Pol. J. Food Nutr. Sci., 2012, Vol. 62, No. 4, pp. 251-258
DOI: 10.2478/v10222-012-0058-4
http://journal.pan.olsztyn.pl

Original paper
Section: Food Technology

The Effect of Royal Jelly on Growth and Short-Chain Fatty Acid Production of Probiotic Bacteria and Activity of Bacterial Procarcinogenic Enzymes in Rat Faeces

Malik S.Y. Haddadin[1], Jamal Haddadin[2], Rachida Benguiar[1]*

[1]Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan, Amman, Jordan
[2] Department of Food Technology and Nutrition, College of Agriculture, Mutah University, Jordan

Key words: royal jelly, probiotic bacteria, short-chain fatty acids, faecal enzymes

The present study was done to evaluate the effect of three different royal jelly samples on the kinetic growth of two isolates of lactic bacteria; *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. The results showed that the addition of royal jelly supported and improved the growth of *L. acidophilus* and *B. bifidum*. The highest count of *L. acidophilus* was 9.01 (log_{10} cfu/mL) when 2% (w/v) of the royal jelly sample 3 was added to milk. The highest count of *B. bifidum* was 9.07 (log_{10} cfu/mL) when 5% (w/v) of the royal jelly sample 1 was added to milk. Based on the obtained results, royal jelly showed the capability of prebiotic activity and increasing the activity of *L. acidophilus* and *B. bifidum.* Royal jelly promotes SCFAs productions which are believed to have an antitumor effect. The results showed the presence of significant synbiotic effect of fermented milk and royal jelly on the intestinal microflora. This effect is translated by the reduction in the faecal enzyme activities of β-glucuronidase, arylsulphatase, and β-gluconsidase which are involved in colon carcinogenesis.

INTRODUCTION

Consumers are no longer appreciating foods only in terms of their taste and immediate nutritional requirements, but also in terms of their ability to provide specific health benefits. Functional foods became an important food sector promoting health benefits *via* functional ingredients in these products. Functional food targets are improving the balance and activity of the intestinal ecology and currently provide the largest sector of functional food market [Saarela *et al.,* 2002b; Verschuren, 2002]. Functional foods, also known as nutraceutical, designer food, medicinal food, and therapeutic food, are defined as foods that contain some health-promoting compounds beyond traditional nutrients. Foods can be modified by addition of phytochemicals, probiotic and/or prebiotic to become functional [Nagai & Inoue, 2004].

In the last two decades, several studies have supported the idea that our health can be affected positively by the daily consumption of specific bacteria that are marketed as probiotics such as Lactobacillus, Bifidobacterium and also, some Propionibacterium strains [Roberfroid, 2000; Saxelin *et al.,* 2005]. In general, these species, which have been introduced as probiotics in food products due to their growing evidence of health benefits [Saxelin *et al.,* 2005; Bernardeau *et al.,* 2008; Guarner & Malagelada, 2003; Saarela *et al.,* 2002a; Shanahan, 2002; Pan *et al.,* 2009a], have low activities of the enzymes involved in carcinogen formation and metabolism (β-glucosidase, β-glucuronidase, urease, azoreductase, and nitrate reductase) by comparison to other major anaerobes strains in the gut such as bacteroides, eubacteria and clostridia [Pool-Zobel *et al.,* 1996]. This proposed that increasing the proportion of lactic acid-producing bacteria in the gut could modify, beneficially, the levels of xenobiotic metabolising enzymes. The ability of the colonic microbiota to generate a wide range of carcinogens, mutagens and tumor promoters from dietary and endogenously-produced precursors is well documented [Liong, 2008].

The dietary supplements of lactic acid bacteria (LAB) as a preventive of colon cancer have received special attention [Roberfroid, 2000]. Carcinogenicity has always correlated with modification of gut bacterial activities. However, it has been reported that certain bacteria in the colon convert procarcinogens to carcinogens [Saarela *et al.,* 2002a].

Prebiotics are selective non-digestible carbohydrate food sources that promote the proliferation of bifidobacteria and lactobacilli [Roberfroid, 2001]. Lactulose, inulin, fructooligossacharide (FOS), soybean oligosaccharide, transgalactosylated oligosaccharides and polysaccharides are the widely used prebiotics [Bezkorovainy, 2001]. The addition of these oligosaccharides as parts of the nutritional diets may be of benefit to the gastrointestinal tract [Pan *et al.,* 2009b].

Royal jelly (RJ) is a bee product secreted from the hypopharyngeal glands of young worker bees to be used in the feeding of young larvae and the adult queen bee. RJ consists of mainly proteins, carbohydrates, fats, free amino acids,

* Corresponding Author: Tel.: 00962777346489
E-mail address: hadadinmalik@yahoo.com (M.S.Y. Haddadin)

vitamins, minerals and other components such as royalisin and apisin [Watanabe *et al.,* 1996, 1998] and large amount of the unsaturated fatty acid 10-hydroxy-trans-2-decenoic acid (10H2DA) [Yang *et al.,* 2010]. RJ has a variety of biological or medical purposes such as life-span elongating [Inoue *et al.,* 2003], anti-fatigue [Kamakura *et al.,* 2001], anti-allergic [Okamoto *et al.,* 2003], antitumor [Bincoletto *et al.,* 2005], anti-hypercholesterolemic, antihypertensive [Matsui *et al.,* 2002; Lichtenthaler & Marx, 2005], and anti-inflammatory [Kohno *et al.,* 2004], anti-bacterial, antioxidant [Nagai & Inoue, 2004; El-Nekeety *et al.,* 2007], DNA-protective [Inoue *et al.,* 2003], and hepatoprotective effects [Zimmermann, 2002].

