ISOLATION AND CHARACTERIZATION OF SOME BACTERIAL STRAINS FROM DIPTERAN LARVAE

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Abstract: The isolation of agronomically beneficial bacteria usually involve the collection of samples from various natural sources, followed by the subsequent use of selective culture techniques. This is not always a straightforward process, due to various reasons, including their reduce concentration level in nature, the difficulty of separating them from the consortia in which they are naturally integrated in the environment, the overgrowth of other competing microorganisms, or even their fastidious way of *in vitro* multiplication. The aim of this work was to identify six bacterial strains that could have potential biotechnological applications, either as agro-inoculants or in environmental protection. These bacterial strains, named LvD1, LvD2, LvD3, LvD4, LvD5, and LvD6 were isolated from dead larvae of *Calliphora vomitoria*. Their identification at specie level was made based on their physiological profile using Biolog GEN III technique. The LvD1 strains, was identified as *Acinetobacter guillouiae*, a bacterial species highly valuable in bioremediation and biocontrol. Another strain, LvD3 was identified as *Proteus mirabilis*, a bacterial species mentioned as biocontrol agent, as well as a plant growth promoter, while LvD4 was identified as *Enterococcus faecalis*, another bacterial species with plant growth promoting features. However, complementary studies are needed to evaluate the activity of these strains and their attributes in biotechnological processes.

Keywords: *bacteria, Calliphora vomitoria, fly larvae, phenotypic identification, plant growth promotion*

INTRODUCTION

The diversity of biocontrol bacteria is known to be quite extensive. Numerous studies revealed entomopathogenic bacterial species, capable of infecting pests and causing insect disease (El-Deen et al., 2019, Bondarchuk et al., 2021, Baazeem et al., 2022), especially within forestall and agricultural ecosystems. Other biocontrol bacteria are studied for plant pathogens control (Liu et al., 2021; Lee et al., 2023), as well as for their ability to enhance natural plant resistance mechanism (Yu et al., 2022), or to promote plant growth (Amaresan et al., 2021; Fasusi et al., 2021; Vishnupradeep et al., 2022). For instance, certain strains of *Bacillus subtilis* have been proved to assist plants to recover from biotic stress (Hashem et al., 2019), while strains of *Bacillus pumilus* induced resistance against bacterial spot in pepper (Yi et al., 2013). Various *Pseudomonas* spp. and *Streptomyces* spp. strains are also described as biocontrol agents, or plant growth-promoting agents (Ghadamgahi et al., 2022; Nonthakaew et al., 2022). This diversity is influenced by various factors, including soil composition, moisture levels, pH, temperature, and the presence of potential insect or plant hosts. Variability in soil ecosystems can harbor distinct bacterial communities, which implicitly leads to variations in bacterial diversity. At species level, among the biocontrol strains, there can be considerable genetic variation. For example, different biocontrol strains, with entomopathogenic activity, may have different virulence factors, insecticidal toxin profiles, and other genetic traits that influence their pathogenicity towards insects.

There are several steps to follow for the isolation of entomopathogenic bacteria, as well as various test procedures, helpful in selecting efficient and competitive bioinsecticide strains (Sharma et al., 2021).

Generally, entomopathogenic bacteria of the Bacilli class are more likely isolated from soil and dead insects (Gobatto et al., 2010; Karshanal and Kalia, 2023). *Bacillus thuringiensis* (*Bt*) and other Bacillus related species are endospores producing bacteria. These heat-resistant bacterial spores enhance the subsistence of the bacterium in harsh natural environments, and increase the chances of isolating such biocontrol bacteria from the soil. However, in the soil there are complex microbial consortia which sometime makes the isolation process a little difficult. Therefore, the use of selective media and appropriated isolation procedures are very important to increase the chance of harvesting competitive biocontrol strains. If the isolation is intended from insects, the challenge is just as high, because insects are colonized by a wide variety of microorganisms that compile their microbiota. The most inhabited areas of the insect body are the external cuticle and the gut (Douglas, 2015). The insect gut microbiota is influenced mainly by the insect's diet and habitat (Maleki-Ravasan et al., 2020).

