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Chemical Characterization and Antibacterial Activity of Royal Jelly Against Multidrug-Resistant Pathogens

Running head: Royal Jelly Against Multidrug-Resistant Pathogens

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SUMMARY

Research background. Given the known antibacterial properties of royal jelly (RJ), we hypothesized that RJ could inhibit priority multidrug-resistant (MDR) bacteria, including different strains of vancomycin-resistant *Enterococcus faecium* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Klebsiella pneumoniae* (CRKP), and *Acinetobacter baumannii*

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(CRAB). We further proposed that RJs antibacterial efficacy may be influenced by its chemical composition and by the inter- and intraspecies variability among MDR pathogens.

Experimental approach. RJ samples were collected from five beekeepers (RJ1–RJ5) in Mediterranean and continental regions of Croatia. Chemical profiling was performed using solid-phase microextraction gas chromatography–mass spectrometry (HS-SPME/GC-MS), Fourier-transform infrared (FTIR) spectroscopy, along with separate assays to measure antioxidant capacity (ABTS) and quantify the content of bioactive compounds. Antibacterial activity was assessed by agar well diffusion assay and by determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against 20 MDR strains of VRE, MRSA, CRKP and CRAB, selected from 85 isolates using rep-PCR genotyping. MDR status was confirmed through standard susceptibility testing.

Results and conclusions. All RJ samples showed strong antioxidant activity and high levels of bioactive compounds, with RJ1 consistently exhibiting the highest content of ABTS, polyphenols, flavonoids, and proteins. FTIR analysis revealed variation in carbohydrate and lipid composition among samples, while protein content remained relatively uniform, and indicated the highest concentrations of sugars, lipids, and proteins in RJ1. GC-MS identified octanoic acid (48.09–83.07 %) as the predominant volatile compound, particularly abundant in RJ1 and RJ4. Despite some variability in chemical profiles, both chemical composition and antibacterial activity were comparable between samples from Mediterranean and continental regions. All RJ samples inhibited MDR bacteria, suggesting a potential synergistic effect of crude RJs, with inhibition zones ranging from 11.8 mm (CRKP) to 16.8 mm (MRSA). *A. baumannii* was most susceptible (MIC/MBC=27.2 µg/mL), while *E. faecium* was the most resistant (MIC=96.6 µg/mL, MBC=126.4 µg/mL). Beyond interspecies differences, pronounced strain-level variability in antibacterial response was also observed.

Novelty and scientific contribution. This is the first study to simultaneously evaluate RJ's antibacterial activity against multiple strains of clinically relevant MDR pathogens alongside comprehensive chemical profiling. Importantly, it reveals for the first time that RJ's efficacy varies not only between species but also among strains within the same species, emphasizing the need to consider strain-level differences in future assessments.

Keywords: multidrug-resistant bacteria; royal jelly; antibacterial activity; gas chromatography–mass spectrometry (GC-MS); Fourier-transform infrared (FTIR) spectroscopy; antioxidant capacity and bioactive compounds

INTRODUCTION

Multidrug-resistant (MDR) bacteria are deadly pathogenic microorganisms that pose a serious threat to human health. In 2019 alone, an estimated 6.27 million people died from infections caused by antibiotic-resistant pathogens, and the World Health Organization (WHO) warns that this number could continue to rise (*1*). MDR bacteria are characterized by an increased level of acquired resistance to multiple classes of antibiotics and are involved in a variety of life-threatening healthcare-associated infections. In the past, these types of antibiotic-resistant bacteria were rare and limited to nosocomial infections, but today they are very common. In addition to hospitals, they are frequently found in various environmental samples, including soil, food (such as vegetables, fruit and animal products), water, plants and sewage (*2*). In 2024, WHO revised the Bacterial Priority Pathogens List (BPPL) which includes bacterial pathogens of public health importance (*1*). In this updated BPPL, Gram-negative bacterial pathogens maintain their critical status. Carbapenem-resistant *Acinetobacter baumannii* (CRAB), carbapenem-resistant Enterobacterales (CRE) and third-generation cephalosporin-resistant Enterobacterales (3GCRE) received the highest scores, confirming their inclusion in the critical priority category. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) remain in the high-priority pathogen category. Infections caused by both categories of priority pathogens are extremely difficult to treat, as they not only exhibit multidrug resistance but also show resistance to last-resort antibiotics including carbapenems (*1*). This severely restricts treatment options and leads to a longer duration of illness, a higher burden on the healthcare system and a higher mortality rate (*2*). The global rise of antibiotic resistance has intensified research efforts into alternative therapeutic strategies against MDR pathogens, with particular interest in natural bioactive compounds such as royal jelly (RJ).

RJ is a yellowish, acidic secretion produced by the hypopharyngeal and mandibular glands of honey bees (*Apis mellifera* L.) (worker bees) for the nourishment of the brood (larvae) and the queen bee. It is composed mainly of water, proteins, sugars, and lipids and is highly sensitive to heat and light (*3*,*4*). In recent years, the use of RJ has increased due to its beneficial properties and health-promoting effects, with great potential in medical and pharmaceutical applications, cosmetics, and as a functional food. The antibacterial properties of RJ against both Gram-positive and Gram-negative bacteria have been well documented (*3*), with a stronger inhibitory effect observed against Gram-positive pathogens (*5*). In addition to its antibacterial properties, RJ also exhibits anti-inflammatory and immune-stimulating effects (*5*). The antibacterial activity of RJ is primarily attributed to the protein royalisin, major royal jelly proteins (MRJPs), jelleines I–III, and the fatty acid 10-hydroxy-2-decenoic acid (10-HDA) (*3*). However, the overall antibacterial effect of whole RJ remains significant, as its complex composition may contribute synergistically to its bioactivity. Notably, the antibacterial

potency of RJ is influenced by several factors, including the geographical origin, the genetic and physiological characteristics of the bee colony and the botanical composition of the harvested plant species (*3*). Despite the proven antibacterial effect, the number of conducted studies of RJ against priority MDR bacterial pathogens is very low. Studies have shown that RJ has antibacterial activity against methicillin-resistant *S. aureus* (MRSA; MIC 37.5-75 mg/mL) (*5*), and the effect is highly dependent on the RJ sample applied. However, there is no data on the effect of crude RJ on other priority pathogens such as carbapenem-resistant *A. baumannii* (CRAB) or Enterobacterales (CRE) as well as on vancomycin-resistant *E. faecium* (VRE). Moreover, to conclude the influence of an active compound on a particular bacterial species, the intraspecies variability should be taken into account. The importance of variability within the species has been particularly well studied in the context of pathogenicity, human microbiome (*6*) and environmental samples (*7*). However, the effect of RJ on different MDR strains within the same species has not been evaluated at all.

Therefore, this study aimed to explore the efficacy of RJ against genetically diverse strains of priority MDR pathogens, including both Gram-positive (vancomycin-resistant *E. faecium* and methicillin-resistant *S. aureus*) and Gram-negative (carbapenem-resistant *K. pneumoniae* and carbapenem-resistant *A. baumannii*) clinical and environmental isolates, complemented by comprehensive chemical characterization of RJ samples. To achieve this aim, RJ samples were collected from five beekeepers across two geographic regions of Croatia, continental and Mediterranean. Antibacterial activity was assessed through agar well diffusion method followed by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Chemical profiles were obtained through headspace solid-phase microextraction (HS-SPME) followed by gas chromatography–mass spectrometry (GC-MS) analysis, Fourier-transform infrared (FTIR) spectroscopy, while bioactivity assessment included evaluation of antioxidant capacity and quantification of bioactive compound content. To the best of our knowledge, this is the first comprehensive study to simultaneously investigate the antibacterial effects of RJ against different strains of priority MDR pathogens alongside detailed chemical profiling of the samples.

MATERIALS AND METHODS

Sampling

RJ samples were collected in Croatia during the production season 2022, directly from the beekeepers maintaining colonies of Carniolan honey bees (*Apis mellifera carnica*, Pollmann, 1879). Two samples, RJ1 and RJ2, were obtained from Konšćica, Zagreb County and RJ3 from Petrovsko, Zagorje County, both located in the continental part of Croatia. The remaining two samples, RJ4 and RJ5, originated from farms located near Obrovac Sinjski, Dalmatia County, in the Mediterranean

region of Croatia. To preserve their integrity, all RJ samples were stored in dark containers and immediately frozen at -20 °C after collection. They were transported to the laboratory in a frozen state and maintained at -20 °C until analysis.

Determination of antioxidant capacity, total polyphenolic content (TPC) and total flavonoid content (TFC)

A total of 0.5 g of RJ was thoroughly homogenized with 5 mL of distilled water. The subsequent suspensions were then filtered using Whatman No. 4 filter paper (Sigma-Aldrich, St. Louis, MO, USA) (*8*). The results obtained were calculated based on a 100 g RJ sample.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, St. Louis, MO, USA) was used to measure the antioxidant potential of the RJ samples, according to the procedure of Re *et al*. (*9*). The data obtained are expressed as μmol Trolox equivalents (μmol TE/100 g).

A modified Folin-Ciocalteu's method (*10*) was used for determination of total polyphenolic content (TPC). Briefly, a volume 0.1 mL of RJ samples was mixed with 7.9 mL of distilled water. Then, 0.5 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) (diluted 1:2 with distilled water) and 1.5 mL of 20 % Na_2CO_3 (Sigma-Aldrich, St. Louis, MO, USA) were added. After 2 h, absorbance was measured at 765 nm using a UV-Vis spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). The calibration curve was established using gallic acid (Sigma-Aldrich, St. Louis, MO, USA) and results were expressed as mg gallic acid equivalents (GAE) per 100 g of RJ.

The total flavonoid content (TFC) was determined using the previously reported method (*11*). A 1 mL volume of the RJs was added to a 10 mL volumetric flask containing 4 mL of distilled water. Next, 300 μL of a $NaNO_2$ (Sigma-Aldrich, St. Louis, MO, USA) solution (0.5 g/L) was added to the suspension. After 5 min a volume of 300 μL of $AlCl_3$ (Sigma-Aldrich, St. Louis, MO, USA) (1 g/L) was added. Six minutes later, 2 mL of NaOH (Sigma-Aldrich, St. Louis, MO, USA) (1 mol/L) was added to the mixture. The final volume was adjusted to 10 mL using distilled water. Absorbance was measured at 360 nm against a blank (distilled water) with a UV-Vis spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). A calibration curve was generated using the quercetin (Sigma-Aldrich, St. Louis, MO, USA) standard, and results were expressed as mg of quercetin equivalents (QE) per 100 g of RJ.

