Pulse ingredients supplementation affects kefir quality and antioxidant capacity during storage.

Linda Ould Saadi[1,3,4], Farid Zaidi[1], B. Dave Oomah[2], Monika Haros[3], Maria Jesus Yebra [3], Farah Hosseinian[4*]

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

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

[3] *Institute of Agrochemistry and Food Technology (IATA-CSIC) Valencia, Spain.*

[4]*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.

E-mail: farah_hosseinian@carleton.ca (F. Hosseinian).

Abstract

Changes in kefir storage (4 °C, 28 days) were evaluated every week in response to pulse (whole faba bean [*Vicia faba* L. *minor*] and its dehulled fractions – hulls and cotyledon; whole chickpea [*Cicer arietinum* L.)] and its crude mucilage) supplementation. Each supplement offered different profile of microbial count that was optimal at 14 days refrigerated storage. Bacterial growth was insignificant for faba bean hull and cotyledon supplemented kefirs between 7 – 21 days storage. Titratable acidity (TTA) of kefirs decreased for the first week then increased with increased storage time at different rates for each supplement. Kefir pH decreased linearly with storage time with significant differences observed among samples after 14 days storage. Inulin and other supplementations improved the production of Lactate and increased proteolytic activity with fermentation time. Antioxidant activity of kefir depended solely on the phenolic content and antioxidant activity of the supplements independent of storage time. Moreover, pulse supplements were superior to commercial inulin in maintaining kefir stability during refrigerated storage.

Keywords: Kefir, Antioxidant, Survival Bacteria, Proteolytic activity, Organic acid.

1. Introduction

Kefir is gaining prominence because of the numerous health benefits attributed to the prominent probiotic effects especially on gut health. Moreover, kefir can improve cardiovascular disease risk profile of young adults (18 – 24 yr) by attenuating C-reactive protein increase due to enhanced kefir digestibility resulting from lactose reduction by fermentation (O'Brien et al., 2015). Probiotic treatment also rescued neurogenesis and cognitive function in antibiotic treated mice by predominantly promoting progenitor cell survival in the brain (Möhle et al., 2016). These developments have spurred the food industry to invest in kefir beverages as the second generation of probiotic products. Thus, recent novelty in kefir is the new line of protein kefir drinks (20 g protein /8 oz serving) with reduced fat containing a combination of inulin and pectin as fat mimetics to bulk viscosity and increase satiety (Shelke, 2016). The veggie (beets, cucumber or tomato purees) kefir line took advantage of the vegetable's cellular matrices to reduce the amount of added sugar. Various protein-and polysaccharide-based ingredients have been developed to replace the physicochemical and sensory properties provided by fats. These ingredients, typically made of indigestible dietary fibers with relatively low-calorie contents can provide added health benefits and some are believed to induce greater satiety than fats(Shelke, 2016).

Pulses play important role in food and nutrition because of numerous health benefits and are being incorporated into many popular food categories. This promotes domestic demand of pulses as a strategy to contain the soaring healthcare costs, enhance long-term health outcomes and accelerate the nutritional improvements of industrial food products. Pulses have yet to make inroads into the probiotic food category due to limited research studies. For example, lactic acid fermentation has been successfully applied to pulse flours including faba bean and chickpea, resulting in reduced antinutritional compounds, increased free essential amino acids and improved *in vitro* protein digestibility (Coda et al., 2015). In yogurt production,

pulse ingredients including chickpea flour favored acidification by probiotic bacteria by improving lactobacilli growth (Zare, Champagne, Simpson, Orsat, & Boye, 2012).

