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ORIGINAL ARTICLE

# Impact of carob (*Ceratonia siliqua* L.) pulp flour supplementation on probiotic viability, milk fermentation and antioxidant capacity during yogurt storage

**Radia Arab [1 *], Christophe Hano [2], Dave Oomah [3], Fouzia Yous [1], Siham Ayouaz [1], Khodir Madani [4] and Lila Boulekbache-Makhlouf [1]**

1. Université de Bejaia. Faculté des Sciences de la Nature et de la Vie. Laboratoire de Biomathématiques, Biophysique, Biochimie, et Scientométrie (L3BS) ; 06000 Bejaia, Algeria. E-mail: radiaarab06@gmail.com / yousfou01@gmail.com / sihamayouaz@gmail.com / lilaboulekbachemakhlouf@yahoo.fr /
2. Department of Biological Chemistry, Orleans University, Eure et Loir Campus, 28000 Chartres, France. Bioactifs et Cosmétiques, CNRS GDR3711, CEDEX 2, 45067 Orléans, France. E-mail: hano@univ-orleans.fr
3. Formerly with Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, BC, V0H 1Z0, Canada. E-mail: oomahd@gmail.com
4. Université de Bejaia. Centre de recherche en technologie Agro-Alimentaire, 06000 Bejaia, Algeria. E-mail: madani28dz2002@gmail.com

## ABSTRACT

**Aim:** The aim of this study was to investigate the effect of carob pulp flour addition on probiotic viability, milk fermentation (pH, acidity and syneresis) and antioxidant activity, during yogurt cold storage (4 °C, 28 days). **Methods:** Four types of yogurts were prepared: plain yogurt (Y); yogurt with carob pulp flour (YC); yogurt with probiotic (YP) and yogurt with carob pulp flour and probiotic (YPC). **Results:** *Ceratonia siliqua* L. pulp flour supplementation (4% w/v) increased probiotic survival (15.96 %), titratable acidity (27.65 %) and syneresis (30.13 %). Carob pulp flour improved antioxidant activity for both DPPH (62.8 %) and iron chelating test (35.81 %), where yogurt containing probiotic and carob exhibited the highest antioxidant activity. Carob pulp flour had a selective effect on probiotic growth implying its prebiotic potential. Probiotic bacteria were viable and available at high concentration (> $10^{6}$ CFU/mL) at 28 day storage to sustain human health. **Conclusion**: Our results show that Algerian Carob can be considered as a very potential prebiotic, stimulating the growth of beneficial bacteria and exert strong antioxidant activity due to the presence of polyphenols.

## Article information

*Corresponding author: Radia Arab. E-mail: radiaarab06@gmail.com

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Yogurt, *Ceratonia siliqua* L., probiotic, acidity, antioxidant activity.

## 1 Introduction

It is well known that fermented dairy products, such as yogurt, are staple foods. In recent years, their consumption has grown strongly and steadily, with these products enjoying a strong nutritional image among consumers particularly with lactose intolerance. Yogurt is the most widely consumed fermented milk in the world, possessing a considerable nutritional value. Yogurt is obtained as a result of lactic fermentation of fresh/ reconstituted milk through the action of two lactic acid bacteria: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* [1]. Occasionally, yogurt can be strengthened by the addition of other ingredients such as prebiotics and probiotics. The beneficial effects of yogurt are correlated with the presence of viable alive microorganisms such as lactobacilli, streptococci, bifidobacteria or their combinations [2]. Probiotic are "Live microorganisms which when consumed in adequate amounts as part of food confer a health benefit on the host" [3]. Several studies have reported the beneficial intestinal health of probiotics including antimicrobial activity, improvement of lactose metabolism, cholesterol reduction, immune system stimulation, antimutagenic property, anti-diarrhea and reduction of *Helicobacter pylori* infection [4]. For example, the consumption of probiotic yogurt (300 g/day) containing *L. acidophilus La5* and *B. lactis Bb 12* displayed improvement of the antioxidant status and fasting blood glucose in Type 2 diabetes mellitus patients [5]. Prebiotics are non-digestible substances that induce a beneficial physiological effect on the host by specifically stimulating the growth and/or activity of a limited number of already established bacterial populations (microbiota) in the colon such as bifidobacteria. The probiotic effect of bifidobacteria depends on their survival not only in food but also in the gastrointestinal tract [4]. It is generally recommended that the consumption of probiotic yogurt should be greater than 100 g per day and that the yogurt should contain at least 106 CFU/mL [6].

Carob (*Ceratonia siliqua L.*) (family *Leguminosae*) is a typical tree of the Mediterranean climate, with high economic and ecological interests. Native to the Middle East, this species can grow as a shrub or tree [7] often with considerable longevity (up to 200 years); it produces fruits 5 or 6 years after plantation [8]. The mature fruit is made up of about 90% of pod and 10% of seed. The pods are composed of 40–60 % sugar, 3 to 6 % ash, protein (3–4%) and lipids (0.4–0.8%) and also contain a high amount of dietary fibers and polyphenols [9]. The carob pods are used in the production of animal feed and substitute for cocoa in human foods [9]. Pod meal has been used in human nutrition for centuries; i.e., to prepare homemade cakes. The seeds are utilized in food industry for their gum content (galactomannan). Carob possesses remarkable pharmacological properties and several beneficial effects on health such us laxative, antidiarrheal, cholesterol lowering activities [10], antibacterial, antifungal, antiproliferative and antioxidant properties in different in vitro test systems [11]. Furthermore, Carob pods contain a large amount of condensed tannins [12].