Total proteins (TP) were determined by using the Lowry method (*12*). Two reagents were prepared: reagent A (2 % (*m/V*) Na_2CO_3 (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mol/L NaOH (Sigma-Aldrich, St. Louis, MO, USA)) and reagent B (0.5 % (*m/V*) $CuSO_4 \cdot 5\ H_2O$ (Sigma-Aldrich, St. Louis, MO, USA) in 1 % (*m/V*) $KNaC_4H_4O_6 \cdot 4H_2O$ (Sigma-Aldrich, St. Louis, MO, USA)). Reagent A (50 mL) was mixed with reagent B (1 mL) to obtain reagent C. A volume of 0.2 mL of Folin-Ciocalteu reagent (1:2 parts of water, *V/V*) was added to 0.4 mL of water-solubilized RJ in a test tube. Finally,

2 mL of Reagent C was added and 50 min after the start of the chemical reaction, absorbance was measured at 740 nm against a blank using a UV-1700 Spectrophotometer (Shimadzu, Kyoto, Japan). A bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) standard was used for the calibration curve and results were expressed as mg of bovine serum albumin equivalents (BSAE) per 100 g of RJ.

Fourier transform infrared spectroscopy (FTIR) - attenuated total reflectance (ATR)

Samples of RJ were analysed by Fourier transform infrared spectroscopy (FTIR) coupled with attenuated total reflectance (ATR) recording technique, according to the Hu *et al*. (*13*) general instrumentation requirements and methodology modified for the acquisition of infrared spectra of RJ samples in its genuine form (as obtained). FTIR-ATR spectra of studied RJ samples were recorded by Cary 660 Fourier transform mid-infrared spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled with single-reflection diamond Golden Gate ATR accessory (Specac Ltd, Orpington, UK). The FTIR-ATR spectra of RJ were acquired in a mid-infrared region (4000–400 cm^{-1}) using the nominal recording resolution of 4 cm^{-1}. Two replicate spectra of each RJ sample were recorded using different aliquots (32 scans were collected for each spectrum). Spectra of RJ samples were recorded at room temperature (24±2 °C). Raw spectral data were stored and pre-analysed using Resolutions Pro version 5.3.0 (*14*) FTIR software (Agilent Technologies, Santa Clara, CA, USA), while further qualitative spectral data analysis was performed using Spectragryph optical spectroscopy software (version 1.2.15) (*15*) and Origin 8.1 (Origin Lab Corporation) (*16*).

Headspace solid-phase microextraction (HS-SPME) and gas chromatography and mass spectrometry (GC-MS)

The portion of 0.2 g of each sample was put separately in 20 mL headspace vial, sealed with PTFE-silicon septum, and the headspace was extracted by a manual holder (Supelco Co., Bellefonte, PA, USA) using two fibres: divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS; 50 µm (DVB layer) and 30 µm (CAR/PDMS layer); fibre length 1 cm) and polydimethylsiloxane/divinylbenzene (PDMS/DVB; 65 µm (PDMS/DVB), fibre length 1 cm) purchased from Supelco Co. (Bellefonte, PA, USA). The fibres were conditioned according to Supelco instructions. Equilibration of the sample was carried out for 15 min at 60 °C, and the sample was extracted for 40 min. Thermal desorption of the fibre was performed directly to the GC column for 7 min at 250 °C.

The gas chromatography mass spectrometry (GC-MS) analysis was performed using an Agilent Technologies (Santa Clara, CA, USA) gas chromatograph model 7820A equipped with a mass

selective detector (MSD) model 5977E (Agilent Technologies, Santa Clara, CA, USA) and a HP-5MS capillary column (5 % phenylmethylpolysiloxane, Agilent Technologies, Santa Clara, CA, USA). The GC conditions were: the oven temperature was isothermal at 70 °C for 2 min, then was increased from 70 to 200 °C at 3 °C/min, and hold isothermal at 200 °C for 15 min; carrier gas was He (1.0 mL/min). The MSD (EI mode) was operated at 70 eV with the mass range 30-300 amu. The GC-MS analyses were performed in triplicate, and mean values of the area percentages were determined. The identification of the compounds involved comparison of their mass spectra with the mass spectral libraries: Wiley 9 (Wiley, New York, NY, USA) and NIST 14 (National Institute of Standards and Technology, Gaithersburg, MD, USA) with the selectivity index (the highest probability of the experimental mass spectrum matching the mass spectrum from the reference library) set up to 95 %. Additionally, retention indices (RI) calculated based on the retention times C_9-C_{25} *n*-alkanes for each compound were compared with those reported in the literature (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Origin of multidrug-resistant bacterial isolates

Bacterial isolates of *K. pneumoniae* (*N*=40) and *E. faecium* (*N*=9) used in this study were previously isolated from treated municipal and untreated hospital wastewater in Zagreb, Croatia, as described by Puljko *et al.* (*17*), and were kindly provided by the Laboratory for Environmental Microbiology and Biotechnology, Ruđer Bošković Institute. *Acinetobacter baumannii* (*N*=10), *Staphylococcus aureus* (*N*=15) and *E. faecium* (*N*=11) were isolated from clinical specimens and were kindly provided by the Clinic for Infectious Diseases “Dr. Fran Mihaljević”, Zagreb, Croatia. All environmental isolates were identified by MALDI-TOF (Bruker Daltonik, Bremen, Germany) whereas clinical isolates were identified by VITEK 2 System (BioMerieux, Marcy-l'Étoile, France).

Extraction of DNA and genotyping of bacterial isolates

Genomic DNA was extracted from all bacterial isolates (*N*=85) using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Genotyping was then performed using repetitive element PCR (rep-PCR) with the $(GTG)_5$ primer (Microsynth Austria,, Wien, Austria) (*18*). Obtained rep-PCR patterns were analysed in BioNumerics 7.6.1. software (Applied Maths, Sint-Martens-Latem, Belgium) (*19*), and the genetic similarity of the isolates was calculated based on the Dice coefficient. Isolates were then clustered by the Unweight Paired Group Arithmetic Average (UPGMA) method and the dendrograms were created with a 1.0 % tolerance level and a 0.5 % optimization. Based on the obtained profiles, representative isolates (*N*=5) were selected from each group for further analysis.

Antibiotic susceptibility testing

All representative strains were screened for antibiotic susceptibility using Kirby-Bauer disk diffusion method to prove their MDR profile. McFarland standard 0.5 which contains approximately $1.5 \cdot 10^8$ CFU/mL was reached by adding individual colonies of each strain to a sterile 0.85 % saline solution (DEN-1 densitometer, Biosan, Riga, Latvia). Mueller-Hinton (MH) agar plates (Biolife, Monza, Italy) were then inoculated with $1.5 \cdot 10^8$ CFU/mL bacterial suspension, followed by the addition of antibiotic discs (BD BBL™ Sensi-Disc™, Becton Dickinson and Company, Franklin Lakes, NJ, USA). After 24 h incubation at 37 °C, zones of inhibition were measured and interpreted according to CLSI guidelines (*20*,*21*). Antibiotics were selected based on CLSI recommendations for each species and are listed in Table S1.

Screening of antibacterial activity using the agar well diffusion method

Antibacterial activity was evaluated first using a modified Kirby-Bauer agar well diffusion method (*20*). For each RJ sample, a working solution (1 g/mL) was prepared by dissolving 6 g of RJ in 6 mL of sterile MH broth (Biolife, Monza, Italy) using a vortex mixer. To protect the RJ from light degradation, the solutions were wrapped in aluminum foil immediately after preparation. MH agar plates were inoculated with a bacterial suspension adjusted to $1.5 \cdot 10^8$ CFU/mL, as described above. Wells of 9 mm in diameter were aseptically punched into the agar using a sterile cork borer. Each well was filled with 0.2 mL of the RJ working solution, applied in duplicate. As a negative control, 0.2 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used. The plates were then incubated at 37 °C for 24 h, after which the zones of inhibition, including the diameter of the well, were measured.

Determining MIC and MBC

MIC was determined using the microdilution method with the addition of resazurin (Santa Cruz Biotechnology, Dallas, TX, USA) (resazurin microplate assay, REMA; *22,23*). This method involves the serial dilution of royal jelly in a 1:2 ratios in MH broth. Testing was performed in 96-well microtiter plates, where RJ samples were initially diluted aseptically (7.81, 15.63, 31.25, 62.50, 125.00, 250.00, and 500.00 mg/mL) in MH broth.

Each bacterial inoculum ($1.5 \cdot 10^8$ CFU/mL) was diluted in sterile saline solution to $1.5 \cdot 10^6$ CFU/mL and subsequently added to microtiter wells to reach $1.5 \cdot 10^5$ CFU/mL per well. A control included MH broth with bacterial inoculum but without RJ. After inoculation, 15 µL of resazurin (0.02 % solution) was added to each well as a bacterial growth indicator. Plates were incubated at 37 °C under constant shaking (90 rpm) for 24 h. A blue to pink colour change indicated bacterial growth. The lowest RJ concentration at which no colour change occurred (*i.e.* no visible growth) was recorded

as the MIC. For MBC, contents of each well were transferred onto Brain Heart Infusion (BHI) agar plates (Biolife, Monza, Italy) in quadruplicate and incubated at 37 °C for 24 h, after which bacterial colonies were counted. The percentage of dead cells following RJ treatment was calculated according to Eq. 1.

$$\text{Dead bacterial cells} = \left(1 - \frac{CFU2}{CFU1}\right) \cdot 100 \qquad /1/$$

where CFU1 represents the number of bacteria initially added to the wells, and CFU2 represents the number of bacteria that survived RJ treatment.

To confirm the CFU1, an additional serial microdilution was performed in a 96-well plate using a sterile 0.85 % saline solution. The prepared bacterial inocula ($1.5 \cdot 10^6$ CFU/mL) were aseptically diluted 1:10 from row A to row H, and the contents were transferred onto BHI agar in quadruplicate. After incubation at 37 °C for 24 h, CFU/mL was calculated. Finally, the RJ concentration at which more than 99.9 % of bacterial cells were killed was recorded as the MBC, while the concentration at which more than 99.5 % of bacterial cells were inhibited was recorded as the MIC (*24*).