The short chain fatty acids (SCFAs) are the products of colonic bacterial metabolism of prebiotic in large bowel, which had different effects on colon morphology and function such as supply of energy to the intestinal mucosa, lowering of the pH, and stimulation of sodium and water absorption. The short-chain fatty acid butyrate is produced *via* anaerobic bacterial fermentation within the colon and is thought to be protective in regard to colon carcinogenesis. Although butyrate (C4) is considered the most potent of the SCFA, a variety of other SCFAs also exist in the colonic lumen. Butyrate is thought to exert its cellular effects through the induction of histone hyperacetylation [Hinnebusch *et al.,* 2002].

By combining the rationale probiotics and prebiotics in what has been called a synbiotic could beneficially affect the host by improving 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]. It is the case when combining the anticarcinogenic effects of inulin and bifidobacteria in experimental animals. The effects of probiotics, prebiotics and synbiotics on gut bacterial enzymes activities and metabolic end-products in laboratory animals and in humans are well documented [Burns & Rowland, 2000].

The objectives of this research were to study the effect of royal jelly from different sources on the growth kinetics of two probiotics bacteria, their ability to produce short chain fatty acids (SCFAs) and effect of synbiotic of royal jelly and probiotic bacteria (*L. acidophilus* and *B. bifidum*) on faecal bacterial procarcinogenic enzymes activities in rats.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study had been isolated from the faeces of breast-fed infants and identified in the laboratory of food biotechnology, University of Jordan [Awaisheh, 2003] as *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. The *Bifidobacterium bifidum* isolates were cultivated in de Man Rogosa Sharpe (MRS) broth (Sigma, St. Louis, MO, USA) supplied with cysteine-HCl (5 g/L) for 20 h at 37°C under anaerobic conditions (Oxoid, Basingstoke, Hampshire, England). The *Lactobacillus acidophilus* isolates were cultivated in MRS broth for 20 h at 37°C under anaerobic conditions (Sigma, St. Louis, MO, USA). The isolates were maintained by subculturing weekly using 1% inoculums of the previous subculture and then incubated for 16 h at 37°C.

Royal jelly samples

Three samples of royal jelly from different origins were collected. Sample 1 was taken from hives on the campus of the University of Jordan in Amman city, sample 2 and sample 3 are commercial royal jellies originating from China, and Australia, respectively.

Before adding royal jelly to the heat-treated milk, stock solutions of each royal jelly sample were prepared with deionised distilled water. Royal jelly samples were sequentially filtered using, Grade No. 1 Filter Paper, and Grade No. 40 filter paper (Whatman membranes, England). Finally, sterilisation was performed *via* microfiltration unit using 0.2 μm sterile cellulose-ester membranes (Advantec MFS, Japan) [Haddadin *et al.,* 2007]. Reconstituted skimmed milk (9%) (Régilait, France) was prepared with distilled water and was heat-treated at 70°C for 30 min in a water bath. The sterilised royal jelly solutions were aseptically added to the pasteurised milk previously cooled to 37°C in a way to obtained final royal jelly concentrations of 1, 2, 5, 7 and 10 g/100 mL.

Evaluation of bacterial growth

The milk/royal jelly samples were inoculated with 1% of *L. acidophilus* or *B. bifidum*. The cultures were then incubated at 37°C for 16 h under anaerobic conditions. After incubation, serial dilutions (10^{-1} – 10^{-7}) were realised 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 in the case of *B. bifidum* and plated on MRS agar and incubated at 37°C for 48 h under anaerobic condition in the case of *L. acidophilus*. The results were recorded as CFU/mL of culture.

Production of short chain fatty acids (SCFAs)

The milk cultures supplied with royal jelly at concentrations of 1 and 2% were inoculated with *L. acidophilus* (1%). Whereas milk cultures supplied with royal jelly at a concentration of 5% were inoculated with *B. bifidum* (1%). The cultures were then incubated at 37°C for 16 h under anaerobic conditions and each assay was performed in triplicate. The short chain fatty acids (acetic, propionic and butyric acids) in fermented milk were measured using the method previously proposed [Marsili *et al.,* 1981]. High performance liquid chromatography (HPLC) Jasco system was used. The chromatographic system was equipped with a manual 20 μL loop injector, a variable wavelength ultraviolet/visible detector (Jasco model 875, Japan) using an integrator recorder (Shimadzu C-R2AX, Japan) and an insulated column oven (Jasco model 865, Japan). Acetic, propionic and butyric acids at concentrations of 50, 100, 200, 500 and 800 ppm were used as standard in the HPLC analysis (Sigma, USA). From the area under the curve (AUC) for the concentration of the three acids, linear correlation was obtained, characterised by correlation coefficients of 0.999, 0.998 and 0.999 for acetic, propionic and butyric acid, respectively. The recovery percentages of the acids were 101%, 98% and 95.5% for acetic, butyric and propionic acid, respectively. Eight samples (in triplicates) were injected into the HPLC, the chromatogram col-

TABLE 1. Compositions of experimental diet (g/kg diet).

