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Original article (Orijinal araştırma)

Assessing the antiparasitic potential of *Bifidobacterium* sp. ERSapi20 isolated from the cuticle of honeybees against *Varroa destructor* Oudemans, 1904 (Acari: Varroidae)[1]

Bal arılarının kutikulasından izole edilen *Bifidobacterium* sp. ERSapi20 suşunun *Varroa destructor* Oudemans, 1904 (Acari: Varroidae)'a karşı antiparazitik potansiyelinin değerlendirilmesi

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Abstract

The mite *Varroa destructor* Oudemans, 1904 (Acari: Varroidae) is a major contributor to honeybee colony losses worldwide. Actinobacteria are known for their wide distribution and production of biologically active compounds effective against various pathogens, including bacteria and parasites. This study aims to investigate the bioactive compounds produced by actinobacterial symbionts associated with *Apis mellifera* L., 1758 (Hymenoptera: Apidae) and evaluate their acaricidal potential against *V. destructor*. In 2022, a strain of *Bifidobacterium* sp. ERSapi20 was isolated from worker bees in Antalya, Türkiye, using a culture-based method. The antiparasitic activity of the extracellular solution and intracellular extract of the strain was assessed through direct spraying on *V. destructor* at varying concentrations of insect Ringer solution (0%, 25%, 50%, 75%, and 100%). The extracellular solution achieved 90% mite mortality within 16 hours, while the intracellular extract resulted in 100% mite mortality within 18 hours. This is the first report demonstrating acaricidal activity against *V. destructor* using intracellular extracts of actinobacterial strains. These findings support the hypothesis that honeybee-associated actinobacteria produce compounds effective against varroa mites, highlighting the potential of natural products for controlling *V. destructor* in honeybee colonies.

Keywords: Actinobacteria, antiparasitic activity, *Apis mellifera*, secondary metabolite, *Varroa destructor*

Öz

Varroa destructor Oudemans, 1904 (Acari: Varroidae), bal arısı kolonilerinin kaybına ciddi katkıda bulunduğu bilinmektedir. Aktinobakteri türleri ise geniş bir dağılıma sahiptir ve bakteriler ve parazitler gibi çeşitli patojenlere karşı biyolojik olarak aktif bileşikler üretirler. Bu temele dayanarak, bu çalışma *Apis mellifera* L., 1758 (Hymenoptera: Apidae) ile ilişkili aktinobakteri simbiyontları tarafından üretilen biyolojik olarak aktif bileşikleri araştırmayı ve bunların *V. destructor*'a karşı akarisit potansiyelini değerlendirmeyi amaçlamaktadır. Kültür bazlı bir yöntem kullanılarak işçi arılardan *Bifidobacterium* sp. ERSapi20 suşu Antalya, Türkiye lokasyonundan 2022 yılında izole edilmiş ve tanımlanmıştır. Antiparazitik aktiviteyi belirlemek için, suşun ekstraselüler ve intraselüler ekstraktı, böcek Ringer çözeltisinin değişen konsantrasyonlarında (%0, %25, %50, %75 ve %100) püskürtme yöntemi kullanılarak *V. destructor*'a uygulanmıştır. Ekstraselüler çözeltinin 16 saat içinde %90 oranında akar ölümüne yol açtığı, intraselüler ekstraktın ise 18 saat içinde %100 oranında akar ölümüne neden olduğu belirlenmiştir. Bu, aktinobakteriyel suşların intraselüler ekstraktı kullanılarak *V. destructor*'a karşı akarisit aktivitesinin rapor edildiği ilk çalışmadır. Bu araştırma, bal arılarının varroa akarlarına karşı etkili bileşikler üreten aktinobakterilerle ilişki kurduğu hipotezini desteklemekte ve *V. destructor*'u kolonilerde kontrol etmek için doğal ürünlerin kullanılmasına dair bilgiler sunmaktadır.

Anahtar sözcükler: Aktinobakteri, antiparazitik aktivite, *Apis mellifera*, sekonder metabolit, *Varroa destructor*

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Introduction

Honeybee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae), is one of the most important invertebrates in the agriculture, playing a crucial role in pollination and the production of honey and other bee products. However, honeybee colonies have been experiencing significant losses globally (Simon-Delso et al., 2014). Various factors contribute to these declines, including habitat loss, inadequate nutrition, numerous pathogens and parasites, reduced genetic diversity, climate change, introduction of alien species, and intensive agricultural practices (Meixner, 2010; Crotti et al., 2013; Goulson et al., 2015; DeGrandi-Hoffman et al., 2017; Muñoz-Colmenero et al., 2020). Among these factors, parasitic infections, particularly by the mite *Varroa destructor* Oudemans, 1904 (Acari: Varroidae), are highly detrimental, affecting honeybee performance, productivity, and overall colony health (Evans, 2003; Zemene et al., 2015; Tutun et al., 2018). This mite not only compromises honeybee health but also acts as a biological vector for various viruses, thereby exacerbating colony losses worldwide (Shen et al., 2005; Hussain et al., 2018; Reyes-Quintana et al., 2019). Efforts to control *V. destructor* over the last few decades have included physical, chemical, and biological methods. However, none of the current mite mitigation strategies are fully effective, and the varroa mite remains a significant challenge for beekeeping (Rosenkranz et al., 2010).

The increasing resistance of varroa mites to commonly used acaricides, such as flumethrin, coumaphos, fluvalinate, and amitraz, has further complicated control efforts, leading to chemical residues in bee products and necessitating alternative management approaches (Martin, 2004; Pettis, 2004; Lodesani & Costa, 2005; Maggi et al., 2010; Rosenkranz et al., 2010; Higes et al., 2020).Consequently, there is an immediate necessity for alternative management approaches, including microbial control. Among them, actinobacteria may offer promise for microbial control of the varroa mite because they are capable of producing a variety of bioactive molecules and are known to be sources of various metabolites with their complex biochemical processes (Khasabuli & Kibera, 2014; ul Hassan & Shaikh, 2017). In this regard, actinobacteria may be the critical bacterial group to resolve varroa mite control.

