Modelling of *Listeria monocytogenes* growth and survival in presence of royal jelly, a promising anti-biofilm agent

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Summary
Royal jelly is a natural bee product with well-known antimicrobial properties and prominent clinical uses. The main objective of this study was to reveal the response of *L. monocytogenes* to royal jelly. Firstly, the influence of royal jelly on the growth kinetic parameters of *L. monocytogenes* ATCC 7644 was studied with the modified Gompertz mathematical equation. Results indicated that royal jelly retarded the growth of *L. monocytogenes* by acting mainly on the lag phase duration. Microdilution assay on *L. monocytogenes* ATCC 7644 performed with two-fold serial dilution method showed that minimal inhibitory concentration (*MIC*) was 41.67 mg·ml^{-1}. However, the estimated *MIC* value modelled with Gompertz survival curve (23.85 mg·ml^{-1}) and the validated value (24 mg·ml^{-1}) were notably lower than in the microdilution assay. It is noteworthy that the predictive microbiology approach has good performance in determining the optimum dose and cost-effective of antimicrobial agents. To our knowledge, biofilm prevention by royal jelly has not yet been studied and a limited number of studies regarded the effects on *Listeria monocytogenes*. The ability to reduce the formation of biofilm with royal jelly may allow further studies to explore the use of apiculture products in the struggle with *L. monocytogenes*.

Keywords
royal jelly; *Listeria monocytogenes*; growth kinetic parameters; anti-biofilm ability; bee product

Listeria monocytogenes is a food-borne pathogen with a mortality rate of 20–30 %, which is the highest among pathogens causing food-borne diseases [1]. The Center for Disease Control and Prevention (Atlanta, Georgia, USA) estimated that there are 1 600 cases of listeriosis per a year in the United States, of which 1 500 are hospitalized and 260 cases result in death [2]. According to the report published by European Food Safety Authority (EFSA) in 2017, 2 480 cases of listeriosis which of 225 cases resulted in death were reported in 28 European countries [3]. Three of the 13 known serotypes (1/2a 1/2b and 4b serotypes) are responsible for food-borne outbreaks and the serotype 4b has been the most frequently reported serotype [4].

L. monocytogenes enters food processing plants through the soil on workers' shoes or clothing, vehicles, raw vegetables, animal tissues and with human carriers. The high humidity and nutrient levels of processing areas promote growth of the bacterium. Therefore, *L. monocytogenes* is often isolated from floor drains, condensed and stagnant water, floors, residues and processing equipment [5]. In case of lack of hygiene and sanitation and the presence of organic matter on the surface, it would inevitably be transmitted to food. Since *L. monocytogenes* does not survive heat processing at 50 °C, ready-to-eat foods can pose a high listeriosis risk for immune-compromised individuals, elderly, babies, organ transplant patients and pregnant women. Currently, dairy products, cheese, meat and poultry products, seafood, fresh fruits and vegetables remain as reservoirs for *L. monocytogenes*. Despite acquired consciousness and legal restrictions against food-borne diseases, the listeriosis case rate has steadily increased with the expansion of the global food supply chain. European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Con-

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trol (ECDC) [6], in October 2018, reported that a multi-country outbreak of listeriosis linked to the consumption of ready-to-eat cold-smoked salmon occurred including Denmark (6 cases), Germany (5 cases) and France (1 case).

L. monocytogenes can persist for a long time in various locations of a processing facility making use of its ability to attach to stainless steel, glass or rubber. The survival of this microorganism in a processing plant may be strongly associated with the ability to form biofilm. That is defined as a form of microorganisms embedded in self-secreted extracellular polymeric substances (EPS) that adheres to a biotic or abiotic surface [7]. The bacteria in the biofilm structure show a different survival capacity in harsh growth and environmental conditions. This structure provides an advantage for bacterial transmission of virulence genes, nutrient supply and quorum sensing. Moreover, the cells in the structure are more resistant to cleaning agents and disinfectants than planktonic cells.