The use of phenolics in popular foods is a viable approach to increase the daily intake of this beneficial compound, therefore yogurts enhanced with value-added ingredients gain positive consumer perception and has been widely studied [13]. Due to their pharmacological values, abundance and low cost in Algeria, carob was selected for this study.

The purpose of the present study was to evaluate the effect of the presence of *Ceratonia ciliqua L* pulp flour on probiotic and starter culture viability, antioxidant activity (DPPH inhibition and Fe+2 chelating ability), changes of milk fermentation parameters (titratable acidity, pH, and syneresis) and phenolic content during 28 days of yogurt cold storage.

# 2 Materiel and Methods

## 2.1 Materials

Carob pods were harvested from trees in Taskriout, South of Bejaia, Algeria, (36°34′N latitude, 5°15′E longitude, and 841 M altitude) at the end of August, the period that corresponds to the total maturation of the pods. Microbial cultures used in this study were mixed of starter yogurt culture consisted of *Lactobacillus delbrueckii* ssp. *bulgaricus* (B-548; USDA) and *Streptococcus salivarius* ssp. *thermophilus* (14485; ATCC) (Yogotherm M 133, Abiasa Inc., Saint Hyacinthe, Quebec, Canada). The probiotic culture used is *Bifidobacterium animalis* ssp. *lactis*, BB-12, DSM15954, (CHR Hansen, France).

## 2.2 Carob powder preparation

The pods were washed with distilled water then manually husked to remove seeds. The pulp was air dried in a dryer ventilated and shaded place during 20 days at 25°C. After drying, the pulp was grounded (5 min, 28000 rpm) (IKA A11 basic, IKA Werke GmbH & Co. KG, Staufen, Germany) then sieved (Tap sieve shaker AS 200, Retsch GmbH, Haan, Germany) through a 500 µm screen. The humidity of the carob pulp flour was 5.6 % and it was stored in sealed plastic bags at -20 °C until the use.

## 2.3 Basic chemical determinations

Carob pulp flour was analyzed for chemical composition (moisture, protein, fat and ash) according to the AOAC methods, 1995. Moisture content was determined by the oven drying (105 °C, until the stabilization of the sample mass) method; the difference between the results of two last determinations was 0.1 g of moisture per 100 g of sample. Lipid by Soxhlet extraction for 6 hrs. with petroleum ether, ash by incineration of 1 g of sesame powder in a muffle furnace at 550 ° C for 5 hrs. and protein (% N x 6.25) by Kjeldahl method. Carbohydrates content was determined by the colorimetric phenol-sulphuric acid method [14].

## 2.4 Yogurt preparation

Milk (3 % fat) with 12 % total solids was purchased locally from a commercial source Candia (Bejaia, Algeria). The milk was pasteurized (90 °C, 15 min) on a stirring hot plate (Benchmark H 4000, Sayreville, New Jersey; USA) cooled to 42 °C and yogurt starter culture was added at (2g/L), stirred and poured into 50 mL plastic sterile cups. The initial concentration of starter culture was approximately 6.9 log CFU/ mL. Carob pulp flour was incorporated at 4% (w/v) simultaneously with probiotics (as specified by the provider) to reach the same dry matter content as the control (for samples without carob: Y and YP, 4 % milk powder was added so that all yoghurts were at 16 % w/w). This concentration was chosen based on a previous study with green lentil flour [15]. Freeze-dried culture of *B. animalis* ssp. *Lactis* ($10^8$ CFU/g) 2 g/100 g (w/w) was incorporated. The samples were vigorously shaken up such as to ensure efficient homogenization of the flour suspensions. Yogurt batches were prepared in triplicates and incubated for fermentation at 41°C, the fermentation was interrupted at pH 4.5 [16]. After fermentation, yogurt samples were stored at 4°C. The samples codification is presented in Table 1.

**Table 1.** Recipe of standard or control yogurt (Y), probiotic yogurt (YP), carob yogurt (YC) and carob probiotic yogurt (YPC)

| Yogurts | Total solid % | Lactic ferment (g/L) | Probiotic (g/ L) | Carob pulp % |
|---|---|---|---|---|
| Y | 16 | 2 | 0 | 0 |
| YP | 16 | 2 | 2 | 0 |
| YC | 16 | 2 | 0 | 4 |
| YPC | 16 | 2 | 2 | 4 |

## 2.5 Microbiological analyses

Colony counts were determined weekly for a total of four weeks. For enumeration of starter cultures, both of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarus* ssp. *thermophilus* were enumerated (37 °C, 48 hrs.) under aerobic incubation on M 17 Agar [17]. The probiotic was enumerated on MRS agar containing L-cysteine hydrochloride (0.5 g/L) and incubated (37° C, 48 hrs.) under anaerobic conditions with incubation the plates in jar of anaerobiosis [18]. Plates containing 30-300 colonies were enumerated and the results expressed as log colony forming units per milliliter (log CFU/ mL) using equation (1):

CFU= average number of colonies/plate factor x dilution factor ….. (1)

Where the plate and dilution factors refer to the amount of sample pipetted and the dilution series of the yogurt sample, respectively.

