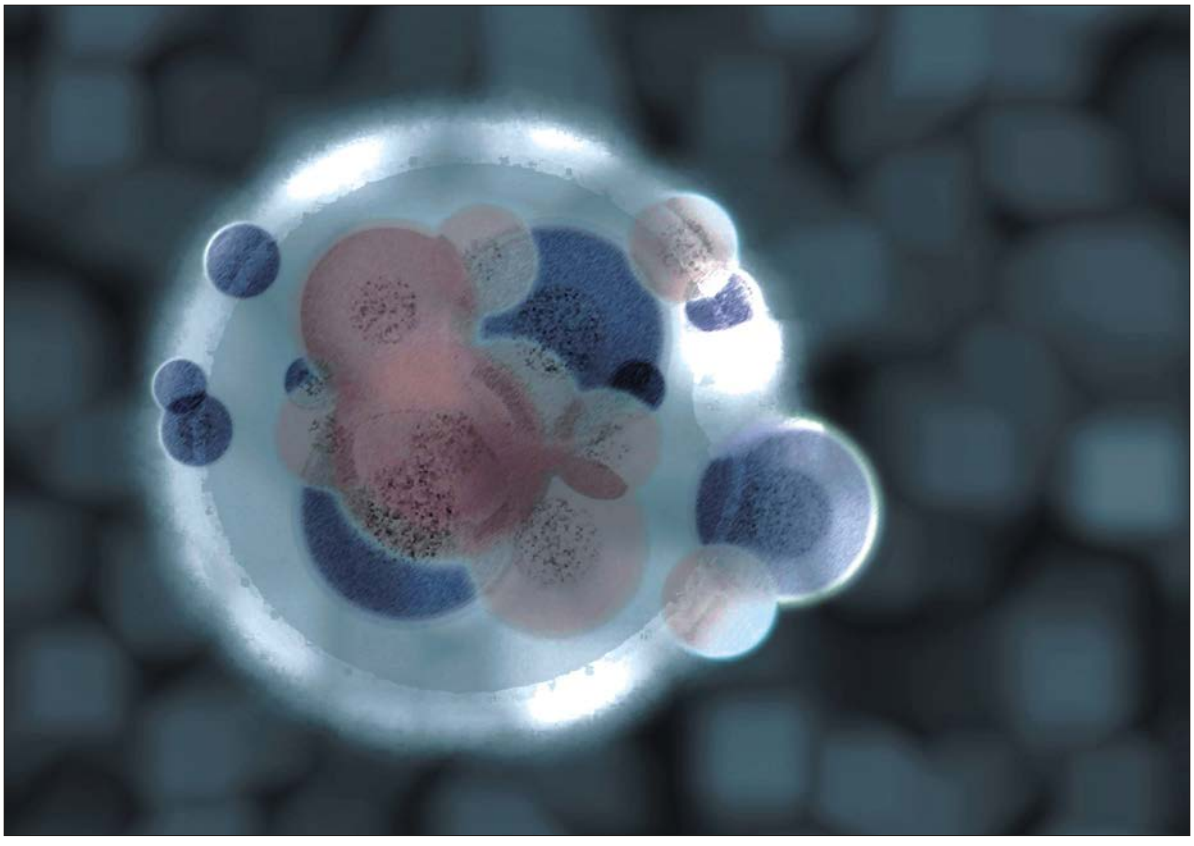

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Research Article
Amelioration of Diabetes in a Rat Model Through Yoghurt Supplemented with Probiotics and Olive Pomace Extract

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Abstract

Background and Objective: Olive pomace (OP) is the by-product of olive oil production. The OP contains large amount of phenolic compounds. The aim of the present study was to investigate the anti-diabetic effect of yoghurt supplemented with probiotics and olive pomace extract (OPE) in diabetic rats. **Materials and Methods:** Ultrasonic-assisted method was used for extraction of OP. Total phenolic content of OPE was estimated. The antibacterial activity of OPE was determined. Yoghurt supplemented with probiotics and OPE was prepared. Antioxidant activity, titratable acidity, water holding capacity (WHC) and organoleptic properties were evaluated in yoghurt. Anti-diabetic effect of yoghurt against type-2 diabetes was evaluated in rats. Blood samples were collected for determination of plasma glucose, insulin, lipid profile, plasma markers of oxidative stress, tumor necrosis factor-α, liver and kidney functions. Data were analyzed statistically using the one-way analysis of variance (ANOVA) followed by Duncan's test. **Results:** The OPE contains 720 mg gallic acid equivalent g^{-1} dry weight. The HPLC analysis revealed the presence of many phenolic compounds such as vanillin and sinapic acid in OPE. OPE possess antibacterial effect against pathogenic bacteria. Antioxidant activity of yoghurt increased with the increment in OPE concentration. During storage the pH and acidity of yoghurt showed continuous decrease and increase, respectively. The WHC of fresh yoghurt decreased slightly with increasing OPE concentration, while WHC increased during storage. Hardness of yoghurt was affected non-significantly with increasing the concentration of OPE and storage periods until the 14th day. Organoleptic results revealed that yoghurt could be supplemented with OPE up to 1.5%. Diabetic rats showed significant changes in plasma glucose, insulin, lipid profile, TNF-α, oxidative stress markers and kidney function. Oral administrations of yoghurt supplemented with probiotic and OPE to diabetic rats showed significant improvement in all the studied biochemical parameters with different degrees. **Conclusion:** Yoghurt supplemented with probiotics and 1.5% OPE was the most promising in improving type-2 diabetes in diabetic rats.