Actinobacteria are gram-positive, filamentous bacteria with high guanine-cytosine (G+C) content in their genomes, characterized by their ability to produce a wide array of bioactive molecules (Barka et al., 2016). Actinobacterial genomes display heterogeneity, which is presumed to contribute to their biodiversity (Daffe et al., 1990). Generally, actinobacteria are free-living microorganisms that are widely distributed in various ecosystems and are able to utilize numerous nutritional sources, including complex polysaccharides (Zimmermann, 1990). These phyla are a crucial bacterial group in the field of biotechnology (Běhal, 2000; Barka et al., 2016). These microorganisms are well-known for their production of secondary metabolites with diverse biological activities, including antibacterial, antifungal, anticancer, antitumor, cytotoxic, cytostatic, anti-inflammatory, antiparasitic, antimalarial, antiviral, antioxidant, antiangiogenetic, immunosuppressive and enzyme effects (Kanbe et al., 1992; Chaudhary et al., 2013; Manivasagan et al., 2014). In this regard, actinobacteria, particularly the genus Bifidobacterium, have shown potential as biological control agents against arthropod pests, including mites.

Specifically, Bifidobacterium species have been highlighted for their acaricidal potential against *V. destructor*. For instance, Saccà & Lodesani (2020) reported that *Bifidobacterium asteroides* strains were 90-95% effective as acaricides against varroa mites. This study underscores the potential of Bifidobacterium species in managing varroa populations, providing a foundation for further exploration of these bacteria as viable biocontrol agents. Given the critical need for alternative and sustainable varroa control strategies, exploring the efficacy of Bifidobacterium strains, such as ERSapi20 isolated from honeybee surfaces, is of great importance.

To fully assess the antiparasitic potential of bacteria such as *Bifidobacterium* sp., it is essential to evaluate both extracellular and intracellular extracts. Extracellular extracts can contain secreted bioactive molecules, including enzymes, toxins, and secondary metabolites, which may directly impact parasite viability. In contrast, intracellular extracts may unveil additional bioactive compounds that are stored within the cells and not secreted into the surrounding environment. Analyzing both types of extracts helps in developing a comprehensive understanding of the mechanisms through which these bacteria exert their antiparasitic effects. This approach ensures that all relevant bioactive components, whether secreted or retained within the cell, are accounted for in the assessment of antiparasitic efficacy. This comprehensive evaluation is crucial, as the variability in bacterial efficacy against varroa mites can be influenced by the specific metabolites present in different extracts.

This study aims to build on these findings by investigating the antiparasitic potential of *Bifidobacterium* sp. ERSapi20 isolated from honeybee surfaces. By assessing the impact of this strain on varroa mite mortality under laboratory conditions, this research seeks to contribute to the development of novel microbial control strategies that can effectively mitigate varroa mite infestations and support honeybee health.

Materials and Methods

Sample collection

Worker honeybees (*Apis mellifera*) were collected individually from different beehives of an apiary in the spring of 2022. All bees were selected based on their vitality and collected separately to ensure the consistency of the bacterial source. Sampling was conducted in the middle-west of Antalya (Latitude: 36.8919 N, Longitude: 30.5666 E), located in the southwest of Türkiye. Five worker honeybees were selected from each beehive, resulting in a total of 20 bees used as bacterial sources. Each bee was placed in a separate sterile container to avoid cross-contamination before being transferred to a combined peptone water solution (0.95%) and stored at +4ºC under aseptic conditions. The parasitic mite *V. destructor* samples were selected according to physical and morphologic features, and were collected from different beehives located in the same apiary as described above.

Selective isolation and preliminary identification of actinobacteria

The surfaces of freshly collected honeybees were subjected to a 0.95% peptone water solution to selectively isolate actinobacteria. Sampling was incubated overnight in International Streptomyces Project-II (ISP-2) broth at 27ºC in a shaker at 150 rpm. ISP-2 broth was prepared as follows: Yeast extract (4.0 g/l), malt extract (10.0 g/l), dextrose (4.0 g/l), pH 7.3±0.2 (Shirling & Gottlieb, 1966). Then the fluid was inoculated on the petri dish containing Actinomycetes Isolation Agar (AIA, BD DIFCO) supplemented with cycloheximide (50 µg/ml) and nalidixic acid (75 µg/ml). The inoculated plates were incubated at 27ºC for 14 days (Roshan, 2013). All experiments were conducted in triplicate.

The isolates were selected according to their colony morphology, the color of mycelium, and diffusible pigments and subcultured three times to obtain pure isolates (Shirling & Gottlieb, 1966). The Gram staining method was used to determine for gram reactions of isolates (Beveridge, 2001). The Gram-positive isolate was selected and used for further analyses. The selected isolate was stored at -20ºC in Luria-Bertani broth (LB broth, Merck) with 20% (v/v) glycerol.

DNA extraction, amplification, and identification of actinobacteria.

Based on morphological characterization and anti-parasitic potential, the selected isolate was identified based on the 16S rRNA sequencing. Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit following the manufacturer's instructions (Thermo Scientific, USA). The purity of the product resulting from genomic DNA isolation was determined using a nanodrop device (NanoDrop 200c, Thermo Scientific). The 16S rRNA gene was amplified with 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGCTACCTTGTTACGACTT-3' universal primer set (Hong et al., 2014). PCR amplification was performed using the 5X PCR Dye Master Mix II (GeneMarkBio) following the manufacturer's instructions. Thermal cycling was programmed as follows: initial denaturation at 95ºC for 5 min, 40 cycles of denaturation at 95ºC for 1 min, annealing at 57ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. Thermal cycling was performed in a final volume of 25 µl using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, USA). The amplified products were determined by 2% (w/v) agarose gel electrophoresis. The PCR products (around 1,300 bp lenght) were sequenced by Aquatayf Biotechnology LLC (Türkiye). The sequencing results were analyzed using the GENEIOUS software (https://www.geneious.com/). The software-generated 16S rRNA consensus sequences were analyzed for homology to type strains in the database via NCBI BLAST (https://blast.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using neighbour-joining method (Saitou & Nei, 1987) by MEGA11 software and was bootstrapped using 1000 replicates for each sequence (Stecher et al., 2020; Tamura et al., 2021).