### pH and Total Titratable Acidity (TTA)

The pH values were measured with a pH meter 211 HANNA (Taufkirchen, Germany). In an Erlenmeyer flask, yogurt (1 mL) was mixed with distilled water (9 mL) with 3-5 drops of phenolphthalein 0.1 % (w/v). The titration was carried out with a solution of NaOH (0.1N) until the persistence of a pink color. The volume of NaOH used was then noted. The titratable acidity (TTA %) was expressed as a percentage and calculated according to equation (2):

$$TTA\ (\%) = V_{NaOH} \times 0.1\ N \times 100\ \% \times 0.009 \times 10\ (\text{dilution factor})$$ [19] ….. (2)

$V_{NaOH}$: Volume of NaOH in mL used for titration; 0.0090: correction factor of NaOH; 10: dilution factor.

## 2.6 Syneresis

The syneresis was estimated by the procedure proposed by Aprianita et al. [20]. Yogurt (10g) from different days storage was centrifuged (Bench-top centrifuge NF 200, Belgium) 700×g at 8 °C for 10 min. The clear supernatant was weighed, and syneresis was expressed as percent mass of supernatant relative to the original yogurt mass using equation (3):

Syneresis (%) = (mass of collected whey/weight of yogurt) × 100 ….. (3)

## 2.7 Analysis of total phenolic content

The extraction, for determining the total phenolics of carob pulp flour, was performed using the method of Oomah et al.[21], in case of yogurt samples, the extract was prepared using the method of Amirdivani & Baba [22].

The total phenolic content was determined as described previously [22]. Briefly, water carob extract or water yogurt extract (1 mL), from different storage days (1, 7, 14, 21, and 28), was mixed with 1 mL of 95% ethanol and 5 mL of distilled water. Folin–Ciocalteu reagent (0.5 mL) was added followed by thorough mixing. After 5 min, 5 % $Na_2CO_3$ (1 mL) was added and the reaction mixture was allowed to stand for 60 min at room temperature. The absorbance was measured at 725 nm and total phenolics expressed as µg of gallic acid equivalents per g of sample (µg GAE/g).

## 2.8 Antioxidant activity of yogurts

### 2.8.1 DPPH inhibition assay

First, water yogurt extracts were prepared using the method described by Amirdivani & Baba [22]. Yogurt (10 g) was mixed with 2.5 mL of distilled water; the mixture was stirred, and adjusted to pH 4 with HCl solution (0.1 M). The mixture was heated in a water bath (45 °C, 10 min) and centrifuged (5000 x g, 10 min, 4 °C) to remove precipitated proteins. The supernatant was adjusted to pH 7 with 0.1 M NaOH and centrifuged (5000 x g, 10 min) to remove residual proteins and salts then stored at -20 °C.

The antioxidant capacity of yogurt extracts was determined as described previously [16]. Yogurt water extract (250 µL) was mixed with ethanol solution of 60 µM DPPH (3 mL). The mixture was agitated vigorously and then incubated for 20 min at room temperature in the dark. The absorbance was measured at 517 nm. The readings were compared with the control which contained distilled water instead of yogurt extract. The percentage inhibition was determined using equation (4):

$$\%\ \text{inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$ ….. (4)

### 2.8.2 $Fe^{+2}$ chelating ability

The ability of the extracts to chelate iron (II) was determined according to the procedure of Ersoy et al. [23]. $Fe^{+2}$ chelating rate (FCR) was calculated using equation (5):

$$FCR\ (\%) = (A_{control} - A_{sample})/A_{control} \times 100$$ ….. (5)

## 2.9 Statistical analysis

Three determinations were made for all assays. Analysis of variance was performed by the general linear models (GLM) procedure, means comparison by Duncan's test, and Pearson correlation according to Statistical Analysis System, SAS 9.1 for Windows.

# 3 Results and Discussion

## 3.1 Basic chemical determinations

Moisture, ash, protein, fat, and carbohydrate contents of the carob pulp flour are shown in Table 2. The composition was similar to or within the range of carob flour data previously reported [24, 25]. The ash content was 3.66 %, protein 4.45 % and lipid 0.84 %. Carbohydrates constituted two third of the dry mass exceeding values found in the literature making it an appropriate energetic snack material. This high carbohydrate, low fat and low protein characteristics of the carob pulp have been reported by Ayaz et al. [26].

The carob pulp contain considerable amounts of dietary fiber and polyphenols (hydrolysable tannins, derived from gallic acid and condensed tannins, derived from flavan-3-ol, anthocyanidines, and flavan-3,4-diol) [8]. In investigated flour, total phenols reached 6.10 mg GAE/g DW. Our findings were in accordance with previous studies for carob species from different origin [27, 28]. The analyzed carob flour was characterized with high carbohydrate content, relatively moderate protein and ash content and low fat. Additionally, it is well established that carob is rich in polyphenols [24, 28].

