Chitinolytic bacteria associated to *Oestrus ovis* L. larvae (Diptera: *Oestridae*)

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SUMMARY

The objectives of this study were: I. To culture, isolate, identify and characterize bacterial species obtained from *O. ovis* L3 larval tissues and the nasosinusal mucosa of goat hosts and, II. To test the *in vitro* larvicidal effects of selected chitinolytic bacteria against *O. ovis* L2 and L3 larvae. A total of thirty three bacteria species were isolated and identified by molecular (16S RNA sequence) and biochemical techniques, and phylogenetic trees were constructed. *Aeromonas veronii* was the most frequent (30%) species isolated from larvae and mucosa tissues. Four bacteria: *Pantoea calida, Pantoea gaviniae, Serratia marcescens* and *A. veronii* showed high *in vitro* chitinolyitic activity. These bacteria were experimentally inoculated in the rearing media of groups of L2 and L3 larvae. Mortality rates in L2 larvae exposed to isolates of *Pantoea gaviniae* (100%), *Aeromonas veronii* (100%), and *Serratia marcescens* (100%) were statistically higher ($P < 0.05$) than in the control group (12%) at 48 h post-incubation. In L3 larvae, mortality rate at 48 h post-incubation was found higher ($P < 0.05$) in larvae exposed to *Pantoea calida* (35%), than in larvae exposed to the other bacteria and control group (12.5%). In conclusion, this is the first report dealing with the identification of bacterial species associated to *O. ovis* larvae, showing potential effects to cause damage and mortality in larval instars. The use of these bacteria as biological control agents against *O. ovis* deserves further investigation.

Keywords: Myiasis, larvicidal effect, parasite, goat, sheep nose bots, bacteria, Oestridae

RESUME

Bactéries chitinolytiques associées aux larves d´*Oestrus ovis* L. (Diptera: Oestridae)

Les objectifs de cette étude étaient les suivants : I. Culture, isolement, identification et caractérisation des espèces bactériennes obtenues à partir des larves *O. ovis* L3 et de la muqueuse nasosinusale des hôtes (caprins). II. Tester les effets larvicides *in vitro* de certaines bactéries chitinolytiques contre les larves *O. ovis* L2 et L3. Au total, trente-trois espèces de bactéries ont été isolées et identifiées par voie moléculaire (séquence d'ARN 16S) et par techniques biochimiques. Des arbres phylogénétiques ont été construits. *Aeromonas veronii* était l'espèce la plus fréquente (30%) isolée à partir de larves et des muqueuses. Quatre bactéries : *Pantoea calida, Pantoea gaviniae, Serratia marcescens et A. veronii* ont montré une forte activité chitinolytique *in vitro*. Ces bactéries ont été inoculées expérimentalement dans les milieux d'élevage de larves L2 et L3. Les taux de mortalité des larves de L2 exposées aux isolats de *Pantoea gaviniae* (100%), Aeromonas veronii (100%) et Serratia marcescens (100%) étaient statistiquement plus élevés ($P <0,05$) que dans le groupe témoin (12%) à 48 h d'incubation. Pour les larves L3, le taux de mortalité à 48 heures après l'incubation a été plus élevé ($P <0,05$) chez les larves exposées à Pantoea calida (35%) que chez celles exposées aux autres groupes de bactéries et aux témoins (12,5%). En conclusion, il s'agit de la première identification d'espèces bactériennes associées aux larves d'*O. ovis* présentant des effets potentiels létaux sur les stades larvaires. L'utilisation de ces bactéries comme agents de lutte biologique contre *O. ovis* mériterait des recherches approfondies.