Statistical analysis

All data is shown as mean values with standard deviations. Based on the normal distribution and homogenous variance of the data, significant differences in antioxidant activity, TPC, TFC and TP were assessed by ANOVA, and between group differences were identified by *post hoc t*-tests, where p values were adjusted by Bonferroni correction. Given the non-normal distribution and heterogeneous variance of the data, statistically significant differences in inhibition zones, MIC and MBC values were assessed using Kruskal-Wallis test. To determine statistically significant differences between the groups, the multiple pairwise comparisons were performed using *post hoc* Dunn's test, with p values adjusted for multiple comparison using the Bonferroni correction. For all analysis, differences with $p<0.05$ were considered statistically significant. All statistical analysis waere performed in R environment version 3.0.2 (*25*).

RESULTS AND DISCUSSION

Antioxidant activity, total polyphenolic content, total flavonoid content and total proteins in royal jelly

Significant differences in antioxidant activity were observed for the tested RJs (Table 1), irrespective of the region (Mediterranean or continental). RJ4 and RJ5 collected from the Mediterranean region had similar ABTS values but were not significantly different compared to other tested RJs. Interestingly, significant differences were observed for samples collected from continental areas (RJ1 and RJ2). The antioxidant activity of RJs can be described by the wide range of bioactive compounds they contain. For example, the major fatty acid showing antioxidant activity in RJ is 10-

hydroxydecanoic acid (10-HDA) and is only present in RJ, but not in other natural raw materials or apiculture products (*26*). Furthermore, since polyphenolic compounds are generally recognized as key contributors to antioxidant activity, it is likely that they are also responsible for the antioxidant activity of our RJ samples. The TPC in RJ ranged from 355.66 to 465.27 mg GAE/100 g and the lowest value was recorded from the continental location Konšćica (RJ2) and was significantly lower compared to RJs collected from Dalmatia County. Similar values were reported from six different RJ samples collected from Morocco, Portugal and Spain, ranging from 300–900 mg GAE/100 g (*27*). With the same method of preparation, Pavel *et al*. (*8*) reported much higher values for TPC determined in Romanian RJ, with averages from 2325 mg GAE/100 g for commercial to 2349 mg GAE/100 g for locally produced RJ. Nabas *et al*. (*28*) also report very similar and higher values, with an average of 2330 mg GAE/100 g. Čeksterytė *et al*. (*29*) reported lower values of 1070 mg GAE/100 g for Lithuanian RJ solubilized in methanol/water. Harvesting time has been shown to significantly affect RJ´s antioxidant compound content, including TPC (*30*). Özkök and Silici (*31*) reported a significantly lower TPC value (59.16 mg GAE/100 g) in Turkish RJ, likely due to use of pure methanol as a solvent. This indicates the impact of solvent choice, with water (or methanol/water mixture) appearing more effective for TPC solubilization. Overall, TPC data in RJ remains scarce and are markedly different. Regarding the total flavonoids in this work, it can be observed that they ranged from 14.44 to 18.20 mg QE/100 g, aligning with values reported by El-Guendouz *et al*. (*27*), who found 10-50 mg QE/100 g in RJ samples from Morocco, Portugal, and Spain. In contrast, Nabas *et al*. (*28*) reported significantly higher values (128 mg rutin/100 g) in Jordanian RJ, which also showed significantly higher values of TPC, as abovementioned. The highest total protein content herein was recorded for RJ1 (16.48 %), significantly exceeding all other RJ samples, while the lowest was in RJ2 (12.8 %), despite both being from the same area. These values align with literature reports, typically ranging from 11.4 % to 15.8 % (*26*). For example, in 19 local Romanian RJ samples, protein content ranged from 9.58–16.38 % using the Lowry method and 8.15–17.73 % by Bradford method (*8*). Nabas *et al*. (*28*) reported 13.15 % of total proteins in RJs produced in Jordan. The protein content reported in the literature is variable and depends on the method of protein determination, but overall, reported protein values tend to be more consistent and reliable than TPC or TFC. Generally, literature on RJ is scarce and values for compounds like polyphenolics and flavonoids, as well as antioxidant activity, are largely underreported. One of the goals of this work is to expand the knowledge of bioactive compounds and the antioxidant activity of RJs collected from Croatian continental and Mediterranean sites. The observed variability in values among samples from the same area indicates that, beyond geographic origin, other parameters likely influence the quality of RJ.

Table 1

Chemical characterisation of RJ by FTIR-ATR spectroscopy

Fig. 1 shows the FTIR-ATR spectra of fresh RJ samples representing comparative spectral features with assignation of major underlying molecular vibrations reflect the overall chemical composition of analysed RJ samples. According to Sabatini *et al*. (*4*), fresh RJ is typically composed of 60–70 % of water, 9–18 % of proteins, 7–18 % of carbohydrates (*i.e.* fructose+glucose+sucrose content), 3–8 % of lipids, >1.4 % of 10-hydroxy-2-decenoic acid (10-HDA), and 0.8–3 % of ash. Fructose, glucose and sucrose are predominant sugars in RJ, with an average content of 3–13, 4–8 and 0.5–2 %, respectively. As royal jelly represents a complex biological matrix comprising water, major macromolecules (proteins, carbohydrates, lipids), and unique compounds such as 10-HDA and antimicrobial peptides (*4*), its FTIR-ATR spectrum exhibits a wide variety of absorption bands arising from the molecular vibrations of these constituents.

As presented in Fig. 1a, the most intensive absorption band in the RJ spectra with an absorption maximum at 3320 cm^{-1} is attributed to the stretching vibrations of the hydroxyl groups (O–H) of water and carbohydrates (CHO) (*32*-*34*), as well as N–H stretching vibrations of proteins, known as Amide A band (*33*,*35*). These overlapping effects are explained by the fact that Amide A band of proteins typically absorb IR radiation in the spectral range from 3600–3000 cm^{-1} (similar to water and CHO), but it can be assumed that commonly less intensive Amide A band is overlapped by more intensive vibrations of O–H groups of water and CHO. The most intensive absorption band at 3320 cm-1 is followed by a low-intensity IR signal appearing at 2930 cm^{-1}. According to Tarantilis *et al*. (*36*), a low-intensity IR signal observed in RJ spectrum at 2930 cm^{-1} corresponds to the C–H stretching vibrations of the -CH_2 groups and secondary amines. However, it can be assumed that this band corresponds to the stretching vibrations of the CH_2 groups of both amines (and/or proteins) and lipids, primarily fatty acids given that they are the most abundant component of the lipid fraction in RJ (*4*,*36*) and they typically absorb IR radiation strongly in this region (*33*,*37*,*38*); this is represented by the asymmetric and symmetric stretching vibrations of the CH_2 groups of lipids (aliphatic chains).

The spectra region between 1800 and 800 cm^{-1} is populated by a number of absorption bands related to various RJ constituents, i.e. proteins, lipids, and carbohydrates (primarily glucose, fructose and sucrose, as predominant sugars in RJ), The most prominent band in this region is a medium-intensity vibration at 1645 cm^{-1} assigned to C=O and C–N stretching vibrations (Amide I band) of RJ proteins. Amide I band primarily comprises stretching vibrations of the C=O (70–85 %) and to a lesser extent C–N groups (10–20 %) (*33*,*35*). Besides being typical for protein β-sheet structures, this band position is also characteristic for well-known molecular vibration of water (H–O–H deformation), thus reflecting an overlapping spectral effect related to these RJ constituents. Similar findings were

reported by Tarantilis *et al*. (*36*). A peak observed at 1545 cm^{-1} is attributed to Amide II band of proteins which comprises N–H bending and C–N stretching vibrations (*33*,*35*,*36*).

A series of less intense absorption bands in the spectral region from 1480 to 1135 cm^{-1} (at 1453, 1412, 1343, 1320, and 1238 cm^{-1}) are primarily attributed to vibrations of the functional groups of proteins and amino acids, and to a lesser extent, fatty acids (*33*,*37*), as denoted in Fig. 1b. The signal at 1238 cm^{-1} represents a band position characteristic for Amide III of proteins, which comprises 30 % of N–H bending, 30 % of C–N stretching, 10 % of C–O stretching, and 10 % of O=C–N bending vibrations (*33*,*35*). The overlapping of CH_2 bending of fatty acids and CH_3 bending of aminoacids (both exhibiting weak signals in this region) is likely for the signal at 1412 cm^{-1}. In the spectral region from 1160 to 965 cm^{-1}, a medium-intensity band with an absorption maximum at 1078 cm^{-1} can be attributed to the C–O stretching vibrations of lipids (although it is overlappping with C–O stretchings of fructose and glucose as explained below), while the stretching vibrations of the C–O bonds of fructose and glucose are represented at 1060 cm^{-1} and 1036 cm^{-1}, respectively (*32*). An absorption band arising at 992 cm^{-1} is assigned to sucrose-specific C–O–H bending / ring vibration (*34*). Sugars are further represented by an absorption band at 1152 cm^{-1} which is attributed to *out-of-plane* CH_2 bending vibration (wagging) of both fructose and glucose, while bands at 1103 and 1078 cm^{-1} can be assigned to C–O endocyclic stretching vibrations of mentioned monosaccharides (*32,33*).

