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Proceeding Paper

Isolation and Identification of Culturable Gut Microbiota in the Larval Stage of Lesser Mealworm (*Alphitobius diaperinus*) †

Gisele Ivonne Antonuccio [1,2,*] and Diego Herman Sauka [1,3]

[1] Instituto Nacional de Tecnología Agropecuaria (INTA), Instituto de Microbiología y Zoología Agrícola (IMYZA), Hurlingham, Buenos Aires, Argentina; email1@email.com
[2] Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Buenos Aires, Argentina
[3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
* Correspondence: antonuccio.gisele@inta.gob.ar
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Abstract: The highly prevalent pest *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) causes significant structural damage in poultry farms. Despite previous investigations on its carriage of pathogenic microorganisms, our understanding of its microbiome remains limited. This study aimed to analyze the diversity of culturable gut microbiota in *A. diaperinus* obtained from laboratory breeding. Fifteen seventh instar larvae underwent a 24-h starvation period, followed by surface disinfection. Dissected midguts were homogenized and plated on nutrient agar (NA), brain heart infusion agar (BHI), and Bacillus cereus agar (BC). The cultured isolates were subjected to gram staining, phylogenetic analysis, biochemical property evaluation, and metabolic activity assessment. Bacterial counts were higher in BHI (2.51×10^5 CFU/gut) than in NA (2.25×10^5 CFU/gut), possibly due to nutrient richness. NA exhibited a dominant colony morphology of gram-negative bacilli, while BHI displayed additional distinct colonies of gram-positive cocci. Surprisingly, yeast-like colonies were observed on BC plates. Based on 16S rRNA gene sequences, eight bacterial isolates were identified as *Enterobacter* sp., and two as *Staphylococcus* sp. Using RNA gene ITS region sequences, two yeast isolates were identified as *Debaryomyces* sp. and *Hyphopichia* sp. A preliminary species-level identification of bacteria (*Enterobacter cloacae*, *Staphylococcus gallinarum*, and *Staphylococcus succinus*) was achieved using API systems and complementary biochemical tests. Discrepancies between phylogenetic analysis and phenotypic data suggest the potential existence of new species or subspecies. Further comprehensive studies are required to confirm this hypothesis.

Keywords: *Alphitobius diaperinus*; gut microbiota; culturable microorganisms; bacterial diversity; yeast

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1. Introduction

Alphitobius diaperinus, commonly known as the lesser mealworm, is an insect species classified under the order Coleoptera and the family Tenebrionidae. This species, originally described by Panzer in 1797, has its origins in the African continent but has since achieved a widespread global distribution. In addition to its significance in research as a potential protein source for humans [1,2] and its role in exploring environmentally friendly waste disposal solutions [3,4], *A. diaperinus* stands out as a notorious pest within poultry environments.

Within the context of poultry farming, *A. diaperinus* presents a significant challenge due to the substantial damage inflicted by both its larvae and adult individuals [5]. These pests can cause extensive harm to poultry facilities, affecting not only the well-being of the birds but also the economic viability of poultry production operations. Consequently,

effective pest control strategies are essential to mitigate the negative impact of *A. diaperinus* on the poultry industry.

Although previous investigations have explored the potential carriage of pathogenic microorganisms by *A. diaperinus*, our understanding of its microbiome remains limited. This knowledge gap is substantial because the insect's gut microbiota can play a crucial role in various aspects of its biology and ecology. Therefore, this study aims to examine the diversity of culturable gut microbiota present in *A. diaperinus* specimens obtained from laboratory rearing.

By shedding light on the microbiome of *A. diaperinus*, we aspire to contribute to a better understanding of the biology of this pest and identify potential vulnerabilities that can be targeted for more effective pest management in poultry facilities. Additionally, this research may have broader implications for pest control strategies and could potentially lead to more sustainable and environmentally friendly solutions for managing *A. diaperinus* infestations in various agricultural settings.

2. Materials and Methods

2.1. Isolation and Count of Culturable Bacteria and Yeast from the Gut of A. diaperinus

The Instituto de Microbiología y Zoología Agrícola (IMYZA) at the Instituto Nacional de Tecnología Agropecuaria (INTA) in Hurlingham, Buenos Aires, Argentina, has established a unique laboratory rearing system for *A. diaperinus* for research purposes, which, to the best of our knowledge, is the only one of its kind in the country.