Ingredients	Basal diet[1]	
	g	kcal
Casein	200	720
Beef tallow	0	0
Methionine	3	12
Starch	150	540
Sucrose	500	2000
Cellulose	50	0
Corn oil	50	450
Salt mixture[2]	35	30.8
Vitamin mixture[3]	10	39
Choline bitartrate	2	0
Total	1000	3791.8

[1]: (AIN-76A diet #100000). [2]: AIN-76 Salt mix; Dyets Inc., Bethlehem, PA, USA. [3]: AIN-76, Vitamin mix; Dyets Inc., Bethlehem, PA, USA

lected has shown the presence of butyric, propionic and acetic acid based on retention time for each acid. In addition, each acid has an area under the curve, in which concentration was calculated according to the regression equation of each organic acid estimated concentration for each treatment.

Effect of pro-prebiotic on faecal enzyme activity

Twenty male albino rats (*Rattus norvegicus* UJ-1) aged 6 weeks and weighing 120 to 150 g were used in this study. The rats were fed on basal diet (AIN-76A #100000, Dyets Inc., Bethlehem, PA, USA) (Table 1) for 5 days *ad libitum* before the treatment. The study comprised three consecutive periods. During periods one and three the rats were fed with their basal diet. In the period two, which lasted for four weeks, the rats were fed orally with a blend of fermented milk (inoculated with *L. acidophilus* or *B. bifidum*) and royal jelly. The rats were divided into two groups of ten rats each. The first group was used for the experiments with *L. acidophilus* and the second group was used for the experiments with *B. bifidum*. The faecal samples were collected before, during and after treatment. β-Glucosidase, β-glucuronidase and arylsulphatase activities were determined with chromogenic substrates as previously described [Marteau *et al.,* 1990]. The substrates were P-nitrophenyl-glucopyranoside, P-nitrophenyl-β-D-glucuronide and P-nitrophenylsulfate, respectively (Sigma, Germany). Fresh faecal samples were suspended in cold 0.1 mol/L potassium phosphate buffer (pH 7.4), then the faecal suspension was homogenised and disrupted by sonication for 3 min at 4°C. The samples were centrifuged at 5000 ×*g* for 15 min. The supernatant was collected and immediately used for the enzyme essay. The enzyme reaction was run at 37°C (pH 7.4). One milliliter of the extract faecal was combined with 0.5 mL of 1 mmol/L substrate. The reaction was run for 30 min at 37°C then stopped by the addition of 0.5 mL of cold 1 mL/L Na_2CO_3 solution. The reading of absorbance at 420 nm and the amount of nitrophenol released was determined by comparison with a standard curve. Enzyme activities are expressed as μg substrate per gram of faecal weight.

pH and titratable acidity

Ten mL of fermented milk were used to measure the pH at 23°C (digital pH meter / Hanna instrument model HI 8519, Italy). Titratable acidity (TA) was determined after adding three drops of phenolphthalein as an indicator to the previous samples used in the pH measurement and titrated with 0.1 N NaOH. After titration, titratable acidity was calculated as a lactic acid percentage (%).

Statistical analysis

The general linear model (GLM) produced by SPSS 15 version was used to analyse the data. Differences between the means of treatments were tested using the Least significant difference (LSD) test at $p<0.05$. Factorial analysis was used to separate the significances between all the royal jelly samples experiments. Results of enzymes activities are expressed as means values ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Dunettes t-test were used to report p-value ($p<0.05$) and significance of differences between results with respect to control.

RESULTS

Effect of royal jelly on bacterial growth

The results of the influence of royal jelly on growth kinetics of *L. acidophilus* and *B. bifidum* are expressed as the total viable counts (log_{10} cfu/mL), (Figures 1 and 2). The growth

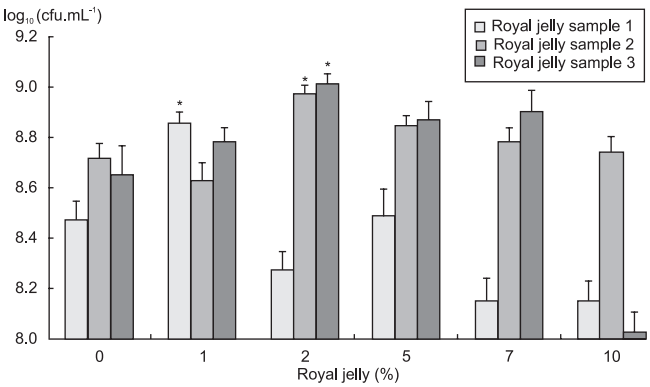

FIGURE 1. Total concentration of *Lactobacillus acidophilus* at different concentrations of the three royal jelly samples 1, 2 and 3 in skim milk culture. * Significantly different at $p<0.05$.

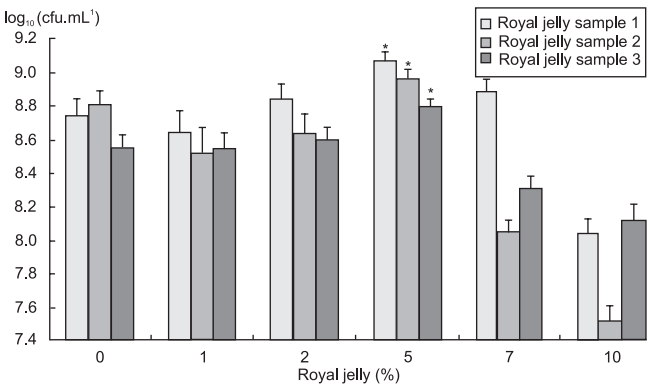

FIGURE 2. Total concentration of *Bifidobacterium bifidum* at different concentrations of the three royal jelly samples 1, 2 and 3 in skim milk culture. * Significantly different at $p<0.05$.

profile of each isolate in the different cultures of royal jelly had a similar mode. The concentration of the tested royal jelly samples at which we obtained the highest counts of *L. acidophilus* and *B. bifidum* is subsequently used for further studies.