Key words: Olive pomace extract, phenolic compounds, yoghurt, type-2 diabetes, probiotics

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Olive pomace (OP) is one of the most prevalent agro-industrial by-products in Mediterranean area. OP considered a rich source of phenolic compounds, which possess different activities such as antioxidant, anti-microbial, anti-inflammatory and anticancer[1-3]. The amount and quality of phenolic compounds in olive pomace depend on the source of the fruits and its maturity and the environmental conditions[4]. Addition of natural antioxidants such as phenolic compounds may be a method for enhancement functional foods and improve their activity in promoting human health and also increased shelf life of food products. So, it would be desirable to find a method for extraction of compounds with antioxidant activity from olive pomace as way for maximizing utilization of food processing wastes. One of the best methods for phenolic extraction from OP is ultrasound-assisted extraction (UAE), which improve efficiency and decrease extraction time compared to conventional extraction techniques[5]. Diabetes mellitus is becoming a serious international public health problem[6]. Type-2 diabetes (T2D) pathogenesis depends on genetic and environmental factors[7]. Gut microbiota play an important role in diabetes[8]. Human gut contains trillions of microorganisms, which plays many biological functions in human metabolism[9,10]. The direct role of gut bacteria in insulin resistance was firstly demonstrated by Cani *et al.*[11]. Gut microbiota play an important role to glucose hemostasis through numbers of different bacterial metabolites[12]. Probiotics inhibit gluconeogenesis in type 2 diabetic mice[13]. Addition of phenolic compounds and probiotics in a food product may be one of the important ways to treat T2D. So, in the present study a functional food containing probiotics and phenolic compound rich source was prepared and evaluated in diabetic rats. The T2D was induced in rats by single dose of streptozotocin (STZ).The aim of the present study was to investigate the anti-diabetic effect of yoghurt supplemented with probiotics and three different concentration of OPE as rich source of phenolic compounds. The content of phenolic compound of OPE was determined through Folin–Ciocalteu reagent and HPLC. The antibacterial activity of OPE was determined. Antioxidant activity, titratable acidity, water holding capacity (WHC) and organoleptic properties of yoghurt were evaluated.

MATERIALS AND METHODS

Materials: Olive pomace from a two-phase olive oil extraction was obtained from Americana Factory for olive oil processing (6 of October City, Giza Egypt) season 2016. The utilization of 2-phase processing technique generates olive oil and by-product which is a mixture of liquid and solid waste, called "Alperujo or two-phase olive mill waste". This by-product is characterized by high moisture content with thick sludge consistency that contains about 80% of the olive fruit, including skin, seed, pulp and pieces of stones. The pomace was stored at -18°C until use.

Fresh buffalo's skim milk and sweet cream (~40.0% fat) were obtained from the farm of Faculty of Agriculture, Cairo Univeristy, Egypt. Skim milk powder (low heat), made in the USA was purchased from the local market at Cairo, Egypt. Strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were obtained from stock cultures of Dairy Microbiology Lab., National Research Centre, Dokki, Cairo, Egypt.

Animals: Male albino rats, of 135.3±4.551g as (Mean±SD) were used. Animals were obtained from the animal house of National Research Centre, Cairo, Egypt. The animals were kept individually in stainless steel cages at room temperature. Water and food were given *ad libitum*.

Diets: A balanced diet composed of 10% protein supplemented from casein, 10% corn oil, 23.5% sucrose, 47% maize starch, 5% fiber, 3.5% salt mixture[14] and 1% vitamin mixture[15] was prepared for feeding rats all over the experimental period.

Methods

Preparation and ultrasound-assisted extraction of olive pomace: Olive pomace was dried in an oven at 40°C for about 24 h until constant moisture content was achieved and ground using a laboratory mixer; then dried pomace treated with n-hexane to remove the residual pomace olive oil. Ultrasound-assisted extraction method was used for extractions of phenolic compounds from olive pomace and was performed following the method of Lafka *et al.*[16], using a solid:liquid ratio of 1:5. A mixture of isopropanol/water (50:50 v/v) was used as solvent; pH was adjusted to 2-3. The extracted samples were filtrated and centrifuged at 4,000 rpm for 10 min. Then, solvent was evaporated at 40°C and the extract was stored at -18°C until use.

Determination and identification of phenolic compounds: Total phenolic compounds of OPE were determined according to the method of Jayaprakasha *et al.*[17]. The OPE (10 µL) containing phenolic compounds were injected in HPLC for determination and identification of phenolic compounds. A Shimadzu LC-10A apparatus (Kyoto, Japan) equipped

with a SPD-M10A photodiode array detector (PDA) was used for analytical HPLC separations. Reversed-phase chromatography was performed with 250×4.6 mm Kromasil 100 C-18 column packed with 5 µm particles (Teknokroma, Barcelona, Spain), fitted with a security guard C18 ODS (4×3.0 mm i.d). Gradient were formed with He-degassed solvent. Solvent A was H_2O containing 0.1% formic acid and solvent B was MeCN by applying different elution conditions. Separation was accomplished starting with 5% A for 2 min at pressure of 115 bar, followed by a linear gradient was performed for 10 min from 5% B to 95% A and subsequent linear gradient from 20-95% A in 5 min. The flow rate was 0.5 mL min^{-1},and the operating temperature was 40°C. The chromatogram was recorded at 286 nm (phenolic acids).

Determination of antibacterial activity: The antimicrobial activity of 5 different concentrations of olive pomace extract was determined by the agar well diffusion method[18]. The seven pathogenic indicator bacteria strains were obtained from the stock cultures of the Dairy Microbiological Lab, National Research Centre: *Escherichia coli* 0157: H7 ATCC 6933, *Bacillus cereus* ATCC 33018, *Staphylococcus aureus* ATCC 20231, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027, *Listeria monocytogenes* ATCC 7644 and *Yersinia enterocolitica* ATCC 9610. Each strain was activated in tryptone soy broth by fermentation at 37°C for 24 h. One milliliter culture of the activated indicator strain (10^5 cells mL^{-1}) was inoculated into 20 mL of Mueller-Hinton agar (Becton Dickinson, USA) and poured in petri dishes. After solidification of the agar, wells of 5 mm in diameter were cut from the agar with a sterile borer and 50 µL of isolates delivered in each well.

The antimicrobial activity was expressed as the diameter of the zone of inhibition (ZOI); whereby a diameter >1mm around the well was considered as a positive result and the greater the diameter of the ZOI, the higher is the antimicrobial activity. The % inhibition was calculated according to National Committee for the Clinical Laboratory (NCCLS). The zone diameter of wells cut in Mueller-Hinton agar was 5.0 mm and the diameter of inhibition zone (DIZ) of negative a control for each bacterium was also 5.0 mm. If the DIZ value is 5.0 mm (*), that means the sample has no inhibitory activity against that bacterium. Determination of the minimum inhibitory concentration (MIC) to the prepared Mueller-Hinton agar plates containing specific bacteria on which different wells have been made, different dilutions of the OPE was introduced. The plates were incubated at 37°C for 24 h. Zones of inhibition were measured at the end of the incubation period. The MIC was taken to be the lowest dilution inhibiting the growth of the organism.

Starter activation for yoghurt preparation: *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were activated individually by three successive transfers in sterile 10% reconstituted skim milk powder and incubated at 37°C under anaerobic conditions.