Royal jelly is a creamy, yellow-white, pungent and nutritious product. It is secreted by mandibular and hypopharyngeal glands in the heads of five to ten days old worker bees [8]. Recently, it has been stated that the royal jelly and/or some of the bioactive compounds have antibacterial, antidiabetic, anticancer and antihypertensive activities [9–11]. The use of royal jelly as a functional product and health enhancer was investigated since the early 60s, but interaction between its bioactive compounds and microorganisms has gained attention over the past two decades. Undoubtedly, the reason for this interest is that much public health and scientific organizations around the world regard antibiotic resistance of pathogens the most serious crisis threatening the human race. As well, the increasing demand for use of natural preservatives to enhance the safety and shelf-life of foods promoted investigation of natural antimicrobials.

Predictive microbiology estimates the microbial growth response to environmental factors by mathematical models. This field has become more necessary because of the legal restrictions, the loss of financial and bad reputation of recalls and the enforcement of lawsuits and legal penalties in cases of outbreaks. Besides, due to the expansion of public awareness and of the global food market, the need for predictive microbiological studies for food quality and safety increased. More recent applications are focused on the use of modelling to reduce or completely inhibit pathogenic bacteria *i*n vitro or in vivo. A fundamental aspect of predictive microbiology is the shape of the microbial growth curve [12]. The response to the change is determined by taking into account the obtained kinetic parameters such as lag time, growth rate and maximum bacterial population. Huang [13] also emphasized that the length of the lag phase and the rate of growth are critical to the evaluation of microbiological safety of foods.

The current work aimed to investigate the growth behaviour of *L. monocytogenes* in the presence of royal jelly through the implementation of a mathematical modelling procedure. Also, this study aimed to evaluate the effect of royal jelly on the biofilm formation of *L. monocytogenes* and investigate the alternative antilisterial or anti-biofilm agent. Results obtained in the present study demonstrated, for the first time, the anti-biofilm activity of royal jelly against *L. monocytogenes*.

MATERIALS AND METHODS

Material and sample preparation

Royal jelly samples were obtained from Bursa-Inegol region, in Turkey. According to our analysis, it contained 19 $g \cdot kg^{-1}$ of 10-hydroxy-2-decenoic acid. The royal jelly was fresh, obtained in 2019 season. It was stored at –80 °C in a sterile plastic cup until use. The stock solution of royal jelly (666 $mg \cdot ml^{-1}$) was dissolved in tryptic soy broth supplemented with 6 $g \cdot l^{-1}$ yeast extract (TSBYE) and 0.5 $ml \cdot l^{-1}$ Tween 80 (all from Merck, Darmstadt, Germany).

Bacterial strain and culture conditions

Listeria monocytogenes ATCC 7644 (serotype 1/2c; American Type Culture Collection, Manassas, Virginia, USA) was cultured in 10 ml of sterile TSBYE and incubated at 37 °C for 18–24 h [14]. After incubation, the bacterial suspension was adjusted to a turbidity of 0.5 McFarland units.

Growth study

The growth of *L. monocytogenes* ATCC 7644 was followed in TSBYE containing different royal jelly concentrations for a period of 30 h at 37 °C. The culture density was assessed by measuring the absorbance at 600 nm (Epoch; Bio-Tek Instruments, Winooski, Vermont, USA) in 30 min intervals. In a 96-well plate, each well was filled with 80 μl TSBYE and 100 μl of royal jelly diluted in TSBYE, at final concentrations from 0.33 $mg \cdot ml^{-1}$ to 333 $mg \cdot ml^{-1}$, and 20 μl of bacterial suspension (6.5 log $CFU \cdot ml^{-1}$ final inoculum in well) was added.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (*MIC*) was determined using broth microdilution technique as described by FERREIRA and DOMINGUES [15]. Briefly, two-fold serial dilutions of diluted were prepared in a concentration range from 333 mg·ml^{-1} to 0.33 mg·ml^{-1} in TSBYE and added into the well. Then, 20 μl bacterial suspension (6.5 log CFU·ml^{-1} final inoculum in well) inoculated into each well and plates were incubated 37 °C for 24 h. The minimum concentration capable of inhibiting the *L. monocytogenes* ATCC 7644 growth to 90 % (MIC_{90}) compared to the growth control was determined by measuring the absorbance at 600 nm.

