

## Chemical Composition of Royal Jelly and Effects of Synbiotic with Two Different Locally Isolated Probiotic Strains on Antioxidant Activities

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This study was carried out to investigate the antioxidant properties of synbiotic product, *Lactobacillus acidophilus* supplemented with 2.5% royal jelly in skim milk and *Bifidobacterium bifidum* supplemented with 7.5% royal jelly in skim milk, using DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay, reducing power, total antioxidant in linoleic acid system and formation of diene-conjugation assay. Results showed that the synbiotic effect of royal jelly and probiotic bacteria provided substantial antioxidant activities. Milk samples fermented by *B. bifidum* supplemented with 7.5% royal jelly and *L. acidophilus* supplemented with 2.5% royal jelly exhibited high scavenging activity with 96.8 and 93.3%, respectively, at a concentration of 500 µg/mL. IC<sub>50</sub> values were estimated at 226.7 µg/mL for *B. bifidum* supplemented with 7.5% royal jelly and at 210.2 µg/ml for *L. acidophilus* supplemented with 2.5% royal jelly. On the other hand, *L. acidophilus* supplemented with 2.5% royal jelly and *B. bifidum* supplemented with 7.5% royal jelly exhibited significantly high reducing power at a concentration of 1000 µg/mL. The percentages of peroxide inhibition of *L. acidophilus* supplemented with 2.5% royal jelly and *B. bifidum* with 7.5% royal jelly were 52% and 42%, respectively. Significant inhibitions were found in the formation of conjugated diene at 66.9% and 65.8% for *L. acidophilus* with 2.5% royal jelly and *B. bifidum* with 7.5% royal jelly, respectively. These results were compared with standards BHT, ascorbic acid and Trolox.

### INTRODUCTION

At present days, remarkable changes were observed in understanding the role of foods in human health. Enormous interest of consumers has been directed toward healthy foods which help in preventing the initiation of diseases and their development; hence, a new term “functional food” was suggested [Hardy, 2000; Grajek *et al.*, 2005].

Functional food is considered to be any food or food component that provides health benefits beyond basic nutrition. Recently, a great deal of interest has been paid by the consumers towards natural bioactive compounds as functional ingredients in the diets due to their various health beneficial effects [Vo & Kim, 2013]. A functional food may be natural or be obtained by the elimination or the modification of one or more of its basic components [Perez-Alvarez *et al.*, 2003]. Selective non-digestible oligosaccharides and polysaccharides, some peptides and proteins, and certain lipids, are the most common prebiotics [Gibson & Roberfroid, 1995]. Prebiotics are known to promote the proliferation of bifidobacteria and lactobacilli [Roberfroid, 2000]. Among foods that can be considered as functional foods, we may include all those originating in the beehive: honey [Haddadin *et al.*, 2007; Mei

*et al.*, 2010], propolis [Haddadin *et al.*, 2008], and royal jelly [Haddadin *et al.*, 2012; Nabas, 2012].

Royal jelly (RJ) is a natural substance considered as one of the most important products of honeybees with high nutritional, functional and biological properties. It is secreted from the hypopharyngeal glands of worker bees which serve as food for the queen bee and to the young larvae [Grout, 1992]. In general, royal jelly is relatively acidic (pH 3.1–3.9) with a high buffering capacity ranges between 4 and 7 [Sauerwald *et al.*, 1998].

It is composed of proteins, lipids, sugars, vitamins and amino acids [Howe *et al.*, 1985], B complex vitamins such as B1, B2, B6 [Moreschi & Almeida-Muradian, 2009], and biotin [Nandhasri *et al.*, 1990]. Moreover, it contains different minerals (P, S, Ca, Mg, K, Na, Zn, Fe, Cu, Mn) and trace elements with biological functions such as (Al, Ba, Sr, Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Ni, Ti, V, Co, Mo) [Stocker *et al.*, 2005]. The main fatty acid present in RJ is 10-hydroxy-trans-2-decenoic acid (10-HDA) [Yang *et al.*, 2010], it plays an important role in boosting immune system, anticancer activity. It contains other components such as royalisin and apisin [Watanabe *et al.*, 1996, 1998]. Royal jelly can be included directly in our food and dietary supplements, moreover, royal jelly is traditionally known to have some biological or medical purposes such as life-span elongating [Inoue *et al.*, 2003], anti-fatigue [Kamakura *et al.*, 2001], anti-

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allergic and immunoregulatory effect [Okamoto *et al.*, 2003], and anti-inflammatory [Kohno *et al.*, 2004], anti-bacterial, and antioxidant activity [Nagai & Inoue, 2004; El-Nekeety *et al.*, 2007; Eshraghi & Seifollahi, 2003; Barnutiu *et al.*, 2011; Guo *et al.*, 2005; Liu *et al.*, 2008].

A probiotic is a beneficial living microbial food ingredient whose growth and activity are stimulated by prebiotics [Salminen *et al.*, 1998]. According to Chandan [1999], the most widely used probiotics in foods, especially in dairy products, are the members of the genera *Enterococcus*, *Lactobacillus*, and *Bifidobacterium*. Probiotic bacteria proved to have desirable clinical benefits, therapeutic actions and protective effects against oxidative damages [Amaretti, *et al.*, 2013; Bernardeau *et al.*, 2008; Osuntoki & Korie, 2010; Pan *et al.*, 2009]. Recently they are being recognized for their remarkable role in regulating the host metabolic processes, weight gain and obesity [Thomas & Versalovic, 2010].

In practice, combined mixtures of probiotics and prebiotics are often used because their synergic effects are conferred onto food products. For this reason, such mixtures are called synbiotics [Gibson & Roberfroid, 1995]. Synbiotic products may have beneficial effects on the host by enhancing survival and implantation of live microbial dietary supplements in the gastrointestinal microbiota, by selectively stimulating the growth or activating the catabolism of one or a limited number of health-promoting bacteria in the gastrointestinal tract, and by improving the intestinal tract's microbial balance [Wollowski *et al.*, 2001]. Moreover, probiotic and prebiotic effects might be additive or even synergistic [Roberfroid, 2000].

The objective of this study was to evaluate the antioxidant properties of the synbiotic effect of royal jelly with two different locally isolated strains *Lactobacillus acidophilus* and *Bifidobacterium bifidum* using different *in vitro* assays.