Bacterial growth curve

Growth curve of *Bifidobacterium* sp. ERSapi20 was performed for the characterization of the death phase or the phase of decline (Monod, 1949; Zwietering et al., 1990). The strain was incubated in 250 ml flasks with 100 ml

of LB broth at 27ºC with aeration in a shaker at 150 rpm. Each 24 h of incubation, 1.5 ml of fluid was taken from flasks, and transferred to eppendorf tubes. The fluids were washed using distilled water and centrifuged at 10.000 rpm for 10 minutes. The growth phases of the isolate were tracked by measuring optical density (OD) at 600 nm under aerobic conditions. The distilled water serves as the control. All experiments were conducted in triplicate.

Screening for antiparasitic activity

In this step, the antiparasitic activity test was conducted using the extracellular and intracellular solutions of the actinobacterial isolate. The approach was modified based on the study by Tsagou et al. (2004). Adult female *Varroa destructor* mites were collected aseptically from capped brood cells and placed in petri dishes (90 mm × 10 mm), with each dish containing ten mites and five bee pupae (two mites per pupa). To prepare the bacterial solutions, the extracellular and intracellular extracts of *Bifidobacterium* sp. ERSapi20 were obtained based on the bacterial growth curve analysis. Solutions were prepared in varying concentrations (250 µl, 500 µl, 750 µl, and 1000 µl) and diluted with 1 ml of insect Ringer solution (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$). For the spray application, a fine mist sprayer calibrated to deliver a consistent 1 ml volume was used. Each petri dish containing the mites and pupae was placed on a clean, sterile surface, and the lid was partially opened to avoid contamination while allowing access for spraying. The sprayer nozzle was held approximately 15 cm above the mites to ensure even coverage without causing physical disturbance to the mites or pupae.

The solution was sprayed gently in a sweeping motion across the surface of the petri dish to uniformly coat the mites and pupae. After spraying, the petri dishes were immediately covered to minimize evaporation and prevent external contamination. The sprayed petri dishes were then placed in an incubator set at 31±1.0°C. Mite survival and activity were monitored every two hours for 24 hours post-application. For the extracellular solution test, Amitraz was used as a positive control, and insect Ringer solution served as a negative control. In the intracellular solution test, sterilized LB broth and Tris/HCl buffer were used as controls, sprayed in the same manner as the test solutions. Each assay was conducted in triplicate to ensure consistency and reliability of the results.

For the experiment estimating the effect of the extracellular broth, the strain was cultivated in 250 ml flasks with 100 ml of LB broth at 27ºC in a shaker at 150 rpm for 6 d. The cultures (45 ml) were transferred to sterilized falcon tubes and centrifuged at 5000 rpm for 30 min at 4ºC. Then, the supernatant was filtered using Whatman No.1 filter paper. The filtered supernatant was sprayed at a dose of 1 ml per dish containing 10 mites. For analyzing the effect of the intracellular extract, the harvested bacterial cells (corresponding to 121.1 mg of dried biomass) were suspended in 5 ml of Tris/HCl buffer, pH 7.5, and were ruptured by four 45 s sonic bursts (20 kHz) on ice using a sonicator (Bandelin Sonopuls HD 2070, Bandelin electronic, Berlin, Germany). Sonicated solution was centrifuged at 5000 rpm for 30 min at 4ºC. Afterward, the supernatant was passed through Whatman No.1 filter paper and utilized in the bioassay (1 ml per petri dish), as outlined above. The bioassays were performed at 31±1ºC in the dark. Varroa mite mortality was recorded per 2 hours for 24 hours. Varroa mites treated with fermentation medium (LB broth), sterilized insect ringer solution, and Tris/HCl buffer served as negative controls. Amitraz was used in this study as a positive control (data not shown). The assay was replicated 3 times.

Assessing the ımpact of extracellular and ıntracellular *Bifidobacterium* sp. ERSapi20 solutions on honeybee mortality

To determine the effect of extracellular and intracellular solution of *Bifidobacterium* sp. ERSapi20 strain on *A. mellifera*, bacterial solutions were sprayed on the bees in laboratory cages. The honeybees were housed in standard laboratory cages constructed with wire mesh to prevent escape, ensure ventilation, and equipped with feeders containing sugar syrup for nutrition. The bees (20-25 individuals) were collected from different beehives located in the same apiary described above. The groups of 20 bees were sprayed with 1 ml extracellular and intracellular solution, separately. The extracellular and intracellular solutions obtained from the isolate were prepared at varying concentrations (0%, 25%, 50%, 75%, and 100%) by mixing with 1 ml of Insect Ringer solution and were applied to the bees using a spraying method (De Guzman et al., 1993; Tsagou et al., 2004). The Insect Ringer solution used in the study was composed of 130 mM NaCl, 5 mM KCl, and 1 mM CaCl2 (Gliński & Jarosz, 1990). The bee mortality was recorded per 2 hours for 24 hours, as described above. The bees in the control laboratory cages were sprayed with bacteria-free mediums.

Data analysis

The sequence of *Bifidobacterium* sp. ERSapi20, along with sequences from closely related species obtained from the GenBank database (NCBI), was aligned using multiple alignment in Clustal W through GENEIOUS (https://www.geneious.com). Pairwise distances were computed using the Tamura-Nei model, and a phylogenetic tree was constructed using the neighbor-joining method as implemented in MEGA11 software (Tamura et al., 2021). The tree was bootstrapped with 1000 replicates, using resampled alignments of the 16S rDNA sequences.