Preparation of yoghurt: Buffalo's skim milk was divided into five equal portions for preparation of four types of yoghurt. Type one contains 0% of OPE and served as yoghurt control, while type two, three, four and five of yoghurt supplemented with four different concentration of OPE 0.5, 1, 1.5 and 2%, respectively. All mixtures were heated to 60°C and adjusted to have the similar total solids (~15.0%) and milk fat (~3.2%) contents using skim milk powder and sweet cream. Heating was raised to 90°C, held for 5 min and followed by cooling to 45°C. This was followed by inoculation with 1.0% w/v of *S. thermophilus* and *L. bulgaricus*. The inoculated mixtures were mixed thoroughly, dispensed in 100 mL polystyrene cups and incubated at 42°C until a uniform coagulation was formed[19]. The produced yoghurt treatments were analyzed when fresh and after 3, 5, 7, 10 and 15 days of storage at 4±2°C. Three replicates were manufacturing from each type of yoghurt.

Bacteriological analysis: Yoghurt samples were diluted and subsequently plated in duplicate onto selective media. The MRS agar medium was used to enumerate *L. Bulgaricus*[20] and the plates were incubated at 37°C for 48 h. *S. thermophiles* was enumerated on M17 agar and the plates were incubated at 37°C for 48 h[21]. The aerobic bacterial count were enumerated according to FDA[22], using plate agar count medium after aerobic incubation at 48±2 h incubation at 35±1°C, colony forming units were counted and calculated per g of sample. Coliforms group were enumerated according to Harrigan and McCance[23], using violet red bile agar medium, incubated at 37°C for 24 h. Mould and yeast were determined using potato dextrose agar medium and the plates were incubated at 30°C for 5 days. *Staphylococcus aureus* was enumerated according to FDA[22]. Enumeration of *S. aureus* in yoghurt samples was carried out by spreading 0.1 mL of each of sufficient (expected) dilution onto the surface agar medium. Baird Parker media supplemented with egg yolk and potassium tellurite solution. Plates were incubated at 37°C for 48 h. *Listeria monocytogenes* was enumerated according to FDA[22]. A plate of selective oxford agar base supplemented with *Listeria* supplement was streaked from each of an enrichment flask and incubated at 35°C for 48 h.

Determination of total phenolic content of yoghurt: Total phenolic content (TPC) of yoghurt samples was determined according to Jayaprakasha *et al.*[17] using Folin-Ciocalteu reagent. About 0.5 mL of extract was mixed with 0.5 mL of 10-fold-diluted Folin–Ciocalteu reagent. After 3 min, 4 mL of 7.5% sodium carbonate was added. The mixture was allowed to stand for 30 min in the dark at room temperature before the absorbance was measured at 765 nm using a spectrophotometer (model 2010, Cecil Instr. Ltd., Cambridge, UK). The final results were expressed as milligrams gallic acid equivalent per gram of dry weight (GAE/DW).

DPPH radical scavenging activity: Free radical scavenging activity (RSA) of the yoghurt samples was measured using the method of Brand-Williams *et al.*[24]. An aliquot 100 µL of the sample solution was mixed with 2.9 mL of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) in methanol. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm by a UV-visible spectrophotometer. The antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity (\%)} = 1-\frac{A_{bs}\ \text{sample}}{A_{bs}\ \text{control}}\times 100$$

Titratable acidity and pH: Titratable acidity was expressed as % lactic acid AOAC[25]. The pH was measured using digital pH meter HANNA-211.

Water holding capacity: Water holding capacity (WHC) was determined according to Arslan and Ozel[26]. Twenty grams sample of native yogurt (NY) was centrifuged for 10 rpm at 669 g and supernatant was removed and weighted (whey expelled (WE). The samples were analyzed at 0, 7, 14 and 21 days. The WHC (%) was defined as:

$$\text{WHC (\%)} = \frac{\text{NY-WE}}{\text{NY}}\times 100$$

Textural evaluation: Texture profile analysis (TPA) performed on the yoghurt samples (hardness, cohesiveness, springiness, gumminess and chewiness) using the double corporation, Slinfold, W. Sussex, UK. From the force time curve, the following parameters were evaluated by TPA according to the definitions by International Dairy Federation[27].

Sensory evaluation: The yoghurt samples were organoleptically evaluated by some panelists from the staff members of the Dairy Science Department, National Research Center, Egypt. Each yoghurt sample was evaluated and used a quality rating score card for evaluation of flavor (60 points) and body and texture (30 points) and appearance (10 points) as described by Nelson and Trout[28].

Design of experimental study: Thirty rats were divided into five groups, each of six rats. The first group was considered as the normal healthy group. The remained rats were served as diabetic rats. After an overnight fasting, hyperglycaemia and type-2 diabetes were induced in twenty-four rats by an intraperitoneal injection of a single dose of freshly prepared streptozotocin (STZ) (45 mg kg^{-1}) in citrate buffer (0.09 M, pH 4.8)[29], diabetic rats given glucose solution (5%) for 48 h after STZ injection to prevent hypoglycemia. Animals were monitored by testing of fasting glucose by taking blood from tail veins and only those with blood glucose levels exceeded 200 mg dL^{-1} at the 7th day after STZ administration, were considered diabetic and included in the study. Diabetic rats were divided into four groups, each of six rats. The first group was control STZ diabetic rats (STZ control). Rats of group two, three and four where rats were given oral administration of yoghurt supplemented with probiotics and three different concentration of OPE (0.5, 1 and 1.5 mL extract/100 mL yoghurt) for 21 days after induction of diabetes. All rats groups were feed on balanced diet all over the study period. During the experiment, body weight and food intake were recorded weekly. After 21 days (end of the study) total food intake, body weight gain and feed efficiency ratio (Body weight gain/total food intake) were calculated. Blood samples were collected from all rats after an overnight fast for the determination of plasma total cholesterol (T-Ch)[30], high density lipoprotein cholesterol (HDL-Ch)[31], low density lipoprotein cholesterol (LDL-Ch)[32] and triglycerides (TG)[33]. T-Ch/HDL-Ch ratio was calculated as indicator of cardiovascular risk. Plasma malondialdehyde (MDA) was estimated as an indicator of lipid peroxidation[34]. Plasma tumor necrosis factor-α (TNF-α)[35] was determined as an inflammatory biomarker. The activity of plasma aspartate transaminase (AST) and alanine transaminase (ALT)[36] were estimated as indicator of liver function. Plasma levels of creatinine[37] and urea[38] were determined to study any possible changes in kidney function. Fasting plasma glucose and insulin were determined according to Trinder[39] and Turkington *et al.*[40], respectively. This study has been carried out according to the Medical Research Ethics Committee, National Research Centre, Cairo, Egypt.