Curve fitting

The optical density (*OD*) at 600 nm (marked as OD_{600}) values were converted to bacterial cell populations based on the standard curve, which was constructed using enumeration assay in tryptic soy agar (Merck) supplemented with 6 g·l^{-1} yeast extract (TSAYE).

In order to calculate the calibration curve, *OD* readings and counts of bacteria were obtained from control (without royal jelly) at 1 h intervals. In order to verify that the addition of royal jelly had no effect on reading accuracy, samples were taken from the wells containing all different royal jelly concentrations at the 3rd hour. Then, bacterial counts and respective *OD* values were compared.

Each growth curve was further treated using the modified Gompertz equation [16]. The non-linear modified Gompertz equations were fitted to growth data by non-linear regression with the Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm. BFGS uses quadratic Taylor approximation and it steers the search through variable space by using an estimate of the inverse Hessian matrix. The aim of using the algorithm is to achieve a minimum error rate by minimizing the difference between the estimated and measured values. Maximum specific growth rates (μ_{max}), lag phase duration (λ) and maximum bacterial population (N_{max}) of 6 replicates were estimated from the growth kinetics.

The generation time (t_g) was calculated by the Eq. 1.

$$\mu_{max} = \frac{\ln 2}{t_g} \tag{1}$$

The dose-dependent population curve was modelled with the Gompertz survival curve with the 24th-hour data obtained using each dose-growth model. The MIC_{90} value was estimated using the generated dose-effect model. Finally, a validation experiment was performed by a plate counting method in triplicates to verify the predicted MIC_{90} value.

Mean squared error was used to find the optimum parameters of the model. To determine the goodness-of-fit of models, we used *r*-squared (R^2) statistical measure [17]. OCTAVE programming tool (Free Software Foundation, Boston, Massachusetts, USA) was used for all modelling and coding studies.

Biofilm assay

The inhibitory effect of royal jelly on *L. monocytogenes* ATCC 7644 biofilm formation was assessed using crystal violet staining assay at MIC_{90} and sub-MIC_{90} concentrations of royal jelly [18]. Briefly, 20 μl of an overnight-grown bacterial culture (6.5 log CFU·ml^{-1} final inoculum in well) were added to TSBYE in the absence or presence (0.33–41.67 mg·ml^{-1}) of royal jelly. After 96 h at 37 °C, the planktonic cells were discarded and the wells were gently rinsed five times with sterile distilled water. The plates were air-dried in the inverted position for 45 min at room temperature. Subsequently, the wells were stained with 125 μl of 10 g·l^{-1} crystal violet (for microscopy; Merck) during 15 min. After pouring out the staining solution, the plate was washed five times with sterile distilled water. The bound dye was re-solubilized in 150 μl of 95% ethanol. One hundred microlitres from each well were transferred to a new microtiter plate and the absorbance was determined at 595 nm. The assay was carried out in 6 replicates. No outlier value has been determined.

Percentage inhibition (*PI*) was calculated using Eq. 2.

$$PI = \frac{OD_{nc} - OD_e}{OD_{nc}} \times 100 \tag{2}$$

where OD_{nc} was obtained from the samples treated with water as a negative control and OD_e was obtained from the samples treated with royal jelly as experimental values.

Also, the biofilm-forming capacity of *L. monocytogenes* ATCC7644 was analysed and classified as weak ($OD_{nc} < OD \leq 2\times OD_{nc}$), moderate ($2\times OD_{nc} < OD \leq 4\times OD_{nc}$) or strong ($OD > 4\times OD_{nc}$) biofilm producer, according to STEPANOVIĆ et al. [19].

In the biofilm-forming capacity assay, OD_{nc} represented the absorbance value of solutions from wells treated with TSBYE and *OD* represented the absorbance of solutions from wells treated with the bacterial culture.