All the bioassays were conducted in triplicate and subjected to statistical analysis ($p < 0.05$). The differences in the lethal effects of intracellular and extracellular solutions of ERSapi20 at different concentrations on Varroa were analyzed using R-Studio. (https://www.rstudio.com/). The impact of bioactive compounds produced by *Bifidobacterium* sp. ERSapi20 on varroa mite survival was assessed using a survival probability test conducted in R Studio (https://CRAN.R-project.org/view=Survival), a package utilized for data analysis. The analysis involved estimating the survival function using the Kaplan-Meier method, generating survival curves, and determining the statistical significance between the negative control groups. Statistical analysis further involved conducting log-rank tests or Cox proportional hazards models to assess differences in survival rates between treatment and control groups (Weerahandi & Yu, 2020).

RESULTS

Isolation and identification of ERSapi20

The strain was grown on the AIA medium. The colony of the strain is regular, circular, milky, and smooth (Figure 1a). The gram staining result showed that the isolate is a gram-positive bacteria and a curved shape in purple (Figure 1b).

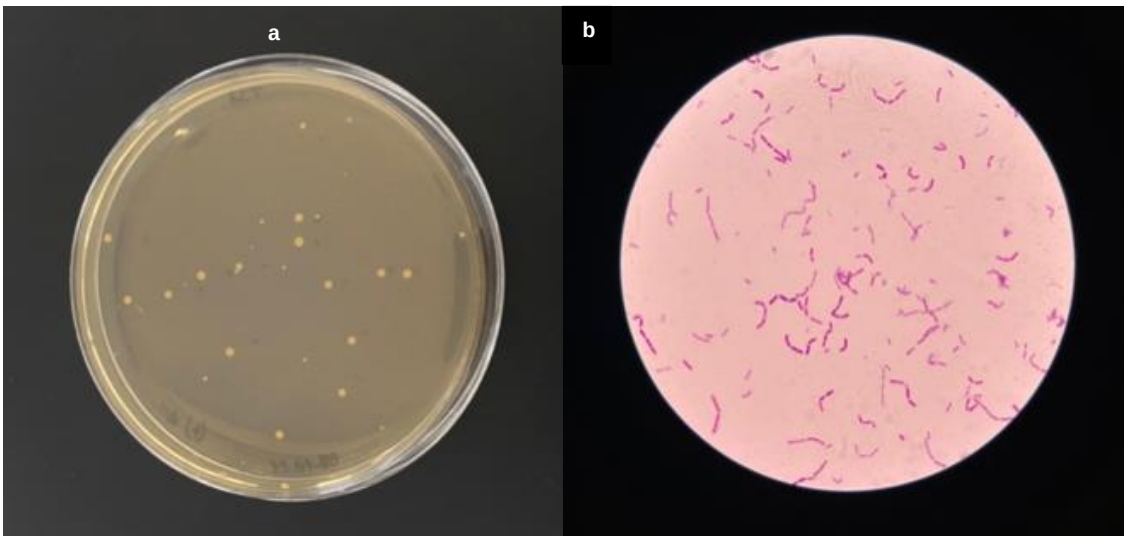


Figure 1. Morphological characterization of *Bifidobacterium* sp. ERSapi20: a) colonizing form of strain on Actinomycete Isolation Agar, b) gram staining result.

The partial 16S rDNA sequences of the isolate have been deposited in the GenBank database under the following accession number: OQ073505. The sequence size of the strain was 1397 bp in length, possessing an average G + C% content of 60.56%. The 16S rDNA sequence of the strain ERSapi20, is having a 99.57% similarity with *Bifidobacterium asteroides* DSM 20089, as analyzed with NCBI. Phylogenetic analysis with the neighbour joining algorithm using the Tamura-Nei model shows that the isolate originated from the same ancestor as *B. asteroides* strains. However, based on the branch length with *Bifidobacterium* sp. strains, it can be assumed that the isolate might be quite different from its closest relative (Figure 2).

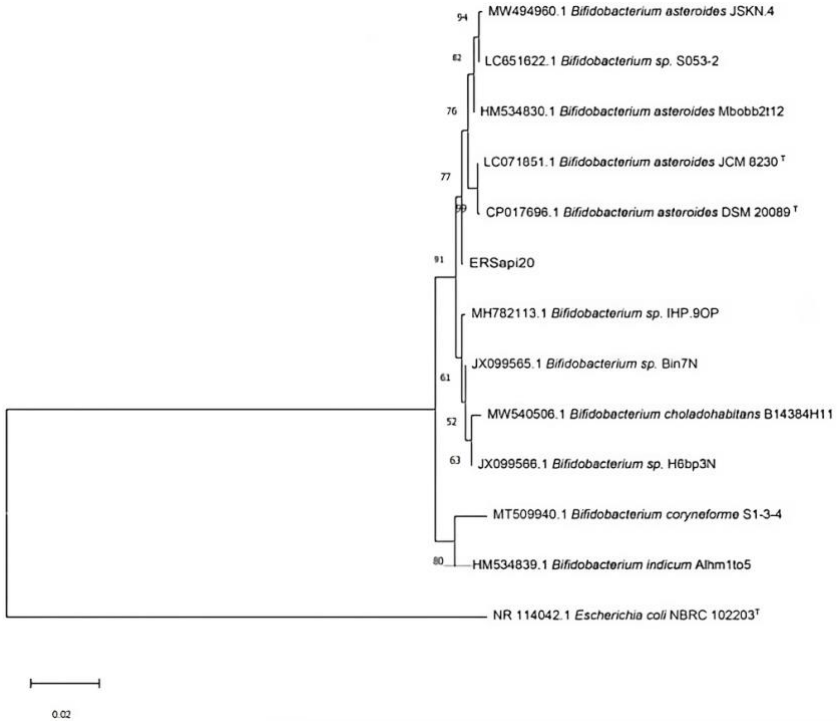


Figure 2. Phylogenetic tree of actinobacteria isolated from honeybee surfaces and their closest type strains, constructed using the neighbour joining method based on 16S rDNA sequences with the Tamura-Nei model. Bootstrap values, indicating the confidence level of each branch, were derived from 1,000 replicates and are shown at the branch nodes.

