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Some Biochemical Parameters of *Pisum sativum* Extracted with *Lactobacillus lactis*

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This study analyzed the effects of inulin containing *Pisum sativum* on *Lactobacillus lactis*. In the study, fatty acid, flavonoid, resveratrol content, vitamin, phytosterol levels and the antimicrobial activities of *P. sativum* and the fatty acid, flavonoid, resveratrol content, vitamin, phytosterol levels and antimicrobial activities of *L. lactis* extracts treated with *P. sativum* were determined and compared. For this purpose, the control (*P. sativum*), *L. lactis* treated with *P. sativum* (Ps+Ll) and *L. lactis* only cultures (Ll) were used. According to experimental results the fatty acid and vitamin levels of the *P. sativum* extracts significantly increased after being treated with *L. lactis* significantly increased whereas in the controls that the flavonoid and resveratrol levels decreased while the antimicrobial activity showed variations.

Key Words: Probiotic bacteria, Prebiotic, *L. lactis*, Pea.

INTRODUCTION

Probiotics are beneficial microorganisms for nutrition and health they maintain microbial balance in the intestinal system and affect the host by adjusting the mucosal and systemic immunity. These microorganisms are found in intestinal system in large numbers[1,2]. The best known probiotics are Lactobacilli. Lactobacilli functions in several ways that are known to be beneficial such as reducing lactose intolerance, positively effecting intestinal flora, preventing intestinal damage and intestinal system infections, strengthening the immune system, reducing inflammations or allergic reactions, lowering the risk of colon cancer and urogenital infections, reducing the occurrence of *Helicobacter pylori* infections, reducing blood lipids and the risk of heart failure and antimicrobial activity[3,4]. As a result of the understanding of the importance of probiotics and the number of studies carried out on probiotics, the field of probiotics has developed. Probiotics are nutrients that can increase bacterial activity and they are found in various nutritional sources. One of these sources is inulin a polysaccharide which has a considerable effect on bacterial growth.

In this study using specified biological parameters we tried to determine how and to what extent *Pisum sativum* (pea) effected the development of *L. lactis*. *Pisum sativum* (pea), is a prebiotic with a high inulin content which increases probiotic activity and is also known as a peptic. In this study, which is important in terms of plant-based nutrition, we aimed to analyze the importance of a plant-based diet on *L. lactis*, by drawing attention to plant-probiotic relationship.

EXPERIMENTAL

The fresh pea samples used in the study were supplied from the Elazig province of Turkey. *P. sativum* grains were kept in deep freezer at -20 ºC until extraction.

Extraction of lipids: Cell pellets whose wet weights were determined were homogenized with 3/2 (v/v) hexane + isopropanol mixture. The homogenate was centrifuged at 5000 rpm for 5 min at 4 ºC and cell pellet remnant was precipitated. The supernatant part was used in the vitamin and fatty acid analysis[5].

Preparation of fatty acid methyl esters: An aliquot was taken from the supernatant part of the cell pellet and 5 mL of 2 % methanolic sulphuric acid was added. The mixture was vortexed and then kept at 50 ºC for 12 h. Then, after being cooled to room temperature, 5 mL of 5 % sodium chloride was added and then it was vortexed. Fatty acid methyl esters were extracted with 2 × 5 mL hexane. Fatty acid methyl esters were treated with 5 mL 2 % $KHCO_3$ solution and then the hexane phase was evaporated by the nitrogen flow and then by dissolving in 1 mL fresh hexane, they were taken to auto sampler vials[5].

Gas chromatographic analysis of fatty acid methyl esters: Methyl esters were analyzed with the SHIMADZU GC 17 Ver. 3 gas chromatography (Kyoto, Japan). For this analysis, 25 m of long Machery-Nagel (Germany) capillary colon with an inner diameter of 0.25 µm and a thickness of 25 micron film was used. During the analysis, the colon temperature was kept at 120-220 ºC, injection temperature was kept

at 240 ºC and the detector temperature was kept at 280 ºC. The colon temperature program was adjusted from 120 to 220 ºC and the temperature increase was determined to be 5 ºC/min until 200 ºC and 4 ºC/min from 200 to 220 ºC. It was kept at 220 ºC for 8 min and the total duration was set as 35 min and nitrogen gas was used as the carrier gas. During the analysis, before the analysis of fatty acid methyl esters, mixtures of standard fatty acid methyl esters were injected and the residence time of each fatty acid was determined. After this process, the necessary programming was made and the fatty acid methyl esters mixtures of the samples were analyzed[5].