Fifteen seventh-instar larvae were subjected to a 24-h starvation period and then underwent surface disinfection. This disinfection procedure involved a series of steps, including immersion in 70% ethanol, exposure to a 15% bleach solution, and three rinses in physiological solution, following the protocol outlined by Fang Lu et al. [6]. The third rinse served as a control before proceeding with the dissection of the midguts. These 15 midguts were kept hydrated in physiological solution and subsequently homogenized in a sterile mortar. The resulting midgut suspension underwent serial dilution. We inoculated 100 µL of four dilutions (ranging from 10^{-3} to 10^{-6}), each in quintuplicate, on nutrient agar (NA) and brain heart infusion agar (BHI). Additionally, 100 µL of the undiluted midgut suspension was inoculated in triplicate on Bacillus cereus agar (BC). The plates were then incubated for 72 h at 28 °C. Selections of colonies displaying distinct morphologies were isolated from the various culture media until pure cultures were obtained, facilitating subsequent identification processes. The cultured isolates underwent plate counting of the colonies to estimate the colony forming units (CFU) per gut, followed by Gram staining.

2.2. Bacterial and Yeast Identification

The isolated bacteria were identified at the genus level by amplifying and subsequently sequencing the 16S rRNA gene using primers as described by Weisburg et al. [7]. For yeast identification, we amplified and sequenced a fragment comprising the internal transcribed spacer (ITS) 1, the 5.8S rRNA gene and ITS 2, along with a segment of the large subunit rRNA gene, utilizing primers following the protocol outlined by White et al. [8].

The PCR products underwent sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit and were subsequently analyzed on the ABI PRISM 3100 Genetic Analyzer Sequencer (Applied Biosystems). Sequence alignment was carried out using the BLASTN algorithm (version 2.0; National Center for Biotechnology Information) and compared against sequences from reference strains available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic analyses were performed using MEGA11.

Following this initial identification, the bacterial isolates were further identified to the species level by employing the API 20E, API STAPH, and/or API 20A systems

(bioMérieux) as per the manufacturer's instructions, in addition to other conventional biochemical tests.

3. Results and Discussion

In this study, our initial analysis involved counting colony-forming units, which revealed higher bacterial counts in BHI (2.51×10^5 CFU/gut) compared to NA (2.25×10^5 CFU/gut), possibly due to the greater nutrient richness in the former. Subsequently, we subjected a selected group of microbial isolates to a comprehensive series of analyses, encompassing gram staining, phylogenetic analysis, evaluation of biochemical properties, and assessment of metabolic activity.

Gram staining yielded intriguing results, leading to the isolation of a total of eight gram-negative bacilli (Figure 1): four from NA (designated as INTA AN1-1, INTA AN1-5, INTA AN1-10, and INTA AN1-15) and an additional four from BHI (referred to as INTA AC1-3, INTA AC1-6, INTA AC1-9, and INTA AC1-14). These bacilli were identified as dominant members of the gut microbiota in seventh stage *A. diaperinus* larvae. Furthermore, we observed two distinct colonies of gram-positive cocci from BHI (named INTA AC1-4 and INTA AC1-8) (Figure 1). An unexpected finding in our assay was the isolation of two yeasts from macerated intestines inoculated directly, undiluted, on BC, a medium typically used for the isolation of gram-positive bacteria belonging to the *Bacillus cereus* group. These yeasts stained as gram-positive, with one presenting pseudohyphae and the other not (referred to as INTA AB1-1 and INTA AB1-4, respectively) (Figure 1).

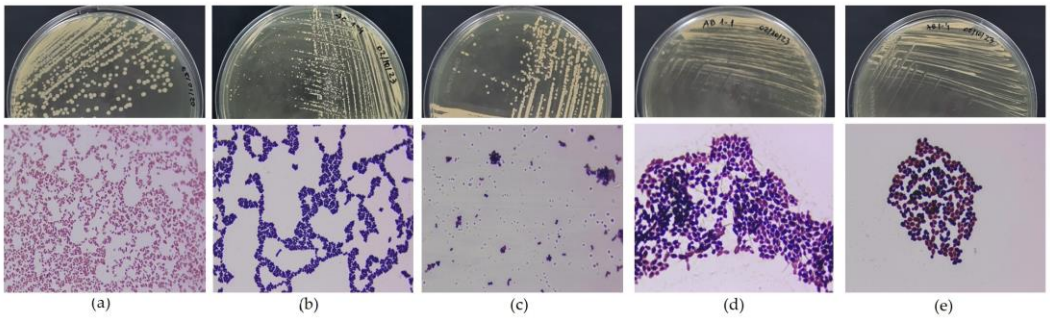

Figure 1. Pure cultures of the isolates were grown in NA medium and incubated for 24 h at 29 °C. Gram staining was performed and observed under a 1000x magnification immersion lens, revealing the following isolates: INTA AN1-1 (**a**), INTA AC1-4 (**b**), INTA AC1-8 (**c**), INTA AB1-1 (**d**), and INTA AB1-4 (**e**). It's important to note that only one representative gram-negative isolate is included in the figure for clarity.

