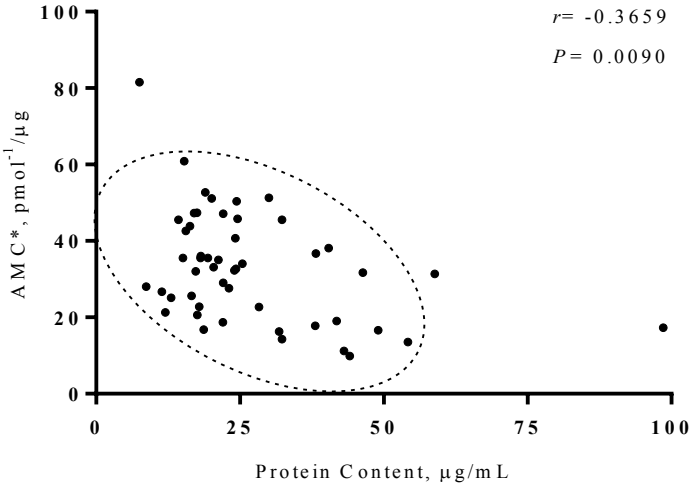

Chart 7: Exploration of the potential relationship between the total protein content and the specific chymotryptic activity (release of fluorescent AMC*) of LAP collected from BB-50 devices (*n*= 50). An emerging significant inverse correlation was observed (dotted line).

Relationship: protein content and larval number

No relationship between protein content and larval number was observed, using the Spearman correlation coefficient for non-parametric data (*r*= 0.2419, *P*= 0.0626, **Chart 8**).

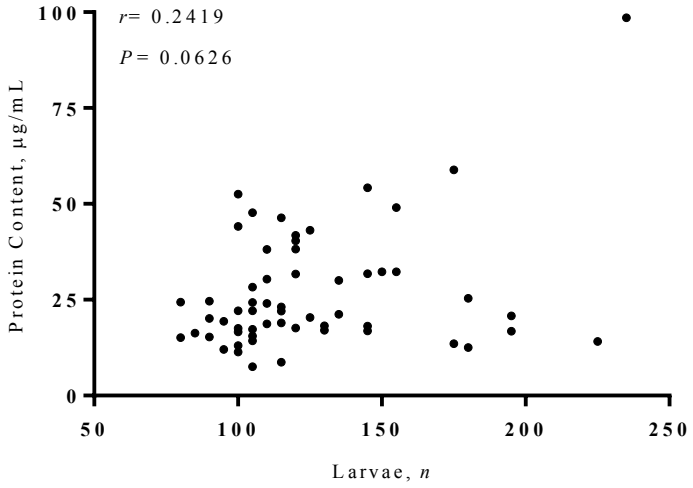

Chart 8: Exploration of the potential relationship between the total protein content and the larval number of LAP collected from BB-50 devices (*n*= 60). No statistically significant correlation was observed.

Relationship: chymotryptic activity and larval number

A weak but significant inverse result was obtained between chymotryptic activity and larval number when tested using the Pearson parametric correlation (*r*= -0.2911, *P*= 0.0402). This was repeated using the Spearman non-parametric correlation, on account of the larval number parameter being skewed (*r*= -0.3051, *P*= 0.0312, **Chart 9**).

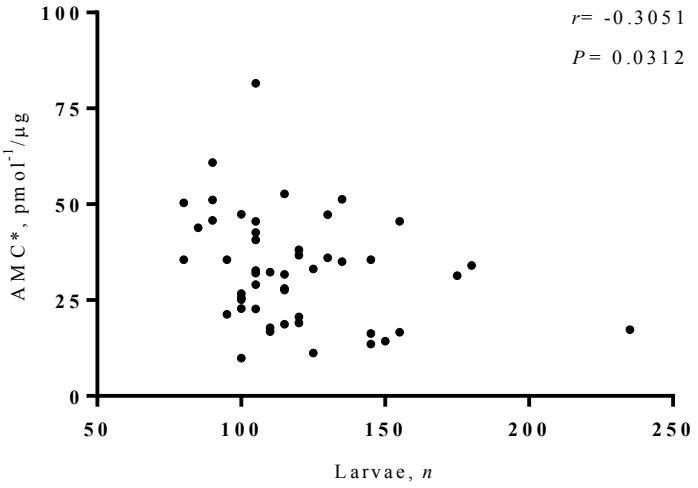

Chart 9: Exploration of the potential relationship between the specific chymotryptic activity (release of fluorescent AMC*) and the larval number, of LAP collected from BB-50 devices (*n*= 50). A weak, but significant correlation was observed.

2.2.3 Significance of findings

Of the total number of BioBag-50 devices received during the course of this study (*n*= 66), all were shown to contain significantly more maggots than the denoted value. BioMonde state that each bag will hold a minimum of 50 larvae, and this was exceeded in all cases. The relevance of this finding is that many bags are over-loaded, based on the recommendation of 5 to 10 maggots per cm^2 of wound surface (Sherman, 2003). Each BB-50 device covers an area of 10 cm^2, however, only 16 devices held ≤ 100 larvae (24.2%; range: 80 to 290). Larvae aggregate around the spacer (**Section 2.1.3**), and the larvae are unlikely to be feeding effectively when numbers are increased (Wilson *et al*., 2016), particularly when tightly confined. Alongside having cost implications during production, excess maggots in the wound are associated with undue pressure and pain (BTER, 2005).

Using the developed procedure, which reflected the conditions of the bio-footprint visualisation (24 hours), LAP was passively collected from 60 loaded BioBags. This simplified method was utilised to examine the potential relationships between the evaluation parameters. The strongest result was the emergence of an inverse correlation between protein content and chymotryptic activity (*r*= -0.3659, *P*= 0.0090), which provides further support for the assertion that potency of this enzyme is not linked to elevated protein levels. It is interesting to note that the larvae were not feeding at the

time of LAP collection, excluding the presence of any residual hatching substrate (*Lucilia* Agar, **Section 1.3.1**) in the gut. It is assumed that these values would change, and become more complex with the advent of feeding. Protein content could not be linked to larval number (r= 0.2419, P= 0.0626), but an inverse association was found between chymotryptic activity and the number of larvae (r= -0.3051, P= 0.0312). An increase in the number of maggots per BioBag appears to reduce the chymotryptic action, which provides an additional evidence that over-loading the device is not likely to improve feeding (and therefore debridement) actions.

As the specific chymotryptic activity is expressed in terms of protein content (**Section 2.2.1.2**), and chymotrypsin is only one of several enzymes known to be present in LAP (**Section 1.4.1**), larval number was considered as the parameter of most relevance. Unlike the other parameters, which are continuous variables, the maggot counts are discrete, independent integers. Once again, the conditions of the bio-footprint (**Section 2.1**) were utilised, and the total gelatinase activity of LAP was explored in the development and validation of a Quality Control assay. A summary of the key findings is shown in **Figure 5**.

Key Findings: Standardisation

- Standardised LAP is a suitable model for bio-footprint capture
- Enzymatic activity of LAP is retained in solution
- Protein content of LAP is variable, but generally low
- Enzymatic activity and protein content are not linear
- Larval number is the only non-continuous variable for assessment
- BB-50 devices are significantly over-loaded with larvae

Figure 5: Summary of the key findings associated with the collection of the bio-footprint components (as LAP) from BioBag devices.

2.3 Development of a Quality Control assay for BioBag activity

Following on from the collection and evaluation of LAP and the suitability of the agar/gelatin arrangement to produce a visual indication of the enzymatic output of the BioBag device, a simple technique suited to non-analytical laboratories was developed for quality assessment. The initial methodology was trialled whilst working with Master of Pharmacy students, to ensure that the gelatin substrate was amenable to the punching and removal of test wells, and to confirm the diffusion of LAP from the wells into the

substrate. The final methodology was optimised for this phase of the study and developed further during future applications (**Section 2.4**).

Drawing from anti-microbial zone of inhibition studies (Abraham *et al*., 1941) and immuno-diffusion methodologies (Mancini *et al*., 1965), this variation of a radial diffusion assay (Bowles *et al*., 1990) produced a measurable indication of gelatinolytic activity within 6 hours. The final method, incorporating the standardised LAP collection methodology, was validated and subsequently published (Pickles & Pritchard, 2017b).

2.3.1 Materials and methods

All reagents were obtained from Sigma-Aldrich Ltd, unless otherwise stated. Larval alimentary products were collected in accordance with the developed SOP (**Section 2.2.2**) and subjected to protein content and chymotryptic activity evaluation, and larval counts (**Section 2.2.3**). Additionally, a mortality assessment was undertaken, by recording the number of discoloured and distorted larvae which were dead prior to preservation. Twenty microlitres of the molten agar/gelatin solution as prepared for the bio-footprint (**Section. 2.1.1**), was added to 10 × 5 × 0.5 cm radial immuno-diffusion plates (kindly supplied by the Binding Site, Birmingham, UK), on a level surface. Following solidification and overnight refrigeration, test wells were punched using a 4 mm metal cork-borer and the contents removed using a sterile lancet. The diffusion-plates are manufactured to test 14 samples and previous experimentation determined the optimum volume of LAP per test well (10 μL) to avoid overlap or distortion of the digestion halos (**Figure 6**). A number of controls were also implemented and tested in RDEA (radial-diffusion enzymatic assay) system, as listed below.

Gelatin digestion: A collagenase standard from *Clostridium histolyticum* (Type VII) was prepared as a positive control (10 μg/mL, in TBS) to confirm enzymatic action upon the substrate gel.

Plate substrate: Agar-only diffusion-plates were produced and tested against LAP solutions with known chymotryptic activity, to verify that digestion was acting upon the gelatin substrate only.

Plate buffer: The TBS solution used to prepare the RDEA system gel was utilised as a negative control and to confirm its inactivity.

Larval device: Control BioBag devices (devoid of larvae) were tested to confirm that the packaging surrounding the larvae was not contributing to the enzymatic action of LAP.

Larval buffer: The PBS solution used for the collection of LAP was tested to confirm that the solution was not contributing to the enzymatic action of LAP.

Larval food: The *Lucilia* Agar hatching plate (**Section 1.3.1**) was tested to exclude the possibility that diet constituents were contributing to the enzymatic action of the LAP. Test solutions were prepared by surface irrigation (1 mL sterile PBS), gel-punch (4 × 10 mm discs created and macerated by sterile pipette tip) and gel-punches (as described previously, macerated in 500 μL sterile PBS). The elution of the diet constituents was confirmed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing conditions. Samples were prepared to a final volume of 10 μL and combined with 0.5 M dithiothreitol (DTT) and NuPAGE LDS (lithium dodecyl sulphate) sample buffer (Life Technologies; California, USA) at a ratio of 65:10:25. Samples were heated for 5 minutes at 98°C and centrifuged for 1 min at 3000 *g* prior to loading on pre-cast NuPAGE 4-12% gradient Bis-Tris midi-gels. Gels were run for 60 minutes at 200 volts, with MES-SDS (2-(N-morpholino) ethanesulphonic acid) running buffer and Novex Sharp Prestained protein standards (5 µL). Upon completion, gels were fixed (50:40:10, water:methanol:glacial acetic acid) for 30 minutes, followed by staining with Coomassie Brilliant Blue G-250 for 2 hours.

2.3.1.1 Validation of RDEA for LAP analysis

From each individual BioBag (*n*= 18, from 3 batches obtained over a 10-month period) 10 µL of collection solution was added to the upper row of each diffusion-plate (wells 1 to 6), alongside the controls as required (wells 7 and 8) of the same volume. To the lower row of the same plate (wells 9 to 14) collection solutions from the corresponding control devices were added (**Figure 6**) and all diffusion-plates (with lids in position, prepared in triplicate) were incubated at 37°C for 6 hours. Visible 'digestion halos' were observed when the lids were removed following incubation, upon completion of the assay. The diffusion-gels were fixed within the plates for 30 minutes, prior to staining and photographing as aforementioned (**Section 2.1.1**).

Halo diameters were measured (± 0.2 mm) using calibrated carbon-fibre digital callipers (Fisher Scientific; Pennsylvania, USA) as recommended in zone of inhibition studies (Hombach *et al*., 2013), on a transmitted light box, and values were corrected to exclude the well diameter (4 mm). Data obtained was used to express the findings in terms digestion activity, as the mean ± SD halo diameter (mm); coefficient of variation (CV%) values and statistical analyses were performed using GraphPad Prism. Data normality was confirmed using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) and Pearson correlation tests were used to compare the digestion activity of the BioBag batches and the relationship with the internal evaluation parameters for possible quantitation. Statistical significance was determined as aforementioned (**Section 2.2.1.1**).

2.3.2 RDEA Results

Collection solutions were obtained from three batches of six BioBags and corresponding control devices. Total protein content estimation and specific chymotryptic assays were conducted, alongside larval counts and mortality assessment (**Table 3**). The collection procedure did not adversely affect larval survival (1.94% mortality) and the values obtained for protein concentration and chymotryptic activity reinforces the suggestion that the extensive enzymatic action is not directly related to elevated protein content. No values were obtained for the control devices, TBS or PBS blanks.

Table 3: Characterisation of larval alimentary products obtained from BB-50 debridement devices (*n*=6 per batch) across 3 batches, mean (± SD).

	Batch 1	**Batch 2**	**Batch 3**
No. of Larvae	109 (± 12)	94 (± 19)	153 (± 49)
Mortality (%)	1.347 (± 1.24)	0.548 (± 0.93)	3.918 (± 1.98)
Protein Concentration	26.27 (± 13.48)	22.75 (± 4.93)	52.46 (± 24.33)
Chymotryptic Activity	33.67 (± 18.07)	51.10 (± 5.28)	33.44 (± 9.45)

Protein concentration estimation (µg/mL), in duplicate.
Specific chymotryptic activity (pmol AMC released/min/µg protein), in duplicate.

The RDEA system was used to assess each BioBag and corresponding control device (both, *n*= 18), in triplicate, across three separate assays. Digestion activity was observed with the collagenase standard and LAP samples only. (**Figure 6**). A summary of the controls follows below.

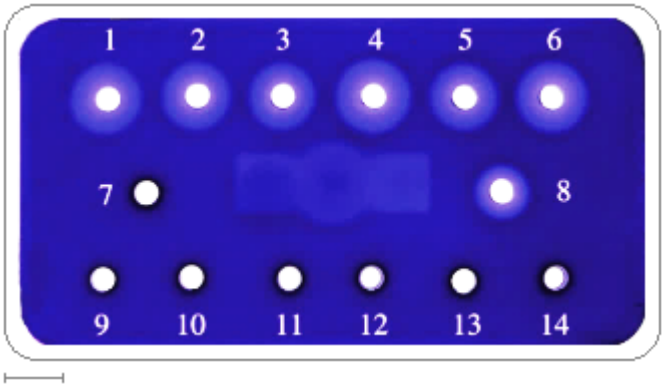

Figure 6: Example RDEA for the digestion activity of LAP for six BB-50 debridement devices (wells 1-6) and corresponding control devices (wells 9-14) with larval food control (macerated in PBS, well 7) and collagenase standard (well 8) following incubation at 37ºC for 6 hours and Coomassie staining. Darker 'edge artefacts' around negative wells demonstrate the lack of digestion in the presence of diffusion, post-staining. Scale bar: 10 mm.

Gelatin digestion: Digestion activity was observed with all collagenase positive controls (**Figure 6**), confirming enzymatic action upon the substrate.

Plate substrate: No digestion activity was observed with any agar-only plates (not shown), confirming enzymatic action upon only the gelatin component of the substrate.

Plate buffer: No digestion activity was observed with any of the TBS negative controls (not shown), confirming inactivity of the system buffer.

Larval device: No digestion activity was observed with any of the control devices devoid of larvae (**Figure 6**), confirming inactivity of the BioBag construction materials and delivery tube.

Larval buffer: No digestion activity was observed with any of the PBS blanks (not shown), confirming inactivity of the collection buffer.

Larval food: No digestion activity was observed with the larval media (**Figure 6**). No chymotryptic activity was associated with the larval media. Protein concentration was estimated at approximately 3.5 mg/mL and constituents were resolved using SDS-PAGE (**Figure 7**), indicating the release of the food components, and any potentially enzymatic properties.

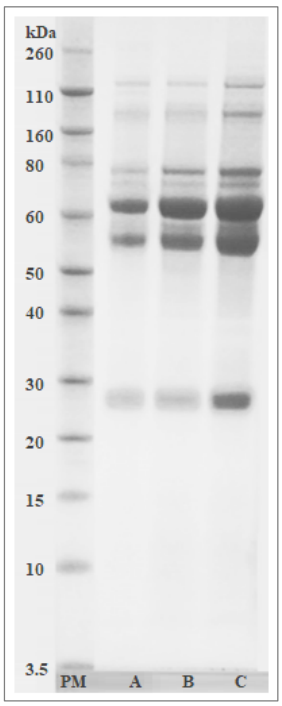

Figure 7: Food constituents of the *Lucilia* Agar hatching plate resolved using SDS-PAGE (reducing conditions, 4-12% Bis-Tris gradient gel with Coomassie staining). Samples (3 μL) represent extraction procedures: A: surface irrigation (1 mL PBS), B: 4 × 10 mm gel punches (macerated, 500 μL PBS) and C: 4× 10 mm gel punches (macerated). PM: protein marker (5μL).

With larval-loaded BioBags, digestion activity was observed in all instances and individual device replicates produced digestion halos with low standard deviation (0.06 to 0.27 mm) and coefficient of variation (CV: 0.75 to 4.31%) values (**Chart 10**). No statistically significant difference in mean values were observed when tested using one-way ANOVA (P= 0.0986). A measurement range of 5.77 to 8.43 mm was noted in the total dataset (n= 18), despite the otherwise consistent results, and notable peaks in activity corresponded with an increase in larval number (**Chart 11**).

The potential relationship between larval counts and digestion activity was explored for linearity (R^2= 0.5790) and was deemed to be extremely significant (P= 0.0002; **Chart 12**).

(Continued after **Chart 12**)

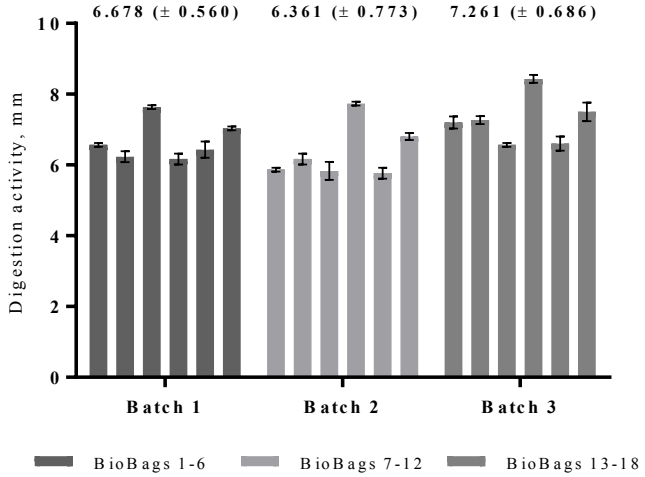

Chart 10: Digestion activity (mm, in triplicate) of LAP enzymatic activity against gelatin substrate by RDEA (6 hours, 37ºC). Text in bold relates to mean values (± SD, *n*= 6 BioBags per batch).

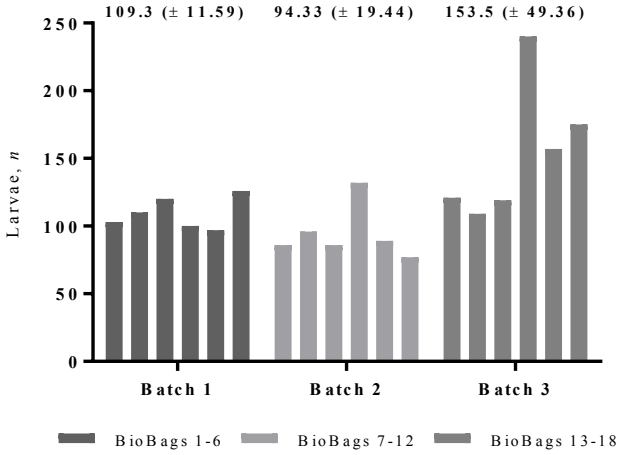

Chart 11: Corresponding larval numbers per BB-50 device (hand-counted). Text in bold relates to mean values (± SD, *n*= 6 BioBags per batch).

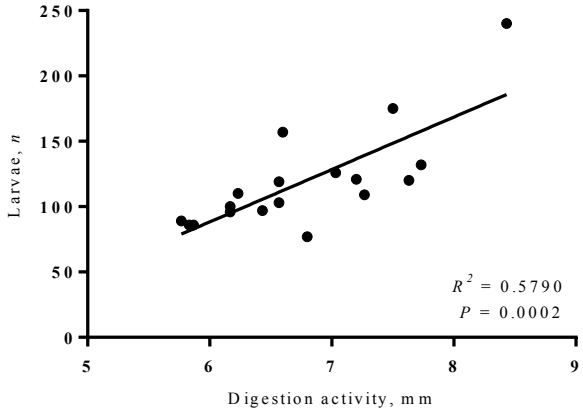

Chart 12: Emerging linear relationship between RDEA digestion activity and number of larvae contained in each BioBag (*n*= 18). Significance of the correlation determined using Pearson coefficient for parametric data.

2.3.3 Significance of findings

As discussed previously (**Sections 1.2** and **1.3**, **Section 2.1**) meat-based assays are not suited to high throughput analysis and do not lend themselves well to standardisation, due to the inhomogeneous and variable nature of animal tissues. General handling is cumbersome, and although the assays are utilised for behaviour and developmental study, they are limited in terms of their potential for modification. Furthermore, it is not possible to harvest the substances produced by the larvae during feeding, as they are lost into the surrounding decomposing matter. Preparing a solution of LAP is common practice in research relating to larval therapy and the method has been used successfully to characterise substances produced by *L. serciata* larvae (i.e. lipase, DNAse, glycosidases, matrix metallopeptidases, trypsin and chymotrypsin (**Section 1.3**). A standardised collection method (**Section 2.2.2**) has been employed in the development of the RDEA system to determine total gelatinase activity, by means of a wound-relevant substrate incorporated into an inert agar base. The system may test up to 14 samples (meat-based assays are singular) using basic laboratory materials and is completed within 6 hours (37ºC), as opposed to 2 days (32ºC), as currently utilised by BioMonde (Wilson *et al*., 2016). With the added advantage of LAP and substrate homogeneity, the reproducibility of the system is high (CV: 0.75 to 4.31%) and larval survival is not adversely affected (mortality: < 2%). The system conforms to good manufacturing and laboratory processes and may be scaled up for production. Furthermore, the liquid/gel arrangement of the RDEA is open to modification, such as the incorporation of other substrates which are known to be degraded by LAP (i.e. fibrin, laminin, fibronectin), or for the exploration of new enzymatic substances. Inhibitors may be readily included, and the system is suited to antimicrobial studies.

The variation of feeding substrates and LAP collection methodologies result in variation in the composition and quality of the solution, particularly where meat-based diets are utilised (**Section 1.5**) Therefore, harvesting LAP under standardised conditions, from insects reared under regulated methods and fed initial on homogenous synthetic diets prior to starvation, will produce a clean, consistent solution, suitable for more sophisticated analysis (**Figure 5**). It is however possible, that components of the

diets, particularly the *Lucilia* Agar, contribute to the composition of LAP, and therefore the dietary preparations developed by BioMonde were interrogated further. A summary of the key findings is shown in **Figure 9**.

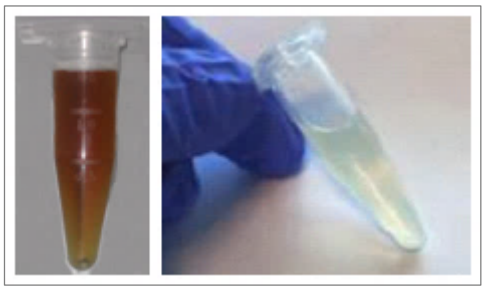

Figure 8: Example of LAP collected from larvae reared on liver by Nigam (2013) and from larvae collected under standardised conditions during this study. (Image scale: 1.5 mL micro-centrifuge tubes.)

Key Findings: Standardisation

- RDEA is a suitable alternative to meat-based models
- RDEA is a suitable alternative to the chymotryptic assay
- RDEA is rapid and with a homogenous substrate
- LAP SOP does not affect larval survival
- Standardised, regulated LAP is a 'clean' solution
- Published findings: Pickles & Pritchard (2017b)

Figure 9: Summary of the key findings associated with the development of the RDEA system and standardised LAP collection methodology.

Chapter Three: Application

3.0 Abstract

This chapter, 'Application' follows on from the standardised collection and testing of larval alimentary products, to consideration of the procedures leading to the production of the BB-50 device. By utilising the enzymatic assay, it was possible to interrogate the substrates developed to support the insect colonies of BioMonde (the 'Carnival Diet' preparation) and to hatch the sterile larvae (the '*Lucilia* Agar' formulation) for inclusion into the BioBags. It was established that neither substrate, or any of the individual ingredients possess enzymatic activity, but the diets are not optimised for blow-fly larvae. Furthermore, it is likely that the alimentary products of the larvae contain components from the hatching substrate, alongside contributions from symbionts present in the gut of the larvae.

3.1 Utilisation of the RDEA QC Assay

In order to be confident in the material, it was first necessary to investigate the composition of the diets used in the production of the larvae. Following the initial analysis of the *Lucilia* Agar larval media in the QC validation, this and the adult rearing media (the Carnival Diet, **Section 1.3.1**) were further interrogated to determine their contribution to the LAP solution, and ultimately the bio-footprint.

Two artificial diets have been formulated by BioMonde to support the mass-rearing of the fly colonies used in the production of BioBags, and to address concerns raised by the European Commission (2011) that animal tissues may harbour infectious agents which could be transmitted to patients via medicinal products. The Carnival Diet completely supports the stock colonies, as a liver replacement on which the flies feed and oviposit, and in which the resultant larvae develop. The *Lucilia* Agar preparation, is a sterile media for the hatching of disinfected eggs and the initial feeding of the maggots, destined for inclusion into the devices. The RDEA system was employed to demonstrate that *Lucilia* Agar was not contributing to the enzymatic action of LAP (**Figure 6**) and was subsequently utilised to test both the Carnival Diet and the *Lucilia* Agar formulations, and each of their individual components. Furthermore, the RDEA was used in conjunction with an endotoxin assay to explore the possibility of symbiont presence.

3.1.1 RDEA: Carnival Diet analysis

To assist mass-rearing efficiency and consistency, and to remove the possibility of the transmission of infectious agents from animal tissues, BioMonde developed and implemented the 'Carnival Diet' as an alternative to porcine liver. The etymology of the word 'Carnival' originates from the Latin words for meat (*carne*) and goodbye (*vale*), and translates directly into '*farewell to the flesh*' (Goldberg, 2013). Nevertheless, the artificial preparation does contain animal-derived products (chicken egg, porcine serum and gelatin) which were incorporated to suit the necrophagous behaviour of the insect. These components have, however, been processed and approved for pharmaceutical use, and are incorporated with the remaining ingredients (soy, wheat and yeast-based nutrients) to create a substrate suitable for adult feeding, oviposition and larval rearing. The presence of soy (Kunitz, 1947) and serum (Borth, 1992) were of immediate interest, as these are known to contain compounds which inhibit larval enzymes (Bowles *et al*., 1990; Telford *et al*., 2011), raising the concern that the diet may not be optimal for *Lucilia sericata*, and these could possibly have an impact on the efficacy of the treatment *in vivo*.

3.1.1.1 Materials and methods

Solutions for testing

Larval alimentary products were obtained from six BB-50 devices as described previously (**Section 2.2.4.2**), prior to pooling and storage at -20°C. Individual food components were supplied by BioMonde, as produced by the manufacturers and routinely used in diet formulation. The Carnival Diet preparation (**Table 4**) is not heat-treated or autoclaved; however, the components are prepared in accordance with the regulatory bodies overseeing ingredient production as suitable for human consumption. Specific pathogen-free eggs (VALO BioMedia; Ringstedt, Germany) were used to create an 80% solution from full chicken eggs (diluted with water). Upon receipt, aliquots of the solution were frozen (-20°C) until use, alongside the heat-inactivated, pre-filtered and gamma-irradiated porcine serum (Kraeber & Co GmbH; Ellerbek, Germany). Powdered porcine gelatin (Weishardt International; Graulhet, France) derived from acid-extracted pig skin was received, and a 2% solution (diluted with water) was prepared, prior to the freezing of aliquots. Non-genetically modified soy peptone (Organotechnie, SAS; La Courneuve, France), wholemeal wheat flour

(SchapfenMühle GmbH & Co. KG; Ulm-jungingen, Germany) and brewers' yeast from *Saccharomyces cerevisiae* (Lallemand; Salutaguse, Estonia) were received as dry products and stored at room temperature.

The complete Carnival Diet was created according to the recipe and the individual components were prepared in the RDEA system buffer (TBS) in representative quantities (**Table 4**), prior to vortex and centrifugation (3360 *g*, 8 minutes). Supernatants were removed and further centrifuged (13,000 *g*, 4 minutes) for two cycles. All supernatants were stored at -20°C until use. Constituent elution was confirmed using SDS-PAGE analysis under reducing conditions, as previously described (**Section 2.3.1**).

Table 4: The Carnival Diet composition, showing the proportion of each food component combined with TBS elution buffer, to give a final composition which is proportionally representative of that used to rear flies in the BioMonde colonies.

Diet Component	Quantity	TBS
Egg solution (80% in H_20)	1 mL	2 mL
Gelatin solution (2% in H_20)	1 mL	2 mL
Porcine serum	1 mL	2 mL
Soy peptone	1 g	3 mL
Wholemeal wheat flour	1 g	3 mL
Brewer's yeast	1 g	3 mL

Digestibility of the diet and its components by LAP using SDS-PAGE

The protein profiles of the food components and the Carnival Diet, before and after the addition of LAP, were compared using SDS-PAGE analysis. All samples were prepared to a final volume of 10 μL as aforementioned (**Section 2.3.1**). The volume of each sample required for optimum lane comparison had been previously determined, and with the exception of gelatin, sample aliquots were further diluted with ultrapure water as follows: egg solution and porcine serum, 1.5 μL; soy peptone, 2 μL; Carnival Diet, 3 μL; wheat flour and yeast, 4 μL. Control LAP pellets (following 4× cold acetone precipitation and centrifugation for 10 minutes, at 13,000 *g*) were suspended in 6.5 μL of sample buffer and prepared as above, to verify that the addition of larval products was not adding significantly to the dietary profiles.

RDEA analysis of LAP in the presence of the diet and its components

Diffusion-plates were prepared as described previously (**Section 2.3.1**). The digestion activity of the food components before and after the addition of LAP, were compared

using the RDEA system. Test solutions were prepared as described below. For proportional analysis, aliquots of each test sample supernatant were diluted to 50% with TBS to provide benchmark comparisons, as they would later be combined with LAP in equal quantities, as neat solutions (50:50, LAP:supernatant) for subsequent digestion/inhibition analysis. The following samples were incorporated as listed below.

Background activity: Aliquots of dietary supernatants were diluted to 50% with TBS to determine the presence of any existing enzymatic properties.

Plate buffer: The TBS solution used to prepare the RDEA system gel was utilised as a negative control and to confirm its inactivity.

Gelatin digestion: Pooled LAP was prepared as a positive control (50%, in PBS collection buffer) to confirm enzymatic action upon the substrate and for the size comparison of digestion halos.

Diet digestion: Aliquots of dietary supernatants were combined with an equal volume of undiluted stock LAP solution, with a known chymotryptic activity of 38.64 (± 7.79, in duplicate) released AMC* $pmol^{-1}/\mu g$.

Titration of activity: Aliquots of dietary supernatants were diluted with TBS as follows: 50:0, 40:10, 30:20, 20:30, 10:40 and 5:45, prior to combining with a constant volume of undiluted LAP (50% of total volume).

Ten microlitres per test solution was added to each well; replicates were conducted in triplicate, across three separate diffusion-plates. Lids were positioned accordingly, and plates were incubated at 20°C for 24 hours (to replicate adult feeding conditions). Each solution tested was prepared in individual micro-centrifuge tube for subsequent SDS-PAGE analysis, which remained alongside the RDEA plates throughout the duration of the assay.

RDEA visualisation of LAP in the presence of the diet and its components

Upon completion of the assay, RDEA gels were fixed, stained and subsequently photographed with calibrated scale (**Section 2.3.1.1**). Digestion was considered viable upon the clarity of the halo (colourless post-staining) and its visibility to the underside of the gel. Images were processed using ImageJ software (v.1.41o, National Institutes of Health; Maryland, USA), as 8-bit (greyscale) images, and calibrated against the scale in each image (mm). Digestion activity was measured as halo area (mm^2) using the

software and corrected to exclude the exact area of the corresponding well, whereas digital callipers were utilised previously and the size of the cork-borer (4 mm) subtracted from each value. This modification was introduced to improve measurement precision and accuracy, as the area of the digestion halos were to be compared between each component, as well as to the LAP control. All statistical analyses were performed using GraphPad Prism, and digestion activity is expressed as the mean halo area (± SD, n= 3, mm^2). Data normality was confirmed using the Shapiro-Wilk test, and the significance between compared groups was determined using the paired t-test ($P \leq 0.05$). Assay reproducibility was determined using the coefficient of variation (CV), and a level of ≤ 15% was considered acceptable.

3.1.1.2 Carnival Diet results

Background activities of test samples

The existing enzymatic activity of the LAP had been confirmed using the chymotryptic assay in duplicate, with 38.64 (± 7.79) released AMC* pmol^{-1}/μg. No values were obtained for the TBS blank and the complete Carnival Diet. Upon RDEA analysis, the LAP positive control produced digestion reactions on all gel plates, with a mean area of 57.89 (± 3.49) mm^2 (n= 12 assays). The coefficient of variation value (6.02%) demonstrated a high level of assay reproducibility. Reactions were not produced by the TBS control (n= 12 assays), the Carnival Diet or any of the components (n= 3 assays). These analyses confirmed that all subsequent digestion activity recorded was LAP-derived (**Figure 10**).