Mots-clés : Myiasis, effet larvicide, parasite, chèvres, bactéries, Oestrus ovis, Oestridae

Introduction

Oestrus ovis (Diptera: Oestridae) causes obligate myiasis in small ruminants worldwide. Sheep and goats infected with *O. ovis* larvae showed reduced performance, and their welfare was frequently impaired, affecting growth and reproduction [3, 4]. It is known that bacteria are ubiquitous microorganisms symbiotic of internal and external parasites. It has been pointed out [7] that effective bacteria are capable to infect and to cause disease and even death in parasites or insects. These bacteria can be cultured and isolate from the own tissues of the parasite, especially found in moribund or dead individuals. A well-documented mechanism of the larvicidal effect of microorganisms on parasites or insects include chitinolytic enzymes that are able to degrade the integumentary cuticle of the insect [18]. At the same time, cuticular damage leads to the entry of fungi or bacteria, loss of homeostasis and death of the infected parasite [15]. This approach has been previously used to develop strategies of parasite control [8, 9, 24, 25]. However, bacterial species with anti-parasitic or larvicidal activity have been little explored so far [5]. Also, elucidation of the taxonomic relationships of symbiotic bacteria may help to improve our understanding about the phylogenetic relationships and their association with parasite hosts. Therefore the aims of this study were: I. To culture, isolate, identify and characterize bacterial species obtained from *O. ovis* larval tissues and the nasosinusal mucosa of goat hosts and II. To test the *in vitro* larvicidal effects of the bacteria species proven to be chitinolytic against *O. ovis* larvae.

Materials and Methods

O. OVIS LARVAE COLLECTION

All *O. ovis* larvae used in these studies were collected from the head of goats (*n*= 21) slaughtered in the municipal slaughterhouse at La Paz, B.C.S., Mexico. Live second (L2, *n*=25) and third (L3, *n*=42) stage larvae were immediately stored in 1.5 mL sterile tubes in an ice bath and transported to the laboratory.

LARVAL DISSECTION FOR BACTERIA ISOLATION

In order to isolate the *O. ovis* associated bacteria (S), L3 larvae (*n*= 22) were selected on the basis of apparent viability (absence of integumentary fungal harm, lesions onto the cuticle, and presence of normal vigorous forward movements). Then, each one L3 larva was washed with peptone salt solution (PSS pH 7.0, 0.1%). Later on, larval dissection was carried out under sterile conditions as it has been described [3], with slight modifications. Briefly, each live larva was fixed with entomological pins on a sterile paraffined Petri dish and dissected inside a disinfected (ethanol 96%) Class I cabinet, aided with stereomicroscope and sterile ophthalmic surgical equipment (Moria Inc., France). Larval dissection was done on an ice bath using sterile cold (4–10 °C) physiological saline solution (0.9% NaCl) as dissecting medium. Then, BD Ultra-Fine insulin syringes (1 mL) were used for hemolymph (H) collection (50 µl) and inoculation into PSS (0.1%). Then, the larval ventral coelomic wall, as well as the surrounding fat body, tracheal and nervous tissues were removed to collect the entire salivary gland (SG) and the digestive tract (DT). Thereafter, SG and DT were separately stored into PSS (1 mL) in a 2 mL conical tube and homogenized. In addition, dead L3 larvae (*n*=6) (with the characteristic dark color, flaccid, unresponsive to tactile stimulation, and showing signs of internal tissue degradation) were completely homogenized. Then, the homogenate of each larva was diluted in 1 ml of PSS. At the same time, 1 g samples of goat nasosinusal mucosa were collected from *O. ovis*-infected (*n*=16) and not infected (*n*=5) goats and transferred to sterile tubes. Both tissue types were macerated by separate and then 0.1 g of homogenates were suspended in 900 µL of PSS until processed.

BACTERIAL CULTURE, COUNT, AND ISOLATION

To determine de number of colony forming units (CFU) of each homogenized tissue, serial dilutions (10^{-1} to 10^{-7}) were plated (100 µL) on plate count agar and incubated for 24 h at 37 °C. For bacterial isolation, homogenized samples were inoculated (100 µL) in nutrient broth medium for enrichment or plated directly in nutrient, macconkey and blood base agar media. All samples were incubated for 24 h at 37 °C, and then the CFUs were picked up and placed on new corresponding media plates.