## MATERIALS AND METHODS

### Probiotic bacterial strains

*L. acidophilus* and *B. bifidum* isolates used in this research were previously isolated from new born breast fed infants stool [Haddadin, 2004], at Queen Alia hospital. One gram of each freeze-dried powders of these isolates was transferred aseptically into 50 mL sterile MRS broth supplemented with 5 g/L L(+) cysteine-HCL (99.6% purity, Sigma, USA), then incubated at 37°C for 20 h in an anaerobic jar (Oxoid, UK). Repeated streaking onto MRS agar plates were used for purification of both isolates, the isolates of *L. acidophilus* and *B. bifidum* were identified physiologically and biochemically according to Bergey's Manual [Kandler & Weiss, 1986] and Prokaryotes [Hammes *et al.*, 1992]. The isolates were activated making sub-culturing twice in MRS broth containing 0.5% L(+) cysteine-HCL, using 1% inoculum and 18–20 h of incubation at 37°C. Each isolate was sub-cultured three times prior to every test [Walker & Gilliland, 1993].

### Royal jelly

Royal jelly samples were collected during the spring and summer of the year 2011 from Langstroth hives with colonies of the most common honeybee species *Apis mellifera* located at the University of Jordan Apiary – Faculty of Agri-

culture, using the artificial cups method according to Grout [1992]. Samples of royal jelly were diluted with distilled water and filtered under a vacuum, using, Grade No.1 filter paper, and Grade No. 40 filter paper (Whatman membrane, England), and 0.45  $\mu\text{m}$  nylon membrane. Cold sterilization was performed *via* micro-filtration unit using 0.20  $\mu\text{m}$  sterile cellulose-ester membranes (Advantec MFS, Japan) [Haddadin *et al.*, 2007]. A 9% skimmed milk was reconstituted in distilled water and sterilized at 115°C for 10 min. After cooling to 37°C, five different concentrations of sterilized royal jelly (0, 2.5, 5, 7.5, 10% m/v) were added to reconstituted milk in 100 mL Duran bottles. A control sample was used without the addition of royal jelly [Haddadin *et al.*, 2012; Nabas, 2012]. Royal jelly/milk samples with highest counts of each probiotic bacteria were chosen to examine the antioxidant activities. The pH of RJ was measured using a digital pH meter (Hanna instrument model HI 8519, Italy). The pH was directly measured after adding 2 g of RJ to 4 mL of distilled water (pH 7.00).

### Evaluation of bacterial growth

One percent % (v/v) of *L. acidophilus* or *B. bifidum* was added to the prepared milk/royal jelly samples. The cultures were then incubated at 37°C in anaerobic jars for 24 h. After incubation, serial dilutions (10<sup>-1</sup> – 10<sup>-7</sup>) were realized using sterile 0.1% peptone broth and plated on MRS agar supplied with cysteine-HCl (5 g/L) and incubated at 37°C for 48 h under anaerobic condition. The results were recorded as CFU/mL of culture [Harrigan & McCane, 1996].

### Determination of total flavonoids of royal jelly

The total flavonoid content of RJ was determined according to Ja *et al.* [1999]. Five grams of RJ were added to 50 mL of distilled water and filtered using Grade No. 1 Filter Paper (Whatman membranes, England). Then, 0.5 mL of the solution was mixed with 0.3 mL of sodium nitrite solution (5 g/L). After 5 min, 0.3 mL of aluminum chloride (1 g/L) was added. After another 5 min, 2 mL of 1 mol/L of sodium hydroxide was added to the mixture. Total volume was made up to 10 mL with distilled water and the sample was sonicated immediately after preparation. The absorbance was measured at 510 nm against water blank using UV/Visible Spectrophotometer (Jasco-V-530, Japan). Calibration curve was plotted by preparing a solution of rutin (0–200  $\mu\text{g}/\text{mL}$ ). Concentrations are expressed as ( $\mu\text{g}$  rutin equivalent /mg RJ).

### Determination of total phenolic content of royal jelly

Total phenolic content of RJ was determined by Folin-Ciocalteu method according to Singleton *et al.* [1999]. To this end, 0.5 mL from the previous solution of RJ used in flavonoid assay was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagents (Sigma –Aldrich, USA) for 5 min and then 2 mL of 7.5% sodium carbonate were added. The absorbance was measured at 760 nm after 2 h of incubation at room temperature against methanol blank using UV/Visible spectrophotometer (Jasco-V-530, Japan). The concentration between 0.01–0.05 mg/mL of gallic acid was used as standard for the calibration curve. The total phenolic content was expressed as  $\mu\text{g}$  gallic acid equivalent mg RJ.

### Moisture content of royal jelly

For the determination of moisture content, 2.5g of royal jelly was placed in a crucible and dried in the oven at 105°C for three hours, covered and then cooled in a desiccator. The process was repeated at a one hour drying intervals until a constant weight was obtained [Adebiyi *et al.*, 2004].

### Ash content of royal jelly

For the determination of ash, 2.5 g of royal jelly was placed in a crucible and dried in the oven at 105°C for three hours, after cooling the sample was ashed in a muffle furnace (carbolite model ELF 11/14) at 550°C for 8 h; then cooled and weighed [Adebiyi *et al.*, 2004].

### Total nitrogen content of royal jelly

To determine the total nitrogen content, the Kjeldahl method for the determination of organic nitrogen was used. One gram of royal jelly was mixed with 15 mL sulphuric acid and placed in a digestion tube, followed by distilling and titrating. The nitrogen content (N % w/w) was then calculated using 6.38 as the factor for converting nitrogen to protein [AOAC, 1980].

### Mineral content of royal jelly

Calcium (Ca), iron (Fe), and manganese (Mn) were determined directly in the ash solution by atomic absorption spectrometer (Perkin Elmer Instrument). Five grams of royal jelly were ashed in a furnace at 550°C until the appearance of white-grey ash. The ash was dissolved in nitric acid and boiled, and the residue was dissolved in distilled water with 1 mL compensating solution to prevent any possible interference with measurement of different metals. The concentration of each metal was extrapolated from standard curves [Vinas *et al.*, 1997].

### Total lipid content of royal jelly

Lipid content was determined by Soxhlet procedure using diethylether as a solvent according to a standard AOAC procedure [1980].

### Total carbohydrates content of royal jelly

Total carbohydrate was obtained by difference [AOAC, 1980], using the formula: Total carbohydrate (CHO) = (100 – moisture% + protein% + lipids% + ash%).

### Antioxidant activities

The highest count of *B. bifidum* was 9.89 log<sub>10</sub> CFU/mL when 7.5% (w/v) royal jelly was added to skimmed milk, while the highest count of *L. acidophilus* was 8.93 log<sub>10</sub> CFU/mL when 2.5% (w/v) of royal jelly was added [Nabas, 2012]. Fermented milk samples were centrifuged at 3000×g for 15 min. Supernatants were used in all experiments.

### DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity

The free radical scavenging activity of fermented milk was measured according to Sanchez-Moreno *et al.* [1998]. A 0.5 mL dose of different concentrations of fermented milk (50, 100, 200, 300, 400, 500 µg/mL) was mixed with 0.5 mL

of 0.1 mmol/L DPPH (Sigma-Aldrich, USA), and 2 mL of methanol, shaken vigorously by rotomixer (Velp-Scientifica, Italy) and incubated in dark for 30 min at room temperature. Absorbance was measured using UV/VIS spectrophotometer at 517 nm against a blank. The sample concentration that caused a decrease in the initial DPPH concentration by 50% was calculated from the graph of scavenging effect percentage against the sample concentration. The DPPH reduction ( $G_R$ ) in 30 min was calculated using the following equation:

$$G_R(\%) = [(1 - A_f/A_0) \times 100]$$

where:  $A_f$ : Absorbance of sample after 30 min;  $A_0$ : Absorbance of sample at the moment of solution preparation.

The inhibition activity was calculated using the following equation:

$$DPPH \text{ radical scavenging activity } (\%) = [(1 - A_i/A_0) \times 100]$$

where:  $A_0$ : Absorbance of control;  $A_i$ : Absorbance of sample.

### Determination of reducing power

The reducing power of fermented milk supplied with royal jelly was determined according to Oyaizu [1986]. Two and one-half mL of different concentrations of fermented milk samples (50, 100, 200, 500 and 1000 µg/mL) were mixed with 2.5 mL of sodium phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of potassium ferricyanide (1%) solution. The mixture was incubated at 50°C for 20 min followed by the addition of 2.5 mL of trichloroacetic acid (10%) and centrifuged at 1400×g for 10 min. Next, 1 mL of the supernatant was mixed with 1 mL distilled water and (0.1%) ferric chloride. The absorbance was measured at 700 nm using UV/VIS spectrophotometer (Elico, SL 150, India). Ascorbic acid was used as a standard and phosphate buffer as blank.

### Total antioxidant activity in linoleic acid system

The total antioxidant activity of fermented milk supplemented with royal jelly was measured by the use of a linoleic acid system [Mitsuda *et al.*, 1996]. The linoleic acid emulsion was prepared by mixing 0.28 g of linoleic acid (Sigma-Aldrich, USA), 0.28 g of Tween-20 emulsifier (Sigma-Aldrich, USA), and 50 mL of phosphate buffer (0.2 mol/L, pH 7.0). The mixture was then homogenized (Janike@Kunkel, IKA® homogenizer, Germany). A 0.5 mL of 100 µg/mL of fermented milk or standard sample (in methanol) was mixed with linoleic acid emulsion (2.5 mL, 0.2 mol/L, at pH 7.0) and phosphate buffer (2 mL, 0.2 mol/LM, pH 7.0). The reaction mixture was incubated at 37°C in the dark to accelerate the peroxidation process. The levels of peroxidation were determined according to the thiocyanate method by sequentially adding ethanol (5 mL, 75% v/v), ammonium thiocyanate (0.1 mL, 30% v/v), sample solution (0.1 mL), and ferrous chloride (0.1 mL, 20 mmol/L in 3.5% HCl). After mixing for 3 min, the peroxide values were determined by reading the absorbance at 500 nm, using UV/Vis spectrophotometer. 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich,

USA) was used as positive control. The percentage inhibition of peroxide value was calculated using the following formula:

$$\text{Inhibition (\%)} = [(1 - A_1/A_0) \times 100]$$

where:  $A_0$ : Absorbance of control;  $A_1$ : Absorbance of sample.

#### Diene-conjugation formation method

The effects of fermented milk supplied with royal jelly on the formation of conjugated diene hydroperoxides were determined according to the method of Tee *et al.* [2002]. Linoleic emulsion system was prepared as described in the previous section. Fermented milk with the most effective concentrations of royal jelly (50, 100, and 200  $\mu\text{g/mL}$ ), BHT (100  $\mu\text{g/mL}$ ) and 100  $\mu\text{g/mL}$  of Trolox (Sigma - Aldrich, USA) were added to each system, respectively. The emulsion was homogenized for 30 sec and incubated at 40°C. The formation of conjugated diene was monitored daily for seven days by solubilizing 0.2 mL of the linoleic acid emulsion in 5 mL of absolute methanol. The absorbance was measured at 234 nm using UV/Vis spectrophotometer. The percentage inhibition of conjugated diene value was calculated using the following formula:

$$\text{Inhibition (\%)} = [(1 - A_1/A_0) \times 100]$$

where:  $A_0$ : Absorbance of control;  $A_1$ : Absorbance of sample.

#### Statistical analysis

The general linear model (GLM), produced by the Statistical Analysis System (SAS) version 7 [SAS® system for Microsoft® Windows®, 2001], was used to analyse the data. All the data of this research were designed to apply two replicates of each level for every experiment. Differences between the means of treatments were tested using the least significant difference (LSD) test at ( $p < 0.05$ ). Repeated measures analysis of variance (ANOVA) was used to analyse the data within the same experiment. Split – Split-Plot, CRD, with repeated measurement design was used for conjugated diene formation test, CRD with repeated measurement design for peroxide values and Complete Randomized Design for reducing power and DPPH scavenging activity. Results are shown as the mean values  $\pm$  standard deviation.

## RESULTS AND DISCUSSION

Royal jelly is a viscous jelly substance, often not homogeneous due to the presence of undissolved granules of varying size. It is partially soluble in water with a density of 1.1 g/mL. Its colour is whitish to yellow, the yellow colour increasing upon storage. Its odour is sour and pungent, the taste being sour and sweet [Lercker, 2003].

The amounts of constituents in royal jelly determined in this study are reported in Table 1. The composition of the main constituents of RJ, carbohydrates, proteins and lipids is reported in the literature [Pourtaillier *et al.*, 1990; Lercker, 2003; Garcia-Amoedo & Almeida-Muradian, 2007]. The differences observed are probably due to the different number of samples taken in different places and at differ-

TABLE 1. General chemical composition of Royal jelly produced in Jordan.

Chemical analysis	Content
pH	3.42 $\pm$ 0.02
Moisture (%)	61.5 $\pm$ 0.25
Ash content (%)	1.10 $\pm$ 0.05
Protein content (%)	13.15 $\pm$ 0.60
Fat (%)	10.18 $\pm$ 3.48
CHO (%)	14.07 $\pm$ 0.0
Total phenolics ( $\mu\text{g}$ galic acid/mg RJ)	23.3 $\pm$ 0.92
Total flavonoids ( $\mu\text{g}$ rutin/mg RJ)	1.28 $\pm$ 0.09
Ca (ppm)	64.5 $\pm$ 6.50
Fe (ppm)	0.63 $\pm$ 0.10
Mn (ppm)	0.03 $\pm$ 0.01

Results are mean values of duplicates determinations  $\pm$  standard deviation.

ent times of production. Besides to that different methods of sampling and analysis were used. Moreover, royal jelly is a naturally heterogeneous product.