As presented in Fig. 1b with emphasized fingerprint region (1500–800 cm^{-1}), the most prominent differences in the composition of analysed RJ samples were related to different proportions of carbohydrates and lipids, while the protein fraction and water content found to be less variable components of analysed RJ samples. Based on spectral data, it was observed that RJ1 contains higher amounts of predominant sugars (fructose, glucose and sucrose), lipids, as well as proteins, RJ2 and RJ4 showed similar spectral features reflecting the lowest amounts of mentioned constituents, while RJ3 and RJ5 samples revealed medium concentrations. The most variable segment of analysed RJ spectra was predominantly sugar-based spectral envelope (from 1150 to 950 cm^{-1}) reflecting various proportions of fructose, glucose and sucrose, as emphasized on Fig. 1b.
Figure 1

The headspace composition of royal jelly

The headspace composition of RJ samples is presented in Table 2. Overall, six compounds were identified by HS-SPME/GC-MS by two fibres. The predominant headspace compound was octanoic acid (48.09–83.07 % (PDMS/DVB fibre); 52.30–80.55 % (DVB/CAR/PDMS fibre) and it was the most present in the headspace of the samples RJ1 and RJ4 while its lowest abundance was in the sample RJ5. Octanoic acid abundance in the volatile fraction of RJ was found by early studies of

Boch *et al*. (*39*). More detailed study by headspace solid-phase microextraction (HS-SPME) followed by diethyl ether and methanol extraction (the extracts were silanized) identified 185 organic compounds (by GC-MS) from 17 samples of RJ (*39*). HS-SPME/GC–MS of fresh RJ detected 25 compounds (*40*) and octanoic acid was the major aliphatic acid, while abundant carbonyls were: heptan-2-one, acetone, nonan-2-one, and benzaldehyde. Therefore, the similarity with present results is noticed and the differences can be attributed to much higher amount of investigated RJ samples (1.5–2 g) as well as to RJ different origin. Octanoic acid was quantified as the major volatile component of RJ (113 to 252 μg/g) and its concentration is much lower in drone and worker larval food (3.2–7.6 and 2.1–7.3 μg/g) as determined by the extraction with diethyl ether after the quantitative GC-MS analysis of the trimethylsilyl (TMS) derivative (*41*). Boch *et al*. (*39*) suggested a possible biological role for this compound. Octanoic acid, at a concentration similar to that found in royal jelly, appeared to be as repellent for *Varroa destructor* (both under lab and field conditions) as royal jelly itself (*41*). In addition, octanoic acid exhibited antimicrobial activity for oral microorganisms (*42*) and exhibited significant anti-*Candida* activity and therefore could contribute to the observed antibacterial activity against MDR pathogens in present research.

Two other lower aliphatic ketones were found with minor abundance (Table 2): heptan-2-one (2.93–11.60 % (PDMS/DVB fibre); 1.06–6.74 % (DVB/CAR/PDMS fibre)) and nonan-2-one (1.19–22.31 % (PDMS/DVB fibre); 1.08–19.20 % (DVB/CAR/PDMS fibre)). Heptan-2-one and nonan-2-one were the most abundant in the sample RJ5. Heptan-2-one is produced by bee mandibular glands (*43*) and acts as a mild anxiety pheromone (*44*). The repelling activity of heptan-2-one in RJ from queen cells can be directed against *Varroa destructor* (*45*) and it can also enhance a repellent action of octanoic acid. Among carbonyl compounds, benzaldehyde was detected in all samples and with higher abundance only by DVB/CAR/PDMS fibre (2.51–9.60 %).

Table 2

Genotyping and strain selection

All isolates in this study were grouped based on similarity patterns obtained by rep-PCR, which showed a high degree of intraspecies variability. *E. faecium* isolates (*N*=20) were grouped into nine unique (monophyletic) profiles and four groups comprising two or more strains (Fig. S1). *S. aureus* isolates (*N*=15) formed five monophyletic clusters and four multi-strain groups (Fig. S2), while *K. pneumoniae* isolates (*N*=40) were grouped into five monophyletic and eleven multi-strain groups (Fig. S3). *A. baumannii* strains (*N*=10) were classified into two monophyletic profiles and three multi-strain groups (Fig. S4). Representative strains (*N*=5) from each species were selected for further analysis based on the following criteria: representatives were primarily chosen from groups containing two or

more strains and for the species exhibiting higher intraspecies diversity or with fewer available isolates, additional representatives were randomly selected from monophyletic clusters identified in the dendrograms. The multidrug-resistant (MDR) phenotype of the representative strains, confirmed through antibiogram analysis (Fig. 2), revealed carbapenem resistance in *K. pneumoniae* and *A. baumannii*, methicillin resistance in *S. aureus*, and vancomycin resistance in *E. faecium*. The combination of the MDR phenotype and the high virulence potential of these species is known to be a major cause of potentially fatal human infections (*46*), making them bacterial pathogens of significant public health concern (*1*).

Figure 2

Antibacterial properties of RJ samples

The antibacterial activity of the RJ samples against MDR pathogenic bacterial strains, assessed by measuring inhibition zone diameters, revealed that RJ at 1 g/mL exhibited antibacterial activity against the tested bacteria, with inhibition zones ranging from (11.8±2.7) mm for *K. pneumoniae* to (16.8±6.1) mm for *S. aureus* (Table 3). Royal jelly samples from the continental (RJ1 and RJ3) and Mediterranean (RJ4) regions of Croatia inhibited all tested bacteria, while RJ2 and RJ5 inhibited all bacteria except for two MRSA strains (RJ2) and one strain each of *A. baumannii* (RJ5) and *K. pneumonia* (RJ5) (Table 3).

Table 3

To obtain more accurate quantitative results, the well diffusion test was supplemented with the serial microdilution method, as the well diffusion test alone is limited. This is because it relies on uniform diffusion of the tested substance, a property uncommon in most natural compounds, and there is no direct correlation between the antibacterial concentration and inhibition zone size (*47*). Additionally, Osés *et al*. (*48*) noted that the agar well diffusion method has relatively low sensitivity because the tested samples become diluted upon diffusion into the agar. Consistent with these observations, some RJ samples did not produce inhibition zones in the agar well diffusion assay but did show antibacterial activity when evaluated by the broth dilution method. Despite minor methodological variations, both approaches confirmed the antibacterial potential of RJ and its broad-spectrum activity against MDR Gram-positive and Gram-negative bacteria. However, bacterial susceptibility varied depending on the species and strains, regardless of the method used (Table 3 and Table 4).

Notably, *S. aureus* and *A. baumannii* exhibited significantly higher susceptibility to RJ, while *E. faecium* and *K. pneumoniae* showed the lowest susceptibility (Table 4). The antibacterial properties

of RJ are attributed to its bioactive compounds, including proteins, peptides, phenolic compounds, and fatty acids (*49*). While certain constituents, such as royalisin, exhibit stronger activity against Gram-positive bacteria (*50*), most bioactive compounds exert a broad-spectrum, non-selective effects on bacterial cells, mainly by disrupting membranes and lysing intracellular contents or by inhibiting the synthesis of various essential cellular components or by inhibiting transcription and translation (*51*,*52*). Although the peptidoglycan layer is not an effective permeability barrier in Gram-positive bacteria compared to the outer membrane of Gram-negative bacteria (*5*), no significant differences between Gram-positive and Gram-negative bacteria in terms of inhibition zone diameter and MICs were observed in our study (Table 5). A significant effect was found only for MBC values, indicating a higher susceptibility of Gram-negative bacteria, which contradicts previous findings (*5*,*50*). Interestingly, Gram-negative *A. baumannii* was highly susceptible to RJ (MIC/MBC (27.2±15.5) μg/mL), regardless of the strain tested, while Gram-positive *E. faecium* strains exhibited the highest resistance (MIC/MBC=(96.6±36.0)/(126.4±58.2) μg/mL) among the four species tested.

Additionally, the antibacterial activity of RJ against certain MDR pathogens varied not only between different bacterial species, but also among different strains within the same species. For example, *K. pneumoniae* strain SE_SC_COL_68 was inhibited at a concentration of 15.6 μg/mL, while a concentration of 500 μg/mL was required to inhibit strain SE_SC_COL_46. Similarly, *A. baumannii* strains exhibited MIC/MBC values between 7.8 and 62.5 μg/mL, while *E. faecium* strains were found to have MIC/MBC values between 31.3 and 250 μg/mL. The most consistent antibacterial effect was observed against *S. aureus*, with MIC values of the tested strains ranging from 15.6 to 62.5 μg/mL (Table 4).

In general, the MIC and MBC values required to suppress the growth of MDR pathogens in this study were between 7.8 and 500.0 μg/mL (Table 4). These values indicate significantly stronger antibacterial activity than in most previous studies testing various RJ samples against non-MDR strains of *K. pneumoniae*, *S. aureus*, and *E. faecium*, with effective concentrations ranging from 3.7 to 14.5 mg/mL (*3*). Similar to our results, Moselhy *et al*. (*53*) reported comparable MIC/MBC values of RJ against non-MDR *Bacillus cereus* and *S. aureus* ranging from 7.8 to 500.0 μg/mL and 15.6 to 500.0 μg/mL, respectively. However, there are only a few studies that specifically investigate the activity of RJ against MDR bacteria. For example, Uthaibutra *et al*. (*5*) observed MIC/MBC values between 37.5 and 75 mg/mL for RJ against methicillin-resistant *S. aureus.* When jelleine I, a peptide derived from RJ, was used to inhibit the growth of carbapenem-resistant *A. baumannii* (*54*) and MRSA (*55*), a much lower concentration was required: between 0.32 and 0.6 μg/mL and 8 and 138 μg/mL, respectively. However, to the best of our knowledge, the effect of RJ or its individual components on different MDR strains within the same bacterial species has not yet been investigated.

Consistent with the chemical profiling, no significant differences in the antibacterial efficacy of RJ samples from different geographical regions of Croatia (Mediterranean vs. continental) were observed (Table 5), despite considerable variation in their chemical composition. Nevertheless, all RJ samples demonstrated consistent antibacterial activity. This suggests a stable and reliable antibacterial profile, likely influenced by local microclimatic conditions rather than broader regional factors. Furthermore, our study supports the hypothesis of a synergistic effect of crude RJ against MDR pathogens, as no significant differences in antibacterial activity were observed among the samples, although the levels of certain bioactive compounds (*e.g.* proteins, lipids and octanoic acid) were higher in RJ1. This concordance is an important aspect for the potential therapeutic use of RJ, especially in the treatment of bacterial MDR infections.

Table 4

Table 5

CONCLUSIONS

This study highlights the broad-spectrum antibacterial activity of royal jelly (RJ) against several strains of clinically relevant Gram-positive and Gram-negative multidrug-resistant (MDR) pathogens, including vancomycin-resistant *E. faecium*, methicillin-resistant *S. aureus* and carbapenem-resistant *K. pneumoniae*, as well as *A. baumannii*. Although some RJ samples contained higher levels of bioactive compounds, their superior chemical profile did not consistently result in higher antimicrobial efficacy, regardless of regional origin (Mediterranean *vs.* continental Croatia). Such an observation may indicate that the antibacterial activity of crude RJ arises from complex interactions among its components rather than from individual compounds alone, a hypothesis that requires further targeted investigation.

