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**Title**

Endotoxin testing of a wound debridement device (BioBag) containing medicinal *Lucilia sericata* larvae.

**Authors**

Miss Samantha F. Pickles (MSc) and Professor David I. Pritchard (PhD)

University of Nottingham, Division of Regenerative Medicine and Cellular Therapies, School of Pharmacy, Nottingham, UK.

**Correspondence**

E-mail: samantha.pickles@nottingham.ac.uk and david.pritchard@nottingham.ac.uk

Tel: 0115 9515395

Fax: 0115 9515102

The University of Nottingham

Division of Regenerative Medicine and Cellular Therapies

School of Pharmacy, Boots Science Building

University Park, Nottingham, NG7 2RD

**Running Title**

Endotoxin screening of larval alimentary products.

**Keywords**

Endotoxins, enzyme, medical device, debridement therapy, larval alimentary products.

**Abstract**

Alimentary products of medicinal *Lucilia sericata* larvae are studied to determine their mechanisms of action, particularly in the contexts of wound debridement and disinfection. Furthermore, the larvae can be applied to patients in contained devices, such as the BioBag (BioMonde). Here, we tested the materials and larval content of the most commonly used BioBag (the “BB-50”) to explore the possibility that endotoxins may be contributing to the bio-activity of the product, given that endotoxins are potent stimulants of cellular activation. Using standardised protocols to collect larval alimentary products (LAP), we proceeded to determine residual endotoxin levels in LAP derived from the BioBag, before and after the neutralisation

of interfering enzymatic activity. The BB-50 device and its associated larval content was not a significant source of LPS activity. However, it is clear from these experiments that a failure to remove the confounding serine proteinase activity would have resulted in spuriously high and erroneous results. The residual LPS levels detected are unlikely to be active in wound healing assays, following cross-referencing to publications where LPS at much higher levels has been shown to have positive and negative effects on processes associated with wound repair and tissue regeneration.

## Introduction

The BioBag (BioMonde), a medicinal product containing larvae of the greenbottle blow-fly *Lucilia sericata*, debrides and disinfects chronic wounds<sup>(1, 2)</sup>. However, the molecular basis for its bio-activity is not yet fully understood. Although enzymes (serine proteinases, DNase, glycosidases) are strongly implicated in the debridement activity of the product, and in the promotion of fibroblast motogenesis, and a defined antibiotic (Lucifensin) has been identified<sup>(3)</sup> little is known of other factors which may be present in relation to wound repair and regeneration.

In the present study, we begin to explore the scenario that symbiont-derived endotoxin/lipopolysaccharide (LPS) in larval alimentary products (LAP) from larvae within BioBags could, if present, have a downstream effect on cell behaviour in wound-healing assays, given the potent stimulatory effects of LPS on cell activation<sup>(4, 5)</sup>. Lipopolysaccharide arises from the outer membrane of Gram-negative bacteria; species of which are known to be harboured by *Lucilia sericata* larvae as symbionts<sup>(6)</sup>.

To detect the presence of authentic endotoxin activity, we used the *Limulus* amoebocyte lysate (LAL) chromogenic assay. As this, and other tests for LPS, is subject to interference by serine proteases<sup>(7)</sup>, it was necessary to prove that we had first denatured those known to be endogenous to LAP<sup>(8, 9)</sup> using heat-treatment and the recently-developed radial diffusion enzymatic assay (RDEA)<sup>(10)</sup>.

Once this was successfully achieved, it was then possible to demonstrate clearly that the BioBag was devoid of significant authentic endotoxin activity. However, future investigators should be wary that residual LPS levels could compromise the interpretation of data pertaining to the wound-healing attributes of LAP in cellular assays associated with wound repair and regeneration, unless other steps had first been taken to identify the active ingredient<sup>(11)</sup>. Based on our findings, we would recommend routine testing for endotoxin activity prior to any cell-based wound-healing assay utilising LAP, and guide the reader to

protocols designed to remove residual activity. This recommendation is particularly relevant in situations where larvae have been obtained from unregulated sources, and LAP collected using reagents and consumables which have not been certified as endotoxin-free.

## Materials and Methods

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

### *Preparation of larval alimentary products (LAP)*

Larval alimentary products were collected from six sterile BB-50 debridement devices (2.5 × 4 cm) as supplied by BioMonde (Bridgend, UK) under the standardised conditions as described previously <sup>(10)</sup> using 2.5 mL sterile PBS (phosphate-buffered saline). Aliquots of LAP solution (200 µL) from individual BioBags were prepared for testing, in duplicate pairs of native and heat-treated material (90°C for 10 minutes). Aliquots from the control devices were not heat-treated.