**Table 2.** Proximate chemical composition of carob pulp flour

| Components | Concentration |
|---|---|
| Moisture (g/100 g of FW) | 12.17 ± 0.62 |
| Ash (g/100 g of FW) | 3.66 ± 0.09 |
| Proteins (g/100 g of FW) | 4.45 ± 0.12 |
| Total fat (g/100 g of FW) | 0.84 ± 0.02 |
| Carbohydrates (g/100 g of FW) | 65.64 ± 5.78 |
| Phenolics (mg GAE/g DW) | 6.10 ± 0.18 |

## 3.2 Microbial viability in yogurts during the cold storage

Probiotic growth was within 6.76 - 7.98 log CFU/mL range in the unsupplemented yogurt (YP), with optimum at day 14, and 7.25 to 8.26 log CFU/mL in the supplemented yogurt (YPC) with an optimum at day 7 (Figure 1). The viable cells in the first week increased by 1.08 and 1.07 log CFU/mL, which represent 13.77 and 12.22 % respectively. This implies that during the first week there was an availability of growth factors and nutrients. Bacterial count on day 1 was significantly higher for carob supplemented yogurt (YPC) compared to unsupplemented one (YP). The Carob significantly ($p < 0.05$) increased (2-7 %) probiotic (*B. animalis* ssp. *lactis*) counts demonstrating its prebiotic activity.

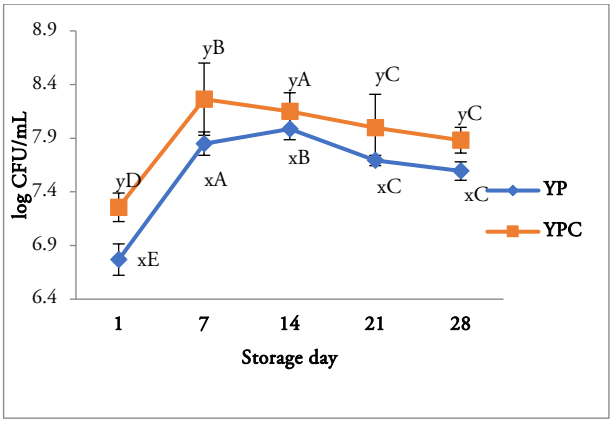


**Figure 1.** Bacterial viability of yogurts probiotics (anaerobic bacteria) over 28 days of storage at 4°C

Means with different lower and upper case letters are significantly different ($p < 0.05$).

Probiotic counts increased initially for the first week storage for yogurt with carob supplementation accelerating the increase rate, followed by a linear reduction during prolonged storage (7-28 days), (YPC = 8.405 - 0.0189 x; $r^2 = 0.998$).

Probiotic bacteria were viable at high concentration (> $10^6$ CFU/mL) at the end of the storage period to benefit human health [4]. The behavior of carob reflects those of its sugars that are unstable during storage due to sucrose inversion; fructose and glucose (readily usable carbohydrates) levels increase during the 1st week storage followed by glucose reduction, while fructose and sucrose levels remain almost constant up to 30 days storage [27].

The viability reduction/loss of probiotic organisms may be due to post-acidification or the acid produced during refrigerated storage and/or sensitivity to antimicrobial substances produced by yogurt bacteria [4]. However, *B. animalis* ssp. *Lactis* is known to withstand harsh conditions compared to other strains and hence its common use in probiotic yogurt. Nevertheless, phenolic compounds of carob pulp, tannins particularly, can exert antimicrobial effect reducing bacterial viability [29].

*B. animalis* ssp. *lactis* (Figure 1) exhibited good survival (≥ 90%) throughout the refrigerated storage period in the presence of yogurt starter culture except on the 1st day of storage (6.76 and 7.25 log CFU/mL for YP and YPC, respectively). Carob addition increased *B. animalis* ssp. *lactis* survival (relative to YP) in the presence of yogurt starter culture at the 7th day, it decreased thereafter to 7.88 log CFU/mL at the end of storage. Probiotic bacteria were able to maintain the recommended viable cell concentration ($10^6$ CFU/mL) until the end of the storage time.

Excellent viability of the microbial starter culture is the first and the most important criterion to ensure a good and

healthy quality of yogurt. Viability of yogurt bacteria was significantly different among yogurt samples, particularly after 14 days storage. Probiotic increased yogurt bacterial ($p < 0.001$) count by 1.06 log CFU/mL and generally maintained its stability during storage except for the viability surge at 21 days (6.94 log CFU/mL for Y and 7.1 log CFU/mL for YC). The highest viable bacteria numbers (8.58 and 8.21 log CFU/mL) of starter culture were in the samples with probiotic YP and YPC, over 28 days storage (Figure 2). Significant enhancement of *L. delbrueckii ssp. Bulgaricus* viability has been reported in the presence of a mixture of probiotic bacteria *Lactobacillus acidophilus, Bifidobacterium lactis and L. paracasei* in yogurt [30]. This can be explained by a competition between yogurt starter culture and probiotics for the carbon source by increasing the number of bacterial cells.