The total lipid fraction in the tested royal jelly is likewise present in reasonably modest concentration, 10.18%, (Table 1), but indeed represents the third most important component of RJ after carbohydrate and proteins. The lipid portion in fact consists primarily of organic acids (80–90%), most of which free, with a rather unusual structure rarely encountered in nature: they are in fact mono- and dihydroxy acids and dicarboxylic acids with 8 and 10 carbon atoms, which show a characteristic arrangement [Lercker *et al.*, 1993]. Hydroxy acids with 10 carbon atoms (10 hydroxydecanoic and 10-hydroxy-2-decenoic acid) considered the main factor for antimicrobial activity [Fujiwara *et al.*, 1990]. Eshraghi & Seifollahi [2003] examined the antibacterial effect of RJ on *Escherichia coli* (ATCC 29532), *Staphylococcus aureus* (ATCC 14776), *Streptomyces griseus* (ATCC 11746), and three different *Streptomyces sp.* (S.46) (S.F8) and (S.66). They found that the application of ether soluble fraction of RJ was more effective than pure RJ. On the other hand, it was found that the inhibitory effect of 30 mg/mL ether soluble fraction of RJ was stronger than the same concentration of ether non soluble fraction which has no effect even at 300 mg/mL of RJ. The identification of this fraction and specially the arrangement and quantitative ratios of free organic acids, is believed to represent the criteria of choice for recognizing the authenticity of RJ and for confirming and quantifying the claimed existence of royal jelly in other products. Besides the free fatty acids, the lipid fraction consist of some neutral lipids, sterols (including cholesterol) and an unsaponifiable fraction of hydrocarbons similar to beeswax extracts [Lercker *et al.*, 1981, 1982, 1984, 1993].

The total carbohydrate content of royal jelly was 14.07% (Table 1) and mostly consisted of fructose and glucose. Fructose is prevalent. In many cases fructose and glucose together account for 90% of the total sugars. Sucrose is always present but often in highly variable concentrations. It is also possible to detect other oligosaccharides such as trehalose, maltose, gentiobiose, isomaltose, raffinose, erlose, melezitose [Garcia-Amoedo & Almeida-Muradian, 2007]; though present in very

small quantities they are useful for identifying a characteristic pattern, which is comparable to that of honey and in some cases indicative of the genuineness of the product [Sabatini *et al.*, 2009].

The values of crude protein content in royal jelly are presented in (Table 1). The obtained data are in full compliance with findings of other authors [Krell, 1996; Wongchai & Ratanavalachai, 2002; Nagai & Inoue, 2004; Garcia-Amoedo & Almeida-Muradian, 2007], who reported the range from 12 to 15%.

In general, water content in royal jelly is fairly uniform and makes up about two third of fresh royal jelly. Table 1 shows that royal jelly consists of 61.5% of water which agreed with the range of 57–70% reported by Krell [1996] and 60–70% by Garcia-Amoedo & Almeida-Muradian [2007]. The constancy of the water content is basically assured, inside the hive, by the continuous provision of fresh supplies of this substance by nurse bees, by the natural hygroscopicity of royal jelly and the entire colony's efforts to maintain a level of ambient humidity; moreover the non-solubility of some compounds can explain the variations in moisture content [Sabatini *et al.*, 2009].

The total amounts of phenolic and total flavonoids contents in the tested royal jelly are presented in Table 1. The phenolic and total flavonoids content of the RJ were as follows: 23.3  $\mu\text{g}/\text{mg}$  of RJ and 1.28  $\mu\text{g}/\text{mg}$  of RJ, respectively. These quantities of phenolic compounds were very similar to that found by Nagai & Inoue [2004] which is 21.2  $\mu\text{g}/\text{mg}$  of RJ. The total phenolic contents of 219.2  $\mu\text{g}/\text{g}$ , were found in royal jelly obtained from Taiwan collected 24 h after grafting, while the total phenolic contents of royal jelly collected after 48 and 72 h was 194.4  $\mu\text{g}/\text{g}$  and 131.7  $\mu\text{g}/\text{g}$ , respectively [Liu *et al.*, 2008].

Liu *et al.* [2008] indicated that regardless of initial larval age, RJ collected 24 h after the larval transfer contained higher total polyphenolic contents than the RJ collected 48 or 72 h after the transfer.

These compounds have antimicrobial activity and explain the antimicrobial capacity of royal jelly. Moreover, it has been reported that phenolics are efficient antioxidant, immunomodulator and anti-inflammatory agents [Nagai & Inoue, 2004]. The variation of the total phenolic contents is attributed to several factors such as climate and floral source. The phenolic compounds of royal jelly could originate from plants where they are widely distributed in nature [Bravo, 1998; Wongchai & Ratanavalachai, 2002; Liu *et al.*, 2008]. The main groups of phenolic compounds present in plants, whether in free form or as glucosides, are derivatives of cinnamic acid, coumarins, and flavonoids [Manthey & Grohmann, 2001]. In royal jelly, most of the phenolic compounds are in the form of flavonoids whose concentration depends on various factors, including plant species used by the bees, health of the plant, season, environmental factors, and so on [Kucuk *et al.*, 2007].

The pH value of RJ was 3.42, which agreed with the pH value reported by Krell [1996]. Percentage of ash in royal jelly sample was 1.10%, this value is comparable to the mean ash content of Thailand royal jelly with 1.14%. On the other hand, it was reported that the ash content of fresh royal jelly reached

0.8–3.0% [Wongchai & Ratanavalachai 2002; Garcia-Amoedo & Almeida-Muradian, 2007].

Concentrations of minerals detected in this research varied, calcium was the most abundant element compared with other determined minerals with 64.5 ppm followed by Fe (0.63 ppm) and very low concentration of Mn (0.03 ppm). These results agree with those reported by Stocker *et al.* [2005], who detected different trace and mineral elements in royal jelly that could be attributed to an external factor such as environment, different botanical and geological origins and to some extent internal factors such as biological factors related to the bees [Stocker *et al.*, 2005; Garcia-Amoedo & Almeida-Muradian, 2007].

The proton radical scavenging has been reported to be a major mechanism for antioxidation. The assay for the assessment of proton radical-scavenging activity with DPPH is relatively simple and enough reproducible; this compound has been reported to be stoichiometrically decolorized by antioxidants. The reduction in the DPPH would be observed as its absorbance at a characteristic wavelength is decreased when it is treated with proton radical scavengers. The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant [Lu & Foo, 2001]. It has been widely used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts and foods [daPorto *et al.*, 2000].