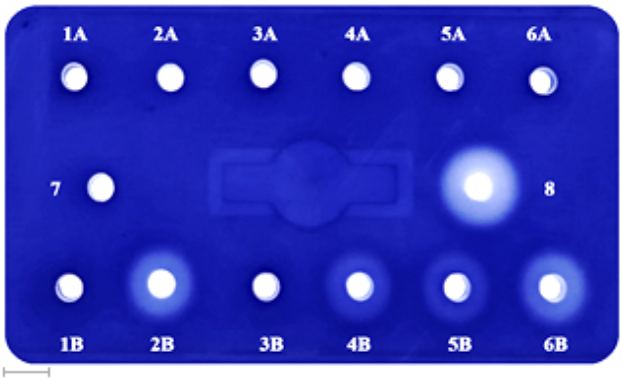

Figure 10: Example of the RDEA system for the assessment of the Carnival Diet and its components (incubation at 20ºC for 24 hours). Individual ingredients are shown from left to right before (A) and after (B) digestion with LAP: egg solution (1), gelatin solution (2), porcine serum (3), soy peptone (4), wholemeal wheat flour (5) and brewers' yeast (6). Controls are shown in the centre panel: TBS blank (7)

and positive LAP (8). The Carnival Diet formulation had no background activity, and completely inhibited the enzymatic action of the LAP (not shown). Scale bar: 10 mm.

Diet digestibility by LAP

The formulation of the Carnival Diet was diluted to 50% with TBS, to produce a background protein profile using SDS-PAGE analysis. This was then compared to the profile obtained following the combination of undiluted diet with an equal volume of LAP. The profiles revealed that the Carnival Diet was only slightly digested by the larval enzymes, with major bands remaining between 40 and 80 kDa (**Figure 11**). The process was repeated for each individual component and it became evident that the degree of digestibility varied between the ingredients, with the gelatin and yeast components showing the greatest change after the addition of LAP. The components which remained undigested, were considered as possible 'anti-nutrients', particularly as the presence of proteinase inhibitors have been reported in egg (Réhault, 2007), serum (Borth, 1992) and soy (Kunitz, 1947) products, which account for half of the ingredients of the Carnival Diet.

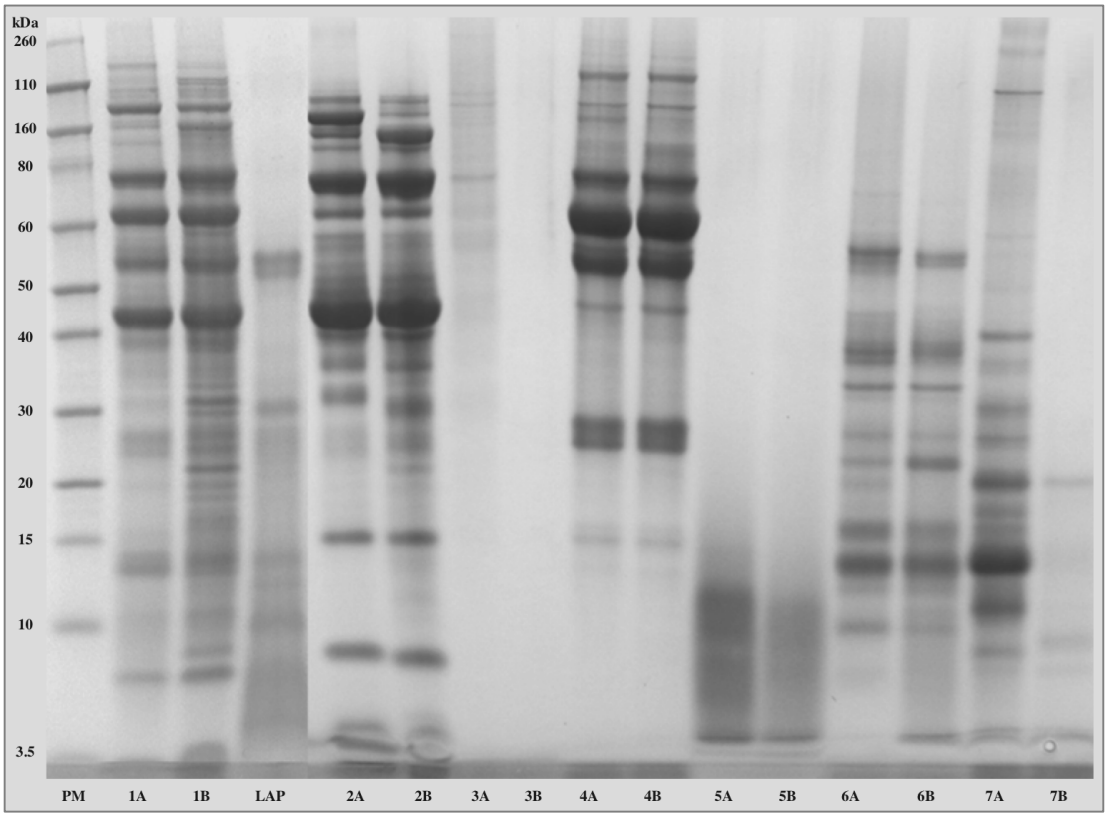

Figure 11: Digestibility of the Carnival Diet and its components by LAP, resolved using SDS-PAGE (reducing conditions). Individual dietary components are shown from left to right before (A) and after (B) digestion with LAP: complete Carnival Diet (1) egg solution (2), gelatin solution (3), porcine serum (4), soy peptone (5), wholemeal wheat flour (6) and brewers' yeast (7). PM: Protein marker, LAP: following acetone precipitation.

LAP inhibition by diet

The RDEA system was used to assess the impact of the Carnival Diet on the enzymatic action of the LAP, and this demonstrated clearly that the digestion activity had been inhibited completely (**Figure 10**). The subsequent analysis of each component revealed that the egg solution and porcine serum were likely to be responsible for the inhibitory action of the complete Carnival Diet (**Chart 13**).

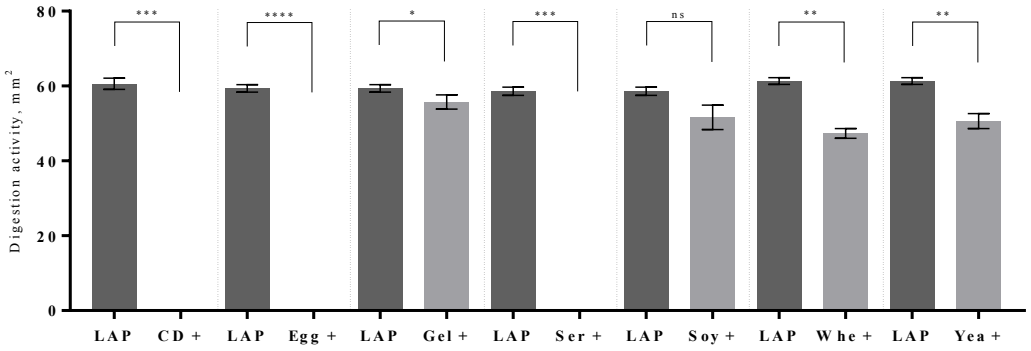

Chart 13: Analysis of the Carnival Diet and its components, against a LAP control using the RDEA system for inhibitory action (mean area ± SD mm^2, *n*= 3 assays), following combination with an equal volume of LAP (+). The Carnival Diet (CD), egg solution (Egg) and porcine serum (Ser) completely inhibited LAP activity, whereas no significant inhibition was seen by soy peptone (Soy). Gelatin solution (Gel), wholemeal wheat flour (Whe) and brewers' yeast (Yea) had relatively less significant inhibitory effects, determined using paired *t*-test: ns= no significant difference.($P > 0.05$), * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

Dilution of diet and components

The Carnival Diet and each component were sequentially diluted to determine the resilience of the inhibitory action, against a constant volume of LAP in the RDEA system. Even with a reduction in quantity, the enzymatic activity of the LAP failed to recover in the presence of the complete diet (**Chart 14**). The individual analysis of each component showed that four of the ingredients (gelatin, soy, wheat and yeast) retained consistent LAP activity throughout the titres, and that the inhibitory property of the egg solution diminished when diluted to 10% and 5%. However, inhibition by porcine serum was highly resistant, and as the complete preparation also showed the same degree of inhibition, it was considered likely that this component was predominantly responsible for the suppression of larval enzymes in the Carnival Diet. The SDS-PAGE titration profiles supported these findings, and both the complete preparation and the serum component remained poorly digestible, even when the ratio of LAP to component was increased considerably. Additionally, constituents in the egg solution

remained resistant to digestion, and at its current quantity, this component may also represent a poor choice for diet inclusion (**Figure 12**).

(Continued after **Chart 14** and **Figure 12**)

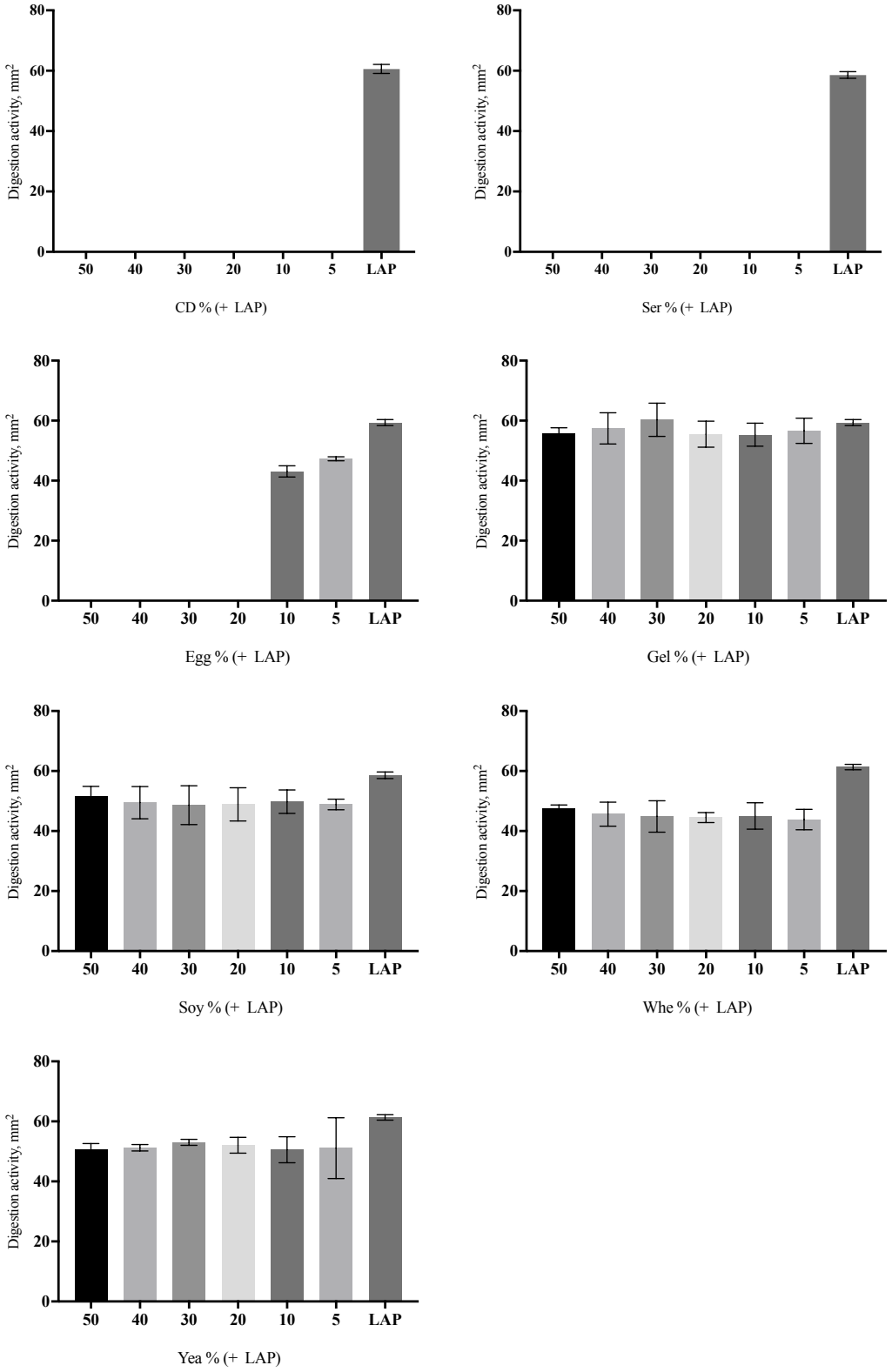

Chart 14: Inhibitory effects of the Carnival Diet and its components following their dilution and subsequent combination with a constant concentration of LAP (+) using the RDEA system (mean area ± SD mm^2, *n*= 3 assays). The inhibitory effect of the egg solution (Egg) was removed following dilution, with enzyme recovery at 10%, whereas the inhibitory effects of the porcine serum (Ser) and the Carnival Diet (CD) were completely resistant to dilution. The gelatin solution (Gel), soy peptone (Soy), wholemeal wheat flour (Whe) and brewers' yeast (Yea) components were not inhibitory at any of the dilutions used.

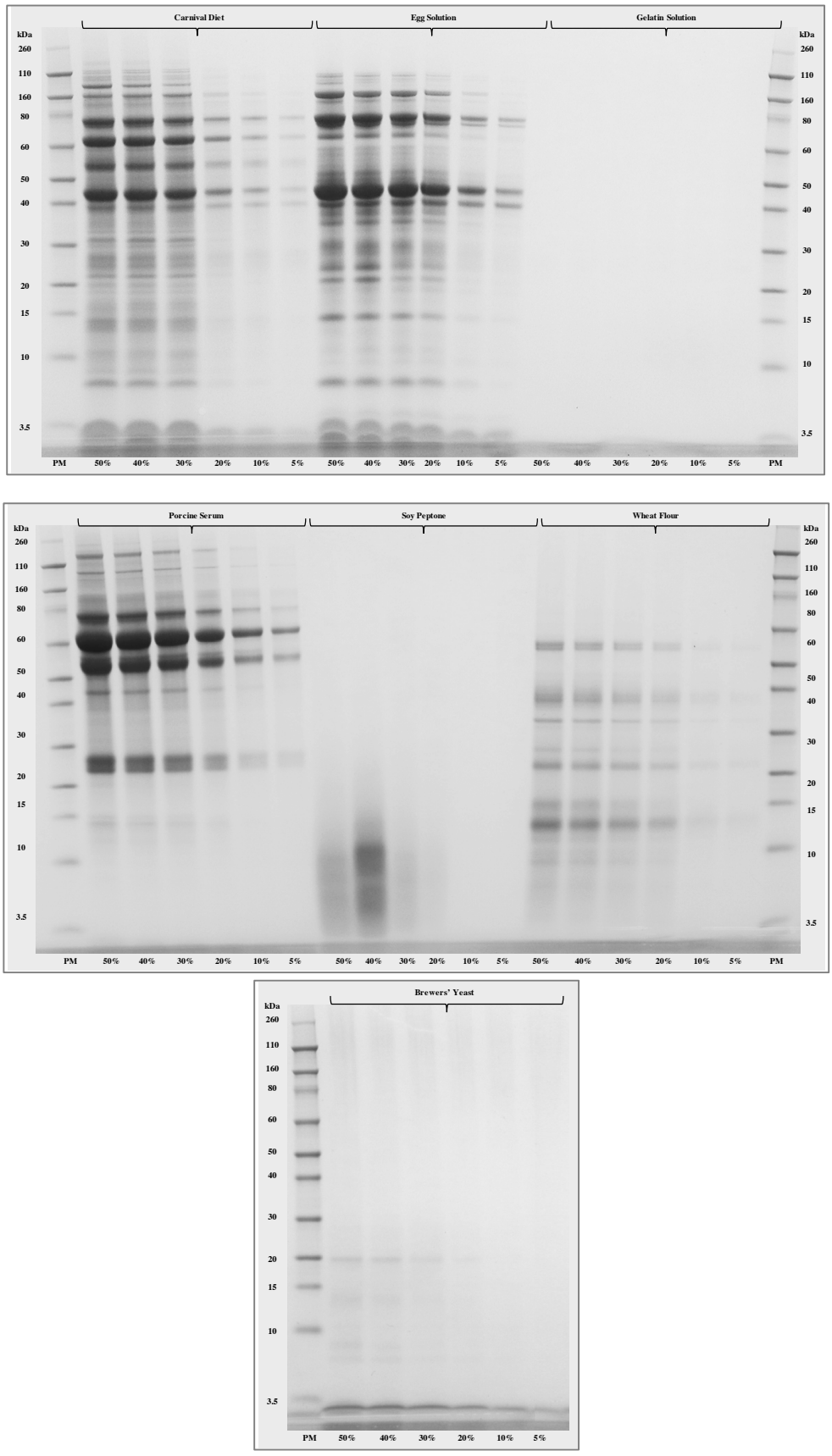

Figure 12: Protein profile (SDS-PAGE) of the LAP-resistant Carnival Diet and the individual components, alongside protein markers (PM). Samples were loaded in the concentrations shown following a 24 hour incubation with a constant concentration of enzymatically active LAP.

3.1.1.3 Significance of findings

By using a combination of the RDEA system to measure the enzymatic action of LAP with SDS-PAGE to assess diet digestibility, it has been established that the Carnival Diet is poorly digested by larval enzymes and that it contains components which inhibit the gelatinase activity of *Lucilia sericata* larvae *ex vivo*. As the larvae feed extra-corporeally (**Section 1.4**), the findings suggest that the formulation is not optimal for this species, and may be producing anti-nutrient effects. The examination of the individual ingredients revealed that those components which were poorly digested, were also the most inhibitory, namely the egg solution and porcine serum. It is therefore possible to recommend adjustments to the current preparation, which may then be evaluated using traditional growth studies for insect survival, fitness and fecundity (Chaudhury, 2009a). The exclusion of unnecessary components is considered to be the first step of insect diet modification (Parker, 2005) and the degree of LAP inhibition by porcine serum and its continued presence in the Carnival Diet preparation, suggest that this ingredient is a candidate for removal.

Serum is known to contain α2-macroglobulin, α1-anti-trypsin and α1-anti-chymotrypsin, and as such inhibits collagenase (gelatinase), elastase, trypsin and chymotrypsin (Werb *et al*., 1974; Westrom, 1979) and more specifically, larval enzymes (Bowles *et al*., 1990; Telford *et al*., 2011). Hobson (1933) reported that *Lucilia sericata* larvae could not develop on sterile serum, and it is not a common ingredient of blow-fly diets. Furthermore, the effects seen with this component occur even after the heat-treatment used in the manufacturing process (**Section 2.4.1.1**). The egg solution was also shown to initially inhibit LAP, and despite possessing a range of proteinase inhibitors, whole chicken eggs and derivative preparations have frequently been incorporated in blow-fly diets (Taylor, 1988; Rueda *et al*., 2010; Shefa *et al*., 2013; Chaudhury *et al*., 2015).

Egg-white also contains a homologue of α2 macroglobulin, ovostatin (also known as ovomacroglobulin), which shares the broad-spectrum activity of its mammalian counterpart (Miller & Feeney, 1966; Nielsen & Sottrup-Jensen, 1993). Additionally, ovoinhibitor and ovomucoid are present throughout the egg, both of which possess anti-tryptic and anti-chymotryptic properties (Nagase *et al*., 1983; Stadelman *et al*., 1995; Desert *et al*., 2001; Réhault, 2007; Li-Chan & Kim, 2008). However, by diluting the

raw whole egg component of the Carnival Diet, the inhibitory effects are reduced, and digestibility is improved. As this ingredient represents a complex nutrient source (Seuss-Baum, 2007) and as it also assists as a binding agent, its presence in the Carnival Diet may improve palatability, texture and moisture content, which are essential factors to encourage insect feeding and oviposition (Moore, 1985). It is therefore recommended that the egg component be diluted as opposed to removed, in order to retain the textural and nutritional benefits, including the presence of vitamins and minerals, which are often overlooked in dietary preparations for insects.

The remaining components of the Carnival Diet did not significantly inhibit LAP, and all have been incorporated into various insect diets previously, with wheat and yeast appearing as common ingredients for blow-fly preparations (Tenquist, 1971; Sherman & My-Tien Tran, 1995; Tachibana & Numata, 2001; Green *et al*., 2003). These may be considered as equivocal ingredients, with quantity adjustments being used to manage any deficits encountered by the alterations of serum and egg, for example. In particular, the amount of wheat flour may be reduced, as the digestibility by LAP was minimal. Acting predominantly as a bulking agent, lower proportions would still assist the passage of the Carnival Diet through the larval alimentary tract (Cohen, 2015). The brewers' yeast component could be increased if required, as digestibility by LAP was high, but this ingredient may be redundant for *Lucilia sericata* if its nutritional benefits are met by other ingredients (Daniels *et al*., 1991; Sherman & My-Tien Tran, 1995). Similarly, soy is not frequently used in blow-fly diets, and it has been reported as an unsatisfactory alternative to blood preparations for the parasitic New World screwworm *Cochliomyia hominivorax* (Diptera: Calliphoridae) (Chaudhury *et al*., 2015). This suggests that the inclusion of soy peptone as a source of amino acids, lipids and carbohydrates may be extraneous in the presence of the other Carnival Diet components (Chaudhury, 2009a). Although the soy component was initially considered as a potential inhibitor of LAP, the enzymatic hydrolysis used to produce the peptone, appears to have suppressed the reported anti-proteases (Sessa & Nelsen, 1991), particularly SBTI (soy bean trypsin inhibitor), which is often utilised to retard the growth of blow-fly larvae (Casu *et al*., 1994; Reed *et al*., 1999; Telford *et al*., 2011; Wilson *et al*., 2016). As the gelatin component was proven to be the most readily digested by LAP, this ingredient could be increased in the formulation. It may be used as an inexpensive alternative to agar, which is frequently used as a solidifier and base

for other ingredients, often in combination with animal blood for blow-flies (Daniels *et al*., 1991; Blenkiron *et al*., 2015). Furthermore, agar itself is considered non-nutritional (Reinecke, 1985) and is not degraded by LAP, as demonstrated by its use as the RDEA system substrate base (**Section 2.3.1**).

In summary, the findings have led to a review of the composition of the Carnival Diet and manuscript of this work is ready for journal submission. In order to evaluate these suggestions, this work is being complemented by traditional rearing studies within the BioMonde laboratories. For the purposes of this study, it seems unlikely that the Carnival Diet preparation would adversely affect the growth of the larvae destined for inclusion into debridement devices, as the eggs laid upon the Carnival Diet are removed prior to hatching, ahead of sterilisation. Eggs are then transferred to the *Lucilia* Agar substrate for hatching and initial larval feeding (≤ 24 hours), prior to inclusion within the BioBag pouches (**Section 1.3.1**). Therefore, the dietary analysis was repeated to determine the digestibility of *Lucilia* Agar formulation, and the presence of any inhibitory ingredients. A summary of the key findings is shown in **Figure 13**.

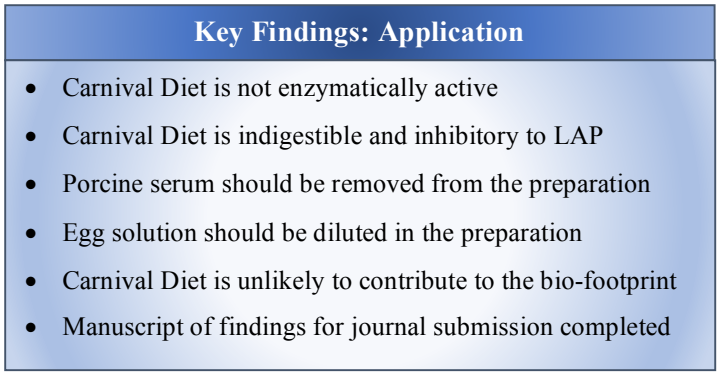

Figure 13: Summary of the key findings associated with the interrogation of the Carnival Diet, to determine its likely contribution to LAP and the bio-footprint.

3.1.2 RDEA: *Lucilia* Agar analysis

As with the Carnival Diet, BioMonde developed and implemented '*Lucilia* Agar' to avoid the use of animal tissues, in response to standardising mass-rearing and to conform to regulatory issues. Unlike the Carnival Diet, which is used to maintain the stock colonies, *Lucilia* Agar is a sterile medium used only for the hatching of eggs and the initial feeding of the larvae, which are destined for inclusion within the BioBag device, and remain under sterile conditions throughout. As the name suggests, this diet

is predominantly agar-based, although it is also supplemented with serum, soy peptone, yeast extract alongside potato starch. The serum originates from foetal calves, raised in New Zealand, where bovine spongiform encephalopathy (BSE) has not been detected, and is therefore approved for use in this capacity by the European Commission (2011). It was however, hypothesised that this component would also be inhibitory to larval enzymes.

3.1.2.1 Material and methods

Solutions for testing

Larval alimentary products were prepared as described previously (**Section 2.2.4.2**). The *Lucilia* Agar hatching plates are manufactured externally (Oxoid; Hampshire, UK), therefore the complete preparation and the individual components were obtained from BioMonde. All ingredients were supplied in powdered form (Thermo Scientific; Leicestershire, UK) and stored at ambient temperature, with the exception of the foetal calf serum (Life Technologies) which was stored in aliquots at -20ºC. *Lucilia* Agar plates were refrigerated upon receipt (4ºC).

The components were prepared in TBS (RDEA system buffer) in quantities representative of the compete formulation (**Table 5**). The components were autoclaved under 'sterile run' conditions (121ºC, 1.5 hours), with the exception of the foetal calf serum, which was incubated at 40ºC for 10 minutes. During manufacture, the serum is added to the molten solution containing the other ingredients, following autoclaving and cooling to 40ºC.

Table 5: The *Lucilia* Agar composition, showing the proportion of each food component combined with TBS elution buffer, to give a final composition which is proportionally representative of that used in the production of larvae for incorporation into BioBag debridement devices.

Diet Component	Quantity	TBS
Agar	130 mg	10 mL
Foetal calf serum	1 mL	9 mL
Potato starch	10 mg	10 mL
Soy peptone	150 mg	10 mL
Yeast extract	50 mg	10 mL

Six grams of *Lucilia* Agar were removed from three individual plates, and no additional buffer was added for component extraction. This method was in-keeping with that used for the preparation of the Carnival Diet (**Section 2.4.1.1**) and had been shown to be

suitable during earlier experimentation (**Section 2.3.2**). All material was vortexed and centrifuged (3360 *g*, 8 minutes), prior to the removal of supernatant for two cycles of micro-centrifugation (13,000 *g*, 4 minutes). Individual supernatants were stored at -20°C until use. Constituent elution was confirmed using SDS-PAGE analysis under reducing conditions as detailed previously (**Section 2.3.1**).

Inhibition and digestibility studies using RDEA and SDS-PAGE analysis.

The protein profiles of the food components and the complete *Lucilia* Agar formulation, before and after the addition of LAP, were compared using SDS-PAGE analysis. All dietary samples and LAP pellets were prepared and analysed as aforementioned (**Section 2.4.1.1**). With the exception of the agar component, sample aliquots were further diluted with ultrapure water as follows: foetal calf serum, 1.5 μL; soy peptone, 2 μL; *Lucilia* Agar, 3 μL; potato starch and yeast extract, 4 μL. All subsequent stages of the study were conducted as described for the Carnival Diet assessment.

3.1.2.2 *Lucilia* Agar results

Background activities of test samples

The existing enzymatic activity of the LAP had been confirmed using the chymotryptic assay in duplicate, with 38.64 (± 7.79) released AMC* $pmol^{-1}/\mu g$. No values were obtained for the TBS blank and the complete *Lucilia* Agar formulation. Upon RDEA analysis, the LAP positive control produced digestion reactions on all gel plates (*n*= 9 assays), with a mean area of 89.31 (± 3.09) mm^2 (*n*= 3 replicates, **Figure 14**).

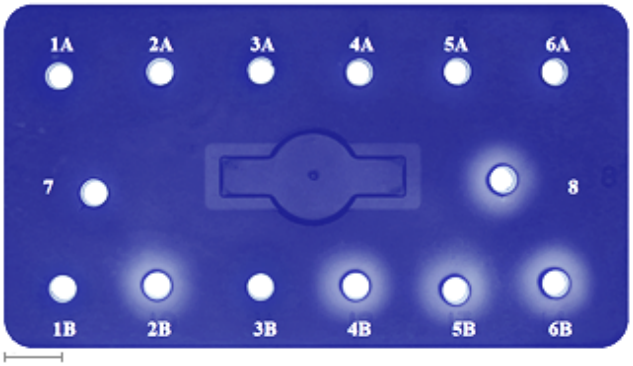

Figure 14: RDEA system for the assessment of the components of the *Lucilia* Agar hatching plate (scale bar = 10 mm). The upper row demonstrates the lack of background enzyme activity for *Lucilia* Agar (1A) and its components: agar (2A), foetal calf serum (3A), potato starch (4A), soy peptone (5A) and yeast extract (6A). Controls are shown in the centre panel: Tris-buffered saline blank (TBS, 7) and

positive larval alimentary products (LAP, 8). The lower row reveals the impact of combining each component with an equal volume of LAP (wells 9B to 14B, order as previously denoted).

The coefficient of variation value (3.46%) demonstrated a high level of assay reproducibility. Reactions were not produced by the TBS control (*n*= 9 assays), the Carnival Diet or any of the components (*n*= 3 assays). These analyses confirmed that all subsequent digestion activity recorded was LAP-derived.

Diet digestibility by LAP

The complete *Lucilia* Agar preparation was diluted to 50% with TBS, to produce a background protein profile using SDS-PAGE analysis. This was then compared to the profile obtained following the combination of the undiluted preparation with an equal volume of LAP. The profiles revealed that the *Lucilia* Agar hatching plate was only slightly digested by the larval enzymes between 110 to 160 kDa and 30 to 50 kDa (**Figure 9**). The process was repeated for each individual component, which revealed that the protein content of all ingredients, with the exception of the foetal calf serum, was minimal (**Figure 9**). The preparation appears to be dominated by this ingredient, and almost identical profiles are produced, before and after the addition of LAP.

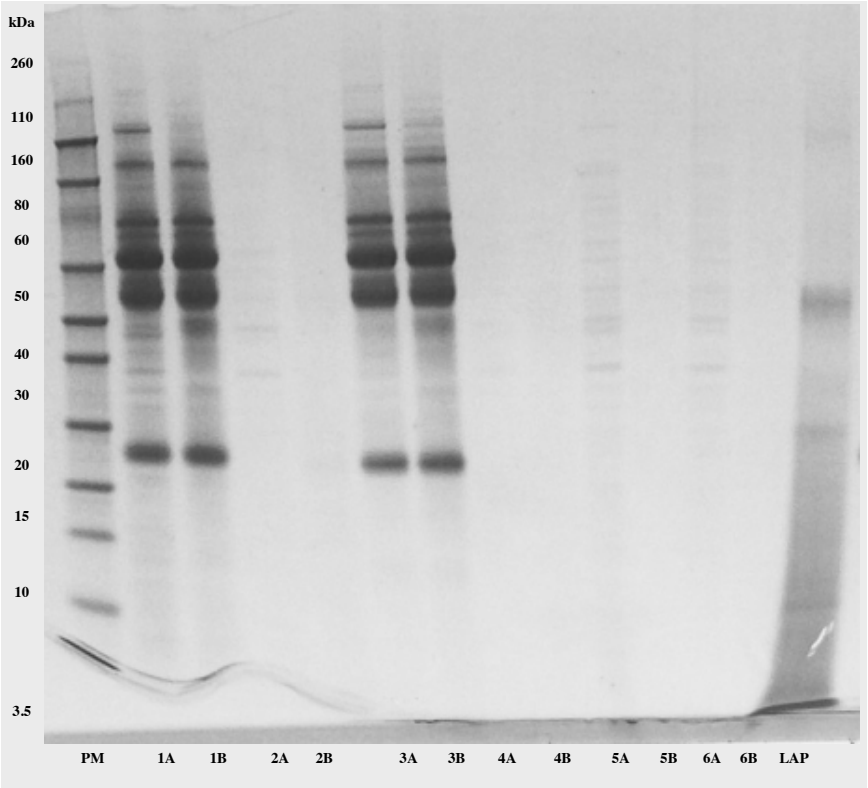

Figure 15: Digestibility of the *Lucilia* Agar hatching plate and its components by LAP, resolved using SDS-PAGE (reducing conditions). Individual dietary components are shown from left to right before (A) and after (B) digestion with LAP: complete *Lucilia* Agar preparation (1) agar (2), foetal calf serum (3),

potato starch (4), soy peptone (5), and yeast extract (6). PM: protein marker (5μL), LAP: following acetone precipitation.

LAP inhibition by diet

The RDEA system was used to assess the impact of the *Lucilia* Agar hatching plate on the enzymatic action of the LAP, and demonstrated clearly that the digestion activity had been inhibited completely (**Figure 14** and **Chart 15**). Individual analysis of each component revealed that the foetal calf serum was likely to be responsible for this observation.

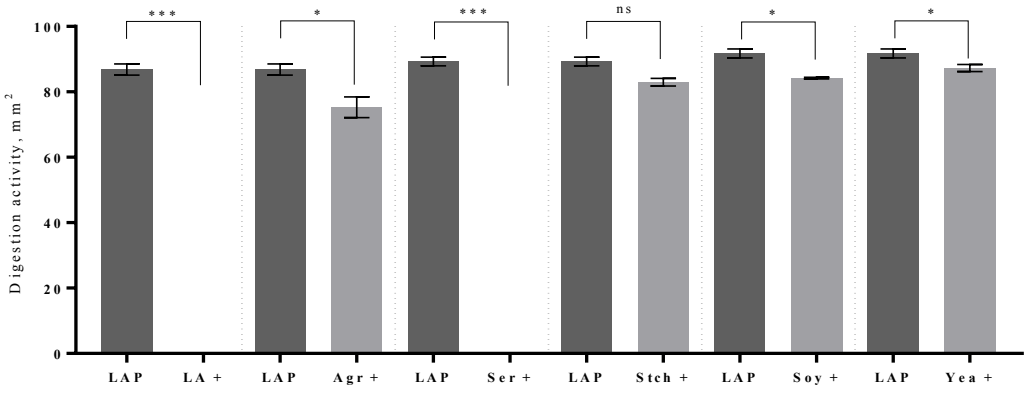

Chart 15: Analysis of the *Lucilia* Agar hatching plate and its components, against a LAP control using the RDEA system for inhibition (mean area ± SD mm^2, n=3 assays). When combined with an equal volume of LAP (+), *Lucilia* Agar (LA) foetal calf serum (Ser) completely inhibited LAP activity, whereas no significant inhibition was seen by potato starch (Stch). Agar (Agr), soy peptone (Soy) and yeast extract (Yea) had relatively less significant inhibitory effects, determined using paired t-test: ns = no significant difference (P >0.05), * = $P\leq$ 0.05, ** = $P\leq$ 0.01, *** = $P\leq$ 0.001 and **** = $P\leq$ 0.0001.