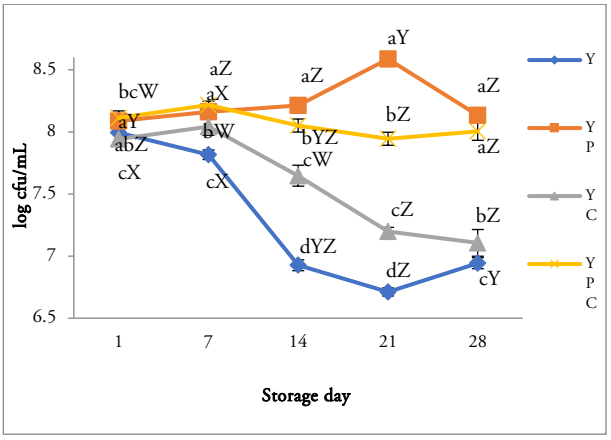


**Figure 2.** Bacterial viability of yogurts starter culture (aero anaerobic bacteria over 28 days storage at 4°C.

Means with different lower and upper case letters are significantly different ($p < 0.05$).

Viable counts of the starter culture decreased significantly in control yogurt after the first day storage (Figure 2). The control yogurt also displayed the highest reduction (1.05 log CFU/mL, 13 %) in yogurt bacteria among all samples during storage with viable counts below the satisfactory acceptable standard ~$10^7$ cfu/mL at the end of storage (28 days). Some researchers have observed an increasing of bacterial population for a few first days of refrigerated storage, and later reduction in the population [30].

Statistical analysis, revealed that addition of carob flour had no effect on numbers of *S. thermophilus* and *L. delbrueckii ssp. bulgaricus*, while the survival of *B. animalis* ssp. *lactis* was improved due to a possible prebiotic effects of carob carbohydrates (48-57 %) [31].

## 3.3 Post-acidification (pH) and titratable acidity (TA) in yogurts during cold storage

Yogurt pH was significantly affected by the presence of carob pulp flour ($p< 0.001$) and storage time ($p < 0.01$). After the first week of cold storage, pH values varied from 4.1 to 4.33. These value are lower than that found in yogurts enriched with various ingredients particularly pulses (lentils, faba beans, and chickpea) [15, 32, 33] and fruits [34, 35]. pH decreased on average from 4.33 to 3.62, whereas titratable acidity increased from 0.9 to 1.74%. The pH of control yogurt decreased during short storage (up to 14 days) and remained constant thereafter suggesting a buffering effect (Figure 3). Following fermentation, the pH of the yogurts was significantly more acidic in carob containing yogurts relative to yogurts without carob. The same observation has been reported from kefir added with carb flour and sesame seed yogurt [32]. Probiotic maintained pH of the control yogurt without the buffering effect. Changes in pH of control and probiotic yogurt occurred in two distinct steps with inversion point at 14 days storage. Rapid linear ($Y = 4.385 - 0.311$ x; $r^2 = 0.932$) pH reduction at the early stage (1-14 days storage) was followed by almost constant linear pH ($Y = 3.95 - 0.0014x$; $r^2 = 1$ and $YP = 4.0183 - 0.0064$ x; $r^2 = 0.907$; 14-28 days storage). Comparing acidification between Y and YC, carob addition significantly ($p< 0.05$) reduced pH of yogurt after 7 and 14 days indicating high acidification activity with or without probiotic. Probiotic had no effect on pH except a significant reduction at 28 days storage. In fact, the pH was identical in yogurts containing carob at all storage period with the lowest pH recorded at 4 weeks storage.

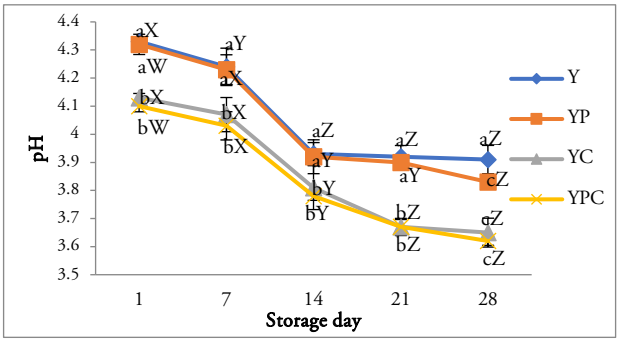


**Figure 3.** Post-acidification (pH) of yogurts over 28 days storage at 4°C

Means with different lower and upper case letters are significantly different ($p < 0.05$).

Control and probiotic yogurts had significantly lower titratable acidity than those containing carob (Figure 4). Probiotic significantly ($p < 0.0001$) increased titratable acidity of control yogurt, particularly at 7 days or longer storage, presumably due to acetic and lactic acids production. Moreover, carob addition

maintained titratable acidity during storage (≥ 7 days) of probiotic yogurt (prevented post acidification) confirming the gradual acid production over 20 days storage due to carob fermentation. The decline in TTA and simultaneous microbial growth during the first week of storage (d1 – d7) corresponds to the microbial community behavior, particularly the lactic acid and acetic acid bacteria. This finding was observed in the first week of Brazilian kefir fermentation [36].