The highest biomass concentrations of *L. acidophilus* were obtained when 2% of royal jelly sample 2 and royal jelly sample 3 were used with a viable count of 8.97 ($\log_{10}$ cfu/mL) and 9.01 ($\log_{10}$ cfu/mL), respectively (Figure 1), which were significantly higher than the other tested concentrations of royal jelly. While with 1% of royal jelly sample 1, *L. acidophilus* gave the highest biomass concentration 8.86 ($\log_{10}$ cfu/mL), (Figure 1).

It was observed that with a concentration of 5% for the three royal jelly samples, the biomass concentration of *B. bifidum* was significantly higher than with the other tested concentrations of royal jelly. The viable bacterial counts were 9.07, 8.96 and 8.79 ($\log_{10}$ cfu/mL) for the royal jelly samples 1, 2, and 3, respectively (Figure 2). The results revealed that the royal jelly sample 3 had significantly better effect on the growth of *L. acidophilus* than royal jelly samples 1 and 2 (Figure 1), while local royal jelly sample 1 had significantly the greatest growth-promoting effect on *B. bifidum*, followed by royal jelly samples 2 and 3 (Figure2).

Short chain fatty acids production (SCFAs)

It was found that the addition of royal jelly samples increased significantly ($p<0.05$) the concentrations of total SCFAs compared with the control group (Table 2). Furthermore, acetic acid had a higher concentration than the other SCFAs in all the treatments (Table 2). Moreover, in the experiments where *L. acidophilus* was used, royal jelly sample 3 gave the highest concentrations of the SCFAs, while in the experiments where *B. bifidum* was used royal jelly sample 1 gave the highest concentrations of the SCFAs. In the experiments where *L. acidophilus* was used there were significant differences ($p<0.05$) between the concentrations of acetic acid. It can be observed that royal jelly sample 3 gave the higher amount of acetic acid production (2590.8 ppm/mL) followed by royal jelly sample 1 with a concentration of acetic acid of 1486.1 ppm/mL. For propionic acid there were significant differences ($p<0.05$) between all the treatments and the control. The highest concentration was observed in the presence of royal jelly sample 3 with a concentration of 148.21 ppm/mL. It was also observed the same phenomenon for butyric acid, in which royal jelly sample 3 enhanced the production of significant amount of butyric acid of 471.35 ppm/mL, which was higher than that obtained in the presence of the royal jelly sample 1 and royal jelly sample 2. On the other hand, in the experiments with *B. bifidum*, acetic and butyric acid had significant difference ($p<0.05$) between the treatments and the control, whereas propionic acid concentrations varied between the treatments but with no significant difference. Fermented milk with royal jelly sample 1 produced significantly the highest amount of acetic and propionic acids, while royal jelly sample 3 promoted significantly the highest production of butyric acid compared with the other treatments (Table 2). Fermented milk with royal jelly sample 1 produced significantly the highest amount of acetic and propionic acids (Table 2). On the contrary, royal jelly sample 3 promoted significantly the highest production of butyric acid compared with the other treatments (Table 2).

The titratable acidity values of all the experiments in the presence of *L. acidophilus* and *B. bifidum* were higher than these of the control cultures. Moreover, the pH values of all the experiments in the presence of *L. acidophilus* and *B. bifidum* were lower than these of the control cultures (Table 2).

Faecal enzyme activities

The activities of faecal enzymes (β-glucosidase, β-glucuronidase and arylsulphatase) were evaluated in rats after feeding the animals with a blend of royal jelly and fermented milk with *L. acidophilus* or with *B. bifidum*. The results are presented in Tables 3 and 4. The modifications of enzyme activities were observed during the second period of the study. During the period 2, after feeding the rats with a blend of royal jelly and milk treated with *L. acidophilus*, the enzyme β-glucosidase activity was significantly ($p<0.05$) decreased to 3.19 μg/g after one week of treatment and to 0.95 μg/g after 4 weeks of treatment (Table 3). In the case of rats nourished with a blend of royal jelly and milk treated with B. *bifidum*, the enzyme β-glucosidase activity was significantly ($p<0.05$) decreased from 3.69 to 1.19 μg/g after 4 weeks of treatment (Table 4). These results

TABLE 2. Concentration of short chain fatty acids, pH and titratable acidity of milk samples treated with *L. acidophilus* or *B. bifidum* and supplied with royal jelly.