Dilution of diet and components

The *Lucilia* Agar preparation and each component were sequentially diluted to determine the resilience of the inhibitory action, in the presence of an equal volume of LAP in the RDEA system (**Chart 16**). Despite a reduction in quantity, the enzymatic action of the LAP failed to recover in the presence of the complete formulation. The foetal calf serum component continued to inhibit larval enzymes, until the penultimate titration, whereby digestion activity began to recover. The remaining components (agar, potato starch, soy peptone and yeast extract) retained their original activities throughout the titres. It was therefore considered likely that the foetal calf serum was responsible for the suppression of larval enzymes, just as the porcine serum in the Carnival Diet inhibited LAP (**Section 2.4.1.2**). The SDS-PAGE titration profiles of the *Lucilia* Agar hatching plate and the serum components demonstrates the lack of digestibility

(**Figure 16**) and support the observations seen with the RDEA analysis (other components not shown).

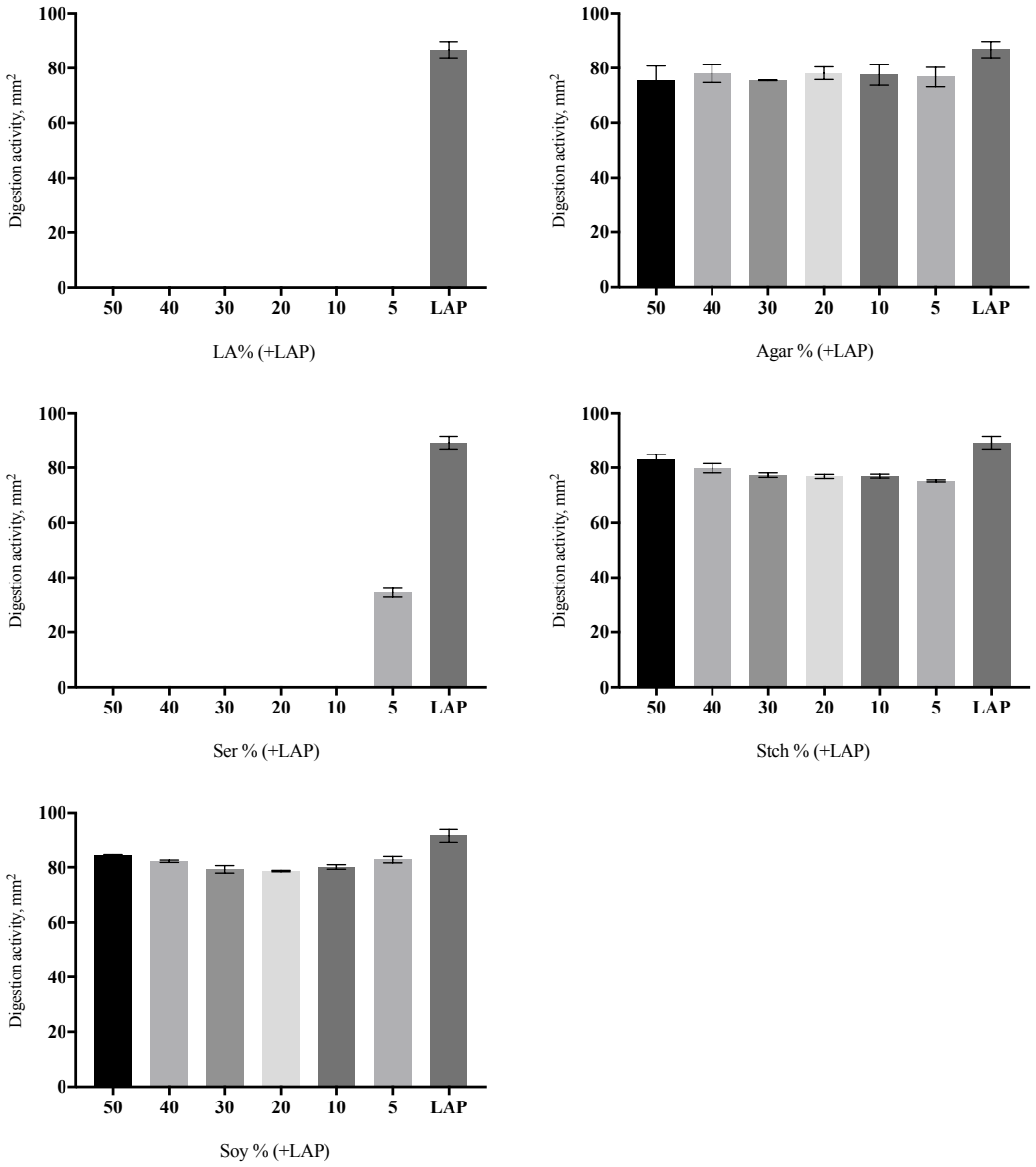

Chart 16: Inhibitory effects of the *Lucilia* Agar hatching plate and its components following their dilution and subsequent combination with a constant concentration of LAP (+) using the RDEA system (mean area ± SD mm^2, *n*= 3 assays). The inhibitory effect of the foetal calf serum (Ser) was removed following dilution, with enzyme recovery emerging at 15%, whereas the inhibitory effect of the *Lucilia* Agar (LA) was completely resistant to dilution. The agar (Agr), potato starch (Stch) and soy peptone components were not inhibitory at any of the dilutions used.

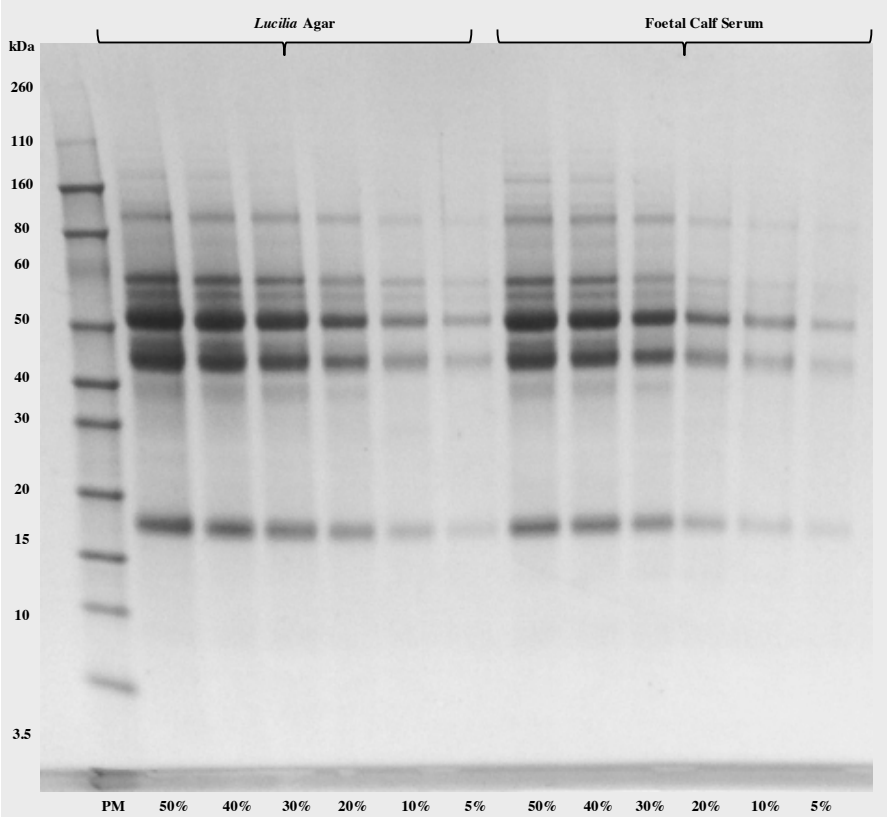

Figure 16: Protein profile (SDS-PAGE) of the LAP-resistant the *Lucilia* Agar hatching plate and the foetal calf serum component, alongside protein marker (PM). Samples were loaded in the concentrations shown following incubation (24 hours, 20°C) with a constant concentration of enzymatically active LAP.

3.1.2.3 Significance of findings

The *Lucilia* Agar hatching plate has been shown to be poorly digested by, and inhibitory to, larval feeding enzymes. As with the Carnival Diet, the presence of animal serum dominants the formulation, although in this instance, foetal calf serum (1 mL in 9 mL TBS) is already diluted in comparison to the porcine serum (1 mL in 2 mL TBS) and this may explain the recovery of the LAP at the final titre. Should this ingredient be necessary in the final preparation, a reduction in the quantity is recommended. However, based upon the findings of the Carnival Diet investigation (**Section 2.4.1.3**), the removal of the foetal calf serum is suggested. This may be shown to improve larval growth, by means of traditional rearing studies, and is currently being explored by BioMonde.

Alongside the earlier recommendation of increasing the amount of gelatin in the Carnival Diet, this could also be used to replace agar in the *Lucilia* Agar hatching plate, as these studies have shown that gelatin is readily digested by LAP, but agar is not.

Therefore, agar represents an expensive option for mass-rearing, particularly as it is non-nutritional and acts only as a gelling agent. The remaining components of the *Lucilia* Agar hatching plate did not significantly inhibit LAP, and the use of soy and yeast ingredients in blow-fly diets has been discussed previously (**Section 2.4.1.3**). Regarding potato starch, this not a common ingredient in Dipteran formulations, as such flies are unable to utilise starch as dietary sugar (Chaudhury, 2009a). Furthermore, sugars form only a small part of blow-fly nutrition, required infrequently as an energy source for the adults (Roberts & Kitching, 1974). It should be borne in mind that the *Lucilia* Agar formulation is employed for only ≤ 24 hours, to ensure maximum numbers of hatchlings from eggs of various ages. Therefore, the nutritional requirement of the hatching plate is minimal, and coupled with the inhibitory effects of the foetal calf serum, it is unlikely that the current formulation is optimal for *Lucilia sericata* larvae.

If the *Lucilia* Agar hatching plate was shown to inhibit the feeding of larvae, there is a concern that this could have an impact on treatment efficacy *in vivo*. Upon administration, the larvae are introduced into a new feeding environment, which may include other stimulants and inhibitors present in the wound, which have not been taken into account during these studies, and may vary with complex pathologies of chronic wounds and patients (Guo & DiPietro, 2010; Eming *et al*., 2014). Although it may be anticipated that once the larvae have been administered, they will commence feeding effectively, it has been shown that development rates are variable, upon different food substrates (Clark *et al*., 2006; Day & Wallman, 2017). The findings have led to a review of the composition of *Lucilia* Agar and manuscript of this work is ready for journal submission.

With regard to optimising the efficacy of the treatment, an investigation into the suppression of subsequent feeding activity following various rearing substrates may be worth consideration in future studies. It is also possible that the larvae, and/or the dietary components harbour symbionts, which may be released with the action of LAP, thus contributing to the total bio-footprint. A summary of the key findings is shown in **Figure 17**.

Key Findings: Application
• *Lucilia* Agar is not enzymatically active • *Lucilia* Agar is indigestible and inhibitory to LAP • Bovine serum should be removed from the preparation • *Lucilia* Agar is likely to contribute to the bio-footprint • Manuscript of findings for journal submission completed

Figure 17: Summary of the key findings associated with the interrogation of *Lucilia* Agar, to determine its likely contribution to LAP and the bio-footprint.

3.1.3 RDEA: Endotoxin analysis

It is known that the many insect species, including members of the *Lucilia* genus harbour internal symbionts, some of which have been shown to be associated with digestion and feeding of the larvae (Hobson, 1932a; Greenberg, 1965; Singh *et al*., 2015). It is therefore possible that such bacteria are liberated from the gut during the release of LAP. Acknowledging the potential for harm should pathogenic and/or pyrogenic bacteria be transmitted to a patient, particularly those with existing medical conditions (Kiers *et al*., 2017) and that lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria, can act as a potent cellular activator (Kisch *et al*., 2015) in LAP assays, it was deemed prudent to screen the LAP for the presence of endotoxins.

The Food and Drug Administration (FDA, USA) permit 20 endotoxin units (EU) per medical device, following rinsing with non-pyrogenic water (FDA, 2012). Although the construction materials of the BioBag are unlikely to exceed this level, and the larvae are reared under sterile conditions, the content of LAP post-dispatch is unknown. The *Limulus* amoebocyte lysate (LAL) chromogenic assay was selected for use, as this is approved by the FDA, MHRA and the European Pharmacopoeia (EMA, 2013). Although a number of variations are available, they are all based on a cascade of enzymatic reactions, it was discovered that these are subject to interference by serine proteases (Ding & Ho, 2010). It was therefore necessary to denature those known to be endogenous to LAP (Casu *et al.*, 1996; Chambers *et al.*, 2003). To assess the degree of false positivity, native and heat-treated LAP were subjected to testing and the efficacy of the enzyme denaturation was confirmed using the RDEA system.

3.1.3.1 Material and methods

All material was handled inside a Class II microbiological safety cabinet unless otherwise stated. All reagents and consumables obtained from Sigma-Aldrich Ltd unless otherwise stated and certified as endotoxin-free, and unopened before use.

Solutions for testing

Collection solutions from six complete BB-50 devices and corresponding controls were prepared as described previously **(Section 2.2.4.2).** Aliquots of LAP solution (200 μL) from individual BioBags were prepared for testing, in duplicate pairs of native and heat-treated material (90ºC for 10 minutes). Aliquots from the control devices were not heat-treated.

RDEA assay

All samples were tested in the RDEA system in duplicate and incubated for 6 hours at 37ºC. Following fixation and staining, gel plates were photographed, and the images were prepared and measured using ImageJ software as outlined in **Section 2.4.1.1.**

Endotoxin assay

Endotoxin quantification was undertaken using a chromogenic *Limulus* amoebocyte lysate (LAL) assay kit (Pierce Biotechnology), whereby bacterial endotoxins trigger a cascade of enzymatic reactions which culminates in the release of yellow p-nitroaniline (pNA) from the colourless substrate (acetyl-isoleucine-glutamic acid-alanine-arginine-pNA). Test samples and endotoxin standards (derived from *Escherichia coli*) were prepared according to manufacturer's guidelines, and modified for incubation using 1.5 mL centrifuge tubes, to ensure the assay was performed under closed conditions when not inside the safety cabinet. Absorbance was measured at 405 nm using Tecan Spark 10M microplate-reader, and the assay was performed in duplicate.

Data analysis

All data were analysed using GraphPad Prism and normality was examined using the Shapiro-Wilk test. For the RDEA, gelatinase activity is expressed as the mean halo area (mm^2, in duplicate) ± SD, and corrected to remove the test well. For the endotoxin assay, data were corrected to exclude background readings, and the values obtained as endotoxin units (EU/mL) for each test sample were interpolated using the standard curve produced for each assay replicate. Endotoxin content per device (EU/device) was

extrapolated, based on the elution volume of 2.5 mL PBS. The significance of LAP-denaturation was determined by the Wilcoxon matched-pairs test (for non-parametric comparisons). The relationship between larval number and endotoxin presence in denatured LAP, was examined for linearity and correlation using the Spearman coefficient for non-parametric data. In all cases, $P< 0.05$ was considered significant.

3.1.3.1 Results

In the RDEA system, the LAP samples from all larval-loaded BioBags (n= 6) produced digestion halos, with a mean area of 80.8 (± 6.66) mm^2. The control devices and TBS blanks did not exhibit any digestive activity (**Chart 17**). Enzyme denaturation of the LAP samples was confirmed following heat-treatment (**Figure 18**).

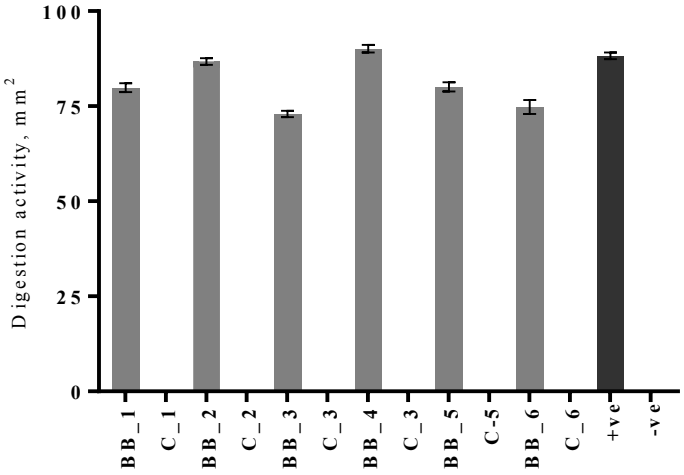

Chart 17: Gelatinase activity of individual larval-loaded BioBags (BB) and control devices (C) as tested in the RDEA system, alongside positive collagenase (+ve) and negative TBS buffer (-ve) controls. Activity determined by digestion area (mean ± SD, in duplicate, mm^2), n= 6 devices per group.

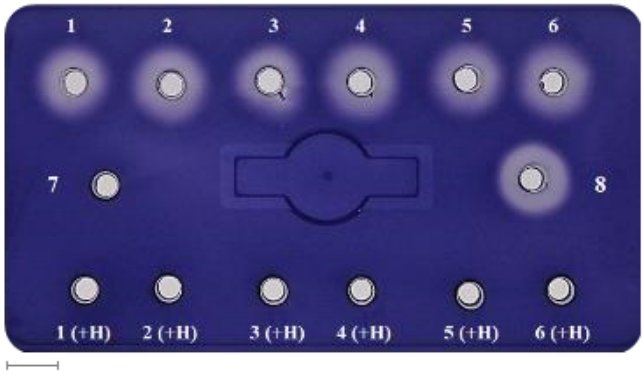

Figure 18: Example of RDEA system for visual confirmation of existing gelatinase activity of individual larval-loaded BioBags (wells 1-6) and subsequent denaturation by heat (+H) of matched samples, alongside negative TBS buffer (well 7) positive collagenase (well 8). Scale bar = 10 mm.

The larval-loaded BioBags (*n*= 6) all tested positive for the presence of endotoxins, with a mean value of 1.62 (± 0.29) EU/device, prior to denaturation. Following heat-treatment, a reduction of 24 to 60% was observed throughout the group (**Chart 18**), with a mean value of 0.92 (± 0.36) EU/device. This difference was shown to be statistically significant (*P*= 0.031). Control devices were not heat-treated and a background level of 0.15 (± 0.02 EU/device) was recorded throughout (**Chart 19**).

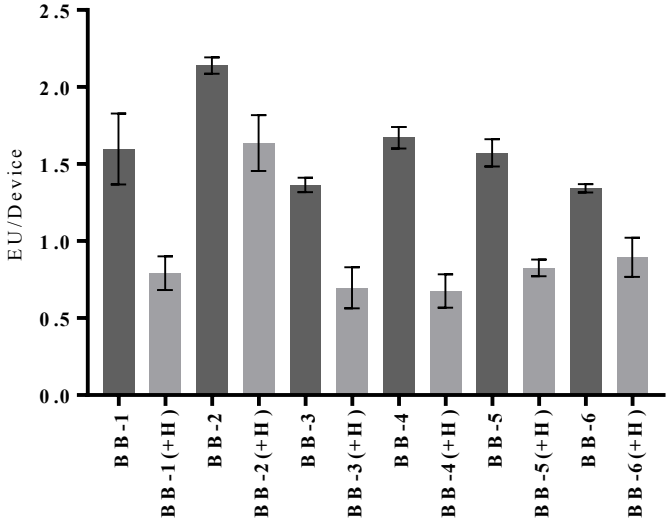

Chart 18: Endotoxin content of individual larval-loaded BioBags before (BB) and after heat treatment of matched samples (+H), as determined by LAL chromogenic assay (± SD, in duplicate, EU/device). A reduction of 24 to 60% was observed post-denaturation.

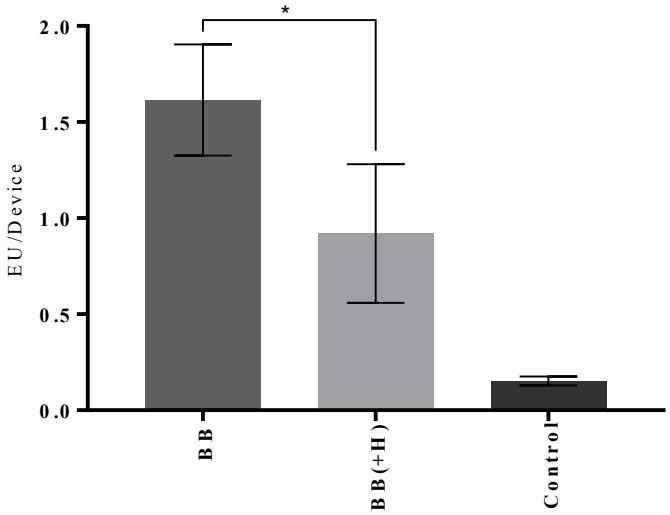

Chart 19: Mean endotoxin content of larval-loaded BioBags before (BB) and after heat treatment of matched samples (+H), alongside control materials (*n*= 6 devices per group), as determined by LAL chromogenic assay (± SD, in duplicate, EU/device). Statistical significance (*, *P*= 0.0031) determined using Wilcoxon matched-pairs test.

Following conversion, the residual levels of LPS detected post-denaturation were determined to be 0.04 (± 0.02) ng/mL, where one EU/mL equals approximately 0.1 ng endotoxin/mL (Unger *et al.*, 2014).

The relationship between the residual endotoxin levels in denatured LAP was examined against the number of larvae within the corresponding BioBag (**Chart 20**). The dataset is small, but a linear relationship emerged (R^2= 0.6802), and this was deemed statistically significant using the Spearman coefficient for non-parametric data (*P*= 0.0167).

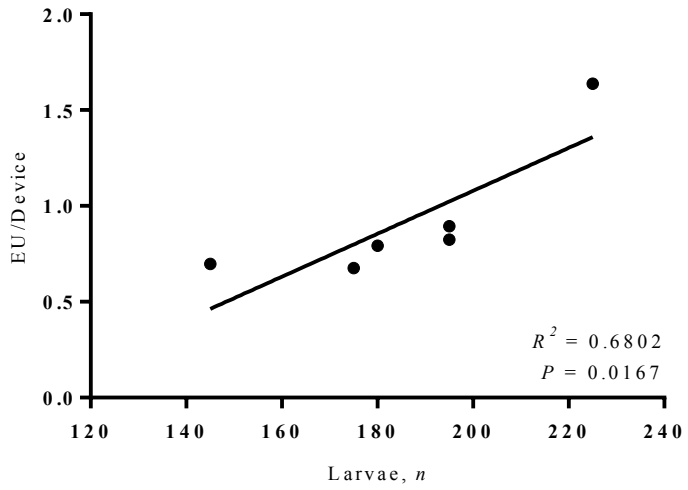

Chart 20: Emerging linear relationship between endotoxin units (EU) per BB-50 debridement device (*n*= 6) and the number of larvae contained in each BioBag. Significance of the correlation determined using Spearman coefficient for parametric data.

3.1.3.1 Significance of findings

The materials used in the construction of the BioBag, and LAP derived from the larvae contained within the device, fall below the endotoxin limit as required by the FDA (2012). Although this finding may be anticipated, when investigating a regulated medical product, a low-level of residual endotoxin presence was detected. During the course of study, none of the BioBags were recalled on the basis of contamination or failure during microbial screening. It is therefore likely that endogenous bacterial symbionts of *Lucilia sericata* larvae are the source (Hobson, 1932a; Singh *et al.*, 2015). Lipopolysaccharides (LPS) arising from the outer membrane of such Gram-negative bacteria, have the potential to act as febrile agents (Kiers *et al*., 2017), and also as a potent stimulator of cellular activation (Koff *et al.*, 2006; Kisch *et al.*, 2015). This

finding is relevant to LAP-based cellular assays, as used in investigations of the wound healing attributes of larval material (Prete, 1997; Sun *et al.*, 2016) whereby activity may be credited to LPS associated with otherwise 'sterile' larvae. Investigators should be aware of the potential for LPS-mediated confounding effects, especially where the effects observed were not neutralised by other means, such as with fibroblast migration mediated by larval enzymes (Horobin *et al.*, 2005). This is a particular concern where non-regulated rearing methods are employed. The recommendation that larval material should be denatured in such assays, alongside regular endotoxin testing was published as a result of this work (Pickles & Pritchard, 2017a).

An alternative scenario, is the incomplete denaturation of all LAP enzymes. The full spectrum of substances produced by the larvae is not known and the RDEA system examines gross gelatinolytic properties. As a simple, semi-quantitative assay, it is not suited to investigations requiring high sensitivity. However, the denaturation step (90^{o}C, 10 minutes) was effective for the purposes of the RDEA assessment, but a reduction of only 24 to 60% was observed in the LAL assay post-heating. However, it must be noted that all BioBags were over-loaded, and there was a linear relationship between larval number and endotoxin units. The heating step is not adequate for endotoxin destruction, as depyrogenation requires temperatures in excess of 200^{o}C, for sustained periods (Sandle, 2011). It is possible that the carry-over from the diet ingredients may also have contributed to the bio-profile, such as the plant-based components, as these are known to harbour many symbionts (Thajuddin *et al.*, 2015). Although the *Lucilia* Agar preparation is autoclaved, the temperatures rarely exceed 200^{o}C and maximum temperatures are usually for only brief periods. Conventional autoclaving (moist heat treatment, 121^{o}C) is not adequate for depyrogenation (Sandle, 2013).

The range of residual LPS levels remaining, suggest dynamic variation, although there appears to be a linear relationship between endotoxin presence and larval number. Whether these findings are due to symbiont presence, differential enzyme expression or larval feeding, is not yet known. For the purposes of this study, all factors may be relevant, when considering the results of more sophisticated analyses. A summary of the key findings is shown in **Figure 19**.

Key Findings: Application
• BioBag construction materials are endotoxin-free • LAP enzymes must be denatured prior to LAL testing • Larval-loaded BioBags possess a residual endotoxin presence • Endotoxin presence increases with increased larval numbers • Bacterial symbionts are the likely source of LPS • Bacterial symbionts are likely to contribute to the bio-footprint • Published findings: Pickles & Pritchard (2017a)

Figure 19: Summary of the key findings associated with the endotoxin investigation of LAP, to determine their potential contribution to LAP to the bio-footprint.

3.2 Bio-footprint conclusions

The BB-50 is the most commonly used contained debridement device. The physical separation of the maggots from the patient, confirms that substances released by the larvae are responsible for the clinical outcomes. It has been possible to collect these alimentary products into a solution suitable for testing, and to visualise the impact of these products on the wound environment. Methodologies have been designed to create standardised material, from a regulated source, to investigate the bio-footprint of the device and to determine what a patient may be exposed to at the onset of treatment. Until now, no such studies have been concerned with the impact of larvae within the first 24 hours of administration.

Visualisation of the bio-footprint not only confirmed the enzymatic action of the larvae during this time period, but also suggested that the maggots behave normally whilst confined within the BioBag. However, a concern regarding the over-loading of devices has become apparent, and this is likely to affect larval feeding and survival, particularly with the exponential growth of the larvae when actively feeding. Although the dosage recommendation is 5 to 10 maggots per cm^2 of wound surface (Sherman, 2003), it has been shown that 5 to 7.5 larvae (assume 6 to 8 whole insects) is the optimal loading for BioBags (Wilson *et al*., 2016). However, it should be noted that the pouch size of the BB-100 was used (4 × 5 cm), and counted larval-loading was specific to the study. Herein, no BB-50 contained 50 maggots, and the smallest count was 80 larvae, across 66 BioBags, which were received as dispatched to practitioners, i.e. as manufactured. Undue pain and pressure is associated with increased numbers of maggots in the wound,

and the exponential growth of the larvae, could cause the pouch to rupture. The initial spread of LAP in the presence of excess saline as received (**Figure 1**), demonstrated that the solution would be appropriate for the collection of alimentary products, and that gelatin is a suitable, wound-relevant substrate for the assessment of enzymatic activity. These findings led to the development of a SOP for the collection of LAP, and a QC assay, the RDEA.

The initial parameters used in the evaluation of LAP, demonstrated wide variation and although significance was assigned to the relationships of protein content versus chymotryptic activity, and larval number versus. chymotryptic activity, no clear linearity or discrete correlations were observed. It is believed that the potency of specific chymotryptic activity is not directly related to total protein content, and this is reinforced by the findings of the RDEA validation study. Here, a linear relationship between larval number and gelatinase activity emerged, which was deemed to be extremely significant (**Chart 12**). Although chymotrypsin is the most well-characterised enzyme, LAP is known to contain a suite of biochemically distinct enzymes (Telford *et al*., 2012) many of which may degrade gelatin, acting as a model for collagen. The RDEA offers the opportunity to identify further enzymes, by the incorporation of other substrates or inhibitors into the agar base.

The RDEA system has shown to be versatile, by its application to diet interrogation and heat denaturation study for the endotoxin investigation. Both the Carnival Diet for colony maintenance and the *Lucilia* Agar hatching/feeding medium were shown to contain inhibitory components, predominantly the porcine and bovine sera respectively. Both formulations could be improved, with a view to not only reducing the cost of unnecessary ingredients, but simple modification of the existing recipes could improve digestibility. Although it could be argued that upon introduction to a wound, the larvae are exposed to a more complex substrate, it is possible that LAP is under-performing at the outset. Accepting that maggot-containment is a less effective form of treatment and larvae are re-ingesting their own products, alteration of the *Lucilia* Agar formulation to create an optimal preparation may improve feeding, and therefore debridement.

In order to progress to the next phase of the study, it was necessary to have confidence in the quality of the LAP. Acknowledging the contribution of the diets, in particular the *Lucilia* Agar to the composition of LAP, and the possibility of bacterial symbionts in

both the larvae and also the plant components, LAP was screened for the presence of endotoxins. As an enzymatic assay, subject to interference by serine proteases (Ding & Ho, 2010) it was necessary to denature those known to be endogenous to LAP (Casu *et al*., 1996; Chambers *et al*., 2003). Without this step, spurious and misleading results would have been obtained. Upon completion, endotoxin presence was still detected, but it must be borne in mind that all BioBags were over-loaded (145 to 225 larvae) and there appears to be a linear relationship between larval number and endotoxin units (**Chart 14**). Therefore, if the number of maggots per BB-50 was in line with its numerical denotation and the recommended dosage for treatment, endotoxin content would be reduced.

Regardless of the variation with these parameters, the devices received were as those dispatched for patient use. Therefore, the findings of this study are authentic, and the LAP model is a suitable substitute for the whole organism. Additionally, attempts at conducting this work with meat-reared maggots would likely create complications in analysis and interpretation, and this is best demonstrated by the difference in LAP solutions as shown in **Figure 5**. Having achieved confidence in the quality of the standardised LAP solution, it was possible to test its suitability for more specific exploration, using instrumental analysis.

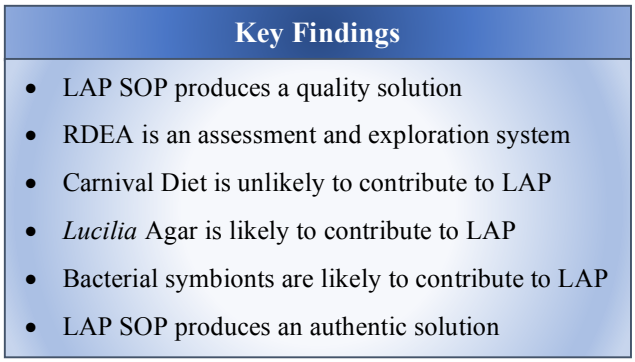

Figure 20: Summary of the key findings associated with the interrogation of LAP, to determine likely contributors to the total bio-footprint.

Chapter Four: Protein Exploration

4.0 Abstract

This chapter, 'Protein Exploration' details the high molecular weight investigation of the bio-footprint, by subjecting complete larval alimentary products to peptide mass fingerprinting methods. To attempt such analyses, using a total protein mixture would not have been possible, without first having confidence in the quality of the solution, which was achieved following the 'Standardisation' and 'Assessment' phases of the study. Although it was possible to putatively identify proteins from the fragments produced, it became evident that the proteome of *Lucilia sericata* larvae is largely uncharacterised. Despite this, it was possible to link the functional proteins to wound healing activities.

4.1 Protein exploration methods

Once confidence in the quality of the LAP samples had been achieved, it was possible to subject them to more sensitive analyses. Although the protein content of LAP is low, it can be surmised that with actively feeding larvae this will vary and become more complex. In order to establish a baseline profile of the initial bio-footprint proteins, LAP was subjected to peptide mass fingerprinting (PMF), whereby proteins are cleaved by enzymatic digestion into smaller peptides, prior to analysis (Henzel *et al.*, 2003; Yates, 2013). Such whole proteome studies tend to be conducted with a pure protein or very simple mixtures.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was employed for a 'bottom-down shotgun' approach. Here, the peptides released from the proteins are separated and subsequently detected by mass, and the spectra generated can then be searched against available databases for the identification of proteins. This differs from the 'top-down' methods which utilise gel electrophoresis for separation, and bands or spots are isolated and individually excised from the gels for further analysis (Zhang *et al.*, 2013). As can be seen from the SDS-PAGE images relating to diet investigation (**Section 2.4.1.2** and **Section 2.4.2.2**), precipitated LAP appears as a smear, with very few distinct bands. Therefore, the methodology was trialled with LAP collected from the existing protocol (**Section 2.2.1.1**) and then repeated with samples obtained using the SOP (**Section 2.2.2.1**).

4.2 Pilot study: initial protocol LAP

Prior to this, no studies pertaining to whole proteome analysis of LAP appeared to have been published. It is possible that the complexities arising from larval feeding on animal tissues may have prevented others attempting this approach.

4.2.1 Materials and methods

All reagents were obtained from Sigma-Aldrich Ltd (Missouri, USA), unless otherwise stated.

Preparation of LAP

Collection solutions were prepared from larval-loaded BioBags and control devices (n= 6 per group), and assessed as described previously (**Section 2.2.1**). Chymotryptic activity was confirmed in all LAP samples. The three LAP solutions with the highest protein concentrations ($\bar{x}$= 7.4 ± 1.12 μg/mL) from the batch received were selected for further analysis, alongside the corresponding controls.

Peptide acquisition

All stages were undertaken by Dr Lina Dahabiyeh from the Division of Molecular and Cellular Science (University of Nottingham); however, the complete process was observed and documented throughout.