Our phylogenetic analysis enabled us to determine the genera of each of the microbial isolates and construct phylogenetic trees with the most closely related species within each genus (Figure 2). Based on 16S rRNA gene sequencing, the eight gram-negative bacilli were classified as *Enterobacter* sp., closely related to *E. hormaechei* subsp. *hormaechei*. The two gram-positive cocci were identified as *Staphylococcus* spp., one closely related to *S. hominis* subsp. *hominis* and *S. hominis* subsp. *novobiosepticus*, and the other to *S. succinus* subsp. *casei* and *S. succinus* subsp. *succinus*, respectively (Figure 2).

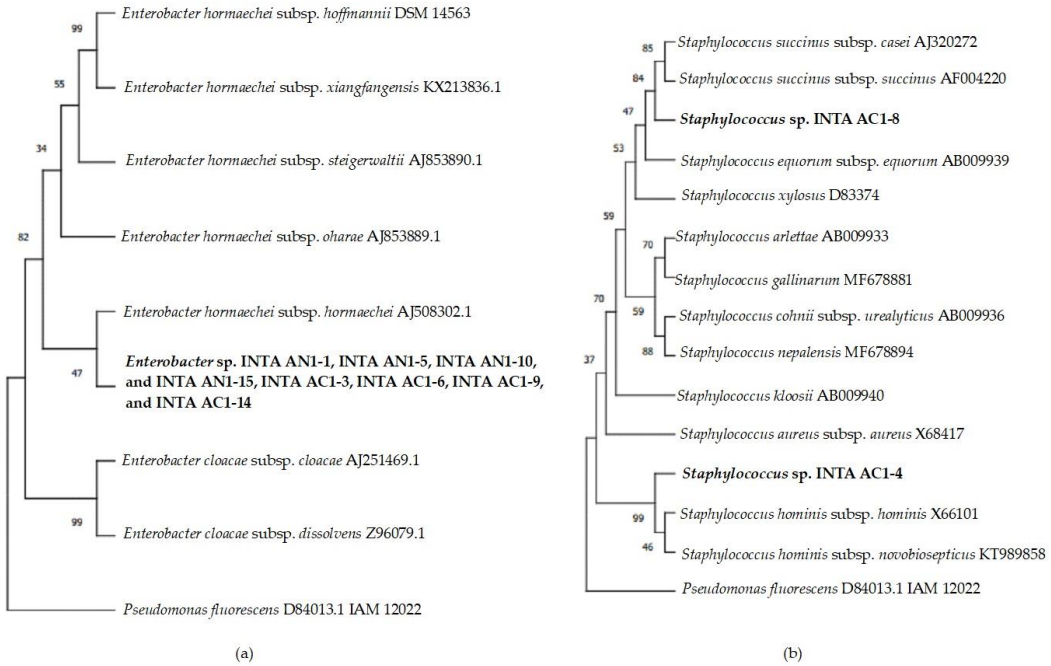

Figure 2. The phylogenetic tree of 16S rRNA sequences illustrates the relationships among the gram-negative isolates and type strains of *Enterobacter* species (**a**), as well as among the two gram-positive isolates and type strains of *Staphylococcus* species (**b**). The tree was constructed using the neighbor-joining method. The numbers displayed at specific nodes indicate consensus bootstrap values based on 1000 replications.

The resulting 16S rRNA gene sequences have been deposited in the GenBank database (Table 1).

