Selective antimicrobial activity of maggots against pathogenic bacteria

Domen Jaklič,¹ Aleš Lapanje,^{1,2} Klemen Zupančič,¹ Dragica Smrke³ and Nina Gunde-Cimerman¹

Correspondence

Nina Gunde-Cimerman

nina.gunde-cimerman@bf.uni-lj.si

¹Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

²Institute of Physical Biology, Veliko Mlačevo 59, 1290 Grosuplje, Slovenia

³University Medical Centre Ljubljana, Zaloška cesta 2, 1000 Ljubljana, Slovenia

Maggot therapy, also known as biosurgery, is an ancient method for the healing of chronic infected wounds. Although clinicians have reported on the beneficial activities of the *Lucilia sericata* larvae that have been used for healing chronic wounds, the selectivity of this therapy against the different pathogenic micro-organisms that are found in chronic wounds has never been analysed. In the present study, we have investigated the *in vitro* activities of larval excreta/secretata both against selected bacterial strains that frequently occur in chronically infected wounds, and against bacteria isolated directly from the larvae and their excreta/secretata. Additionally, the antibacterial activities were investigated in *in vivo* studies, by comparing bacterial diversity in wounds before and after the application of *L. sericata* larvae. In conclusion, larval therapy is highly recommended, particularly for the treatment of wounds infected with Gram-positive bacteria, like *Staphylococcus aureus*, but less so for wounds infected with Gram-negative bacteria, especially *Proteus* spp. and *Pseudomonas* spp. strains. Bacteria from the genus *Vagococcus* were resistant to the maggot excreta/secretata.

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INTRODUCTION

Maggot therapy, also known as biosurgery, is an ancient method for the healing of chronically infected wounds. Although the beneficial actions of fly larvae on wounds has long been known by the Australian aborigines (Lee, 1968) and by Central American Maya Indians (Weil *et al.*, 1933), the maggots of the blow fly *Lucilia sericata* were first used clinically only five centuries later (Pechter & Sherman, 1983). It was only after the appearance of bacterial strains with antibiotic resistance that maggot therapy was reintroduced for the second time in many hospitals in the USA and Europe (Sherman *et al.*, 2000). Of particular interest was the finding that larval excreta/secretata are potent agents against meticillin-resistant *Staphylococcus aureus* (Bexfield *et al.*, 2004).

Successful clinical experiences led to the initiation of several studies that have focused on the basic healing principles of maggot therapy. This healing can be attributed to the synergistic actions of four main activities: (i) the mechanical stimulation of wound drainage (Livingston & Price, 1932; Weil *et al.*, 1933) and the destruction of bacteria in the larval intestine (Greenberg, 1968; Lerch *et al.*, 2003; Mumcuoglu *et al.*, 2001), (ii) the enzymic degradation of necrotic tissue (Hobson, 1931; Vistnes *et al.*, 1981), (iii) the antibacterial activity of

maggot excreta/secretata (Bexfield *et al.*, 2004; Livingston & Price, 1932; Robinson & Norwood, 1933; Simmons, 1935), and (iv) the stimulation of granulation tissue formation (Livingston & Price, 1932; Prete, 1997; Robinson, 1935).

In terms of research, it is the antibacterial properties of the larval excreta/secretata that have been studied most extensively. Although *in vitro* studies of the antibacterial activities have revealed that they are potent against many pathogenic species of bacteria (Pavillard & Wright, 1957; Robinson & Norwood, 1933; Simmons, 1935; Vistnes *et al.*, 1981), the effects of maggot therapy have not been determined for the diverse groups of micro-organisms that can infect chronic wounds.

The main focus of our investigation was thus to monitor bacterial diversity in the wounds of 30 patients *in vivo*, before and after the application of maggots. Additionally, selected species of bacteria were exposed to maggot excreta/secretata *in vitro*, and intrinsic bacterial resistance to the larval excreta/secretata was investigated by isolating bacteria directly from the excreta/secretata and from the surface of non-sterile maggots. Based on the determined susceptibilities of the bacteria isolated and the selective actions on the bacteria present in a wound, the microbiological criteria for the selection of wounds that should be most appropriate for efficient healing with classical larval therapy were determined.