Seventy microlitres of each sample solution was denatured (6 M urea) prior to reduction (50 mM DTT) and alkylation (200 mM iodoacetamide) reactions at 37°C in the dark (30 minutes), within 10 kDa Amicon Ultra 0.5 mL centrifugal filters for purification. Upon completion, samples were washed twice (50 mM ammonium bicarbonate) at 14,000 *g* for 8 minutes, to remove excess reagents. The protein mixtures were prepared to a volume of 100 μL (50 mM ammonium bicarbonate) prior to the addition of M/S grade porcine trypsin (Promega; Hampshire, UK) for overnight proteolysis at 37°C. The reaction was quenched (1% formic acid) prior to centrifugation at 11,000 *g* for 5 minutes, and the upper protein layer transferred to chromatography vials for analysis.

An ion trap mass spectrometer (Thermo Fisher LTQ Velos, Thermo Fisher Scientific; California, USA), coupled to an ultra-performance liquid chromatograph (Advance Chromatography Technologies Ltd; Aberdeenshire, Scotland) with electrospray ionisation (ESI) running in the positive ion mode, was used to analyse the tryptic

digests. Peptides were separated by reverse-phase LC and eluted at a flow rate of 300 μL/min. Tandem MS spectra were acquired and analysed using Xcalibur 2.2 software (Thermo Fisher Scientific).

Raw data files were converted to Mascot generic format (MSConvert; available from www.proteowizard.sourceforge.net) for ion searching using the Mascot interface (v.2.3.01, Matrix Science Inc.; available from www.matrixscience.com). The National Center for Biotechnology Information (NCBI) non-redundant database was selected, in order to search the obtained peptides against *Drosophilia* (fruit-flies) entries, as this was the only entomological genus available. Peptides were divided into two sets: those which were matched to *Drosophilia* proteins, and those which were not matched. The peptide sets were supplied for subsequent analysis and interpretation.

Data analysis

All data were handled using GraphPad Prism v.7.0b. Individual sequences were searched against the BLASTp suite (basic local alignment search tool, for proteins), supplied by the NCBI for searching against non-redundant protein sequences (available from: www.blast.ncbi.nlm.nih.gov/Blast.cgi). Expectation values (E) of ≤ 1 were considered significant. Potential functions of named proteins and regions of larval expression in *Drosophila* were obtained from the FlyBase resource (v. FB2017_05; available from: www.flybase.org) as supplied by The Genetics Society of America.

4.2.2 Results

The supplied dataset was comprised of 50 peptides, of which the average length was 11 (± 3) amino acids (range: 6 to 18 residues). Thirty-eight peptides were associated with the BioBag samples (76%) and 12 with the control devices (24%). Potential biomarkers based on sequence similarity were absent, as all the peptides within each group were different. Of the 50 peptides, only 10 had matches within the Mascot *Drosophila* protein database (**Table 6**), none of which were associated with named, functional proteins.

(Continued after **Table 6**)

Table 6: Numbers of peptides detected following a pilot study of the use of LC-MS/MS proteomic analysis of BioBag devices. Solutions from larval-loaded BioBags (BB) and control devices (C) were subjected to tryptic digest prior to separation. Of the 50 peptides, only 10% of the sequences were found in the Mascot *Drosophila* database (Matched), the remaining peptides (Non-Matched) accounted for the majority of the results.

	BB 3	**BB 5**	**BB 6**	**C 3**	**C 5**	**C 6**
Matched	4	3	3	0	0	0
Non-Matched	7	12	9	5	7	0
Total	11	15	12	5	7	0

Each sequence was submitted to the BLASTp suite, for searching against non-redundant protein sequences of the entire GenBank database. As of September 2017, the GenBank database held over 200,000,000 sequences (NCBI, 2017b). As a collection of all publicly-available sequences, it offered the opportunity for further exploration of the dataset, in comparison to the limited sequences held in the Mascot database.

The BLASTp results were capped at 100 matches per peptide. Potential protein matches are scored and assigned a level of significance based on the expectation (E-value) of finding other similar matches in the database. Although potential matches were found for each peptide (including those initially assigned by the Mascot database as non-matched), only 11 (22%) were associated with a named, functional protein. The remaining top hits were either 'hypothetical' (16%) or 'uncharacterised' (62%) proteins (**Chart 21**), suggesting that these have no known biological function. Furthermore, only half of the dataset had an E-value of ≤ 1. This value describes the number of hits that can be expected by chance; the lower the figure, the more significant the match (NCBI, 2017a). The E-value is calculated from an algorithm which incorporates the length of the query sequence and the number of residues in the database. The latter will change over time, as more sequences are submitted and the same query can yield different results at a later stage (Kerfeld & Scott, 2011). An E-value of 1 can be interpreted as meaning that one match with a similar score, may be expected by chance in the selected database.

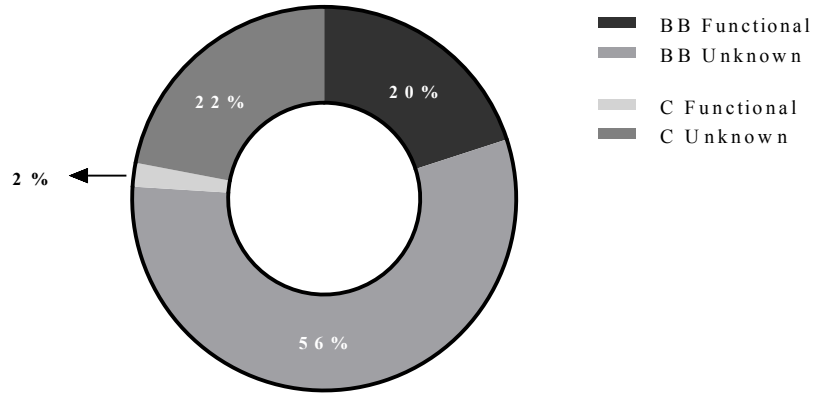

Chart 21: Initial overview of the number of peptides (*n*= 50) detected during a pilot study of LC-MS/MS proteomic analysis of BioBag devices and their putative assignment to matched proteins in the complete GenBank database. The majority of the peptides were assigned to proteins of unknown function in both the larval-loaded BioBags (BB; 56%) and the control devices (C; 22%).

As GenBank permits taxon-specific searching, the 50 peptides were re-submitted to the BLASTp suite, against the *Lucilia* genus database (taxid: 7374), which comprises approximately 25,000 sequences. As two *L. cuprina* and one *L. sericata* genomes have been added since 2014, it was anticipated that this database would improve the likelihood of protein identification. Once again, 11 functional proteins were named (**Chart 22**), and three had been identified by searching against the complete GenBank database. Using the *Lucilia* database, 54% of the total peptide set produced a top hit with an E-value of ≤ 1.

Protein matches with an E-value ≤ 1 generated from each database were selected for further consideration. Of the 25 sifted-hits obtained from the complete GenBank database, 18 were associated with the larval-loaded BioBags (72%), and seven with the control devices (28%). Only three of the hits were matched to named proteins.

(Continued after **Chart 22**)

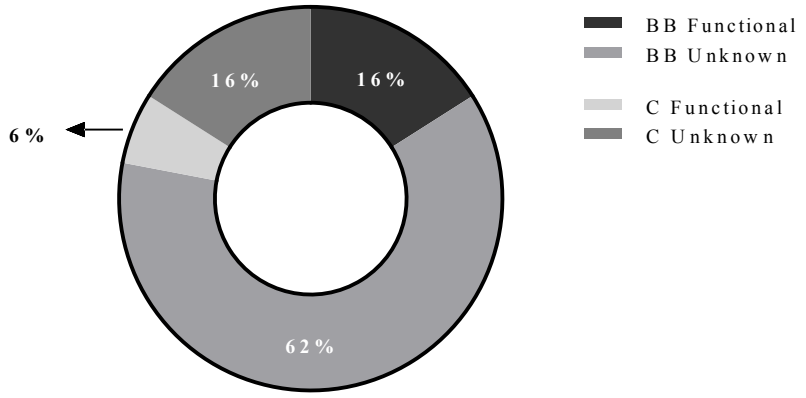

Chart 22: Initial overview of the number of peptides (*n*= 50) detected during a pilot study of LC-MS/MS proteomic analysis of BioBag devices and their putative assignment to matched proteins in the GenBank *Lucilia* genus database. The majority of the peptides were assigned to proteins of unknown function in both the larval-loaded BioBags (BB; 62%) and the control devices (C; 16%).

Of the 27 sifted-hits obtained from the *Lucilia* sub-database, 20 were associated with the larval-loaded BioBags (74.1%), and seven with the control devices (25.9%). Seven of the hits were matched to named proteins, with one sequence being found in both databases (**Table 7**).

Table 7: Putative protein identifications following BLASTp analysis of 50 peptides detected during a pilot study of LC-MS/MS proteomic analysis of BioBag devices. Sample origin and peptide sequence are shown for results obtained from the complete GenBank database (upper, all matched to *Drosophila obscura*) and the *Lucilia* genus sub-database (lower, all matched to *Lucilia cuprina*). One peptide was common to both sets of results (bold text).

Sample	Peptide Sequence	Associated Protein [species]
BB 5	**VIFTPAAPHTHARIMVI**	Dopamine N-acetyltransferase-like [*D. obscura*]
BB 5	NREGLGIPLK	Proteasome maturation protein-like [*D. obscura*]
C6	SAQDAVVLGTAKR	Ectopic P granules protein 5 homolog [*D. obscura*]
BB 3	NSRPLSTAVPGSSIEEIK	E3 ubiquitin-protein ligase mind-bomb [*L. cuprina*]
BB 5	**VIFTPAAPHTHARIMVI**	Dopamine N-acetyltransferase [*L. cuprina*]
BB 6	EALVLISDAIAK	Enolase [*L. cuprina*]
BB 6	NLEAAASTK	Laminin subunit alpha [*L. cuprina*]
BB 6	LSIDLSDVPR	Rho GTPase-activating protein 92B [*L. cuprina*]
C 3	GQNTRTLSSLPPR	PDZ and LIM domain protein Zasp [*L. cuprina*]
C 3	LLSNGDAGRPK	mRNA-decapping protein 3 enhancer [*L. cuprina*]

4.2.3 Significance of findings

Of the 50 peptides detected during the proof of principle study, 10 were putatively matched to proteins of known functions. Although the peptide sequences are varied between replicates and the identifications are unconfirmed, confidence in the method was achieved by obtaining 25 results with an E-value ≤ 1 from the complete GenBank

database. All but one of the matches were assigned to *Drosophila* species and the remaining peptide was matched to *Lucilia cuprina*. The outcome of searching a database of this size, using short sequences obtained from a relatively unstudied species, was uncertain. However, the methodology employed has produced results, and for the purposes of this investigation, it will be assumed that the identifications are reliable, in order to demonstrate the potential of the technique (**Table 8**). Furthermore, the detection of peptides in the control samples (n= 3 peptides) and the subsequent protein assignments will be considered as contamination or carry-over, at this stage (discussed further in **Section 3.3.3** and **Section 3.4**).

By searching the named proteins against the *Drosophila* FlyBase resource, it is possible to speculate as to the potential role and location of expression of the proteins within *L. sericata* larvae. Although the fruit-fly is the most well-characterised genetic model, and it shares much of its biochemistry with *Lucilia*, caution must be exercised on account of the feeding preferences of the two insects. However, it can be seen from **Table 8** that many of the proteins are associated with the excretory system (gut and Malpighian tubules), which again provides confidence in the method.

Dopamine N-acetyltransferase may be considered as the protein with the highest putative identification confidence, as it was detected in both databases, and with the greatest significance in *Drosophila*. It is involved with the regulation of daily melatonin biosynthesis (Cheng *et al.*, 2012), which acts a hormonal mediator of circadian cycles and photoperiodism (responses to the length of day or night), and is synthesised from serotonin. These three proteins have been associated with positive wound healing effects: dopamine and serotonin for neovascularisation and epithelial growth (Shome *et al.*, 2011; Mann & Oakley, 2013) and serotonin and melatonin for anti-inflammatory properties (Pugazhenthi *et al.*, 2008). Each of these proteins has been linked to angiogenesis, which in turn has been related to LAP studies (Bexfield *et al.*, 2010), and numerous clinical observations (**Section 1.3** and **Section 1.4.3**).

Table 8: Putative protein identifications following BLASTp analysis of 50 peptides detected during a pilot study of LC-MS/MS proteomic analysis of BioBag devices. Sequences are shown with associated genus and E-value (selected for exploration if ≤ 1). One peptide was common to both sets of results (bold text). Peptides matched to proteins with known functions in *Drosophila* species were used to identify the primary regions of expression in the larvae (source: FlyBase). The potential role these proteins may play in the wound environment have been speculated (individual references cited in main body of text).

Detected Peptide (E ≤ 1)	**Protein function and location of peak larval expression**	**Potential relevance to wound healing**
VIFTPAAPHTHARIMVIK (*Drosophila,* E= 3×10^{-10}) (*Lucilia,* E= 0.29)	Dopamine N-acetyltransferase (midgut): Regulation of the circadian sleep/wake cycle. Biosynthesis of melatonin from serotonin.	Dopamine: Angiogenesis and vascular endothelial growth regulation. Serotonin: Inflammation, vasoactivity and epithelial regeneration. Melatonin: Anti-inflammatory and immunomodulatory effects.
NREGLGIPLK (*Drosophila,* E= 0.61)	Proteasome maturation protein (throughout): Involved in aggregation, arrangement and bonding together of a mature, active complex, and proteolysis.	Proteasome maturation protein: Essential for proteasome biogenesis. Proteasomes: Degrades and recycles proteins (ubiquitin tagging). May be activated by bacterial challenge.
SAQDAVVLGTAKR (*Drosophila,* E= 0.004)	Ectopic P granules autophagy protein 5 (Malpighian tubules): Involved in autophagy (degradation of cellular components), the creation of lysosomes and endosome recycling.	Autophagy: Involved in inflammation and wound healing regulation. Lysosomes: Involved in autolytic debridement and disinfection. Endsomes: Precursors to lysosomes, some respond to bacteria.
NSRPLSTAVPGSSIEEIK (*Lucilia,* E= 0.21)	E3 ubiquitin-protein ligase mind-bomb (throughout): Involved with proteasomes, protein ubiquitination (modification) and endocytosis.	Ubiquitin: Role in inflammation and bacterial response, reduction in erythema and oedema. E3 ligase: Involved in regulation of inflammatory cascade.
EALVLISDAIAK (*Lucilia,* E= 0.003)	Enolase (throughout, esp. hindgut): Metalloenzyme involved primarily in glycolytic and glucose metabolic processes.	Alpha enolase: Multifunctional enzyme, inflammatory response. Plasminogen receptor, stimulating plasmin activity (proteolytic activity). Involved in ECM remodelling and cell migration.
NLEAAASTK (*Lucilia,* E= 0.056)	Laminin subunit alpha (hindgut): Binding receptor, mediates migration and organisation of cells into tissues. Interacts with ECM components.	Laminin subunit alpha-1: Structural constituent of ECM. Contributes to re-epithelialisation and angiogenesis. Not expressed in adult tissues, interest in induced expression for therapeutics.
LSIDLSDVPR (*Lucilia,* E= 0.82)	Rho GTPase-activating protein 92B (fat body and midgut): Binds to and increases GTPase activity (GTP; guanosine triphosphate). Epithelial wound repair functions.	Rho: GTPase sub-family involved in cytoskeletal functions. GTPase: Regulator of G-proteins (molecular switch). Rho-family GTPases: Essential role in blood vessel tubulogenesis.
GQNTRTLSSLPPR (*Lucilia,* E= 0.063)	PDZ and LIM domain protein Zasp (larval hindgut): Scaffold protein, regulates adhesion of cells to ECM and activates integrins.	Integrins: ECM adhesion receptors, includes collagen, laminin and fibronectin. Various forms and functions, interested in up and/or downregulating expression for therapeutics.
LLSNGDAGRPK (*Lucilia,* E= 1)	Enhancer of mRNA-decapping protein 3 (throughout): Involved in degradation of mRNA, hydrolytic removal of 5' cap (protected from RNases prior to decapping).	Decapped RNA: Exposure to other enzymes. Interest in RNA decay via RNases to hijack exogenous matter, as a novel target for antimicrobial action.

Other putative identifications linked to inflammation, which may play positive and negative and negative roles in wound repair (Kapp & Miller, 2011), include autophagy proteins (Guo *et al.*, 2016), ubiquitin-protein ligase (Goru *et al.*, 2016; Henderson *et al.*, 2016) and α-enolase (Ji *et al.*, 2016). Enolase is also associated with extracellular matrix (ECM) remodelling, alongside laminin (Perrin *et al.*, 2017) and the integrins (Koivisto *et al.*, 2014; Longmate & DiPersio, 2014), both of which have gained attention as therapeutic targets. Additionally, the Rho-family GTPases (guanosine triphosphate) control lumen morphogenesis by mediating vessel collapse and regression (Barry *et al.*, 2016).

Similarly, ubiquitin is connected with bacterial responses, as are proteasome maturation proteins (Qureshi *et al.*, 2003) and mRNA-decapping proteins (Eidem *et al.*, 2012), which share this feature with autophagy proteins. The link between debridement and disinfection is also demonstrated with these proteins, by the degradation and recycling of proteins by the proteasome by ubiquitination (Tanaka, 2009), which may be activated by bacterial challenge (Qureshi *et al.*, 2003). Autophagy proteins create lysosomes, which are involved in autolytic debridement and disinfection (Schultz *et al.*, 2003) and they also recycle endosomes, the precursors to lysosomes which respond to bacteria (Singh *et al.*, 2016). Indeed, all the proteins appear to overlap in terms of their possible functions, suggesting that debridement, disinfection and wound repair may not be multifactorial, and that individual compounds may contribute to one, or all of these therapeutic outcomes.

Despite the potential relevance of these finding, the lack of any compounds known to LAP, in particular the enzymes (**Section 1.4.1**) was noted. It was hypothesised that the volume of PBS (15 mL) used in the initial protocol collection (**Section 2.2.1.1**) may have swamped the low-level proteins. Following the promising findings of the pilot study, the work was repeated using standardised LAP (**Section 2.2.2.1**).

4.3 Standardised LAP

Fifty peptides were obtained from LAP collecting using the initial protocol, in which larvae were agitated in 5 mL of PBS for 30 minutes, across 3 cycles. It was anticipated that the SOP created for LAP collection, whereby larvae were exposed to 2.5 mL of PBS for 24 hours, would produce a solution of greater protein content.

4.3.1 Materials and Methods

Preparation of LAP

All reagents were obtained from Sigma-Aldrich Ltd (Missouri, USA), unless otherwise stated. Collection solutions were prepared from larval-loaded BioBags and control devices (n= 6 per group), and assessed as previously described (**Section 2.2.2.1**). Chymotryptic activity was confirmed in all LAP samples. The LAP solutions with the two highest and two lowest protein concentrations ($\bar{x}$= 40.58 ± 11.18 μg/mL) from the batch received were selected for further analysis, alongside the corresponding controls. Two millilitres of each test solution were concentrated using individual 10 kDa VivaSpin 2 centrifugal filters (GE Healthcare Ltd; Buckinghamshire, UK) at 3360 *g* for 8 minutes, to prepare a protein-rich retentate.

All processes were undertaken in a Class II microbiological safety cabinet, with the exception of centrifugation (within the sealed filtration tubes), and upon completion, all solutions were transferred to individual sterile 1.5 mL micro-centrifuge tubes. Approximately 250 μL of LAP retentate was produced for each BioBag sample and only residual volumes for each control device was obtained. Preparation for proteomic analysis occurred the same day, to avoid repeated freeze-thaw cycles which can degrade proteins (Bhatnagar *et al*., 2007).

Peptide acquisition

All peptide acquisition stages were under taken as described in **Section 3.2.1**.

Data analysis

All data were handled as described in **Section 3.2.1**.

4.3.2 Results

The initial data set was comprised of 101 peptides, of which the average length again was 11 (± 3) amino acids (range: 6 to 24 residues). Seventy-one peptides were associated with the BioBag samples (70.3%) and 30 with the control devices (29.7%). As a general comment, the results represent approximately double those obtained from the initial LAP collection protocol.

None of the peptides found in the pilot study (**Table 7**) were detected in this phase. Peptides were however found in common between BioBag replicates (sequence:

YMALIYDR, in samples BB29 and BB30; sequence: DEVTMLGFK, in samples BB25 and BB26), but also in control replicates (sequence: DEVTMLGFK, in sample C25; sequence ALRPAVGLPR, in samples BB25 and C26). Therefore, no potential biomarkers based on sequence were present, and the possibility of contamination or carry-over was again considered, particularly in light of only residual volumes of the control retentate solutions. Of the 101 peptides, only 31 had matches within the Mascot *Drosophila* protein database (**Table 9**), and again, none were associated with named, functional proteins.

Table 9: Numbers of peptides detected following the second study of the use of LC-MS/MS proteomic analysis of BioBag devices, whereby 10 kDa centrifugal filters were used to concentrate the proteins prior to tryptic digest. Of the 101 peptides, 30.7% of the sequences were found in the Mascot *Drosophila* database (Matched), the remaining peptides (Non-Matched) accounted for the majority of the results.

	BB 25	**BB 26**	**BB 29**	**BB 30**	**C 25**	**C 26**	**C 29**	**C 30**
Matched	2	4	7	10	1	4	0	3
Non-Matched	11	10	14	13	8	7	0	7
Total	13	14	21	23	9	11	0	10

No further distinction between the Mascot matches were made, and all peptides were submitted individually to the BLASTp suite (**Section 3.2.2**), for searching against the complete database. Potential matches were found for each peptide and 28 were associated with a named, functional protein, of which 24 originated from larval-loaded BioBags (**Chart 23**). From 101 peptides, 62.4% of the top hits had an E-value of ≤ 1, and 16 functional proteins were subsequently named (15.8%).

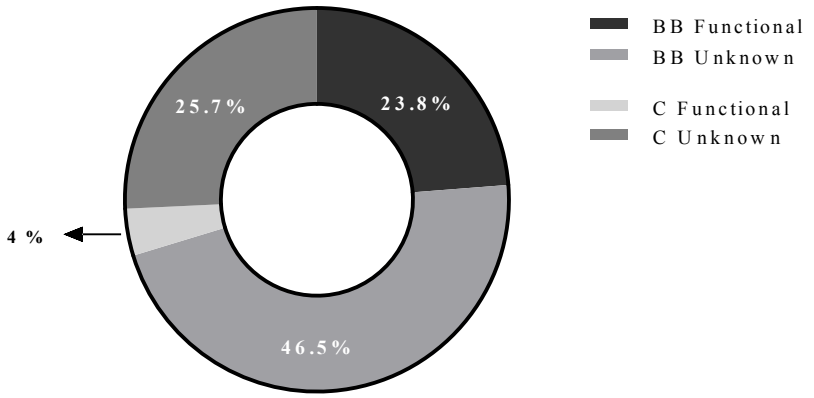

Chart 23: Initial overview of the number of peptides (*n*=101) detected during the second study of LC-MS/MS proteomic analysis of BioBag devices and their putative assignment to matched proteins in the complete GenBank database. The majority of the peptides were assigned to proteins of unknown function in both the larval-loaded BioBags (BB; 46.5%) and the control devices (C; 25.7%).

The process was repeated using the *Lucilia* genus sub-database and potential matches were found for each peptide. Twenty-seven sequences were associated with a named, functional protein, of which 20 originated from larval-loaded BioBags (**Chart 24**). Of the complete dataset, 59.4% of the top hits had an E-value of ≤ 1, and 28 functional proteins were subsequently named (27.7%).

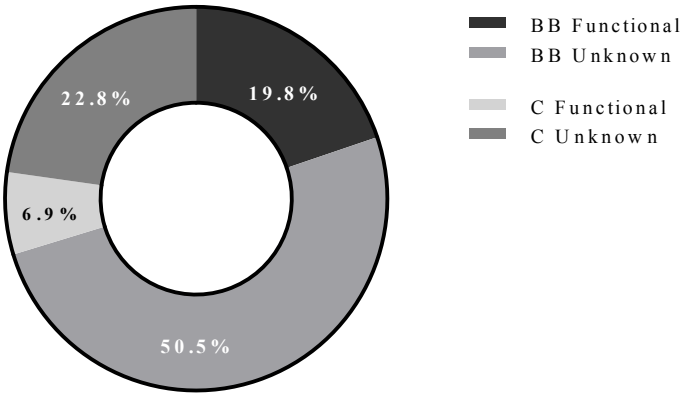

Chart 24: Initial overview of the number of peptides (*n*=101) detected during the second study of LC-MS/MS proteomic analysis of BioBag devices and their putative assignment to matched proteins in the GenBank *Lucilia* genus database. The majority of the peptides were assigned to proteins of unknown function in both the larval-loaded BioBags (BB; 50.5%) and the control devices (C; 22.8%).

Top hits with an E-value > 1 were excluded from further consideration, resulting in a combined final list of 27 named, functional proteins, of which six were found in common between each dataset (**Table 10**). Overall, four proteins were identified from the complete database (14.8%), with the remainder (85.2%) originating from the *Lucilia* sub-database.

4.3.3 Significance of findings

Of the 101 peptides detected during the second phase of this study, 27 were putatively matched to proteins of known functions. All but one of the matches from the complete GenBank database were assigned to the *Drosophila* genus, with a greater diversity of species. The detection of peptides in the control samples (*n*= 5 peptides) was again considered as contamination or carry-over, at this stage (discussed further in **Section 3.4**). The location of peak expression in *Drosophila* larvae of the proteins, and their potential relevance to wound healing are shown in **Table 11**. Eleven proteins are associated with excretory systems and ten with the nervous system; the latter is known to be intrinsically linked to alimentary regulation, which is particularly relevant for

juvenile holometabolous insects, who stockpile the resources required to fuel complete metamorphosis (Copenhaver, 2007; Douglas, 2013; Schoofs *et al.*, 2014).

Of the 27 named proteins, none matched any of those in the pilot study (**Table 8**), although there was shared relevance regarding the proteasome and GTPase activity. Six of the peptides were matched to proteins in *Drosophila* and *Lucilia*, which provides confidence in the results. Of the speculated wound-relevant actions, the majority of the proteins could be linked to healing, in particular regarding cellular activity and the restoration of tissue structure. Again, potential anti-infective properties were common, with over-lap between the potential therapeutic actions and the natural roles of the proteins.

Enzymatic proteins

The expected debridement enzymes, such as chymotrypsin and trypsin were not detected using the approaches herein. However, a phospholipase (**Table 11b**) with hydrolase activity relevant to lipid degradation and metabolism was matched using the *Lucilia* database. This is also known to possess slight peptidase activity, and its action results in the formation of lysolecithin and fatty acids, which have been linked to tissue repair and antimicrobial responses (Magalhaes *et al.*, 2008; Ali *et al.*, 2016). With the presence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, **Table 11b**), which is involved with lipid and carbohydrate metabolism (part of the glycolysis pathway leading to the production of adenosine triphosphate, ATP), the energy for cutaneous wound healing is provided (Demling, 2009). Furthermore, GAPDH has attracted attention in therapeutic administration, with anti-inflammatory functions, when using a lipopolysaccharide-induced, sepsis-related lung injury model (Takaoka *et al.*, 2014). Of particular interest is the presence of the tolloid (metallopeptidase, **Table 11c**) which has a human homologue with known serine-type peptidase activity, and is related to the bone morphogenetic proteins with various proteolytic (Shimell *et al.*, 1991) and regenerative actions (Vadon-Le Goff *et al.*, 2015). Tolloid is linked with the *Drosophila* Toll pathway, which is induced by bacteria (Gram positive) and fungi (Valanne *et al.*, 2011), as will be discussed in the following sub-section.

(continued after **Table 10** and **Table 11**)

Table 10: Putative protein identifications following BLASTp analysis of 101 peptides detected during the second study of LC-MS/MS proteomic analysis of BioBag devices. Sample origin and peptide sequence are shown for results obtained from the complete GenBank database (upper, matched to *Drosophila obscura*) and the *Lucilia* genus sub-database (lower, matched to *Lucilia cuprina*). Six peptides were common to both sets of results (bold text).

Sample	Peptide Sequence	Associated Protein [species]
BB 25	**CDLGMDDLK**	Solute carrier family 22 member 7-like isoform X1 [*D. obscura*]
BB 25	LAQALVQGVARGIWMK	Helical factor [*D. yakuba*]
BB 25	THVAGPNIASALK	Helicase domino [*D. serrata*]
BB 25	**IWDTSGQER**	Rab family small GTPase [*Naegleria gruberi*]
BB 26	FLFAEEAPEPMA	Aquaporin-like [*D. arizonae*]
BB 26	GKISQQIAQYR	Dicer-2 [*D. yakuba*]
BB 29	TNGSSPDGDGTQPKTPLTPR	GTP-cyclohydrolase 1 [*D. melanogaster*]
BB 29	**ILMLGLDAAGKTTILYR**	ADP-ribosylation factor 3 [*D. melanogaster*]
BB 29	CLRELSSPESIAGDLQK	Odorant-binding protein 83ef [*D. simulans*]
BB 29	**APPPPSKEQSKPER**	Heat shock protein 27 [*D. serrata*]
BB 29	**IRSAEMLGFSEK**	Protein 18w [*D. yakuba*]
BB 30	VFKIGDIVDIK	60S ribosomal protein L21 [*D. takahashii*]
BB 30	**LPKPSGLKVPQMPIK**	Non-claret disjunctional [*D. melanogaster*]
C 30	QVSLHGISSNPRK	Methylosome subunit pICln [*D. arizonae*]
C 30	AAVIRLGLMK	Glycerol 3 phosphate dehydrogenase [*D. sucinea*]
BB 25	**CDLGMDDLK**	Protein onecut [*L. cuprina*]
BB 25	**IWDTSGQER**	Rab-family small GTPase [*L. cuprina*]
BB 25	AAEGGIGGGRSFGGGR	H/ACA ribonucleoprotein complex subunit 1 [*L. cuprina*]
BB 25	NDMKAIAMR	Phospholipase B-like lamina ancestor [*L. cuprina*]
BB 26	AIPLILGVGGR	ATP-dependent RNA helicase Ddx1 [*L. cuprina*]
BB 26	ATPTEPDMSHLR	Protein expanded [*L. cuprina*]
BB 26	LFRSSASHSHGGAGMGR	JNK-interacting protein 3 [*L. cuprina*]
BB 26	THSNQMYIKFVSDGSVQK	Dorsal-ventral patterning protein tolloid [*L. cuprina*]
BB 29	**ILMLGLDAAGKTTILYR**	ADP-ribosylation factor 3 [*L. cuprina*]
BB 29	**APPPPSKEQSKPER**	Heat shock protein 27 [*L. cuprina*]
BB 29	GENNIIMVNK	Inhibin beta chain [*L. cuprina*]
BB 29	**IRSAEMLGFSEK**	Protein toll [*L. cuprina*]
BB 29	APPQHSPHK	Protein empty spiracles [*L. cuprina*]]
BB 30	**LPKPSGLKVPQMPIK**	Protein claret segregational [*L. cuprina*]
BB 30	LTQMNIVPKPLK	DNA-directed RNA polymerase III Rpc2 [*L. cuprina*]
C 26	IKSNNVSGMLK	26S Proteasome regulatory subunit Rpn2 [*L. cuprina*]
C 26	CMQSPTDPYIR	Protein bowel [*L. cuprina*]
C 30	FDYILGMDQSILASLK	DNA topoisomerase 2 [*L. cuprina*]

Table 11a: Putative protein identifications (*n*= 27) following BLASTp analysis of 101 LAP peptides detected during the second study of LC-MS/MS proteomic analysis of BioBag devices. Sequences are shown with associated genus and E-value (selected for exploration if ≤ 1). Six peptides were common to both sets of results (bold text). Peptides matched to proteins with known functions in *Drosophila* species were used to identify the primary regions of expression in the larvae (source: FlyBase). The potential role these proteins may play in the wound environment have been speculated (individual references cited in main body of text).

Detected Peptide (E ≤ 1)	Protein function and location of peak larval expression	Potential relevance to wound healing
CDLGMDDLK (*Drosophila,* E= 0.067) (*Lucilia,* E= 0.9)	Solute carrier 22 [*Drosophila*] (fat body): Membrane transporter. Onecut [*Lucilia*] (nervous system): Transcriptional regulator.	Transporters: Toxic mediators, may affect many cellular properties. Transcriptional regulators: Roles in inflammation and epithelialisation.
LAQALVQGVARGIWMK (*Drosophila*, E= 1×10^{-6})	Helical factor (fat body): Putative cytokine, involved in immune responses, leading to antifungal and antibacterial peptide production.	Cytokines: Cell communication and recruitment, inflammation. Destruction of bioburden and polymicrobial populations, for disinfection.
THVAGPNIASALK (*Drosophila,* E= 0.002)	Helicase domino (nervous system): Drives the unwinding of DNA or RNA helices, ATPase. Roles within cell communication and the immune system.	Linked roles with helical factor. Interest in DNA and RNA decay as a novel target for antimicrobial action, and ATPases as drugs targets.
IWDTSGQER (*Naegleria,* E= 0.66) (*Lucilia*, E= 0.0009)	Rab-family small GTPase (nervous system): Involved in regulating membrane traffic and proliferation of cells. Binds GTP and GDP, acting as a molecular switch.	Rab: GTPase sub-family involved in cytoskeletal functions. GTPase: Regulator of G-proteins (molecular switch). Rab-family GTPases: Possible role in inflammation and immunity.
FLFAEEAPEPMA (*Drosophila,* E= 0.0006)	Aquaporin (malpighian tubules and hindgut): Involved in water channel activity and transmembrane transport (including ammonium and bicarbonate).	Positive regulation of fibroblast proliferation, angiogenesis and cell migration. Role in skin hydration, glycerol transportation and proliferation of keratinocytes (epidermal cells).
GKISQQIAQYR (*Drosophila,* E= 0.022)	Dicer-2 (throughout): RNAase, with helicase activity, to bind and remodel RNA. Involved in the detection of, and response to, viruses.	Interest RNAses and helicases as possible antimicrobial targets. RNA helicases: Involved in collagen synthesis. Linked to other similar compounds.
TNGSSPDGDGTQPKTPLTPR (*Drosophila*, E= 9×10^{-10})	GTP-cyclohydrolase 1 (fat body and hindgut): Binds GTP. Required for production of eye pigment. Involved in the regulation of epithelial cell migration.	GTP-cyclohydrolase: Involved in vitamin biosynthesis. Interest in GTP-cyclohydrolase 1 as a novel anti-infective. Over-expression accelerates diabetic wound healing.
ILMLGLDAAGKTTILYR (*Drosophila,* E= 2×10^{-8}) (*Lucilia,* E= 6×10^{-11})	ADP-ribosylation factor 3 (hindgut): GTP-binding protein, involved in trafficking of proteins and cell movement.	ADP ribosylation factor: Members of Ras GTP-binding proteins. Switch for genes involved in cell growth, survival, differentiation and proliferation. May induce wound epidermal wound closure.
CLRELSSPESIAGDLQK (*Drosophila,* E= 5×10^{-8})	Odorant-binding protein 83ef (fat body): Binds volatile or odorant molecules for transportation. Involved in the sensory perception of chemical stimuli.	Odorant-binding protein: Member of lipocalin family. Lipocalins: May modulate fibroblast response, expression of healing mediators and synthesis of ECM proteins.