The antioxidant capacity of fermented milk (*L. acidophilus* supplemented with 2.5% royal jelly and *B. bifidum* supplemented with 7.5% royal jelly) was evaluated in different *in vitro* tests. The percent of DPPH radical scavenging activity of fermented milk samples containing royal jelly, with ascorbic acid and butylated hydroxytoluene (BHT) as reference samples are listed in Figures 1 and 2. The scavenging effects of fermented milk with *B. bifidum* and standards on DPPH radicals were 8.6, 17.1, 18.9 and 48.5% at a concentration of 50  $\mu\text{g}/\text{mL}$  for *B. bifidum*, *B. bifidum* supplemented with 7.5% RJ, BHT and ascorbic acid, respectively (Figure 1). A significant DPPH radical scavenging activity was observed when fermented milk with *B. bifidum* supplemented with 7.5% RJ was used at 500  $\mu\text{g}/\text{mL}$  with an effect of 96.8% (Figure 1). Moreover, the value of the antioxidant capacity of fermented milk with *L. acidophilus* supplemented with 2.5% RJ was 12.6% at a concentration of 50  $\mu\text{g}/\text{mL}$ . This value was increased significantly to 93.2% at a concentration of 500  $\mu\text{g}/\text{mL}$  (Figure 2). As presented in Figures 1 and 2, the scavenging abilities of different samples were clearly demonstrated dose-dependent antioxidant activity against DPPH. These findings agree with observations indicated by other researchers [Nagai & Inoue, 2004; Sowndhararajan & Kang, 2013; Zhang *et al.*, 2011]. Nagai & Inoue [2004] demonstrated that the radicals scavenging activities of royal jelly samples tended to decrease with a decreasing concentration of the sample.

The maximum  $G_R$  percentage for fermented milk was obtained at a concentration of 500  $\mu\text{g}/\text{mL}$  with values of 28.4, 92.0, 91.8 and 80.5% for *B. bifidum*, *B. bifidum* supplemented with 7.5% RJ, ascorbic acid and BHT, respectively. On the other hand, the maximum  $G_R$  percentage for fermented milk was obtained also at a concentration of 500  $\mu\text{g}/\text{mL}$  with values of 15.4, 93.2, 91.8 and 80.5% for *L. acidophilus*, *L. acidophilus* supple-

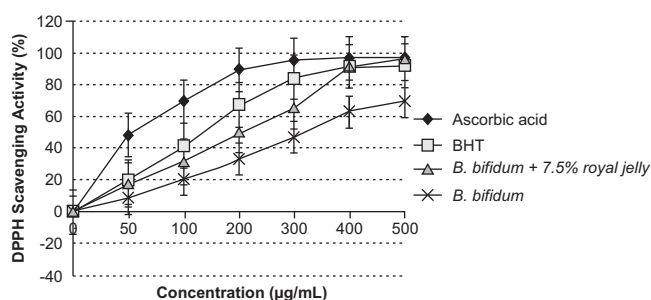


FIGURE 1. Scavenging activity of different concentrations of ascorbic acid, BHT, *B. bifidum* supplemented with 7.5% royal jelly and *B. bifidum*.

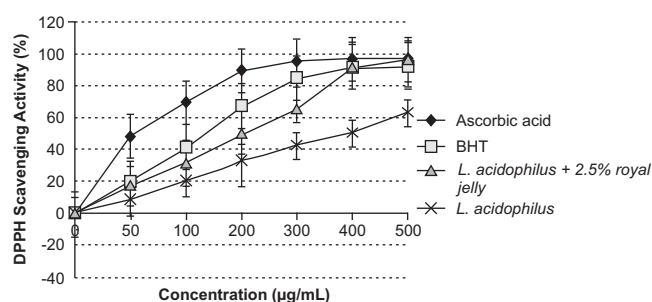


FIGURE 2. Scavenging activity of different concentrations of ascorbic acid, BHT, *L. acidophilus* supplemented with 2.5% royal jelly and *L. acidophilus*.

TABLE 2. The reduction of DPPH ( $G_R$ ) of ascorbic acid, BHT, *B. bifidum* and *B. bifidum* supplemented with 7.5% RJ, *L. acidophilus* and *L. acidophilus* supplemented with 2.5% RJ.

Concentration (µg/mL)	$G_R$ (%)					
	Ascorbic acid	BHT	<i>B. bifidum</i>	<i>B. bifidum</i> supplemented with 7.5% RJ	<i>L. acidophilus</i>	<i>L. acidophilus</i> supplemented with 2.5% RJ
50	33.3 ± 0.04 <sup>a</sup>	3.2 ± 1.2 <sup>b</sup>	2.2 ± 0.33 <sup>b</sup>	6.2 ± 1.6 <sup>b</sup>	2.5 ± 1.2 <sup>b</sup>	3.2 ± 1.3 <sup>b</sup>
100	55.7 ± 0.64 <sup>a</sup>	17.6 ± 1.4 <sup>b</sup>	1.7 ± 1.53 <sup>c</sup>	13.3 ± 0.8 <sup>bc</sup>	1.1 ± 0.4 <sup>d</sup>	9.0 ± 0.8 <sup>c</sup>
200	80.3 ± 1.61 <sup>a</sup>	43.9 ± 0.4 <sup>b</sup>	6.9 ± 0.57 <sup>d</sup>	28.5 ± 2.12 <sup>c</sup>	3.7 ± 1.11 <sup>d</sup>	17.0 ± 1.2 <sup>c</sup>
300	90.9 ± 1.30 <sup>a</sup>	72.6 ± 0.11 <sup>b</sup>	16.0 ± 3.2 <sup>d</sup>	44.8 ± 1.3 <sup>c</sup>	7.9 ± 0.3 <sup>d</sup>	48.3 ± 1.23 <sup>c</sup>
400	89.9 ± 0.9 <sup>a</sup>	76.1 ± 1.2 <sup>c</sup>	27.3 ± 1.8 <sup>d</sup>	83.1 ± 0.8 <sup>b</sup>	3.7 ± 0.7 <sup>c</sup>	79.4 ± 0.9 <sup>b</sup>
500	91.8 ± 1.1 <sup>a</sup>	80.5 ± 0.8 <sup>b</sup>	28.4 ± 2.2 <sup>c</sup>	92.0 ± 0.73 <sup>a</sup>	15.4 ± 2.4 <sup>c</sup>	93.2 ± 1.4 <sup>b</sup>