### *Enzyme assay*

The existing and subsequently denatured gelatinase activity of LAP was determined using the radial-diffusion enzyme (RDEA) system in duplicate, as detailed previously <sup>(10)</sup>. The system buffer, TBS (tris-buffered saline) was utilised as a blank sample, and a positive 10 mg/mL collagenase (gelatinase) control was prepared from *Clostridium histolyticum* (Type VII) in TBS. Plates were incubated for 6 hours at 37°C, prior to fixation for 30 minutes (acetic acid, methanol and water; 10:25:65) at room temperature. Gels were stained within the assay plates using 0.05% Coomassie Brilliant Blue R-250 to confirm protein digestion.

Gel plates were photographed at a fixed height on a transmitted light-box with calibrated scale and processed using ImageJ software (v.1.41o, National Institutes of Health; Maryland, USA). Images were calibrated against the scale in each 8-bit (greyscale) image, and gelatinase activity was determined by measuring each halo (area of digestion, mm<sup>2</sup>), and corrected by subtracting the area of the test well.

### *Endotoxin assay*

Endotoxin quantification was undertaken using a chromogenic *Limulus* amoebocyte lysate (LAL) assay kit (Pierce; Illinois, USA). Test samples and standards were prepared according to manufacturer's guidelines, and modified for incubation using 1.5 mL centrifuge tubes.

Absorbance was measured at 405 nm using Tecan Spark 10M microplate-reader (Grödig, Austria). Data were corrected to exclude background readings, and the values obtained as endotoxin units (EU/mL; one EU/mL equals approximately 0.1 ng endotoxin/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.

### Data analysis

All data were analysed using GraphPad Prism (v.7.01; California, USA) and normality was examined using the Shapiro-Wilk test. Gelatinase activity is expressed as the mean halo/digestion area (mm<sup>2</sup>, in duplicate) with standard deviation ( $\pm$  SD). Endotoxin presence is expressed as mean endotoxin units per device (EU/device;  $n=2$ ,  $\pm$  SD), following elution in 2.5 mL of buffer. The statistical significance of LAP-denaturation on the LAL assay was determined using the Wilcoxon matched-pairs signed rank test; a probability value ( $P$ ) of  $< 0.05$  was considered significant.

## Results

Prior to heat-treatment, LAP from all larval loaded BioBags ( $n=6$ ) demonstrated gelatinase activity with a mean digestion halo area of  $80.8 \pm 6.66$  mm<sup>2</sup>. Control devices did not produce any digestion halos (**Figure 1a**). Confirmation of enzyme denaturation was achieved following the complete removal of the initial activity, as demonstrated by the before and after test wells (**Figure 1b**).

**Figure 1.** (a) Gelatinase activity of individual devices determined by the RDEA assay (mean  $\pm$  SD,  $n=2$  replicates), for larval loaded devices (BB) and control materials (C). Positive collagenase (+ve) and negative TBS buffer (-ve) controls are included. (b) Visual confirmation of existing gelatinase activity (1–6) and subsequent denaturation by heat (1H), against TBS blank (7) and collagenase positive control (8). Scale bar, 10 mm.

Fig. 1a

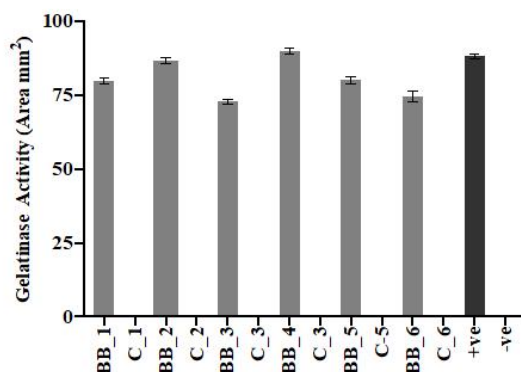
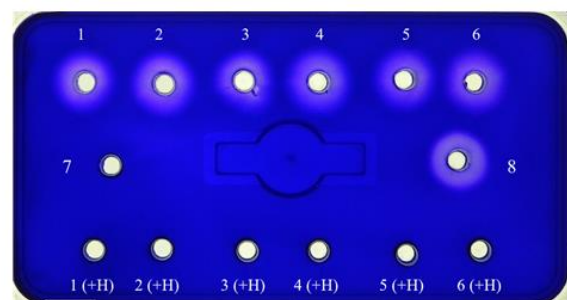


Fig. 1b



Prior to heat-treatment, LAP from all larval loaded BioBags ( $n=6$ ) tested positive for the presence of endotoxins, with a mean value of  $1.62 \pm 0.29$  EU/device. Following enzymatic denaturation, each LAP sample remained positive, but with a reduction of 24 - 60% throughout the group (**Figure 2a**), resulting in a mean value of  $0.92 \pm 0.36$  EU/device. The difference between the two sample groups was determined to be statistically significant ( $P = 0.031$ ). Control devices were not heat-treated and a background level of  $0.15 \pm 0.02$  EU/device was recorded throughout (**Figure 2b**). The residual levels of LPS detected following enzymatic neutralisation ( $0.04$  ng/mL after conversion from EU/mL) indicate that the device is medically benign<sup>(12)</sup> in terms of contributing LPS- mediated effects to the wound environment.