(Continued overleaf)

Table 11b: Continuation of **Table 11a.** Putative protein identifications following BLASTp analysis of LAP peptides detected during the second study of proteomic analysis of BioBag devices. Sequences are shown with associated genus and E-value (selected for exploration if ≤ 1).

Detected Peptide (E ≤ 1)	Protein function and larval location	Potential relevance to wound healing
APPPPSKEQSKPER (*Drosophila,* E= 0.0003) (*Lucilia,* E= 1×10^{-6})	Heat shock protein 27 (fat body): Chaperone involved in protein folding, responses to heat and bacteria (recognition of mis-folded proteins).	Roles in platelet aggregation and angiogenesis. Promotes endothelial cell and keratinocyte migration. Stabilises actin filaments and contributes towards wound contraction.
IRSAEMLGFSEK (*Drosophila,* E= 0.15) (*Lucilia,* E= 0.0001)	18-Wheeler/Toll (trachea/midgut): Co-expressed/similar proteins. Roles in tissue morphogenesis, antibacterial/antifungal responses and Rho-GTPase pathway.	Antimicrobial action, epidermal regeneration and serine protease activator. Interest in toll-like receptors in wound healing. Rho-family GTPases: Essential role in blood vessel tubulogenesis.
VFKIGDIVDIK (*Drosophila,* E= 0.023)	60S ribosomal protein (throughout): Structural component of ribosome, involved in cytoplasmic translation and RNA binding.	Extra-ribosomal functions, including cellular growth, differentiation and proliferation, and DNA repair. Interest as a novel drug target for various diseases and inflammatory responses.
LPKPSGLKVPQMPIK (*Drosophila,* E= 0.15) (*Lucilia,* E= 0.004)	Non-claret disjunctional (nervous system): Claret segregational synonym, cytoskeletal protein required for chromosomal segregation. Kinesin, with ATPase activity.	Cytoskeletal response during wound repair, filament contraction and cell migration, regulated by Rho-family GTPases. Interest in ATPases as drugs targets.
QVSLHGISSNPRK (*Drosophila,* E= 0.0007)	Methylosome subunit pICln (nervous system): Chaperone that regulates assembly of spliceosome. Regulates cellular volume and transport. RNA binding.	Spliceosome: Removal of introns from mRNA precursors, essential for gene expression. Of interest as a biomarker and target for drug discovery.
AAVIRLGLMK (*Drosophila,* E= 0.59)	Glycerol 3 phosphate dehydrogenase (mid- and hindgut): Link between lipid and carbohydrate metabolism. Redox reactions and sodium current regulation.	Carbohydrate and lipid metabolism provides the energy required for cutaneous wound healing and tissue repair. Range of functions, including lubricating, enzymatic and immunological responses.
AAEGGIGGGRSFGGGR (*Lucilia,* E= 0.019)	H/ACA ribonucleoprotein complex subunit 1 (trachea): Ribosome biogenesis for protein production. Involved in pseudouridylation of rRNA and RNA binding.	Ribosome biogenesis linked to many cellular process, including growth and division. Interest in RNA-based therapeutics for wound healing, particularly angiogenesis and, re-epithelisation.
NDMKAIAMR (*Lucilia,* E= 0.002)	Phospholipase B-like lamina ancestor (midgut): Hydrolase, role in lipid degradation and metabolism. Required for adult development and behaviour.	Phospholipase action forms lysolecithin and fatty acids relevant to wound healing. Production of lipids for energy and tissue repair. Slight peptidase activity, relevant to debridement.
AIPLILGVGGR (*Lucilia,* E= 0.17)	ATP-dependent RNA helicase Ddx1 (nervous system): Helicase able to unwind RNA and DNA duplexes, involved in ribosome and spliceosome formation.	DEAD-box (Ddx) proteins may modulate endothelial growth factors. Roles in the rearrangement and degradation of RNA-protein interactions, chaperone activity.

(Continued overleaf)

Table 11c: Continuation of **Table 11a** and **Table 11b.** Putative protein identifications following BLASTp analysis of LAP peptides detected during the second study of proteomic analysis of BioBag devices. Sequences are shown with associated genus and E-value (selected for exploration if ≤ 1).

Detected Peptide (E ≤ 1)	Protein function and larval location	Potential relevance to wound healing
ATPTEPDMSHLR (*Lucilia,* E= 0.061)	Expanded (throughout): Regulator of organ size. Role in cytoskeleton regulation and involvement with Ras GTPases and kinases.	A FERM-domain containing protein, a superfamily with an actin-binding domain. Promotes endothelial cell and keratinocyte migration, leading to angiogenesis, re-epithelisation and closure.
LFRSSASHSHGGAGMGR (*Lucilia,* E= 0.25)	JNK-interacting protein 3 (nervous system): Scaffold protein, c-Jun N-terminal kinase. Involved in mitogen-activated protein kinase signalling and inflammatory response.	JNK regulates many processes, including cell death/survival, stress responses. Critical for normal re-epithelisation and keratinocyte migration.
THSNQMYIKFVSDGSVQK (*Lucilia*, E= 4×10^{-11})	Dorsal-ventral patterning protein tolloid (nervous system and gut): Metallopeptidase required for normal dorsal development.	Related to human tolloid-like protein (protease) and bone morphogenetic protein 1 (collagenase). Involved in assembly of ECM and cartilage, tissue regeneration, angiogenesis and growth factors.
GENNIIMVNK (*Lucilia,* E= 0.07)	Inhibin beta chain (nervous system): Controls neuronal morphogenesis, with cytokine and growth factor activities. Roles in cell growth, development and death.	Inhibin: Antagonist to closely-related activin. Activin: Involved in re-epithelisation and wound closure. Over-expression can lead to dermal thinning and delayed closure.
APPQHSPHK (*Lucilia,* E= 0.23)	Protein empty spiracles (trachea): Homeotic selector gene, involved in development of trachea and head appendages, and in DNA binding.	Related to human transcription factor Sp1 which regulates genes related to immune response, cell growth/death and DNA damage and wound healing.
LTQMNIVPKPLK (*Lucilia,* E= 0.052)	DNA-directed RNA polymerase III Rpc2 (mid- and hindgut): Catalyses transcription of DNA to RNA, core component of RNA polymerase III. Role in cell growth.	Interest in RNAs and role in normal wound healing and in complications. Target for future therapeutics. Involved in inflammation, proliferation, remodelling and angiogenesis. Biogenesis requires polymerase III.
IKSNNVSGMLK (*Lucilia,* E= 0.086)	26S Proteasome regulatory subunit Rpn2 (hindgut): Regulator of 26S proteasome with peptidase activity. Removes or repairs misfolded, damaged or non-required proteins.	Proteasomes: Degrades and recycles proteins (ubiquitin tagging). May be activated by bacterial challenge. Activation of 26S mediates growth factors and fibrosis.
CMQSPTDPYIR (*Lucilia,* E= 0.71)	Bowel (salivary gland): Transcription factor, involved in gut morphogenesis and cell fate. Zinc finger gene, stabilises protein folds and binds DNA.	Transcription factors linked with JNK pathway associated with re-epithelisation and keratinocyte growth factor, to modulate inflammation and the survival of epithelial cells.
FDYILGMDQSILASLK (*Lucilia,* E= 0.61)	DNA topoisomerase 2 (nervous system): Breaks and re-joins double-stranded DNA when over- or under-wound. Regulates many biological processes.	Bacterial type II topoisomerases contain DNA gyrase, a target for novel antimicrobial development, especially fluoroquinolones and aminocoumarins, by inhibiting gyrase.

Enzymatic proteins (continued)

The proteasome, as aforementioned (**Section 3.2.3**) is relevant as it degrades and recycles proteins, and the 26S regulatory subunit (**Table 11c**) possesses peptidase activity (Ferrell *et al.*, 2000). The spliceosome (regulated by subunit pIC1n, **Table 11b**) has received interest as a disease biomarker and target for drug discovery (Le *et al.*, 2015). The remaining protein identifications with enzymatic action are predominantly related to GTPase, ATPase and helicase activity. Their roles in the insect are varied, and each has been reported to have multiple therapeutic actions, as discussed further in the relevant sub-sections.

Antimicrobial proteins

Eight of the identified proteins are linked to antimicrobial actions, many of which share additional relevance to wound repair. The helical factor, helicase domino and Dicer-2 (**Table 11a**) have related roles and are involved with immune, antibacterial and antifungal responses (Sanyal & Doig, 2012; Cowan *et al.*, 2013). The remodelling of DNA and RNA, particularly by helicases has been of medical interest as new drug targets, and they have also been described in collagen synthesis (Eidem *et al.*, 2012; Manojlovic & Stefanovic, 2012). Similarly, the GTP-cyclohydrolases (**Table 11a**) have been identified as novel anti-infectives (Grawert *et al.*, 2013) and are purported to accelerate wound healing (Tie *et al.*, 2009). Toxicity mediators are associated with solute carriers (**Table 11a**), such as Slc22 (Sweet, 2005).

The constitutive heat shock proteins (HSP, **Table 11b**) include chaperones and proteases which function primarily to overcome the changes associated with protein denaturation by thermal stress in insects (King & MacRae, 2015). However, some members, including HSP27 may be induced following bacterial challenge or starvation (Pundir *et al.*, 2017). The 18-Wheeler/Toll proteins (**Table 11b**) as previously referred to, are involved with a number of potentially relevant actions, beyond their antibacterial and antifungal roles, including enzyme activation, and tissue and vascular morphogenesis (Capilla *et al.*, 2017). Their related receptors are of interest in wound healing research, due to their involvement with inflammation, macrophage activation and the recognition of invading microbial entities and damaged host cells (Dasu & Isseroff, 2012).

The regulatory subunit of the 26S proteasome (**Table 11c**) possesses peptidase activity, as aforementioned. It may also be activated by bacterial challenge (Qureshi *et al.*, 2003). The DNA topoisomerase (**Table 11c**) regulates many biological properties in insects, by the breaking and reforming of DNA and the hydrolysis of ATP. With regard to bacterial replication, some topoisomerases contain DNA gyrase (Sanyal & Doig, 2012), which has received attention as target for antibacterial drug discovery.

Wound healing proteins

Of the 27 putatively identified proteins, 20 may be potentially associated with wound repair. Many have multiple roles in inflammation, epithelisation and angiogenesis, as can be seen in **Table 11a**, such as the protein Onecut (Haertel *et al.*, 2014), the aquaporins (Hara-Chikuma & Verkman, 2008), the Rab-family small GTPases (Johnson & Chen, 2012) and GTP-cyclohydrolase; the latter of which has been reported to accelerate diabetic wound healing (Tie *et al.*, 2009). The RNA helicases (which includes Dicer-2) are reportedly connected with collagen synthesis (Manojlovic & Stefanovic, 2012), which may work in association with members of the lipocalin family (i.e. odorant-binding proteins) which synthesise extracellular matrix proteins (Miao *et al.*, 2014) and Ras GTP-binding proteins (i.e. ribosylation factors) to promote wound closure (Tscharntke *et al.*, 2005).

Similarly, in **Table 11b**, angiogenic activities have been reported with heat shock proteins, and over-expression of HSP27 promotes wound contraction, and endothelial cell and keratinocyte migration (Atalay *et al.*, 2009). Toll proteins have been linked with epidermal regeneration (Capilla *et al.*, 2017), which are associated with the Rho-GTPase pathway (**Section 3.3.2**) and tubulogensis (Barry *et al.*, 2016). This family also regulates the claret disjunctional/segregrational proteins, which are involved with filament contraction, leading to wound repair (Abreu-Blanco *et al.*, 2012). Favourable cellular responses, including proliferation, re-epithelisation and related growth factors are associated with 60S ribosomal proteins (Wang *et al.*, 2015) and ribonucleoprotein complexes (Banerjee & Sen, 2015) and DEAD-box proteins (de Vries *et al.*, 2013).

Almost all of the proteins in **Table 11c** have relevance to wound healing. Angiogenesis, re-epithelisation and wound closure dominate, as with the protein Expanded (Bosanquet *et al.*, 2014), protein Tolloid (Vadon-Le Goff *et al.*, 2015), the balance between inhibin/activin (Antsiferova & Werner, 2012) and RNA polymerase (Fahs *et*

al., 2015). Keratinocyte migration is linked to JNK (c-Jun N-terminal kinases) (Zhang *et al.*, 2012) interacting proteins and the transcription factor protein Bowel (Pearson *et al.*, 2009), and activation of the 26S proteasome also mediates keratinocyte growth factors (Braun *et al.*, 2002). Protein empty spiracles is related to the human transcription factor Sp1, which regulates immune response genes and cellular growth (Shirasaki *et al.*, 1999).

4.4 Summary

Whole proteome shotgun analysis of LAP was attempted to determine the baseline protein profile of the BB-50 bio-footprint. What is evident at the outset, is the variation observed between the two phases of the study and within each replicate. Based on the methodologies applied, it appears that the specific content of LAP is highly variable. It is, however, acknowledged that this work has been applied to a small sample size (n= 7), and other factors may also be responsible for the disparity between LAP replicates (to be further discussed).

No repeat biomarkers were apparent and none of the key enzymes known to be present in LAP were detected (**Section 1.4.1**), despite specific chymotryptic activity being confirmed in all LAP samples. Given the low protein content values obtained throughout the study (**Section 2**), it is possible that the relative concentration of chymotrypsin in LAP is minimal, and below the level of detection of the instruments, particularly in the absence of any optimisation studies, which would normally be required (Hustoft *et al.*, 2012). Referring to the SDS-PAGE images showing LAP (**Figure 11** and **Figure 15** chymotrypsin (or its precursor zymogen chymotrypsinogen) would be expected at c. 25 kDa, but its presence is not visible. Again, this provides support for the assertion that the LAP protein mixture is more complex than initially assumed. Therefore, the 'top-down' approach (**Section 3.1**), for LAP analysis, incorporating the separation of intact proteins by means of two-dimensional SDS-PAGE for example, may be more informative than peptide fragments.

It is acknowledged that protein identification from peptides obtained by enzymatic digest is problematic, especially where small, non-specific sequences are used for database searches. In both phase of the study, the average peptide length was 11 (± 3) amino acids, and the majority of the hits were assigned to unnamed proteins (**Charts 15** to **18**). This demonstrates that characterisation of the proteome is incomplete in both

the *Drosophila* and *Lucilia* genus. Protein identifications were determined by statistical match, and with small peptides, the chances of obtaining a significant result based on E-value are reduced. It is therefore possible that in either the uncharacterised hits, after the first 100 results, or where E > 1 (as capped, **Section 3.2.2**) that familiar compounds may be found.

In total 37 named, functional proteins were putatively identified using the shotgun approach and the employed data curation methods. Of these, eight were associated with the control solutions, and it is believed that some form of contamination, or instrumentation carry over, may be responsible. This is particularly evident in the second phase of the study, whereby MWCO filters were utilised to obtain protein-rich retentates (**Section 3.3.1**) and barely any solution was recovered from the control samples (**Section 3.3.2**). Protein roles and their potential relevance to chronic wound care were suggested (**Table 8** and **Table 11**) and the findings are of interest, particularly in relation to antimicrobial effects and wound repair. Furthermore, many of the proteins are being considered as alternative therapeutics, or new drug targets. Although it is accepted that the substances are unconfirmed, and may not be present in the quantities required to elicit the therapeutic response, the findings suggest that exploration of the *L. sericata* proteome is required to determine the full content of LAP. An overlap of activities is evident, and although three modes of larval action are acknowledged (debridement, disinfection and healing promotion, (**Section 1.1**) a multifactorial contribution may be provided by many of the proteins in LAP. This is particularly evident with the enzymes associated with GTP (*n*= 6, **Table 8** and **Table 11**). Guanosine triphosphate is a guanine nucleotide, which alongside its role in energy provision for DNA and RNA synthesis, activates the signal transduction of G-proteins, which are involved in cellular proliferation, vesicle formation and kinase cascades (Wishart *et al.*, 2013).

The LAP protein mixture is complex, and harbours extensive enzymatic activity, despite the low protein concentration. Alimentary products were collected from larvae produced under regulated conditions, however, additional factors must be considered in relation to this study. Although the larvae are produced under regulated conditions, they are not all of the same age, and therefore do not represent the same biological state. Eggs are harvested in bulk from the stock colony, and these may be between 1 to 24 hours old (**Section 1.2.1**). Subsequently, these hatchlings of different ages may feed for

up a further 24 hours on the *Lucilia* Agar media and therefore each BioBag may contain larvae of mixed instars, and of differing ages (in hours) within the same instar. Although none of the peptides were linked to any of the dietary components (**Table 4** and **Table 5**) in the complete GenBank database (**Section 4.2.1**), it is possible that the dietary components (and symbionts, where present) contributed to the peptides obtained. Although this represents the authentic product as administered to patients, the processes leading to LAP production are too diverse to study using non-optimised shotgun methodologies. However, the potential for LAP exploration in this manner has been demonstrated and although it would be of biological interest to attempt proteomic studies of LAP collected from larvae of the same egg-hatch age and which have not fed, BioBag devices are not produced in this manner. Although high molecular weight proteins and mid-range peptides have been identified previously in larval research, the metabolic study of an organism specifically utilised to feed upon necrotic material has never been attempted. Following the detection of peptides in LAP, the characterisation of such low molecular weight bio-footprint components was undertaken. A summary of key findings is shown in **Figure 21**.

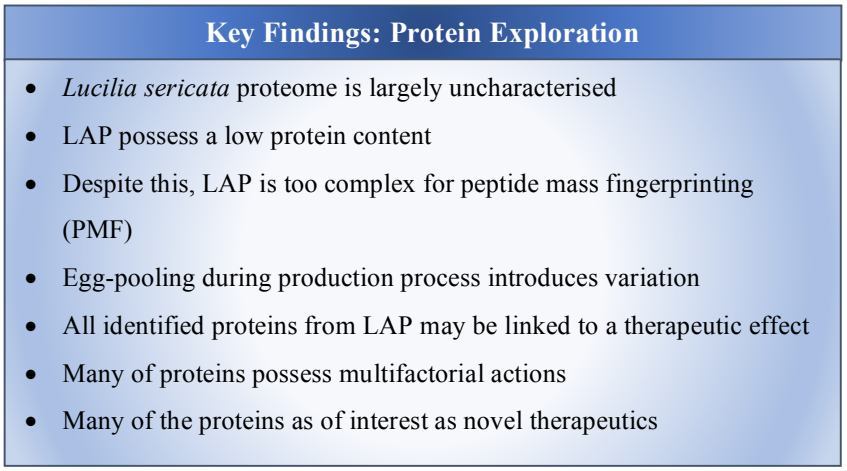

Figure 21: Summary of the key findings associated with the exploration of LAP using peptide to protein identification, to explore the high molecular weight content of the bio-footprint.

Chapter Five: Metabolite Profiling

5.0 Abstract

This chapter, 'Metabolite Profiling' details the low molecular weight investigation of the bio-footprint, by subjecting complete larval alimentary products to hydrophilic interaction chromatography methods. Following the putative identifications from the 'Protein Exploration' section and the consideration of the contributions identified in the 'Assessment' section, the first characterisation of the metabolome of *Lucilia sericata* larvae was undertaken. Significant compounds were detected, and it was possible to link these to each other by metabolic pathways, and also to wound healing activities.

5.1 Metabolite profiling

Following on from the protein exploration of the bio-footprint, the detection of the LAP metabolome was attempted. Metabolomics methods aim to identify low molecular weight substances (< 1.8 kDa) in a biological organism, (Zhou *et al.*, 2012). It offers the advantage of quantification over proteomic study, and again, various approaches may be employed, such as the untargeted system to obtain a global metabolite profile (Theodoridis *et al.*, 2008), or the targeted methodology to identify selected metabolites (Lu *et al.*, 2008). Here, the untargeted approach was employed to obtain a small molecule inventory from standardised LAP, incorporating a liquid solvent extraction procedure, to capture a broad range of metabolites whilst excluding the proteinaceous content. In order to obtain the maximum information from the LAP samples, an optimised metabolomic service, supplied by the Polyomics facility of the University of Glasgow was utilised.

As with the proteomic study, the methodology was first trialled with LAP collected from the existing protocol (**Section 2.2.1.1**) and then repeated with samples obtained using the developed SOP (**Section 2.2.2.1**). Additionally, two varieties of LC-MS technique were utilised. With the pilot study, hydrophilic interaction chromatography (HILIC) was used to separate and detect polar compounds, and for the standardised LAP samples, this was coupled with a reversed-phase liquid chromatography (RPLC) column. By utilising the reverse-phase hydrophilic interaction liquid chromatography (RP-HILIC) approach, the results of the pilot study could be verified, whilst the presence of non-polar compounds which may have been missed in the pilot study could be investigated. By profiling the metabolome of LAP, it was possible to identify larval compounds which have the potential to be detected in the wound environment. If such

biomarkers could be linked to healing outcomes, it may be possible for low molecular weight compounds to be characterised in the same manner as chymotrypsin (c. 25 kDa) and lucifensin (c. 4 kDa), as discussed in **Section 1.4.2**.

5.2 Pilot study: initial protocol LAP

Prior to this, no studies pertaining to whole metabolome analysis of LAP appeared to have been published. It is possible that the complexities arising from larval feeding on animal tissues may have prevented others attempting this approach. Traditionally, analytic approaches have been incorporated into investigations to identify the substances responsible for antimicrobial effects, detected within 'cuts' of varying molecular weights. Furthermore, metabolomic studies are often utilised in targeted studies, and/or to document changes in response to specific stimuli.

5.2.1 Materials and methods

All reagents were obtained from Sigma-Aldrich Ltd (Missouri, USA), unless otherwise stated.

Preparation of LAP

Collection solutions were prepared from larval-loaded BioBags (n= 6) and control devices (n= 6), and assessed as previously described (**Section 2.2.2.**1). Three LAP solutions from the batch received were selected for analysis alongside the corresponding controls and concentrated using individual VivaSpin 20 centrifugal filters (GE Healthcare Ltd; Buckinghamshire, UK). Sequential concentration was achieved using 30, 10, 5 and 3 kDa molecular weight cut-off (MWCO) filters at 3360 *g*, for the duration as specified by the manufacturer for each size. The final filtrate solutions (≤ 3 kDa) from the BioBags and control devices were prepared for metabolomic analysis in accordance with guidelines supplied by the University of Glasgow Polyomics facility for the submission of liquid material. Aliquots of the corresponding non-filtered, native LAP were also prepared, to determine if analysis would be possible without the use of concentrator filters.

Potential metabolites were extracted from all samples (250 µL) using 2 mL of cold solvent mix (4ºC) of chloroform, methanol and 18.2 MΩ water (Millipore; Massachusetts, USA) at a ratio of 1:3:1. Samples were cool-vortexed for 5 minutes,

prior to centrifugation (13,000 × *g*; 3 minutes) at 4°C. Supernatants were removed to cryotubes and a pooled quality control (QC) sample was prepared using 50 μL aliquots from the test samples, alongside a solvent blank (500 μL). All processes were undertaken in a Class II microbiological safety cabinet. All samples (*n*= 11) were stored at -80°C prior to transportation on dry ice to the University of Glasgow Polyomics facility.

LC-MS analysis and data processing

All samples were submitted for analysis, which was undertaken the at University of Glasgow (methodology details were obtained). All instruments were provided by Thermo Fisher (California, USA) unless stated otherwise.

A rapid separation liquid chromatograph (RSLC; UltiMate 3000) coupled with an Orbitrap Exactive mass spectrometer (MS) operating with electrospray ionisation (ESI) running in the positive and negative ion modes was used to analyse the collected samples. The compounds were separated using hydrophilic interaction liquid chromatography (HILIC), LC–MS spectra were acquired using Xcalibur v2.2 software (Thermo Fisher) and processed by the IDEOM interface (available from www.mzmatch.sourceforge.net). The IDEOM data files were supplied for subsequent analysis and interpretation.

Data analysis

Principal component analysis (PCA) was used for modelling the differences between test samples, based on peak area, mass and retention time data from LC-MS spectra, using SIMCA P+14 software (Umetrics AB; Umeå, Sweden), as conducted by Dr Lina Dahabiyeh of the University of Nottingham. Statistical analyses were performed using IDEOM interface for Microsoft Excel or GraphPad Prism v.7.0b (GraphPad Software Inc; San Diego, USA). Peak intensities are presented as the mean abundance intensity ($\times 10^3$) with standard deviation (± SD). Significance was determined using unpaired *t*-test, a probability value (*P*) of < 0.05 was considered significant.

5.2.2 Results

The IDEOM dataset consisted of 292 detected compounds in total; 27 had been identified in comparison with the mass and retention time of authentic standards (9.3%). The remainder were identified using the following databases linked to IDEOM: Kyoto

Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Lipid Maps and MetaCyc. Group differentiation was achieved using principal component analysis from peak intensity, mass and retention time data from the combined ESI modes (**Chart 25**). As an unsupervised method (i.e. the test groups were not specified at the outset), the plot provides an unbiased overview of data trends, demonstrating good separation between the test groups, and consistency within replications, which provides confidence in the methodology.

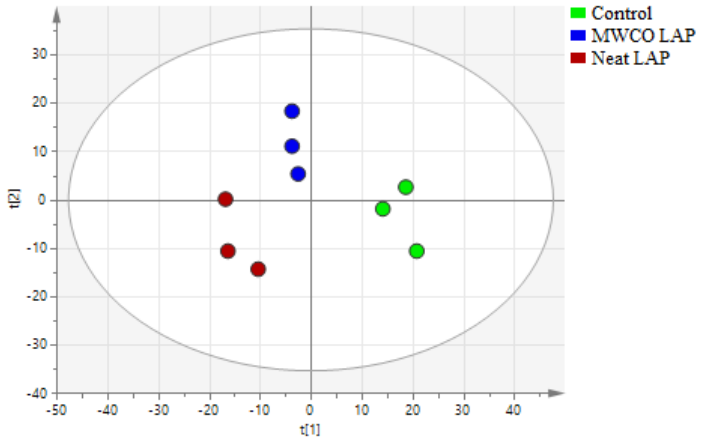

Chart 25: Unsupervised principal component analysis for test sample separation, based on peak intensity, mass and retention time data (combined positive and negative mode).

Step-wise data curation was undertaken (**Figure 22**), with individual replicates examined to determine the presence of potential larval biomarkers. Initially, 32 compounds of interest were detected in all LAP samples (n= 6 per group), as these were not found in any of the control (n= 3) and blank replicates (n= 6). Seven of the metabolites were identified by comparison with authentic standards.

(Continued after **Figure 22**)

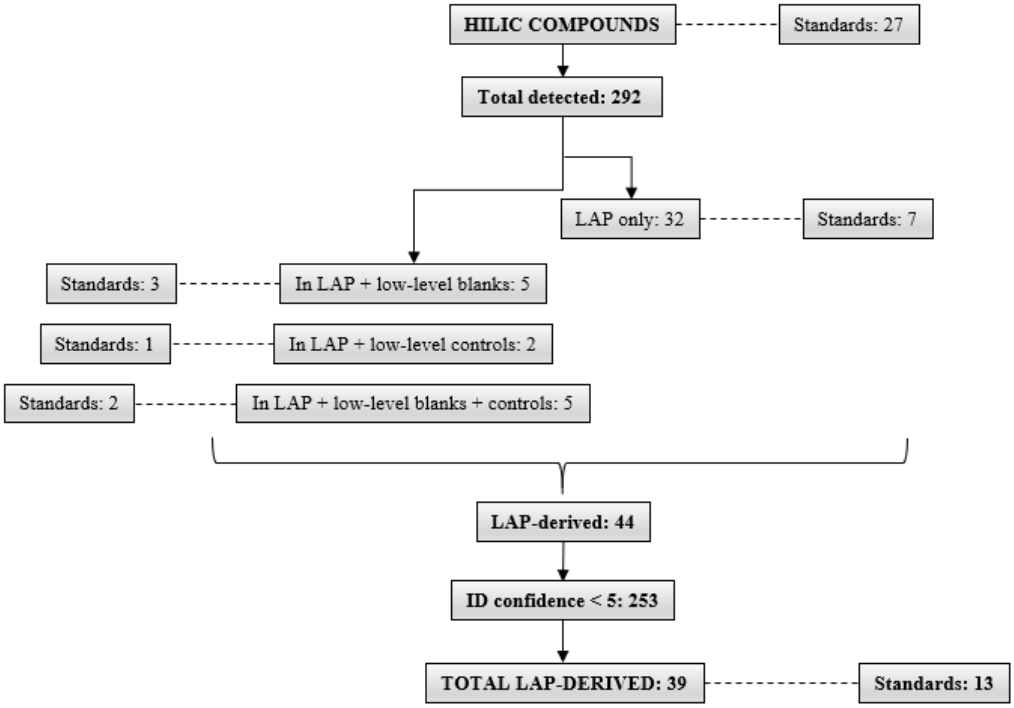

Figure 22: Flow-chart summarising data curation method relating to 292 compounds detected during a pilot study of LAP metabolome profiling, using HILIC.

A further five metabolites were detected in all LAP samples, and although these were not present in any of the controls, a low-level presence was observed in either one or two of the blank replicates. Within the blank samples, these compounds were under the signal intensity threshold for detection (< 1000) and were considered as noise or carry-over, given their absence in the controls and significant presence in all LAP replicates. Of these, three were identified by comparison with standards. Two additional metabolites (including one standard) were detected in all LAP samples, and although these were absent in the blanks, a low-level presence was observed in one of the control replicates. Again, these were under the base detection threshold and therefore the compounds were included for consideration as potential larval biomarkers. Lastly, five substances (including two standards) which were detected in all LAP samples, were also observed in all control replicates and in two or more of the blanks, but always under the threshold limit.

Within IDEOM, the identification of metabolites is scored from 1 to 10, and any compounds with a confidence value of < 5 were excluded for further examination. In summary, 39 metabolites from the original dataset (13.4%), including 13 standards (4.5%) were selected as compounds of interest (**Chart 26**), and chromatogram peak quality was verified based on size, spread and consistency between replicates. The

remaining 253 substances did not meet these stringent criteria, and were either not observed in the individual LAP replicates, had low-level detection, or the relative intensity between the control samples were not significant (*t*-test, $P \geq 0.05$).

According to the Metabolomics Standards Initiative, compounds matched to standards may be referred to as Level 1 identified metabolites (Salek *et al.*, 2013). These represent the most authoritative results and can be considered with confidence as larval biomarkers. Thirteen Level 1 metabolites were detected in the LAP samples and the signal intensities were higher in the Neat group (**Chart 26**). Identifications not supported by authentic standards may be referred to as putatively annotated compounds (Level 2) or putatively characterised compound classes (Level 3). With the exception of tryptophan hydroxamate (**Chart 26b**) and the Gly-Arg di-peptide (**Chart 26c**), all compound signal intensities were higher in the Neat LAP group.

(Continued after **Chart 26**)

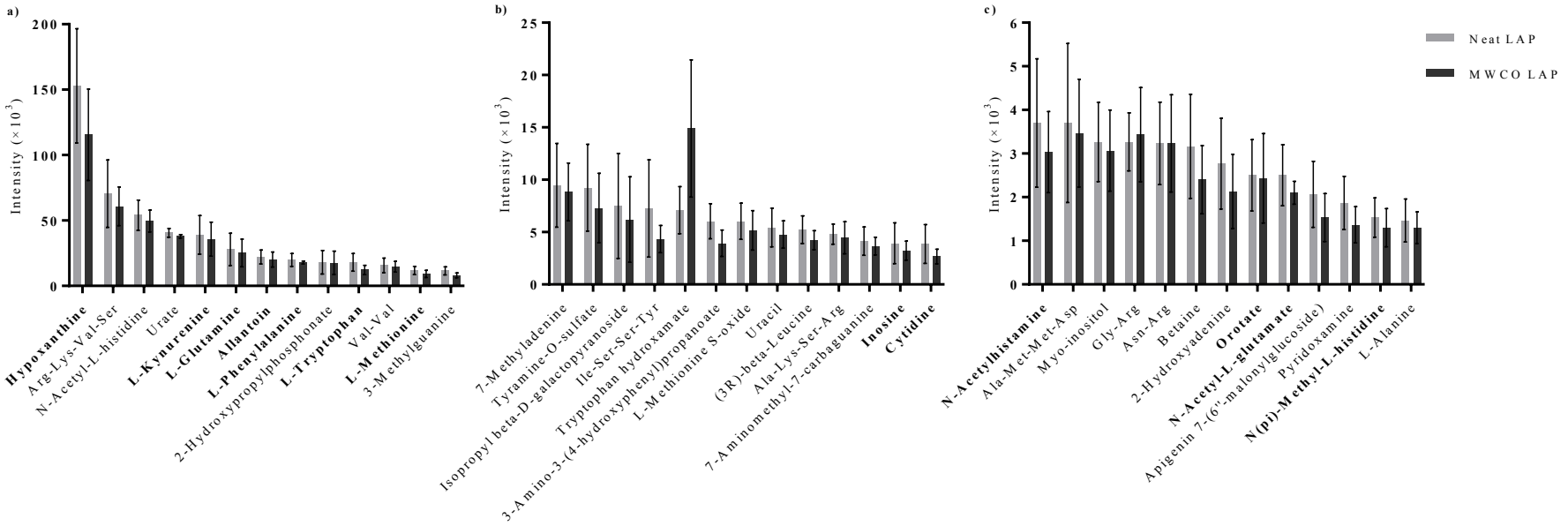

Chart 26: Identification and intensities (×10^3) of 39 compounds detected during a pilot study of HILIC metabolomic analysis of LAP collected from BioBag devices (*n*= 3). Compounds in bold have been identified in comparison with authentic standards (Level 1 metabolites), the remainder are classed as Level 2 putatively annotated compounds having been matched to databases (using mass and retention time). The LAP samples were prepared as neat material (grey shading, *n*= 3) and following sequential fractionation (using MWCO filters to < 3 kDa, black shading, *n*= 3). With the exception of tryptophan hydroxamate (**b**) and the glycine-arginine di-peptide (**c**), signal intensities were higher in the neat group.