Statistical analysis

Biofilm assay results and counts of bacteria were represented as mean values ± standard deviation (*SD*) of replicates ($n = 6$). The growth kinetic parameters and biofilm inhibition assay results were evaluated using one-way ANOVA (SPSS Statistics Version 22.0; IBM, Armonk, New York, USA) considering the distribution as normal. Significance of differences between the means was further analysed by Duncans' multiple range tests. For all tests, the confidence level for significance was 95% ($P < 0.05$).

RESULTS AND DISCUSSION

The present comprehensive study evaluated for the first time the potential of *L. monocytogenes* to grow in the presence of royal jelly. Also, the current study provided a new approach for the determination of *MIC* through the value estimated from obtained growth data. Based on promising results, the research focused on the biofilm prevention ability of royal jelly against *L. monocytogenes* test strain.

Growth in the presence of royal jelly

To construct the calibration curve, simple linear regression was applied to OD and microbial counts values. The calibration formula and fitting accuracy was given (Eq. 3),

$$CL_{\mathrm{m}} = (1.13 \times 10^{9} OD) - (1.06 \times 10^{8})$$
$$R^2 = 0.974 \quad (3)$$

where CL_{m} is count of *L. monocytogenes* obtained by the plate counting method and *OD* is optical density.

Growth data were fitted to modified Gompertz equation and lag time (λ), maximum specific growth rate (μ_{max}) and maximum bacterial population (N_{max}) parameters were obtained at a range of royal jelly concentrations (Tab. 1). The output with the modified Gompertz equation at all concentrations of royal jelly fitted well with a high R^2 value (0.972–0.996). The mean square error is a goodness-of-fit indicator. After fitting, the mean square errors were found close to zero.

The addition of royal jelly appeared to alter λ, μ_{max}, t_g, and N_{max} significantly ($P < 0.05$) compared to control. Fig. 1 illustrates that *L. monocytogenes* ATCC 7644 exhibited lag, exponential and stationary phases in all growth curves.

The λ-value of control was calculated as 1.13 h. This result was comparable with that (1.40 h) obtained by Huang [13], who compared three models for description of growth of *L. monocytogenes* (a different strain than in the present study) in tryptic soy broth at 37 °C. Our growth curve showed that the addition of 20.83 mg·ml^{-1} royal jelly prolonged the lag phase of *L. monocytogenes* ATCC 7644 up to 2.64 h. This means that, in the presence of royal jelly, bacteria required more time for adaptation to the new environment. Similarly, a dose-dependent extension of the lag time was reported by some authors who studied natural alternative antimicrobial compounds [20, 21]. Since prolongation of lag phase delays the entry of bacteria to the exponential growth phase, combined food preservation methods to be applied in this time range may be more successful.

Wytock and Motter [22] emphasized that the growth rate is one of the most important and most complex phenotypic characteristics of unicellular microorganisms. As shown in Tab. 1, the μ_{max} values of *L. monocytogenes* ATCC 7644 ranged between 0.54 log CFU·ml^{-1}·h^{-1} and 0.23 log CFU·ml^{-1}·h^{-1}. The μ_{max}-value of *L. monocytogenes* in TSBYE at 37 °C without royal jelly

Tab. 1. Estimated growth kinetic parameters of *L. monocytogenes* at various concentrations of royal jelly.

Growth kinetic parameter	Royal jelly concentration [mg·ml^{-1}]											
	Control	0.33	0.65	1.30	2.60	5.21	10.42	20.83	41.67	83.33	166.67	333.33
λ [h]	1.13^{f}	1.30^{e}	1.63^{d}	1.75^{c}	1.82^{c}	1.95^{b}	1.97^{b}	2.64^{a}	nd	nd	nd	nd
μ_{max} [log CFU·ml^{-1}·h^{-1}]	0.46^{b}	0.52^{a}	0.51^{a}	0.51^{a}	0.53^{a}	0.53^{a}	0.54^{a}	0.23^{c}	nd	nd	nd	nd
t_g [h]	1.51^{b}	1.33^{c}	1.36^{c}	1.34^{c}	1.32^{c}	1.31^{c}	1.27^{d}	3.06^{a}	nd	nd	nd	nd
N_{max} [log CFU·ml^{-1}]	8.48^{a}	8.29^{b}	8.30^{b}	8.32^{b}	8.32^{b}	8.39^{b}	8.20^{c}	8.05^{d}	nd	nd	nd	nd
Goodness of fit statistics												
R^2	0.985	0.996	0.991	0.972	0.984	0.992	0.995	0.982	nd	nd	nd	nd
MSE	0.012	0.027	0.025	0.028	0.025	0.039	0.006	0.045	nd	nd	nd	nd

