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Carob (*Ceratonia siliqua L.*) Supplementation Affects Kefir Quality and Antioxidant Capacity during Storage

Rosa Mahtout[1], Farid Zaidi[1], Linda Ould Saadi[1], Souhila Boudjou[1], B.Dave Oomah[2], Farah Hosseinian[3*]

[1]*Departement des Sciences Alimentaires, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie*

[2]*Formerly with the National Bioproducts and Bioprocesses Program, Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, BC, Canada V0H 1Z0*

[3]*Food Science and Nutrition Program, Chemistry Department, 1125 Colonel By Drive, Carleton University, Ottawa, Ontario, Canada, K1S 5B6*

**Corresponding author. Tel: 1-613-520-2600 x 2048; Fax: 1-613-520-3749.*

Abstract:

Carob (pod, pulp, seed) flours and crude pulp mucilage supplementation were investigated for their effects on kefir properties and antioxidant activity during 4 weeks cold (4 °C) storage. Carob flours differed in composition, mucilage yield, antioxidant activity and phenolic components analyzed by HPLC. Bacterial count increased during the first week of kefir storage with carob supplementation stimulating bacterial growth similar to inulin. Bacterial viability and titratable acidity of carob supplemented kefir increased, whereas pH decreased during storage. The antioxidant activity of kefirs generally increased during storage attaining maximum level at 14 days with the supplemented kefirs exhibiting higher activity compared to the control plain kefir.

Keywords *— **Ceratonia siliqua L.,* **Pod, Pulp, Seed, Mucilage, Prebiotic, Kefir, Antioxidant activity.***

I. INTRODUCTION

The carob tree (*Ceratonia siliqua* L.) pod was a traditional food that declined in consumption in the last decades in the Mediterranean countries. However, consumption of manufactured products from carob has increased due to extensive research on processing, utilization and health benefits of the pod and/or its components. The seed, an industrial source of gum (locust bean gum-LBG), can be refined by calibrated grinding into seed flour listed in the European food additive [1]. The seedless pod (pulp) ground into flour is used as a chocolate or cocoa substitute. Many health benefits have been attributed to carob pod, pulp and/or its fiber constituent. For example, the carob pulp powder has been recommended in the regimen of obese/overweight, hyperlipidemic and/or hypercholesterolemic diets due to its beneficial effects in the blood lipid profile and histopathology of the heart and kidney [2]. Carob pod exhibits potent antioxidant activity and its aqueous extract exerted gastro-protective effects against EtOH-induced oxidative stress, partly due to its antioxidant properties [3]. Fiber obtained from carob pod reduced total cholesterol levels in young (<30 years old) hypercholesterolemic adults when administered in a milk matrix (400 ml of dairy product containing 20 g of carob fiber for 4 weeks) [4]. Carob pod fiber also attenuates diet induced atherosclerosis by reducing endothelial function, inflammation and fibrosis in rabbits [5].

Locust bean gum sourced from carob seed is a globally approved food additive (as E 410 in Europe). However, its pricing fluctuates and has increased recently due to the poor carob pod harvest (20 % lower than average) in the Mediterranean and record low stock volumes [6]. Unfortunately, obesity and diabetes are widespread in this (Middle East and North Africa-MENA) region and consumers are inclined to revert to their traditional diets toward general wellbeing using functional foods and/or nutraceutical foods to meet nutritional deficiencies prevalent in the population [7]. In this regard, it is opportune to develop functional beverages and/or fortified dairy convenience foods containing carob and/or its products, thereby providing great potential for improving health and market opportunity of this region. Therefore, we investigated the benefits of supplementing kefir with carob pod, pulp, seed and crude pulp mucilage during cold storage.

The effects of carob products supplementation have previously been investigated in various dairy products. For example, carob juice concentrate supplementation reduced yoghurts pH, viable bacterial (*S. thermophilus* and *L. bulgaricus*) counts and increased titratable acidity during 14 days storage, with higher pH and no changes in titratable acidity at the end of the storage period compared to those of plain yoghurts [8]. Carob molasses also reduced set-type yoghurts pH and increased volatile fatty acid production with no significant changes in titratable acidity during storage [9]. Titratable acidity of low-fat yoghurts was also greater with high levels of locust bean gum (from carob seed) [10]. Furthermore, the high polyphenolic content of roasted carob pod powder reflected the antioxidant activity of carob-based milk beverages [11].