	Experiment (W/V)		Acetic acid (ppm/mL)	Propionic acid (ppm/mL)	Butyric acid (ppm/mL)	pH	T.A. (%)
L. acidophilus (1% v/v)	*Control	(0 %)	22.5±0.23^{c}	ND	ND	4.47±0.3^{b}	0.7±0.09^{c}
	Royal jelly 1	(1 %)	1486.1±1.05^{b}	95.94±0.35^{b}	203.5±0.58^{b}	4.12±0.13^{a}	1±0.05^{a}
	Royal jelly 2	(2 %)	1158.3±0.95^{b}	50.5±0.65^{c}	188.34±0.65^{b}	4.3±0.20^{b}	0.85±0.06^{b}
	Royal jelly 3	(2 %)	2590.8±1.1^{a}	148.21±0.41^{a}	471.35±0.32^{a}	4.02±0.12^{a}	1.2±0.11^{a}
B. bifidum (1% v/v)	*Control	(0 %)	35±0.12^{d}	ND	ND	4.79±0.11^{c}	0.65±0.10^{d}
	Royal jelly 1	(5 %)	1027.26±1.12^{a}	62.5±0.21^{a}	60.9±0.36^{b}	4.0±0.10^{a}	1.1±0.12^{a}
	Royal jelly 2	(5%)	550.15±1.05^{b}	56.07±0.20^{a}	40.56±0.19^{c}	4.41±0.09^{b}	0.9±0.08^{b}
	Royal jelly 3	(5 %)	409.01±0.26^{c}	58.16±0.32^{a}	83.71±0.45^{a}	4.57±0.13^{b}	0.83±0.06^{c}

ND = not detected. Means with different superscript within the same column and for the same bacteria are significantly different ($p<0.05$). T.A.: titratable acidity. *: No addition of royal jelly.

TABLE 3. Effect of feeding with a blend of fermented milk with *L. acidophilus* and royal jelly on the faecal enzyme activities in rats.

Enzymes	Enzyme activities (μg/g)					
	Period 1 (Control) 1week	Period 2				Period 3 (1 week)
		week 1	week 2	week 3	week 4	
β-Glucosidase	3.76±0.41	3.19±0.27	2.02±0.61*	1.23±0.2*	0.95±0.13*	2.99±0.66
Arylsulphatase	2.61±0.56	1.17±0.2	0.87±0.12*	0.81±0.18*	0.77±0.19*	2.5±0.14
β-Glucuronidase	3.43±0.29	2.8±0.49	1.75±0.13*	1.26±0.24*	0.92±0.19*	3.41±0.99*

* = significantly different from period 1, $p<0.05$. Results are expressed as Mean ± SD. n = 10.

TABLE 4. Effect of feeding with a blend of fermented milk with *B. bifidum* and royal jelly on the faecal enzyme activities in rats.

Enzymes	Enzyme activities (μg/g)					
	Period 1 (Control) 1week	Period 2				Period 3 (1 week)
		week 1	week 2	week 3	week 4	
β-Glucosidase	3.80±0.1	3.69±0.27	2.95±0.58	2.33±0.12*	1.19±0.25*	2.65±0.24
Arylsulphatase	1.83±0.27	1.77±0.2	1.23±0.35	0.98±0.15*	0.83±0.12*	1.79±0.37
β-Glucuronidase	3.21±0.29	3.2±0.56	1.8±0.43*	1.45±0.52*	0.82±0.61*	3.07±0.20*

* = significantly different from period 1, $p<0.05$. Results are expressed as Mean ± SD. n = 10.

are significantly lower than that in the control period (period one), (Tables 3 and 4). In period 3, β-glucosidase regained its normal activity level once the treatment was stopped. A significant ($p<0.05$) decrease in the arylsulphatase activity from 2.61 to 0.77 μg/g was obtained for the blend with *L. acidophilus* (Table 3), and from 1.77 to 0.83 μg/g in the group receiving the blend with *B. bifidum* (Table 4). During period 3, after stopping the treatment, the arylsulphatase activity showed a progressive increase in the two groups. A significant ($p<0.05$) decrease in β-glucuronidase concentration during the four weeks of the period 2 in the group receiving the blend with *L. acidophilus* and reached a value of 0.92 μg/g (Table 3) and 0.82 μg/g in the group receiving the blend with *B. bifidum* (Table 4), which is significantly ($p<0.05$) lower than that obtained by the control samples (3.43 and 3.21 μg/g, respectively).

DISCUSSION

Results revealed that the addition of the royal jelly at certain concentrations enhanced the growth of the tested probiotic bacteria, *L. acidophilus* and *B. bifidum*. Lactobacilli and bifidobacteria need complex nutritional requirements such as carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acid derivatives and vitamins, which vary markedly between one species to another [Saarela *et al.,* 2002a]. Royal jelly can provide these nutrients to *L. acidophilus* and *B. bifidum.* Higher counts of *L. acidophilus* and *B. bifidum* were related to certain royal jelly concentrations. Lower concentrations of royal jelly samples (1 and 2 %) gave the maximal biomass concentration of *L. acidophilus*. Whereas *B. bifidum* attained its maximal biomass concentration in the presence of 5% royal jelly samples. Bifidobacteria species such as *B. bifidum* are fastidious organisms that require specific growth factors for optimal growth. Furthermore, it could be that *B. bifidum* needs higher amount of specific sugars or oligosaccharides for its growth than *L. acidophilus*. Perez-Conesa *et al*. [2005] reported that oligosaccharides increased the growth, activity and viability of *Bifidobacterium ssp*. in milk.

In contrast, declines of *L. acidophilus* and *B. bifidum* growth were observed at a higher concentration (10%) of royal jelly samples. This observation could be attributed to the high level of antibacterial activity [Sauerwald *et al*., 1998]. The antibacterial activity can be attributed to the phenolic compounds present in the royal jelly samples. Furthermore, it seems that *L. acidophilus* is more sensitive to the antibacterial effect of royal jelly than *B. bifidum*. Royal jelly samples 1 and 3 seem to contain certain components that favour the growth of *B. bifidum* and *L. acidophilus*.