METHODS

Preparation of sterile *L. sericata* larvae. Continuous cultures of *L. sericata* flies were maintained under 12 h/12 h day/night cycles at room temperature under 60 % humidity. To obtain embryos from the flies, a piece of beef liver was exposed to the flies for 2 h. Oviposited eggs, containing the developing embryos, were transferred into sterile 0.5 % sodium sulphite. After their dispersion in sodium sulphite, the eggs were immediately sterilized using the procedure described by Horn *et al.* (1976). The sterilized embryos were rinsed with sterile distilled water and incubated on blood agar plates for 11 h at 30 °C. For their transportation and further experimental use, the sterile freshly hatched larvae were transferred to sterile containers.

Production of larval excreta/secret. Approximately 500 2–3-day-old larvae were used for the collection of excreta/secret. Over the 4 h needed for the collection, the larvae were rinsed every hour with 1 ml distilled water. The crude excreta/secret were filter-sterilized through 0.22 µm filters. Their sterility and activity were tested on brain heart infusion (BHI) and blood agar plates (both from Biolife) as well as in liquid BHI media.

In vitro activity of larval excreta/secret. The antibacterial activities of sterile larval excreta/secret were tested on the K57 *Escherichia coli* strain, the V102 *S. aureus* strain and the K198 *Pseudomonas aeruginosa* strain, all of which were obtained from the Ex Culture Collection of the Biotechnical Faculty, University of Ljubljana, and also on the LM1 *Vagococcus* sp. strain, which was isolated from the collected larval excreta/secret (see below) and deposited with the Ex Culture Collection. Bacterial cultures were grown in 25 ml BHI liquid nutrient medium at 37 °C until a cell density of approximately 5×10^8 cells ml⁻¹ was reached. Each culture was further diluted to 10⁴ and 10² cells. From each bacterial dilution, 0.1 ml was introduced into the test tubes containing 2 ml BHI and 2 ml larval excreta/secret. For positive growth control, 2 ml 0.9 % NaCl was added instead of the larval excreta/secret, while for the negative control the tubes contained only 4 ml 0.9 % NaCl and 0.1 ml bacterial culture from previously prepared dilutions. Following an overnight incubation of the samples at 37 °C, 0.1 ml was spread on BHI agar plates. Bacterial colonies were counted after 1 day of incubation at 37 °C.

The duration of excreta/secret antibacterial activity over time was also followed. The procedure was similar to that described above, with 0.1 ml each sample spread over BHI agar plates every hour, over 9 hours. After overnight incubations at 37 °C, the bacterial colonies were counted.

Selection of patients. Seventeen female and thirteen male patients (total of thirty) were treated with classical applications of the larvae. Their wounds were classified according to Falanga (2000). The wound-bed appearance was classified according of the percentage of granulation tissue as type A, B and C for 100, 50–100 and <50 % covered with granulation tissue, respectively. If any eschar was present, they were scored as type D. The wound exudates were graded as 1 if changes of the wound dressing were needed only weekly, as 2 if changes were required every 2–3 days and as 3 if they were required daily. The 30 patients with chronic wounds were classified according to Falanga (2000) as types B2 and C3. Further details are shown in Table 1.

Application of sterile larvae to the wounds. The larvae were administered within a cage-like dressing, as described by Sherman (1997). Briefly, a cage into which sterile larvae (5–8 larvae cm⁻²) were placed, was made of a ring of hydrocolloid (cut from Granuflex; ConvaTec) and Tegapore mesh (3M Health Care). The mesh was topped with a light gauze pad to absorb the necrotic drainage, which was replaced at least every 4–6 h. The maggots were left in place

Table 1. Treated patients and their wounds

Clinical observation	No. of wounds/ patients
Wound type	
Venous ulcer	1
Arterial–venous combinations	14
Diabetic foot ulcers	7
Pressure ulcers	3
Postoperative chronic wounds	5
Mobility of patient	
Normal	10
Immobilized	3
On crutches	17
Patients with simultaneous antibiotic therapy	13

within the cage dressing for 24 to 60 h. With this protocol, 32 wounds of 30 patients were treated, since 2 patients developed additional secondary wounds during the study.

Bacteria identification in samples from wound tissue smears.

Smears from the wounds and from larvae were taken separately before and after every single maggot application. They were placed in aerobic and anaerobic transportation media (Bibby Sterilin; Copan Innovation). Bacterial identification was based on standard methods used for the identification of clinically important isolates (Murray *et al.*, 2003), and also on antibiograms performed at the Institute of Microbiology and Immunology at the Medical Faculty, University of Ljubljana, Slovenia.