HPLC analysis of A, D, E & K vitamins and sterol amount: The five mL supernatant was taken to 25 mL tubes with caps and 5 % KOH solution was added. After it was vortexed, it was kept at 85 ºC for 15 min. The tubes were then taken and cooled to room temperature and 5 mL of pure water was added and mixed. Lypophlic molecules that did not saponify were extracted with 2 × 5 mL hexane. The hexane phase was evaporated with nitrogen flow. It was dissolved in 1 mL (50/50, v/v) acetonitril/methanol mixture and then was taken to auto sampler vials and was analyzed. The analysis was made with the Shimadzu brand HPLC device. In the device as the pump LC-10 ADVP UV-visible, as the detector SPD-10AVP, as column oven CTO-10ASVP, as auto sampler SIL-10ADVP, as degasser unit DGU-14A and Class VP software (Shimadzu, Kyoton Japan) was used and during the mobile phase the acetonitril/methanol (60/40, v/v) mixture was used. The mobile phase flow rate was determined to be 1 mL A UV detector was used for the analysis and as a column the Supelcosil LC 18 (15 × 4.6 cm, 5 µm; Sigma, USA) column was used. For vitamin A and β-charoten, detection of wavelength 326 nm, for vitamin D and K, 265 nm, for vitamin E, 202 nm was used[6].

Statistical analysis: The SPSS 10.0 software program was used. The comparison between experimental groups and the control group was made using ANOVA and LSD tests[5].

DPPH radical scavenging activity: A methanolic solution of 25 mg/L free radical DPPH was prepared. During the tests, plant samples of 25, 50, 100 and 250 µL concentrations were added to the methanolic solution of 3.9 mL DPPH radical. The mixture was vortexed and incubated for 0.5 h at room temperature in a dark medium. The absorbance values were measured using a spectrophotometer at 517 nm against a blank[7,8]. Radical scavenging activity was calculated as a %. The DPPH radical scavenging activity was calculated using the following formula: (%) = [(Controlλ-Samplingλ)/(Controlλ)] × 100.

Identification of resveratrol and flavonoid content: The flavonoid and resveratrol analysis was performed in an HPLC device and all procedures were performed at 25 ºC[9].

Extraction and analysis of phytosterols: To the plant sample which was homogenized with hexane/isopropyl alcohol mixture (in proportion of 3/2 v/v) 5 % KOH was added and it was hydrolyzed at 85 ºC. The extraction was treated with *n*-heptane and analyzed in an HPLC device[10].

Sugar analysis: The 10 g plant sample was homogenized with distilled water. Then, the pellet and supernatant sections were separated. After the identification of the volume of the total filtrate, it was analyzed in an HPLC device and a Shim-Pack HRC NH_2 (150 × 4.6 mm, 5 µ) colon was used. Acetonitrile/water (v/v) (75/25) mixture was used as the mobile phase[10].

Test microorganisms: A total of 4 bacteria (*Bacillus megaterium* DSM 32, *Staphylococcus aureus* COWAN 1, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* FMC 5), 2 yeasts (*Candida albicans* FMC 17 and *Candida glabrata* ATCC 66032) and 2 dermatophytes (*Trichophyton* spp. and *Epidermophyton* spp.) were used in the study. The microorganisms were supplied by Microbiology Laboratory Department of Biology, Faculty of Science, Firat University.

Preparation of microorganisms cultures and cultivation: Bacteria strains were inoculated with a nutrient broth and were incubated at 35 ± 1 ºC for 24 h. Yeast strains were incubated in Malt Extract Broth-Agar, while the dermatophyte fungi were incubated in Sabouraud Glucose Broth at 25 ± 1 ºC for 48 h. The culture growing in the liquid medium was transferred to the broth tubes after performing turbidity adjustment according to a McFarland (0.5) standard tube. Müller Hinton Agar, Sabouraud Dextrose Agar and Potato Dextrose Agar which were sterilized in an Erlenmayer flask and cooled to 45-50 ºC were inoculated with the cultures of bacteria, yeast and fungi in the broth at 1 % proportion (10^6 bacteria/mL, 10^4 yeast/mL, 10^4 fungus/mL). After shaking well, a homogenous medium was obtained and 15 mL of the mixture was placed in petri dishes of 9 cm diameter. The soaked discs were placed on solidified agar and lightly pressed. The petri dishes prepared in this manner were maintained at 4 ºC for 1.5 to 2 h. The plates inoculated with bacteria were incubated at 37 ± 1 ºC for 24 h; the plates inoculated with yeast and dematophyte were incubated at 25 ± 1 ºC for 3 days. At the end of these periods, the inhibition zones that formed on the medium were evaluated as mm[11].