Different letters indicate statistical differences between values ($P < 0.05$).
λ – lag phase duration, μ_{max} – maximum specific growth rate, t_g – generation time, N_{max} – maximum bacterial population, R^2 – coefficient of determination, *MSE* – mean square error, nd – not determined.

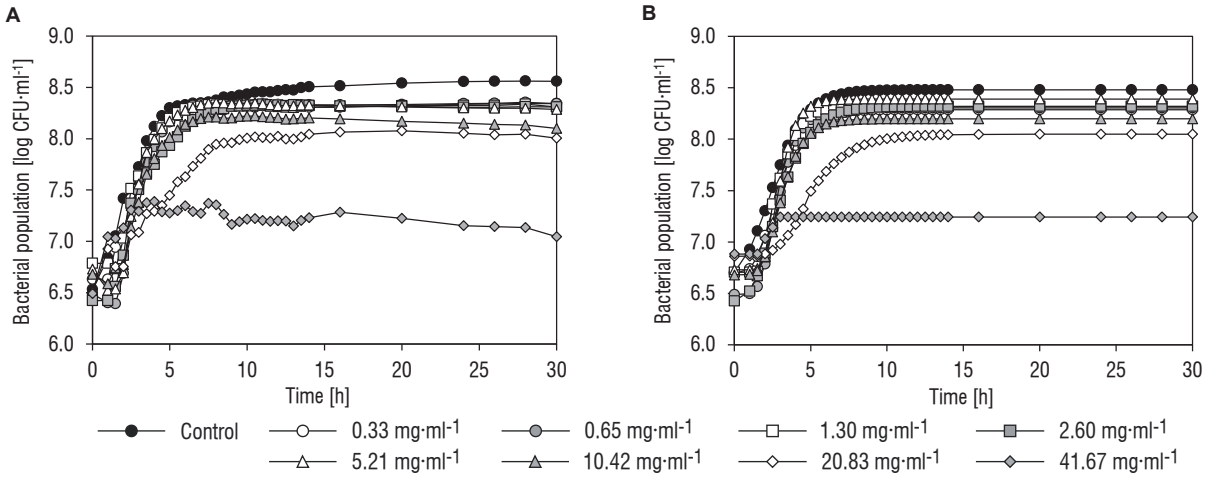

Fig. 1. Growth curves of *L. monocytogenes* at various royal jelly concentrations.

A – experimental values, B – fitted values in tryptic soy broth supplemented yeast extract at 37 °C.

(control) was found to be 0.46 log CFU·ml^{-1}·h^{-1}. Surprisingly, whereas the μ_{max}-values of *L. monocytogenes* ATCC 7644 remained constant up to the royal jelly concentration of 10.42 mg·ml^{-1}, the agent at 20·83 mg·ml^{-1} reduced it by almost half compared to the control.

The μ_{max}-value of the control was found to differ statistically significantly from samples with royal jelly at concentrations not greater than 10.42 mg·ml^{-1}. Therefore, we hypothesize that, at low concentrations, royal jelly may act as a nutrition source for *L. monocytogenes* ATCC 7644. As indicated in the review by FRATINI et al. [8], royal jelly contains 110–230 g·kg^{-1} of carbohydrates (mainly fructose, glucose and saccharose), 90–180 g·kg^{-1} of proteins and 8–30 g·kg^{-1} of vitamins and minerals, B group vitamins and mainly B_5 being abundant. In addition, free amino acids such as phenylalanine and tryptophan, which stimulate *L. monocytogenes* growth, were reported to be contained in royal jelly [23, 24]. Minimizing the growth of pathogenic bacteria (minimizing μ_{max} or inhibiting the growth completely) is essential for evaluation of antimicrobials.

