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# اسب تروآ در کنترل زیستی؟ به چالش کشیدن خطر گسترش باکتری‌های فرصت‌طلب از طریق نماتدهای بیمارگر حشرات وارداتی

# Trojan Horse in Biocontrol? Challenging the Risk of Spreading Opportunistic Bacteria via Imported Entomopathogenic Nematodes

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## چکیده

Entomopathogenic nematodes (EPNs) are widely used as biocontrol agents, yet their non-symbiotic bacterial communities which may pose underexplored biosafety risks remain poorly characterized. This study isolated and phylogenetically identified bacteria associated with infective juveniles of native EPN species (*Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, and *Steinernema feltiae*) collected from northwestern Iran. Our analysis, based on 16S-rDNA revealed a community of clinically significant bacteria. Isolates were identified within genera known for their roles as opportunistic pathogens, including *Enterobacter* (clustering with *E. huaxiensis*), *Citrobacter* (closely related to *C. braaki*), *Morganella* (grouping with *M. morganii*), *Staphylococcus* (showing high similarity to *S. succinus*), and *Acinetobacter* (phylogenetically proximate to *A. schindleri*). The presence of *Acinetobacter* is of particular concern due to the genus's global notoriety for multidrug resistance and environmental persistence. These findings demonstrate that native EPN populations can harbor bacteria with documented associations to nosocomial infections and livestock diseases. Consequently, the expanding international trade of EPN-based biocontrol products creates a potential pathway for the cross-border dissemination of such opportunistic pathogens. Our results underscore the urgent need for enhanced regulatory oversight, harmonized international biosafety standards, and the implementation of culture-independent screening protocols to mitigate unintended public health risks associated with these otherwise beneficial biological control agents.

**Keywords:** *Acinetobacter schindleri*, *Citrobacter freundii, Enterobacter huaxiensis, Morganella morganii, Staphylococcus succinus*

## Abstract

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**خلاصه**

نماتدهای بیمارگر حشرات به طور گسترده به عنوان عوامل کنترل زیستی آفات مورد استفاده قرار می‌گیرند، با این حال این‌که چگونه اجتماعات باکتریایی غیرهمزیست آن‌ها ممکن است خطرات ایمنی‌زیستی را به همراه داشته باشند، هنوز به خوبی شناسایی نشده است. در این مطالعه، باکتری‌های همراه با مرحله لارو عفونت زا (Infective Juvenile) گونه‌های بومی نماتدهای بیمارگر حشرات (شامل *Heterorhabditis bacteriophora*، *Steinernema carpocapsae* و *Steinernema feltiae*) که از شمال‌غرب ایران جمع‌آوری شده بودند، جداسازی و بر اساس تبارزایی مبتنی بر داده‌های مولکولی شناسایی شدند. بررسی ماکه مبتنی بر توالی ناحیه s-rDNA ۱۶ بود، اجتماعاتی از باکتری‌های دارای اهمیت بالینی را آشکار ساخت. جدایه‌های شناسایی‌شده درون جنس‌های متعلق به پاتوژن‌های فرصت‌طلب *Enterobacter* (هم گروه با *E. huaxiensis*)، *Citrobacter* (خویشاوند با *C. braaki*)، *Morganella* (هم گروه با *M. morganii*)، *Staphylococcus* (با شباهت زیاد به *S. succinus*)، و *Acinetobacter* از نظر تبارزایی نزدیک به *A. schindleri*. حضور جنس *Acinetobacter* به دلیل شهرت جهانی این جنس در زمینه مقاومت چندداروی و پایایی در محیط زیست، نگرانی خاصی ایجاد می‌کند. این یافته‌ها نشان می‌دهند که جمعیت‌های نماتدهای بیمارگر حشرات می‌توانند حامل باکتری‌هایی باشند که ارتباطات مستندی با عفونت‌های بیمارستانی و بیماری‌های دامی دارند. در نتیجه، تجارت رو به گسترش محصولات کنترل زیستی مبتنی بر-EPNها، مسیری بالقوه برای انتشار چنین بیمارگرهای فرصت‌طلبی ایجاد می‌کند. نتایج ما بر ضرورت تقویت فرایندهای نظارتی، هماهنگ‌سازی استانداردهای بین‌المللی ایمنی‌زیستی و اجرای دستورالعمل های غربالگری مستقل از کشت (culture-independent) برای کاهش خطرات پیش‌بینی‌نشده سلامت عمومی مرتبط با این عوامل کنترل زیستی، تأکید دارد.

**کلمات کلیدی**: *Acinetobacter schindleri*، *Citrobacter freundii*، *Enterobacter huaxiensis*، *Morganella morganii*، *Staphylococcus succinus*

## Introduction

**مقدمه**

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae (Poinar, 1976) and Steinernematidae (Travassos, 1927) are obligate insect parasites that have gained widespread use as effective biocontrol agents against a broad spectrum of insect pests (Kaya & Gaugler, 1993). Their remarkable pathogenicity is fundamentally rooted in mutualistic symbioses with entomopathogenic bacteria of the genera *Photorhabdus* and *Xenorhabdus* (Symbionts of Heterorhabditidae Steinernematidae, respectively) (Boemare et al., 1993; Thomas & Poinar, 1979). These primary bacterial symbionts are indispensable for nematode reproduction, playing a multifaceted role in killing the insect host by overcoming its immune system, creating a nutrient-rich environment through bioconversion and protecting the cadaver from microbial competitors via the production of a diverse arsenal of antimicrobial compounds (Akhurst & Dunphy, 1993; Akhurst, 1982).