\*Means with different superscript within the same row are significantly different ( $p < 0.05$ ) according to LSD. Significant differences between various treatments within each experiment are indicated by superscript letters (a, b, c and d). Results are mean values of duplicates determinations  $\pm$  standard deviation.

mented with 2.5% RJ, ascorbic acid and BHT, respectively (Table 2). Also, the *in vitro* antioxidant activity was expressed in terms of  $IC_{50}$  value, the  $IC_{50}$  value is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour). The  $IC_{50}$  was determined by linear regression equation for fermented milk with *B. bifidum* supplemented with 7.5% RJ and for fermented milk with *L. acidophilus* supplemented with 2.5% RJ. The  $IC_{50}$  value of fermented milk with *B. bifidum* supplemented with 7.5% RJ reached at a concentration of 226.7  $\mu\text{g/mL}$  ( $y = 0.188x + 7.38$   $R^2 = 0.968$ ), while the value of  $IC_{50}$  of *L. acidophilus* supplemented with 2.5% RJ reached 210.2  $\mu\text{g/mL}$  ( $y = 0.181x + 11.95$   $R^2 = 0.979$ ).

Reducing power indicates the ability of enzymes (catalase, NADH oxidase, and NADH peroxidase) or non-enzymatic compounds (ascorbate, tocopherol, and glutathione) to reduce oxygen radicals or iron that then become unavailable for oxidative reactions [Warriner & Morris, 1995; Liu *et al.*, 2008]. Several researches have reported that the reducing power of a compound may be used as a significant indicator of its potential antioxidant activity [Meir *et al.*, 1995]. In reducing power assay, fermented milk containing *L. acidophilus* supplemented with 2.5% royal jelly and *B. bifidum* supplemented with 7.5% royal jelly reduced  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by reducing oxygen radical that is usually considered as promotive to oxidative reactions. However, the amount of  $\text{Fe}^{2+}$  complex can be then monitored by measuring the formation of Perl's Prussian blue at 700 nm [Dorman *et al.*, 2003; Ara & Nur, 2009]. Fermented milk containing *B. bifidum* supplemented with 7.5% RJ

exhibited significantly higher reducing power than ascorbic acid or fermented milk containing *B. bifidum* alone with values of 1.253, 0.759 and 0.330, respectively at a concentration of 500  $\mu\text{g/mL}$  on dose dependent basis (Figure 3). Fermented milk containing *L. acidophilus* supplemented with 2.5% RJ exhibited higher reducing power than ascorbic acid or fermented milk with *L. acidophilus* alone with reducing power values at 1.535, 0.943 and 0.441, respectively, at 1000  $\mu\text{g/mL}$  as dose dependent manner (Figure 4). Based on the results, in order to get the effect of reducing power of the mixture (RJ and probiotic) the dose should not be less than 200  $\mu\text{g/mL}$ . This could be related to a sufficient amount of reductive substances, with the capacity to react with free radicals to stabilize and terminate the radical reactions. Wang *et al.* [2009] reported that lactic acid bacteria exhibited a strong reducing power ability.

The development of lipid peroxidation of fermented milk containing royal jelly over a fermentation period of 24 h is shown in Figures 5 and 6. The total antioxidant capacity was determined by thiocyanate method. This method measures the total amount of peroxide produced during the initial stages of oxidation. The % inhibition of *B. bifidum* supplemented with 7.5% royal jelly and *L. acidophilus* was 42% and 52%, respectively. The process of lipid peroxidation is initiated by an attack on a fatty acid or fatty acyl side chain by any chemical species featuring sufficient reactivity to take a hydrogen atom away from a methylene carbon in the side chain. The resulting lipid radicals then undergo molecular rearrangement, followed by reacting with oxygen to produce peroxy radicals, which are

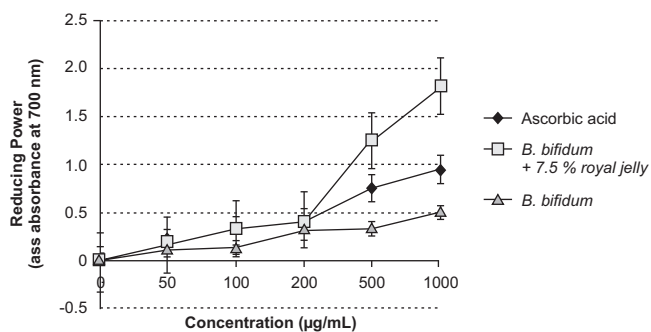


FIGURE 3. Reducing power of different concentrations of ascorbic acid, *B. bifidum* supplemented with 7.5% royal jelly and *B. bifidum*.

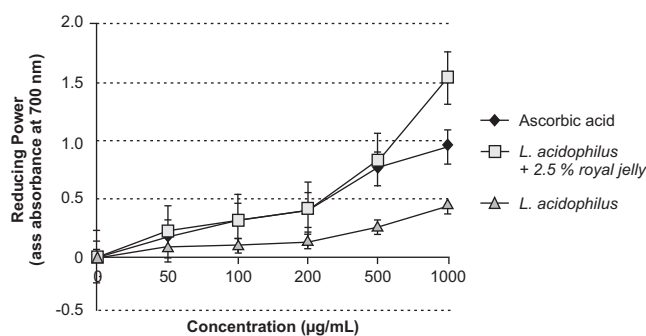


FIGURE 4. Reducing power of different concentrations of ascorbic acid, *L. acidophilus* supplemented with 2.5% royal jelly and *L. acidophilus*.

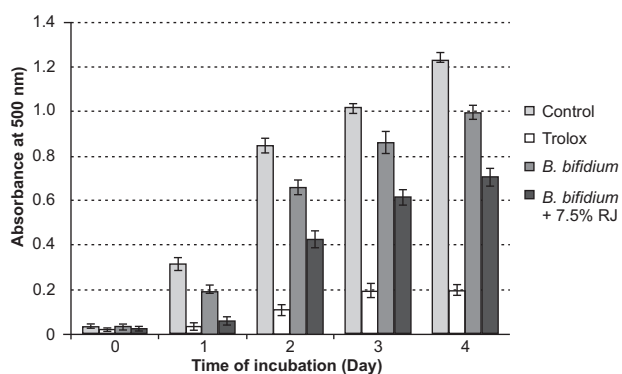


FIGURE 5. Antioxidant activity of Trolox, *B. bifidum* and *B. bifidum* supplemented with 7.5% royal jelly measured by thiocyanate method during different time intervals (day).