BACTERIAL IDENTIFICATION

Molecular identification of the bacteria

Total DNA extraction and molecular identification was carried out on each isolate as by procedures published [6]. The 16S rRNA gene sequence of LAB was amplified using pA (5'-AGAGTTTGATCCTGGCTCAG-3') as forward and PH* (5'-AAGGAGGTGATCCAGCCGCA-3') as reverse primers. The PCR reaction mixture consisting of 1x PCR enzyme buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP's mix, 0.5 µM of each primer, 0.03 U/µL of Taq polymerase and 1 ng/µL of template DNA. The thermocycler program was as follows: 95°C for 4 min; 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min; and a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis in a 1.0 % agarose gel stained with SYBR green in 1X TAE buffer at 100 V for 45 min. The bands were visualized under an UV trans-illuminator. The sizes of DNA fragments were estimated (~1.6 kb) using a standard 1kb DNA ladder (Norgen biotek Corporation, Canada) and sequenced (Macrogen, Korea). The 16S rDNA sequences of isolated strains were compared to sequences from type strains held in the GenBank DNA database using BLAST.

Biochemical characterization

All bacterial isolates obtained were characterized considering the following biochemical tests: Colony shape and color, catalase, cytochrome C oxidase, methyl red, Voges–Proskauer, indol, motility, sulphydric acid production in SIM media, phenylalanine deaminase, urease, citrate, nitrate reduction, glucose and lactose fermentation in TSI media, ornitine decarboxylase, gelatin hydrolysis (25 °C), hemolysis, malonate and maltose fermentation, and growth in nutrient broth plus 10% and 15% of NaCl.

Phylogenetic analyses

Phylogenetic analysis was performed using molecular evolutionary genetics analysis, Version 7.0 (MEGA 7.0). Multiple alignments of 16S rRNA nucleotide sequences were done with CLUSTAL W. The tree topologies were generated using the neighbor-joining method and evaluated by bootstrap analyses based on 1,000 replicates.

CHITINASE PRODUCING BACTERIA

Preparation of colloidal chitin

Colloidal chitin was prepared as previously described [14]. In brief, pure chitin powder (20 g) was slowly added to 300 mL of concentrated HCl and incubated at 30 °C for 60 min under vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 1 L of water at 4 °C. This suspension was collected by filtration on Whatman filter paper No. 3, and washed by resuspending it in 5 L of distilled

water until pH was 3.5. After this process, colloidal chitin was dried off and used later as a substrate.

Screening chitinase producing bacteria

In order to screen the potentially chitinase producing bacteria, agar medium containing colloidal chitin (1% colloidal chitin, 0.01% $MgSO_4$.7H2O, 0.01% K_2HPO_4, 0.05% yeast extract, 0.7% $(NH_4)2SO_4$, 0.1% NaCl and 1.5% bacto agar) was used. Each bacterial isolate was grown and adjusted to 1 x 10^8 cells/mL. Then, 50 µL of bacteria was inoculated in the colloidal chitin agar medium and incubated at 37 °C. Bacterial isolates were analyzed on the basis of formation of a chitin hydrolysis zone after 96 h incubation. Bacterial isolates forming a 0.2 mm or a wider zone of chitin activity in the agar media were considered as chitinolytic and chosen for further studies.

Determination of chitinase activity in chitinolytic bacteria

Based on the previous screening results, some bacterial isolates (*n*=6) were selected and used to assess their chitinase activity, which was carried out by reducing sugar released from colloidal chitin. In short, 1 mL of bacteria (1 x 10^8 cells/mL) was inoculated into 50 mL chitin medium at 37°C for 96 h. Samples were taken at 12 h intervals and centrifuged at 10, 000 *g* for 10 min (at 4 °C) to obtain the supernatant with extracellular chitinase enzyme (CE). CE (150 µL) was added to a mixture consisting of 300 µL of 0.3% colloidal chitin and 150 µL of 0.1 M phosphate buffer pH 8.0. After incubation at 55 °C for 10 min, the reaction mixture was centrifuged (4 °C) at 8,000 *g* for 5 min. The supernatant (100 µL) was added to 1 mL of DNS (3,5-Dinitrosalicylic acid 96 mM) and incubated at 100 °C during 10 min. The absorbance of the solution was measured with a light spectrophotometer at 595 nm. One unit of chitinase activity was defined as the amount of enzyme that yielded 1 µmol of reducing sugar as N-acetyl-D-glucosamine-equivalent (GlcNAc) per minute. Also, the growth curve of each bacteria culture was determined by measuring the optical density at 600 nm.