The KEGG database linked to IDEOM assigns each compound to a metabolic map where relevant (available from http://www.genome.jp/kegg/kegg2.html). Twenty-four compounds were assigned, across 16 pathways (excluding peptides, *n*= 7), with purine metabolism being the most frequent (**Table 12a**). The remaining compounds (*n*= 8) were not linked to any map or pathway (**Table 12b**).

5.2.3 Significance of findings

Of the 292 compounds detected during the pilot metabolomics study, 39 were selected as LAP-derived compounds of interest. Of these, 13 were additionally identified in comparison with authentic standards and therefore their presence in LAP may be treated with confidence. Additional confidence in the method was supplied by the recognition of allantoin and urate, which have been associated previously with LAP (**Section 1.4.3**) and are known to be metabolic products of *L. sericata* larvae (Brown, 1938; Dow, 2013).

The metabolism of amino acids represented the largest map proportion (*n*= 14, **Table 12a**) and this group contained the largest number of Level 1 metabolites (*n*= 8). The remainder of the Level 1 metabolites were associated with nucleotide metabolism (*n*= 5). These Level 1 metabolites will be considered further in relation to their presence in LAP and their relevance to healing in the forthcoming Summary section (**Section 5.5**).

The initial outcome of the HILIC study suggested that the use of MWCO filters is unnecessary for the preparation of regulated LAP material. Indeed, 21 compounds were common to MWCO LAP and the controls (also processed using MWCO filtration), but were not found in the neat LAP, suggesting that the filters are erroneously contributing to the profile. Filtration techniques are commonly encountered in the existing literature, although these studies tend to use larvae reared on animal tissues, such as meat or fish (Huberman, *et al.*, 2007b; Kruglikova & Chernysh, 2011). As no attempts at characterising the full LAP content have been conducted prior to this study, it is likely that filtration and concentration methods were employed due to the assumption that low molecular substances represented a minimal component of LAP, and that the dietary source complicated the mixture. This again provides support in the proposition that only larvae from regulated sources should be utilised when endeavouring to elucidate LAP

content. As a result, non-fractionated standardised LAP was used for the second phase of the study. All findings will be discussed conjointly in **Section 5.4**.

(Continued after **Table 12a** and **Table 12b**)

Table 12a: Identification and relative intensities ($\times 10^3$) of 31 larval compounds known to be associated with metabolomic maps and pathways. In total, 39 LAP-derived compounds were detected during a pilot study of HILIC metabolomic analysis of BioBag devices (*n*= 3), prepared from neat and fractionated (MWCO, < 3 kDa) material. Compounds in bold have been identified in comparison with authentic standards.

Map	Compound	Neat LAP	MWCO LAP	Primary Pathway
Amino Acid Metabolism	L-Alanine	1.47	1.31	Alanine and aspartate metabolism
	N-Acetyl-L-glutamate	2.48	2.07	Arginine biosynthesis
	L-Glutamine	27.97	25.17	Glutamine and glutamate metabolism
	Betaine	3.16	2.42	Glycine, serine and threonine metabolism
	N-Acetylhistamine	3.70	3.01	Histidine metabolism
	N(pi)-Methyl-L-histidine	1.53	1.30	Histidine metabolism
	L-Methionine	11.80	9.19	Methionine metabolism
	L-Methionine S-oxide	6.02	5.15	Methionine metabolism
	L-Phenylalanine	19.78	17.80	Phenylalanine metabolism
	2-Hydroxypropylphosphonate	18.04	17.62	Phosphonate and phosphinate metabolism
	L-Kynurenine	39.08	35.70	Tryptophan metabolism
	L-Tryptophan	17.93	12.16	Tryptophan metabolism
	3-Amino-3-(4-hydroxyphenyl)propanoate	6.05	3.95	Tyrosine metabolism
	(3R)-beta-Leucine	5.23	4.23	Valine, leucine and isoleucine degradation
Nucleotide Metabolism	7-Methyladenine	9.46	8.84	Base excision repair
	Hypoxanthine	152.89	115.49	Purine metabolism
	Urate	40.60	37.92	Purine metabolism
	Allantoin	22.06	20.18	Purine metabolism
	Inosine	3.93	3.22	Purine metabolism
	Uracil	5.43	4.79	Pyrimidine metabolism
	Cytidine	3.88	2.66	Pyrimidine metabolism
	Orotate	2.52	2.44	Pyrimidine metabolism
Peptide	Arg-Lys-Val-Ser	70.52	60.76	Basic tetra-peptide
	Ala-Lys-Ser-Arg	4.79	4.51	Basic tetra-peptide
	Ala-Met-Met-Asp	3.70	3.49	Basic tetra-peptide
	Asn-Arg	3.22	3.20	Basic di-peptide
	Gly-Arg	3.25	3.44	Basic di-peptide
	Ile-Ser-Ser-Tyr	7.29	4.37	Hydrophobic tetra-peptide
	Val-Val	15.63	14.68	Hydrophobic di-peptide
Carbohydrate Metabolism	Myo-inositol	3.27	3.08	Galactose metabolism
Metabolism of Cofactors and Vitamins	Pyridoxamine	1.86	1.37	Vitamin B6 metabolism

Table 12b: Identification and relative intensities ($\times 10^3$) of eight larval compounds which have not been associated with any metabolomic maps or pathways. In total, 39 compounds were detected during a pilot study of HILIC metabolomic analysis of BioBag devices (*n*= 3), prepared from neat and fractionated (MWCO, < 3 kDa) material.

Map	Compound	Neat LAP	MWCO LAP	Overview
No map or pathway	N-Acetyl-L-histidine	53.96	49.58	Histidine derivative, no known function
	3-Methylguanine	11.53	8.03	Guanine derivative
	Tyramine-O-sulfate	9.20	7.28	Tyrosine derivative
	Isopropyl beta-D-galactopyranoside	7.53	6.23	Carbohydrate derivative, unknown mechanism
	Tryptophan hydroxamate	7.08	14.88	Tryptophan derivative
	7-Aminomethyl-7-carbaguanine	4.13	3.67	Precursor in queuosine (nucleoside) biosynthesis
	2-Hydroxyadenine	2.77	2.12	Adenine derivative
	Apigenin 7-(6"-malonylglucoside)	2.07	1.57	Flavonoid biosynthesis, plant metabolite

5.3 Second study: standardised LAP

Leading on the from the HILIC pilot study, the first modification for this phase was the use of LAP collected following the standard procedure. Due to the results obtained from the pilot study, MWCO filters were omitted, therefore only native LAP and PBS were prepared for analysis. Six biological replicates were utilised to examine sample consistency. Furthermore, the RP-HILIC method was employed to verify the initial findings and to capture additional non-polar compounds.

5.3.1 Materials and methods

Preparation of LAP

All reagents were obtained from Sigma-Aldrich Ltd (Missouri, USA), unless otherwise stated. Collection solutions were prepared from larval-loaded BioBags and control devices (n= 6 per group), and assessed as described previously (**Section 2.2.2.1**).

Potential metabolites were extracted from all samples (25 µL) using 1 mL of cold solvent mix (4ºC) of chloroform, methanol and 18.2 MΩ water (Millipore; Massachusetts, USA) at a ratio of 1:3:1. Samples were cool-vortexed for 5 minutes, prior to centrifugation (13,000 × g; 3 minutes) at 4^{o}C. Supernatants were removed to cryotubes and a pooled quality control (QC) sample was prepared using 25 µL aliquots from the test samples, alongside a solvent blank (500 µL). All processes were undertaken in a Class II microbiological safety cabinet. All samples (n= 14) were stored at -80^{o}C prior to transportation on dry ice to the University of Glasgow Polyomics facility.

LC-MS analysis and data processing

All samples were submitted for analysis, which was undertaken the at University of Glasgow. A rapid separation liquid chromatograph (RSLC; UltiMate 3000) coupled with an Orbitrap Exactive mass spectrometer (MS) operating with electrospray ionisation (ESI) running in the positive and negative ion modes was used to analyse the collected samples. A single run, comprising dual separation for polar and non-polar substances was employed based on the method of Haggarty *et al* (2015). All subsequent steps were undertaken as described in **Section 5.2.1**. Data files were supplied for subsequent analysis and interpretation.

Data analysis

All data were handled as described in **Section 5.2.1**.

5.3.2 Results

The initial RP-HILIC IDEOM dataset consisted of 195 detected compounds in total; 35 had been identified in comparison with the mass and retention time of authentic standards (17.9%). The remainder were identified using the linked databases (**Section 4.2.2**). Group differentiation was again achieved using unsupervised principal component analysis from the peak intensity, mass and retention time data, from the combined ESI modes (**Chart 27**). The control samples were clustered, showing consistency and the distinction between the LAP and control samples was clear.

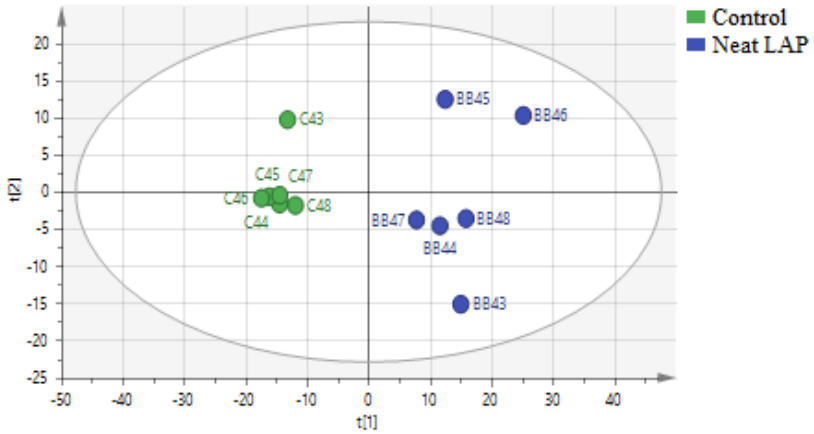

Chart 27: Unsupervised principal component analysis for test sample separation, based on peak intensity, mass and retention time data (combined positive and negative mode).

Step-wise data curation was undertaken (**Figure 23**), individual replicates were examined for the presence of potential larval biomarkers. Initially, 99 compounds with a confidence score ≥ 5 were selected for exploration (50.8%). It was however, noted that in almost all instances, the chromatogram peak quality was poor (**Chart 28**). As a combined detection method, this may be expected to some degree, but as non-polar compounds and compounds matched to standards also produced sub-standard peaks, data curation was undertaken conservatively. Therefore, the complete HILIC and RP-HILIC datasets were compared to determine commonality in each dataset, by examining the molecular formulae of all detected compounds. Before considering any selection parameters, 54 shared compounds were found (peak quality was superior in the HILIC dataset) and of these, 21 were identified as Level 1 metabolites and 15

compounds had been named according to alternative isomers. For example, in the HILIC dataset $C_{11}H_{12}N_2O_2$ appeared as the Level 1 metabolite L-tryptophan, but in the RP-HILIC dataset, it was annotated as the Level 2 compound D-tryptophan. Due to lowered confidence with the RP-HILIC identifications, these alternative isomers were subsequently re-assigned according to the HILIC results, for the purposes of this study. These data curation steps were undertaken to verify the findings of the two methods and to improve confidence in the results.

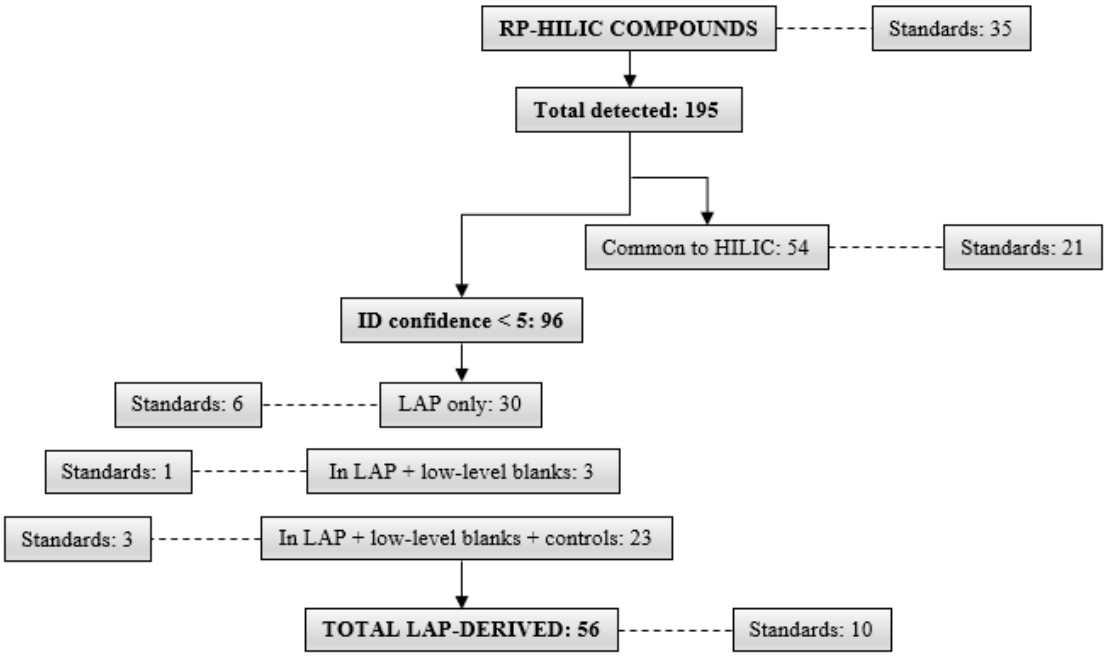

Figure 23: Flow-chart summarising data curation method relating to 195 compounds detected during a metabolome-profiling study of standardised LAP, using RP-HILIC.

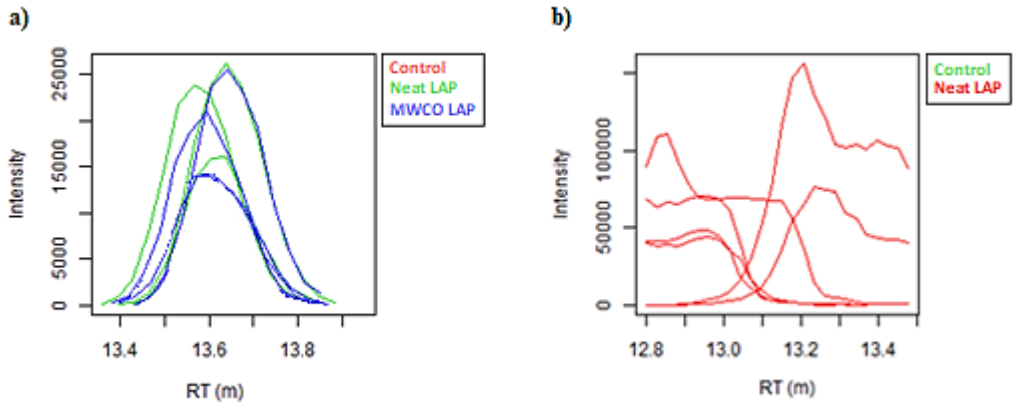

Chart 28: Example of peak quality comparison between two metabolomic profiling methods, demonstrated with the Level 1 metabolite allantoin as detected in all LAP samples using HILIC (**a**) and RP-HILIC (**b**) detection methods.

All significant compounds of commonality were selected for exploration if they were present in all LAP replicates, in both the HILIC and RP-HILIC datasets (and below the detection threshold of 1000, if present in the control or blank, as in **Section 5.2.2**). Fourteen mutual compounds were identified, which included six Level 1 metabolites, and the tetra-peptide Arg-Lys-Val-Ser (**Chart 29**).

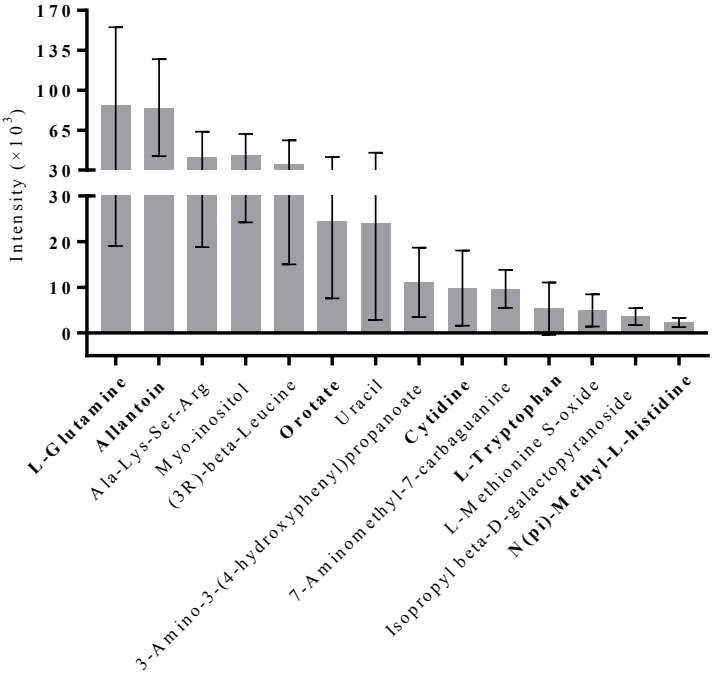

Chart 29: Identification and intensities ($\times 10^3$) of 14 compounds detected using RP-HILIC metabolomic profiling, which were also detected during the HILIC pilot phase, as used in the analysis of standardised LAP collected from BioBag devices. Compounds in bold have been identified in comparison with authentic standards (Level 1 metabolites).

Following the isolation of common compounds, the original RP-HILIC dataset was subjected to further stringent examination; compounds of interest had to be present in all LAP replicates, and the minimum intensity per replicate had to be at least double the base detection threshold if the compound also had a low-level presence in the controls or blanks. Thirty-six LAP-only compounds were detected in all replicates (n= 6), of which six were identified as Level 1 metabolites. A further three compounds were detected in all LAP samples (including one Level 1 metabolite), but these were also present in low-level quantities in the blanks (n= 7 blanks). Additionally, 23 compounds were detected in all LAP samples (including three Level 1 metabolites), but these were also present in low-level quantities in the blanks (n= 7 blanks) and the controls (n= 6 blanks). In total, 56 compounds from the original dataset were detected in all LAP

replicates (28.7%), ten of which were identified according to authentic standards (5.1%).

By using the KEGG database to assign each compound to a metabolic map, it can be seen that 42 compounds, across 21 pathways were identified (excluding peptides, $n = 4$), with pyrimidine metabolism being the most frequent (**Table 13a**). The primary pathways are shown, but there is overlap with many compounds. The remaining compounds ($n = 14$) were not linked to any map or pathway (**Table 13b**).

5.3.3 Significance of findings

Of the 195 compounds detected during the RP-HILIC metabolomics study, 56 were selected as LAP-derived compounds of interest. Of these, 10 were additionally identified in comparison with authentic standards and therefore their presence in LAP as Leve1 metabolites may be treated with confidence. Additional confidence in the method was supplied by the recognition of allantoin (**Section 1.4.3** and **Section 5.2.3**), and 13 other LAP-only substances which were also detected in the HILIC pilot study (**Chart 29**).

Once again, the metabolism of amino acids represented the largest map proportion ($n = 22$, **Table 13a**) and this group contained the largest number of Level 1 metabolites ($n = 6$). The remainder of the Level 1 metabolites were associated with nucleotide metabolism ($n = 3$) and carbohydrate metabolism ($n = 1$). These Level 1 metabolites will be considered further, alongside those detected using the HILIC method, in relation to their presence in LAP and their relevance to healing in the forthcoming Summary (**Section 5.4**).

The initial outcome of the RP-HILIC study confirmed that the use of MWCO filters is unnecessary for the preparation of regulated LAP material for such studies. Despite the inter-study and intra-replication variability (as observed with the proteomics phase), commonality and consistency has been observed within the LAP samples and potential larval biomarkers can be identified.

(Continued after **Table 13b**)

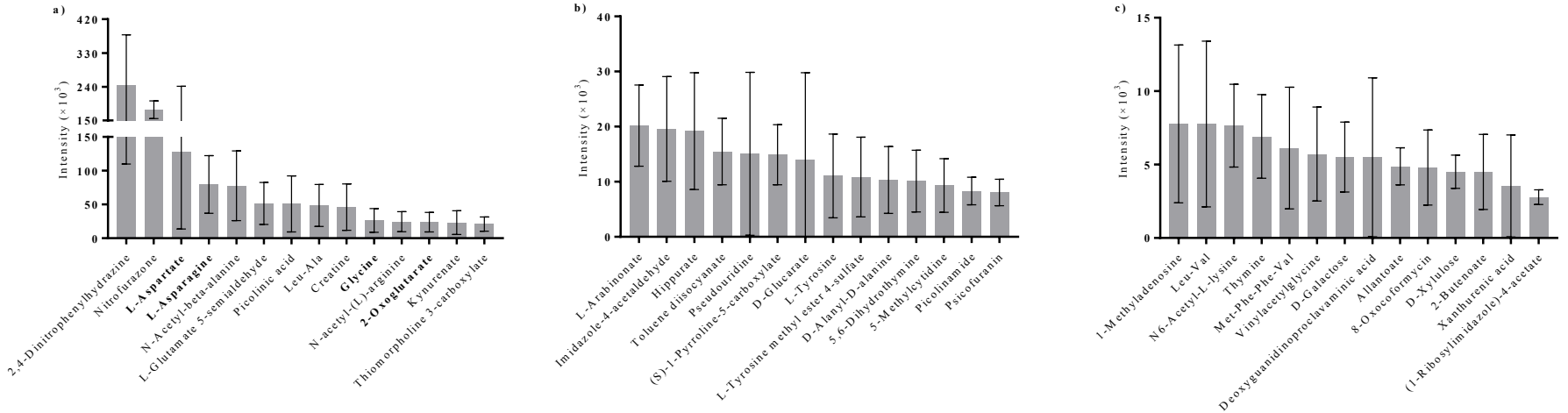

Chart 30 (a-b): Identification and intensities ($\times 10^3$) of 42 additional larval compounds detected during RP-HILIC metabolomic analysis of LAP collected from BioBag devices (*n* =6). In total, 56 compounds of interest were identified. Compounds in bold have been identified in comparison with authentic standards (Level 1 metabolites), the remainder are classed as Level 2 putatively annotated compounds having been matched to databases (using mass and retention time).

Table 13a: Identification and relative intensities ($\times 10^3$) of 44 larval compounds known to be associated with metabolomic maps and pathways. In total, 56 LAP-derived compounds were detected during RP-HILIC metabolomic analysis of BioBag devices. Compounds in bold have been identified in comparison with authentic standards.

Map	Compound	Neat LAP	Primary Pathway
Amino Acid Metabolism	**L-Aspartate**	127.52	Alanine and aspartate metabolism
	L-Asparagine	79.58	Alanine and aspartate metabolism
	L-Glutamate 5-semialdehyde	51.39	Arginine and proline metabolism
	Creatine	46.13	Arginine and proline metabolism
	(S)-1-Pyrroline-5-carboxylate	14.90	Arginine and proline metabolism
	N-Acetyl-beta-alanine	77.63	Beta-alanine metabolism
	D-Alanyl-D-alanine	10.31	D-alanine metabolism
	L-Glutamine	87.17	Glutamine and glutamate metabolism
	Glycine	26.39	Glycine, serine and threonine metabolism
	Imidazole-4-acetaldehyde	19.59	Histidine metabolism
	(1-Ribosylimidazole)-4-acetate	2.78	Histidine metabolism
	N(pi)-Methyl-L-histidine	2.30	Histidine metabolism
	N6-Acetyl-L-lysine	7.65	Lysine degradation
	Hippurate	19.17	Phenylalanine metabolism
	L-Methionine S-oxide	4.95	Methionine metabolism
	Picolinic acid	50.80	Tryptophan metabolism
	Kynurenate	23.22	Tryptophan metabolism
	L-Tryptophan	5.31	Tryptophan metabolism
	Xanthurenic acid	3.55	Tryptophan metabolism
	L-Tyrosine	11.33	Tyrosine metabolism
	3-Amino-3-(4-hydroxyphenyl)propanoate	11.06	Tyrosine metabolism
	(3R)-beta-Leucine	35.35	Valine, leucine and isoleucine degradation
Nucleotide Metabolism	**Allantoin**	84.72	Purine metabolism
	Allantoate	4.88	Purine metabolism
	Orotate	24.56	Pyrimidine metabolism
	Uracil	24.10	Pyrimidine metabolism
	Pseudouridine	15.06	Pyrimidine metabolism
	5,6-Dihydrothymine	10.11	Pyrimidine metabolism
	Cytidine	9.82	Pyrimidine metabolism
	Thymine	6.92	Pyrimidine metabolism
Carbohydrate Metabolism	L-Arabinonate	20.18	Ascorbate and aldarate metabolism
	D-Glucarate	14.03	Ascorbate and aldarate metabolism
	2-Oxoglutarate	23.65	Citrate cycle
	myo-Inositol	42.94	Galactose metabolism
	D-Galactose	5.51	Galactose metabolism
	D-Xylulose	4.52	Pentose and glucuronate interconversions
Secondary Metabolites	Deoxyguanidinoproclavaminic acid	5.49	Clavulanic acid biosynthesis
Lipids: Fatty Acyls	2-Butenoate	4.50	Fatty acids and conjugates
Peptide	Ala-Lys-Ser-Arg	41.29	Basic tetra-peptide
	Met-Phe-Phe-Val	6.13	Hydrophobic tri-peptide
	Leu-Ala	48.40	Hydrophobic di-peptide
	Leu-Val	7.76	Hydrophobic di-peptide

Table 13b: Identification and relative intensities ($\times 10^3$) of 14 larval compounds which have not been associated with any metabolomic maps or pathways. In total, 56 LAP-derived compounds were detected during RP-HILIC metabolomic analysis of BioBag devices. Compounds in bold have been identified in comparison with authentic standards.

Map	Compound	Neat LAP	Overview
No map or pathway	2,4-Dinitrophenylhydrazine	244.18	Secondary metabolite
	Nitrofurazone	178.83	Pharmaceutical compound
	N-acetyl-(L)-arginine	24.48	Guanidino compound
	Thiomorpholine 3-carboxylate	20.96	Dehydrogenase substrate
	Toluene diisocyanate	15.48	Plasticiser derivative
	L-Tyrosine methyl ester 4-sulfate	10.85	Tyrosine derivative
	7-Aminomethyl-7-carbaguanine	9.66	Precursor in queuosine (nucleoside) biosynthesis
	5-Methylcytidine	9.31	Nucleoside
	Picolinamide	8.31	Picolinic acid derivative
	Psicofuranin	8.05	Nucleoside
	1-Methyladenosine	7.77	Nucleoside
	Vinylacetylglycine	5.72	Fatty acid minor metabolite
	8-Oxocoformycin	4.80	Oxidoreductase substrate
	Isopropyl beta-D-galactopyranoside	3.58	Carbohydrate derivative, unknown mechanism

5.4 Metabolic pathways of interest

All primary pathways (**Tables 12** and **Table 13**) were examined (available from http://www.kegg.jp/kegg/pathway.html) to demonstrate the potential associations and origins of the detected LAP components. Those of particular interest or significance are detailed in the following sections. Although many of the pathways may overlap, speculation as to probable reactions may be bolstered by the occurrence of linked routes.

5.4.1 Pyrimidine metabolism

Regarding the LAP compounds of commonality, those relating to pyrimidine metabolism were the most dominant (*n*= 9); however not all bases were represented in the dataset. Uracil was detected using both methods, thymine was only detected in LAP using RP-HILIC and cytosine did not occur in either total dataset. The positions of the LAP compounds and the related reactions can be seen in **Figure 24** (as isolated from the full map), and not all the compounds are linked.

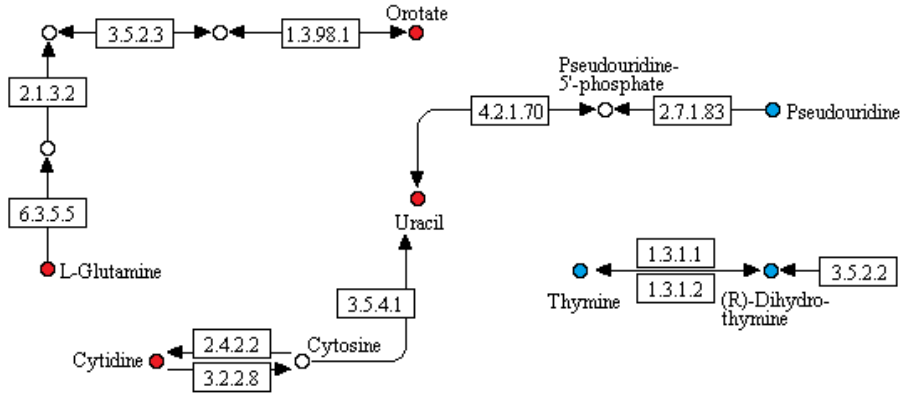

Figure 24: Sections of the pyrimidine metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). Circles in red denote that the compound was detected using both HILIC and RP-HILIC methods, circles in blue denote that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Thymine and 5,6-dihydrothymine [shown as (5R)-dihydrothymine in **Figure 24**] may each give rise to the other by means of dehydrogenase activity [enzyme commission (EC) numbers shown boxed], and cytidine may lead to the production of uracil, but the base cytosine did not occur (even as an excluded compound) in either of the complete datasets. Pseudouridine, orotate and glutamine may be in close proximity to other compounds, but they do not appear to be linked within this metabolic pathway. Neither

pseudouridine or orotate feature in any other map, and glutamine is primarily associated with the combined glutamine and glutamate metabolism pathway (**Section 5.4.3**).

5.4.2 Purine metabolism

Neither of the purine bases adenine or guanine occurred in the RP-HILIC dataset; however, adenine was detected in the LAP samples using HILIC, but also above the base threshold in the blanks and the control samples. Therefore, adenine was not initially included for consideration as a larval biomarker, but has been included in the consideration of possible metabolic routes, as shown in **Figure 25**. An isomer of guanine, 2-hydroxyadenine was detected using HILIC, although this is not linked to any known pathways (**Table 12b**) and as guanine, it was associated with a poor identification confidence level (2). Neither occurred in the RP-HILIC dataset.

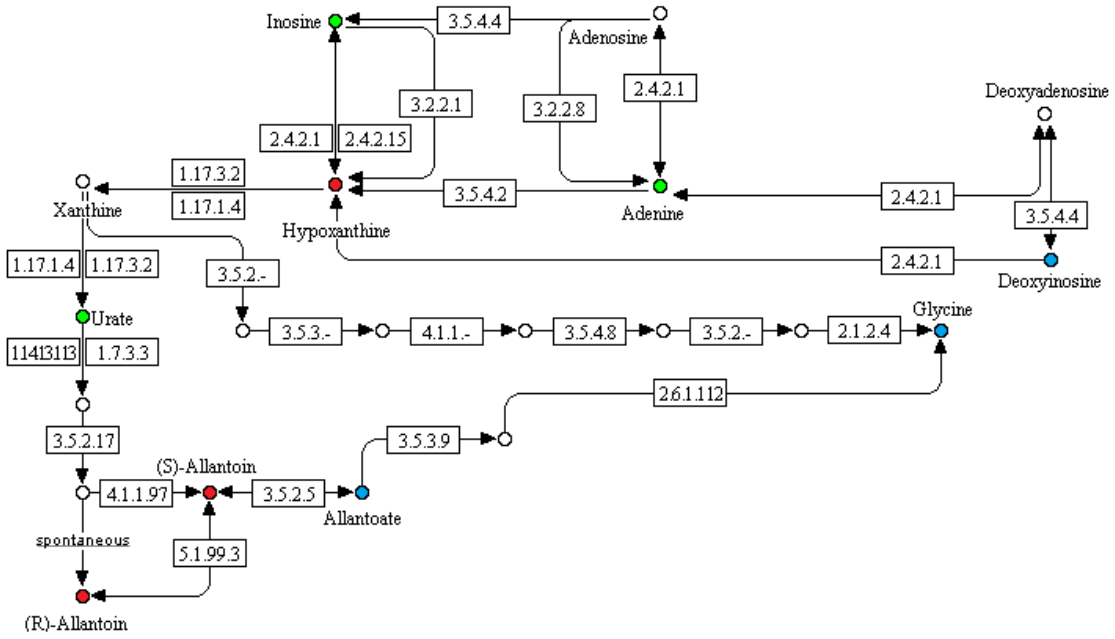

Figure 25: Sections of the purine metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). Circles in red denote that the compound was detected using both HILIC and RP-HILIC methods, circles in blue denote that the compound was detected using RP-HILIC only and those in green denote that the metabolite was detected using HILIC only. None of the other named compounds occurred in either dataset (white circles). Boxes contain the commission numbers as used in enzyme classification.

The most abundant component detected using the HILIC method, was the purine derivative hypoxanthine (**Chart 26a**). In the RP-HILIC system, it occurred as the isomer allopurinol, but as it was present in only four of the six replicates, it was not initially included for consideration as a larval biomarker. Additionally, the RNA

nucleoside inosine was also detected using HILIC, and in the RP-HILIC dataset, the DNA nucleoside deoxyinosine also occurred, but it was not included initially as a significant LAP-only compound (it was detected in only five of the six replicates). Again, these have been included in the consideration of possible metabolic routes, as shown in **Figure 25**.

Between hypoxanthine and allantoin (the only original compound of commonality in this pathway), is urate, which was detected using the HILIC method. Conversely, only RP-HILIC detected allantoate which is produced from allantoin (by means of the hydrolase enzyme allantoinase, EC 3.5.2.5) and leads to the production of the amino acid glycine, which was also only detected using the reverse-phase method (primarily part of the combined glycine, serine and threonine metabolism map, **Section 5.4.3**). None of the other named compounds shown in **Figure 25** occurred in either dataset. Had deoxyadenosine been present, a complete route would have been demonstrated.

Related to this pathway, is the presence of 7-methyladenine (as part of base excision repair, **Table 12a**) which was detected using the HILIC method, alongside the unassigned nucleobase derivatives, 2-hydroxyadenine and 3-methylguanine (**Table 12b**).

5.4.3 Amino acid metabolism

Individual pathways linked to amino acid metabolism were the most dominant overall (*n*= 15 individual pathways). In terms of commonality, glutamine and tryptophan were the only native amino acids detected using both methods.