Antiparasitic activity of ERSapi20 against varroa mites

The production of secondary metabolites occurs during the stationary phase (Duquesne et al., 2007). Thus, bacterial growth was observed for ERSapi20 strain in LB broth at 27 ºC. The observation was performed once every 24 h until the strain entered the death phase. The strain entered the stationary phase after 48 h, with OD600 values of 2.5122, whereas it entered the death phase after six days with OD600 values of 2.3241 (Figure 3). Varroa mite mortality was determined after treatment with fermentation solution of ERSapi20 strain isolated from the surface of worker honeybees (Figure 4). For each experiment mite susceptibility to the fermentation solutions of six-day-old strain (according to growth curve result) within different concentrations were determined by calculating the time for 50% and 90% mortality of the varroa mites (LT50 and LT90, respectively) (Table 1). At 31±1ºC in the dark, mite mortality in the treatments of intracellular extract of ERSapi20 strain reached 100% within 18 h, whereas 90% mortality of the mites occurred using the extracellular solution at 16 h.

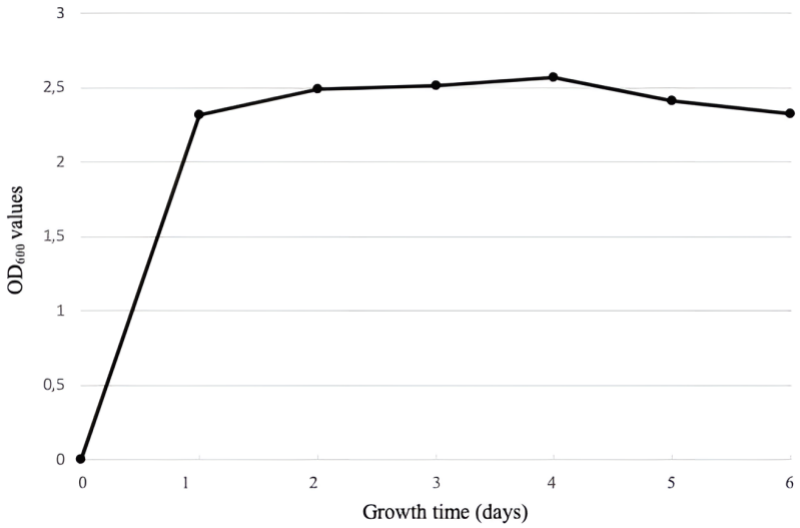
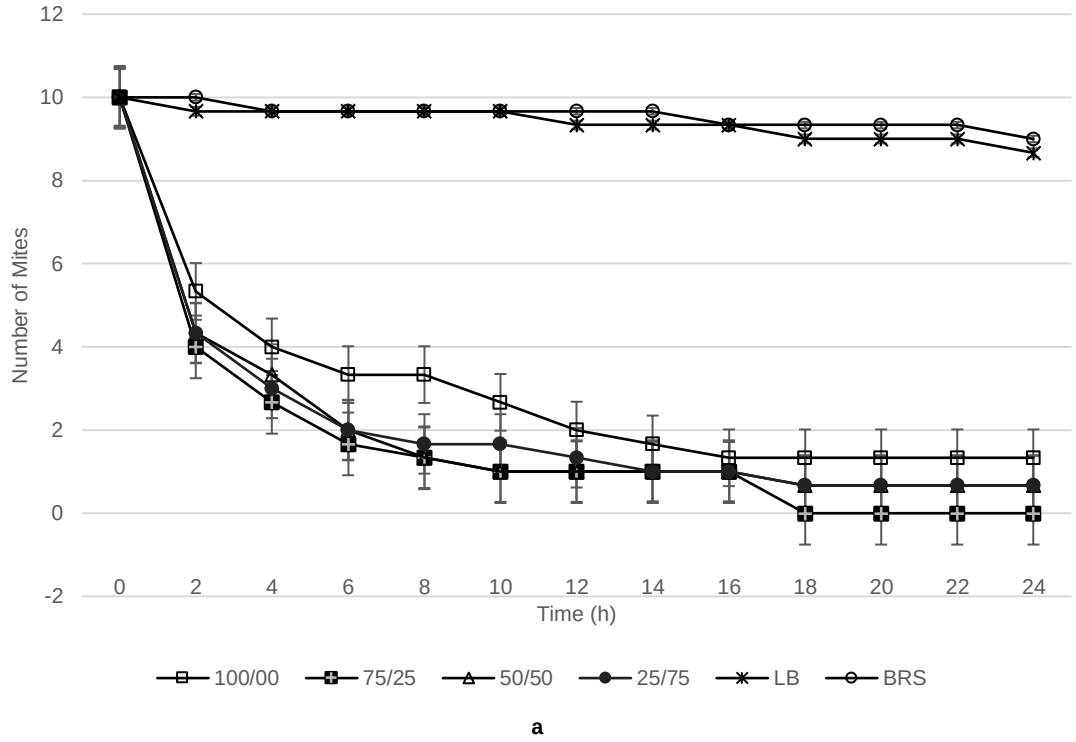
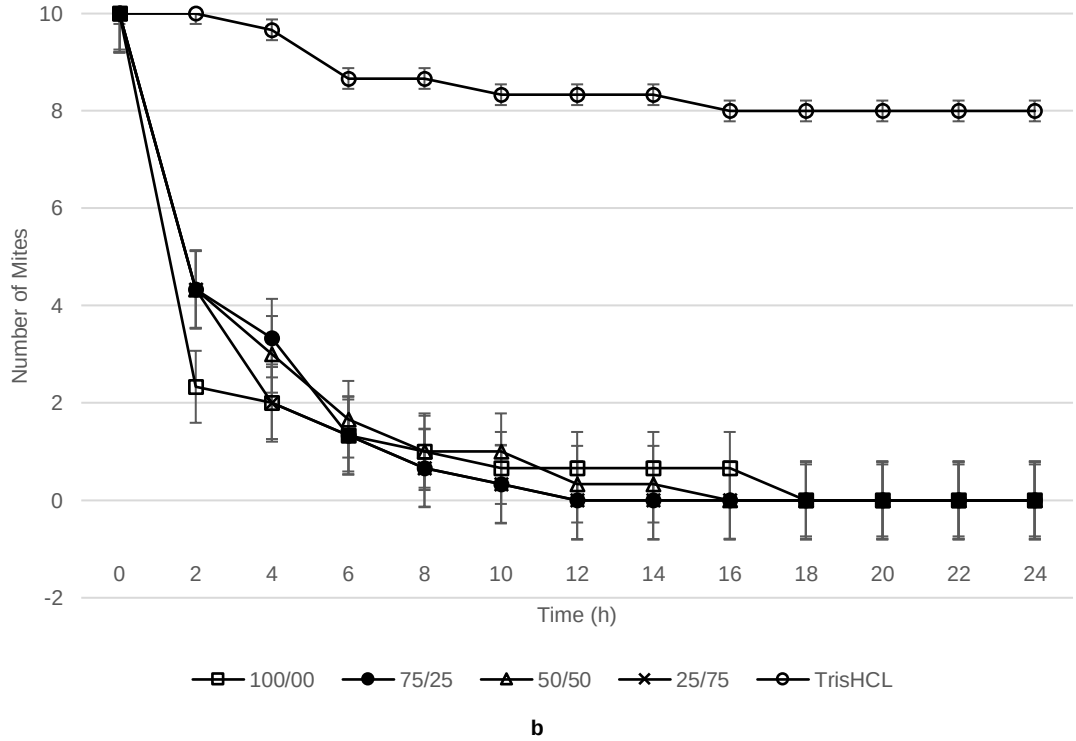