Kefir is an excellent vehicle to deliver pulse ingredients to consumers; however, viability of probiotic organisms must be maintained within an appropriate shelf-life to be beneficial to health. Previously, we found that faba bean flour supplementation (4%) stimulated bifidogenic microbial growth, increased titratable acidity linearly from day 1 to 21, and reduced pH during kefir storage for 28 days (Boudjou, Zaidi, Hosseinian, & Oomah, 2014). Subsequent studies with air-classified faba bean fractions demonstrated more efficient *Lactobacillus plantarum* growth in the starch rich than in the fiber fraction; protein enriched fraction exerted the highest lactic acid and acetic acid production and TTA indicating strong buffering capacity (Coda et al., 2015). Our investigation therefore aimed at evaluating the effects of supplementing whole faba bean flour, its cotyledon and hull fractions, chickpea flour and its mucilage on kefir stability during refrigerated storage for 28 days. Chickpea mucilage was included in the study because water-soluble polysaccharide extracted from chickpea flour has been reported to display good anti-hypertensive activities and can be used as a thickening or functional agent in food systems (Mokni Ghribi et al., 2015). Inulin was also included in our investigation since it has been extensively studied, granted blood glucose claim in Europe and provides the best evidence of prebiotic effects in human (Crane, 2016). The development of pulse-based kefir is contingent on demonstrating the prebiotic effects of the pulse ingredient/s relative to commercially available prebiotic such as inulin, the capacity of these ingredients to maintain their prebiotic effect during storage and enhance other bioactivities that can confer additional human health benefits.

2. Materials and method

Faba bean (*Vicia faba* L.) subspecies *minor* and chickpea (*Cicer arietinum* L.) samples were from Skikda and Oeud Amizour Wilaya of Bejaia, Algeria, respectively. Faba bean seeds were cleaned, air dried, and manually separated into hulls and cotyledons. All samples were initially crushed in a traditional stone mill followed by an electric coffee mill (Moulinex, France) then sieved (Tap sieve shaker AS 200; Retsch GmbH, Haan, Germany) to pass a 500 µm screen. The powders were stored in the fridge in sealed plastic bags until analysis.

2.1. Chickpea crude mucilage extraction

Ground chickpea was extracted with distilled water (1:40, w/v), stirred for 3 h at 60 °C, extracts allowed to cool to room temperature then centrifuged (4000*g*, 20 min; Sorvall Legend XTR centrifuge, Thermo Scientific, Ashville, NC, USA). The supernatant was considered as the crude mucilage and used for further analysis. *2.2. Phenolic extraction and analysis*

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Phenolics were extracted with 95% acidified (1N HCl) methanol as described previously (Hosseinian & Mazza, 2009). Briefly, defatted samples (1 g) were extracted with methanol (20 ml) by magnetic stirring for 6 h at room temperature. The extract was centrifuged (4000g, 15 min; Sorvall Legend XTR, Thermo Scientific, Ashville, NC), the supernatant recovered and stored in the fridge until analysis.

Total phenolics of the methanol extracts were determined by the Folin-Ciocalteau method (Singleton & Rossi, 1965). Absorbance of samples and gallic acid standards (0 – 0.9 mg/ml prepared in 80% ethanol) was monitored at 725 nm (Cary 50 Bio UV-visible Spectrophotometer, Varian, Mulgrave, Australia). Samples were analyzed in triplicates and results expressed in mg gallic acid equivalents (GAE)/g sample.

The $AlCl_3$ method (Lamaison & Carnet, 1990) was used for determination of total flavonoid content of the methanol extracts. Aliquots (2 ml) of extracts were added to equal

92 volumes of a solution of 2% $AlCl_3.6H_2O$ (2 g/100 ml methanol). The mixture was vigorously
93 shaken, and absorbance was monitored at 430 nm after 15 min incubation using quercetin (0–
94 0.013 mg/ml in 80% ethanol) as standard. Flavonoid content was expressed in mg quercetin
95 equivalents/g sample.