Growth of *L. lactis* and treatment with plant extraction: After growing *L. lactis*, in the MRS broth and measuring it at spectrophotometer against a blank at 517 nm, it was cultivated in the environment containing the prepared minimal medium (0.019 M NaCl, 0.022 M KH_2PO_4, 0.049 M Na_2HPO_4, 0.019 M NH_4Cl, 0.002 M $MgSO_4$, 0.011 M glucose)[12] and the pea extract (the plant sample was treated with a solvent and evaporated in evaporator, favourable growth media was prepared for the bacteria) at a level of 10^6 bacteria/mL under sterile conditions and an appropriate value was achieved. It was measured at spectrophotometer against blank at 517 nm. After incubation, the extracts which had been grown in the minimal medium were collected and measured in spectrophotometer against a blank at 517 nm for 6, 12, 24, 36, 48, 60 and 72 h. After the measurements, the extracts were cultivated and incubated in MRS agar for the cell and plate count. The samples were centrifuged and pellets were collected at the point where growth was about to end. The fatty acid, vitamin, flavonoid, resveratrol and antimicrobial activities of these pellets were analyzed. The same procedures were applied to the *L. lactis* and pea control group grown only in the minimal medium. In conclusion, the control (only pea) and *L. lactis* extract which was treated with pea were compared in terms of fatty acid, vitamin, flavonoid, resveratrol and antimicrobial activity.

RESULTS AND DISCUSSION

Free radical (DPPH) neutralization activity: It was found that the effect of 250 µL concentration of P.sativum extracts on the DPPH radical was higher than those of 50 µL and 100 µL ($P < 0.0001$) (Fig. 1).

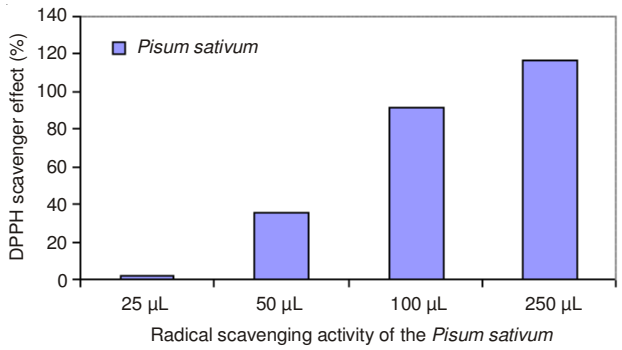

Fig. 1. DPPH free radical neutralization activity of *P. sativum* (%)

Sugar contents: According to the sugar analysis results, *P. sativum* had the highest saccharose sugar content followed by fructose, maltose and glucose (Fig. 2).

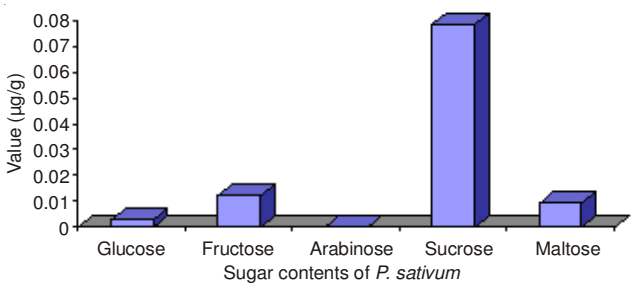

Fig. 2. Sugar contents of *P. sativum* (µg/g)

Fatty acid contents: Fatty acid analysis indicated that the pea extract contained 16:0, 18:0, 18:1, 18:2, 18:3 fatty acids and that 18:1 and 16:0 were found at the highest level. In the pea extracts which were treated with *L. lactis*, it was found that 16:0, 18:0, 18:2 and 18:3 fatty acid levels significantly increased and it was thought that the factor causing this increase was bacterial (Table-1). We believe that the mechanism which caused this increase is associated with the activating effect of the existing carbon source in the pea on the enzymes which control fatty acid synthesis. In other words, we believe that fatty acid synthesis in the cell is affected by the carbon source in the medium[13,14]. The fact that the fatty acid level in *L. lactis* extract which was treated with the pea extract increased when compared to the fatty acid levels of the pea and *L. lactis* indicates that the pea extract is a medium that activates the growth of *L. lactis*.