As expected, since the t_g-value was inversely proportional to μ_{max}, it remained constant up to 5.21 mg·ml^{-1} but was statistically lower than the control. In control, it was found that *L. monocytogenes* ATCC 7644 doubled every 1.51 h. BARBOSA et al. [25] found that t_g of *L. monocytogenes* (a different strain than in the present study) was (1.1 ± 0.5) h, which is consistent with our result.

The N_{max} values were not considerably affected by royal jelly, as it apparently stronger affected the lag phase duration (λ). Nevertheless, N_{max} value of the lowest royal jelly concentration was found significantly lower than N_{max} value of control sample. Tab. 1 shows that N_{max}-values remained constant (8.29–8.39 log CFU·ml^{-1}) until royal jelly concentration reached 5.21 mg·ml^{-1}, but N_{max} gradually decreased at higher concentrations and was reduced by almost 0.5 log CFU·ml^{-1} at 20.83 mg·ml^{-1}. HUANG [13] reported that the low initial inoculum of *L. monocytogenes* (< 7 log CFU·ml^{-1}) did not affect N_{max} and μ_{max} values, and N_{max} value reached 9.68 CFU·ml^{-1} with the modified Gompertz equation in TSB at 37 °C. Consistently, N_{max} and μ_{max} values obtained with different initial inocula (1, 2 and 6 McFarland units) were found to produce the same results in the current study (data not shown). In this study, the reason for not reaching the level of N_{max} values of Huang's study [13] was probably associated with strain difference.

Determination of minimum inhibitory concentration

MIC_{90} values of royal jelly were determined on planktonic cells of *L. monocytogenes* ATCC 7644. In the current study, the growth of *L. monocytogenes* ATCC 7644 was inhibited at 41.67 mg·ml^{-1} of royal jelly. However, the MIC_{90}-value obtained from growth curves by the Gompertz survival function was 23.85 mg·ml^{-1} (Fig. 2). This difference can be explained by the use of two-fold serial dilution. By the nature of the two-fold serial dilution method, a range can be determined, not a single value of *MIC* [26]. Instead of repeating the experiments, MIC_{90} prediction was carried out mathematically and validation was done experi-

Tab. 2. Minimum inhibitory concentrations of royal jelly.

Evaluation method	MIC_{90} [mg·ml^{-1}]
Two-fold analysis	41.67
Predictive	23.85
After validation	24.00

MIC_{90} – minimum inhibitory concentration capable of inhibiting the *L. monocytogenes* ATCC 7644 growth to 90 %.

Tab. 3. Results of the validation experiment.

Concentration [mg·ml^{-1}]	Count of bacteria [log CFU·ml^{-1}]
0.00	8.55 ± 0.07
22.00	7.90 ± 0.03
24.00	7.53 ± 0.11
26.00	7.30 ± 0.03
28.00	7.23 ± 0.04

mentally (Tab. 2, 3). Consequently, MIC_{90} of royal jelly against *L. monocytogenes* ATCC 7644 was determined as 24 mg·ml^{-1} (Tab. 2).

Even though the antimicrobial activity of royal jelly and its bioactive components against both Gram-positive and Gram-negative bacteria has been studied, only few studies involved *L. monocytogenes*. In a study by Attalla et al. [27], the efficacy of bee venom, propolis and royal jelly in inhibiting some pathogenic bacteria including *L. monocytogenes* were investigated. Their obtained results indicated that antimicrobial activity of royal jelly on *L. monocytogenes* ranged from 32 mg·ml^{-1} to 128 mg·ml^{-1}, the values being higher than our findings. A possible explanation is that the main bioactive components of royal jelly (*trans*-10-hydroxy-2-decenoic acid), royalisin and jelleines) vary depending on geographical, seasonal and botanical origin of the product. Moreover, microorganisms show strain-specific susceptibility to antimicrobial agents. In another study, Romanelli et al. [28] showed that Jelleine II, which is one of the antimicrobial peptides of royal jelly, inhibited *L. monocytogenes* growth at 200 μg·ml^{-1}.