Statistical analysis: The results of animal experiments were expressed as the Mean±SE and they were analyzed statistically using the one-way analysis of variance ANOVA followed by Duncan's test. In all cases $p<0.05$ was used as the criterion of statistical significance.

RESULTS

Total phenolic content of OPE was 720 mg GAE g^{-1} DW. Injection of OPE using HPLC (Table 1) revealed the presence of many phenolic compounds. Vanilin was the highest one (585.26 mg L^{-1}) followed by sinapic acid (243.39 mg L^{-1}), while quercetin (9 mg L^{-1}) was the lowest one.

Bacteriological analysis: Table 2 shows the minimum inhibitory concentration of OPE. It ranged from 0.1% (v/v) to 0.5% (v/v) for *Escherichia coli* 0157: H7, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium, Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Yersinia enterocolitica.* All OPE concentration tested, inhibited the growth of the organisms with diameter of zones of inhibition ranging from 6 mm for *Escherichia coli* 0157:H7 and *Salmonella typhimurium* to 8 mm for *Staphylococcus aureus* at 0.4% (v/v) of the extract.

Table 3 summarized the obtained results of analysis of yoghurt manufacturing with 0, 0.5, 1 and 1.5% of olive pomace extracts and stored in the refrigerator for 15 days. As seen from Table 3 counts of *L. bulgaricus* detected on MRS agar were ranged from 43×10^8-89×10^6, counts of *S. thermophilus* detected on M17 agar were ranged from 89×10^7-78×10^6. Counts of total bacterial count detected on plate count bacteria were ranged from 14×10^8-81×10^6. Count of coliform bacteria detected on violet red agar did not detected through storage period. Count of mould and yeast detected on potato dextrose agar were ranged from 94×10^3-5×10^1.

Results obtained in the present study indicated that OPE did not influence on population of *L. bulgaricus*, *S. thermophiles* and total bacterial count, while it affect the appearance of mould and yeast. Mould and yeast were not detected after 3 days of yoghurt storage period; this may be due to the synergistic effect of OPE components, on the contrary the count of mould and yeast increased in this control through the period of storage because it didn't contains OPE.

Total phenolic content and radical scavenging activity of yoghurt: Figure 1 and 2 revealed that total phenolic content and antioxidant activity of yoghurt samples increased with the increment in the concentration of OPE. The TPC was presented by 153 mg GAE g^{-1} in the fresh control sample and 245.53 mg GAE g^{-1} in the yoghurt supplemented by 2% of OPE. Fresh yoghurt sample showed the lowest (12.03%) RSA%, while yoghurt supplemented with 2% of OPE showed the highest RSA% (23.6%). Both TPC and RSA were increased in all yoghurt samples with storage period.

Table 1: Identified phenolic compound in olive pomace extract (mg L^{-1}) by HPLC

Phenolic compound	Concentration (mg L^{-1})
3-4-Dihydroxybenzoic Acid	116.30
4-Hydroxybenzoic Acid	18.75
Caffeic Acid	31.72
Catechin	129.21
Chlorogenic Acid	109.29
Ellagic Acid	15.02
Epicatechin	115.34
Ethyl-3-4-dihydroxybenzoate	106.12
Ferulic Acid	58.49
Fumaric Acid	0.00
Gallic Acid	114.00
p-coumaric Acid	124.11
Quercetin	9.00
Rutin	83.44
Sinapic Acid	243.39
Syringic Acid	85.77
t-cinnamic Acid	75.33
Vanillic Acid	0.00
Vanilin	585.26
Floridzin	0.00
Anthocyanin	112.11

Table 2: Antibacterial activity (MIC) of different concentration of olive pomace extract measured as zone of inhibition (mm)

	Zone of inhibition (mm) for concentration of olive pomace extract				
Microorganisms	0.1%	0.2%	0.3%	0.4%	0.5%
List.	Nil	Nil	Nil	7	8
Staph	Nil	Nil	Nil	8	10
B.c	Nil	Nil	Nil	Nil	Nil
E.c	Nil	Nil	Nil	6	7
Yerse	Nil	Nil	Nil	Nil	Nil
Sal.	Nil	Nil	Nil	6	7
Pse	Nil	Nil	Nil	Nil	Nil

List.: *Listeria monocytogenes* ATCC 7644, Staph: *Staphylococcus aureus* ATCC 20231, B.c: *Bacillus cereus* ATCC 33018, E.c: *Escherichia coli* 0157: H7 ATCC 6933, Yerse.: *Yersinia enterocolitica* ATCC 9610, Sal. *Salmonella typhimurium* ATCC 14028, Pse: *Pseudomonas aeruginosa* ATCC 9027, Nil: Non detected

Table 3: Microbial evolution in yoghurt with olive pomace extracts within 15 days storage in the refrigerator

Days storage	Treatment	*L. bulgaricus*	*S. thermophilus*	T.C	Coliform group	Mould and yeast
Zero	T1 (Control)	86×10^7	53×10^7	82×10^7	Nil	28×10^1
	T2	18×10^7	29×10^7	33×10^7	Nil	13×10^1
	T3	40×10^7	22×10^7	55×10^7	Nil	19×10^1
	T4	22×10^7	61×10^7	79×10^7	Nil	32×10^1
3	T1 (Control)	43×10^8	89×10^7	14×10^8	Nil	17×10^2
	T2	49×10^7	58×10^7	60×10^7	Nil	5×10^1
	T3	63×10^7	47×10^7	53×10^7	Nil	7×10^1
	T4	36×10^7	73×10^7	62×10^7	Nil	14×10^1
5	T1 (Control)	15×10^8	61×10^7	94×10^7	Nil	10×10^3
	T2	68×10^7	49×10^7	51×10^7	Nil	Nil
	T3	40×10^7	28×10^7	35×10^7	Nil	Nil
	T4	19×10^7	46×10^7	26×10^7	Nil	Nil
7	T1 (Control)	92×10^7	33×10^7	82×10^7	Nil	22×10^3
	T2	43×10^7	31×10^7	39×10^7	Nil	Nil
	T3	22×10^7	11×10^7	21×10^7	Nil	Nil
	T4	96×10^7	28×10^7	17×10^7	Nil	Nil
10	T1 (Control)	73×10^7	17×10^7	73×10^7	Nil	63×10^3
	T2	29×10^7	14×10^7	65×10^7	Nil	Nil
	T3	7×10^7	93×10^6	26×10^7	Nil	Nil
	T4	82×10^7	9×10^7	8×10^7	Nil	Nil
15	T1 (Control)	28×10^7	92×10^6	40×10^7	Nil	94×10^3
	T2	12×10^7	86×10^6	29×10^7	Nil	Nil
	T3	89×10^6	78×10^6	3×10^7	Nil	Nil
	T4	66×10^7	90×10^6	81×10^6	Nil	Nil

