

# Evaluation the effect of royal jelly on the growth of two members of gut microbiota; *Bacteroides fragillis* and *Bacteroides thetaiotaomicron*

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**Objective** In this study the effect of Royal jelly on the growth of two important members of *Bacteroides* spp.; *Bacteroides fragillis* and *Bacteroides thetaiotaomicron*, was evaluated. Also the physicochemical properties and cytotoxicity effects of Royal jelly on Caco-2 cell line as gastrointestinal epithelial cell model, was assessed.

**Methods** Bacteria, *B. fragillis* and *B. thetaiotaomicron* were grown on brain heart infusion (BHI) broth medium supplemented with Royal jelly in three different concentrations (2.5%, 5% and 10% v/v), both of the bacteria ( $1.5 \times 10^8$  cfu/mL) were inoculated to BHI broth contained Royal jelly in anaerobic condition. To calculate the bacterial optical density (OD), the absorbance was measured at 600 nm after an overnight. Also Caco-2 cells, was used to study the effects of Royal jelly on epithelial cell viability, and the physicochemical properties consist of total proteins, polysaccharides, phenolic compounds, total lipids, ash and moisture by UV-Vis spectrophotometric and gravimetric methods were evaluated.

**Results** The growth of *B. fragillis* and *B. thetaiotaomicron* were increased by Royal jelly (2.5%, 5% and 10% v/v concentrations) and the results indicated that Royal jelly increased the growth of bacteria in a dose dependent manner ( $p < 0.001$ ). In addition MTT assay showed more than 95% viability of Caco-2 cells treated with Royal jelly. The Iranian Royal jelly sample contains 59.01% water, 11.57% proteins, 12% lipids, 12.63% polysaccharide, and 5% mineral.

**Conclusion** This study showed that Royal jelly has a potential effect in the preserving gut microbiota and it is suggested that Royal jelly as a complementary and alternative medicine can be used to treatment diseases are associated with gut microbiota–host interactions and immune regulating. Although we need to expand our knowledge by designing clinical trials to confirm the therapeutic effects of Royal jelly on gut microbiota modulation as a barrier function.

**Keywords** *bacteroides fragile*, *bacteroides thetaiotaomicron*, gastrointestinal tract microbiota, gut microbiota, royal jelly, traditional medicine

## Introduction

Royal jelly (RJ) as a natural product and healing compound has been widely used since ancient times in the treatment of diseases and health promotion in many countries. This substance is a complex mixture of water, proteins, fats, carbohydrates, amino acids, mineral salts, vitamins, enzymes, hormones, trace elements and antibiotics. RJ has been used as a supplement due to its rich carbohydrate, protein, and mineral ingredients.<sup>1-3</sup> According to the recent pharmacological studies, RJ has antioxidant, antitumor, anti-inflammatory, antiallergic, anti-aging, and antihypertensive activities. It has been indicated that oral ingestion of RJ ameliorated lipoprotein metabolism and decreased serum total cholesterol and low-density lipoprotein levels. This product may play an important role in the maintenance of the gastrointestinal function due to its components and antioxidant activity. The bacteria are the major part of the adult gut microbiota with an essential metabolic function to degrade polysaccharides of plants. Further, formation of the intestinal mucosal barrier and stimulation of angiogenesis within a gut are other beneficial properties of the bacteria for human.<sup>1-3</sup> The microbial community of the digestive tract is known as microbiota which is a combination of various micro-organisms and thousands of bacterial species.<sup>4,5</sup>

Gut microbiota are involved in maintaining the gastrointestinal tract function. They also modulate the synthesis of vitamins, metabolism of substances, inhibition of pathogenic

bacteria growth, stimulation of mucosal IgA production, modulation of immune system, and maintenance of human hemostasis.<sup>6-9</sup> *Bacteroides fragillis* and *Bacteroides thetaiotaomicron* belong to the phylum of Bacteroidetes. *Bacteroides* spp. are abundance in gut microbiota and have significant roles in complex community. *Bacteroides* spp. mostly colonizes in the distal of GI where fermentation of indigestible carbohydrates occurs.<sup>10,11</sup>

*Bacteroides* spp. have significant role on the host metabolism and immunity through degradation of proteins and carbohydrate complex and activating the regulatory T cells. Furthermore, gut microbiota especially *Bacteroides* spp. have an important role in regulation of immune system and maintenance of homeostasis.<sup>5,11,12</sup> The patterns in the growth of bacterial in the gastrointestinal tract can be affected by various diseases, drugs, and diet. Recent studies have indicated that the effect of enteral nutrition on the reduction of infectious morbidity in critically ill patients was significantly higher than parenteral nutrition.<sup>14</sup> Therefore, the modification of gut microbiota–host interactions by Royal jelly (viable benefited bacteria) is controversial.