In addition to the variability between species, significant differences in antimicrobial activity at the strain level were also observed.

To the best of our knowledge, this is the first study investigating antibacterial effect of RJ against multiple MDR strains and simultaneously providing a comprehensive chemical characterization. Our results show for the first time that efficacy of RJ varies not only between species but also between strains of the same species, emphasizing the need to consider both the chemical composition and the intraspecies diversity of pathogens when assessing its therapeutic potential.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

M. Mrkonjić Fuka and L. Svečnjak contributed to the design of the study, performed the analyses, interpreted the data, and drafted the manuscript. I. Tanuwidjaja, V. Odorčić, S. Jurić, and I. Jerković contributed to performing the analyses and to data interpretation. I. Tanuwidjaja performed the statistical analyses. M. Vinceković contributed to sample preparation. N. Udiković-Kolić collected multidrug-resistant pathogens and reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

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Table 1. Antioxidant capacity (ABTS) and bioactive compounds as total polyphenolic compounds (TPC), total flavonoid content (TFC) and total proteins (TP) share in royal jelly samples (RJ1-RJ5)

Royal jelly	Antioxidant capacity	Bioactive compounds		
	ABTS/(μmol TE/100 g)	TPC/(mg GAE/100 g)	TFC/(mg QE/100 g)	TP/(g BSAE/100 g)
RJ1	(352.92±8.30)^{a}	(415.51±6.67)^{ab}	(18.20±0.16)^{a}	(16.48±0.12)^{a}
RJ2	(285.28±5.73)^{b}	(355.66±15.25)^{a}	(15.95±0.26)^{b}	(12.80±0.07)^{b}
RJ3	(293.19±4.84)^{b}	(402.28±11.30)^{ab}	(14.44±0.19)^{c}	(13.37±0.18)^{b}
RJ4	(326.22±11.21)^{ab}	(465.27±12.69)^{b}	(15.74±0.27)^{bc}	(15.57±0.16)^{c}
RJ5	(305.44±11.23)^{ab}	(406.50±7.19)^{b}	(16.47±0.08)^{b}	(14.68±0.18)^{d}
Origin				
Continental	(310.46±32.75)^{a}	(390.82±29.58)^{a}	(16.20±1.66)^{a}	(14.21±1.72)^{a}
Dalmatia	(315.83±16.75)^{a}	(435.89±24.12)^{b}	(16.11±0.46)^{a}	(15.12±0.52)^{a}

The results are shown as a mean value±standard deviation. a-c=different letters in a column indicate significant differences in antioxidant capacity, TPC, TFC and TP based on *t*-tests with Bonferroni adjusted p values ($p<0.05$)

Table 2. Headspace compounds of RJ samples extracted by headspace solid-phase microextraction (HS-SPME) with two fibres and analysed by gas chromatography and mass spectrometry (GC-MS)

Compound	RI	PDMS/DVB fiber GC area percentage/%					DVB/CAR/PDMS fiber GC area percentage/%				
		RJ1	RJ2	RJ3	RJ4	RJ5	RJ1	RJ2	RJ3	RJ4	RJ5
Heptan-2-one	<900	2.93	3.36	10.22	2.47	11.60	1.06	1.39	6.57	2.20	6.74
Benzaldehyde	950	N.D.	N.D.	2.88	3.31	N.D.	2.51	3.38	8.15	9.60	4.46
2-Metoxyphenol	1094	N.D.	3.08	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Nonan-2-one	1095	1.19	3.06	1.57	3.64	22.31	1.08	4.20	4.66	5.79	19.20
Octanoic acid	1183	83.07	69.82	66.43	73.53	48.09	80.55	70.37	65.22	68.33	52.30
2-Methoxy-4-methylphenol (*p*-Creosol)	1196	N.D.	2.99	N.D.	0.54	N.D.	N.D.	0.85	N.D.	0.27	N.D.

N.D.=not detected, RI=retention index, standard deviation among the triplicates was <8 %

Table 3. The antibacterial activity of royal jelly (RJ1-RJ5) towards selected multidrug-resistant (MDR) pathogens as shown by disc diffusion method

Species	Strain	IZ/mm				
		RJ1	RJ2	RJ3	RJ4	RJ5
E. faecium						
	SE_SC_COL_40	$(14.0\pm0.0)^{a}$	$(11.5\pm0.7)^{b}$	$(14.0\pm0.0)^{a}$	$(13.5\pm0.7)^{ab}$	$(12.0\pm0.0)^{ab}$
	SE_SC_COL_73	$(15.0\pm0.0)^{a}$	$(11.5\pm0.7)^{b}$	$(14.5\pm0.7)^{ab}$	$(15.0\pm0.0)^{a}$	$(15.0\pm0.0)^{a}$
	SE_SC_COL_119	$(16.5\pm0.7)^{ab}$	$(14.5\pm0.7)^{ab}$	$(18.5\pm0.7)^{a}$	$(13.5\pm0.7)^{b}$	$(13.5\pm0.7)^{b}$
	193/0	$(14.0\pm0.0)^{ab}$	$(11.5\pm0.7)^{a}$	$(15.0\pm0.0)^{b}$	$(14.5\pm0.7)^{b}$	$(14.0\pm0.0)^{ab}$
	560/2	$(12.0\pm0.0)^{ab}$	$(10.0\pm0.0)^{a}$	$(13.0\pm0.0)^{ab}$	$(13.5\pm0.7)^{b}$	$(13.0\pm1.4)^{b}$
S. aureus						
	SA_6	$(15.5\pm0.7)^{ab}$	$(0.0\pm0.0)^{a}$	$(26.0\pm0.0)^{b}$	$(26.0\pm0.0)^{b}$	$(13.0\pm1.4)^{ab}$
	SA_7	$(16.5\pm0.7)^{ab}$	$(0.0\pm0.0)^{a}$	$(17.0\pm1.4)^{b}$	$(15.5\pm0.7)^{ab}$	$(17.0\pm0.0)^{b}$
	SA_8	$(16.0\pm0.0)^{ab}$	$(15.0\pm0.0)^{a}$	$(18.5\pm0.7)^{ab}$	$(20.0\pm1.4)^{b}$	$(17.5\pm0.7)^{ab}$
	SA_9	$(16.0\pm1.4)^{ab}$	$(16.5\pm0.7)^{ab}$	$(20.0\pm1.4)^{a}$	$(15.5\pm0.7)^{b}$	$(16.0\pm0.0)^{ab}$
	SA_14	$(19.5\pm0.7)^{ab}$	$(16.0\pm0.0)^{a}$	$(24.5\pm0.7)^{b}$	$(23.0\pm0.0)^{b}$	$(19.5\pm0.7)^{ab}$
K. pneumoniae						
	SE_SC_COL_46	$(13.0\pm0.0)^{abc}$	$(12.0\pm0.0)^{ac}$	$(15.0\pm0.0)^{b}$	$(14.0\pm0.0)^{ab}$	$(11.0\pm0.0)^{c}$
	SE_SC_COL_68	$(12.0\pm0.0)^{ab}$	$(10.0\pm0.0)^{a}$	$(13.0\pm0.0)^{b}$	$(12.0\pm0.0)^{ab}$	$(11.5\pm0.0)^{a}$
	SE_SC_COL_96	$(12.0\pm0.0)^{ab}$	$(10.0\pm0.0)^{a}$	$(13.5\pm0.0)^{b}$	$(12.5\pm0.0)^{b}$	$(11.5\pm0.0)^{a}$

	SE_SC_COL_173	(11.5±0.0)^{a}	(11.0±0.0)^{a}	(14.0±0.0)^{b}	(13.5±0.0)^{ab}	(13.5±0.0)^{ab}
	H2_COL_79	(12.5±0.0)^{abc}	(10.5±0.0a)^{c}	(13.5±0.0)^{b}	(12.5±0.0)^{ab}	(0.0±0.0)^{c}
A. baumannii						
	AB_6	(12.0±0.0)^{a}	(12.0±0.0)^{a}	(16.0±0.0)^{ab}	(17.0±0.0)^{b}	(14.0±0.0)^{ab}
	AB_7	(13.0±0.0)^{a}	(13.5±0.0)^{ab}	(17.0±0.0)^{bc}	(19.5±0.0)^{c}	(16.5±0.0)^{ac}
	AB_8	(14.0±0.0)^{abc}	(10.5±0.0)^{ac}	(16.5±0.0)^{ab}	(18.0±0.0)^{b}	(0.0±0.0)^{c}
	AB_9	(14.0±0.0)^{ab}	(13.0±0.0)^{a}	(16.5±0.0)^{b}	(16.5±0.0)^{b}	(14.0±0.0)^{ab}
	AB_10	(13.0±0.0)^{abc}	(12.0±0.0)^{a}	(17.0±0.0)^{b}	(18.5±0.0)^{b}	(15.5±0.0)^{ab}

IZ=inhibition zone, the inhibition zones are shown as a mean value±standard deviation (*N*=3). a-c=different letters in a row indicate significant differences in the antibacterial activity of royal jelly based on Dunn's test with Bonferroni adjusted p values ($p<0.05$)

Table 4. Minimum inhibitory concentration and minimum bactericidal concentration of royal jelly (RJ1-RJ5) against selected multidrug-resistant (MDR) pathogens

Species	Strain	MIC (MBC)/(μg/mL)				
		RJ1	RJ2	RJ3	RJ4	RJ5
E. faecium						
	SE_SC_COL_40	125.0 (125.0)	125.0 (125.0)	62.5 (62.5)	62.5 (62.5)	125.0 (125.0)
	SE_SC_COL_73	125.0 (125.0)	125.0 (125.0)	125.0 (125.0)	62.5 (125.0)	62.5 (125.0)
	SE_SC_COL_119	31.3 (31.3)	125.0 (125.0)	62.5 (125.0)	125.0 (125.0)	31.3 (62.5)
	193/0	125.0 (125.0)	125.0 (250.0)	62.5 (62.5)	62.5 (125.0)	125.0 (125.0)
	560/2	125.0 (250.0)	250.0 (250.0)	125.0 (250.0)	250.0 (250.0)	250.0 (250.0)