For decades, research focused predominantly on this binary partnership between EPNs and their symbionts. However, it is now unequivocally recognized that EPNs, conceptualized as holobionts, harbor a more complex microbial community that extends beyond their primary symbionts. A diverse array of non-symbiotic bacteria can be isolated from both infected insect cadavers and from within or on the surface of surface-sterilized infective juveniles (IJs). Early culture-dependent studies provided the first glimpses of this diversity, reporting associations with bacteria such as *Alcaligenes* spp., *Pseudomonas aureofaciens*, *P. fluorescens*, *Enterobacter agglomerans* and *Serratia liquefaciens* from *S. carpocapsae* (Gouge & Snyder, 2006; Lysenko & Weiser, 1974). Subsequent work isolated *Ochrobactrum anthropi*, *Paracoccus denitrificans* and *P. maltophilia* from *Steinernema scapterisci*, *Heterorhabditis indica* and *H. bacteriophora* (Aguillera, 1993; Babic et al., 2000). More recent investigations have continued to identify a variety of non-symbionts, including *Flavobacterium* spp., *Providencia vermicola*, *P. rettgeri*, *C. freundii*, *Staphylococcus succinus* and *A. faecalis* from species like *S. abbasi*, *S. feltiae* and *Rhabditis blumi* (Eivazian Kary & Alizadeh, 2016; Park et al., 2011; Sharifi & Eivazian Kary, 2016; Somvanshi et al., 2006).




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Furthermore, non-symbiotic bacteria including *Flavobacterium* spp., *Ochrobactrum cytisi* and *Schineria larvae* have been identified in EPN-infected cadavers (Razia et al., 2011).

The advent of molecular tools and high-throughput sequencing has revolutionized our understanding, revealing that EPNs possess a characteristic core microbiota comprising bacterial taxa that are consistently associated with them, alongside a variable microbiota that is more fluid and influenced by host genotype, diet, and environment (Gulcu et al., 2017; Ogier et al., 2020). This refined perspective is critical, as it shifts the focus from random, transient contaminants to stable, ecologically significant associations. Of paramount concern is the finding that this core microbiota frequently includes bacterial genera with well-established roles as opportunistic pathogens in clinical and veterinary settings (Gulcu et al., 2017). For instance, the genus *Enterobacter* contains numerous clinically relevant species, with *E. cloacae* and *E. aerogenes* frequently implicated in hospital-acquired infections including urinary tract infections, pneumonia and neonatal sepsis (Mezzatesta et al., 2012). In agricultural contexts, these organisms are associated with bovine mastitis and poultry infections (Bradley & Green, 2000), suggesting potential zoonotic transmission routes. Similarly concerning are *Citrobacter* isolates, with *C. freundii* recognized as an emerging nosocomial pathogen capable of causing meningitis in neonates and septicemia in immunocompromised patients (Plakkal et al., 2013). The genus *Enterobacter* demonstrates remarkable environmental persistence and frequently exhibits multidrug resistance (Liu et al., 2018). *Morganella* isolates present additional challenges, as *M. morganii* is increasingly recognized as a cause of complicated urinary tract infections and bacteremia which often displaying resistance to multiple antibiotic classes (Kara et al., 2024), and its presence in poultry production systems creates potential transmission bridges (Paudel et al., 2022). *Staphylococcus* isolates represent potential risks given the capacity for horizontal gene transfer of virulence factors among staphylococcal species (Tong et al., 2015). Perhaps most alarming is the association with *Acinetobacter* species, which have emerged as formidable opportunistic pathogens. The *A. calcoaceticus-baumannii* complex, particularly *A. baumannii*, causes severe hospital-acquired infections including pneumonia and bacteremia, often exhibiting multidrug resistance (Antunes et al., 2014). In animals, these bacteria are associated with mastitis in cattle, septicemia in poultry, and urinary tract infections in companion animals (Pomba et al., 2017), and their ability to persist in diverse environments and develop antibiotic resistance makes them particularly concerning (Berendonk et al., 2015).

The potential for EPNs, especially those produced in masse and traded internationally for biocontrol, to act as vectors for the dispersal of such bacteria is a significant and understudied biosafety consideration. The expanding international trade of EPN-based biocontrol products creates a potential pathway for the cross-border dissemination of these opportunistic pathogens through commercial shipments. While current quality control measures in EPN production typically screen for known symbionts, routine testing for opportunistic pathogens is not standard practice. This regulatory gap is particularly worrying given that these bacteria may persist as surface contaminants on the infective juvenile (IJ) cuticle, protected by biofilms or establish transient associations within the nematode digestive tract.

A critical first step in risk assessment is accurate genus- and species-level identification of cultured isolates. While multi-locus sequence analysis (MLSA) provides superior resolution for closely related taxa, a robust phylogenetic analysis based on the 16S ribosomal RNA (rRNA) gene remains a powerful and entirely appropriate tool for the initial taxonomic placement of bacterial isolates and for identifying potential pathogenic relatives, particularly when the goal is a broad survey of diversity (Janda & Abbott, 2007; Johnson et al., 2019). The 16S rRNA gene has proven highly effective in flagging clinically relevant bacteria in environmental studies and is standard for determining phylogenetic relationships at genus level (Shin et al., 2015). Its utility is well-established in preliminary biosafety screenings in order to determine whether an isolate falls within a clade containing known pathogens, thereby prioritizing it for further, more detailed genomic characterization.