However, it is suggested that RJ could modulate gut microbiota, the balance between species of living bacteria in human gastrointestinal tract and effect on homeostasis and host functions.

In this study the physicochemical properties of Iranian Royal jelly and its effect on the growth of *B. fragillis* and *B. thetaiotaomicron*, as important parts of gastrointestinal microbiota and also the cytotoxicity effect of Royal jelly on Caco-2 cell line as gastrointestinal epithelial cell model evaluated.

## Materials and Methods

### Evaluation the Physicochemical Properties of Royal Jelly

#### Royal jelly preparation

Freshly harvested Royal jelly applied from beekeepers of Damavand reign, Tehran province (35.7013° N, 52.0586° E) was used.

#### Royal jelly composition

The composition of RJ is depended on seasonal and regional conditions; 100 mg of RJ sample were put on small amount of water and to dissolve using ultrasonic homogenizer, subsequently the volume reached to 100 mL with deionized water in a volumetric flask to obtain concentration of 1 mg/mL. Total phenol was measured using UV-Vis spectrometer (Optizen, Korea) according to the method of Folin-Ciocalteu.<sup>15</sup> The total polysaccharide was analyzed using UV-Vis spectrometer (Optizen, Korea) according to the previous study conducted by Vazirian et al.<sup>16</sup> Bradford method was applied to determine total protein content of the sample spectrophotometrically.<sup>17</sup> Total lipid was measured based on the described method by Müller et al. (2000).<sup>18</sup> Moisture and ash content of the sample was measured according to the previous study conducted by Horwitz and Latimer (2000).<sup>19</sup>

### Evaluation the Cytotoxicity Effect of Royal Jelly

#### Cell culture

The human epithelial cell line IBRC C10094 Caco-2 was purchased from Iranian Biological Resource Center. The cells were grown in Dulbecco's modified eagle medium (high glucose; Gibco™, USA), supplemented with 10% fetal bovine serum (Gibco™, USA), 1% penicillin/streptomycin (Gibco™, USA) and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were routinely subcultured every 3 or 4 days by trypsinization until they reached approximately 80% confluence.<sup>20</sup>

#### MTT assay

Caco-2 cells were cultured at the density of  $2 \times 10^4$  cell/well in a 96-well culture plate and incubated overnight, before RJ treatment. Cells were treated with RJ and incubated for 24 h. Then, the cell culture medium was changed. Cells were incubated with 100 µL MTT contained medium for 4 h, the medium was removed and then 100 µL DMSO was added. The absorbance was measured at 550 nm, using a microplate spectrophotometer (Epoch™, USA).<sup>20</sup>

### Evaluation the Effect of Royal Jelly on Bacterial Growth

#### Bacterial strains and growth condition

*Bacteroides fragillis* ATCC 23745 and *B. thetaiotaomicron* ATCC 10774 were grown on blood agar plates containing brain heart infusion (BHI) broth supplemented with hemin (5 µg/mL) and menadione (1 µg/mL). Incubation performed at 37°C under anaerobic conditions provided 80% N<sub>2</sub>, 10% CO<sub>2</sub>, and 10% H<sub>2</sub> atmosphere.<sup>21</sup>

#### Treatment bacteria with medium contained Royal jelly

Samples of RJ solution were prepared by deionized distilled water. Sterilization of RJ solution was performed using a 0.22-µm pore size polyvinylidene difluoride filter (Millipore, Billerica, MA, USA). This sterile solution was aseptically added to BHI broth at different concentrations (2.5%, 5% and 10% v/v). After preparation of  $1.5 \times 10^8$  cfu/mL bacterial suspension, 100 µL of *B. fragillis* and *B. thetaiotaomicron* suspension were inoculated to BHI broth and RJ contained BHI broth. Incubation was performed overnight in anaerobic condition. Finally, the bacterial optical density was calculated by measuring the absorbance at 600 nm after an overnight.

#### Ethics Statement

This work was part of the research that has been performed in accordance with the ethical rules of the Helsinki Declaration and all the experiments and procedures used in this research were approved by the Ethics Committee of Tehran University of Medical Sciences (No.IR.TUMS.REC.1395.2643).