Glutamine and glutamate metabolism

Glutamine is primarily associated with the glutamine/glutamate metabolism pathway, and although the latter did occur in each dataset, it was not included as a potential biomarker as it was either present above the threshold limit in the blanks (HILIC) or in only four of the six LAP replicates (RP-HILIC). Although 2-oxoglutarate (RP-HILIC) is considered primarily as part of the citrate cycle (**Table 13a**) it may be formed from glutamate (**Figure 26**). Each of the metabolites occur in several other pathways, but a simple link between them is evident by the route shown overleaf.

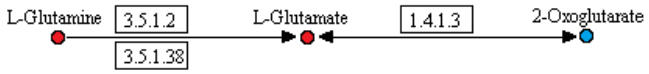

Figure 26: Section of the glutamine and glutamate metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). Circles in red denote that the compound was detected using both HILIC and RP-HILIC methods, the circle in blue denotes that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Tryptophan metabolism

Components of the tryptophan metabolism were more numerous; beyond being detected using both methods, each system provided further insight (**Figure 27**). Kynurenine and kynurenate were initially detected using HILIC and RP-HILIC respectively, although the origin of the latter is kynurenine and can only be produced along this route. In the RP-HILIC dataset, kynurenine was not included as a significant compound, as it was present in only four of the six replicates. At the same confidence level, is the alternative isomer formyl-5-hydroxykynurenamine. In this form, it can only be produced downstream of tryptophan with serotonin as an intermediary. This has relevance to the proteomics phase of this study, as a peptide was matched to a dopamine N-acetyltransferase-like protein (**Section 4.2.2**, **Table 7**) and this is the enzyme represented by EC 2.3.1.87 in **Figure 27**; and both forms of the isomer are also shown. Additionally, xanthurenic acid (as xanthurenate in **Figure 27**) was also detected using RP-HILIC, which again, can only be produced downstream of kynurenine. Furthermore, the reverse-phase method detected picolinic acid (as picolinate in **Figure 27**) and its derivative picolinamide (**Table 13b**, not linked to any pathway), although these did not occur in the HILIC dataset.

No other intermediate compounds in the routes shown featured in either dataset. Tryptophan hydroxamate (**Table 12b**), is classed as a derivative of tryptophan, but is not associated with any metabolic pathway, and was detected by HILIC only. It may be surmised that kynurenine (and possibly the related compounds) may also be a suitable larval biomarker, alongside tryptophan itself.

(Continued after **Figure 27**)

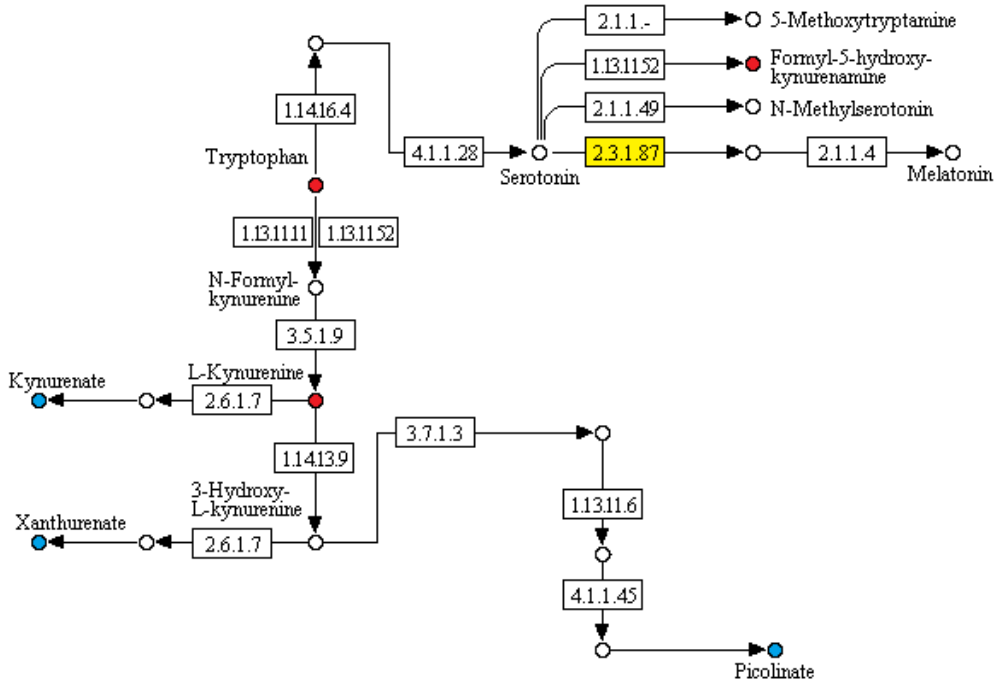

Figure 27: Section of the tryptophan metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). Circles in red denote that the compound was detected using both HILIC and RP-HILIC methods, circles in blue denote that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification, the enzyme highlighted in yellow, dopamine N-acetyltransferase (2.3.1.87) was identified using peptide mass fingerprinting.

Histidine metabolism

Common to both datasets was the presence of the intermediate metabolite N(pi)-methyl-L-histidine (shown as 1-methyl-L-histidine in **Figure 28**), which can only be formed from histidine via alternative routes, one of which includes a di-peptidase (3.4.13.5) which acts upon N-acetyl-L-histidine. Although the latter is not part of any metabolomic pathway, this compound was detected using the HILIC method (**Table 12b**).

Histidine itself occurred in both datasets, but as it was always present in the control and blank samples, it was not considered as a LAP-only compound; however, due to its repeated (and elevated) presence it is shown within the pathway in **Figure 28**. Histamine did not occur in either dataset, but it can only be formed from the histidine metabolite in the presence of a decarboxylase (EC 4.1.1.22) and this is the only route which gives rise to N-acetylhistamine [shown as 4-(beta-acetylaminoethyl)imidazole in **Figure 28**]. This end-product was detected using HILIC, but as it occurred in the RP-HILIC dataset in only five of the six LAP replicates, it was not initially considered as a LAP-only compound.

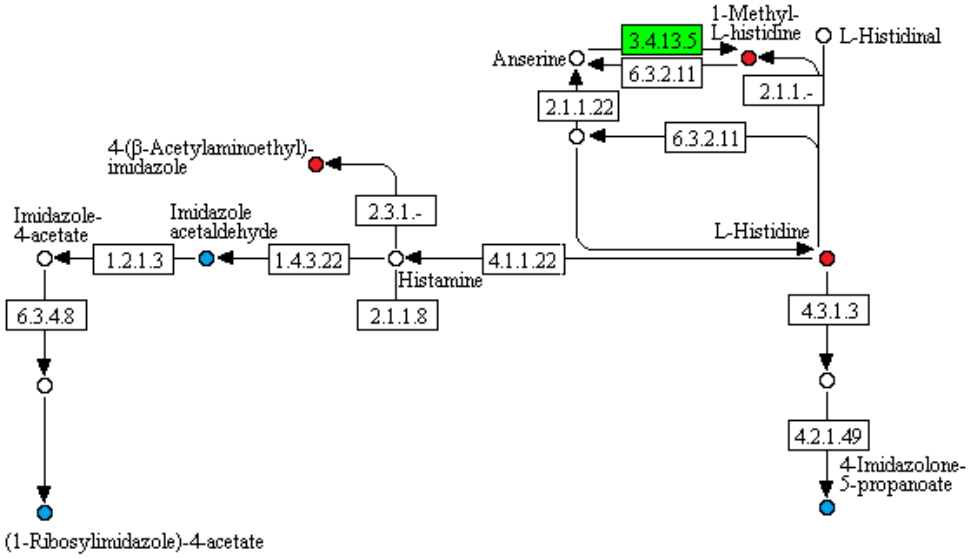

Figure 28: Section of the histidine metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). Circles in red denote that the compound was detected using both HILIC and RP-HILIC methods, circles in blue denote that the compound was detected using RP-HILIC only. The green box (with commission number) denotes the enzyme which acts upon N-acetyl-L-histidine (not part of any pathway) which was detected using HILIC. Note that 4-(β-acetylaminoethyl)imidazole is a synonym of N-acetylhistamine, and that 1-methyl-L-histidine is a synonym of N(pi)-methyl-L-histidine.

Furthermore, imidazole-4-acetaldehyde was detected using the reverse-phase method (shown as imidazole acetaldehyde in **Figure 28**), which again, can only be formed from histamine, and was detected using RP-HILIC only. This compound gives rise to (1-ribosylimidazole)-4-acetate which was also detected using the reverse-phase method. Regarding 4-imidazolone-5-propanoate, this was detected using RP-HILIC, but it was not included initially as a LAP-only compound as it occurred in three of the six replicates. They have all been included in **Figure 28** to demonstrate the possible links.

Tyrosine metabolism

Tyrosine was detected using RP-HILIC, alongside its isomer, 3-Amino-3-(4-hydroxyphenyl)propanoate, which was also detected using both systems (**Chart 29**). The latter is an end-product which can only be produced from tyrosine by a single isomerase reaction (EC 5.4.3.6), and it does not feature in any other pathway. Derivatives were also detected using HILIC and RP-HILIC, as tyramine-O-sulfate (**Table 12b**) and L-tyrosine methyl ester 4-sulfate (**Table 13b**) respectively, but these also do not form part of any other metabolic pathway. Otherwise components of this particularly complex pathway appear to be under-represented in LAP (**Figure 29**).

Figure 29: Section of the tyrosine pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The red circle denotes that the compound was detected using both HILIC and RP-HILIC methods, and the blue circle denotes that the compound was detected using RP-HILIC only. The box contains the commission number of a isomerase enzyme.

Alanine and aspartate metabolism

The HILIC method detected alanine, which occurred in the RP-HILIC dataset, but it was excluded as a LAP-only compound as it was present in all the blank replicates, above the threshold. It is shown in **Figure 30** as a mutual metabolite, alongside aspartate and asparagine, which were detected only by the reverse-phase method. Each of the metabolites occur in several other pathways, but a simple link between them is evident by the route shown.

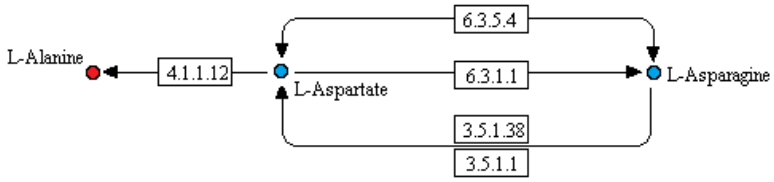

Figure 30: Section of the alanine and aspartate metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circle in red denotes that the compound was detected using both HILIC and RP-HILIC methods, circles in blue denote that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Methionine metabolism

Methionine was detected using HILIC, but it did not occur in the RP-HILIC dataset; however, its precursor L-methionine S-oxide was present in both datasets and it does not appear in any other pathway, as it is considered as an oxidation product of methionine. Stereoisomer reductase enzymes (EC 1.8.4.13 and 1.8.4.14) facilitate its conversion to methionine (**Figure 31**).

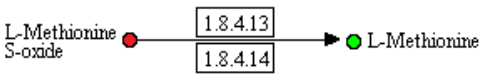

Figure 31: Section of the methionine metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circle in red denotes that the compound was detected using both HILIC and RP-HILIC methods, the circle in green denotes that the compound was detected using HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Glycine, serine and threonine metabolism

Glycine was detected using RP-HILIC only, and it did not occur at all in the HILIC dataset. Likewise, serine was not detected using HILIC, and was present in all reverse-phase LAP samples however, it was not included as a LAP-only metabolite as it had a low-level presence in one of the replicates. Threonine was present in four of the six LAP samples in the RP-HILIC dataset and its isomer allothreonine was detected using HILIC, but it was present in the control samples and at only low-levels in the LAP replicates. Additional compounds were detected, predominantly using RP-HILIC (**Figure 32**), although betaine was also detected using HILIC, which in the RP-HILIC dataset, appeared as an isomer of the amino acid valine. It was not included as a LAP-derived compound as it was present in the control at quantities well above the threshold limit. Sarcosine was detected using RP-HILIC (as an isomer of alanine, and above the base threshold in all controls and blanks), alongside dimethyglycine (also above these thresholds) and creatine, which was selected as a LAP-only compound, but it did not occur in the HILIC dataset.

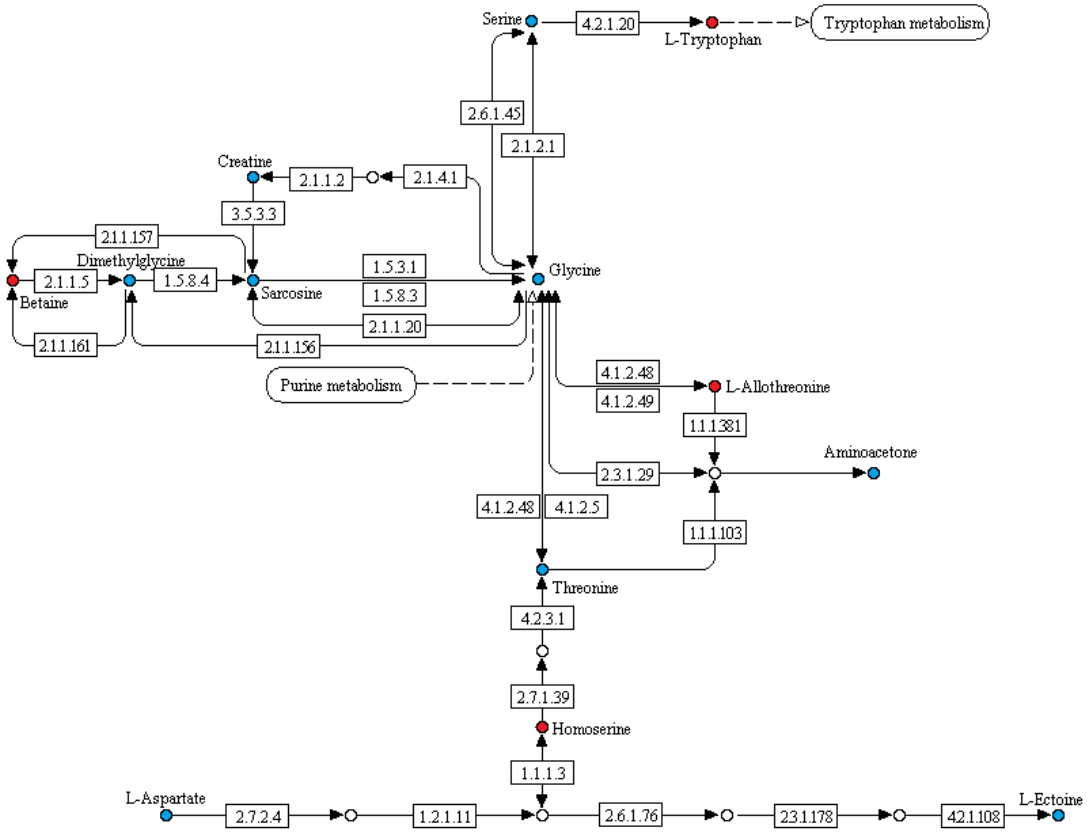

Figure 32: Section of the glycine, serine and threonine metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circle in red denotes that the compound was detected using both HILIC and RP-HILIC methods, the circles in blue denote that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Aspartate (as aforementioned, **Figure 30**) was detected using the reverse-phase method, and may give rise to ectoine, but the latter compound was present in only five of the LAP replicates, one of which was below the minimum threshold for detection. Incorporated into this route, homoserine (HILIC; as an isomer of threonine and allothreonine) may be encountered, which can give rise to threonine, leading to the production of aminoacetone (RP-HILIC; above the base threshold in all controls and blanks). Taking all of these possible metabolites into account, a well-characterised map emerges. This pathway is also linked to tryptophan metabolism, another significant map.

Arginine and proline metabolism

The reverse-phase method detected LAP-only compounds within this pathway (**Figure 33**), which included creatine, L-glutamate 5-semialdehyde, and its precursor (S)-1-pyrroline-5-carboxylate (**Table 13a**). The latter may give rise to glutamate (both methods, as aforementioned), which can be formed from the amino acid arginine; this occurred in both datasets, but always at elevated levels within the control replicates. Creatine, and sarcosine, also feature in the glycine, serine and threonine metabolism pathway (**Figure 32**). The end-product octopine (RP-HILIC) would have been considered as a LAP-only compound, as it was not present in the blanks or the controls, but it was found at low-levels in four of the LAP replicates. In addition, the HILIC method detected the related compound N-acetyl-L-glutamate, which forms part of the arginine biosynthesis pathway and therefore is not shown in **Figure 33**. Regarding proline, this amino acid did not occur in either dataset.

(Continued after **Figure 33**)

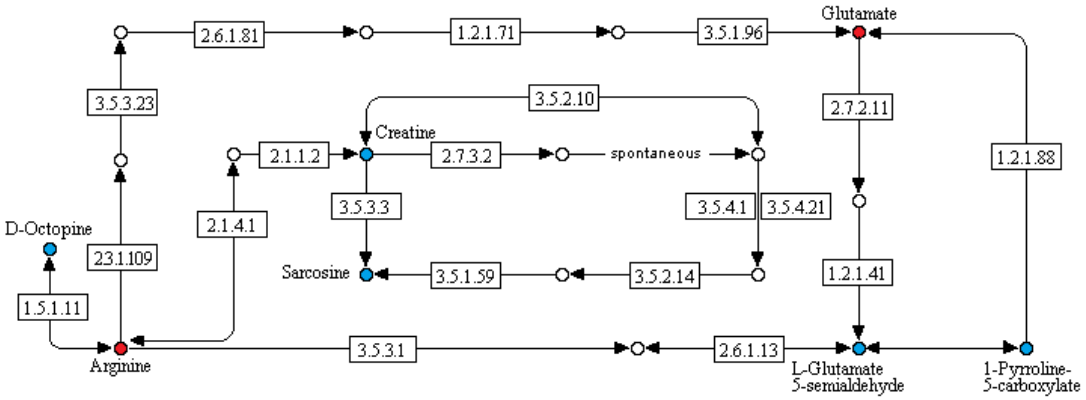

Figure 33: Section of the arginine and proline metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circle in red denotes that the compound was detected using both HILIC and RP-HILIC methods, the circles in blue denote that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Beta-alanine metabolism

Both N-acetyl-beta-alanine and aspartate (as aforementioned) were detected as significant LAP compounds (RP-HILIC, **Table 13a**), both of which are end-products of beta-alanine metabolism (**Figure 34**). This amino acid was present in both datasets, but as the isomer of alanine (as aforementioned). Additionally, the precursor uracil, was detected using both methods (**Figure 34**), suggesting that this portion of the full pathway may be represented in larval compounds.

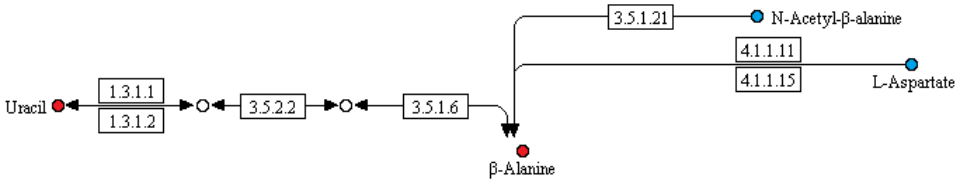

Figure 34: Section of the beta-alanine metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circles in red denotes that the compound was detected using both HILIC and RP-HILIC methods and the circles in blue denote that the compound was detected using RP-HILIC only. Boxes contain the commission numbers as used in enzyme classification.

Satellite metabolites

The remaining compounds associated with amino acid metabolism could not be linked to any routes within the pathways. Most notably, this relates to (3R)-beta-leucine which was common to both datasets, and is an isomer of leucine. According to IDEOM, it is associated with the degradation of valine, leucine and isoleucine (combined pathway), but beta-leucine did not actually feature in the map, and no other components were detected. Likewise, phenylalanine was detected using HILIC, although it also occurred

in the RP-HILIC dataset in only four of the six LAP replicates. It was not linked to any other compounds in the pathway, but the intermediary hippurate was detected in all RP-HILIC replicates; however, it is distant from phenylalanine in the map (not shown). Only 2-hydroxypropylphosphonate, from the phosphonate and phosphinate metabolism map (not shown) was detected, using HILIC. Similarly, N6-acetyl-L-lysine was detected only using the reverse method, and was the only component represented in the lysine degradation map.

5.4.4 Additional pathways

The mutual compound myo-inositol is an end-product of galactose metabolism, although it is associated with many other pathways. It is formed from the sugar by the action of an alpha-galactosidase (EC 3.2.1.22, **Figure 35**), which was detected using RP-HILIC (**Table 13a**).

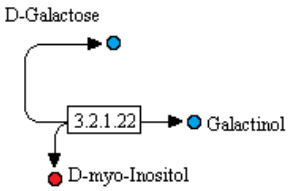

Figure 35: Section of the galactose metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circle in red denotes that the compound was detected using both HILIC and RP-HILIC methods, the circles in blue denote that the compound was detected using HILIC only. The box contains the commission number as used in enzyme classification.

The reverse-phase method also detected glucarate and arabinonate from the combined ascorbate and aldarate metabolism pathway (**Table 13a**), but these were distant from each other (map not shown), and no additional components from either of these carbohydrate metabolism maps were observed. Xylulose may be indirectly linked to 2-oxoglucarate (**Figure 26**) in the pentose and glucuronate interconversions pathway, but overall these maps are under-represented in LAP.

Although IDEOM links peptides to pathways (**Table 12a** and **Table 13a**), these are not actually represented within the KEGG maps. However, the presence of the mutual tetra-peptide Ala-Lys-Ser-Arg, demonstrates that short-sequence peptides may be reproducibly detected, which was not found to be the case in the proteomic study (**Section 4.2.2. and Section 4.3.2**). Furthermore, the peptide did not occur in any of the control or blank replicates, in either dataset. Of additional interest is the presence of five di-peptides, which according to the Human Metabolome Database (HMDB,

available from www.hmdb.ca), these are not only short-lived intermediates, but may also possess physiological or cell-signalling effects (Wishart *et al.*, 2013).

The remaining compounds of pyridoxamine (HILIC), deoxyguanidinoproclavaminic acid and 2-butenoate (RP-HILIC) were not linked to any other detected metabolites.

5.4.5 Unassigned compounds

Twenty-two compounds could not be linked to any primary pathways. Although derivatives of metabolites are present, it was the occurrence of two mutual larval products there were of particular interest. Isopropyl beta-D-galactopyranoside and 7-aminomethyl-7-carbaguanine were detected using both methods, and were classified as LAP-only compounds, as they were not observed in either the blank or control replicates. Although the latter is not listed in the KEGG database as a metabolite, it features in the HMDB as a precursor of queuosine biosynthesis, and it was subsequently found within KEGG as an end-product of folate (folic acid) biosynthesis by searching for the enzyme EC 1.7.1.13. This reductase reaction yields 7-aminomethyl-7-carbaguanine (**Figure 36**), but no other components of the map (including folate) occurred in either dataset. However, a precursor enzyme to this compound was recognised from the proteomic study (**Section 4.3.3**, **Table 10**), GTP-cyclohydrolase 1 (EC 3.5.4.16) which catalyses several steps from GTP to this end product, and is linked to dopamine synthesis and the tryptophan pathway (**Figure 27**). It would appear that 7-aminomethyl-7-carbaguanine cannot be formed by any other means.

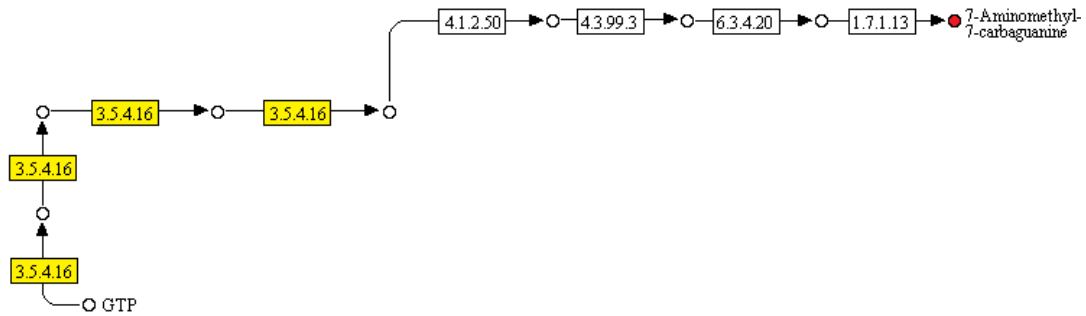

Figure 36: Section of the folate biosynthesis pathway, showing the end-product of this map, which was detected in all LAP samples, using both HILIC and RP-HILIC methods (red circle, additional reactions have been removed for clarity). Although no other components in the map were detected in this phase of the study, GTP-cyclohydrolase 1 (3.5.4.16) was identified using peptide mass fingerprinting.

Isopropyl beta-D-galactopyranoside was identified according to a single match within the MetaCyc database (**Section 5.2.2**) and no alternative isomers were listed in the

IDEOM datasets. In MetCyc, it was listed as an inhibitor of lactose galactohydrolase in 1989 (Caspi *et al.*, 2014). Now known as lactase (EC 3.2.1.108), the enzyme hydrolyses lactose to glucose and galactose, a reaction which can be found in the galactose metabolism pathway and its position is shown (**Figure 37**). Regarding lactose and glucose, these have been included for demonstrative purposes, despite sharing the same molecular formula with 41 and 56 other alternative isomers, respectively. The appeared in the RP-HILIC dataset as maltose and mannose respectively, in only one LAP replicate above the base threshold for inclusion. Lactose 6'-phosphate did not occur in either dataset, but is shown in **Figure 37** for completeness of the route. This evidence may suggest that isopropyl beta-D-galactopyranoside has been active as an inhibitor of lactase.

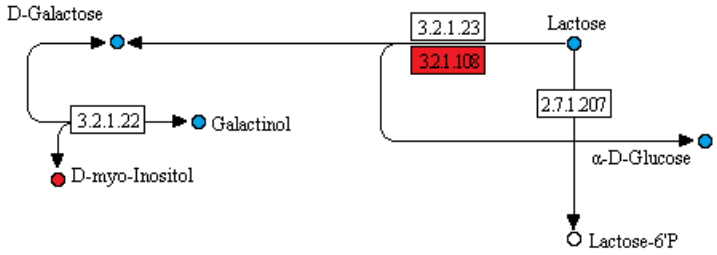

Figure 37: Section of the galactose metabolism pathway, showing compounds detected in LAP samples (additional reactions have been removed for clarity). The circle in red denotes that the compound was detected using both HILIC and RP-HILIC methods, the circles in blue denote that the compound was detected using RP-HILIC only; however, lactose and glucose were detected at low-levels. The red box (with commission number) denotes the enzyme which is known to be inhibited by isopropyl beta-D-galactopyranoside, a significant LAP-only compound which is not linked to any pathway, but was detected in all replicates, using both methods.

Additionally, the reverse-phase method detected the most abundant compounds in the entire dataset, which are not related to any metabolic pathways within KEGG. Regarding 2,4-dinitrophenylhydrazine and nitrofurazone (**Table 13b**), these compounds are isomers, but they appear as two distinct entries within IDEOM. They both occur within KEGG, annotated as a drug, particularly nitrofurazone, which is classed in the HMDB, as metabolite found only in patients which that have used or taken a drug by this name.

5.5 Summary

Untargeted metabolite profiling methods were applied to determine the baseline metabolite profile of the BB-50 bio-footprint. Unlike the proteomic analyses attempted (**Section 4.1**), commonality between replicates, and study phases, was observed.

Despite stringent data curation, 14 compounds were found to be present in every LAP replicate tested (*n*= 9). Furthermore, six of these may be considered as Level 1 metabolites, as their identifications were verified using authentic standards, in both the HILIC and RP-HILIC studies. As such, these metabolites are suitable larval biomarker candidates, and may be targeted for detection in the wound environment, following the initial application of BioBags to patients. By examining each metabolic pathway, support for the occurrence of the compounds in LAP is generated. If the metabolites can be detected in clinically-relevant quantities and linked to a therapeutic effect, they have the potential to be characterised in the manner of chymotrypsin (**Section 1.4.1**) and lucifensin (**Section 1.4.2**). Currently, no such metabolites have been characterised in this way.

Although the identifications would need to be confirmed, the results of this study suggest that many metabolites of *L. sericata* larvae may be associated with specific wound-healing activities. Purines and pyrimidines have long been associated with positive medical benefits (Mabley *et al.*, 2003), from studying their natural roles within healthy humans, their changes associated with disease and the application of exogenous material to exert clinical effects (Gendaszewska-Darmach & Kucharska, 2011). Amino acids have received similar attention, and each of these groups have been subjected to experimental testing, and with positive outcomes, particularly regarding wound healing (Veerabagu *et al.*, 1996; Jin *et al.*, 2014; Jones *et al.*, 2014; Squadrito *et al.*, 2014). As organisms with almost entirely proteinaceous feeding habits, it is expected that the excretory products of the larvae would be composed of degraded proteins and their components (Chen, 1966; Dow, 2013).

Pyrimidine metabolism was under-represented in comparison with purine metabolism. The only complete route was the reversible reaction of thymine to di-hydrothymine, whereas the other routes were lacking the intermediaries. (**Figure 24**). However, within this group, uracil, orotate and cytidine were significant LAP compounds (**Chart 29**), common to both methods and all replicates (*n*= 9 LAP samples). The latter two were identified in comparison with authentic standards and as such, are considered as Level 1 metabolites, possessing a high confidence in their identities. Beyond their roles within RNA structure, the nucleobase uracil is required to support many biochemical reactions, including enzyme synthesis, whilst the nucleoside cytidine acts as a substrate in the salvage pathway of pyrimidine nucleotide synthesis (Wishart *et al.*, 2013). Orotate (or

orotic acid), was previously known as vitamin B-13 (Loffler *et al.*, 2016) and is reported to stimulate and quicken the healing process, by mechanisms which increase the rate of collagen synthesis, fibroblast differentiation and phagocytic leucocytes (Yamaguchi, 1961; Cihak & Reutter, 1981; Dudnikova & Zaidenberg, 1981). It does not appear to have received much interest contemporaneously or to have been associated with larval products.

Of the eight compounds detected which are associated with purine metabolism, only allantoin was initially regarded as a significant LAP-only metabolite (**Chart 29**). Its relevance to maggot therapy has already been introduced and is an excretory product known to be associated with *L. sericata* larvae (**Section 1.4.3**). However, the Level 1 metabolite hypoxanthine was also detected using both methods (unlike the remaining route pathway components) and at excessively high levels. It was not originally regarded as a significant LAP metabolite, as it occurred in only four of the six RP-HILIC replicates. However, it was noted that the same two samples (BB43 and BB46) were consistently responsible for this deviation. Given that hypoxanthine has been shown to accelerate wound closure (Jiang *et al.*, 2006), and is a precursor to allantoin in the nitrogenous excretion pathway of insects, this metabolite may also be of interest in maggot therapy. However, hypoxanthine is located in the muscle tissues, therefore, it may not be suitable as a biomarker following larval treatment. Allantoin is not produced metabolically in humans (uric acid is excreted), although it has been suggested for use as a biomarker of oxidative stress (Gruber *et al.*, 2009). If the levels of these substances are significantly different from those detected in patients, they may hold the potential for use as larval biomarkers.

Amino acid metabolism accounted for the majority of the compounds detected, although only glutamine and tryptophan were initially regarded as significant LAP-only metabolites (**Chart 29**) both were identified in comparison with authentic standards. Glutamine is considered a non-essential amino acid and is abundantly present throughout the body, however it may be administered to patients when the demand increases, including for wound suffers (MacKay & Miller, 2003; Watford, 2015). Although it may be linked to clinical benefits, it is not suitable as a larval biomarker, due to its substantial presence in the body, especially in the blood (Kesici *et al.*, 2015). Tryptophan is an essential amino acid for both humans and insects, and as it cannot be natively formed, it is sourced from food. It is currently used as a medical biomarker,

and although it may not be suitable as a wound-relevant larval biomarker (depending upon relative levels), it has been associated with substantial wound healing effects. These include the reduction of inflammatory responses and the reversal of delayed re-epithelisation, collagen deposition and wound contraction (Bandeira *et al.*, 2015; Barouti *et al.*, 2015). As with hypoxanthine, kynurenine was absent from the same two LAP replicates, and therefore it was not initially considered as a significant LAP compound. However, it has been suggested for use as anti-inflammatory factor (Salimi-Elizei *et al.*, 2015) and is reported to increase the expression of matrix metalloproteinases and reduce wound scarring (Li *et al.*, 2014). Of particular interest is the likelihood of the tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase enzymes occurring in LAP, as shown in **Figure 27** (EC 1.13.11.11 and EC 1.1311.52, respectively). Both are receiving attention in neurodegeneration research as promising therapeutics (Breda *et al.*, 2016), but that latter (IDO) is associated with diverse antimicrobial action and immune responses (Mellor & Munn, 1999; Moffett & Namboodiri, 2003; King & Thomas, 2007). The tryptophan/kynurenine pathway is well documented in insects, for the production of pigments, particularly in the formation of ommochromes which can colour the eye and body of the insect (Vukusic & Chittka, 2013), although the neither of the metabolites have been associated with maggot therapy.

Another compound which has featured in the literature, which has also been detected in this study, is histidine. Initially, only N(pi)-methyl-l-histidine was regarded as significant LAP-only metabolite (**Chart 29**), as it was detected in all replicates and by both methods. Further exploration revealed the presence of histidine within both datasets, but it also occurred in all control replicates at quantities above the base threshold, although the relative abundance values demonstrated that histidine was approximately 50 times higher in the LAP samples. Maggot-derived histidine was reported to increase endothelial cell proliferation, and a role within wound healing by stimulation angiogenesis was suggested (Bexfield *et al.*, 2010). Regarding N(pi)-methyl-l-histidine, it is considered to be a product of peptide bond synthesis and is used as a biomarker, following the consumption of soy products (Wishart *et al.*, 2013). The occurrence of this metabolite demonstrates the importance of standardised rearing media, as soy is one of the dietary components in the *Lucilia* Agar hatching plate

(**Section 3.1.1.1** and **Table 4**), and it was not detected in any of the control replicates throughout the study.

An isomer of beta-tyrosine, 3-amino-3-(4-hydroxyphenyl)propanoate, was a compound of commonality (**Chart 29**). It does not appear to be directly associated with any specific wound healing activities; however, the use of tyrosine kinase inhibitors in tumour treatment has demonstrated that tyrosine residues significantly alter the magnitude of the response of cellular proliferation, activation and signalling (Okuda *et al.*, 1999). As it can only be formed from tyrosine, this end-product or its originator (**Figure 29**), may play a role in the advancement of wound healing.