This study aimed to conduct an initial phylogenetic survey and biosafety risk assessment of the culturable, non-symbiotic bacteria associated with IJ of three EPN species from northwestern Iran. We employed phylogenetic analysis of the 16S rDNA gene as a validated first-tier approach to achieve robust genus-level identification and to determine the phylogenetic proximity of our isolates to clinically significant species. This provides the essential foundational data to evaluate the potential biosecurity implications of these associations and to identify specific isolates that warrant future in-depth genomic and phenotypic analysis.

## Materials and Methods

## مواد و روش‌ها

**Nematode Origin, Multiplication, and Storage:** Infective juveniles (IJs) of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, and *Steinernema feltiae* were sourced from Insect Pathology laboratory culture bank, where they are maintained as live populations. These populations were originally isolated from soil samples collected from agricultural and natural ecosystems in the East Azerbaijan province of northwestern Iran.

For this study, to obtain fresh, physiologically standardized IJs for bacterial isolation, the nematodes were subjected to a single cycle of in vivo multiplication. Briefly, last-instar larvae of the greater wax moth, *Galleria mellonella*, were exposed to





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IJs at a rate of approximately 50 IJs per larva in 6-cm petri dishes lined with moist filter paper. The infection process was allowed to proceed at 25°C in the dark. Upon mortality, the infected cadavers were aseptically transferred to modified White traps and maintained at 25°C to collect the next generation of IJs.

Crucially, only IJs that emerged within the first 48 hours of the emergence window were collected for this study. These newly emerged IJs were rinsed with sterile distilled water and stored in sterile tissue culture flasks containing sterile distilled water at 14°C. The storage period did not exceed 7 days to minimize any stress-induced shifts in the associated microbiota. Immediately prior to bacterial isolation, IJ viability was microscopically assessed and confirmed to be greater than 95% based on spontaneous movement and response to gentle probing.

**Surface Sterilization of IJs and Isolation of Associated Bacteria:** A critical, multi-step surface sterilization protocol was employed to ensure that the isolated bacteria originated from within the nematode body or were so firmly attached that they survived the disinfecting process, rather than being superficial contaminants. Approximately 1,000 IJs per sample were transferred to a 1.5 ml microcentrifuge tube and rinsed three times with 1 ml of sterile 0.9% (w/v) sodium chloride solution to remove adherent soil particles and debris (Dougherty & Calhoun, 1948). After the final wash, the IJs were treated with 1 ml of a 0.1% (w/v) aqueous solution of merthiolate (thiomersal) for 45 minutes with gentle agitation on a rotary shaker. The merthiolate solution was carefully removed, and the IJs were rinsed three times with 1 ml of sterile saline to thoroughly eliminate any residual disinfectant. To empirically confirm the efficacy of the surface sterilization process, 100 µl of the final rinse water was plated onto Nutrient Agar (NA) at petri dishes. The plates were incubated at 30°C for 72 hours and monitored for microbial growth. Only batches for which the final rinse plates showed no bacterial growth were processed further. This step is essential to validate that any subsequent bacterial growth originated from inside the sterilized IJs. Following successful sterility checks, the surface-sterilized IJs were transferred to a new sterile microcentrifuge tube and mechanically crushed using a sterile plastic pestle in 100 µl of sterile saline. The resulting homogenate was serially diluted and streaked onto Nutrient Bromothymol Blue-Triphenyltetrazolium Chloride (NBTA) agar plates. Plates were incubated at 30°C for 48–72 h, and distinct bacterial colonies were selected based on morphology and purified for molecular identification.

**DNA Extraction, PCR Amplification, and Sequencing:** Genomic DNA was extracted from pure bacterial colonies using a freeze-thaw lysis method (Chen et al., 2020). A single colony was suspended in 100 µl of lysis buffer (0.1 M Tris-HCl, 0.1 M KCl, 20 mM EDTA-$Na_2$, pH 8.0). The suspension was subjected to a freeze-thaw cycle involving incubation at –20°C for 10 minutes followed by heat treatment at 95°C for 10 minutes. Cellular debris was pelleted by centrifugation at 10,000 × g for 5 minutes, and the supernatant containing the DNA template was carefully collected.

The 16S ribosomal DNA (rDNA) gene was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) . The PCR reactions were performed in a final volume of 25 µl, containing 12.5 µl of 2X Master Mix (including Taq DNA polymerase, dNTPs, MgCl2), 1 µl of each primer (10 µM), 3 µl of DNA template, and 7.5 µl of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s; with a final extension at 72°C for 7 min. PCR reactions were performed in quadruplicate for each isolate to ensure reproducibility. The amplified products were visualized on a 1% agarose gel, purified, and sent for Sanger sequencing in both directions (Biomagic Co., South Korea).

**Sequence Analysis and Phylogenetic Reconstruction:** Raw sequence chromatograms were assembled, quality-checked, and trimmed using ChromasPro software (Technelysium Pty Ltd). Consensus sequences were generated in BioEdit v7.2.5. Taxonomic affiliation was preliminarily determined by comparing the sequences against the NCBI GenBank non-redundant database using the BLASTn algorithm. For phylogenetic analysis, homologous sequences of the closest type strains and related species were retrieved from GenBank. Multiple sequence alignments were constructed using Clustal X. A Maximum Parsimony (MP) tree was inferred using MEGA X with 1,000 bootstrap replicates to assess the statistical support of the tree nodes. Genetic distances among isolates were calculated using the Maximum Composite Likelihood model in MEGA X.