#### Statistical Analysis

Significant differences between groups were determined by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. The level of statistical significance was  $p < 0.05$ .

## Results

The obtained data showed that the total amounts of phenol and polysaccharide in Iranian Royal jelly sample were  $21.99 \pm 0.41$  µg/mg GAE and 12.63%, respectively. The contents of lipid and protein were 12% and 11.57%. The moisture of Royal jelly was 59.01% and the amount of ash was 0.1% (Table 1). The results demonstrated that the growth of both the bacteria were increased by RJ supplementation (Table 2). The growth of *B. fragillis* and *B. thetaiotaomicron* exposed to all concentration of RJ increased in comparison with control group. This product induced growth of *B. fragillis* higher than *B. thetaiotaomicron* in all of the tested concentrations. RJ with concentration of 2.5% versus 5% of RJ significantly increased the growth of *B. fragillis* (OD;  $0.330 \pm 0.045$ ,  $0.610 \pm 0.098$ , respectively) and *B. thetaiotaomicron* (OD;  $0.299 \pm 0.060$ ,  $0.518 \pm 0.100$ , respectively). The results also indicated that there was a significant difference between the growth of *B. fragillis* (OD;  $1.020 \pm 0.360$ ) and *B. thetaiotaomicron* (OD;  $0.751 \pm 0.250$ ) exposed to RJ at a concentration of 10% v/v in comparison with the growth of them exposed to RJ at concentration of 5% v/v ( $p < 0.001$ ,  $< 0.01$ , respectively).

The MTT assay indicated that RJ had no cytotoxicity effect on Caco-2 cells after 24 h. The cell growth inhibition values for RJ against Caco-2 cells were 5% after 24 h. Result of this study showed that this substance did not reduce cell viability.

Table 1. The chemical composition of Iranian Royal jelly

| Phenol             | Polysaccharide  | Protein         | Lipid           | Moisture        | Ash           |
|--------------------|-----------------|-----------------|-----------------|-----------------|---------------|
| $21.99 \pm 0.41^a$ | $12.63 \pm 0^b$ | $11.57 \pm 0^b$ | $12.00 \pm 0^b$ | $59.01 \pm 0^b$ | $0.1 \pm 0^b$ |

<sup>a</sup>Microgram per milligram Gallic acid equivalent.

<sup>b</sup>Percentage (%).

Table 2. The effect of different concentrations (OD) of Royal jelly on the growth of *B. fragillis* and *B. thetaiotaomicron* versus control (non-exposed bacteria to RJ)

| Bacterial strain           | Control       | Royal jelly concentration (%v/v) |                     |                         |
|----------------------------|---------------|----------------------------------|---------------------|-------------------------|
|                            |               | 2.5                              | 5                   | 10                      |
| <i>B. fragillis</i>        | 0.102 ± 0.020 | 0.330 ± 0.045*                   | 0.610 ± 0.098***,†  | 1.020 ± 0.360***,††,§§§ |
| <i>B. thetaiotaomicron</i> | 0.116 ± 0.020 | 0.299 ± 0.060*                   | 0.518 ± 0.100***,†† | 0.751 ± 0.250***,††,§§  |

\*Significant difference between the control versus others.

†Significant difference between bacteria exposed to RJ 2.5% versus bacteria exposed to other concentrations.

§Significant difference between bacteria exposed to RJ 5% versus bacteria exposed to RJ 10%.

## Discussion

The present findings indicated that RJ could increase the bacterial growth with no cytotoxicity effect on Caco-2 cells. Intestinal epithelial cells are the interface between gut microbiota and host interactions. Therefore, the effect of Royal jelly on Caco-2 cell viability (a human intestinal epithelial cell model) was evaluated. According to our findings, Iranian RJ contains 59% water, 11.57% proteins, 12% lipids, and up to 12.63% polysaccharide. Similar to our study, Johansson (1995) indicated that the moisture of RJ was near to 60%. It was also indicated that RJ contained 12% proteins, 7.6% lipids, and up to 18% polysaccharide.<sup>11</sup> Compared with Johansson's (1995) study, the amount of protein and polysaccharide in our study was low. While, higher lipid contents in the RJ samples was observed in this study.