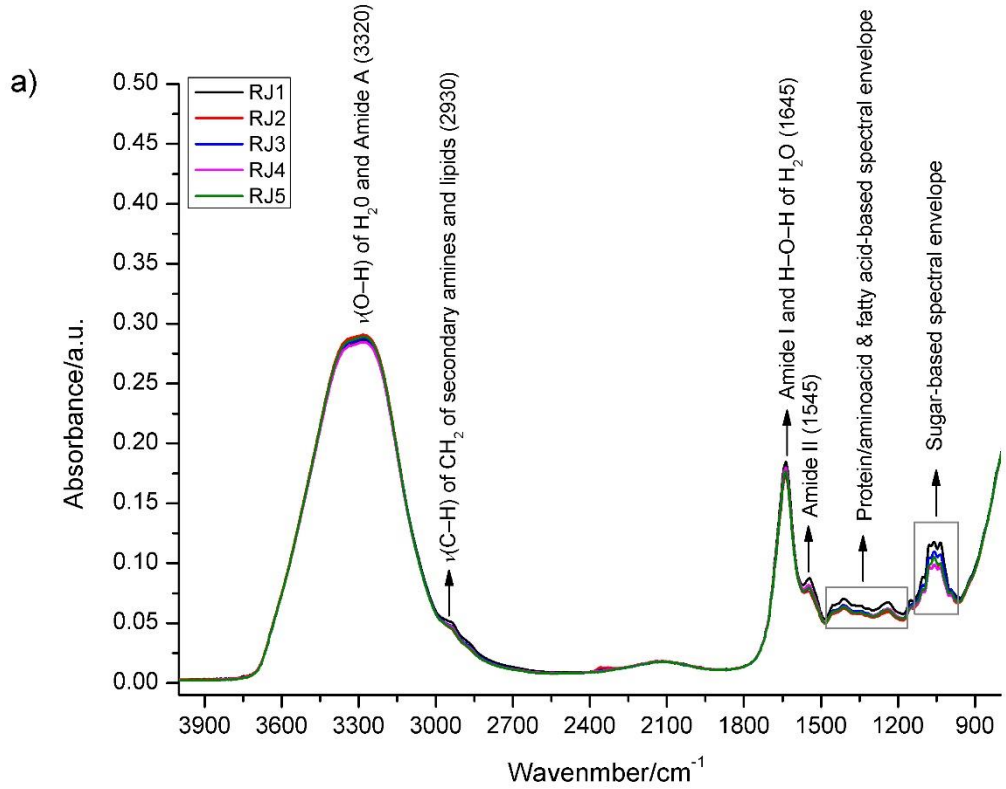
S. aureus						
	SA_6	15.6 (15.6)	62.5 (62.5)	31.3 (31.3)	31.3 (31.3)	31.3 (31.3)
	SA_7	15.6 (15.6)	31.3 (31.3)	31.3 (31.3)	15.6 (15.6)	15.6 (15.6)
	SA_8	62.5 (62.5)	62.5 (62.5)	62.5 (62.5)	62.5 (62.5)	62.5 (62.5)
	SA_9	31.3 (31.3)	62.5 (62.5)	31.3 (31.3)	31.3 (31.3)	31.3 (31.3)
	SA_14	15.6 (15.6)	31.3 (31.3)	31.3 (31.3)	15.6 (31.3)	15.6 (31.3)
	SE_SC_COL_46	125.0 (125.0)	500.0 (500.0)	62.5 (62.5)	125.0 (125.0)	62.5 (62.5)
	SE_SC_COL_68	125.0 (125.0)	250.0 (250.0)	62.5 (62.5)	15.6 (15.6)	62.5 (62.5)
	SE_SC_COL_96	125.0 (125.0)	125.0 (125.0)	62.5 (62.5)	62.5 (62.5)	125.0 (125.0)
	SE_SC_COL_173	125.0 (125.0)	125.0 (125.0)	62.5 (62.5)	125.0 (125.0)	62.5 (62.5)
	H2_COL_79	125.0 (125.0)	250.0 (250.0)	62.5 (62.5)	62.5 (62.5)	125.0 (125.0)
A. baumannii						
	AB_6	62.5 (62.5)	62.5 (62.5)	31.3 (31.3)	31.3 (31.3)	31.3 (31.3)
	AB_7	15.6 (15.6)	31.3 (31.3)	15.6 (15.6)	15.6 (15.6)	31.3 (31.3)
	AB_8	15.6 (15.6)	7.8 (7.8)	15.6 (15.6)	15.6 (15.6)	31.3 (31.3)
	AB_9	15.6 (15.6)	62.5 (62.5)	15.6 (15.6)	15.6 (15.6)	31.3 (31.3)
	AB_10	15.6 (15.6)	31.3 (31.3)	31.3 (31.3)	31.3 (31.3)	15.6 (15.6)

Values in the parentheses correspond to the MBC values

Table 5. The effect of species, Gram stain, and royal jelly origin on antibacterial activity of royal jelly towards selected multidrug-resistant (MDR) pathogens

Factor	IZ/mm	MIC/(μg/mL)	MBC/(μg/mL)
Species			
E. faecium	(13.7±1.8)^{a}	(96.6±36.0)^{a}	(126.4±58.2)^{a}
S. aureus	(16.8±6.1)^{b}	(35.6±18.3)^{b}	(36.9±17.4)^{b}
K. pneumoniae	(11.8±2.7)^{c}	(91.6±35.5)^{a}	(91.6±35.5)^{a}
A. baumannii	(14.4±3.8)^{a}	(27.2±15.5)^{b}	(27.2±15.5)^{b}
Gram staining			
Positive	(15.3±4.7)^{a}	(57.3±41.9)^{a}	(78.8±61.2)^{a}
Negative	(13.1±3.5)^{a}	(64.2±41.4)^{a}	(57.3±41.9)^{b}
Royal jelly			
RJ1	(14.1±2.1)^{a}	(71.1±51.8)^{a}	(77.3±64.6)^{a}
RJ2	(11.1±4.2)^{a}	(74.7±43.2)^{a}	(82.5±60.6)^{a}
RJ3	(16.7±3.5)^{a}	(52.3±31.0)^{a}	(61.7±54.0)^{a}
RJ4	(16.2±3.7)^{a}	(51.0±38.2)^{a}	(58.4±44.1)^{a}
RJ5	(12.9±4.9)^{a}	(56.7±39.7)^{a}	(62.5±41.3)^{a}
Origin			
Continental	(13.9±4.1)^{a}	(65.4±43.2)^{a}	(73.2±59.4)^{a}
Dalmatia	(14.6±4.6)^{a}	(53.9±38.6)^{a}	(60.4±42.2)^{a}

The growth inhibition zone, and MIC and MBC values are shown as a mean value±standard deviation. a-c=different letters in a column indicate significant differences in the antibacterial activity of royal jelly for each factor based on Dunn's test with Bonferroni adjusted p values ($p<0.05$)



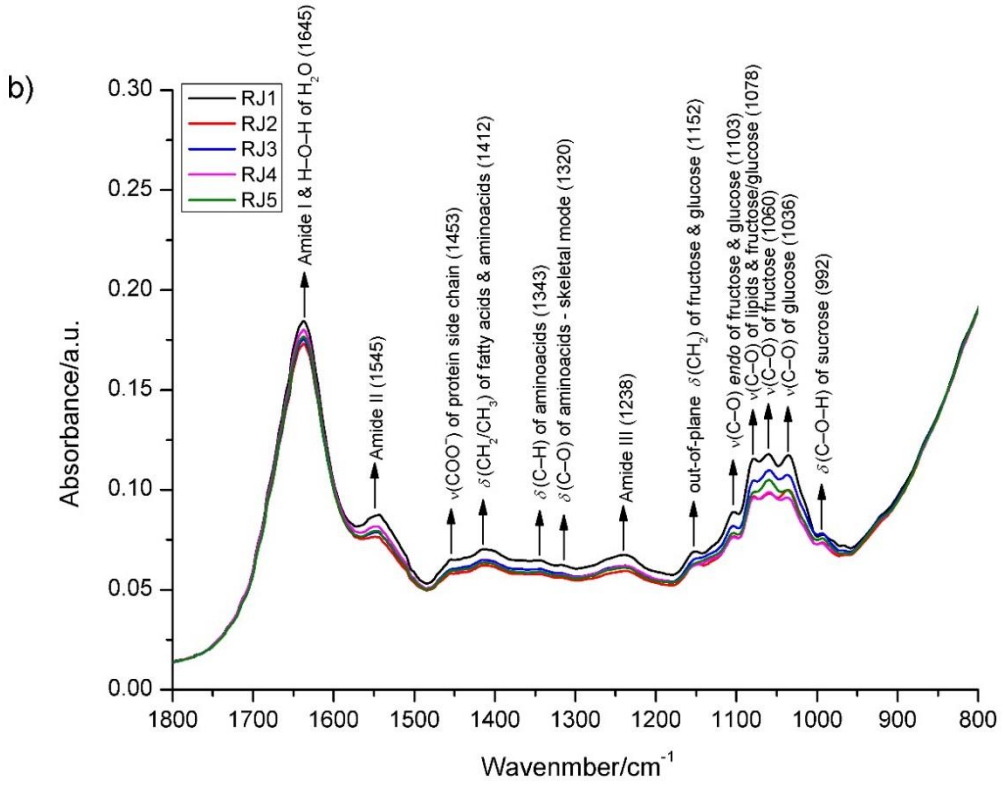


Fig. 1. FTIR-ATR spectra of royal jelly samples (*N*=5; RJ1–RJ5) representing comparative spectral features with assignation of major underlying molecular vibrations: a) whole spectral region (4000–800 cm^{-1}), b) spectral range between 1800 and 800 cm^{-1} with emphasized fingerprint region (1500–800 cm^{-1}). ν=stretching vibration, δ=deformation vibration (bending)