The results presented in Table 2 showed the presence of short chain fatty acids productions (SCFAs) in all the treatments. This could be due to the availability of carbohydrates like oligosaccharides, as this group of carbohydrates is easily metabolised by probiotic bacteria and the end products of this metabolism are SCFAs. The obtained results are in agreement with those reported by Nyman [2002]. In turn, Rossi *et al.* [2005] found that the presence of fructooligosaccharides affected the production of acetate and lactate. The three different samples of royal jelly gave different amounts of SCFAs, and these results could be attributed to the variation in the royal jelly chemical composition. According to Stocker *et al.* [2005], the composition of royal jelly depends on climate and floral sources. It could be concluded that royal jelly samples 1 and 3 have high amounts of oligosaccharides that produced significantly the highest amounts of SCFAs. The results indicate also that *L. acidophilus* produced relatively high amounts of SCFAs than *B. bifidum*. This could be due to the differences in biochemical and physiological properties between these microorganisms.

The milk fermented with *L. acidophilus* in the presence of royal jelly sample 3 had a significantly lower pH value than the control test, which indicates that the amount of lactic acid has been produced in addition to the presence of other produced organic acids. In the case of *B. bifidum*, the fermented milks with royal jelly sample 1 had significantly lower pH and this reduction was probably caused by the high levels of organic acids production.

Milk fermented with *L. acidophilus* or *B. bifidum* and in the presence of royal jelly samples 3 and 1 respectively, had pH values well below that of the control test and this reduction was probably caused by the production of organic acids.

Overall, it is clear that royal jelly has beneficially influenced the growth and metabolism of these two microorganisms and it might be reasonable to assume that royal jelly ingested by a consumer would have a similar effect on the native populations of these species in the lower intestine. Consumption of *L. acidophilus* and *B. bifidum* with royal jelly could promote bacterial growth in the colon and hence produce greater quantities of short chain fatty acids such as butyric acid, which has been shown to have antitumor effects at the cell level [Kailasapathy & Chin, 2000]. Upadhyay & Moudgal [2012] have indicated that there is good evidence to support probiotic use in the treatment of acute diarrheal diseases, prevention of antibiotic-associated diarrhea, and prevention of pouchitis. However, there is insufficient evidence to recommend probiotics for use in other clinical conditions. According to Topping [1996], there is insufficient evidence that the health benefits of probiotics, such as prevention of colon cancer, are exerted through short chain fatty acids.

This study has demonstrated that oral administration of a blend of royal jelly and fermented milk with *L. acidophilus* or with *B. bifidum* can cause an alteration in the metabolic activity of the intestinal microflora. The enzymes, β-glucuronidase, arylsulphatase, and β-gluconsidase, selected in this study are known to be potential mediators of colon carcinogenesis [Hyang *et al.,* 2011]. This synbiotic mixture has been found to reduce significantly the levels of faecal enzymes (Tables 3 and 4).

It can be observed that it requires four weeks of feeding for the effects to be realised (Tables 3 and 4). The faecal enzyme activities remained low as long as the synbiotic feeding was being administered (4 weeks) and returned to reference concentrations one week after stopping the bacterial feeding. There is no proving that the intestine was permanently colonised with *L. acidophilus* and *B. bifidum.* There might be transient colonisation or at least an increase in the counts of lactobacilli during the feeding for four weeks afterward. Moreover, it would seem that the continuous intake of the blend of royal jelly and fermented milk is necessary for maintaining these enzymes effects in the microflora. Goldin & Gorbach [1984] obtained a 2 to 4 fold reduction in the activities of three human faecal enzymes during the 4 weeks of *L. acidophilus* feeding to twenty–one subjects. In other study, it has been observed that prebiotic oligosaccharides may modulate expression of theses enzymes, by reducing the risk of intestinal genotoxicity [Sanz *et al.,* 2005]. β-Glucuronidase is an enzyme responsible for the hydrolysis of glucuronides in the lumen of the gut. This reaction generates toxic and carcinogenic substances which are detoxified by glucuronide formation in the liver and then enter the bowel *via* bile [Leblank & Perdigon, 2005]. It is a good marker of deconjugation activities, a decrease in its activity is generally considered positive in terms of cancer protection because the enzyme helps to restore the toxic properties of some xenobiotics in the colon. Different possible mechanisms have been identified and evaluated but no single protective action has been clearly defined. On the other hand, human epidemiological and interventional studies still do not seem to support the promising results observed in experimental conditions.

One of the main postulated mechanisms is the production of SCFAs especially butyrate *via* the fermentation of prebiotic by gut flora. In addition, the synbiotic products have been found to exert increased benefits compared to the administration of either probiotic or prebiotic alone [Liong, 2008]. Some studies found that a decrease in β-glucuronidase activity may be related to a change in intestinal pH and/or to medication in the composition of intestinal flora [Djouzi *et al.,* 1997]. Other study has shown that rats fed *L. acidophilus* and naphthylamine glucuronide substrate for glucuronidase excreted lower amounts of free amines in their feces compared to rats not receiving *L. acidophilus* [Leblank & Perdigon, 2005]. The significantly decreased enzyme activities of β-glucuronidase, arylsulphatase, and β-gluconsidase were an indicator of positive effects of the consumption of a synbiotic product with the combination of royal jelly and *L. acidophilus* or *B. bifidum*. No other studies are available for comparison.