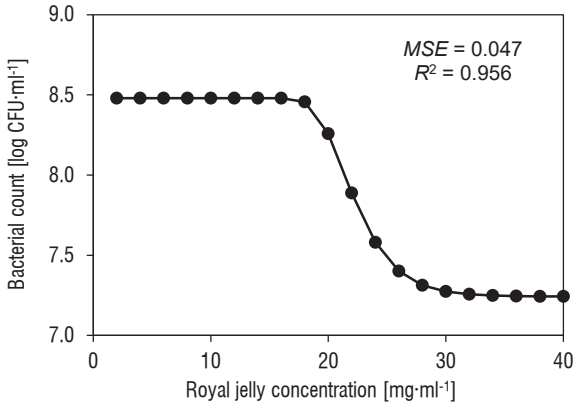

Fig. 2. Royal jelly concentration-dependent bacterial maximum growth values at 24 h.

R^2 – coefficient of determination, *MSE* – mean square error.

The number of studies investigating the effect of royal jelly on *L. monocytogenes* is limited. Thus, we discussed studies that examine the effect of other bee products on *L. monocytogenes*. Todorov et al. [29] suggested that propolis combined with ethylenediaminetetraacetic acid (EDTA) or bacteriocin could be an alternative anti-*Listeria* agent to reduce the use of antimicrobials and chemical substances in food processing. Similar results were also reported by Thamnopoulos et al. [30]. Their findings revealed that the ethanolic extract of propolis (4 mg of dry propolis extract per millilitre), when dissolved in glycerol, exhibited an enhanced antilisterial effect in extended shelf-life milk artificially contaminated with *L. monocytogenes*.

In the work of Ozkalp and Ozcan [31], the antimicrobial activities of methanolic pollen and propolis extracts were investigated on nine foodborne pathogens including *L. monocytogenes* NCTC 5348. They reported that *L. monocytogenes* was the most sensitive microorganism to 50 mg·l^{-1} concentrations of both extracts and the propolis extract had a higher inhibitory effect than the pollen extract.

Fatrcová-Šramková et al. [32] also studied the antioxidant and antimicrobial properties of poppy, rape and sunflower bee pollen on some Gram-positive and Gram-negative bacteria. They revealed that the highest antimicrobial activity of sunflower bee pollen was that of a 70 % methanolic extract after 48 h against *L. monocytogenes* CCM 4699. Also, they demonstrated that Gram-positive bacteria were more sensitive to bee pollen than the Gram-negative bacteria.

An extensive research by Yang et al. [33] was devoted to the effect of ethanolic propolis extract at various incubation temperatures, pH and cell age on *L. monocytogenes*. It was stated that 7.5 μg·ml^{-1} or higher concentrations of ethanolic propolis extract exhibited a bactericidal effect on *L. monocytogenes*. Taking into consideration all these studies, it could be stated that the inhibitory effects of propolis and pollen extracts on *L. monocytogenes* were higher than that of pure royal jelly. However, it is possible that higher antimicrobial

effects can be reached by using royal jelly extracted by organic solvents.

Concentration-dependent inhibition of biofilm growth of *L. monocytogenes*

It is well known that adhered bacterial cells are more resistant to the cleaning agent. According to a study by NORWOOD and GILMOUR [34], a hundred times greater chlorine concentration was required to inhibit *L. monocytogenes* cells in biofilm than planktonic cells.

In the biofilm-forming capacity analysis, the mean *OD* value of negative control (OD_{nc}) was found at 0.112 ± 0.013. The *L. monocytogenes* ATCC 7644 strain was classified as weak biofilm producer ($0.112 < 0.171 < 2 \times 0.112$) according to STEPANOVIC [19]. Similarly, a study where the adhesion of *L. monocytogenes* serotypes to Caco-2 cells was evaluated, it was determined that strains of the serotype 1/2c (ATCC 7644, ATCC 19112) had the lowest level of adhesion [35]. Since the stages of biofilm formation involve reversible and irreversible adhesion, lower level of adhesion may lead to weaker biofilm formation.