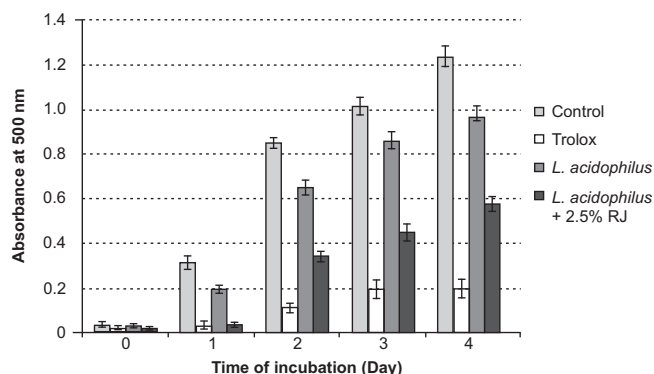


FIGURE 6. Antioxidant activity of Trolox, *L. acidophilus* and *L. acidophilus* supplemented with 2.5% royal jelly measured by thiocyanate method during different time intervals (day).

capable of abstracting hydrogen from adjacent fatty acid side chains and so propagating a chain reaction of lipid peroxidation [Halliwell & Gutteridge, 1986; Prakash & Gupta, 2009]. Lipid peroxidation is not only associated with food deterioration and lowers the nutritional quality of food, but it exerts adverse effects on human health. Liu *et al.* [2008] demonstrated that royal jelly exhibited an inhibitory effect on linoleic acid peroxidation at 27.9%. Another study by Guo *et al.* [2005] ex-

amined the antioxidant capacity of royal jelly peptides and water-soluble protein in linoleic acid peroxidation, where royal jelly peptides had greater effect with 97.4% than water-soluble royal jelly protein with 6.8% inhibition.

The development of diene conjugated compounds in linoleic acid system treated with 50, 100, 200 µg/mL of both different fermented milk and 100 µg/mL of Trolox and BHT are listed in Tables 3 and 4. Fermented milk with *B. bifidum* supple-

TABLE 3. Effect of BHT, Trolox, *B. bifidum* and *B. bifidum* supplemented with 7.5% royal jelly on the formation of diene-conjugation (expressed as inhibition %) using different intervals day in linoleic acid model system.

Treatment (µg/mL)	Inhibition of diene conjugation (%) during different intervals (day)								
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
BHT (100)	42.6 ± 0.27 <sup>e</sup>	57.7 ± 3.8 <sup>d</sup>	54.6 ± 2.5 <sup>c</sup>	59.9 ± 0.74 <sup>b</sup>	57.3 ± 0.4 <sup>b</sup>	50.6 ± 0.20 <sup>b</sup>	49.3 ± 0.44 <sup>b</sup>	47.8 ± 0.23 <sup>b</sup>	
Trolox (100)	61.5 ± 1.7 <sup>a</sup>	74.9 ± 0.7 <sup>a</sup>	68.0 ± 2.8 <sup>a</sup>	68.0 ± 1.5 <sup>a</sup>	67.6 ± 0.35 <sup>a</sup>	61.6 ± 0.11 <sup>a</sup>	59.3 ± 1.2 <sup>a</sup>	58.7 ± 0.45 <sup>a</sup>	
<i>Bifidobacterium bifidum</i>	0% Royal jelly (50)	1.7 ± 0.8 <sup>d</sup>	14.7 ± 2.10 <sup>f</sup>	6.3 ± 2.0 <sup>e</sup>	8.5 ± 0.6 <sup>d</sup>	12.2 ± 1.52 <sup>d</sup>	2.8 ± 0.31 <sup>e</sup>	1.2 ± 1.33 <sup>d</sup>	0.97 ± 1.0 <sup>d</sup>
	0% Royal jelly (100)	5.3 ± 0.23 <sup>d</sup>	16.9 ± 2.01 <sup>f</sup>	8.2 ± 1.98 <sup>e</sup>	9.3 ± 0.32 <sup>d</sup>	13.2 ± 0.83 <sup>d</sup>	5.7 ± 2.6 <sup>d</sup>	1.4 ± 0.66 <sup>d</sup>	0.83 ± 0.73 <sup>d</sup>
	0% Royal jelly (200)	6.2 ± 1.64 <sup>d</sup>	19.6 ± 1.92 <sup>e</sup>	16.5 ± 0.9 <sup>d</sup>	17.4 ± 0.84 <sup>c</sup>	19.3 ± 1.9 <sup>c</sup>	7.2 ± 1.02 <sup>cd</sup>	5.4 ± 0.74 <sup>c</sup>	3.11 ± 1.73 <sup>d</sup>
	7.5% Royal jelly (50)	3.4 ± 0.8 <sup>d</sup>	15.9 ± 1.44 <sup>f</sup>	17.1 ± 0.72 <sup>d</sup>	18.9 ± 0.33 <sup>c</sup>	20.3 ± 0.3 <sup>c</sup>	8.3 ± 0.28 <sup>c</sup>	6.3 ± 0.79 <sup>c</sup>	4.0 ± 1.31 <sup>c</sup>
	7.5% Royal jelly (100)	43.2 ± 0.47 <sup>c</sup>	62.1 ± 1.70 <sup>c</sup>	57.4 ± 2.53 <sup>b</sup>	60.9 ± 0.4 <sup>b</sup>	57.6 ± 0.77 <sup>b</sup>	51.7 ± 0.28 <sup>b</sup>	50.2 ± 0.54 <sup>b</sup>	48.6 ± 0.2 <sup>b</sup>
	7.5% Royal jelly (200)	46.2 ± 0.71 <sup>b</sup>	72.1 ± 1.52 <sup>b</sup>	67.8 ± 0.94 <sup>a</sup>	66.7 ± 0.4 <sup>a</sup>	65.8 ± 1.6 <sup>a</sup>	61.8 ± 2.32 <sup>a</sup>	52.1 ± 0.45 <sup>b</sup>	49.5 ± 0.64 <sup>b</sup>

\*Means with different superscript within the same column are significantly different (p<0.05) according to LSD. Significant differences between various treatments within each experiment are indicated by superscript letters (a, b, c, d, e and f). Results are mean values of duplicates determinations ± standard deviation.

TABLE 4. Effect of BHT, Trolox, *L. acidophilus* and *L. acidophilus* supplemented with 2.5% royal jelly on the formation of diene-conjugation (expressed as inhibition %) using different intervals day in linoleic acid model system.