CONCLUSION

It can be concluded that the three types of royal jelly enhanced the growth, activity and viability of two isolates of probiotic bacteria. Royal jelly has been found to promote the production of a significant amount of short chain fatty acids. The ingestion of fermented milk containing *L. acidophilus* and *B. bifidum* supplemented with royal jelly has influenced the activities of intestinal enzymes. It is likely that the exact composition of royal jelly samples may determine their cellular effects, including their possible beneficial role in the prevention and/or treatment of colon cancer. It is clear that nutraceuticals and probiotics have beneficial effects, but additional carefully designed, mechanistic-based laboratory and clinical studies clearly need to be undertaken to provide scientific evidence for mechanisms of their action and efficacy.

REFERENCES

1. Awaisheh S., *In vitro* studies of the effect of fermented dairy products containing probiotics and neutraceuticals on different characteristics of normal flora. Ph.D. 2003, Thesis, University of Jordan, Amman, Jordan.
2. Bernardeau M., Vernoux J.P., Henri-Dubernet S., Guéguen M., Safety assessment of dairy microorganisms: The Lactobacillus genus. Int. J. Food Microbiol., 2008, 126, SI, 278–285.
3. Bezkorovainy A., Probiotics: determinants of survival and growth in the gut. Am. J. Clin. Nutr., 2001, 73, S399-S405.

4. Bincoletto C., Eberlin S., Figueiredo C.A.V., Luengo M.B., Queiroz M.L., Effects produced by royal jelly on haematopoiesis: relation with host resistance against Ehrlich ascites tumour challenge. Int. Immunopharmacol., 2005, 5, 679–688.
5. Burns A.J., Rowland I.R., Anti-carcinogenicity of probiotics and prebiotics. Curr. Issues Intest. Microbiol., 2000, 1, 13––24.
6. Djouzi Z., Andrieux C., Degivry M.-C., Bouley C., Szylit O., The association of yogurt starters with *Lactobacillus casei* DN 114.001 in fermented milk alters the composition and metabolism of intestinal microflora in germ-free rats and in human flora-associated rats. J. Nutr., 1997, 127, 2260–2266.
7. El-Nekeety, A.A., El-Kholy, W., Abbas, N.F., Ebaid, A., Amra, H.A., Abdel-Wahhab, M.A., Efficacy of royal jelly against the oxidative stress of fumonisin in rats. Toxicon, 2007, 50, 256–269.
8. Goldin B.R., Gorbach S.L., The effect of milk and lactobacilli feeding on human intestinal bacteria enzyme activity. Am. J. Clin. Nutr., 1984, 39, 756–761.
9. Guarner F., Malagelada J.R., Gut flora in health and disease. Lancet, 2003, 361, 512–519.
10. Haddadin M.S.Y., Nazer I., Sara' J.R., Robinson R.K., Effect of honey on the growth and metabolism of two bacterial species of intestinal origin. Pakistan J. Nutr., 2007, 6, 693–697.
11. Hinnebusch B.F., Meng S.F., Wu J.T., Archer S.Y., Hodin R.A., The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. J. Nutr., 2002, 132, 1012–1017.
12. Hyang M.A., Shin Y.P., Do K.L., Jung R.K., Min K.C., Si W.L., Hyung T.L., Kyung J.K., Nam J.H., Antiobesity and lipid-lowering effects of Bifidobacterium spp. in high fat diet-induced obese rats. Lipids Health Dis., 2011, 10, 116.
13. Inoue S., Koya-Miyata S., Ushio S., Iwaki K., Ikeda M., Kurimoto M., Royal jelly prolongs the life span of C3H/HeJ mice: correlation with reduced DNA damage. Exp. Gerontol., 2003, 38, 965–969.
14. Kailasapathy K., Chin J., Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol. Cell Biol., 2000, 78, 80–88.
15. Kamakura M., Mitani N., Fukuda T., Fukushima M., Antifatigue effect of fresh royal jelly in mice. J. Nutr. Sci. Vitaminol., 2001, 47, 394–401.
16. Kohno K., Okamoto I., Sano O., Arai N., Iwaki K., Ikeda M., Kurimoto M., Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. Biosci. Biotechnol. Biochem., 2004, 68, 138–145.
17. Leblank A.D., Perdigon G., Reduction of β-glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. Biocell, 2005, 29, 15–24.
18. Lichtenthaler R., Marx F., Total oxidant scavenging capacities of common European fruit and vegetable juices. J. Agric. Food Chem., 2005, 53, 103–110.
19. Liong M.T., Roles of probiotics and prebiotics in colon cancer prevention: postulated mechanisms and *in vivo* evidence. Int. J. Mol. Sci., 2008, 9, 854–863.
20. Marsili R.T., Ostapenkoo H., Simmons R.E., Green D.E., High performance liquid chromatography determination of organic acids in dairy products. J. Food Sci., 1981, 46, 52–57.
21. Marteau P., Pochart P., Flourie B., Pellier P., Santos L., Desjeux J.F., Rambaud J.C., Effect of chronic ingestion of a fermented dairy product containing *Lactobacillus acido*philus and *Bifidobacterium bifidum* on metabolic activities of the colonic flora in humans. Am. J. Clin. Nutr., 1990, 52, 685–688.
22. Matsui T., Yukiyoshi A., Doi S., Sugimoto H., Yamada H., Matsumoto K., Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. J. Nutr. Biochem., 2002, 13, 80–86.
23. Nagai T., Inoue R., Preparation and the functional properties of water extract and alkaline extract of royal jelly. Food Chem., 2004, 84, 181–186.
24. Nyman M., Fermentation and bulking capacity of indigestible carbohydrates: the case of inulin and oligofructose. Br. J. Nutr., 2002, 87, S163-S168.
25. Okamoto I., Taniguchi Y., Kunikita T., Kohno K., Iwaki K., Ikeda M., Kurimoto M., Major royal jelly protein 3 modulates immune responses in vitro and *in vivo*. Life Sci., 2003, 73, 2029–2045.
26. Pan X.D., Chen F.Q., Wu T.X., Tang H.G., Zhao Z.Y., Prebiotic oligosaccharides change the concentrations of short-chain fatty acids and the microbial population of mouse bowel. J. Zhejiang Univ. Sci. B, 2009a, 10, 258–263.
27. Pan X.D., Chen F.Q., Wu T.X., Tang H.G., Zhao Z.Y., The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. Food Contr., 2009b, 20, 598–602.
28. Perez-Conesa D., Lopez G., Ros G., Fermentation capabilities of bifidobacteria using nondigestible oligosaccharides, and their viability as probiotics in commercial powder infant formula. J. Food Sci., 2005, 70, M279-M285.
29. Pool-Zobel B.L., Neudecker C., Domizlaff I., Schillinger S., Ji U., Rumney C., Moretti M., Vilarini I., Scassellati-Sforzolini R., Rowland I., Lactobacillus- and Bifidobacterium-mediated antigenotoxicity in the colon of rats. Nutr. Cancer, 1996, 26, 365–380.
30. Roberfroid M.B., Prebiotics and probiotics: are they functional foods? Am. J. Clin. Nutr., 2000, 71, 1682–1687.
31. Roberfroid M.B., Prebiotics: preferential substrates for specific germs? Am. J. Clin. Nutr., 2001, 73, 407S-415S.
32. Rossi M., Corradini C., Amaretti A., Nicolini M., Pompei A., Zanoni S., Matteuzzi D., Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl. Environ. Microbiol., 2005, 71, 6150–6158.
33. Saarela M., Mättö J., Mattila-Sandholm T., Safety aspects of Lactobacillus and Bifidobacterium species originating from human oro-gastrointestinal tract or from probiotic products. Microb. Ecol. Health Dis., 2002a, 14, 234–241.
34. Saarela M., Lahteenmaki L., Crittenden R., Salminen S., Mattila-Sandholm, T., Gut bacteria and health foods – the European perspective. Int. J. Food Microbiol., 2002b, 78, SI, 99–117.
35. Sanz M.L., Polemis N., Morales V., Coezo N., Drakoularakou A., Gibson G.R., Rastal R.A., *In vitro* investigation into the potential prebiotic activity of honey oligosaccharides. J. Agric. Food Chem., 2005, 53, 2914–2921.
36. Sauerwald N., Polster J., Bengsch E., Niessen L., Vogel R.F., Combined antibacterial and antifungal properties of water-soluble fraction of royal jelly. Adv. Food Sci., 1998, 20, 46–52.
37. Saxelin M., Tynkkynen S., Mattila-Sandholm T., De Vos W.M., Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol., 2005, 16, 204–211.