Isolation and identification of the intrinsically excreta/secret-resistant bacterial strains.

Bacterial strains that were resistant to the maggot excreta/secret were isolated from non-sterile maggot macerates (4 maggots), from crude non-sterile maggot excreta/secret (5 ml) and from maggot food (200 mg) previously exposed to maggots. For the isolation of these strains, all of the samples were exposed to 10 ml sterile excreta/secret diluted in BHI liquid medium (1:1), and then incubated at 30 °C for 24 h. *E. coli* cultures (approximately 5×10^8 cells) were used as the positive control for excreta/secret activity. Tenfold dilutions of incubated samples in 0.9 % NaCl solution were prepared, inoculated (0.1 ml) on BHI plates and incubated until bacterial colonies appeared. Moreover, 50 ml filter-sterilized excreta/secret was incubated for 2 months at room temperature. In both cases, excreta/secret-resistant bacteria were isolated and axenically reinoculated on new agar plates.

Isolation of DNA, sequencing and sequence manipulations.

DNA was isolated with the Chelex (Bio-Rad)-based procedure, as described by Giraffa *et al.* (2000). The DNA isolated was used for PCR amplification with primers, according to Bianciotto *et al.* (1996). A 1495 bp long part of the 16S rRNA gene was amplified and sequenced by MacroGene. The DNA sequences obtained were compared with those deposited in the RDPII database. DNA sequences were aligned using CLUSTAL_X software (Thompson *et al.*, 1997) and manipulated using BioEdit software (Hall, 1999). Phylogenetic analysis was performed with Mega3 software (Kumar *et al.*, 2004) with the neighbour-joining algorithm based on Kimura two-parameter matrices. Branches of the phylogenetic trees were tested with bootstrapping (1000 replications).

Statistic analyses. Statistical analyses were performed with the ANOVA and chi-square tests.

RESULTS

One of the aims of the study was to determine the *in vitro* susceptibility and resistance of bacteria to maggot excreta/secret. Twelve Gram-negative bacterial strains (Table 2, Fig. 1) were isolated from non-sterile larvae that were grown on liver. Most of the isolates (73 %) were closely related to representatives of the genus *Providencia* and its relatives (98–99 % similarity). The remaining 27 % belonged to the genera *Proteus* (98 % similarity to *Proteus vulgaris* and its relatives) and *Pseudomonas* (99 % similarity to *Pseudomonas fluorescens* and its relatives). Surprisingly, direct incubation of the maggot excreta/secret resulted in the isolation of one Gram-positive strain, identified as *Vagococcus lutrae* (Table 2).

The effects of excreta/secret added to solid BHI medium were not measurable (data not shown), while on liquid

BHI medium inhibitory effects on selected bacterial strain could be observed immediately. *S. aureus* (Fig. 2) completely stopped growing for up to 4 h, and after 8 h of incubation the *S. aureus* cells could not be cultivated anymore. Incubation of maggot excreta/secret with *E. coli* and *P. aeruginosa* cultures was less effective in terms of bactericidal activity; however, a prolonged lag phase that lasted for 5 to 6 h for both bacterial species was seen. After this temporary inhibition, normal growth was resumed. In all three bacterial species (*S. aureus*, *E. coli* and *P. aeruginosa*), an inoculum-size-dependent inhibition was seen (ANOVA, $P < 0.05$), while the growth of *V. lutrae* was not affected at all (data not shown).

Thirty treated patients received a total of fifty-one applications. For 20 wounds (67 %), the larvae were administered only once, for 10 wounds (33 %) an additional treatment followed the first one, for the wounds

Table 2. 16S rRNA sequence similarities between the larval excreta/secret-resistant isolates and their three closest relatives, according to GenBank (National Center for Biotechnology Information)