T1: Control 0% olive pomace extract, T2: Treatment with added 0.5% olive pomace extract, T3: Treatment with added 1% olive pomace extract, T4: Treatment with added 1.5% olive pomace extract. T.C: Total bacterial count

Table 4: pH of yoghurt supplemented with different concentration of OPE

	Storage period (days)			
Concentration (%)	Fresh	7	14	21
Control (0)	4.69±0.006	4.65±0.010	4.60±0.006	4.58±0.012
0.5	4.68±0.015	4.61±0.015	4.59±0.006	4.53±0.026
1	4.66±0.006	4.57±0.021	4.55±0.006	4.52±0.015
1.5	4.60±0.006	4.55±0.015	4.52±0.015	4.49±0.006
2	4.58±0.025	4.53±0.015	4.50±0.006	4.48±0.006

Mean±SE

Table 5: Acidity of yoghurt supplemented with different concentration of OPE

	Storage period (days)			
Concentration (%)	Fresh	7	14	21
Control (0)	1.297±0.015	1.483±0.015	1.557±0.040	1.633±0.035
0.5	1.423±0.025	1.487±0.015	1.623±0.021	1.693±0.015
1	1.477±0.025	1.520±0.020	1.717±0.021	1.763±0.023
1.5	1.500±0.020	1.563±0.015	1.777±0.025	1.803±0.015
2	1.550±0.030	1.603±0.015	1.827±0.025	1.863±0.015

Mean±SE

Titratable acidity and pH: Table 4 and 5 showed a slight decrease in pH and increase in acidity values of fresh yoghurt samples with increasing the concentration of OPE added.

During storage yoghurt samples showed continues decrease in pH and increasing in acidity values. Also, it was noticed that until 21st day of yoghurt storage, the acidity of control and experimental yoghurt samples were nearly the same.

Water holding capacity: As shown in Fig. 3 WHC of fresh yoghurt samples with OPE had a slight decreased with increasing OPE concentration compared to control, while WHC increased during storage for all yoghurt samples.

Textural properties: Table 6 presents the results of texture analysis performed on yoghurt supplemented with OPE and the control sample. Data revealed that hardness of yoghurt

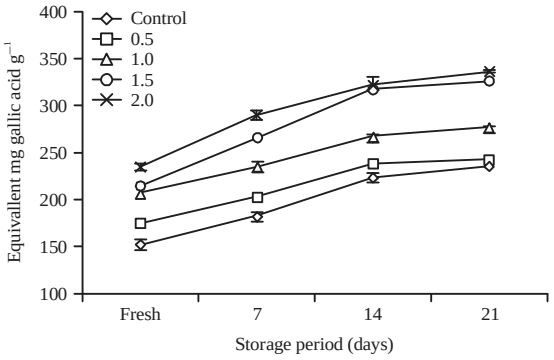

Fig. 1: Total phenolic content of yoghurt supplemented with different concentration of olive pomace extract
Mean±SE

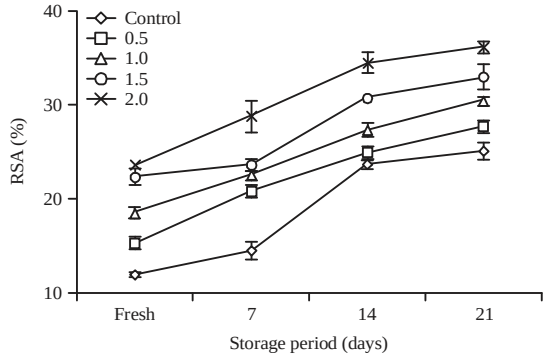

Fig. 2: Radical scavenging activity of yoghurt supplemented with different concentration of olive pomace extract
Mean±SE

samples was not significantly affected with increasing the concentration of OPE and storage periods until the 14th day then decreased significantly with increasing the OPE added (0.5, 1, 1.5 and 2%) at the end of storage (21st day).

Organoleptic properties of yoghurt supplemented with OPE: The results of organoleptic properties of yoghurt supplemented with probiotics and OPE are shown in Table 7. From total scores of sensory evaluation, the results revealed that all yoghurt samples were accepted when fresh. The acceptability of all yoghurt samples were reduced when stored at 5°C till for 21 days. It was clear that addition of OPE up to 1.5% still accepted for panelists till the 21st day, as the yoghurt had pleasant flavor with no unpleasant aftertaste during these days of storage. Yoghurt supplemented with OPE up to 1.5% and kept at 5°C for 21 days was acceptable to panelists, while yoghurt supplemented with 2% OPE was not acceptable to panelists after storage for 21st day. So decided to exclude this ratio of addition from the study and evaluate only the three other additions (0.5, 1 and 1.5%).

Anti-diabetic effect of yoghurt supplemented with olive pomace extract: Yoghurt supplemented with OPE was evaluated for its anti-diabetic effect in rats. Streptozotocin was used for induction of diabetes in rats.