**Data Availability:** The 16S rDNA sequences obtained in this study were deposited in the NCBI GenBank database under the accession numbers PV566946 (*Morganella* sp.), PV565725 (*Citrobacter* sp.), PV562236 (*Morganella* sp.), PV565728 (*Staphylococcus* sp.), PV562236 (*Morganella* sp.) and PV562217 (*Morganella* sp.).

## Results and Discussion

## نتایج و بحث

### Phylogenetic Analysis of Bacterial Isolates

Bacterial isolation from surface-sterilized IJs of *H. bacteriophora*, *S. carpocapsae*, and *S. feltiae* yielded a collection of isolates, which were phylogenetically identified based on 16S rDNA gene sequences. The analysis revealed a range of bacterial genera with clinical significance, as detailed below.





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**Phylogenetic Placement of *Citrobacter* (Braak 1928) Isolates**

The Maximum Parsimony (MP) phylogenetic reconstruction of *Citrobacter* isolates delineated clear evolutionary relationships. The studied isolates, *Citrobacter* sp. Azaruniv12 SF and *Citrobacter* sp. Azaruniv5 HB, formed a well-supported monophyletic clade with *Citrobacter braakii* (Brenner et al. 1993). This clade subsequently grouped with *C. freundii (Braak 1928) Werkman and Gillen 1932*, demonstrating a close phylogenetic affiliation with these species. A separate, distinct clade comprised *C. murliniae* and *C. portucalensis*, indicating clear separation from the isolates identified in this study (Fig. 1).

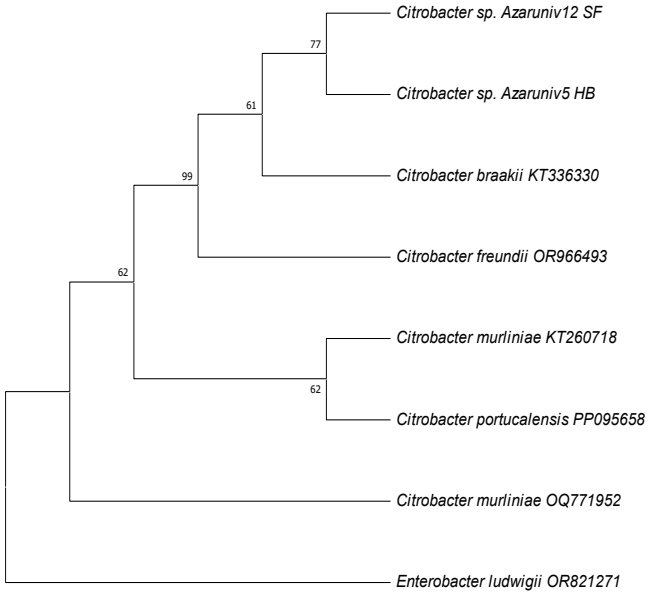


**Figure 1.** Phylogenetic analysis of *Citrobacter* isolates (Azaruniv12 SF and Azaruniv5 HB) and closely related strains based on 16S rRNA gene sequences, with *Enterobacter ludwigii* as the outgroup. Bootstrap support values (based on 1000 replicates) are shown at nodes.

**Phylogenetic Affiliation of *Acinetobacter* Isolate**

Phylogenetic analysis positioned the *Acinetobacter* sp. Azaruniv11 SF isolate within a highly supported clade (bootstrap value 100%) alongside *A. schindleri* strains (KT261413, AJ278311). The *A. schindleri*-Azaruniv11 SF cluster was resolved as a distinct lineage within a larger group containing *A. bouvetii*, *A. johnsonii*, *A. gyllenbergii* and *A. haemolyticus*, from which it was clearly separated (Fig. 2).

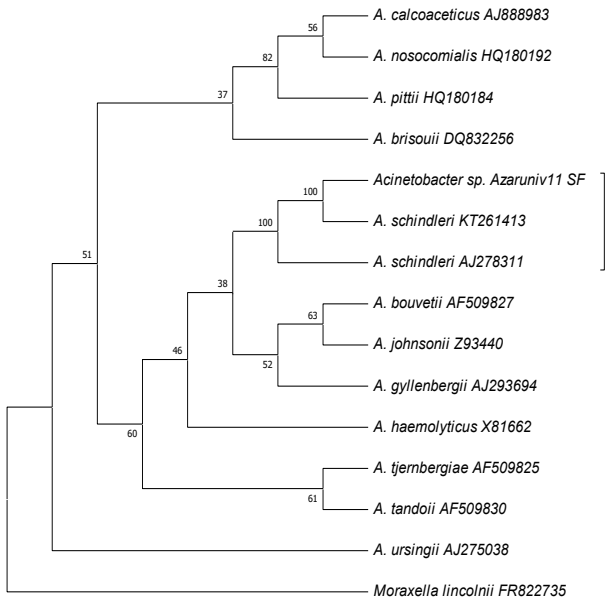


**Figure 2.** Phylogenetic analysis of *Acinetobacter* sp. isolates Azaruniv11 SF and closely related strains based on 16S rRNA gene sequences, with *Moraxella lincolnii* as the outgroup. Bootstrap support values (based on 1000 replicates) are shown at nodes.