Differences in titratable acidity were insignificant during storage except at 14 days for control yogurt (Y) and at the 1st day for YPC. Maximum titratable acidity was observed at 14 days storage for control and probiotic yogurts and at 21 days storage for carob yogurts. In fact, titratable acidity increased linearly up to 14 days storage for control and probiotic yogurt ($Y = 0.8678 + 0.0239\ x$; $r^2 = 0.993$ and $YP = 0.9463 + 0.0373\ x$; $r^2 = 0.953$) and up to 21 days storage for carob containing yogurts ($YC = 1.2173 + 0.0258\ x$; $r^2 = 0.978$ and $YPC = 1.3133 + 0.0209\ x$; $r^2 = 0.930$).

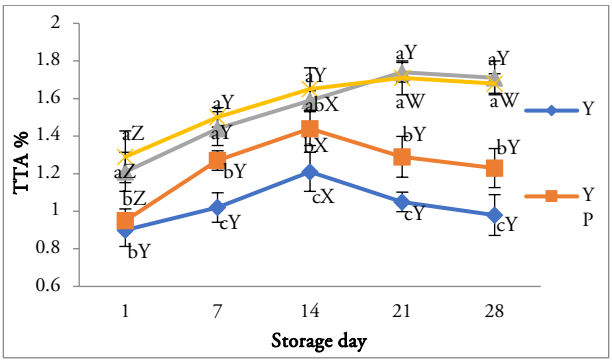


**Figure 4.** Total titratable acidity of yogurts over 28 days storage at 4°C

Means with different lower and upper case letters are significantly different ($p < 0.05$).

## 3.4 Syneresis of yogurts during cold storage

The syneresis or spontaneous whey separation on the surface of set yogurt is regarded as a defec. Syneresis of yogurt samples as affected by carob addition is shown in Figure 5. The susceptibility to syneresis was increased with carob addition compared to control and probiotic yogurts. However, differences in syneresis were insignificant between carob yogurts in the absence or presence of probiotic. At day 1, in our experimental conditions, the syneresis of the sample containing carob flour increased by about 33%, whereas samples prepared with probiotic culture decreased negligibly (~3 %). Some bifidobacteria strains are known to produce exopolysaccharides (EPS) thickening molecules able to retain water molecules. Indeed, yogurts containing EPS-producing cultures had better water holding capacity, which increased during storage and thereby reduced whey syneresis.

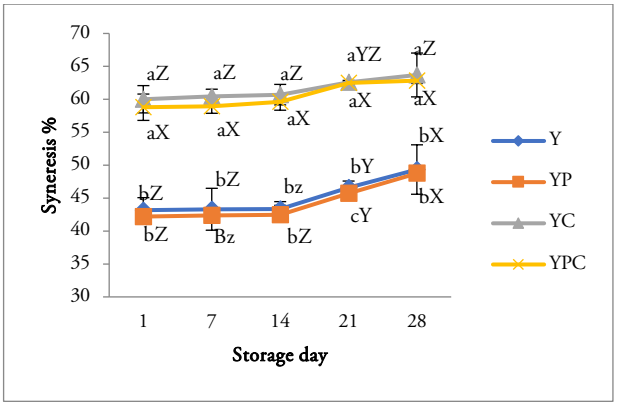


**Figure 5.** Syneresis of yogurts over 28 days of storage at 4°C

Means with different lower and upper case letters are significantly different ($p < 0.05$).

Storage linearly ($YC = 59.485 + 0.1408\ x$; $r^2 = 0.92$) increased syneresis of carob yogurt, although differences among storage days were insignificant. In contrast, storage had no significant effect on syneresis of carob supplemented probiotic yogurt (YPC). Syneresis of control and probiotic yogurt (YP) followed parallel biphasic trends during storage with inflection at 14 days storage. Prolonged storage (14-28 days) linearly ($Y = 37.438 + 0.4279\ x$ and $YP = 36.208 + 0.4493\ x$; $r^2 = 0.99$) increased syneresis of control and probiotic yogurt, respectively, presumably due to structure disintegration related to high proteolytic activity.

The syneresis trend of control and probiotic yogurt (Y and YP) paralleled changes in their pH and acidity during storage, thereby indicating their strong association.

Syneresis mainly occurs due to modification and disruption in the protein network of yogurt. Increase in syneresis is common in supplemented yogurts, in our samples it is due to the composition of carob flour low in proteins (4.45 %). To reduce this phenomenon in supplemented yogurts, industries use whey protein concentrates or caseins, or stabilizers and texture agents that increase viscosity such as polydextrose, starches, gums, gelatins or pectin [37].

## 3.5 Phenolic content (PC) of yogurts during cold storage

Phenolic content of water carob extract was 6.1 mg GAE/g dry weight before incorporation. Carob pods are reported to Our result agree with previous reports: 5.8 mg GAE/g dry matter [27], 0.19 to 9.28 mg GAE/g dry matter [38].