Addition of prebiotics or dietary fiber sources has variable effects on kefir quality during storage. Kefir pH is generally reduced and titratable acidity increased during refrigerated storage and not affected by inulin addition [12]. However, insignificant or no pH changes have been reported with inulin fortified or traditional kefir in other studies throughout 7 days storage [13] and references therein). The changes in Norwegian kefir properties were reported to be less pronounced during short- (0-4 weeks) than long-(4-8 weeks) term storage [14]. Various changes were observed in microbiological and chemical characteristics of Brazilian kefir during fermentation and storage [15]. Kefir changes its fatty acid profile during storage thereby increasing its antimutagenic, anticarcinogenic and nutritional potential [16]. These changes can lead to considerable benefits by improving human health and/or protect injury and diseases [17].

This study based on our previous investigations [18, 19] aims at evaluating the effects of carob pod, pulp, seed and crude pulp mucilage on kefir properties during 4 weeks cold (4 °C) storage. Inulin supplemented kefir was also included in this investigation to compare the effects of carob products with those of inulin, a commonly used prebiotics in human studies. This is the first study comparing carob pod, pulp and seed and their use in a new dairy matrix, kefir with considerable potential health benefits.

II. MATERIALS AND METHODS

Carobs were collected from trees growing naturally in Tazmalt, North of Algeria, (geographic location: latitude 36°38′ N, longitude 4°39′ E, altitude 309 m). The randomly selected samples were harvested during September 2011 and washed carefully; then separated into pod (whole pod with seed), pulp (without seed) and whole seed. The pods, pulp and seeds were ground in a mill (IKA A 11 basic; IKA Werke GmbH & Co. KG, Staufen, Germany) then sieved (Tap sieve shaker AS 200; Retsch GmbH, Haan, Germany) to pass a 500 µm screen. Ground samples (30 g) were stirred for 5 h at 20 °C with 600 mL of hexane, the solvent removed by vacuum filtration (Whatman No. 4), and the defatted residue air dried prior to storage in sealed plastic bags at -20 °C until analysis.

A. Proximate Composition

The contents of moisture, ash lipid, and crude protein (N x 6.25) of the ground samples were determined according to the AOAC procedures [20]. Moisture was determined by the oven drying

(105 °C) method, ash content by incineration in muffle furnace (550 °C), lipid by Soxhlet extraction for 6 h with petroleum ether, and nitrogen by combustion (N analyzer Flash EA 1122, Thermo Scientific, West Palm Beach, FL). Total available carbohydrates (TAC) were extracted using 0.8g sample and 15 mL of 52% (v/v) perchloric acid ($HClO_4$) [21]. TAC was determined by the Anthrone method.

B. Preparation of Crude Mucilage

Crude mucilage was extracted from ground defatted pulp, pod or seed (10 g) with double distilled water (400 mL), stirred for 3 h at 60ºC and cooled to room temperature. After centrifugation (4000g, 20 min; Sorvall Legend XTR, Thermo Scientific, Ashville, NC) the recovered supernatant corresponding to the crude mucilage was used for further analysis.

C. Extraction of Phenolics

Extraction of phenolics was performed according to the procedure described previously [22]. Ground samples (1 g) were extracted with aqueous acidified 85% methanol (15 1N HCl/85 MeOH, v/v) (20 mL) by constant magnetic stirring (RT15 power S1, IKA Werbe GmbH & Co. KG, Staufen, Germany) for 6 h at room temperature (23 °C). After centrifugation (1100g, 20 min; IEC HN-SII Centrifuge, International Equipment Company, Needham Heights, MA), the recovered supernatant was stored at -20 °C in the dark until analysis.

Phenolics were extracted from kefir according to previously described procedure [23] with some modifications. Briefly, kefir (1 g) was stirred (25 °C, 1h) with 10 mL of aqueous ethanol (80%, v/v) and filtered (Whatman No.4). The residue was re-extracted under the same conditions, the combined filtrates centrifuged (1100g, 10 min; IEC HN-SII Centrifuge, International Equipment Company, Needham Heights, MA), and the recovered supernatant stored at 4 °C prior to determination of antioxidant activity.