Figure 3. Growth curve of bacterial culture showing OD_{600} values over time.



a



b

Figure 4. Effect of *Bifidobacterium* sp. ERSapi20 on the survival of *V. destructor* mites over time. The graph presents the mean number of surviving *V. destructor* mites, with standard error values included for each concentration tested: a) effect of extracellular solution of ERSapi20 at different concentrations on the number of living mites. Concentrations were prepared by mixing extracellular solution with insect ringer solution (BRS) in different ratios (extracellular solution / insect ringer solution, v/v). Mites sprayed both sterilized LB broth and BRS. Also, LB and BRS served as negative controls; b) effect of intracellular extract of ERSapi20 on the number of living mites using different concentrations of extract. Concentrations were prepared by mixing intracellular extract with Tris-HCl buffer (intracellular solution / Tris-HCl buffer v/v). Tris-HCl buffer was used as a negative control.

Table 1. Effect of ERSapi20 strain on the*Varroa destructor**

Sprayed agents	LT_{50}[a] (h)	LT_{90}[b] (h)
ERSapi20e*	3.0 ± 1.0	16.0 ± 0.5
ERSapi20e : IRS** (75:25, v/v)	1.5 ± 0.5	10.0 ± 0.5
ERSapi20e : IRS (50:50, v/v)	1.5 ± 0.5	10.0 ± 0.5
ERSapi20e : IRS (25:75, v/v)	2.0 ± 0.5	14.0 ± 0.5
LB broth control	ND	ND
IRS control	ND	ND
ERSapi20i***	1.5 ± 0.25	8.0 ± 0.5
ERSapi20i : TrisHCl (75:25, v/v)	2.0 ± 0.5	4.0 ± 0.5
ERSapi20i : TrisHCl (50:50, v/v)	2.0 ± 0.5	4.0 ± 0.5
ERSapi20i : TrisHCl (25:75, v/v)	2.0 ± 0.5	4.0 ± 0.5
TrisHCl control	ND	ND

[a]: Times for 50% mortality of mites; [b]:Times for 90% mortality of mites.

ND: Not detected. *: extracellular solution of ERSapi20; **: insect ringer solution; ***: intracellular extract of ERSapi20.

The survival probability for all concentrations was found to be close to 1.0 within a 24-hour timeframe. This suggests that the survival rate of the bees exposed to the different concentrations of the bacterial solution was similar to that of the control group. In other words, the bacterial solution did not have a significant adverse effect on the bees' survival within the tested time frame. These findings have important implications, as they indicate that the use of the bacterial solution, even at different concentrations, did not lead to a significant reduction in the bees' survival rate. It supports the potential safety and effectiveness of the bacterial solution as an intervention method against varroa mites without harming the honeybees.

The survival probability test and ggplot revealed significant differences in mite mortality rates between the control group and treatment groups exposed to the extracellular solution and intracellular extract of *Bifidobacterium* sp. ERSapi20 (Figures 5 & 6). These findings suggest that the bioactive compounds derived from the strain have a substantial impact on varroa mite survival ($p < 0.05$).