Phenolic content did not differ significantly between control and probiotic supplemented control yogurt and between the yogurts containing carob (Table 3). The presence of PC in unsupplemented yogurts can result from the phenolics linked to cow milk proteins or to the presence of other reducing

**Table 3. Phenolic content of yogurts over 28 days storage at 4°C**

| Samples | Storage time (days) | | | | |
|---|---|---|---|---|---|
| Yogurts | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
| Y | $27.94 \pm 0.36^{bV}$ | $25.58 \pm 0.61^{bW}$ | $23.53 \pm 0.13^{bX}$ | $21.19 \pm 0.38^{bY}$ | $20.09 \pm 0.26^{bZ}$ |
| YP | $27.46 \pm 0.86^{bV}$ | $25.10 \pm 1.03^{bW}$ | $22.74 \pm 1.12^{bX}$ | $20.83 \pm 0.36^{bY}$ | $19.41 \pm 0.21^{bZ}$ |
| YC | $309.12 \pm 10.3^{aY}$ | $307.56 \pm 10.1^{aYZ}$ | $304.75 \pm 16.13^{aYZ}$ | $301.63 \pm 20.13^{aYZ}$ | $298.51 \pm 18.3^{aZ}$ |
| YPC | $310.69 \pm 20.3^{aY}$ | $306.94 \pm 20.8^{aYZ}$ | $304.13 \pm 24.6^{aYZ}$ | $300.69 \pm 23.13^{aYZ}$ | $298.19 \pm 20.8^{aZ}$ |

Results are expressed in microgram equivalents of gallic acid per gram (µg GAE/g) sample. Means with different lower and upper case letters in the same column or row are significantly different ($p < 0.05$).

substances which respond to the photometric total phenolic estimation; indeed, tyrosine (with a phenolics side chain) was reported to elevate the reading in TPC measurement [4].

Carob flour increased phenolics content of control yogurt with or without probiotic; increasing linearly (Y = 11.086+0.1476 x; $r^2$ = 0.996) with storage time. Similar increase in phenolic content has been reported for mulberry fruit fortified yogurt compared to control [34]. Fortified yogurts with grape seed and garlic water extracts contained more total PC in all the samples compared to their controls [35]. Storage significantly ($p< 0.0001$) reduced phenolic contents of control and probiotic supplemented yogurts linearly (Y = 27.85 - 0.2946 x; $r^2$ = 0.983 and YP = 27.35 - 0.2987 x; $r^2$ = 0.985). At 28 day storage, PC reduction in the four yogurts varied between 3 to 29%. According to Oliveira et al. [34] in strawberry fortified yogurt, reduction in PC were due to their association with serum milk proteins such as β-lactoglobulin (β-LG) and α-lactalbumin. The phenolic reduction observed in our study contrast with the insignificant changes reported during yogurt storage with or without the presence of berries [39].

## 3.6 Antioxidant activity of yogurts during cold storage

DPPH antioxidant activity differed significantly ($p< 0.0001$) among yogurt samples with the carob probiotic (YPC) and control yogurts displaying the highest and lowest values, respectively (Figure 6). DPPH activity of control yogurt increased (3-13 %) with probiotic addition and only minimally (< 3 %) in the presence of carob. Carob containing yogurts exerted (~60 %) higher DPPH activity compared to those without as expected due to their higher phenolic contents. The ability of carob extracts to scavenge different radicals was previously reported *in vivo* and *in vitro* tests demonstrating high antioxidant potency [40]. Similar increase in DPPH scavenging activity ($EC_{50}$ 93 vs 59 mg/mL) was reported for yogurt fortified with aqueous ethanol (80 %) extract of carob kibbles [41]. Storage decreased DPPH activity linearly (Figure 6) with the highest and lowest reduction for YPC and control yogurts (~9 and 16 %, $DPPH_{[day\ 28\text{-}day\ 1]}$), respectively.

Carob yogurts expressed identical DPPH reduction rate during storage. Similar DPPH increase and reduction in its scavenging activity during storage (up to 8 weeks) was reported for berry fortified yogurt [39].

Generally, the percentage of DPPH radical inhibition in our samples was more elevated than that found in a yogurt with olive leaves phenolic extract [42]. The high antioxidant activity observed in our samples may be due to the mixed effect of phenolic compounds provided by the carob and organic acids formed by bacterial fermentation due to the richness of the carob in fermentable sugars which served as a carbon source to the bacteria.

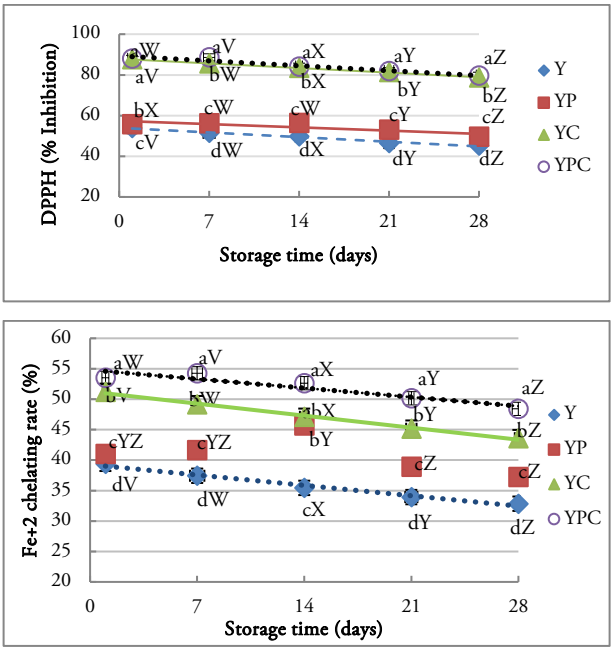


**Figure 6.** Antioxidants activity (DPPH & FCR) of yogurts over 28 days storage at 4°C

Means with different lower and upper case letters are significantly different ($p < 0.05$).