Isolate	Three of the closest matches from GenBank	Similarity (%)	Sequence length (bp)	Accession no.	Classification
M1-27F-EUBAC, M10-27F-EUBAC	<i>Proteus vulgaris</i>	98	719, 580	DQ499636	Gamma-proteobacteria
	<i>Proteus</i> sp. SC-Y1-5	98		DQ319065	
	<i>Proteus</i> sp. SO-Y1-40	98		DQ316135	
M2-27F-EUBAC, M3-27F-EUBAC, M4-27F-EUBAC, M6-27F-EUBAC	Uncultured bacterium clone RL185_aaj72f10	99	750, 748, 746, 690, 766	DQ824750	Gamma-proteobacteria
	Uncultured bacterium clone RL185_aaj71a12	99		DQ824648	
	Uncultured bacterium clone RL185_aaj71g06	99		DQ824692	
	Uncultured bacterium clone RL185_aaj72f10	99		DQ824750	
M5-27F-EUBAC	Uncultured bacterium clone RL185_aaj71a12	98	690	DQ824648	Gamma-proteobacteria
	Uncultured bacterium clone RL185_aaj71g06	98		DQ824692	
	Uncultured bacterium clone RL185_aaj72f10	98		DQ824750	
	Uncultured bacterium clone RL185_aaj71a12	98		DQ824648	
M11-27F-EUBAC, M14-27F-EUBAC	Uncultured bacterium clone RL185_aaj72f10	99	750, 717	DQ824750	Gamma-proteobacteria
	Uncultured bacterium clone RL185_aaj71a12	98		DQ824648	
	Uncultured bacterium clone RL185_aaj71g06	98		DQ824692	
	Uncultured bacterium clone G24	99		DQ480483	
M12-27F-EUBAC	Uncultured bacterium clone SX2-3	99	808	DQ469202	Gamma-proteobacteria
	Uncultured bacterium clone RL185_aaj72f10	99		DQ824750	
	<i>Providencia vermicola</i>	99		EF192136	
M13-27F-EUBAC	Uncultured bacterium clone RL185_aaj71g06	99	826	DQ824692	Gamma-proteobacteria
	<i>Vagococcus lutrae</i> isolate F01595	100		DQ395281	
	<i>Vagococcus</i> sp. SCS12	100		AB211029	
<i>Vagococcus lutrae</i>	<i>Vagococcus</i> sp. CCUG39187	100	826	Y17152	Enterococcaceae

Fig. 1. Phylogenetic positions of the bacterial isolates from the larvae of *L. sericata*. The tree was constructed using the neighbour-joining method of Saitou & Nei (1987) based on a Kimura two-parametric distance matrix. The bootstrap values (out of 1000 trees) are shown adjacent to the nodes.

of 2 patients the larvae were applied three times, and five applications were needed for 1 wound. All except one of the patients included in this study were medically examined after the wounds had healed. The maggot application was efficient in 30 cases (83.2%), with 6.6% of the treated wounds completely healed and 76.6% of the wounds not infected by pathogenic bacteria anymore. Healing with the maggots was effective in treating type B2 wounds as well as type C3. In 21 wounds that were cleaned with the larval therapy, spontaneous epithelialization occurred after additional dressings. A Thiersch graft was applied in four cases. With five patients, the larval therapy was interrupted because of the emergence of new disease

with higher healing priority. The healing effects of larvae are depicted in Fig. 3, where a successfully healed foot is shown before and after 2 months of larval therapy.

The susceptibility of bacteria to maggot excreta/secretaria was tested *in vivo* by monitoring the changes in the pathogenic bacterial diversity, and frequency, before and after the application of the sterile larvae to the wounds. The following pathogenic bacterial species were identified in the wounds before the treatment: coagulase-negative staphylococci, group C streptococci, group G streptococci, *Bacteroides fragilis*, *Citrobacter freundii*, *Citrobacter koseri*, *Klebsiella oxytoca*, *Klebsiella* spp., *Peptococcus* sp., *Prevotella*

Fig. 2. *In vitro* activity of larval excreta/secret on diluted cultures of *S. aureus*. Viability of bacterial cells was checked every hour with plate spreading of growing culture (0.1 ml), while colonies were counted after overnight incubation at 37 °C. ■, 10⁻⁴ *S. aureus* treated with excreta/secret; □, 10⁻⁴ *S. aureus* control; ▲, 10⁻⁵ *S. aureus* treated with excreta/secret; △, 10⁻⁵ *S. aureus* control.

bivia, *Proteus mirabilis*, *Proteus vulgaris*, *P. aeruginosa*, *Serratia marcescens*, *S. aureus* and *Streptococcus agalactiae* (Table 3). After the application of the larvae, the total

Fig. 3. Wound before treatment (a) and 2 months after completion of larval therapy (b).