IN VITRO ASSAY OF BACTERIAL LARVICIDAL EFFECTS

Preparation of the larvicidal cultures

In order to prepare six different larvicidal bacterial isolates, the respective purified isolates were grown by separate in LB at 37° C during 48 h. Then the bacteria counts were adjusted to 1 x 10^8 cells/mL.

Collection of *O. ovis* larvae and rearing for larvicide tests

Healthy *O. ovis* L2 (*n*=42) and L3 (*n*=63) larvae were collected from the heads of infected goats (*n*=26). These larvae were washed with PBS, checked for viability as described previously. Then, the selected larvae were grouped in Petri dishes according to larval stage, to be used later on.

Experimental *in vitro* testing of bacterial larvicidal effects on *O. ovis* larvae

Groups of L2 (*n*=6 larvae per group) and L3 (*n*=9 larvae per group) *O. ovis* larvae were used to test each bacterial isolate as a treatment group. For this purpose, two L2 larvae per Petri dish were assigned and each dish was used as a replicate. In the case of L3 larvae, due to its size, only one L3 larva per Petri dish was included and three L3 larvae represented a treatment replicate. Each bacterial isolate was tested using three replicates. Treatment replicates received a single dose of 500 µL of each individual bacterial isolate (*n*=6). Untreated (contained in sterile PBS media only) L2 larvae or L3 larvae were used as controls by triplicate. After larvicidal bacteria inoculation, the larvae were incubated at 37 °C during 48 h. All culture media were provided with fresh 500 µL of sterile PBS daily. Larval mortality was recorded at 0, 18, 24 and 48 h post inoculation. Larval mortality rate was calculated as the number of dead larvae/total number of larvae per treatment-group × 100.

STATISTICAL ANALYSIS

Descriptive statistics of culturable bacteria counts and larval mortality rates recorded in L2 and L3 *O. ovis* larvae were calculated. Results were expressed as mean mortality rate and standard deviation compared to control (PBS) groups. Data from growth curves were reported as means ± standard deviation (SD). One-way ANOVA was performed to check significant differences in mortality rate among groups and differences between means were analyzed using the Tukey test at value of P= 0.05. All analyses were performed using the SPSS v11 software.

Results

MOLECULAR AND BIOCHEMICAL IDENTIFICATION OF THE ISOLATED BACTERIAL SPECIES

A total of 67 bacteria isolated from *O. ovis* L3 larvae and host's nasosinusal mucosa were correctly identified by the 16S RNA sequence. Nevertheless, only 39 isolates were consistently identified as species by molecular and biochemical tests (Table I). The most frequent species found in the nasosinusal mucosae of *O. ovis* infected and not infected-goats was *Aeromonas veronii* (29 and 30%, respectively). *Bacillus* sp. and *Acinetobacter* sp. were also found in both, infected (12.5 and 6.25%, respectively) and not infected (15.4 and 7.7%, respectively) mucosae. Ten bacterial species were found only in infected mucosae and six bacterial species were only present in uninfected mucosae. Moreover, *Pantoea calida*, *Staphylococcus hominis*, *Terribacillus* sp. and *Bacillus sonorensis* were found in both healthy and dead larvae, although with different frequencies (Table I). In *O. ovis* L3

larvae, eight bacteria were found in dead larvae and eight else were found in healthy larvae. *Staphylococcus pasteuri* was found in either infected mucosae or healthy *O. ovis* larvae. In addition, *P. calida* was found in infected mucosae as well as healthy and dead L3 larvae, and *Staphylococcus arlattae* and *A. veronii* were found both in infected host mucosae and dead L3 larvae (Table I).