Lipophilic vitamin and phytosterol contents: It was found that the pea extract contained vitamins D and K_1 and δ- tocopherol, phytosterols, ergosterol, stigmasterol and β-sitosterol. The vitamin D and δ- tocopherol values were found to increase, vitamin K_1 was found to decrease and ergosterol, stigmasterol and β-sitosterol were found to increase in the *L. lactis* extracts, which were treated with the pea sample (Table- 2). It was found that pea contents in the bacterial growth media had an effect on the vitamins causing an increase in the levels. The results revealed that *L. lactis* synthesized δ-tocopherol, vitamin K_1, ergosterol, stigmasterol and β-sitosterol. It was thought that there was no vitamin D synthesis however the factor which caused the vitamin D increase in the extracts treated with pea resulted from the decomposing characteristic of the bacteria. It was believed that the decrease in vitamin K_1 level was related to the consumption by the bacteria, while the increase in other vitamin values was caused by *L. lactis*. According to these results, it was found that pea effects *L. lactis* growth.

TABLE-1
FATTY ACID CONTENTS (µg/1g)

Fatty acid	Control (Ps)	Ll	Ps + Ll
16.0	99.40 ± 0.26	36.30 ± 2.10	140.30 ± 0^{cd}
18:0	17.90 ± 0	12.91 ± 0.31	62.75 ± 0^{cd}
18.1	67.43 ± 0	31.11 ± 17.26	45.90 ± 0^{c}
18:2	34.80 ± 0	36.30 ± 5,50	207.75 ± 0^{d}
18:3	20.50 ± 0	18.91 ± 0.08	28.50 ± 0^{b}
Toplam (µg/g)	239.68 ± 0.26	135.55 ± 25.25	485.2 ± 0^{cd}

(Ps: *P. sativum*, Ll: *L.lactis*, Ps + Ll: *P. sativum* + *L.lactis*), (c: $P < 0.01$, d: $P < 0.001$, cd: $P < 0.0001$)

TABLE-2
LIPOPHILIC VITAMIN AND PHYTOSTEROL CONTENTS (µg/g)

Vitamin contents	Kontrol (Ps)	Ll	Ps + Ll
δ-Tocopherol	0.016 ± 0.00	0.0001 ± 0	0.0206 ± 0^{cd}
Vitamin D	0.0007 ± 0	-	0.0318 ± 0^{cd}
α-Tocopherol	-	0.102 ± 0.003	0.3877 ± 0^{cd}
Vitamin K_1	0.013 ± 0.00	0.0002 ± 0	0.0014 ± 0^{cd}
Ergosterol	0.456 ± 0.00	0.0030 ± 0.001	0.5776 ± 0^{cd}
Stigmasterol	0.065 ± 0.00	0.0023 ± 0.001	3.582 ± 0^{cd}
β-Sitosterol	0.201 ± 0.00	0.0040 ± 0	0.7604 ± 0^{cd}

(Ps: *P. sativum*, Ll: *L.lactis*, Ps + Ll: *P. sativum*+ *L.lactis*), (c: $P < 0.01$, d: $P < 0.001$, cd: $P < 0.0001$)

Flavonoid contents: It was found that the pea sample contained myricetin, catechin, naringin, morin, kaempferol, naringenin and resveratrol and that among these the catechin content was higher. On the other hand, it was found that these values significantly decreased in the *L. lactis* extract which was treated with pea (Fig. 3). Since flavonoids are compounds produced by the plants. the decrease in these compounds when compared to the pea sample(control) indicates that they were consumed by the bacteria.

Antimicrobial activity: According to the antimicrobial activity results, the methanol extraction of pea (Ps) had an effect on *C. glabrata* and *Epidermophyton* spp. (10 mm) (Table-3). The extracts which were prepared in the pea fatty acid analysis (Ps) most affected *Tricophyton* spp, *Epidermophyton* spp, *B. megaterium* (18 mm, 17 mm, 16 mm) (Table- 4); while the extracts which were prepared in vitamin analysis (Ps) had a considerable effect on all the test microorganisms used in the study (Table-5). When the antimicrobial activity of the *L. lactis* extracts which were treated with pea were analyzed (the extracts which were prepared for the flavonoid analysis), almost no antimicrobial activity was observed (Table-3). It is thought that flavonoid compounds with an