Table 3. Bacterial susceptibility to larval excreta/secret tested *in vivo*

The numbers in the before and after columns represent identified bacterial isolates from all wounds before the application of the sterile larvae and after larval treatment, respectively, while the ratio represents the increase/decrease in the number of certain species.

	Before	After	Ratio
<i>Bacteroides fragilis</i>	2	0	0
<i>Citrobacter freundii</i>	1	0	0
<i>Citrobacter koseri</i>	2	1	0.5
Diphtheroids	4	6	1.5
<i>Enterococcus faecalis</i>	2	4	2
<i>Klebsiella oxytoca</i>	3	1	0.33
<i>Klebsiella pneumoniae</i>	1	1	1
<i>Klebsiella</i> spp.	1	0	0
<i>Morganella</i> sp.	1	2	2
<i>Peptococcus</i> sp.	2	0	0
<i>Peptostreptococcus asaccharolyticus</i>	0	1	–
<i>Porphyromonas</i> sp.	0	1	–
<i>Prevotella bivia</i>	1	0	0
<i>Proteus mirabilis</i>	4	4	1
<i>Proteus vulgaris</i>	1	1	1
<i>Providencia rettgeri</i>	0	1	–
<i>Pseudomonas aeruginosa</i>	10	8	0.8
<i>Serratia marcescens</i>	2	0	0
<i>Staphylococcus aureus</i>	11	7	0.64
Coagulase-negative staphylococci	5	2	0.4
<i>Stenotrophomonas maltophilia</i>	1	1	1
<i>Streptococcus agalactiae</i>	2	0	0
α-Haemolytic streptococcus	1	1	1
Group C streptococci	1	0	0
Group G streptococci	1	0	0
<i>Streptococcus pyogenes</i>	1	1	1
Total	61	43	

number of species decreased for 14 wounds (63.6 %), while for 8 wounds (33.3 %) no differences were seen. This microbiological analysis of the wounds before and after the treatment was not carried out in 10 cases (30.3 %), while an increase in bacterial species was detected in only 1 case. Here, only *Morganella morganii*, a Gram-negative opportunistic invader, was found in one patient after the application of the sterile larvae (Table 3).

As well as from the isolates from wounds, bacteria were also isolated from the larvae used in the therapy. In 70 % of cases (14 out of 20), differences in the bacterial species of the larvae and the bacterial species present in the wounds were detected after the treatment. In addition, in 20 % of cases (4 out of 14), a difference in the ratio of the individual species was seen, although the species composition itself did not change. In 2 cases (10 %), there were no differences (Table 4).

Analysis of bacterial susceptibility to this maggot therapy (Table 3) showed bactericidal effects against group G streptococci, group C streptococci, *B. fragilis*, *C. freundii*,

Table 4. Microbiological characterization of the efficiency of maggot therapy was determined by counting and identifying isolates before as well as after larval application

When microbiological tests were not performed according to the protocol, results were marked as not determined (ND). Microbiological characterization was also made to detect differences between the bacterial isolates from the wounds and isolates from the larvae used for the treatment of the same wounds: +, differences between the isolates from wound and larvae; (+), same isolates from wound and larvae but with different ratios between the isolates. 0, No differences; ↓, decreased number of species after treatment; ↑, increased number of species after treatment.

No. of wound treated	No. of applications for individual wounds	Differences in no. of bacterial species after treatment	Differences between larvae and wounds
1	3	↓	ND
2	3	ND	+
3	1	ND	ND
4	2	ND	ND
5	2	ND	ND
6	1	↓	ND
7	1	ND	ND
8	1	ND	+
9	1	ND	ND
10	2	0	ND
11	2	ND	ND
12	2	ND	ND
13	5	0	+
14	2	↓	(+)
15	1	↓	+
16	2	0	ND
17	1	ND	+
18	1	↓	+
19	1	↓	+
20	2	↓	+
21	2	↓	+
22	2	0	+
23	1	0	0
24	1	↓	+
25	1	↓	(+)
26	1	↓	+
27	1	↓	+
28	1	0	(+)
29	1	0	0
30	1	↓	ND
31	1	↓	ND
32	1	↑	(+)
33	1	0	+