Table 1. Nucleotide lengths and GenBank accession numbers of sequences obtained from selected gut isolates of *Alphitobius diaperinus.*

Isolate	Sequenced Gene/Genes	Nucleotide Length (bp)	GenBank Accession Number
INTA AN 1-1	16S rRNA	1401	OP339834.1
INTA AN 1-5	16S rRNA	1369	OP346784.1
INTA AN 1-10	16S rRNA	1396	OP346981.1
INTA AN 1-15	16S rRNA	1397	OP347118.1
INTA AC 1-3	16S rRNA	1395	OP348220.1
INTA AC 1-6	16S rRNA	1396	OP348874.1
INTA AC 1-9	16S rRNA	1399	OP348886.1
INTA AC 1-14	16S rRNA	1369	OP351273.1
INTA AC 1-4	16S rRNA	1417	OP348929.1
INTA AC 1-8	16S rRNA	967	OP348932.1
INTA AB 1-1	rRNA genes ITS region	446	OP348991.1
INTA AB 1-4	rRNA genes ITS region	612	OP348992.1

Furthermore, based on rRNA gene ITS region sequencing, we classified the two isolated yeasts as *Debaryomyces* sp. and *Hyphopichia* sp., with the latter closely related to *Hyphopichia burtonii* (Figure 3). These sequences have also been deposited in the GenBank database (Table 1).

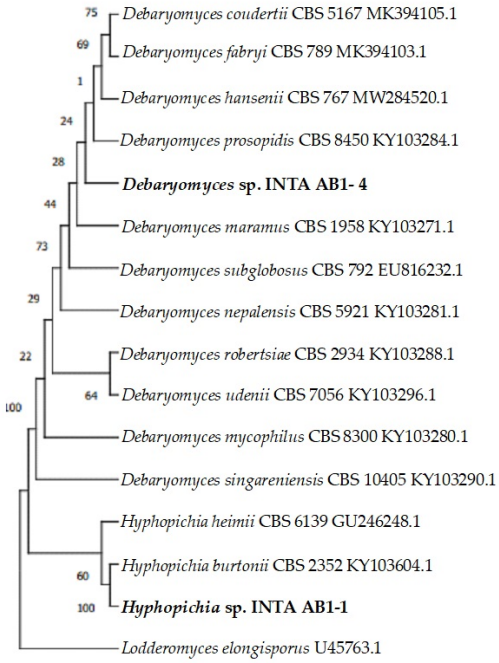

Figure 3. The phylogenetic tree of rRNA gene ITS region sequences illustrates the relationships among the yeast isolates and the type strains of *Debaryomyces* and *Hyphopichia* species. The tree was constructed using the neighbor-joining method, and the numbers shown at specific nodes represent consensus bootstrap values derived from 1000 replications.

In addition, we conducted a preliminary species-level identification of bacteria using API systems and complementary biochemical tests. The isolates INTA AN1-1, INTA AN1-5, INTA AN1-10, INTA AN1-15, INTAAC1-3, INTA AC1-6, INTA AC1-9 and INTA AC1-14 were identified as *Enterobacter cloacae*, INTA AC1-4 as *Staphylococcus gallinarum*, and INTA AC1-8 as *S. succinus*.

Our findings diverged from previous reports in recent years on the bacterial and fungal diversity in the gut of polystyrene fed *A. diaperinus* population [4]. These discrepancies in microorganism genera may be attributed to differences in larval diet. While our laboratory breeding relies on feed for baby chicks, *A. diaperinus* colonies in other studies were exposed to plastic compounds and microorganisms potentially involved in plastic degradation.

A separate Italian master's thesis on the microbiological aspects and chemical composition of *A. diaperinus*, *Tenebrio molitor*, and *Zophobas morio* larvae intended for human consumption supported our findings regarding the presence of Enterobacteriaceae and *Staphylococcus* spp. in the total mesophilic bacterial load, along with enterococci and lactic acid bacteria, all ranging between 5 and 7 log CFU/g [9].

Furthermore, microbial dynamics during an industrial production cycle of *A. diaperinus* intended for human consumption were characterized [10]. While bacterial diversity decreased during rearing, the number of aerobic endospores remained at 4.0 log CFU/g. Coagulase-positive staphylococci were not detected, but fungal isolates from the genera *Aspergillus* and *Fusarium* were recovered.

Our study underscores the potential influence of rearing environments, procedures, hygiene measures, and insect feed on insect microbiota.

The disparities between phylogenetic analysis and phenotypic data for certain isolates suggest the possible existence of new species or subspecies. Further comprehensive studies, including the sequencing of entire genomes for the three kinds of bacteria isolated from the gut microbiota of *A. diaperinus*, are warranted. Bioinformatics analysis currently underway will provide deeper insights into this ecological niche, potentially enhancing insecticidal strategies against beetles with significant poultry impact.

In conclusion, this study sheds light on the diverse microbiota of *A. diaperinus*, revealing potential implications for insect pest management and offering avenues for future research into insect-microbe interactions.

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