Iron chelating activity of yogurt mirrored their DPPH activity with the carob probiotic (YPC) and control yogurts displaying the highest and lowest values, respectively (Figure 6). The three yogurts (YP, YC, and YPC) had higher ($p < 0.05$) chelating rate than plain-yogurt, both at the end and throughout the storage period. At day 1, the highest antioxidant activity was recorded for YPC (53%) followed by YC (51%), YP (41%), and plain-yogurt Y (39%). Probiotic or carob addition increased iron chelating activity of control yogurt with carob inducing the highest (~30 %) increase. Similar increase in reducing power ($EC_{50}$ 39 vs 15 mg/mL) has been reported for yogurt fortified with aqueous ethanol (80 %) extract of carob kibbles [41]. Results of chelating rate showed that our probiotic manifested antioxidant activity; in this context that some probiotic strains (*Bifidobacterium bifidum* WBIN03 and *Lactobacillus plantarum* R315) exert important antioxidant potential due to their exopolysaccharides production [43].

Fe chelating activity differed significantly ($p \leq 0.002$) among yogurt samples and decreased during storage. This reduction was significant ($p < 0.0001$) and linear for carob and control yogurts ($Y = 39.261 - 0.2433\ x$; $r^2 = 0.981$; $YC = 51.251 - 0.2822\ x$; $r^2 = 0.995$ and $YPC = 54.8 - 0.212\ x$; $r^2 = 0.877$). The probiotic yogurt (YP) followed a dibasic trend (a small increase followed by a decrease) in Fe chelating activity during storage with an inflection at 14 days storage. The reduction in antioxidant potential of yogurts simples after 28 days cold storage owing to the destruction of the phenolic compounds during time with fermentation and the effect of yogurt bacteria [42].

### 3.7 Correlations

Correlation among variables showed that the yogurt starter culture viability was moderately associated with TTA and syneresis and highly related to phenolic content and antioxidant activities for all yogurts. As expected, pH was inversely related to TTA that in turn was highly associated with syneresis.

Post acidification (pH) was generally associated with bacterial viability, total phenolic content and antioxidant activities of yogurts, except probiotic yogurt (YP) (Table 4). Moreover, the pH of carob containing yogurts (YC-YPC) was highly and inversely correlated with their titratable acidity and syneresis, implying that changes in these factors were dependent on the presence of carob.

The high correlation of yogurts containing carob related to antioxidant activity is presumably due to carob's considerable natural antioxidant activity [24]. In contrast, probiotic supplementation (YP) nullifies/negates/ attenuates these effects probably due to the relatively high lactic acid production and reduced acidification attributed to probiotics (*Bifidobacterium* sp and/or *Lactobacillus acidophilus*) [44].

**Table 4.** Correlations coefficients for yogurt parameters

| | CFU | TTA | Syneresis | phenolics | DPPH | CFU |
|---|---|---|---|---|---|---|
| PH | | | | | | |
| Y | 0.976* | -0.529*** | -0.634ns | 0.902* | 0.872* | 0.926* |
| YP | -0.390ns | -0.635ns | -0.717** | 0.937* | 0.663** | 0.158ns |
| YC | 0.918* | -0.920* | -0.702** | 0.669*** | 0.936* | 0.946* |
| YPC | 0.749** | -0.780** | -0.674*** | 0.684** | 0.956* | 0.882* |
| YC-YPC[a] | 0.533** | -0.856* | -0.645* | 0.673* | 0.913* | 0.627** |

*, **, and *** correlations at $p < 0.0001$, <0.005, and <0.05, respectively, n=15; ns, not significant; [a] – n=30.

## 4 Conclusions

In this study, we confirm the notion that carob pod flour contains high levels of carbohydrates and phenolics, appreciable amount of proteins, and low levels of fat. Despite its nutritional value for human diet, yogurt, which is widely consumed, is not a source of phenolic compounds. The incorporation of carob flour can overcome this fact and improve effectively the antioxidant potential (62.8% for DPPH test and 35.81% for the chelating capacity test) of yogurts, with preserved functional value. Carob (*Ceratonia siliqua L.*) flour addition not only favored the growth of probiotic (15.96%) but also enhanced acidity (27.65%) of yogurt during cold storage. The enhanced antioxidant potential of carob-supplemented yogurt can benefit human health; this implies that carob can be a very effective alternative for probiotic dairy product concept and phenolic enrichment. Our probiotic yogurt-containing carob can be considered as a solution to increase consumption of dairy products with health benefits and possibly raising the nutritional level among the poorest people, knowing that carob kibbles are cheap. It was easy to obtain a fermented product prepared with cheap natural ingredient, having a desirable impact and nutritional value and high vitality.

**Author Contribution:** R.A. Conceptualization, methodology, writing—original draft and design of the work, drafted and undertook the literature research. D.O. statistical analysis and reviewed the manuscript; C.H. project administration and funding acquisition. Y.F; manuscript correction, S.A revised and corrected the manuscript. L.B and K.M. project administration.

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