Klebsiella spp., *Peptococcus* sp., *Prevotella bivia*, *Serratia marcescens* and *Streptococcus agalactiae*, all of which were not detected after the treatment. A considerable decrease in the incidence of five species was seen: coagulase-negative staphylococci, *C. koseri*, *K. oxytoca*, *P. aeruginosa* and *S. aureus* (Fig. 4). Surprisingly, six species increased in

Fig. 4. Pathogenic bacteria in the wounds of the 30 patients before and after larval treatment.

occurrence as compared to the situation before the treatment: diphtheroids, *Enterococcus faecalis*, *Morganella* sp., *Peptostreptococcus asaccharolyticus*, *Porphyromonas* sp. and *Providencia rettgeri*. In *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Stenotrophomonas maltophilia*, α -haemeolytic streptococcus and *Streptococcus pyogenes* we did not observe any considerable changes.

DISCUSSION

Nowadays, it is generally recognized that maggots can excrete a complex mixture of substances into a wound that can clean the wound and stimulate its healing. One aspect of the complex mode of action of this effect is the antibacterial activity. Despite several reports concerning such antibacterial properties (Greenberg, 1968; Pavillard & Wright, 1957; Simmons, 1935; Thomas *et al.*, 1999) and some attempts to determine what these antibacterial compounds are (Bexfield *et al.*, 2004), the main components of this effective antimicrobial activity are still not known. However, it can be expected that several different antibacterial components, like oligopeptides (Bexfield *et al.*, 2004), disinfectants (Erdmann & Khalil, 1986) and low pH (Baer, 1931), act synergistically, as was proposed for *Phormia terraenovae* (Pavillard & Wright, 1957). Moreover, some of these antibacterial components could also be synthesized by some of the maggots' gut commensals, as proposed by Greenberg (1968). *Proteus mirabilis* is one such commensal, indicating the general occurrence of members of the genus *Proteus* in flies of the family *Calliphoridae*. Therefore, if some bacteria present in the gut of the maggots survive the complex mode of maggot antibacterial action, some antibacterial selectivity of the excreta/secreted mix of substances would be expected.

Since multiple factors are responsible for the antibacterial activity of maggot excretions, it is evident that the efficiency of the excreta/secretata, and hence of larval therapy, depends on one hand on the 'quality' of the maggots, and on the other hand on the microbiological and physico-chemical conditions present in their immediate environment.

To the best of our knowledge, there have been no reports on the diversity and susceptibility of bacteria present in the growth environment of the larvae, such as in larval feed, non-sterile maggot macerates and maggot excreta. Our study has revealed that several Gram-negative bacterial species, in addition to *Proteus mirabilis*, can colonize maggots; however, *V. lutrae* was the only Gram-positive strain that was isolated directly from the excreta/secretata. The bacteria isolated from the homogenized maggots were in the majority closely related to *Providencia*, and additionally to *Proteus* and *Pseudomonas*. Many strains of the genus *Pseudomonas* have developed multiple antibiotic resistances by acquiring resistant determinants and these are often responsible for long-term wound infections (Hsueh *et al.*, 1998; Sekiguchi *et al.*, 2005). Also, *Pseudomonas*, *Proteus* and *Providencia* can form biofilms and produce quorum-sensing signal molecules in the wounds (Schaber *et al.*, 2004; Stickler *et al.*, 1998), which present another barrier for the larvae to conquer. Pathogenic strains of *Proteus mirabilis* can also present a problem in wound infections, in spite of their potential antibacterial activity (Erdmann & Khalil, 1986), since a plasmid-mediated antibiotic resistance was discovered in *Proteus mirabilis* (Wachino *et al.*, 2006). It is noteworthy that *Providencia rettgeri* (previously known as *Proteus rettgeri*) produces an L-amino acid oxidase (Duerre & Chakrabarty, 1975), which could act on the larval antibacterial oligopeptides. When *V. lutrae* (isolated from the maggot excreta/secretata) and the most frequent species infecting chronic wounds (*S. aureus*, *P. aeruginosa* and *E. coli*) were exposed in liquid cultures to the maggot excreta/secretata *in vitro*, we saw that the action on *S. aureus* was bactericidal, while no changes were seen for *V. lutrae*. Initially, the exposure of the selected bacteria to the maggot extracts was performed on solid media. It is of note that no antagonistic effects of the excreta/secretata on the bacterial cultures cultivated on solid media were seen, not even for *S. aureus*. Similar observations have already been reported in the literature (Bexfield *et al.*, 2004; Pavillard & Wright, 1957); although, some studies have identified activities against *S. aureus* cultivated on solid media (Kerridge *et al.*, 2005). It appears that the preparation of excreta/secretata and the standardization of the media that are used for the determination of antibacterial activities could be important, as seen for the media used for MIC evaluation of antibacterial activities of conventional antibiotics (Murray *et al.*, 2003).