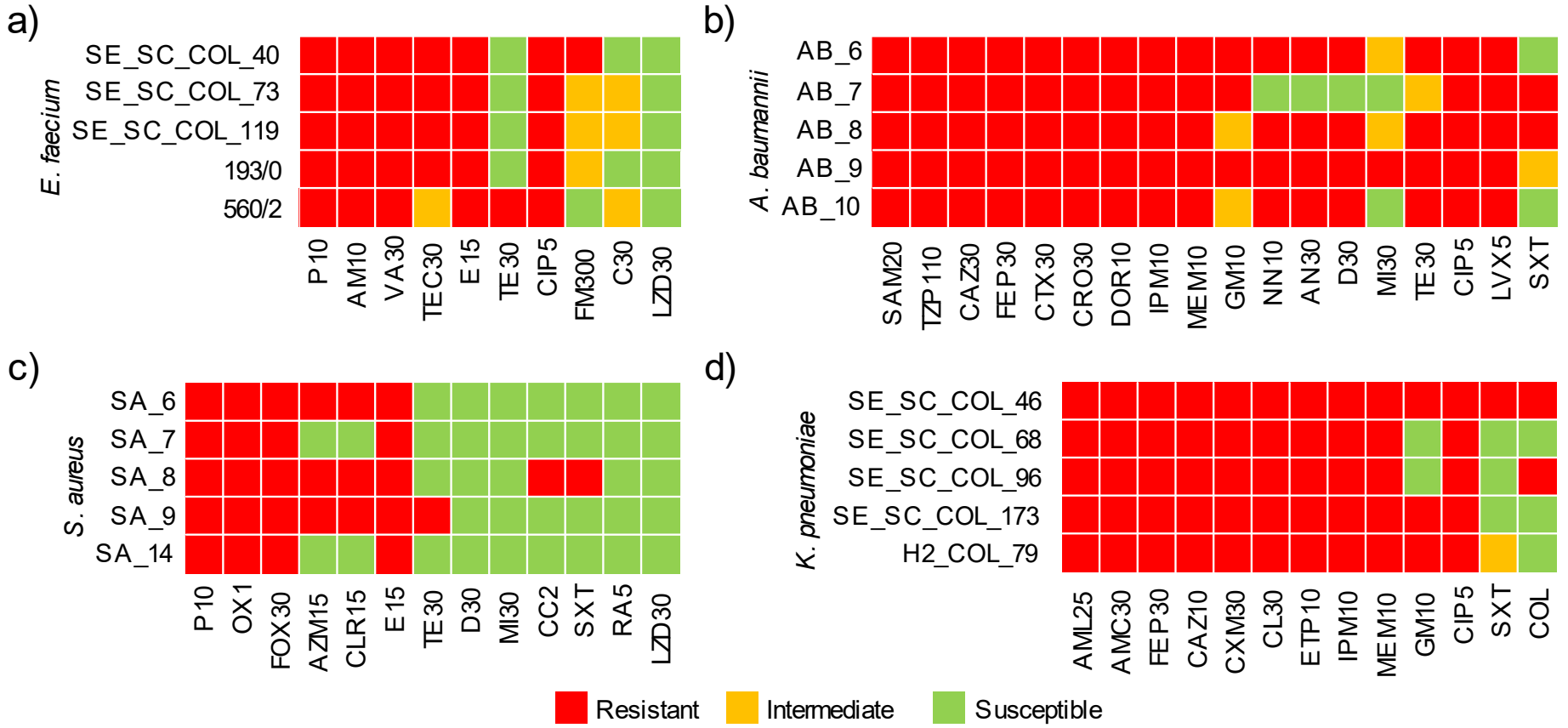


Fig. 2. The multidrug-resistant (MDR) phenotype of representative strains: a) *E. faecium*, b) *A. baumannii*, c) *S. aureus* and d) *K. pneumoniae.* Penicillins: P10=penicillin (10 U), AM10=ampicillin (10 μg), OX1=oxacillin (1 μg), AML25=amoxicillin (25 μg); Penicillins+ß-lactamase inhibitors: SAM20=ampicillin-sulbactam (10/10 μg), TZP110=piperacillin-tazobactam (100/10 μg), AMC30=amoxicillin-clavulanic acid (20/10 μg); Cephems: CAZ30=ceftazidime (30 μg), CAZ10=ceftazidime (10 μg), FEP30=cefepime (30 μg), CTX30=cefotaxime (30 μg), CRO30=ceftriaxone (30 μg), FOX30=cefoxitin (30 μg), CXM30=cefuroxime (30 μg), CL30=cephalexin (30 μg); Glycopeptides: VA30=vancomycin (30 μg); Lipoglycopeptides: TEC30=teicoplanin (30 μg);

Macrolides: E15=erythromycin (15 µg), AMZ15=azithromycin (15 µg), CLR15=clarithromycin (15 µg); Carbapenems: DOR10=doripenem (10 µg), IPM10=imipinem (10 µg), MEM10=meropenem (10 µg), ETP10=ertapenem (10 µg); Aminoglycosides: GM10=gentamicin (10 µg), NN10=tobramycin (10 µg), AN30=amikacin (30 µg); Tetracyclines: TE30=tetracycline (30 µg), D30=doxycycline (30 µg), MI30=minocycline (30 µg); Fluoroquinolones: CIP5=ciprofloxacin (5 µg), LVX5=levofloxacin (5 µg); Nitrofurantoins: FM300=nitrofurantoin (300 µg); Phenicols: C30=chloramphenicol (30 µg); Lincosamides: CC2=clindamycin (2 µg); Folate pathway antagonists: SXT=trimethoprim-sulfomethoxazole (1.25/23.75 µg); Ansamycins: RA5=rifampin (5 µg); Oxazolidinones: LZD30=linezolid (30 µg); Polymyxins: COL=colistin (2 mg/L)

SUPPLEMENTARY MATERIAL

Table S1. The list of antibiotics used in this study for validating the multidrug-resistant (MDR) profiles of *E. faecium*, *A. baumannii*, *S. aureus* and *K. pneumoniae* strains

Species	Class of antibiotics	Antibiotic	Abbreviation	Disc content/U or µg *c*/(mg/L)
E. faecium	Penicillins	Penicillin	P10	10 U
		Ampicillin	AM10	10 µg
	Glycopeptides	Vancomycin	VA30	30 µg
	Lipoglycopeptides	Teicoplanin	TEC30	30 µg
	Macrolides	Erythromycin	E15	15 µg
	Tetracyclines	Tetracycline	TE30	30 µg
	Fluoroquinolones	Ciprofloxacin	CIP5	5 µg
	Nitrofurantoins	Nitrofurantoin	FM300	300 µg
	Phenicols	Chloramphenicol	C30	30 µg
	Oxazolidinones	Linezolid	LZD30	30 µg
A. baumannii	Penicillins+ß-lactamase inhibitors	Ampicillin-sulbactam	SAM20	10/10 µg
		Piperacillin-tazobactam	TZP110	100/10 µg

	Cephems	Ceftazidime	CAZ30	30 µg
		Cefepime	FEP30	30 µg
		Cefotaxime	CTX30	30 µg
		Ceftriaxone	CRO30	30 µg
	Carbapenem	Doripenem	DOR10	10 µg
		Imipinem	IPM10	10 µg
		Meropenem	MEM10	10 µg
	Aminoglycosides	Gentamicin	GM10	10 µg
		Tobramycin	NN10	10 µg
		Amikacin	AN30	30 µg
	Tetracyclines	Doxycycline	D30	30 µg
		Minocycline	MI30	30 µg
		Tetracycline	TE30	30 µg
	Fluoroquinolones	Ciprofloxacin	CIP5	5 µg
		Levofloxacin	LVX5	5 µg
	Folate pathway antagonists	Trimethoprim-sulfomethoxazole	SXT	1.25/23.75 µg
S. aureus	Penicillins	Penicillin	P10	10 U
		Oxacillin	OX1	1 µg
	Cephems	Cefoxitin	FOX30	30 µg
	Macrolides	Azithromycin	AZM15	15 µg
		Clarithromycin	CLR15	15 µg
		Erythromycin	E15	15 µg
	Tetracyclines	Tetracycline	TE30	30 µg

		Doxycycline	D30	30 µg
		Minocycline	MI30	30 µg
	Lincosamides	Clindamycin	CC2	2 µg
	Folate pathway antagonists	Trimethoprim-sulfomethoxazole	SXT	1.25/23.75 µg
	Ansamycins	Rifampin	RA5	5 µg
	Oxazolidinones	Linezolid	LZD30	30 µg
K. pneumoniae	Penicillins	Amoxicillin	AML25	25 µg
	Penicillins+ß-lactamase inhibitors	Amoxicillin-clavulanic acid	AMC30	20/10 µg
	Cephems	Cefepime	FEP30	30 µg
		Ceftazidime	CAZ10	10 µg
		Cefuroxime	CXM30	30 µg
		Cephalexin	CL30	30 µg
	Carbapenem	Ertapenem	ETP10	10 µg
		Imipenem	IPM10	10 µg
		Meropenem	MEM10	10 µg
	Aminoglycosides	Gentamicin	GM10	10 µg
	Fluoroquinolones	Ciprofloxacin	CIP5	5 µg
	Folate pathway antagonists	Trimethoprim-sulfamethoxazole	SXT	1.25/23.75 µg
	Polymyxins	Colistin	COL	2 mg/L