D. HPLC Analysis of Phenolic Acids

Chemicals (acetonitrile, formic acid) used for high-performance liquid chromatography (HPLC) were of chromatographic grade (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Analysis of phenolic acids in the methanol extracts was carried out on an HPLC (Alliance Waters 2695) system equipped with a photodiode array detector (PDA, Waters 2998), Empower software and auto sampler (Waters Corp., Milford, MA). The separation was carried out with a reversed-phase Synergi Max-RP column (250 mm × 4.6 mm, 4 µm particle size; Phenomenex, Torrance, CA). Chromatographic separation was carried out with 10 µL extract using two solvent systems: (A) Milli-Q water: formic acid (99.99:0.01, v/v) and (B) acetonitrile (100%) at 1mL/min and 30 °C. The gradient conditions were as follows: solvent B: 0 min, 90%; 35 min, 50%; 40 min, 90%; 50 min, 90%. The chromatograms were recorded in full scan mode over a wavelength range of 200-700 nm. Identification was achieved by comparing the retention times and spectra with authentic standards. Phenolics were quantified using gallic, protocatechuic, p-hydroxy benzoic, chlorogenic, caffeic, vanillic, syringic, *trans-p*-coumaric, synapic, ferulic and *o*-coumaric acids, myricetin, epicatechin gallate, quercetin 3β glucoside, rutin, (-)-epicatechin, (+)-catechin, pyrogallol, quercetin, apigenin and kaempherol (Sigma, St. Louis, MO) as standards.

E. Determination of Total Phenolic Content (TPC)

Total phenolic content was determined according to the Folin-Ciocalteu colorimetric assay following the method described previously [22]. An aliquot of sample extract (200 µL) was added to 1.9 mL of 10-fold distilled water diluted Folin-Ciocalteu reagent and 1.9 mL sodium carbonate solution (60 g/ L). The test tubes were allowed to stand in the dark at room temperature for 2 h. Absorbance was measured at 725 nm versus the prepared blank (distilled water) with spectrophotometer (Varian Cary 50 Bio UV/Vis, Varian, Mulgrave, Australia). Total phenolic content of samples were expressed as mg gallic acid equivalent per g of dry weight (mg GAE/ g DW) through the calibration curve

with gallic acid. All samples were analyzed in three replications.

F. Oxygen Radical Absorbance Capacity (ORAC)

Antioxidant activity was measured using the oxygen radical absorbance capacity (ORAC-$_{FL}$) described previously [24]. A FLx800™ Multi-Detection microplate fluorescence reader (BioTek Instruments, Ottawa, ON, Canada) was used with excitation and emission wavelength at 485 and 525 nm, respectively. Sample extracts and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid, Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) standards (20 µL) were transferred into a 96-well microplate (Fluotrac 200, Greiner Bio-One Inc., Longwood, FL). A peroxyl radical was generated by AAPH [2, 2′-azobis (2-methylpropionamide) dichloride] (Sigma-Aldrich, St. Louis, MO) after incubation (37 °C, 20 min) with 120 µL of fluorescein solution as substrate. Measurements were taken every 2 min for 60 min upon addition of AAPH. Final ORAC values were calculated using a regression equation between the Trolox concentration (0– 6 µg/mL) and the net area under the curve (AUC in µM Trolox equivalents (TE)/g sample) and expressed as µM Trolox equivalent per g sample.

G. Kefir Preparation

Freeze-dried starter kefir culture (kefir-type B-heterofermentative culture-without CO_2 production, containing *Lb. plantarum* and *Lb. rhamnosus*, *Lactoccocus lactis, L. cremoris*, and *Leuconostoc cremoris*) was purchased from Abiasa Inc., (Saint Hyacinthe, Quebec, Canada). The culture was diluted in pasteurized, homogenized (3.25% fat) milk (commercial source, Ottawa, ON), stirred for 15 min at 85°C, and then cooled to 42 °C. An aliquot was transferred to 50 mL sterile test tubes, and starter culture (kefir grain, 2 g/L of milk) were added to each tube. The carob flours (pulp, pod or seeds) (1.5 g; 3%, w/v) or of the liquid prebiotic solutions (10 mL) of inulin (3%) or crude pulp mucilage were added to the kefir samples. The kefir control consisted of the kefir grain diluted with 10 mL of double distilled water. Treatments were incubated at room temperature overnight and then stored at 4 °C for 28 days. Three replications were conducted for each treatment.