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**Phylogenetic Identification of *Morganella* Isolates**

The MP tree confirmed that the *Morganella* isolates from this study form a monophyletic group with known *M. morganii* strains, robustly supporting their classification within this species. A hierarchical clustering pattern was observed, with isolates Azaruniv9 SC and Azaruniv15 SF forming a tight sub-clade that grouped with Azaruniv3 HB, suggesting potential microdiversity among the studied isolates (Fig. 3).

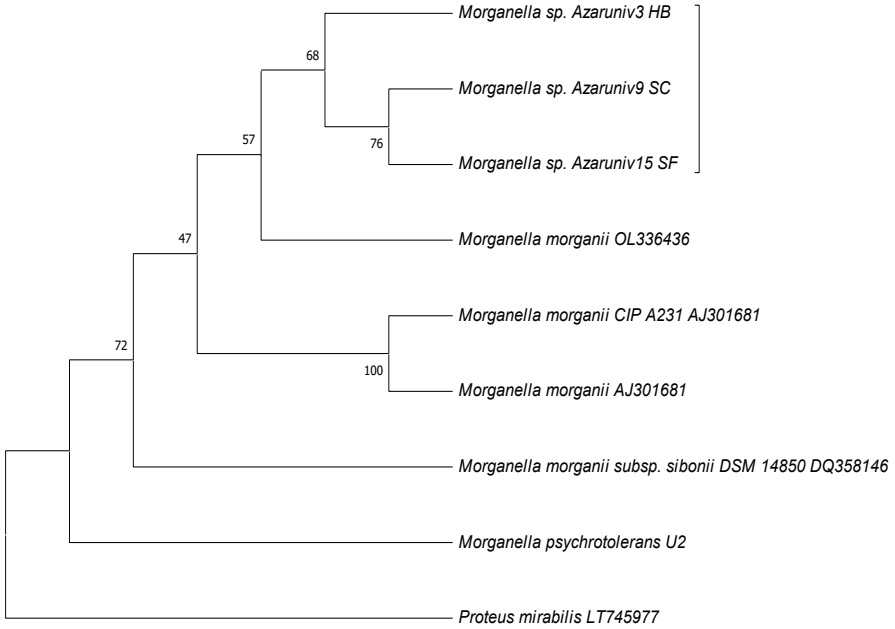


**Figure 3.** Phylogenetic analysis of *Morganella* isolates (Azaruniv3 HB, Azaruniv9 SC and Azaruniv15 SF) and closely related strains based on 16S rRNA gene sequences, with *Proteus mirabilis* as the outgroup. Bootstrap support values (based on 1000 replicates) are shown at nodes.

**Phylogenetic Relationship of *Enterobacter* Isolates**

Phylogenetic reconstruction showed that the *Enterobacter* isolates Azaruniv2 SF and Azaruniv8 SF formed a distinct, well-supported clade with *E. huaxiensis*. This clade was phylogenetically separate from other *Enterobacter* species, including *E. cloacae* and *E. asburiae*, indicating a specific evolutionary relationship with *E. huaxiensis* (Fig. 4).

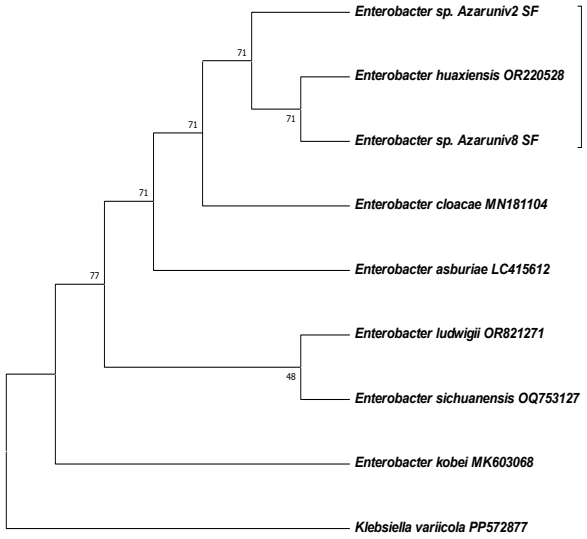


**Figure 4.** Phylogenetic analysis of *Enterobacter* isolates (Azaruniv2 SF and Azaruniv8 SF) and closely related strains based on 16S rRNA gene sequences, with *Klebsiella variicola* as the outgroup. Bootstrap support values (based on 1000 replicates) are shown at nodes.



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**Phylogenetic Classification of *Staphylococcus* Isolate**

The *Staphylococcus* sp. Azaruniv13 HB isolate was phylogenetically placed within a clade of *Staphylococcus* succinus subsp. succinus strains. This clade demonstrated a sister-group relationship to *Staphylococcus xylosus*, clearly delineating it from other coagulase-negative *staphylococci* (Fig. 5).