Oral administration of yoghurt supplemented with probiotics and OPE to diabetic rats showed significant $p<0.05$ reduction in plasma glucose and significant elevation in insulin levels in all treated groups compared with STZ diabetic

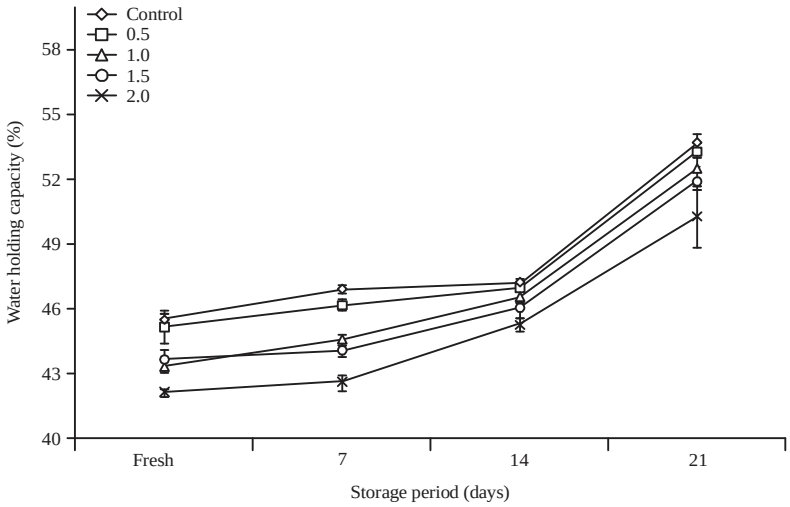

Fig. 3: Water holding capacity of yoghurt supplemented with different concentration of OPE
Mean±SE

Table 6: Textural properties performed for yoghurt supplemental with OPE and the control sample

		OPE concentration (%)				
Analysis		Control (0)	0.5	1	1.5	2
Hardness	0	0.700	0.700	0.700	0.600	0.700
	7	0.710	0.700	0.600	0.600	0.700
	14	0.800	0.820	0.400	0.500	0.600
Cohesiveness area (B/A)	0	0.554	0.683	0.587	0.393	0.436
	7	0.499	0.416	0.688	0.578	0.497
	14	0.528	0.523	0.276	0.575	0.437
Springiness	0	0.612	0.805	0.831	0.510	0.370
	7	0.483	0.504	0.819	0.437	0.600
	14	0.724	0.568	0.418	0.508	0.620
Gumminess	0	0.388	0.478	0.411	0.236	0.305
	7	0.354	0.291	0.413	0.345	0.348
	14	0.422	0.428	0.111	0.288	0.262
Chewiness N.mm	0	0.237	0.385	0.341	0.120	0.113
	7	0.171	0.165	0.338	0.119	0.209
	14	0.306	0.233	0.046	0.146	0.163

Table 7: Organoleptic properties of yoghurt supplemented with OPE during storage

OPE concentration (%)	Storage time (days)	Flavor (60)	Body and texture (30)	Color and appearance (10)	Total (100)
Control (0)	Fresh	58	29	10	97
	7	57	28	9	94
	14	56	27	8	91
	21	56	26	8	90
0.5	Fresh	59	29	9	97
	7	59	28	9	96
	14	55	26	8	89
	21	54	28	7	89
1	Fresh	57	28	8	93
	7	54	28	8	90
	14	50	28	8	86
	21	50	28	8	86
1.5	Fresh	53	28	9	90
	7	52	28	9	89
	14	50	27	9	86
	21	47	28	8	83
2	Fresh	52	25	9	86
	7	51	24	8	83
	14	49	24	8	81
	21	43	24	8	75

Table 8: Plasma glucose and insulin of different experimental groups

Groups	Glucose (mg dL^{-1})	Insulin (mU L^{-1})
Normal	72.50 ± 1.34^{a}	6.18 ± 0.22^{d}
Diabetic	220.67 ± 3.44^{e}	3.92 ± 0.08^{a}
Yoghurt supplemented with 0.5% OPE	157.50 ± 3.82^{d}	4.60 ± 0.17^{b}
Yoghurt supplemented with 1% OPE	141.67 ± 2.17^{c}	5.48 ± 0.18^{c}
Yoghurt supplemented with 1.5% OPE	123.33 ± 2.25^{b}	5.68 ± 0.19^{cd}

In each column same letters means non-significant difference; different letter means the significance among the tested groups at 0.05 probability. Mean±SE

control with different degrees (Table 8). STZ reduced insulin levels in diabetic rats. Rat's group given oral administrations of yoghurt containing 1.5% OPE showed the highest reduction in plasma glucose and elevation in insulin levels.

Table 9 represented plasma lipid profile of all the studied groups. Diabetic control rats (STZ-control) showed dyslipidemia as reflected in significant $p<0.05$ elevation in T-Ch, TG, LDL-Ch and the ratio of T-Ch/HDL-Ch, while HDL-Ch reduced significantly compared with normal rats. Yoghurt supplemented with probiotics and 1.5% OPE was the most promising in improving plasma lipid profile of diabetic rats.

Table 10 showed plasma MDA, total antioxidant capacity and TNF-α of normal and diabetic rats. Oxidative stress was elevated significantly through elevation of malondialdehyde

Table 9: Plasma lipid profile of different experimental groups

Groups	T-Ch (mg dL^{-1})	TG (mg dL^{-1})	HDL-Ch (mg dL^{-1})	LDL-Ch (mg dL^{-1})	T-Ch/HDL-Ch ratio
Normal	73.83±1.81^{a}	81.00±1.32^{a}	39.00±0.97^{b}	20.17±0.60^{a}	1.90±0.07^{a}
Diabetic	106.17±2.09^{d}	102.33±2.19^{d}	29.33±0.71^{a}	24.67±0.88^{b}	3.62±0.06^{e}
Yoghurt supplemented with 0.5% OPE	99.67±2.59^{c}	100.33±1.48^{cd}	30.83±0.79^{a}	24.17±0.60^{b}	3.23±0.06^{d}
Yoghurt supplemented with 1% OPE	99.00±1.81^{c}	95.83±1.58^{bc}	37.67±0.61^{b}	23.33±0.88^{b}	2.63±0.05^{c}
Yoghurt supplemented with 1.5% OPE	86.00±1.65^{b}	92.17±1.58^{b}	39.33±0.71^{b}	20.83±0.60^{a}	2.19±0.06^{b}

T-Ch: Total cholesterol, HDL-Ch: High density lipoprotein cholesterol, LDL-Ch: Low density lipoprotein cholesterol and TG: Triglycerides. In each column same letters means non-significant difference, different letter means the significance among the tested groups at 0.05 probability. Mean±SE

Table 10: Plasma malondialdehyde, total antioxidant capacity and tumor necrosis factor-α of different experimental groups