1) Microbiological Analyses

Bacterial enumerations were performed once a week during the storage period for a total of 4 weeks (1, 7, 14, 21, and 28 days) in triplicate. Each kefir treatment was serially diluted, a 100 µL aliquot plated on MRS agar (Sigma Aldrich Canada Ltd., Oakville, Ontario) as previously described [25], and incubated (37 °C, 48 h). The colony counts were converted to log cfu/ mL (log colony-forming units per mL).

2) Total Titratable Acidity (TTA) and pH

Titratable acidity was determined by titrating a mixture of kefir and sterile water (1:9, v/v) with 0.1 N NaOH using 0.1% (w/v) phenolphthalein indicator. The TTA values were reported as % lactic acid equivalent. The pH of the kefir samples was measured with a Denver Instrument UB-5 pH meter (Denver Instrument, Bohemia, New York, NY).

At least three determinations were made for all assays. Analysis of variance by the general linear models (GLM) procedure, means comparison by Duncan's test, and Pearson correlation were performed according to Statistical Analysis System, SAS 9.1 for windows [26].

III. RESULTS AND DISCUSSION

A. Chemical Composition

The seeds representing 10% of the pod had significantly ($P < 0.0001$) higher ash, protein and antioxidant activity (ORAC) and lower total carbohydrate content than those from the pod or pulp (Table I). The pulp (constituting 90% of the pod), with the lowest protein and lipid contents had the highest total phenolic concentration. Total phenolics of carob pulp was similar to those of the Portuguese cultivar Gasparinha (16.6 ± 1.2 mg GAE/g extract [DW]) that also had the highest inhibition of lipid peroxidation (ILP= 80.9 ± 16.6%) of all pulp extracts from carob trees [27].

The pod and pulp, often referred as deseeded pod did not differ significantly in ash, sugar and antioxidant activity. The higher carob seed protein and lower carbohydrate compared to the pod correspond to previous report [1]. The highest mucilage yield was obtained from the pod, followed by the pulp and lowest for the seed. Mucilage yield from the seed was within the range (27-33%) of locust bean gum extracted from carob seed from various regions of Tunisia [28]. Mucilage from the pod did not differ significantly in phenolic content and antioxidant activity than those from the pulp. The high phenolic content of the seed mucilage was reflected in its high ORAC value. Pearson correlation revealed that ash content was positively associated with protein content and inversely related to total carbohydrate content ($r = 0.962$ and -0.976, respectively; $P < 0.0001$, $n = 9$). This indicated that protein and carbohydrate contents were inversely related ($r = 0.995$; $P < 0.0001$). Antioxidant activity was positively ($r = 0.971$) correlated with ash and protein content and negatively ($r = -0.975$) associated with carbohydrate content ($P < 0.0001$). Total phenolics and antioxidant activity of mucilage were also inversely associated with carbohydrate content ($r = -0.917$; $P = 0.0005$ and -0.995; $P < 0.0001$, respectively).

TABLE I

CHEMICAL COMPOSITION OF CAROB FLOURS AND CRUDE MUCILAGE

	Concentration (g/ kg)							Mucilage		
Sample	Moisture	Ash	Protein	Lipid	TAC	Phenolics	ORAC	Yield	Phenolics	ORAC
Pod	9.48a	2.90b	4.97b	2.65a	69.03a	14.79b	194.7b	60.00a	25.44b	1424b
Pulp	8.40b	3.23b	3.28c	1.63c	68.18a	16.35a	215.8b	46.67ab	26.09b	1368b
Seed	9.44a	4.93a	25.33a	2.26b	30.09b	14.92b	455.9a	31.56b	49.37a	2418a

Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at 5% level. Mucilage yield is expressed in g/kg; total phenolics in g GAE/kg; TAC, total available carbohydrates; ORAC, oxygen radical absorbance capacity, in mmol trolox equivalent/kg.