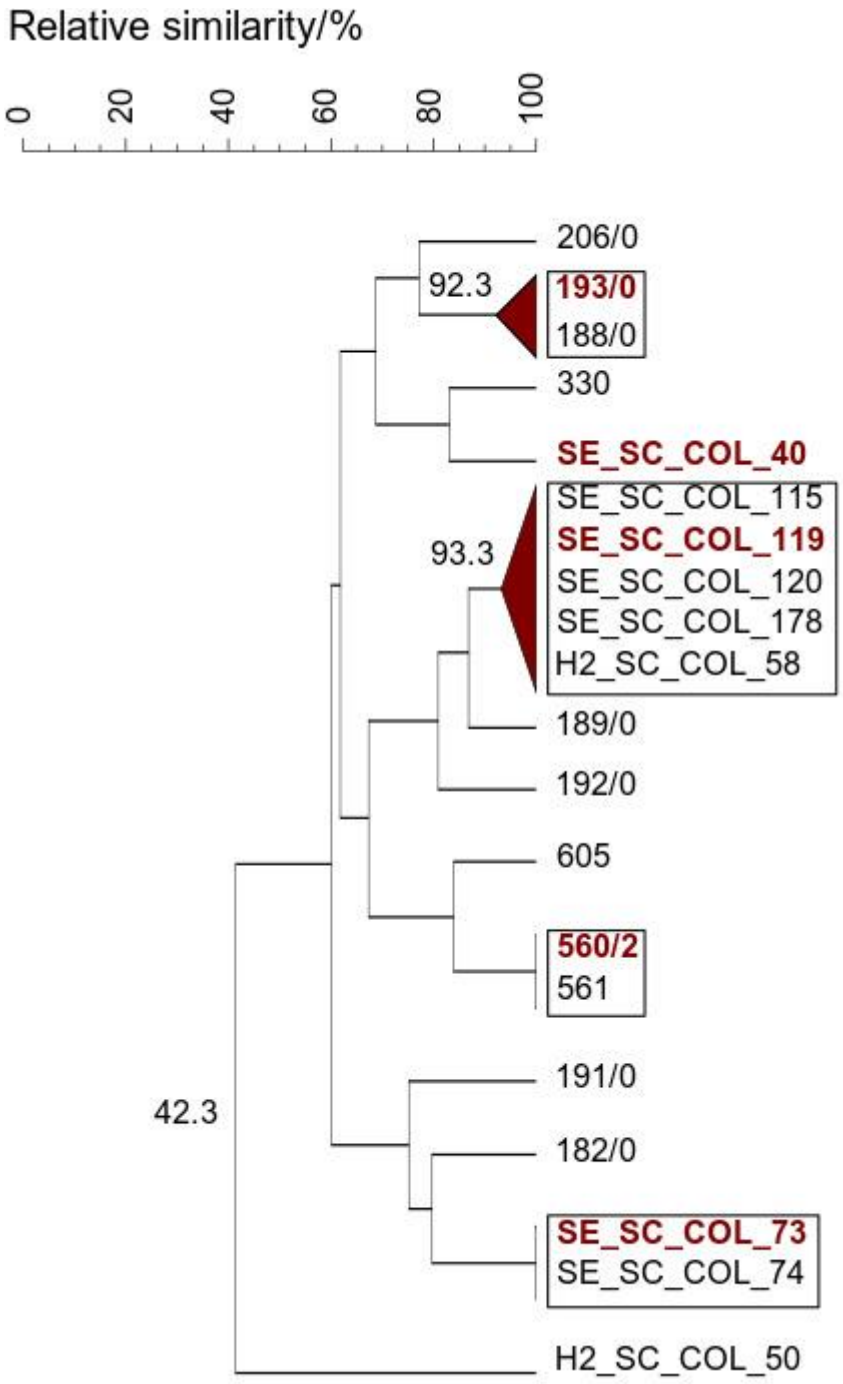


Fig. S1. Intraspecies variability of vancomycin-resistant *Enterococcus faecium* (VRE)

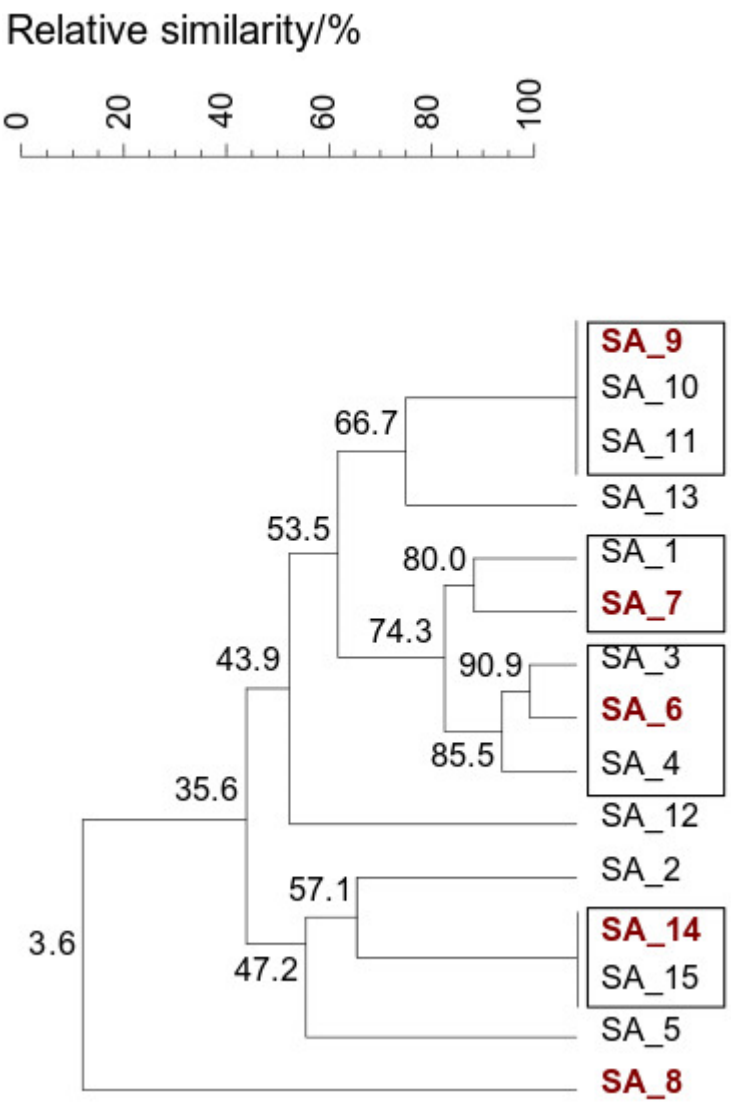


Fig. S2. Intraspecies variability of methicillin-resistant *Staphylococcus aureus* (MRSA)

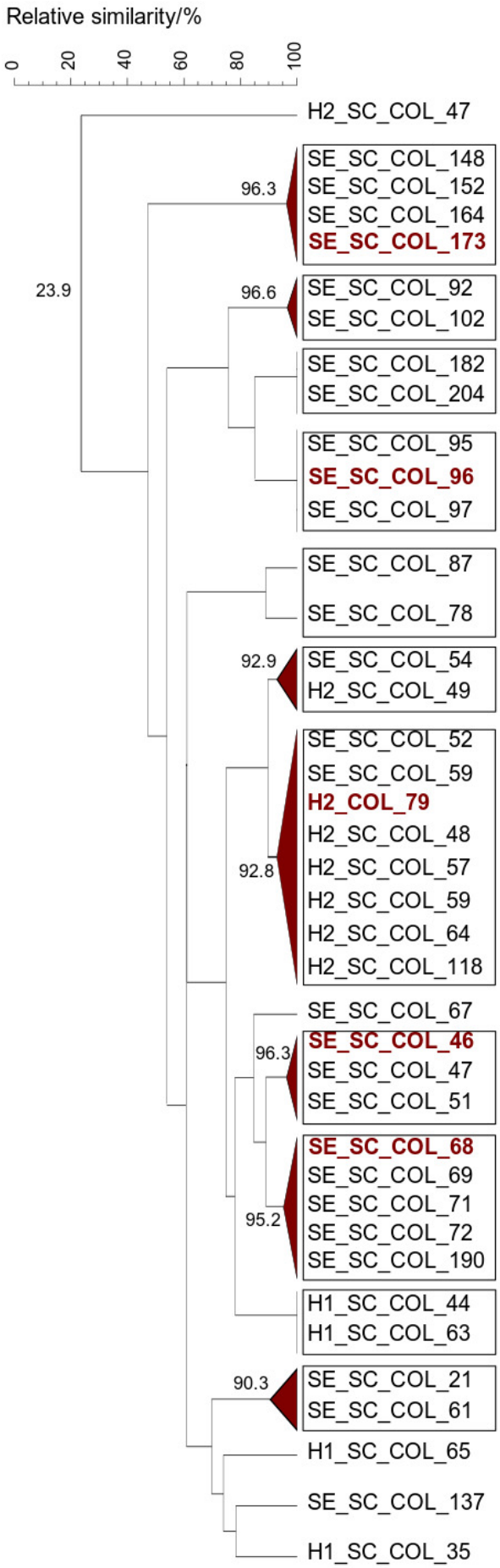


Fig. S3. Intraspecies variability of carbapenem-resistant *Klebsiella pneumoniae* (CRKP)

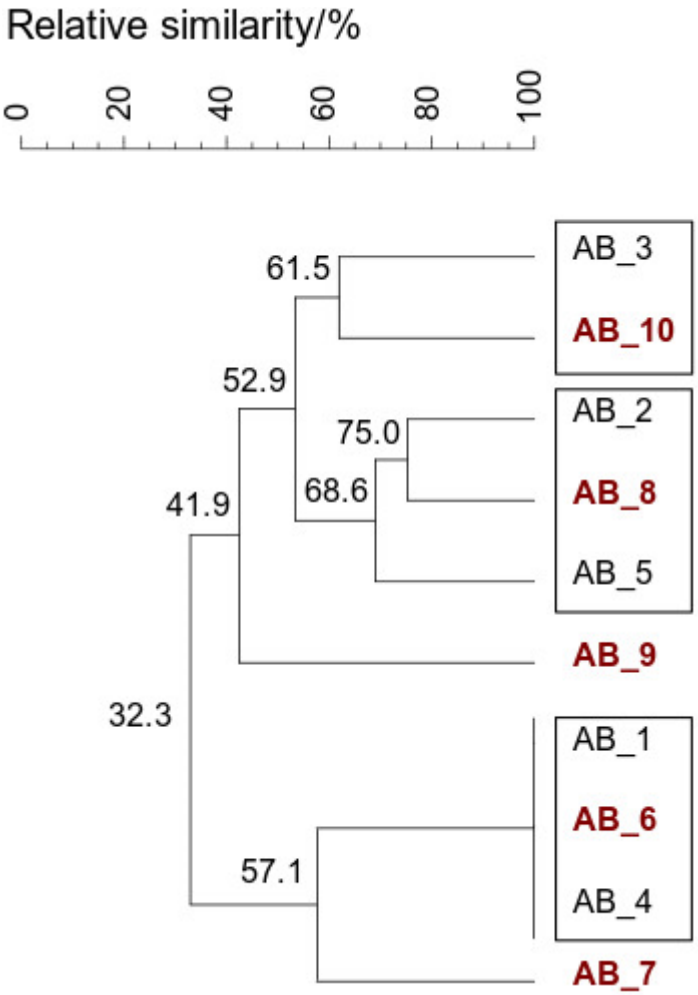


Fig. S4. Intraspecies variability of carbapenem-resistant *Acinetobacter baumannii* (CRAB)