PHYLOGENETIC ANALYSES

Aeromonas and Staphylococcus species were distinctly grouped according to tissue location in *O. ovis* L3 larvae. Other bacterial groups were composed of mixed species, which were found throughout different tissue locations in the larvae (Fig. 1). Similarly, *Aeromonas veronii* isolates and several species of Staphylococcus collected from the goat mucosa were clustered in well-defined groups (Fig. 2). In general, *Bacillus* sp. were grouped and closer related to *Staphylococcus* sp. group. *Pantoea calida* together with *Bacillus* sp. were in a separate group. Other various genera, such as *Pseudomonas* sp. and *Acinetobacter* sp. were separately grouped. In addition, *Kocuria rizophila*, a Gram-positive bacterium commonly found in the ground, was also found in the goat mucosa, forming a unique cluster. In addition,

Bacteria	Bacteria species (%)			
	Mucosae		Larvae	
	Not infected	Infected	Heathly	Dead
1. *Bacillus sp.*	15.4	12.5	5.5	
2. *Staphylococcus epidermidis*		12.5		
3. *Staphylococcus arlattae*		6.25		5.88
4. *Staphylococcus sp.*		6.25		
5. *Pantoea agglomerans*		6.25		
6. *Pseudomonas stutzeri*		6.25		
7. *Psychrobacter sanguinis*		6.25		
8. *Kocurina rhizophila*		6.25		
9. *Aeromonas veronii*	30.08	12.5		29.4
10. *Pantoea calida*		6.25	11	5.88
11. *Staphylococcus sciuri*		6.25		
12. *Staphylococcus pasteuri*		6.25	5.5	
13. *Acinetobacter sp*	7.7	6.25		
14. *Moraxella phenylpyrubica*	7.7			
15. *Staphylococcus hominis*	7.7		22	5.88
16. *Stenotrophomonas chelatiphaga*	7.7			
17. *Pseudomonas sp*	7.7			
18. *Enterococcus durans*	7.7			
19. *Terribacillus sp.*	7.7			
20. *Micrococcus luteus*			16.5	5.88
21. *Micrococcus sp.*			5.5	
22. *Bacillus amyloliquefaciens*			5.5	
23. *Bacillus sonorensis*			5.5	
24. *Escherichia coli*			5.5	11
25. *Roseomonas genomospecies*			5.5	
26. *Serratia marcescens*			11	
27. *Bhargavaea cecembensis*			5.5	
28. *Kocuria palustris*				5.88
29. *Aeromonas caviae*				5.88
30. *Shigella sp.*				5.88
31. *Pantoea gaviniae*				5.88
32. *Enterobacter hormaechei*				5.88
33. *Aeromonas sp.*				5.88

[a]For bacterial isolation, goat nasosinusal mucosae were collected from O. ovis-infected (n=16) and not infected (n=5) goats. All O. ovis larvae used were collected from the head of goats (n= 21) slaughtered in the municipal slaughterhouse at La Paz, B.C.S., Mexico. A total of twenty-two healthy L3 larvae and 6 dead L3 larvae were selected for bacterial isolation

Table I: Bacteria species isolated from goat nasosinusal mucosae and from healthy or dead *O. ovis* L3 larvae[a] (frequency of isolates was expressed as percentage).

it was observed that bacteria identified as *Pantoea calida, P. gaviniae, Serratia marcescens* and *Aeromonas veronii* showed evident larvicidal effects in *O. ovis* larvae.

Figure 1: Phylogenetic tree of 16S gene sequences from bacterial isolates from *O. ovis* L3 larvae.