Although *in vitro* bactericidal activities have been reported for larval excretions for individual species, including *S. aureus* (and also for meticillin-resistant *S. aureus*),

Streptococcus pyogenes, *Enterococcus faecalis*, '*Clostridium welchii*', *Proteus vulgaris*, *S. pneumoniae* and *E. coli* (Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Lerch *et al.*, 2003; Pavillard & Wright, 1957; Simmons, 1935; Wolff & Hansson, 1999), the effects of either sterile maggots or their excretions on mixed bacterial communities present in wounds (Bowler *et al.*, 2001) have not been reported previously.

Infected wounds that harbour different bacterial communities represent a varied, natural environment for maggots, which changes after their application in a relatively consistent and repetitive pattern. Our statistically supported analyses of the microbial diversity of wounds revealed that the bacteria present can, in general, be divided into two main groups. The first group could be described as highly susceptible, since after the treatment its representatives either completely disappeared from the wounds or decreased considerably in occurrence. Within this group are *E. coli*, *P. aeruginosa* and *S. aureus*, for which high susceptibility has already been shown in other *in vitro* studies (Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Simmons, 1935). Some other human pathogens were also considerably affected: *B. fragilis*, *C. freundii*, *C. koseri*, *K. oxytoca*, *Klebsiella* spp., *Peptococcus* sp., *Prevotella bivia*, *Serratia marcescens*, coagulase-negative staphylococci, *Streptococcus agalactiae*, group C streptococci and group G streptococci.

The second group consists of bacteria that either remained unaffected or even increased in occurrence. This group consists mainly of Gram-negative, as well as selected Gram-positive bacteria. It appears that the larvae have little effect on bacteria from *Proteus* spp, which we also isolated from maggot macerates. This might be due to either bacterial adaptation, or their symbiotic relationships as members of the *L. sericata* gut flora. It must be stressed that maggots have partially extracorporeal digestion. Therefore, an association of the bacteria with the animal might be already present outside the body, in the nearest animal environment, and may help in degradation of food particles (Chambers *et al.*, 2003). Moreover, the bacteria that populate the environment close to the maggots might not be tolerated in such high densities inside the maggot gut, as shown by dissimilarities in the ratios of species present in/on the maggots as compared to the wounds.

As shown by *in vitro* studies and as demonstrated by the use of larvae enclosed in permeable Biobags (Grassberger & Fleischmann, 2002), antibacterial components of maggot excretions are mainly responsible for the selective actions on different bacterial species present on wounds. However, it appears that the crawling and wandering of the larvae on the wounds can also considerably affect the bacteria, and particularly those that are anaerobic, that can form biofilms, that are less resistant to the passage through the larval intestine (Lehane, 1997) or that are generally less susceptible to the presence of antibiotic substances (Lerch *et al.*, 2003; Mumcuoglu *et al.*, 2001). The diminished action of antibiotics on bacterial biofilms is due to low

diffusion, the thick extracellular matrix, co-existence of antibiotic-degrading bacteria and a low blood supply. The action of maggots can increase the microcirculation, and probably also destroy the very complex structure of a biofilm, consequentially exposing antibiotic-susceptible bacteria to the action of antibiotics (Costerton *et al.*, 1994).

In certain cases, after the maggot application, the wounds were infected by bacterial species that were not present prior to the treatment. These could have been introduced from the microbial flora colonizing healthy skin around the wound (diphtheroids, coagulase-negative staphylococci, group C streptococci), due to the increased humidity below the bandage (Hutchinson & Lawrence, 1991) or by the crawling of the larvae to the wound edges.

We can conclude that the bacterial diversity present in infected wounds is one of the factors that affect the success of larval therapy. This type of therapy is most appropriate for wounds infected with anaerobic and Gram-positive bacteria, while special precautions are needed when infections are due to Gram-negative bacterial species, and particularly with *Proteus vulgaris* and *Proteus mirabilis*.

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