Treatment ( $\mu\text{g/mL}$ )	Inhibition of diene conjugation (%) during different intervals (day)								
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
BHT (100)	42.6 $\pm$ 0.27 <sup>c*</sup>	57.7 $\pm$ 3.8 <sup>d</sup>	54.6 $\pm$ 2.5 <sup>c</sup>	59.9 $\pm$ 0.74 <sup>b</sup>	57.3 $\pm$ 0.4 <sup>b</sup>	50.6 $\pm$ 0.20 <sup>b</sup>	49.3 $\pm$ 0.44 <sup>b</sup>	47.8 $\pm$ 0.23 <sup>b</sup>	
Trolox(100)	61.5 $\pm$ 1.7 <sup>a</sup>	74.9 $\pm$ 0.7 <sup>a</sup>	68.0 $\pm$ 2.8 <sup>a</sup>	68.0 $\pm$ 1.5 <sup>a</sup>	67.6 $\pm$ 0.35 <sup>a</sup>	61.6 $\pm$ 0.11 <sup>a</sup>	59.3 $\pm$ 1.2 <sup>a</sup>	58.7 $\pm$ 0.45 <sup>a</sup>	
<i>Lactobacillus acidophilus</i>	0% Royal jelly (50)	2.7 $\pm$ 3.1 <sup>h</sup>	15.7 $\pm$ 1.35 <sup>e</sup>	3.1 $\pm$ 1.91 <sup>h</sup>	14.3 $\pm$ 1.13 <sup>f</sup>	16.4 $\pm$ 0.24 <sup>e</sup>	7.61 $\pm$ 0.43 <sup>f</sup>	4.1 $\pm$ 1.67 <sup>f</sup>	1.5 $\pm$ 0.32 <sup>e</sup>
	0% Royal jelly (100)	6.3 $\pm$ 1.83 <sup>e</sup>	15.3 $\pm$ 3.0 <sup>e</sup>	15.7 $\pm$ 1.55 <sup>e</sup>	19.2 $\pm$ 1.61 <sup>c</sup>	22.0 $\pm$ 0.7 <sup>f</sup>	13.5 $\pm$ 1.82 <sup>c</sup>	8.4 $\pm$ 0.85 <sup>c</sup>	5.4 $\pm$ 0.83 <sup>d</sup>
	0% Royal jelly (200)	9.0 $\pm$ 3.2 <sup>f</sup>	18.5 $\pm$ 5.82 <sup>f</sup>	27.9 $\pm$ 1.65 <sup>f</sup>	33.8 $\pm$ 2.7 <sup>d</sup>	24.5 $\pm$ 1.4 <sup>e</sup>	14.9 $\pm$ 0.65 <sup>c</sup>	13.3 $\pm$ 1.15 <sup>d</sup>	10.8 $\pm$ 0.82 <sup>c</sup>
	2.5% Royal jelly (50)	30.0 $\pm$ 4.3 <sup>c</sup>	54.6 $\pm$ 2.52 <sup>c</sup>	48.5 $\pm$ 2.3 <sup>c</sup>	54.3 $\pm$ 0.6 <sup>c</sup>	47.4 $\pm$ 3.06 <sup>d</sup>	21.9 $\pm$ 0.9 <sup>d</sup>	22.5 $\pm$ 1.16 <sup>c</sup>	4.3 $\pm$ 0.10 <sup>d</sup>
	2.5% Royal jelly (100)	60.4 $\pm$ 1.1 <sup>bc</sup>	66.0 $\pm$ 0.44 <sup>c</sup>	57.6 $\pm$ 2.19 <sup>c</sup>	62.1 $\pm$ 0.6 <sup>b</sup>	60.3 $\pm$ 0.3 <sup>b</sup>	52.8 $\pm$ 0.8 <sup>bc</sup>	50.8 $\pm$ 0.33 <sup>b</sup>	48.4 $\pm$ 0.51 <sup>b</sup>
	2.5% Royal jelly (200)	63.0 $\pm$ 0.7 <sup>a</sup>	78.7 $\pm$ 1.16 <sup>a</sup>	74.6 $\pm$ 1.18 <sup>a</sup>	67.1 $\pm$ 1.01 <sup>a</sup>	66.9 $\pm$ 0.9 <sup>a</sup>	60.7 $\pm$ 0.54 <sup>a</sup>	57.3 $\pm$ 1.05 <sup>a</sup>	56.8 $\pm$ 0.07 <sup>a</sup>

\*Means with different superscript within the same column are significantly different ( $p < 0.05$ ) according to LSD. Significant differences between various treatments within each experiment are indicated by superscript letters (a, b, c, d, e, f, g and h). Results are mean values of duplicates determinations  $\pm$  standard deviation.

mented with 7.5% royal jelly and *L. acidophilus* supplemented with 2.5% royal jelly inhibited the formation of conjugated diene in linoleic acid system. The inhibition effects were noticed throughout the induction period compared to control. In general, results show that as the concentration of both fermented milk with *B. bifidum* and *L. acidophilus* increased, the formation of oxidation products decreased and in some concentrations showed close absorbance values and sometimes competitive with Trolox and BHT. The formation of the oxidation products increased gradually in the control system during the induction period and the products started to decompose after the fourth day. The percentage of inhibition for fermented milk with *B. bifidum* were 20.3, 57.6, 65.8, 12.2, 13.3, 19.3, 67.7 and 57.3 percent for 50, 100, and 200  $\mu\text{g/mL}$  of *B. bifidum* supplemented with 7.5% royal jelly; for 50, 100, and 200  $\mu\text{g/mL}$  of *B. bifidum*; 100  $\mu\text{g/mL}$  of Trolox and 100  $\mu\text{g/mL}$  of BHT, respectively (Table 3). While, the percentage of inhibition for fermented milk with *L. acidophilus* were 47.4, 60.3, 66.9, 16.4, 22.0, 24.5, 67.6 and 57.3 percent for 50, 100, and 200  $\mu\text{g/mL}$  of *L. acidophilus* supplemented with 2.5% royal jelly; for 50, 100, and 200  $\mu\text{g/mL}$  of *L. acidophilus*; 100  $\mu\text{g/mL}$  of Trolox and 100  $\mu\text{g/mL}$  of BHT, respectively (Table 4). Tee *et al.* [2002] made the same observations concerning the decreasing of the percentage inhibition of diene-conjugation compounds after four days of incubation.

## CONCLUSION

Royal jelly is a natural substance considered as one of the most important products of honeybees with high nutritional, functional and biological properties. Therefore, it could be concluded from the obtained results that the synbiotic effect of probiotic bacteria *L. acidophilus* and *B. bifidum* with the royal jelly can be used in the dairy industry. This mixture, which is potentially beneficial to enhance human health and protect against oxidative damage and would be considered a strong competitor for the synthetic antioxidants. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and n-propyl gallate (PG) exhibit strong antioxidant activity against several oxidation systems. However, synthetic

antioxidants pose potential risks *in vivo*; their use in food is restricted or prohibited in some countries. Antioxidants from natural sources are likely to be more desirable than those chemically produced because some synthetic antioxidants have been reported to be side effects [Osuntoki & Korie, 2010].

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