B. HPLC Determination of Carob Pod, Pulp and Seed

Twenty one phenolic compounds were identified in carob extracts, with very low levels (< 0.15 mg/g) of apigenin, coumaric and vanillic acids (Table II). Vanillic acid was undetected in the pulp, while chlorogenic acid and apigenin were absent in the seeds. The seed generally had the highest content (17.23 mg/g) of phenolic components analyzed by HPLC except for chlorogenic, gallic and syringic acids, quercetin, kaempherol and apigenin. These phenolic compounds were most abundant in the pod with a total of 10.53 mg/g phenolic compounds. The pulp contained the least amount of phenolic compounds (8.62 mg/g) except chlorogenic, protocatecuic and gallic acids, catechin, myrecetin and apigenin. The gallic acid content of the pod was similar to those reported earlier (1.2 mg/g) [29], whereas the gallic and caffeic acid contents of the pulp were within the range of Portuguese carob cultivars [27]. Chlorogenic acid, gallic acid and rutin (23.9, 15.4 and 12% for the pulp and 20.7, 12.7 and 15.4 % of the pod total phenolics) comprised the major phenolic compounds accounting for 51.3 and 48.8% of the total pulp and pod phenolics, respectively. Pyrogallol, rutin, protocatecuic and *p*-hydroxy benzoic acids were predominant in the seed accounting for 20.8, 17.1, 12.1 and 10.9% of the total phenolic compound, respectively. The seed pyrogallol contributed over twice the amount (9.12%) recently reported for aqueous seed extract from Tunisian carob [30]. Phenolic acids constituted 72, 65 and 61%, whereas flavonoids accounted for 28, 35 and 39% of the total phenolic compounds for the pulp, pod and seed, respectively (Table II).

A. Microbial Analyses

The polynomial equations ($Y = -0.0033x^2+0.1023x+7.6059$, $r^2 = 0.939$; and $Y = -0.0032x^2+0.1033x+7.2959$, $r^2 = 0.803$) best described the total microbial evolution of the supplemented (inulin; K+IN) and control kefir (K), respectively (Fig. 1) during 28 days storage.

TABLE II

PHENOLIC COMPOUNDS (mg/g) OF CAROB PULP, POD AND SEED

Phenolic Compounds	Pulp	Pod	Seed
Phenolic acids			
Caffeic	0.28c	0.29b	0.78a
Chlorogenic	2.06b	2.18a	0c
Ferulic	0.15c	0.17b	0.21a
Gallic acid	1.33b	1.34a	0.84c
o-Coumaric	0.02c	0.03b	0.08a
p-Coumaric	0.00	0.014b	0.06a
p-Hydroxybenzoic	0.27c	0.32b	1.87a
Protocatechuic	1.52b	0.92c	2.09a
Pyrogallol	0.22c	0.94b	3.59a
Sinapic	0.02c	0.26b	0.53a
Syringic	0.08c	0.18a	0.14b
Vanillic	0c	0.002b	0.12a
Flavonoids			
Apigenin	0.04b	0.07a	0c
Catechin	0.24b	0.16c	0.98a
Epicatechin	0.25c	0.31b	0.49a
Epicatechin gallate	0.20c	0.25b	0.47a
Kaempherol	0.13b	0.15a	0.13b
Myricetin	0.22b	0.20c	0.23a
Quercetin	0.51c	0.80a	0.67b
Quercetin 3β-glucoside	0.04c	0.32b	1.01a
Rutin	1.03c	1.62b	2.94a

Means in a row followed by the same letter are not significantly different by Duncan's multiple range test at 5% level.

Bacterial count (7.3 – 7.5 log cfu/mL) on day 1 was significantly ($P < 0.001$) higher for supplemented kefir compared to the control plain kefir. Maximum/optimal increase (7.9 – 10.3%) in bacterial count occurred on day 7 compared to day 1, indicating significant bacterial growth in all kefir samples. The increase in bacterial count was minimal (0.1 – 2.3%) from day 7 to 14 followed thereafter by reduction in bacterial count to the end of the storage period. The linear decrease in bacterial count (day 14 – 28) of the kefir samples was in the following order: control (K) > K+MP > K+Pd = K+SD = K+P > K+IN. Carob pod (Pd), seed (SD), pulp (P) and crude pulp mucilage (MP) addition to kefir stimulated bacterial growth and activity similar to inulin (IN) supplementation suggesting similar prebiotic activity. Inulin addition maximally increased total bacterial counts probably due to improved survival of acetic acid bacteria during kefir storage [12]. A total bacterial count by the 28th day was similar for K+PD and K+SD (8.1 log cfu/mL) and for K+MP and K+IN (7.9 log cfu/mL). During refrigerated storage, total bacterial count of kefir was between 7.3 and 8.4 log cfu/mL.