Another study indicated that RJ from different ecosystems in South America to Europe has the wider standard ranges of components (60–70% water, 8–18% proteins, 9–18% total polysaccharide, 3–11% lipids and 0.3–0.5% ash). The findings of this study also were comparable with those examined samples of different origins. The amount of ash in the sample of our study was lower (0.1%) than those reported previously.<sup>12,13</sup> The amount of phenolic compounds in our sample was near to the findings of Nagai and Inoue (21.2 µg/mg) and Nabas et al. (23.3 µg/mg).<sup>11,13</sup> Several factors including the plants species, health of the plant, season, and environmental factors can affect the amount of phenolic compounds in RJ.<sup>14</sup>

The main biological and pharmacological activities (antioxidant, immunomodulation and anti-inflammatory effects) of natural antioxidants may be related to their phenolic constituents. It has been suggested that consuming foods rich in phenolic compounds can increase the population of *Bacteroides* in the gastrointestinal tract (GIT). Therefore, Iranian RJ could be effective for modulation of several functional organs such as gastrointestinal tract. The GIT contains many important beneficial microbes. For example, *Bacteroides* spp. are one of the micro-organisms present primarily for the sustenance of a healthy GI system.<sup>19</sup> The biological activity and development of this bacterium is further enhanced in the presence of phenolic compounds.<sup>19</sup> Some *in vitro* and *in vivo* experimental studies on polyphenolic compounds have reported that these compounds hastened the growth of *Lactobacillus* and *Bacteroides* and catalyze their probiotic potency in the GIT.<sup>20</sup> Under *in vitro* conditions, polyphenolic compounds promoted the increase in the numbers of *Bacteroides* by 10–100-folds, which was beneficial for the intestinal microbiota. Increasing in *Bacteroides* leads to a decrease in the formation of ammonia,

skatoles and harmful amine procarcinogens in the large intestine, and reduce acid production that raises fecal pH.<sup>21</sup> Since microbiota growth was relatively unaffected by most of the aromatic compounds tested, probiotic colonization in the intestine should continue in the presence of phenolic compounds so as to improve the intestinal microbial balance and inhibit pathogen growth. Previous research has demonstrated that consumption of other plants rich in polyphenolic compounds such as green tea selectively promotes the growth of *Bacteroides* in the gut wall.<sup>18</sup> This data demonstrated that RJ contains a high percent of phenolic compounds. However, phenolic compounds increase the growth of intestinal bacteria; antimicrobial activities of polyphenols have been demonstrated.<sup>21</sup> The level of inhibition varies depending on the bacterial species, the chemical structure of the compound and phenolic concentration. Findings of this study showed that RJ could change the bacterial growth. This effect may be related to the RJ concentrations and the bacterial species. It has been known that, gastrointestinal microbiota composition has significant roles in the determination of health and disease states.<sup>13</sup> As well as scrutiny gut microbiota pattern in conduct an investigation and preclinical studies can be helped in understanding the processes of maintaining gut microbiota by supplementation and prescription of some natural products such as Royal jelly in preserving gut microbiota, especially in ill patients undergoing intensive care and receiving medication and variety of drugs with a microbiota weakening effect. This study was designed based on research to recognizing ways to reinstate gut microbiota composition and results were showed that RJ has a potential effect in the preserving and energy conversation of gut microbiota to improve human health as a defense barrier, and also Royal jelly can be used to formulating preparations for treatment diseases and maintaining health situation. Although this assumption needs to expand our knowledge about the mechanism of effects caused by Royal jelly on the common microbial community includes pathogens and friendly micro-organisms of healthy voluntaries and patients, by designing clinical trials to confirm the preliminary obtained results on the therapeutic effects of Royal jelly on gut microbiota modulation as a barrier function.

## Conclusion

According to the importance of *Bacteroides* spp. in gut microbiota–host interactions, it is suggested that gastrointestinal microbiota pattern could be changed by Royal jelly supplementation in patients receiving variety of drugs with a gut microbiota weakening side effects. Finding of this work indicated that RJ has a potential effect in the growth of gut microbiota

which could improve human health and treatment diseases. However, several clinical trials should be done to cover a large number and wide scope of subjects and confirm the beneficial effects of RJ on gut microbiota. In conclusion, Royal jelly seems to be a very effective approach to treat and, all results of low sample size and short-term studies have to be taken into account. In this purpose, multi-center study to evaluate the comprehensive application of Royal jelly with established treatment modalities in a randomized, controlled trial has been started.

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## Conflict of Interest

The authors declare that there is no conflict of interest. ■

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