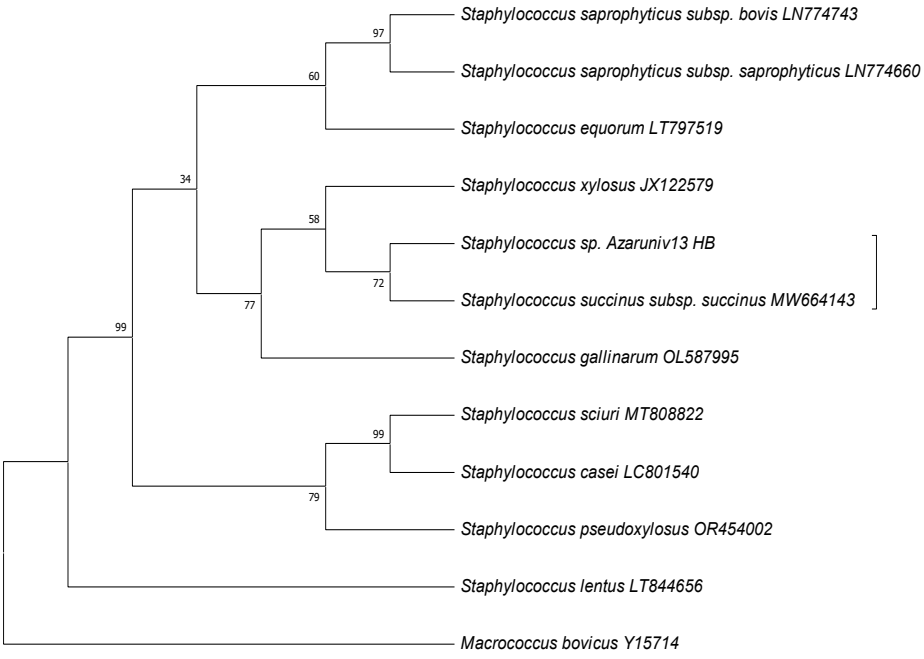


**Figure 5.** Phylogenetic analysis of *Staphylococcus* isolates (Azaruniv13 HB) and closely related strains based on 16S rRNA gene sequences, with *Macrococcus bovicus* as the outgroup. Bootstrap support values (based on 1000 replicates) are shown at nodes.

## Discussion

The genera we identified- *Enterobacter*, *Citrobacter*, *Morganella*, *Staphylococcus*, and *Acinetobacter*- are not merely transient contaminants but are frequently represented in the core microbiota of EPNs as revealed by recent culture-independent studies. For instance, Gulcu et al. (2017) consistently detected members of Enterobacteriaceae, including *Enterobacter* and *Morganella*, as stable associates of *H. bacteriophora*, suggesting these relationships are evolutionarily stable and not accidental. Therefore, our findings are not anomalous but rather confirm, using a culture-dependent approach, that these opportunistic pathogens can be integral members of the EPN microbial community. Among these genera, species such as *Enterobacter huaxiensis* and *Acinetobacter schindleri* are recognized as emerging opportunistic pathogens. *E. huaxiensis*, a novel species first recovered from human blood (W. Wu et al., 2019) is primarily associated with nosocomial infections, including bacteremia, meningitis, pneumonia, and urinary tract infections. *A. schindleri*, while often considered part of the normal skin flora, is an opportunistic pathogen responsible for infections in hospitalized patients, particularly those who are immunocompromised. Clinically significant isolates have been linked to bacteremia, ventilator-associated pneumonia, and wound infections, and they can exhibit resistance to antibiotics, including carbapenems. The presence of such clinically relevant bacteria in native EPN isolates is not an isolated observation but reflects a broader, demonstrable risk inherent to EPN biology. Supporting our concerns, Hernández-Mendoza et al. (2014) genomically characterized *Alcaligenes faecalis* strain MOR02, isolated from an entomopathogenic nematode, which demonstrated a temporary but highly pathogenic association with three different EPN species (*S. feltiae*, *S. carpocapsae*, and *H. bacteriophora*) and caused 96% mortality in insect larvae. This evidence is pivotal, as it confirms that non-symbiotic bacteria can form functional associations with EPNs and act as potent pathogens independently. Furthermore, the additional hazardous genera we identified, *Morganella* and *Citrobacter*, expand the scope of this known problem. *Morganella morganii* is a well-known cause of opportunistic infections, most commonly urinary tract infections, wound infections, sepsis, and, more rarely, central nervous system infections like brain abscesses. It is also notable for its intrinsic resistance to several antibiotics, including penicillin and cephalosporin. The genus *Citrobacter* (particularly *C. freundii* and *C. koseri*) is implicated in urinary tract infections, respiratory tract infections, and sepsis. Furthermore, *C. freundii* is a significant cause of meningitis in neonates and can cause severe central nervous system infections with high mortality rates in adults. Even the less common *C. braakii* has been reported to cause bacteremia and other infections in immunocompromised patients. Therefore, the potential for EPNs to act as vectors is not limited to their primary


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symbionts but extends to a secondary pathobiome. The convergence of evidence—from the temporary, pathogenic symbiosis reported by Hernández-Mendoza et al. (2014) to the diverse clinically relevant genera we report—underscores that the current quality control paradigm, which fails to screen for these non-symbiotic associates, is inadequate. This mandates a systematic re-evaluation of biosafety protocols across the entire EPN industry to account for the full spectrum of associated bacteria with pathogenic potential.