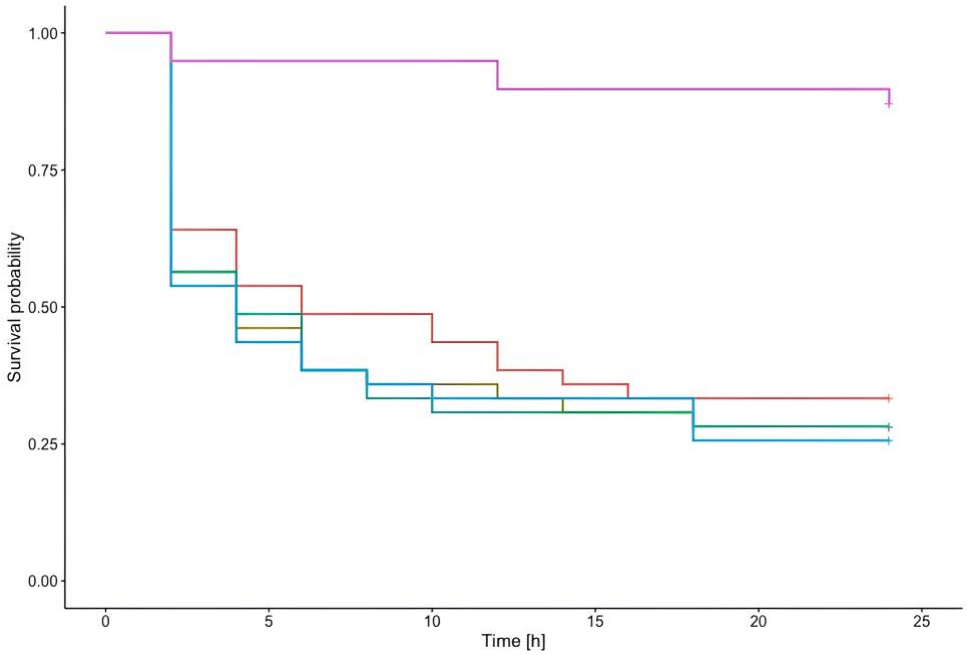


Figure 5. Survival analysis of Varroa mites exposed to varying concentrations of *Bifidobacterium* sp. ERSapi20. The Kaplan-Meier survival curves show no statistically significant differences among the different bacterial solution concentrations tested. Concentrations are represented by different colors: red (100% concentration), brownish (75% concentration), green (50% concentration), blue (25% concentration), and pink (control group).

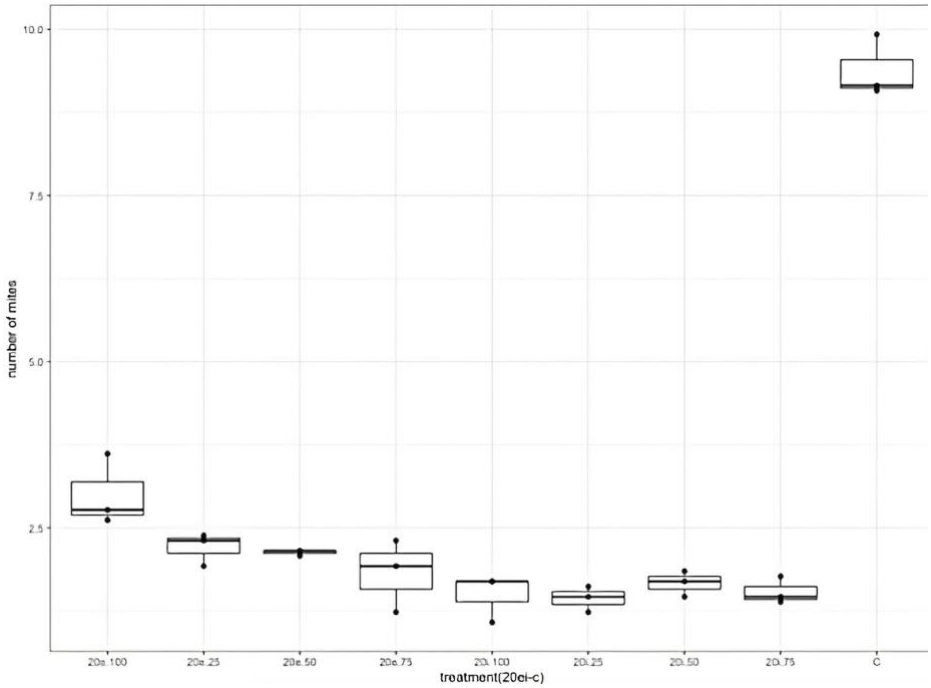


Figure 6. Acaricidal activity of intracellular and extracellular solutions of ERSapi20 against *Varroa destructor* analyzed using ggplot. The figure shows the acaricidal efficacy of different concentrations of intracellular and extracellular solutions compared to the control group. The extracellular solution at 75% concentration exhibited the highest acaricidal activity, significantly reducing mite numbers compared to other tested concentrations and controls.

Discussion

The findings of this study provide a thorough analysis of the actinobacterial strains colonizing the honeybee surface in Türkiye. The findings showed that a strain of *Bifidobacterium* sp. ERSapi20 isolated from worker bees using a culture-based method produced bioactive compounds that exhibited strong acaricidal activity against *Varroa destructor* Oudemans, 1904 (Acari: Varroidae). Moreover, 90% mite mortality rate was achieved by the extracellular solution of the strain within 16 hours, whereas complete mite mortality was observed within 18 hours with the intracellular extract. This is the first report of the acaricidal activity against *V. destructor* using the intracellular extract of actinobacterial strains in Türkiye. This publication also represents the first use of a culture-dependent approach to investigate *Bifidobacterium* strain, making it a significant contribution to the field of bee health regulation. Thus, it was aimed to find new solutions for controlling the varroa mites that harm honeybees. The acaricidal potential of *Bifidobacterium* sp. ERSapi20 (GenBank accession no. OQ073505) was evaluated, focusing on its ability to produce bioactive metabolites effective against mites.