The methionine precursor, L-methionine S-oxide was also a compound of commonality (**Chart 29**). The oxidation of methionine residues, to this compound, is reported to inactivate proteins (Grimaud *et al.*, 2001). The potential relevance of this to larval-facilitated wound healing may be speculated from existing literature, such as by the disruption of bacterial proteins or the destruction of wound-endogenous proteases (Brot *et al.*, 1981; Levine *et al.*, 1996). However, due to the readiness of methionine to be oxidised, the unintentional loss of a range of biological properties is the major concern regarding L-methionine S-oxide (Achilli *et al.*, 2015). The presence of this compound within LAP, and whether it elicits any effects (negative or positive) may prove of interest.

The final mutual compound, relating to amino acid metabolism, (3R)-beta-leucine, could not be linked to any KEGG pathways, but a reaction involving leucine 2,3-aminomutase (EC 5.4.3.7) appears to catalyse the interconversion, although no further information within KEGG was available. According to the HMDB, the presence of beta-leucine and its associated enzymes in the human body is debatable. As with L-methionine S-oxide, further investigations are warranted, and they may represent promising larval biomarkers.

Only one component of carbohydrate metabolism appeared as a mutual compound, myo-inositol, which is a natural sugar component of cell membrane phospholipids and has been considered previously as a B vitamin (Regidor & Schindler, 2016). It is routinely used as a treatment/supplement for patients with polycystic ovary syndrome and gestational diabetes, and is reported to reduce blood glucose levels of those with type-2 diabetes (Corrado *et al.*, 2011; Pintaudi *et al.*, 2016). Although myo-inositol and

diabetic wound treatment do not appear to have been directly linked, compounds incorporating the sugar moiety have been used in the treatment of burns, citing vasodilatory and anti-inflammatory properties as possible mechanisms of action (Tarnow *et al.*, 1996).

The remaining LAP-significant, mutally-detected compounds were not linked to any metabolic pathways, these were: Ala-Lys-Ser-Arg, 7-aminomethyl-7-carbaguanine and isopropyl beta-D-galactopyranoside. The tetra-peptide could not be searched using the BLASTp interface (**Section 4.2.1**) due to its small size of four residues. Isopropyl beta-D-galactopyranoside was identified according to a single match within the MetCyc database (**Section 5.2.2**), but no further information is available, other than being listed as an inhibitor of lactose galactohydrolase in 1989 (Caspi *et al.*, 2014). Also known as lactase (EC 3.2.1.108), the enzyme hydrolyses lactose to glucose and galactose, a reaction which can be found in the galactose metabolism pathway (**Figure 37**).

Isopropyl beta-D-galactopyranoside and 7-aminomethyl-7-carbaguanine were detected using both methods, and were classified as LAP-only compounds, as they were not observed in either the blank or control replicates. The latter is related to the biosynthesis of queuosine, a modified tRNA nucleoside, which improves translation accuracy. It has been documented in plants and animals, but it cannot be synthesised *de novo* and must be formed from the diet or intestinal microflora, either of which could be possible regarding LAP. Queuosine did not occur in either dataset, but it may participate in many cellular functions (Vinayak & Pathak, 2009). The role of isopropyl beta-D-galactopyranoside as an inhibitor of lactase, is unclear, as this enzyme is absent from many insects, including blow-fly larvae (Hobson, 1931b; Fletcher & Haub, 1933). However, it is complementary to the beta-galactosidase, lacZ (EC 3.2.1.23, **Figure 37**), the first gene in the lac operon (the control system of lactose-induced enzymes); both enzymes occur in animal and human intestinal mucosa (Asp *et al.*, 1969), although beta-galactosidase is more widely known and has been detected in the gut epithelium of insects (Nation, 2016).

Although these compounds did not occur in the HILIC dataset, the abundance intensities of 2,4-dinitrophenylhydrazine and nitrofurazone detected by the reverse-phase method, require further comment. The latter has been applied as a topical ointment to combat bacterial infections of the skin, including those related to burns and

wounds (Weiner & Fixler, 1959; Hooper & Covarrubias, 1983; Popiołek & Biernasiuk, 2017) and the alternative isomer has been linked to blow-fly larvae. Isolated from the screw-worm *Cochliomyia hominovorax* (**Section 1.4.2** and **Section 3.1.1.3**), 2,4-dinitrophenylhydrazine was reported to possess antibacterial properties. However, the larval digestive symbiont *Proteus mirabilis* (**Section 1.4.2**) was shown to be the source of this compound (Greenberg, 1968; Erdmann & Khalil, 1986), which is of relevance to endotoxin study (**Section 3.1.3.1**).

The characterisation of the low molecular weight components of the bio-footprint has proven more successful than the proteomic approach employed for high molecular weight exploration. Reproducible results, with high confidence were achieved, and these may be investigated further in the search for potential larval biomarkers. Many other compounds, with potential wound healing benefits have been encountered. Although it is acknowledged that the quantities detected may not occur in clinically significant amounts, and that varying metabolites will be produced by the maggots once they are introduced into the wound environment, (which will also vary over time, and between patients), this aspect of the study has demonstrated the variety of substances which the larvae are capable of producing. This finding is of particular significance in light of the attempts at replacing the live organism with synthetic substances. A manuscript for the publication of this work is currently underway. A summary of key findings is shown in **Figure 38**.

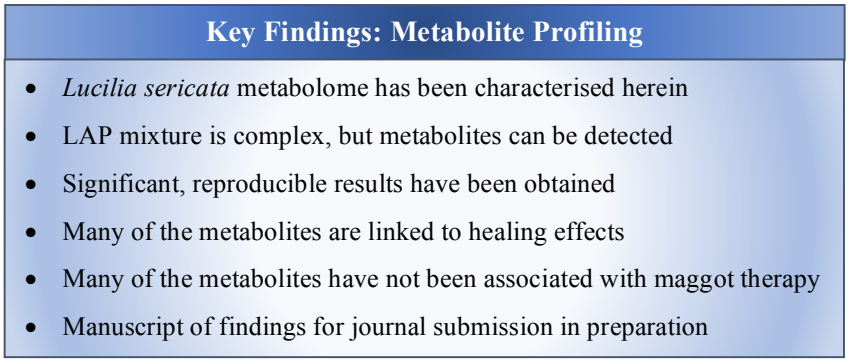

Figure 38: Summary of the key findings associated with the metabolic profiling of LAP, to explore the low molecular weight content on the bio-footprint.

Chapter Six: Discussion

6.0 Purpose of the study

The primary aim of this work was to characterise the bio-footprint produced by the most frequently used debridement device, the BB-50, and all objectives were met (**Section 1.5.1**). The concept of the bio-footprint was introduced in **Section 2.1**, whereby the immediate impact of the BioBag upon a patient was modelled and visualised (**Figure 2**) using the wound-relevant substrate, gelatine. This methodology was developed to collect the substances produced by the larvae contained within the device, into a working solution, essentially capturing the substances of the bio-footprint of what a wound may be exposed to, during the first 24 hours of BioBag administration. By using larvae produced from a consistent and regulated source, LAP was collected under standardised conditions, to determine the true composition of medical-grade LAP. Although a substantial body of work exists regarding maggot therapy, the experimental studies have utilised different methods. Most notably, the conditions related to the rearing of larvae do not reflect those undertaken by producers of medical-grade maggots (**Table 15**). Therefore, the content of LAP and the reported activities vary, and so consequently the findings are not relevant to all patients. Due to strictly enforced regulations regarding standardised rearing substrates (**Section 1.2.1**), all patients receiving BioBags across the UK, Europe and America, will experience the same quality of LAP upon initial administration, as characterised by this study.

Visualisation of the bio-footprint not only led to the establishment of the first standard operating procedure for the collection of LAP from contained maggots, but also the development of an alternative assay for the assessment of larval products. By using the same substrate as that of the bio-footprint, the gelatinolytic property of LAP could again be visualised, and in a semi-quantitative system, known as the RDEA (**Section 2.3** and **Figure 3**). These methods now form the current pinnacle of LAP standardisation (Pickles & Pritchard, 2017b), offering rapidity, consistency and homogeneity, which cannot be achieved with meat-based assays (**Section 1.3**).

Although the larvae within the BB-50 devices were not actively feeding at the time of LAP collection, the contribution of the diets to the LAP solution was investigated, using the RDEA system. It was shown that neither the Carnival Diet (**Section 3.1.1**) or the *Lucilia* Agar (**Section 3.1.2**) formulations, or any of the individual ingredients, possessed enzymatic activity. Despite confirming that the preparations did not

contribute to the gelatinolytic property of LAP, it was acknowledged that previously ingested *Lucilia* Agar could still be present within the larval alimentary tract and that the products of its metabolism would form part of the standardised LAP profile. This was also the hypothesis regarding gut microflora (**Section 1.4.2**), or any symbionts potentially harboured by the dietary components (**Section 3.1.3.1**), therefore the RDEA system was also employed to assist in the elimination of false positives in an endotoxin assay (**Section 3.1.3**). Although the denaturation of LAP was necessary to avoid erroneous LPS readings (Pickles & Pritchard, 2017a), a residual endotoxin presence was demonstrated; however, this was shown to be below the threshold limit stipulated by the FDA (2012), even with BioBags which are over-loaded with larvae.

The potential contribution of *Lucilia* Agar and bacterial symbionts to LAP represent authentic findings. As a product approved by the FDA and the MHRA, all stages of BioBag production are regulated. This is not the case with larvae produced outside of such medical directives. The use of animal offal, from various sources and species (pork, lamb, beef, and chicken livers or minced tissues, are commonly used for blow-fly rearing) introduces extreme variation in terms of homogeneity, content and the potential presence of other symbionts. It has therefore been recommended that the findings of studies using non-regulated material are treated with caution (Pickles & Pritchard, 2017a; 2017b). Having achieved confidence in the quality and origin of the LAP solution, it was possible to explore the bio-footprint using analytical methods.

High and low molecular weight profiling of the LAP solution was undertaken not only to determine the composition, but also to identify possible larval biomarkers of the bio-footprint, which may be responsible for the healing effects observed, following the administration of maggots to chronic wounds. These study phases also established that LAP collected from regulated sources, may demonstrate biological variation, as numerous larvae of varying juvenility may be incorporated into the BioBag device during production (**Section 1.3.1**). Sixty-six BB-50 devices were examined in this study, each containing 80 to 290 larvae per BioBag (n= 8735 larvae in total), across an age range of approximately 24 to 48 hours, prior to LAP collection. It is likely that the production procedure involving the pooling of colony eggs, of various ages, has created the discrepancies observed, as verified by the differing protein content and chymotryptic activity estimations (**Section 2.2.2.2**). This was particularly evident with the proteomic analyses, whereby only three peptide sequences were reproducibly

detected (**Section 4.3.2**). Such studies are useful for the exploration of larger compounds, particularly intact proteins; however, peptide mass fingerprinting of LAP is not suitable, despite the low protein content observed. However, the metabolite profiling of LAP was shown to be a method with greater consistency, with compounds being reproducibly detected in all LAP replicates. Furthermore, the same compounds were detected using two different HILIC-based methods, assessing two different batches of LAP solutions (**Chart 29**). This phase of the study proved suitable for the identification of bio-footprint biomarkers, which may have the potential to be detected in the wound environment, following the administration of larval treatment.

6.1 Characterisation of LAP

The metabolic profiling of LAP has proven particularly informative, and the findings have been compared to published literature, to verify the results herein and to determine the contribution of this study to field of maggot therapy research and practice. The most commonly reported compounds which have been associated with *L. sericata* larvae are shown in **Table 14**, alongside the origin of the substance and the purported relevance to wound care. Of these, the majority are associated with disinfection (*n*= 12), six of which have low molecular weights. None of these were detected in the current study, and **Table 15** highlights the importance of using standardised material, as non-sterile, meat-reared third-instar larvae have been predominantly utilised (**Section 1.4**). Although none of the enzymes shown (*n*= 9) were detected using peptide mass fingerprinting techniques, all the LAP samples tested were shown to possess chymotryptic activity. Despite the low protein content, total LAP is complex, and the mixture should be separated by gel electrophoresis, to facilitate exploration of the proteome, which is currently under-characterised.

Similarly, the low molecular weight profile of LAP was largely unknown, and of the five compounds linked to healing outcomes, as shown in **Table 14**, only allantoin has been detected contemporaneously, as a result of this study. Allantoin was shown to be a significant larval compound (**Chart 29**), and it is commonly cited in modern maggot therapy literature; alongside the other four substances. Of significant interest is the finding that allantoin is produced independently of feeding, which is not the case with ammonia (Brown, 1938a; 1938b).

Table 14: Compounds reported from *Lucilia* larvae and repeatedly cited in current literature as occurring in *L. sericata* 'medicinal maggots'. Compounds have been listed in decreasing molecular weight (divided as high, mid and low molecular weight substances), alongside the larval source and reported benefits associated with wound care.

Detected compound	Source of compound	Reported benefit	Reference
DNAse (45 kDa)	LAP	Debridement and disinfection	Brown *et al.*, 2012
Aspartyl proteinase (20-40 kDa)	LAP	Debridement	Chambers *et al.*, 2003
Metalloproteinase (20-40 kDa)	LAP	Debridement	Chambers *et al.*, 2003
Glycosidases (25-37 kDa)	LAP	Debridement	Telford *et al.*, 2012
Sericase (37 kDa)	LAP	Debridement	van der Plas *et al.*, 2014
Uricase (33 kDa)	Body tissues	Healing (allantoin production)	Baumann *et al.*, 2017
Jonah protease (25-27 kDa)	LAP	Debridement	Pöppel *et al.*, 2016
Trypsin (20-25 kDa)	LAP	Debridement	Chambers *et al.*, 2003
Chymotrypsin (20-25 kDa)	LAP	Debridement and disinfection	Britland *et al.*, 2011
Peptides (6.5 to 9 kDa)	LAP	Disinfection	Kruglikova & Chernysh, 2011
Lucimycin (8.2 kDa)	Body tissues	Disinfection (antifungal)	Pöppel *et al.*, 2014
Lucifensin (4 kDa)	LAP and tissues	Disinfection	Čeřovský *et al.*, 2010
Lucilin (3.8 kDa)	Body tissues	Disinfection	Tellez & Castano-Osorio, 2014
Seraticin (< 500 Da)	LAP	Disinfection	Bexfield *et al.*, 200)
Proline diketopiperazine (194 Da)	Body tissues	Disinfection	Huberman, *et al.*, 2007b
p-hydroxyphenylacetic acid (152 Da)	Body tissues	Disinfection	Huberman, *et al.*, 2007b
p-hydroxybenzoic acid (138 Da)	Body tissues	Disinfection	Huberman, *et al.*, 2007b
Uric acid (168 Da)	Body tissues	Healing (allantoin production)	Brown, 1938a
Allantoin (158 Da)	LAP	Healing	Brown, 1938a
Phenylacetaldehyde (120 Da)	LAP	Disinfection	Arora *et al.*, 2011
Calcium carbonate (100 Da)	LAP	Healing	Stewart, 1934a
Ammonium bicarbonate (79 Da)	LAP	Disinfection (and alkalinisation)	Brown, 1938b
Urea (60 Da)	LAP	Healing	Brown, 1938b

Table 15: Low molecular weight compounds from **Table 14** reported from *Lucilia* blow-fly species and repeatedly cited in current literature as occurring in *L. sericata* 'medicinal maggots'. Compounds annotated by asterisk possess antimicrobial activities (*n*= 6), although the source larvae are often non-sterile and/or have been examined as mature (third instar) larvae. Only allantoin (bold text) was detected as a significant component of LAP in this study.

Reported compound	Detection method	Larval type from literature	Notes from literature
Seraticin*	Not detected in this study	Sterile, third instar	Compound not fully characterised
Proline diketopiperazine*	Not detected in this study	Non-sterile, third instar	Grown on fish residues
p-hydroxyphenylacetic acid*	RP-HILIC, not significant	Non-sterile, third instar	Grown on fish residues
p-hydroxybenzoic acid*	HILIC, not significant	Non-sterile, third instar	Grown on fish residues
Uric acid	HILIC only	Sterile, all instars	Uric acid is not excreted (tissue storage)
Allantoin	**HILIC and RP-HILIC**	**Sterile, all instars**	**Allantoin is independent of feeding**
Phenylacetaldehyde*	Not detected in this study	Non-sterile, third instar	*L. cuprina*, grown on raw meat
Calcium carbonate	Not detected in this study	Sterile, all instars	Limited rearing details provided
Ammonium bicarbonate*	Not detected in this study	Sterile, all instars	Starved larvae do not produce ammonia
Urea	Not detected in this study	Sterile, all instars	Not present in sterile LAP

The odour of ammonia is particularly associated with maggots (**Section 1.4.3**), but this was never encountered during this study and larvae were not actively feeding at any time. Neither ammonia, or its expected excretory product ammonium bicarbonate, were detected herein, which supports Brown's conclusions. Hobson (1932a) reported that juvenile sterile *Lucilia sericata* larvae excrete ammonia, but the quantity does not become appreciable until the sufficient larval growth has been attained after 3 or 4 days of feeding, which again is consistent with the findings of this study. Urea was not encountered and although it does occur in the IDEOM database, its molecular weight is below that of the lowest compound detected (masses of 77 and 73 using HILIC and RP-HILIC, respectively). Urea was reportedly absent from the excreta of sterile *Lucilia sericata* larvae (Brown, 1938b), and in modern entomological literature it is considered as only a minor insect compound and in general is absent from this species (Dow, 2013). This is also true of uric acid (detected using the HILIC method only, *n*= 3 LAP replicates) which was reported to occur only in the tissues of *L. sericata* larvae (Brown, 1938a). It is therefore apparent that only allantoin and ammonia can be reliably associated with medicinal LAP, and the latter is only likely to be encountered following feeding.

6.2 Study contribution

Beyond providing standardised methods of LAP collection and assessment, this study has raised awareness regarding the origin and quality of LAP, and that not all research concerning *L. sericata* can be applied to patient experience. This is perhaps best demonstrated by considering the constitutive and inducible compounds, such as allantoin versus ammonia, and specifically the antimicrobial activity of LAP. As can be seen in **Table 15**, the low molecular weight compounds reported to possess such actions were not detected herein. The characterisation of the mid-molecular weight compounds has purposely been omitted from this study, due to the vastness of the topic and the requirement for physical or bacterial challenge to induce their presence. **Table 15** also demonstrates the importance of examining the larval status and food source when considering the content of LAP. The majority of the low weight antimicrobial or healing compounds reported to be produced by the larvae tend not occur from so-called 'medicinal maggots'. It is acknowledged that challenged larvae represent the 'real' BioBag experience, but studies could be performed on wound material, such as eschar

to recreate this (Telford *et al.*, 2010; Brown, *et al.*, 2012), as opposed to various animal tissues.

In the first instance, larvae from eggs of the same age (in hours), raised fully on the Carnival Diet may be compared to those reared on *Lucilia* Agar, to determine the baseline range of antimicrobial substances produced by regulated larvae. The work could then be expanded to test LAP against a range of relevant bacterial or fungal strains, before utilising wound material from patients (human or animal) and for LAP or larval experimentation. Larvae produced by other regulated manufacturers (such as Monarch, **Section 1.3**) could also be investigated in this manner, to obtain a library of larval compounds.

The chief contribution of this study to the field of maggot therapy are the unique metabolite profiling results. These have shown that substances from many pathways are produced by the larvae, and as with the protein identifications, that they may be linked to healing outcomes. Due to the complexity of protein expression with mixed-age larvae, this phase of the study did not yield reproducible results between LAP replicates; however, the associations of the proteins to the metabolic pathways (**Section 5.4**) have been incorporated, into a 'LAP map' (**Figure 21**). What this schematic demonstrates is the vastness of the low molecular weight contribution to the bio-footprint. Evidence for the complexity of LAP already exists, but this work not only contributes to the current knowledge, but provides further support that no single compound can be created to mimic the effects of live larvae in the wound environment (**Section 1.1**), something which has, and continues to be, of interest (Livingston & Prince, 1932; Vistnes *et al.*, 1981; Prete, 1997; Britland *et al.*, 2011; Čeřovský *et al.*, 2011; Cazander *et al.*, 2012; Pritchard *et al.*, 2012; Sherman *et al.*, 2013; Valachová *et al.*, 2013).

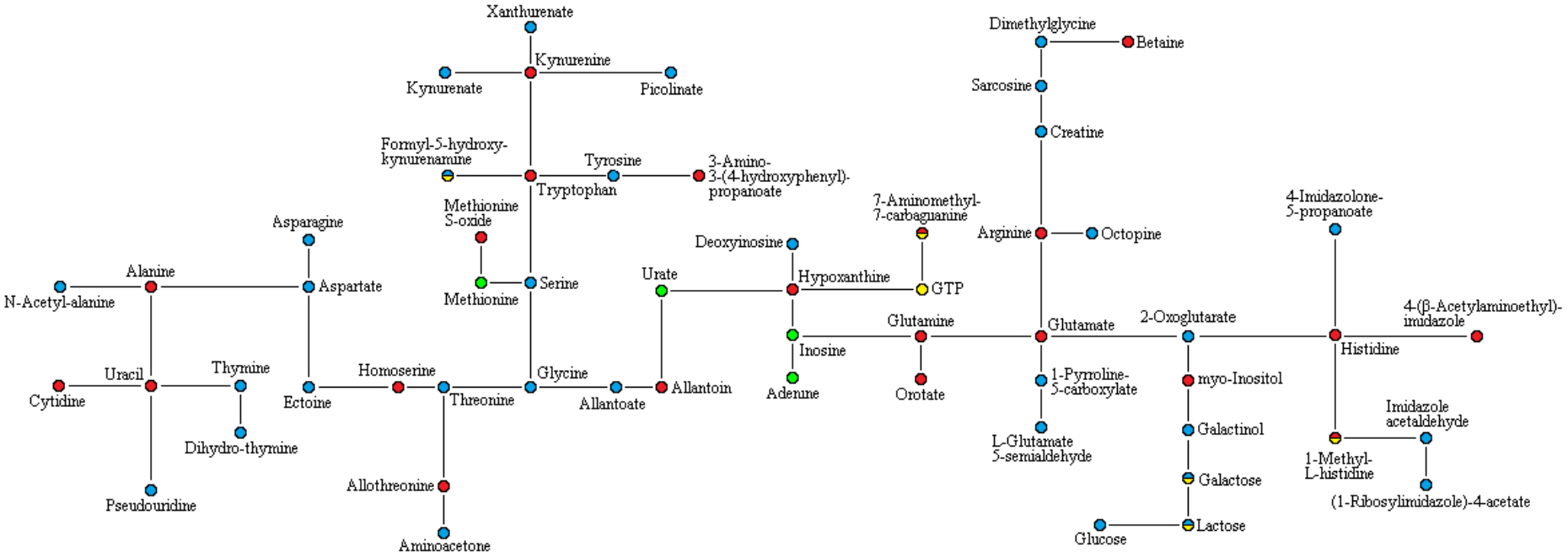

Figure 39: The LAP map, detailing larval components detected during the course of this study (*n*= 58) which have been linked by metabolic pathways. Circles in green denote that the compound was detected using HILIC only (*n*= 4), circles in blue denote that the compound was detected using RP-HILIC only (*n*= 32) and circles in red denote that the compound was detected using both methods (*n*= 21). The full circle in yellow (*n*= 1), and those split with yellow are linked to the high molecular weight protein exploration of LAP. Note that the metabolomic pathways have been linked for illustration purposes and do not detail the specific associations (i.e. in some instances, a series of steps are required to produce the neighbouring compound).

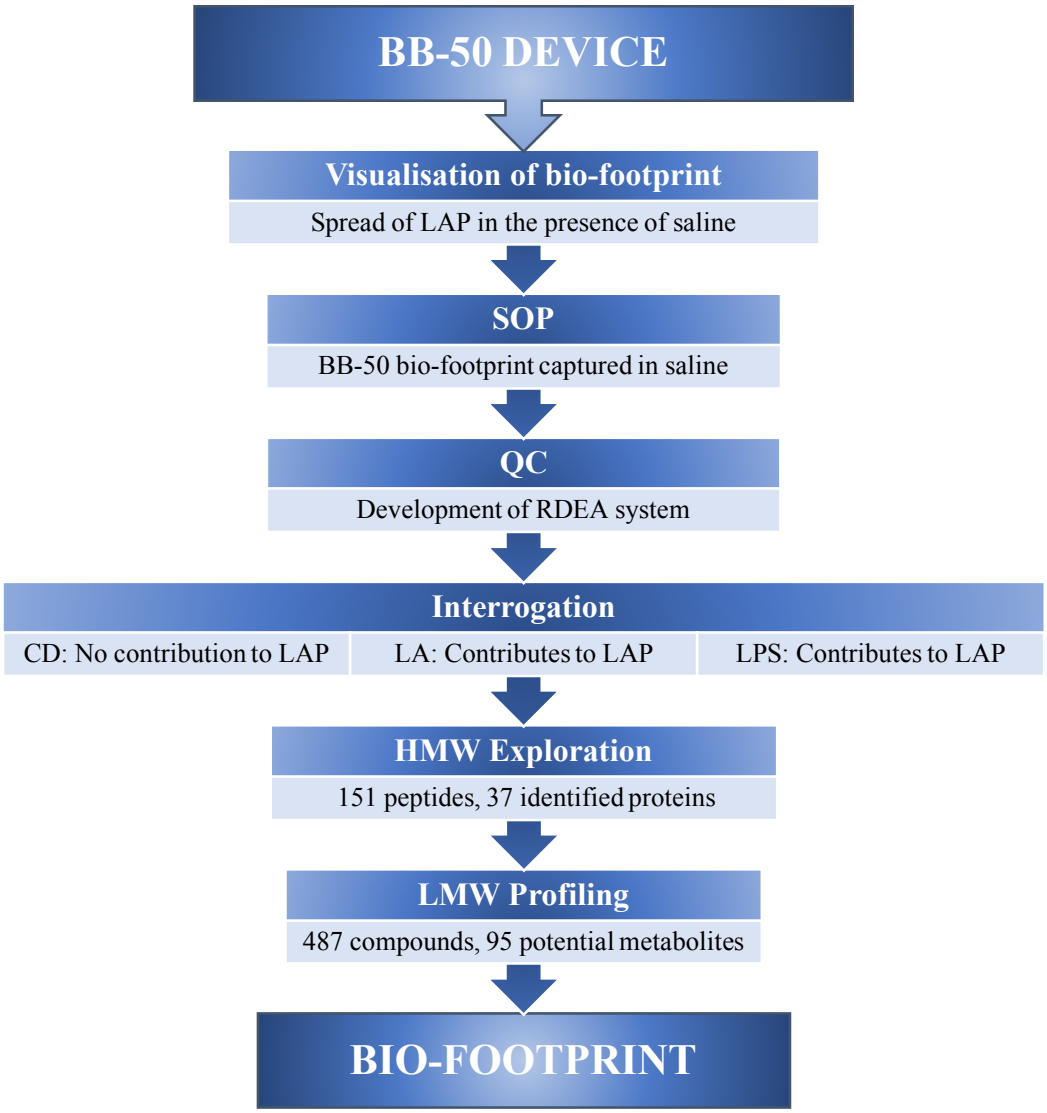

Figure 40: Study overview, detailing the progression of work from the visualisation of the BB-50 bio-footprint, leading to the collection of larval alimentary products (LAP) into a saline solution. A standard operating procedure (SOP) was created to optimise the collection of LAP from live larvae within the device, alongside the development of a radial-diffusion enzymatic assay (RDEA) for Quality Control (QC) assessment. The RDEA system was utilised to support the interrogation of the rearing substrates: Carnival Diet (CD) and *Lucilia* Agar (LA), and the potential presence of lipopolysaccharides arising from larval gut symbionts. Once confidence in the quality of the solution was achieved and the origin of LAP components determined, high molecular weight (HMW) exploration and low molecular weight (LMW) profiling was undertaken to characterise the content of the total bio-footprint.

6.3 Future recommendations

Recommendations relating to each study phase have been raised in the appropriate sections, although suggestions for future working will be summarised accordingly. The findings herein may represent those of an authentic product, and the differential expression of proteins, peptides and metabolites may create an all-encompassing approach to debridement, disinfection and healing; it is proposed that research into

discrete insect ages would enable better characterisation of the bio-footprint. The collection of eggs of a known age (in hours) and larval rearing under controlled conditions, would result in age-specific LAP, which could be used to identify which stage of the life-cycle (**Appendix**) yields which substances. This would then allow the use of untargeted and targeted analytical approaches to discover new compounds, and to isolate those of interest. The search for larval biomarkers should follow this method, and endotoxin testing should be performed routinely. The RDEA system could be widely employed for enzymatic, microbial and immunological explorations. The incorporation of specific substrates and inhibitors would aid macro investigations, which could then be explored on a molecular level, an aspect beyond the scope of the current study. With the growth of the larvae, and the ingestion of the feeding substrate, it is anticipated that LAP would become more complex. Although a baseline has been established herein, the collection of alimentary products from larvae actively feeding on the standardised substrates across each life stage, will create solutions requiring further interrogation. Such actions would capture the full content of LAP, and the true bio-footprint. This could then be attempted with maggots raised on wound material *ex vivo* or *in vivo*.

6.4 Study evaluation

This study represents the most complete and comprehensive work relating to a regulated medical larval device. Although concerns regarding BioBag over-loading and the pooling of unspecified egg ages have been raised, the material investigated represents an authentic product, as experienced by chronic wound sufferers in the UK, and overseas. Furthermore, BioMonde have implemented the findings of the diet evaluations, and the removal of expensive unnecessary components such as animal sera and decreasing the amounts of other ingredients (**Section 3.1.1** and **Section 3.1.2**), will be cost beneficial, as well as producing optimised rearing substrates. Regarding larval loading, it has been shown that decreasing the number of maggot within the BioBag will keep any potential symbiont-contributed LPS well below regulatory limits (**Section 3.1.3**), may improve debridement efficacy (**Section 2.2.3**) and improve pain and pressure responses (BTER, 2005).

In terms of improvement, all LAP samples should have been subjected to endotoxin testing, as part of the SOP development. However, this aspect of the study was not

considered until the results of the metabolomic profiling had been interpreted, which represented a significant portion of the total study.

6.5 Concluding remarks

It is currently accepted that LAP contains a range of substances, many of which are still unknown (Sherman *et al*., 2000; Pritchard *et al*., 2016), which contribute to the three modes of action in larval-assisted wound care: debridement, disinfection and healing. It is hoped that further studies of this nature may bolster the use of maggot therapy and promote its application before the risk of amputation (**Section 1.1**). If any of the metabolites suggested as larval biomarkers (in particular allantoin, tryptophan, kynurenine and orotate) could be detected in the wound post-larval treatment, and characterised in the manner of chymotrypsin and lucifensin, this would represent a substantial contribution to the discipline (**Section 1.4.3**). What has been demonstrated, is that *L. sericata* larvae produce a substantial number of compounds within 24 hours (**Figure 39**), when neither actively feeding or challenged. BioBags will remain within the wound for a further two or three days, prior to their removal. Accepting that feeding, larval moults and the preparations for pupariation occur during this time, it seems unlikely that the isolation of Baer's or Livingston's 'active principle' to replace maggots is a realistic goal (**Section 1.1**, **Section 1.4.1** and **Section 1.4.3**). Given the multifactorial actions of a multitude of compounds, efforts to capture the total bio-footprint from egg to pre-pupae (**Appendix**) may prove more successful.

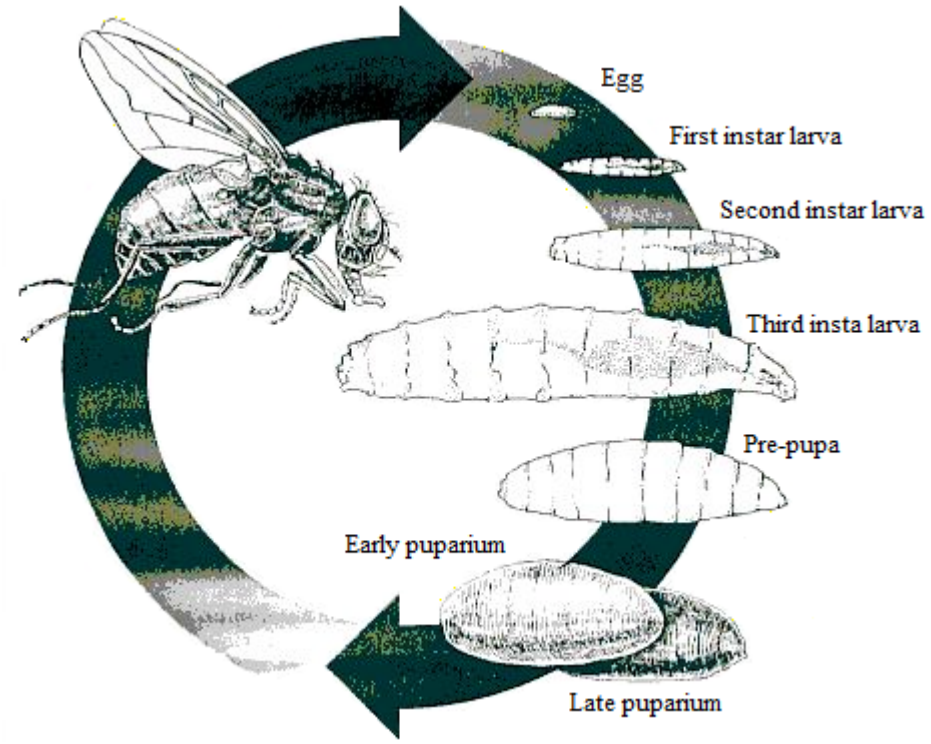

Figure 41: Example of a blow-fly life-cycle. Female flies deposit eggs in clusters of hundreds, usually on a protein-rich substrate. The eggs hatch in less than 24 hours and the resultant first instar larvae commence feeding, passing through two cuticular moults to accommodate growth, over a matter of days. Upon completion of feeding, the larvae move away from the food source to undergo pupariation in preparation for metamorphosis. The cuticle contracts (pre-pupa), shrinks (early puparium), eventually hardening and darkening to form the late puparium. It may be 1 to 2 weeks until the adult fly emerges from the puparium. The time taken depends upon the species of blow-fly and the ambient temperature. At 30^{o}C, it takes approximately 11 days for *Lucilia sericata* to complete the life-cycle (Grassberger & Reiter, 2001). Image modified from Saferstein (2001).

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