Groups	MDA (nmol mL^{-1})	TAC (mM L^{-1})	TNF-α (pg mL^{-1})
Normal	7.85±0.35^{a}	1.74±0.05^{d}	14.98±0.37^{a}
Diabetic	18.17±1.08^{c}	1.08±0.06^{a}	27.83±0.98^{d}
Yoghurt supplemented with 0.5% OPE	16.83±0.54^{c}	1.20±0.03^{a}	24.83±0.79^{c}
Yoghurt supplemented with 1% OPE	14.17±0.60^{b}	1.35±0.04^{b}	20.50±0.76^{b}
Yoghurt supplemented with 1.5% OPE	13.00±0.58^{b}	1.52±0.05^{c}	19.00±0.73^{b}

MDA: Malondialdehyde, TAC: Total antioxidant capacity, TNF-α: Tumor necrosis factor-α. In each column same letters means non-significant difference, different letter means the significance among the tested groups at 0.05 probability. Mean±SE

Table 11: Liver and kidney function of different experimental groups

Groups	AST (IU L^{-1})	ALT (IU L^{-1})	Urea (mg dL^{-1})	Creatinine (mg dL^{-1})
Normal	51.17±1.17^{a}	35.67±1.23^{a}	28.50±1.09^{a}	0.69±0.03^{a}
Diabetic	53.83±1.17^{a}	36.50±1.09^{a}	32.17±0.75^{b}	0.96±0.04^{c}
Yoghurt supplemented with 0.5% OPE	51.17±1.08^{a}	35.50±0.76^{a}	29.83±0.70^{a}	0.85±0.04^{bc}
Yoghurt supplemented with 1% OPE	51.83±1.14^{a}	36.00±0.58^{a}	29.33±0.76^{a}	0.83±0.04^{b}
Yoghurt supplemented with 1.5% OPE	51.67±1.61^{a}	34.00±0.58^{a}	28.67±0.49^{a}	0.80±0.04^{ab}

ALT: Alanine transaminase, AST: Aspartate transaminase. In each column same letters means non-significant difference, different letter means the significance among the tested groups at 0.05 probability. Mean±SE

Table 12: Nutritional parameters of different experimental groups

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Total Food intake (g)	Feed efficiency ratio
Normal	135.50±2.67^{a}	176.83±2.99^{c}	41.33±1.41^{c}	206.67±4.94^{a}	0.20±0.005^{c}
Diabetic	135.33±2.17^{a}	158.33±2.14^{a}	23.00±1.86^{a}	213.17±3.39^{a}	0.11±0.008^{a}
Yoghurt supplemented with 0.5% OPE	135.17±1.45^{a}	165.50±1.48^{b}	30.33±0.33^{b}	204.50±1.71^{a}	0.15±0.001^{b}
Yoghurt supplemented with 1% OPE	135.50±1.82^{a}	166.83±1.01^{b}	31.33±1.45^{b}	209.17a6.25^{a}	0.15±0.006^{b}
Yoghurt supplemented with 1.5% OPE	135.17±1.66^{a}	174.50±2.12^{c}	39.33±0.95^{c}	206.83±2.44^{a}	0.19±0.004^{c}

In each column same letters means non-significant difference; different letter means the significance among the tested groups at 0.05 probability. Mean±SE

and reduction of total antioxidant capacity in diabetic rats compared with normal rats. Yoghurt supplemented with probiotics and 1.5% OPE was the most promising in reduction of oxidative stress through elevation of TAC and reduction of MDA. Plasma levels of TNF-α as inflammatory marker was elevated significantly in diabetic rats compared with normal rats. All yoghurt groups showed significant $p<0.05$ reduction in TNF-α plasma levels, yoghurt containing 1.5% OPE was the promising one.

Liver and kidney functions of all experimental groups are shown in Table 11. The results revealed that plasma activities of AST and ALT showed non-significant changes in all experimental groups. Plasma creatinine and urea elevated significantly in diabetic rats groups compared with normal rats. Oral administration of diabetic rats with yoghurt supplemented with probiotics and 1.5% OPE were the most effective in improvement of kidney function.

Table 12 represented the nutritional parameters of different experimental groups. Diabetic control rats showed significant reduction in final body weight, body weight gain and feed efficiency ratio. Treatment of diabetic rats with yoghurt supplemented with probiotics and different concentration of OPE improved nutritional parameters. Yoghurt containing 1.5% OPE was the promising one in improving nutritional parameters in diabetic rats.

DISCUSSION

In the present study, diabetes was induced in rats by intraperitoneal injection of STZ in rats. Yoghurt supplemented with olive pomace extract and probiotics was used for treatment of diabetic rats. Diabetic rats showed elevation in plasma glucose and reduction in insulin which revealed hyperglycemia. Hyperglycemia was associated with

dyslipidemia. All diabetic rats observed in the study showed elevation in oxidative stress through elevation of MDA, also TNF-α as inflammatory marker was elevated in diabetic rats. Diabetic rats treated with yoghurt showed improvement in hyperglycemia and dyslipidemia which associated with reduction in inflammatory markers and oxidative stress.

Plant-derived raw materials generates enormous by-products during industrial processing, which made a serious seasonally problem. Also they are an abundant source of valuable compounds such as phenolic compounds which may be recovered and used in preparation of functional foods and replace synthetic additives with biologically active compounds[41].

In the present study, olive pomace was used as valuable by-product from olive oil production. Olive oil extraction generates a large amount of residue consisting mainly of the pomace and leaves. Olive pomace is rich in bioactive substance such as phenolic compounds and triterpenic acids that could contribute to the revalorization of this by-products[42]. Phenolic compounds exert several biochemical and pharmacological beneficial effects. Phenolic compounds seem to prevent/improve obesity and metabolic-related disorders[43]. In the present study, UAE method was used for extraction of phenolic compounds from olive pomace. These extracted phenolic compounds was added to yoghurt in accordance with probiotics and evaluated in diabetic rats.

In the present study, phenolic compounds extracted from olive pomace was used as food supplements. Dietary supplement is a source of nutrients or provide phytochemicals which is biologically active. It is used to supplement the normal diet. Olive pomace phenolic compounds were combined with probiotics in the preparation of yoghurt. One example of dietary supplements is probiotics which have a major role in gut through regulation of gut microbiota.

It is well established that a combination of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* have a synergistically role with the traditional yoghurt starter cultures, previous studies have shown that this combination can also antagonistic effects on pathogenic microorganisms[44,45].