A pivotal question arising from our study is the origin of these bacteria. While they may form part of a stable core microbiota, we must critically evaluate whether their presence could be amplified or influenced by laboratory culture practices. In a highly relevant study, it was demonstrated that bacteria such as *C. gillenii*, *E. asburiae*, and *M. morganii* can be isolated from EPNs and likely originate from the gut microbiome of the *Galleria mellonella* larvae used for their propagation. This is a crucial consideration for our work. The close phylogenetic relationship of our isolates to clinically relevant species (e.g., *C. freundii*, *E. huaxiensis*, *M. morganii*) suggests that even if the initial source was the insectary host, the EPN IJs effectively acquire and potentially vector these bacteria. This does not diminish the biosafety concern but reframes it: the mass-rearing process itself, reliant on nutrient-rich insect hosts, may systematically load EPN products with bacteria of clinical concern, regardless of the nematodes' geographic origin.

The phylogenetic analysis revealed close relationships between EPN-related *Citrobacter* isolates (Azaruniv12 SF and Azaruniv5 HB) and established species (*C. braakii* and *C. freundii*), supported by acceptable bootstrap values. The isolation of these strains from EPNs raises important biosafety concerns, as members of this genus are well-documented opportunistic pathogens in both human and veterinary medicine (Samonis et al., 2008). The close relationship of our isolates to *C. freundii* is particularly significant, as this species is increasingly recognized as a cause of nosocomial infections, including urinary tract infections, neonatal meningitis, and bacteremia, especially in immunocompromised patients (Jacobsen et al., 2008). The presence of these potential pathogens in EPNs suggests that nematode-based biocontrol products could serve as unexpected vectors for *Citrobacter* dissemination. In agricultural settings, *Citrobacter* species have been implicated in livestock infections, particularly in poultry and swine, where they can cause enteritis, septicemia, and increased mortality in young animals (Swelum et al., 2021). The ability of *Citrobacter* to persist in soil and water (Rogers et al., 2016) may facilitate its association with EPNs, either through direct interaction or environmental contamination during mass production. Of particular concern is the genus' propensity for acquiring antibiotic resistance genes, with multidrug-resistant *C. freundii* strains increasingly reported in both clinical and veterinary isolates (Oliveira et al., 2016).

The strong clustering of Azaruniv11 SF with *A. schindleri* (bootstrap 100%) suggests a close genetic relationship between them. The clear separation of Azaruniv11 SF from clinically significant species such as *A. calcoaceticus*, *A. nosocomialis* and *A. pittii* supports the notion that not all *Acinetobacter* lineages possess the same pathogenic potential. This observation aligns with previous studies suggesting that environmental *Acinetobacter* isolates often occupy distinct phylogenetic branches compared to their clinical counterparts (Touchon et al., 2014). The isolation of *Acinetobacter* sp. phylogenetically related to *A. schindleri* from IJs of EPNs raises significant biosafety concerns regarding commercial nematode-based products. This finding suggests that IJs may serve as potential vectors for *Acinetobacter* dissemination, a genus notorious for its environmental persistence and clinical importance. The risk is particularly concerning given that *Acinetobacter* species can acquire antibiotic resistance determinants from environmental bacteria (Peleg et al., 2008). If resistant strains are transported via nematode products applied to agricultural fields, they could potentially transfer resistance genes to soil microbiota or directly infect farm workers.

The phylogenetic analysis of *Morganella* isolates, including Azaruniv3 HB, Azaruniv9 SC and Azaruniv15 SF clustered these isolates with clinical *M. morganii* strains suggests that EPNs as environmental reservoirs may harbor strains genetically similar to those found in healthcare settings. This finding aligns with recent reports of *Morganella* species being widely distributed in natural environments (Wang et al., 2017), while maintaining close genetic relationships with clinical isolates. The close phylogenetic relationship between the studied strains and clinical *M. morganii* strains raises important questions about potential pathogenicity. While these environmental strains may lack the virulence factors of clinical isolates (Chakraborty et al., 2000; Mahmud et al., 2024), their genetic similarity suggests they could serve as reservoirs for horizontal gene transfer. This possibility warrants further investigation through comparative genomics to identify potential virulence or antibiotic resistance determinants.

The isolation of two *Enterobacter* strains (Azaruniv2 SF and Azaruniv8 SF) from indigenous EPNs raises important safety considerations for nematode-based biocontrol products. Phylogenetic analysis revealed these isolates form a distinct clade with *E. huaxiensis*, an emerging opportunistic pathogen in clinical settings (Wenjing Wu et al., 2019). This close relationship raises important questions about the potential for EPNs to serve as vectors for pathogenic *Enterobacter* species, particularly through commercial nematode products that are increasingly traded globally.

The phylogenetic placement of *Staphylococcus* sp. Azaruniv13 HB within the *S. succinus* clade provides insights into the environmental diversity of these bacteria. The clustering with *S. succinus* subsp. *succinus* suggests this isolate likely shares ecological and genomic traits with this species, which is frequently recovered from natural habitats like fermented foods, plant materials, and ancient amber (Lambert et al., 1998). This finding aligns with emerging evidence that environmental staphylococci may represent understudied reservoirs of genetic diversity (Becker et al., 2014; Gómez et al., 2016; Nováková et





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al., 2006). The close relationship between Azaruniv13 HB and *S. succinus* is particularly noteworthy given the latter's phylogenetic distinctness from clinically prevalent coagulase-negative staphylococci like *S. saprophyticus* and *S. xylosus*. While coagulase-negative staphylococci have traditionally been considered commensal organisms, accumulating evidence demonstrates the pathogenic capacity of *S. succinus* and *S. xylosus* across clinical and veterinary settings. Originally identified in amber-residing specimens, *S. succinus* has emerged as a clinically relevant pathogen, with documented cases of human bacteremia, particularly in immunocompromised patients, and medical device-associated infections (Nováková et al., 2006). In veterinary medicine, this species has been isolated from bovine mastitis cases, where it demonstrates biofilm-forming capabilities that complicate treatment (Taponen et al., 2008). Of particular concern are reports of mecA-positive *S. succinus* strains exhibiting multidrug resistance patterns similar to those observed in *S. aureus* MRSA isolates (Ruffier d'Epenoux et al., 2024).