The effect of royal jelly on the formation of biofilm by *L. monocytogenes* ATCC 7644 is demonstrated in Fig. 3.

It has been suggested that an ideal anti-biofilm agent should not exert pressure on the growth of the bacterium at sub-*MIC* concentrations [14]. Therefore, we examined the effects of royal jelly against *L. monocytogenes* biofilm also at concentrations below *MIC*.

Our crystal violet staining assays showed that royal jelly inhibited the biofilm formation by *L. monocytogenes* ATCC 7644 at sub-inhibitory concentrations. The anti-biofilm properties of other natural agent were previously studied against *L. monocytogenes*, however, to our knowledge, this is the first study describing the anti-biofilm activity of royal jelly against *L. monocytogenes*. Results obtained in this study demonstrated that royal jelly inhibited formation of *L. monocytogenes* ATCC 7644 biofilm at 0.33 mg·ml^{-1}. As the royal jelly concentration increased (from 0.33 mg·ml^{-1} to 41.67 mg·ml^{-1}), the percentage of biofilm inhibition increased (from 73.8 % to 96.8 %).

Inhibition of *L. monocytogenes* biofilm formation by applying various natural agents is a promising solution approach for the food industry. Hence, culinary herbs, medicinal plants, essential oils, antimicrobial peptides, flavonoids and bacteriocins were investigated with respect to anti-biofilm efficacy against *L. monocytogenes* ATCC 7644. A study by VAZQUEZ-ARMENTA et al. [1] investigated the antibacterial and anti-

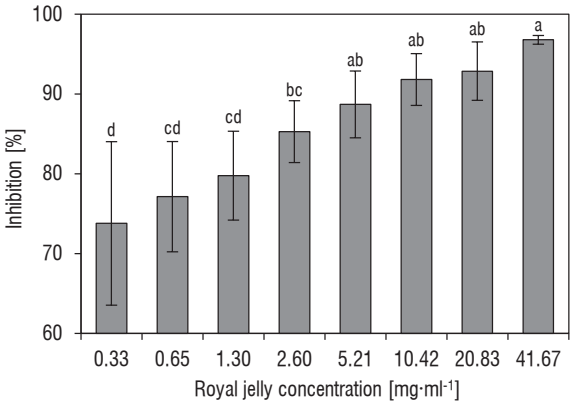

Fig. 3. Anti-biofilm activities of royal jelly on *Listeria monocytogenes*.

Different letters indicate statistical differences between values ($P < 0.05$).

biofilm activity of quercetin against the same strain. They reported that biofilm development was significantly reduced by quercetin at concentrations of 60.44–120.88 mg·l^{-1}. A study by NOSTRO et al. [36] showed that copolymers ethylene vinyl acetate coated with nisin at 1 000 IU·mg^{-1} and 8 000 IU·mg^{-1} inhibited the formation of biofilm of *L. monocytogenes* ATCC 7644.

CONCLUSION

The kinetic data of *L. monocytogenes* ATCC 7644 highlighted the possible effect of royal jelly at certain concentrations on the growth of the strain. Also, based on the findings of the present study, implementation of royal jelly supplies a quite promissing approach for the control of biofilm formation. To our knowledge, this is the first study providing data on royal jelly against the *L. monocytogenes* ATCC 7644 growth and biofilm formation and also suggesting its potential as a natural preservative and anti-biofilm agent. The study demonstrated that the predictive microbiology-based approach is practical to optimize the effective concentration, which allows to explore a novel cost-effective natural preservative. Nevertheless, further investigations are needed to better elucidate the anti-biofilm potential of royal jelly against *L. monocytogenes* strains that are strong biofilm producers. Moreover, new studies examining royal jelly and also other bee products may focus on other bacteria and yeast species forming biofilms. Future investigations proving the effect of royal jelly in food matrix and new for-

mulations regarding the industrial application may encourage the food industry to use bee products as alternative food preservatives.

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