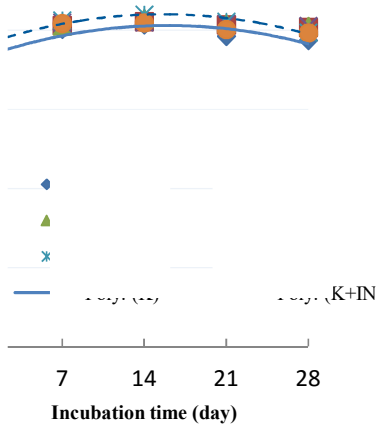

Fig. 1 Total microbial evolution of the supplemented (inulin; K+IN) and control kefir (K) during 28 days storage.

A. Total Titratable Acidity (%TTA) and pH

Lactic acid concentration, a measure of total titratable acidity, ranged from 0.75 to 0.87% at day 1 and did not differ significantly among kefirs (Table III). Furthermore, lactic acid concentration of inulin supplemented kefir at day 1 was similar to previously reported value [31]. At day 7, TTA decreased considerably with maximum reduction (41%) observed for the control plain kefir and 18-22% reduction for supplemented kefirs except carob seed. This contrast with previous studies

where TTA of kefir and inulin supplemented kefir was unaffected during 7 days storage [31]. In fact, TTA increased linearly ($Y = 0.0146x+0.7167$; $r^2 = 0.972$) for carob seed supplemented kefir during the whole storage period. This TTA increase was also reported for kefir fermented with grains during 28 days storage [12], but not with beverages fermented with starter kefir cultures. A similar linear increase in TTA was observed for carob pulp ($Y = 0.0099x+0.6$; $r^2 = 0.989$), pod ($Y = 0094x+0.6$; $r^2 = 0.835$) and inulin ($Y = 0.0111x+0.615$; $r^2 = 0.676$) supplemented kefir during storage from 7 – 28 days.

TTA linear increase also occurred from 14 – 28 days storage for the control kefir ($Y = 0.0043x+0.78$; $r^2 =1$) and for crude carob mucilage supplemented kefir ($Y = 0.0021x+0.785$; $r^2 = 0.75$). The divergent TTA profiles of carob supplemented kefirs probably reflect the amount of available carbohydrates that can be metabolized by lactic acid bacteria during storage.

TABLE III
TOTAL TITRATABLE ACID (TTA) OF DIFFERENT KEFIR FORMULATIONS DURING 28 DAYS STORAGE AT 4° C

Day	1	7	14	21	28
K	0.81a	0.48c	0.84abc	0.87b	0.90b
K+P	0.81a	0.66b	0.75cd	0.81bc	0.87b
K+Pd	0.87a	0.69b	0.72d	0.75c	0.90b
K+SD	0.75a	0.78a	0.93a	1.05a	1.11a
K+IN	0.81a	0.63b	0.87ab	0.84bc	0.90b
K+MP	0.78a	0.63b	0.81bcd	0.84bc	0.84b

Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level. K, kefir type B-heterofermentative culture; P, carob pulp; Pd, carob pod; SD, carob seed; IN, inulin; MP, carob pulp crude mucilage.

Kefir pH (similar [4.3] at day 1) decreased linearly ($r^2 \geq 0.86$) during storage (Table IV) with significant differences among all samples at 28 day storage. This indicated variable rate of pH reduction among the kefir samples. Thus, carob pulp and seed supplemented kefir induced the minimum and maximum pH reduction rate, respectively. Inulin supplementation did not affect the pH reduction rate of the control kefir during storage, confirming similar previous reports [12, 13]. The pH reduction and acidity increase of kefirs during refrigerated storage result from post-acidification induced by the ongoing lactose metabolization by the microbial cultures [12].