In the present study OPE did not influence on population of L. *bulgaricus*, *S. thermophiles* and total bacterial count while affect the appearance of mould and yeast. Mould and yeast did not detected after 3 days of yoghurt storage period, this might be explained as a synergistic effect of the components of olive pomace. Crude extracts constantly having antimicrobial effects more than individual constituents due to synergistic effect of combination of all compounds in the extracts[46].

The present study demonstrated the antimicrobial potential of OPE. The results also indicate that OPE have antibacterial effect on the Gram-positive greater than Gram-negative bacteria. The observed inhibition of Gram-positive bacteria, *Staphylococcus aureus* and *Listeria monocytogenes*, suggested that OPE contains phytochemical compounds which possesses antibacterial activity that suppress the bacterial growth. So from the present results OPE could be used in the preparation of antimicrobial pharmaceuticals. OPE is able to be a good candidate as antimicrobial agent.

Total phenolic compounds content and antioxidant activity of yoghurt samples increased by the increment in the concentration of olive pomace extract. This ascribed to the high level of phenolic compounds in OPE as shown from HPLC results and in agreement with the results of Tsimidou and Papoti[47] and El and Karakaya[48]. They noticed that olive pomace had high amount of simple phenols and acids. Flavonoids may occur in appreciable amounts. The major phenolic compounds present in olive pomace are hydroxytyrosol, oleuropein and tyrosol[49], caffeic acid, p-coumaric acid, vanillic acid and rutin[4].

During cold storage, total phenolic contents and antioxidant activity increased gradually in yoghurt samples. Yoghurt samples supplemented with OPE contains high amount of total phenolic and free radical reducing activity during the storage period compared with control samples. This may be attributed to conversion of phenolic acids (ferulic acids) by lactic acid bacteria to other phenolic acids such as vanillic acid[50].

Water holding capacities of fresh yoghurt decreased slightly with increasing OPE concentration, while water holding capacity increased during storage. Water holding capacity of a protein gel is a crucial parameter in yoghurt industrialization, since it is related to syneresis, which is due to the intrinsic instability of gels. When the protein networks of yoghurt system shows low water holing capacity, syneresis occurs so, lower water holding capacity or whey separation is referring to a weakness of gel network[51].

Supplementation of yoghurt with OPE did not affect its texture until the 14th day, while decreased yoghurt texture with the increment of OPE concentration at the 21st day. This may be attributed to the moisture content of OPE at higher concentration which weakens the protein network resulting in a less firmness[52]. In yoghurt, the protein matrix consists of short interconnected chains; consequently the liquid phase of extract is immobilized in the interstitial spaces in the protein matrix[53]. Values of cohesiveness were relatively lower for yoghurt samples supplemented with OPE than in plain

yoghurt samples, this may due to the strength of protein-protein interaction bonds in control yoghurt rather that in the mixture of milk and OPE which weakened these phenomena. Also, noticed the same results in springiness data. Moreover, chewiness and gumminess of the yoghurt supplemented with OPE were reduced significantly rather than control yoghurt (% OPE).

Yoghurt supplemented with probiotics and OPE was evaluated for its anti-diabetic effect in type 2 diabetes induced in rats. The STZ was used for induction of diabetes in rats. The STZ is generally used for induction of type 1 and 2 diabetes in animals, STZ is preferentially toxic to pancreatic beta cells[54].

Diabetes mellitus is physiological condition associated with hyperglycemia and induce dysfunction and failure of various organs[55]. T2D is usually associated with bad dietary habits and reduction of physical activity. Hyperglycemia in diabetes is associated with insulin resistance[56].

In the present study, oral administration of yoghurt supplemented with probiotics and OPE improved plasma lipid profile, glucose, insulin, MDA, TAC and TNF-α of diabetic rats. Hyperglycemia and dyslipidemia observed in the present study lead to inflammation which reflects by elevation of TNF-α, in the meantime producing free radicals as seen through elevation of plasma MDA and reduction of TAC. For reduction of oxidative stress the body scavenging free radicals such as hydroxyl radical (OH) and Fe^{2+} [57]. As a result of oxidative stress and inflammation in diabetes can induce insulin resistance.

Plasma levels of creatinine and urea as indicator of kidney function were elevated in diabetic rats. Hyperglycemia can lead to long-term damage, dysfunction and failure of some organs such as kidneys[55] as shown in the present study.

This improvement in all the studied biochemical parameters may be attributed to the presence of phenolic compounds and probiotic in the yoghurt.

In diabetes phenolic compounds can suppress carbohydrate absorption through inhibition of amylase absorption[58]. Phenolic compounds modulate carbohydrate and lipid metabolism. These led to improvement in adipose tissue metabolism and alleviate oxidative stress and inflammation processes. Phenolic compounds prevent diabetes complications such as cardiovascular disease, neuropathy and retinopathy[57].

Probiotics may attenuate type 2 diabetes. One possible mechanism of *Lactobacillus casei* protection against diabetes may involve gut flora. *Lactobacillus casei* modified the gut flora-SCFA-inflammation/GLP-1 mechanism to ameliorate type 2 diabetes[59]. It was reported that probiotics supplementation showed significant improvement in hemoglobin A1c and fasting insulin in type 2 diabetic patients[6, 60-62]. Also probiotics intake significantly reduced total cholesterol and LDL-Ch levels[63,64] as observed in the present study. Feeding of *Lactobacillus* GG strains in rats showed significant delay in heightened glucose intolerance and hyperglycemic levels[65]. Probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* significantly delayed the progression of diabetes in the high-fat diet animals by showing biochemical changes[66]. Also Yun *et al.*[67] investigated the effect of *Lactobacillus gasseri* BNR17 on blood glucose levels and body weight in 6 week old male C57BL/KS/J db/db mice of type 2 diabetes.

CONCLUSION

The present study discovered that OPE possess antimicrobial and antioxidant activities. Yoghurt supplemented with probiotics and OPE showed anti-diabetic effect in type-2 diabetes. Yoghurt supplemented with OPE 1.5% was the most promising concentration in reducing type-2 diabetes complications in diabetic rats. Yoghurt as functional food containing phenolic compounds from olive pomace and probiotics may be used to attenuate the complications in type-2 diabetic patients.

SIGNIFICANCE STATEMENTS

This study confirmed that olive pomace as one of the agro industrial by-product rich in phenolic compounds which possess valuable biological activities for human health. It's so important to use this rich source of phenolic compounds in preparation of functional food to enhance human health and reduce the spread of non-communicable disease such as type-2 diabetes and its side effects to human health.

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