38. Shanahan F., Probiotics and inflammatory bowel disease: from fads and fantasy to facts and future. Br. J. Nutr., 2002, 88 (Suppl. 1), S5–S9.
39. Stocker A., Schramel P., Kettrup A., Bengsch E., Trace and mineral elements in royal jelly and homeostatic effects. J. Trace Elem. Med. Biol., 2005, 19, 183–189.
40. Topping D.L., Short chain fatty acids produced by intestinal bacteria. Asia Pac. J. Clin. Nutr., 1996, 5, 15–19.
41. Upadhyay N., Moudgal V., Probiotics: A Review. JCOM, 2012, 19, 76–84.
42. Verschuren P.M., Functional foods: scientific and global perspectives. Br. J. Nutr., 2002, 88, S125-S130.
43. Watanabe K., Shinmoto H., Kobori M., Tsushida T., Shimohara K., Kanaeda J., Yonekura M., Growth stimulation with honey royal jelly DIII protein of human lymphocytic cell lines in a serum-free medium. Biotechnol. Tech., 1996, 10, 959–962.
44. Watanabe K., Shinmoto H., Kobori M., Tsushida T., Shimohara K., Kanaeda J., Yonekura M., Stimulation of cell growth in the U-937 human myeloid cell line by honey royal jelly protein. Cytotechnology, 1998, 26, 23–27.
45. Wollowski I., Rechkemmer G., Pool-Zobel B.L., Protective role of probiotics and prebiotics in colon cancer Am. J. Clin. Nutr., 2001, 73(suppl), 451S–5S.
46. Yang X.Y., Yang D.S., Wei-Zhang, Wang J.M., Li C.Y., Hui Y., Lei K.F., Chen X.F., Shen N.H., Jin L.Q., Wang J.G., 10-Hydroxy-2-decenoic acid from Royal jelly: A potential medicine for RA. J. Ethnopharmacol., 2010, 128, 314–321.
47. Zimmermann A., Liver regeneration: the emergence of new pathways. Med. Sci. Monit., 2002, 8, 3, RA 53–63.

Received January 2012. Revision received and accepted May 2012. Published on-line on the 28th September 2012.