**Figure 2.** (a) Endotoxin content of individually eluted larval loaded devices (BB), as determined by the LAL assay (mean  $\pm$  SD,  $n=2$  replicates), for native material and following the denaturation of enzyme activity by heat (+H). (b) Mean endotoxin content of loaded devices before and after enzyme denaturation by heat (+H), as compared with control materials. Statistical significance \* ( $P = 0.031$ ) determined using Wilcoxon matched-pairs signed rank test ( $n=6$  devices, in duplicate  $\pm$  SD). Following heating, the residual mean endotoxin value was  $0.92$  EU/device. Prior to heating, confounding enzyme activity would have contributed to a spuriously elevated reading. One endotoxin unit/ml (EU/ml) equals approximately  $0.1$  ng endotoxin/ml of solution.

Fig. 2a

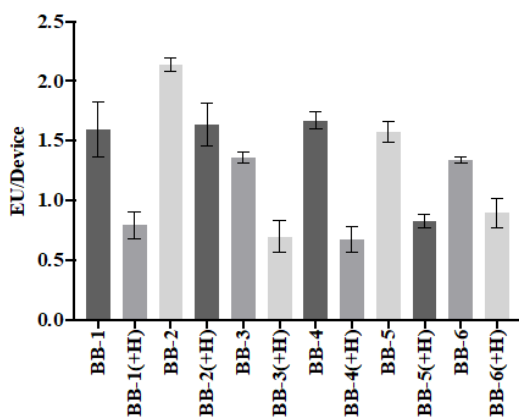
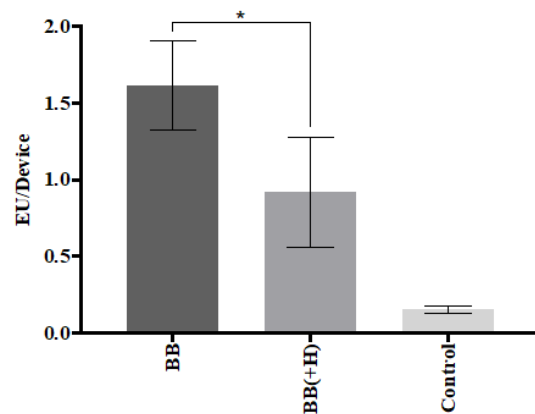


Fig. 2b



These levels are also unlikely to be of biological significance *ex vivo* as they are well below the levels shown to elicit effects in assays for wound healing ( $10 \mu\text{g/mL}$ )<sup>(4)</sup> and tissue regeneration ( $10 \text{ ng/mL}$ )<sup>(5)</sup>. However, investigators using larvae and LAP from unregulated sources are advised to be suitably cautious, as discussed below.

## Discussion

The present study was conducted to determine whether larvae contained within the BB-50 device, used as an active debridement agent, produced LPS; a bio-active molecule which interfere with assays pertaining to wound repair and regeneration. It would appear from this

study that LPS is not a major component of LAP emanating from this device; however, a failure to take into account the serine proteinase activity associated with the LAP would have led to spuriously high and erroneous endotoxin values. It should be noted that the brief heat-treatment applied to LAP for enzymatic denaturation (90°C for 10 minutes) is not adequate for depyrogenation, which requires sustained periods in temperatures in excess of 200°C<sup>(13)</sup>. Therefore, we report authentic endotoxin values, which have not been subjected to interference during the protocol. Furthermore, as the majority of endotoxin screening assays involve the enzymatic cleavage of a substrate and LAP is known to possess a range of enzyme classes,<sup>(8, 9, 14, 15)</sup> it is recommended that an effective denaturation step is incorporated during testing, for comparative purposes.

The data presented here will inform scientists performing experiments using LAP of the potential for LPS-mediated confounding effects on cellular behaviour, particularly where the effects seen were not neutralised by other means, such as the inhibition of larval enzyme-mediated stimulatory effects on fibroblast migration by soy bean trypsin inhibitor<sup>(11)</sup>. In cases where there may be concern that the presence of authentic endotoxin may result in confounding effects, well-documented measures to remove LPS (such as affinity chromatography) should be taken<sup>(16)</sup>. This may be particularly true for larvae (and their products) obtained for experimentation from non-regulated sources. In this case, all larval material should be subjected to routine testing for endotoxin activity, to avoid the possibility of generating spurious experimental data.

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## **Conflict of Interest**

The authors declare no conflict of interest.

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