The aim of this study is to analyze and isolate internal bacterial colonizers of *Calliphora vomitoria* dead larvae, in order to harvest biotechnological important strains with potential use in agricultural or environmental biotehnology.

MATERIALS AND METHODS

Calliphora vomitoria larvae were purchased form a local producer of natural fishing baits. Among the fly larvae, a small number was found dead one day after purchase, while larvae were kept in corn flour, at could temperature, in darkness. Dead larvae were collected, and prepared for microbiological analysis and bacterial harvest (figure 1). The larvae were surface washed and immersed in 5% NaClO for 15 min, under gentile shaking. Then, they were rinsed for five times with sterile distilled water. The disinfected larvae were subsequently dried in sterile air flow. They were dissected, and the content was plated on Luria Bertani (LB) agar. The streak-plate method (Sanders, 2012) was then used for bacterial purification on LB agar medium. This isolation protocol was performed within the Faculty of Biotechnologies, at the University of Agronomic Sciences and Veterinary Medicine of Bucharest, while the bacterial characterization was carried out at the Bacteriology Laboratory, within the Research and Development Institute for Plant Protection.

Once purified, the isolated bacteria are obtained as single strains of genetically homogeneous population. Strains purity was checked by colony morphology on solid media and microscopic examination.

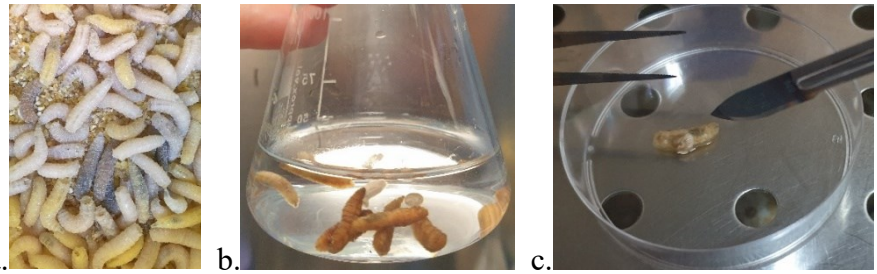

Figure 1. Aspects during the *Calliphora vomitoria* larvae preparation for microbiological analysis and bacterial harvest
a. dead larvae selection, b. disinfection, c. dissection

Bacterial colony morphology was evaluated in pure cultures on LB agar. Colony aspect, profile, edge, color, and biomass consistency was described. Microscopic examination was carried out to reveal the shape and cell arrangement, as well as tinctorial affinity by Gram staining. Tripathi and Sapra (2022) are among the most recent authors to synthesize the principle of the Gram stain method. For this analysis, fresh bacterial biomass was used from overnight grown cultures. The biomass was smeared in one droplet of fresh water on the surface of the slide, by spreading it with circular movements. The microscopic slides were air dried and flame fixed for three times. For Gram staining, each specimens smear mount was flooded for 1-2 min in crystal violet (gentian violet) solution (Figure 2). After removing the excess dye, the slides were covered with Lugol solution for another 1-2 min. This iodine solution acts as a mordant, by fixing the violet dye in the Gram-positive cell wall, rich in peptidoglycan. Fast decolorization step, no longer than 30 seconds, was then performed using a mixture of ethylic alcohol with acetone (7:3 v/v). The slides were then rinsed with water. A last pigmentation step was then performed, using safranin, for 30 seconds, followed by excessive rinsing. The dried microscopic slides were analyzed under a microscope, using the 100X magnitude objective and immersion oil.