The bacterial pre-cultivation step is an important stage to isolate specific groups of microorganisms. In this context, peptone water was the most commonly used diluent for the enumeration of *Bifidobacteria* sp. (Roy, 2001). Therefore, in our laboratory, peptone water (0.95%) was used for the isolation of Bifidobacteria from the surface of honeybees. Additionally, to mimic of bee-keeping field conditions, the incubation temperature was fixed to 31±1ºC in the dark during this study, minimizing the effects of environmental factors on varroa mites. This bioassay strategy was reported for the first time in the literature. In the growth of bacterial cultures, a succession of phases, characterized by a growth curve, may be conveniently distinguished (Monod, 1949). Moreover, certain environmental stresses, among which is starvation, induce toxin–antitoxin (TA) production. During the stationary phase, a range of secondary metabolites, including antibiotics and toxins, are synthesized (Engelberg-Kulka et al., 2006; Duquesne et al., 2007). In this regard, ERSapi20 growth curve was determined to achieve the end of the stationary phase and the beginning of the death phase, which is the most common stage for releasing secondary metabolites into the exhausted media.

Varroa mite has been reported to be susceptible to the entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana*, *Verticillium lecanii*, *Hirsutella* spp., and *Hirsutella thompsonii* (Chandler et al., 2000, 2001; Peng et al., 2002; Shaw et al., 2002; Hamiduzzaman et al., 2012) Moreover, most of the literature has focused on the

bacterial communities of the gut microbiome (Crotti et al., 2013), the gastrointestinal tract or stored food (Anderson et al., 2013; Asama et al., 2015; Kwong & Moran, 2016), and the acquisition of bacteria from the environment (Aizenberg-Gershtein et al., 2013). The present study resulted in a microbial collection of strain belonging to the *Actinobacteria* phyla, which have been frequently found in the pollination environment, alimentary tract, and stored food of honeybees (Martinson et al., 2011; Ganeshprasad et al., 2022).

Although the genera *Bifidobacterium* were commonly found in bees' gut (Ganeshprasad et al., 2022), the isolation of *Bifidobacterium* from the surface of bees was also confirmed in this study, as previously reported by Saccà and Lodesani (2020). Both extra- and intracellular solutions of ERSapi20 caused a significant increase in mite mortality (Figure 4). Strains of the genus *Bifidobacterium* have already been reported to be pathogens of varroa mites. Bioassays done by Saccà & Lodesani (2020) showed strain of *B. asteroides* isolated from an apiary located in Italy. By comparison, our study result was found to be more effective than the finding published by Saccà & Lodesani (2020). This result disclosed that the same species isolated from different regions might have different effects on mites' mortality. On the other hand, some studies have shown the effectiveness of lactic acid bacteria in the biocontrol of varroosis in bees. Tejerina et al. (2020) reported a 50-80% reduction in varroosis levels with the use of *Lactobacillus salivarius* A3iob, and de Piano et al. (2020) found that strains of *L. johnsonii* AJ5 and *Enterococcus faecium SM21* improved bee survival and increased bee protein levels. Saccà & Lodesani (2020) conducted in vitro experiments which showed that bacterial cultures of *L. kunkeei* BO-G12 caused a 95-100% mortality rate of the mite within a period of three days. Nevertheless, the present study results show that members of the actinobacteria group have promising potential in terms of duration and effect of toxicity against the mite.

The present study indicated that intracellular extract of ERSapi20 was more rapidly affected varroa mites than the extracellular solution. This study has shown that adult honeybees inoculated with bacterial suspensions of ERSapi20 by directly applying them onto their bodies had not affected under laboratory conditions. In addition, the survival probability test conducted in R Studio highlighted the significant acaricidal activity of *Bifidobacterium* sp. ERSapi20 against varroa mites. These findings underscore the importance of this research in the development of bee health products and integrated pest management strategies. However, additional research is necessary to validate these results, address limitations, and explore potential avenues for further investigation. Conversely, previous studies have shown that adult honeybees inoculated with spores of entomopathogenic fungi onto their bodies had higher mortality than non-inoculated bees (Hamiduzzaman et al., 2012).

Interestingly, the similar mean and standard deviation values observed for different concentrations of ERSapi20 mixed with Tris-HCl buffer (75:25, 50:50 and 25:75 v/v) suggest that the acaricidal effect reaches a plateau within this concentration range. This indicates that the efficacy of ERSapi20 may not significantly increase beyond a certain concentration threshold. The consistent results imply stability and reproducibility, but further investigation is needed to understand the underlying mechanisms. Additional studies exploring a broader concentration range could help clarify whether these trends hold at different levels and identify the optimal formulation for practical use.

In summary, this study is the first to utilize a culture-dependent approach to investigate actinobacterial strains colonizing the honeybee microbiota in Türkiye, highlighting the uniqueness of our findings in this region. The identification of *Bifidobacterium* sp. ERSapi20 and its acaricidal activity against varroa mites is a significant step forward in the development of novel bee health products and integrated pest management strategies. As similar studies have not been conducted in Türkiye before, our research fills a crucial gap in understanding the potential of actinobacterial strains for controlling varroa mites. Further research is necessary to confirm these findings and address any potential limitations of the study.

Conclusion

Beekeepers constantly look for high-quality products combined with a good quality/price ratio (Alberoni et al., 2016). Therefore, this study may be an opportunity to find a new solution to *V. destructor*. The virulence of *Bifidobacterium* sp. ERSapi20 against varroa mites needs to be reassessed by additional tests conducted under bee-keeping conditions similar to those, as the physical environment of the honeybee colony will be a major factor ensuring the success and sustainability of any prospective biological control agent. This strain holds potential for integration into pest management strategies or development of bee health products. The study findings are

promising, but it is important to address potential limitations and further explore the scope of this research. One potential limitation could be the relatively small sample size used in the study, which may affect the generalizability of the results. Potential confounding factors and limitations of the experimental design that relate to this research could be further investigated in future studies.

Even if this study proved that the bacterial solution of ERSapi20 is harmless to the honeybees, several experiments should be investigated, such as effects on the fecundity of queen. To resolute method against the mite, a comprehensive approach that emphasizes understanding *V. destructor* biology and its interaction with honeybees is essential. This approach should involve in silico, in vivo, semi-field, and field-scale (Vilarem et al., 2021). Further research is necessary to ascertain the prolonged impacts of microbial solutions on bees, both in controlled laboratory settings and field conditions.

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