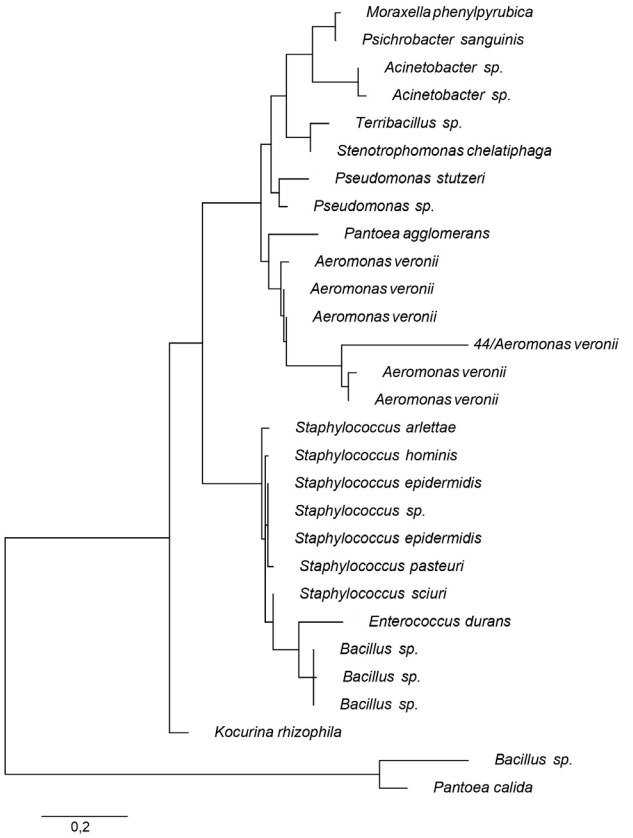

Figure 2: Phylogenetic tree of 16S gene sequences of bacterial isolates from *O. ovis*-infected goat mucosae.

CULTURABLE BACTERIA FROM *O. OVIS* L3 LARVAE AND HOST MUCOSA

The colony morphology analysis through several bacteriological media yielded a total of 21 bacterial isolates, which were obtained from the L3 healthy larvae and 17 isolates obtained from dead larvae. From these, seven isolates were obtained from the cuticle tissue and six isolates from the gut. Only three bacterial isolates were collected from the salivary glands. Furthermore, five bacterial isolates were obtained from the larval hemolymph. By contrast, nine isolates from the cuticle and eight isolates from whole-larva extracts were cultured from dead larvae. Also, the nasal and sinusal mucosa of *O. ovis*-infected goats harbored more culturable (n=16) bacterial isolates than *O. ovis*-uninfected goats (n=13).

CHITINASE ACTIVITY

Ten bacteria species grew up on chitin agar, showing a clear zone in the culture media around the colony spot, which indicated production of extracellular chitinase. From these, six isolates showed the highest chitinolytic activity. Therefore, a chitinase activity assay in broth media using these six isolates was carried out. The highest chitinase activity was observed in *Pantoea calida* followed at 24 h post incubation. Also, *Aeromonas veronii* showed an increase in chitinase activity at 12 and 60 h post-incubation, while the remaining bacteria reached the activity peak at 48 h post-incubation, and then their chitinase activity decreased. Growth patterns of the bacteria were similar during the chitinase activity assay. The highest chitinase activity of the bacteria occurred at 24 h post-incubation, at the middle of the exponential phase of the growth pattern. For *Pantoea gaviniae, Micrococcus luteus, Serratia marcescens* and *Aeromas veronii*, the highest activity occurred during the late exponential phase of growth.

O. OVIS LARVICIDAL *IN VITRO* ASSAY

Larvicidal and damaging effects on the larval external cuticle were observed in L3 and L2 larvae exposed to the chitinolytic bacterial isolates (shown in Fig. 3). Overall, larvae were observed flaccid and/or swollen after bacterial co-incubation; and they developed the typical dark-brown appearance and died. By contrast, control larvae did not show any of the larvicidal or cuticular signs. Mortality rates in L2 larvae exposed to isolates of *Pantoea gaviniae* (100%), *Aeromonas veronii* (100%), and *Serratia marcescens* (100%) were statistically higher ($P < 0.05$) than the control group (12%) at 48 h post-incubation (Fig. 4-A). In L3 larvae, mortality rate was found higher ($P < 0.05$) in larvae exposed to *Pantoea calida* (35%) compared to the other bacteria and control group (12.5%) at 48 h post-incubation (Fig. 4-B).