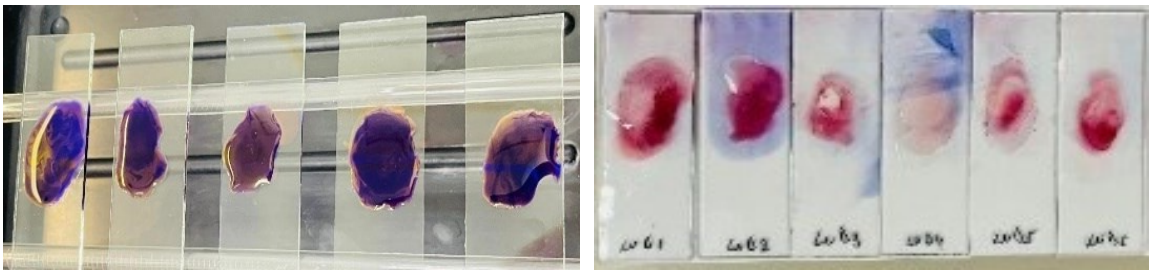

Figure 2. The microscopic slides while performing the Gram staining.

Phenotypic identification of the bacterial isolates was performed using the GEN III microplates system at the Biolog MicroStation. The strains were refreshed on Biolog Universal Growth (BUG) media, at 30°C. The identification protocol A was then followed, according to the manufacturer instructions. Single colonies were suspended in wormed up Biolog type A inoculation fluid, at 94 to 97% turbidity, in 590 nm wavelength light. The homogenous suspensions were equally distributed in prewarmed Biolog GEN III microplates, adding 100 µl suspension volume in each of the 96 wells of the plate. After 24h of incubation at 30°C, the plates were uploaded in the MicroStation and read with the semi-automat Biolog System to reveal the utilization of carbon sources or resistance to inhibitory chemicals, salts, and antibiotics. After software analyses the physiological profile of the strain was compared with the database of bacterial profiles and identified each specimen at specie level.

RESULTS AND DISCISSIOS

Six bacterial strains, LvD1, LvD2, LvD3, LvD4, LvD5, and LvD6, were isolated from the dead fly larvae of *Calliphora vomitoria.* These bacterial isolates revealed different colony morphology (Figure 3).

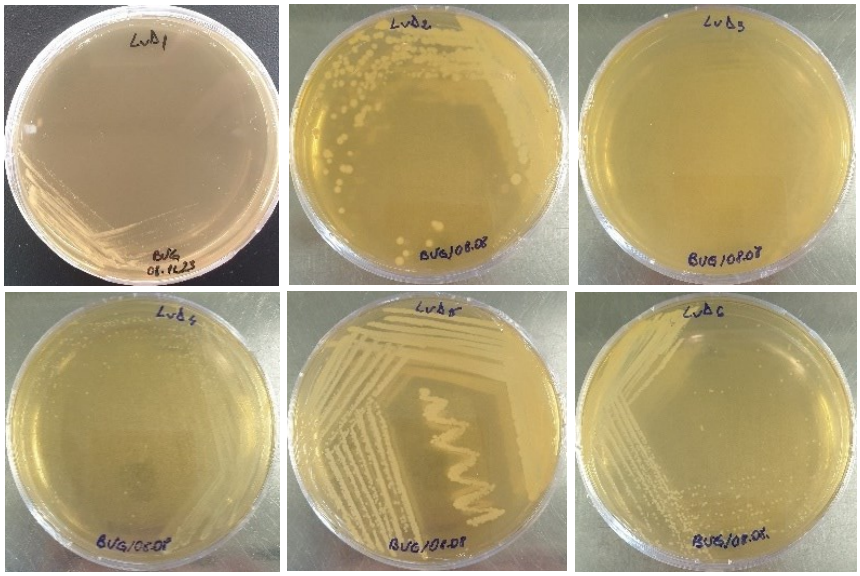

Figure 3. Bacterial strains isolated from dead fly larvae.

The LvD1 strain revealed semi-translucent growth, smooth, mucoid, round, convex, and medium-sized colonies. The LvD2 colonies were roundish, with slight irregular edge, opaque, shiny, smooth and convex. Very similar colony morphology was seen in the LvD5 strain. In contrast, the LvD3 colonies revealed translucid appearance, with swarming tendency. The LvD4 strain revealed abundant small to medium size colonies, with circular shape and smooth appearance, while LvD6 strain also revealed circular, smooth colonies, but shiny and translucent.