Our study provides compelling evidence that EPNs can harbor non-symbiotic bacteria through both facultative symbiotic relationships and environmental contamination. The presence of these opportunistic pathogens in EPNs may result from preliminary symbiotic interactions, where bacteria establish transient but stable associations with nematodes. Species of *Enterobacter* and *Citrobacter*, which share environmental niches with EPNs, may exploit the nematode as a vector for dispersal. These bacteria could potentially benefit from the protective environment of the nematode gut or cuticle during stressful conditions, while not providing the reciprocal benefits characteristic of true symbionts like *Xenorhabdus* or *Photorhabdus*. Alternatively, the contamination may be entirely accidental, occurring during commercial production or environmental exposure. The mass-rearing processes for EPNs often use nutrient-rich media that could facilitate bacterial growth. Furthermore, as IJs move through soil ecosystems, they may mechanically acquire bacteria from contaminated substrates or infected insect cadavers. Several mechanisms could facilitate the distribution of virulent bacteria via nematode-based products. First, these bacteria may persist as surface contaminants on the cuticle of IJs, protected by biofilm formation. Alternatively, they might establish transient associations within the nematode digestive tract, allowing survival during environmental stress.

Our study, coupled with the evidence from the broader literature, demonstrates that the association of EPNs with opportunistic pathogens is a widespread phenomenon, not a risk exclusive to imported non-native species. The potential for EPNs to act as vectors is, in principle, inherent to their biology and their production method. However, it is important to emphasize that our findings, and those in the literature, do not indicate that these nematodes transmit significant primary human, plant, or animal pathogens. Instead, the genera we identified are opportunistic or environmental bacteria that pose a theoretical, rather than a documented acute, risk. Consequently, while the metaphor of a "Trojan horse" may be overly strong and potentially misleading if interpreted as an imminent threat, it does serve to highlight a critical blind spot in current quality control paradigms. Given the absence of evidence for the transmission of major overt pathogens, the metaphor is not strictly applicable to direct environmental harm. Rather, the "Trojan horse" concept, if used, should be limited to the idea of an *undetected* biological load in commercial products, emphasizing that the industry must improve screening protocols. The hazardous vectorization of problematic strains is not a proven reality of EPN use but rather a universal, theoretical biosafety consideration that arises whenever quality controls are absent. Therefore, we suggest that the application of this metaphor requires careful qualification to avoid overstating the risk, and we agree with the assessment that the benefits of EPNs for biological control far outweigh the currently unconfirmed risks of environmental pathogen spread.

Therefore, the focus must shift from a narrow concern about imports to a broader imperative for universal biosafety standards. Current quality control measures, which primarily ensure nematode viability and the presence of primary symbionts, are insufficient to address this hidden microbiome. We advocate for the development and implementation of routine, culture-independent screening protocols for commercial EPN products. Such screenings should specifically target a panel of clinically relevant bacterial genera, including those identified here. Furthermore, sustained surveillance of EPN-associated microbiota is essential to monitor the potential emergence and spread of antibiotic-resistant strains through these biological control agents. In conclusion, our findings from native Iranian EPNs serve as a compelling case study that underscores the urgent need for harmonized international biosafety frameworks to ensure the safe and sustainable use of EPN biocontrol products globally.

While the primary goal of biosafety research is undoubtedly to determine the actual pathogenic impact of nematode-associated bacteria on human, animal, and plant health, concluding that such an impact is negligible or absent based solely on the current lack of reported acute infections would be premature. The genera identified in this study-*Citrobacter*, *Morganella*, and *Acinetobacter*-are well-documented opportunistic pathogens in clinical settings, and their potential risk cannot be definitively dismissed without further investigation. Extensive identification of potential pathogenic companions is certainly a necessary step. However, identification *alone* is insufficient for a comprehensive risk assessment. Simply cataloging species does not reveal whether they persist in the environment, whether they proliferate after field application, or whether they exchange genetic material with resident soil or clinically relevant bacteria. Therefore, the three key questions proposed here are not tangential to assessing harm; rather, they are mechanistically prerequisite to it. First, understanding the ecological dynamics between EPNs and these non-symbiotic bacteria clarifies whether these associations are transient or stable. Second, evaluating their survival and proliferation under field conditions determines whether these bacteria pose a genuine environmental burden, as organisms that fail to establish are inherently less hazardous. Third, investigating horizontal gene transfer is arguably the most critical step; even if these isolates currently exhibit low intrinsic virulence, they may serve as





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reservoirs for antibiotic resistance or virulence genes that could be transferred to clinically relevant strains. Consequently, while harm assessment remains the ultimate goal, an integrated research framework—combining ecological, environmental, and genetic approaches-offers the most rigorous scientific pathway to accurately determine that harm, rather than limiting efforts to extensive but context-free identification.

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