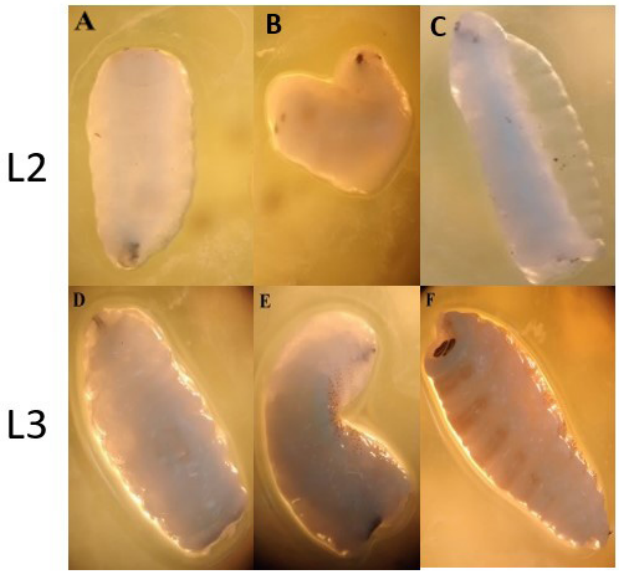

Figure 3: Phylogenetic tree of 16S gene sequences of bacterial isolates from *O. ovis*-infected goat mucosae.

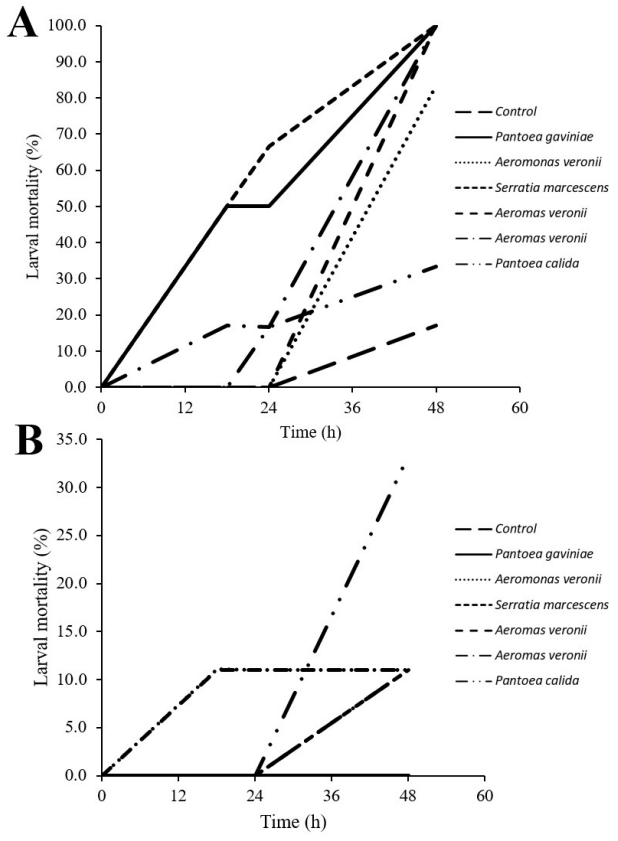

Figure 4: Mortality rate curves of *O. ovis* L2 (A) and L3 (B) larvae, recorded after *in vitro* incubation with different chitinolytic bacteria isolates.

Discussion

For the first time, identification of culturable bacteria associated with *O. ovis* larvae was carried out. Overall, 21 and 16 isolates of bacteria were obtained from *O. ovis* L3 healthy and dead larvae, respectively. These findings may be attributable to the origin of the parasites, physiological stage, and the use of different culture media. It is known that different laboratory conditions may influence certain bacteria associations with insects, resulting in great reduction or nil culturable bacteria. Hence, the application of more than one culture media was recommended [12]. In this study, using three culture media gave a better picture of the culturable bacteria associated to *O. ovis* larvae. Identification of associated bacteria revealed new insights of interaction between Oestrid larvae and symbiotic/larvicidal bacteria, as it has been observed in other insect species [19, 23]. Interestingly, we found several larvicidal bacteria that may be regularly in contact with *O. ovis* larvae, as it has been reported for other parasite species. For instance, the larvicidal bacteriome of the cattle tick (*Rhipicephalus* (Boophilus) *microplus*) was studied using the non-culture based molecular approach [2]. Also, relationships between some bacterial endosymbionts (commensal, mutualistic, or parasitic) and *O. ovis* larvae may exist. Thus, in our study, the bacterial species found in *O. ovis* larvae may represent the microbiota associated to the mucosa of the upper respiratory passages of the host, which has been exposed to various environmental sources. Similarly, studies on ticks have revealed the presence of bacteria truly associated with *R. microplus* that came from environmental origin [2].