Analyzing cell morphology and tinctorial properties, the studied strains revealed to be all Gram-negative rods, except for LvD4 strains whose cells were Gram-negative cocci (figure 4).

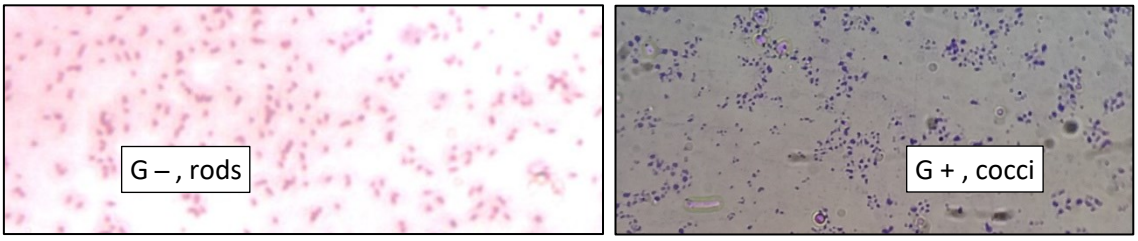

Figure 4. Gram reaction and cell morphology of LvD6 (left) and LvD4 (right).

Following the type A protocol of the GEN III system, the phenotypic fingerprint of each bacterial strain was revealed, and moreover all strains were identified at species level (table 1).

Table 1. Identification of bacterial strains with BIOLOG GEN III system

Isolated bacterial strains	Bacteria identification using the Biolog GEN III system
LvD1	*Acinetobacter guillouiae*
LvD2	*Moellerella wisconsensis*
LvD3	*Proteus mirabilis*
LvD4	*Enterococcus faecalis*
LvD5	*Moellerella winconsensis*
LvD6	*Pseudomonas lundensis*

Although the microbiome of houseflies (Diptera: *Muscidae*) and blowflies (Diptera: *Calliphoridae*) has not been described yet (Junqueira et al., 2017), there are certain surveys that describe some of the colonizing bacteria (Tomberlin et al., 2017; Wohlfahrt et al., 2020; Mondal et al., 2023). Among the identified bacteria, *Acinetobacter, Enterococcus, Proteus* and *Pseudomonas* were mentioned to be associated to *Calliphoridae* flies in various studies (Singh et al., 2015; Brits et al., 2016; Junqueira et al., 2017).

Among the bacterial colonizers of *C. vomitoria* larvae, *Acinetobacter guillouiae* was found. This newly isolated LvD1 strain revealed some differences compared with the physiologic pattern of *A. guillouiae* from the Biolog GEN III Database. The LvD1 strain revealed the capacity to metabolise p-hydroxy-phenylacetic acid and D-lactic acid methyl ester, and be resistant to nalidixic acid and potassium tellurite. However, such properties are not common for *A. guillouiae.* Studies mention this specie to have promising applications in environmental protection and agriculture. The *A. guillouiae* SFC 500-1A strain is used in bioremediation of tannery wastewater (Fernandez et al., 2023), while the *A. guillouiae* NFB2 strain is used as plant growth promoting bacteria for tomato seedlings (Cervantes-Vázquez et al., 2021).

Within this study, beside *A. guillouiae*, *Moellerella winconsensis* was also isolated from dead larvae of *C. vomitoria*. Until present *M. winconsensis* has already been retrieved from insects and parasites, as well as from domestic and wild animals, and from humans (Athanasakopoulou et al., 2022). *M. wisconsensis* is considered a potentially zoonotic pathogen, that can be disseminated by wild birds and flies, including *Calliphora vicina, Musca domestica* and *Lucilia sericata* (Athanasakopoulou et al., 2022; Wiktorczyk-Kapischke et al., 2022).

Proteus mirabilis was isolated from dead larvae of *C. vomitoria* as well. However, the LvD3 strain revealed some biochemical differences to the *P. mirabilis* phenotypic fingerprint. The newly isolated strain was able to metabolize D-turanose and L-rhamnose, but was not able to use citric acid, as it was shown during the Biolog identification.