TABLE IV

pH OF DIFFERENT KEFIR FORMULATIONS DURING 28 DAYS OF STORAGE AT 4° C

Day	1	7	14	21	28
K	4.33a	4.20ab	4.00a	4.00a	3.47f
K+P	4.34a	4.18b	4.06a	3.89b	3.77a
K+Pd	4.31a	4.20ab	4.05a	3.79c	3.66b
K+SD	4.36a	4.21a	4.04a	3.68d	3.61c
K+IN	4.31a	4.12c	3.78b	3.80c	3.52e
K+MP	4.31a	4.06d	3.82b	3.78c	3.56d

Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level. Sample coding are described in Table III.

E. Antioxidant Activity, Oxygen Radical Absorbance Capacity (ORAC) of Kefir Samples

The antioxidant activity of kefirs generally increased during storage attaining maximum level at 14 days with the supplemented kefirs exhibiting higher activity compared to the control (Table V). The highest and least antioxidant activity was elicited by carob seed and crude pulp mucilage supplementation, respectively. Kefir supplemented with carob seed reflected its high antioxidant activity (Table I) and variability in amount and diversity of phenolic components (Table II) relative to the pulp and pod. Furthermore, the antioxidant activity profile of the control, carob seed and crude pulp mucilage supplemented kefirs followed/obeyed the polynomial response ($r^2 \geq 0.91$) with increasing storage time. Carob pulp supplemented kefir had the second highest antioxidant activity followed by carob pod, particularly during 14 – 28 days storage where their activity decreased linearly ($Y = -0.0626x+11.768$; $r^2 = 0.867$ and $Y = -0.1698x+10.282$; $r^2 = 0.969$ for carob pulp and pod, respectively). A linear increase ($Y =0.13x+5.2058$; $r^2 = 0.988$) was observed in antioxidant activity of inulin supplemented kefir during 1 – 21 days storage. At 21 days storage, kefir samples differed significantly ($P < 0.05$) in antioxidant activity from one another. Thus, the supplement dependent increase in antioxidant

activity of kefir was probably due predominantly to the polymeric phenolic degradation/mobilization by active kefir culture [32]. The increase in antioxidant activity of the control kefir was similar to those observed after 32 h incubation [33] and may be partly associated with the transfer of antioxidant components from kefir grains during storage.

The similar bacterial stimulation of carob and inulin supplemented kefir indicate that carob behavior simulates those of known prebiotics, although prebiotic activity was not investigated in this study. Furthermore, the superior antioxidant activity of the carob seed and pulp supplemented kefirs provide evidence of considerably more potent synbiotics relative to inulin supplemented kefir. In this regard, the carob seed and/or pulp supplemented kefir can potentially have beneficial effects similar to other synbiotic demonstrated on adult obesity and children with primary obesity [34].

TABLE V

OXYGEN RADICAL ABSORBANCE CAPACITY (mmol TROLOX EQUIVALENT/ kg) OF KEFIR SAMPLES.

Day	1	7	14	21	28
K	1.37c	2.03c	2.88d	2.15f	1.40e
K+P	6.57b	4.99c	10.79b	10.65b	9.92b
K+Pd	3.04c	3.69bc	8.03c	6.47d	5.65c
K+SD	16.63a	18.51a	19.89a	19.75a	18.19a
K+IN	5.46b	5.94b	7.03c	7.99c	5.73c
K+MP	1.70c	2.70c	3.35d	3.74e	3.49d

Means in a column followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level. Sample coding are described in Table III.

IV. CONCLUSION

To the best of our knowledge, this is the first study comparing carob pod, pulp and seed and their use in a new dairy matrix, kefir with considerable potential health benefits.

During 4 weeks storage, bacterial viability and titratable acidity of carob supplemented kefir increased, whereas pH decreased. The antioxidant activity of kefirs generally increased during storage attaining maximum level at 14 days with the supplemented kefirs exhibiting higher activity compared to the control plain kefir.

Therefore, carob flours/products can be considered efficient prebiotics and natural antioxidants in developing dairy beverages for the functional food and nutraceutical industries.

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