Chitinolytic activity of the isolates was used as a screening test to explore the potential of the bacteria to control *O. ovis* larvae. Three bacteria species (*Pantoea gaviniae*, *Aeromonas veronii*, and *Serratia marcescens*) caused 100% *in vitro* mortality rate in L2 while *Pantoea calida* caused 35% in L3 larvae. *In vitro* mortality rate studies are scarce since oestrid larvae are impossible to grow under laboratory conditions so far. From epidemiological data published [1], it was calculated that *in vivo* L2 mortality is about 7%. It has been reported the *in vitro* 11.1% mortality rate in L3 larvae [10]. These figures match well with the *in vivo* mortality rate 7% in *Hypoderma lineatum* L3 larvae reported [29], and bacteria control may be also important to enhance oestrid larval *in vitro* cultures [11].Likewise, it has been reported that chitinase-producing bacteria strains exerted strong insecticidal toxicity that were useful in biocontrol of agriculturally important pests [16, 22]. The selected chitinolytic isolates (n=6) were identified as *Pantoea calida*, *P. gaviniae, Serratia marcescens* and *Aeromonas veronii*. Similarly, *S. marcescens* and *A. hydrophila* have been reported as chitinase producers [17, 28], while the genus Pantoea has been associated to ticks, lice and fleas [21]. Similarly, *P. gaviniae* was found in *O. ovis* L3 dead larvae only. Moreover, *P. calida* was found in infected sinusal mucosae and also in healthy and dead *O. ovis* L3 larvae, but not in not infected goat mucosae, suggesting that *P. calida* and *P. gaviniae* might be associated to L3 larvae but not to goat mucosae. *Aeromonas veronii* was found in not infected and infected mucosae as well as in dead larvae, but not in healthy larvae. This fact suggests that this bacteria is a specific resident microorganism of the goat mucosa and a pathogen for the larvae. Thus, it would feasible to hypothesize that *A. veronii* has a natural role as biological control agent of the *O. ovis* larval populations into the host. Our results add

evidence about the potential larvicidal effect of *A. veronii* on *O. ovis* larvae. In addition, since *A. veronii* showed potent chitinolytic activity, it may be associated with *O. ovis* larvae mortality, as it has been observed for myxozoan parasites [17]. However, *A. veronii* has not been recommended as a potential biological control agent because of human and animal health concerns associated to this bacterium. In contrast, *S. marcescens* and its chitinolytic enzymes are effective as biological control of *Varroa destructor* (a parasite of honey bees) and Anopheles mosquitoes [27, 28]. Also, *S. marcescens* has been used as a specific biological control agent of parasite-transmitting insects [27], protozoan parasites [13, 20] and nematodes [5, 26]. In addition, there seems to exist a relationship between *O. ovis* L2 larvae mortality and the highest chytinolotic activity patterns of the bacteria. Therefore, the larvicidal potential of these bacteria merits further investigation. Studies in other biological systems have revealed the complexity of such interactions that open the opportunity to develop novel therapeutic approaches for *O. ovis* biological control. In the present study, some bacterial isolates demonstrated to be larvicidal for *O. ovis* larvae, and these bacteria may be used for further control approaches. Finally, analyzing the taxonomic composition of the cultured bacteria may facilitate the understanding of phylogenetic and evolutionary biology traits of the *O. ovis*-host relationships.

To conclude, this is the first report on the identification of bacterial species associated to the sheep nasal bot fly *O. ovis* and their potential effects to cause mortality in parasitic larvae. *A. veronii* was a common bacterium found in the nasosinusal mucosae of goats, while *P. calida*, *S. hominis*, *Terribacillus* sp. and *Bacillus sonorensis* were common residents in healthy and dead *O. ovis* larvae. Among the obtained isolates, *P. calida*, *P. gaviniae* and *S. marcescens* deserves further confirmation of their larvicidal effects on *O. ovis* larvae throughout *in vivo* experiments. Moreover, this study also highlighted the potential role of the sheep nasal bot fly as a reservoir or vector of some zoonotic bacteria. To date, the microbiome of oestrid species causing myiasis remains largely unexplored. This contribution to understanding the larvicidal bacteria-*O. ovis* association may open new control approaches of biological control.

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