Similar to this study, Singh et al. (2015), mention *Proteus* sp. to be associated to the salivary glands of a *Calliphoridae* flies, and also revealed the predominant bacterial families found as insect colonizers during the life cycle of the *Lucilia sericata* and *L. cuprina* flies (Diptera: *Calliphoridae*). Brits et al. (2016), mentioned *Pseudomonas* sp. to be among the abundant bacterial species associated to the studied dipteran, while *Proteus mirabilis* is presented as a colonizer in the flies' species *Chrysomya megacephala* (Diptera: *Calliphoridae*) and *Musca domestica* (Diptera: *Muscidae*). *Proteus mirabilis* found in *Calliphoridae* larvae is mentioned to produce antimicrobial compounds, such as phenylacetic acid and phenylacetaldehyde, that could protect its host from bacterial infections (Erdmann et al., 1986). This is a highly beneficial aspect for the host insect, considering it often feed on the remains of vertebrate tissues (Tomberlin et al., 2017). Barnes et al. (2010) also sustain that *Proteus mirabilis* has beneficial effects on flies' larvae, by reducing the growth of competing or insect pathogenic bacteria.

Enterococcus faecalis can also occur in flies at low prevalence (Lazzaro, 2002; Chandler et al., 2011). Studies on *E. faecalis* isolated from wild *Drosophila melanogaster* hemolymph, caused intermediate levels of mortality in laboratory reared flies at a relatively low dose (Lazzaro, 2002; Lazzaro et al., 2006). Therefore, *E. faecalis* is not only a gut commensal in flies, but rather can cause systemic pathogenic infections towards insects (Chapman et al., 2020).

Among the bacterial isolated from *Calliphoridae* dead larvae, LvD6 strain was identified as *Pseudomonas lundensis*, according to the Biolog identification system. This species is mentioned as a meat, fish and milk spoiling bacteria, due to its high proteolytic activity (Marchand et al., 2009; Bahlinger et al., 2021). To our knowledge, this is the first report

on *P. lundensis* associated to insects, although other *Pseudomonas* species are mentioned to cause lethal infections in insect larvae (Flury et al., 2016).

The studies carried so far on *Calliphoridae* flies microbiome revealed that, in the insect gut and stomach various microbial colonizers were found, both beneficial and harmful to their host. Some of the symbiotic relationship improve insect survival in harsh environments, especially as this fly larvae are feeding with decaying animal remains. Some of the internal colonizing microorganisms play a variety of physiological roles in host metabolism and well-being, by improving food digestion, providing essential nutrients that are scarce in food, degrading toxins and pesticides, producing antibiotics and competing detrimental microorganisms for the host insect, or preventing pathogen invasion by stimulating the host immune system. However, beside such microbial symbiosis, insect pathogenic microorganisms can also be found, especially in dead insects. Such entomopathogenic strains could be used in biotechnological processes for the development of microbial pesticides.

Taking into consideration that *A. gullouiae* and *E. faecalis* are known to be useful for various agricultural and environmental applications, further studies are necessary to reveal the efficacy of the isolated strains in plant growth promotion or pest control. *P. lundensis* LvD6 strain should also be considered in future studies to reveal its entomopathogenic potential.

CONCLUSIONS

The isolation technique used to collect cultivable bacteria from dead larvae of *Calliphora vomitoria* fly have led to the purification of six bacterial strains. The identification through the Biolog GEN III system revealed their phenotypic fingerprint and taxonomic affiliation to *Acinetobacter gullouiae* (LvD1 strain), *Enterococcus faecalis* (LvD4 strain), *Moellerella wisconsensis* (LvD2 and LvD5 strains), *Proteus mirabilis* (LvD3 strain) and *Pseudomonas lundensis* (LvD6 strain). Mors of these species were confirmed to belong in the dipteran microbiome. However, to our knowledge this is the first study revealing *P. lundensis* isolation from insects.

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