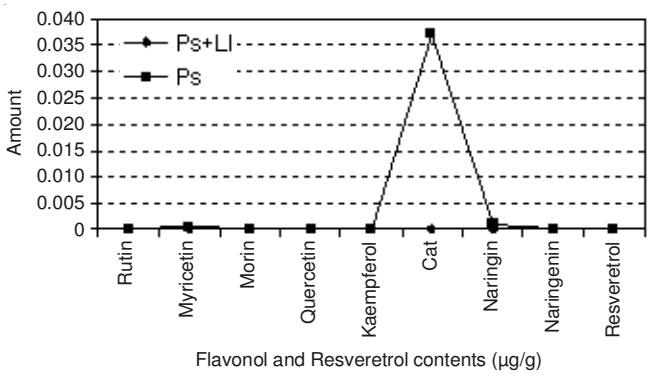

Fig. 3. Flavonoid contents, (µg/g)

antimicrobial effect might have been consumed by the *L. lactis*. This study clearly reveals that *L. lactis* treated with pea supports the flavonoid analysis results. When the antibacterial activities of *L. lactis* extracts which were treated with pea (the extracts which were prepared for the fatty acid analysis) were analyzed, it was found that they had the highest effect on the dermatophyte fungi (Table-4); while the samples which were prepared for vitamin analysis had an effect on *C. albicans* and *B. megaterium* (13 mm, 12 mm), (Table-5). The fact that the *P. sativum* extracts which were prepared for the fatty acid analysis and treated with *L. lactis* (Ps + L1) had a higher antimicrobial activity when compared to the control (Ps fatty acid antimicrobial activity) might result from the fatty acid levels. The results of the fatty acid analysis showed that *L. lactis* affected the fatty acid profile and increased the levels of fatty acid with antimicrobial activity. This result is consistent with the results from the antimicrobial activity. A general analysis of all the antimicrobial activity results revealed that the pea samples which were treated with *L. lactis* showed variations when compared to the control.

TABLE-3
ANTIMICROBIAL ACTIVITY OF FLAVONOID ANALYSIS PREPARED EXTRACTS *(*mm*)*

Sampling	Sa	Bm	Ec	Kp	Ca	Cg	E	T
Ps	8	8	-	8	8	10	10	8
Ps + Ll	-	8	-	-	-	-	8	-

-Ps: *P. sativum*, Ps+Ll: *P. sativum+L.lactis*

TABLE 4
ANTIMICROBIAL ACTIVITY OF FATTY ACID ANALYSIS PREPARED EXTRACTS (mm)

Sampling	Sa	Bm	Ec	Kp	Ca	Cg	E	T
Ps+Ll	13	8	7	-	9	13	17	15
Ll	-	-	14	10	-	9	-	-
Ps	8	16	8	-	11	12	17	18

-Ps: *P. sativum*, Ll: *L.lactis*, Ps + Ll: *P. sativum + L.lactis*

TABLE-5
ANTIMICROBIAL ACTIVITY OF VITAMIN ANALYSIS PREPARED EXTRACTS (mm)

Sampling	Sa	Bm	Ec	Kp	Ca	Cg	E	T
Ps + Ll	-	12	-	-	13	10	-	-
Ll	-	15	-	15	12	17	-	-
Ps	24	24	26	34	15	26	21	22

-Ps: *P. sativum*, Ll: *L.lactis*, Ps + Ll: *P. sativum + L.lactis*

In recent years, the number of studies on plant phenols has increased and gained importance for the treatment of chronic diseases and supporting a healthy life. It was emphasized that plant phenols were of great importance in terms of their anti-tumoral and antimicrobial activities[15]. In the pharmaceutical industry, in particular it is the antimicrobial activities of phenolic substances that are used. These phenolic compounds which have antimicrobial activity are flavonoids, fatty acids and organic acids. It has been reported that the use of plants containing phenolic compounds could be beneficial treatments in modern medicine[16]. In present study, the fact that in addition to the DPPH radical neutralization activity, the pea sample was found to contain some phenolic compounds indicated that the pea plant has a strong antioxidant activity. Previous studies[17,18] also revealed that the pea plant has significant phenolic compounds and antioxidant properties and a rich content of vitamins A, B and C. In addition, it has been reported that pea seeds contained a higher amount of phenolic compounds than coloured pea seeds and that to produce a pea plant with a high antioxidant capacity, favourable media should be provided[17]. In a previous study carried out on pea plants it was reported that the lipid level in plasma concentrations which were treated with cadmium varied according to the created stress factor[19]. The research detailed above support the results from our study. However, these partial differences are caused by the levels of phenolic compounds. Environmental conditions, chemical structure of soil, sunlight, growing area of the plant, maturity period and species differences are among the reasons causing differences in the levels of phenolic compounds. A review of the literature found no studies similar to our study which used probiotic cell culture analysis. Similar studies should be repeated in related laboratories and the results of our study should be scientifically verified.

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