96 *2.3. HPLC analysis of phenolic compounds*

97 Chemicals (acetonitrile, formic acid) used for high-performance liquid chromatography
98 (HPLC) were of chromatographic grade (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada).
99 Analysis of phenolic acids in the methanol-HCl extracts of legume powders was carried out on
100 an HPLC (Alliance Waters 2695) system equipped with photodiode array detector (PDA,
101 Waters 2998), Empower software, and auto sampler (Waters Corp., Milford, MA). The
102 separation was carried out with an Atlantis RT3 column (150 mm x 4.6 mm, 5 μm particle size;
103 Waters, Milford, MA). Chromatographic separation was carried out with 10 μl extract using
104 two solvent systems: (A) water: formic acid (99.99:0.01, v/v) and (B) acetonitrile 100% at 1.23
105 ml/min and 30 °C. The gradient conditions were as follows: solvent B: 0 min, 10%; 35 min,
106 50%; 40 min, 90%. The chromatograms were recorded at 254, 280, 320 and 520 nm for phenolic
107 acids and flavonoids, respectively. Phenolics were quantified using authentic commercial
108 compounds supplied by Sigma Aldrich Chemicals (St.Louis, MO, USA). Concentration of
109 phenolic compounds were determined from the average of three replicate chromatograms and
110 expressed in mg/g sample.

111 *2.4. Oxygen radical absorbance capacity (ORAC)*

112 Antioxidant activity was measured using the radical absorbance capacity ($ORAC_{FL}$)
113 described previously (Agil & Hosseinian, 2012), according to established procedure (Prior et
114 al., 2003). A multi-detection microplate fluorescence reader (BioTek Instruments, Ottawa, ON,
115 Canada) was used with excitation and emission wavelengths at 485 and 525 nm, respectively.
116 Sample extracts and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-

117 Aldrich Canada Ltd., Oakville, ON, Canada) standards were diluted with 75 mM phosphate
118 buffer (pH 7.4) prior to transfer into a 96-well microplate (Fluotrac 200, Greiner Bio-One Inc.,
119 Longwood, FL). A peroxyl radical was generated by AAPH [2,2′-azobis (2-
120 methylpropionamide) dichloride] (Sigma-Aldrich, St. Louis, MO) during measurement, and
121 fluoresceine was used as the substrate. Measurements were taken after 60 min at 37 °C upon
122 addition of AAPH. Final ORAC values were calculated using a regression between the Trolox
123 concentration (0-6 μg/ml) and the net area under the curve and expressed as μM Trolox
124 equivalents (TE)/g sample.

125 For kefir, samples (1 ml) were extracted (25 °C, 1 h) with 80% aqueous ethanol (10 ml),
126 filtered (Whatman No.4), and the residue re-extracted (10 ml 80% aqueous ethanol). The
127 combined extracts were centrifuged (4000g, 10 min; Sorvall Legend XTR centrifuge, Thermo
128 Scientific, Ashville, NC, USA), and the supernatant used for ORAC analysis.

129 *2.5. Kefir preparation*

130 The freeze-dried starter kefir culture (kefir type B-heterofermentative culture-without
131 production of CO_2 containing *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactoccocus*
132 *lactis* subspecies *lactis* and *cremosis* and *Leuconostoc cremosis* (Abiasa Inc., Saint Hyacinthe,
133 Quebec, Canada) was used in this study. The culture was diluted in pasteurized, homogenized
134 (3.25% fat) milk purchased locally from a commercial source (Ottawa, ON, Canada), stirred at
135 85 °C for 15 min, portioned into sterile conical tubes (50 ml), and cooled to 42 °C (Espírito
136 Santo et al., 2010). Seven treatments were prepared containing three faba bean (whole,
137 cotyledon and hull), and chickpea flours (1.5g; 3%, w/v), chickpea mucilage, inulin (10 ml
138 added to 40 ml milk) and the control without any additives. The inoculated milk samples were
139 prepared in triplicate, incubated overnight at room temperature and stored refrigerated (4 °C)
140 for 28 days.

141

142 *2.5.1. Physicochemical and Microbiological analysis*

143 The Kefirs were subjected to physicochemical and microbiological analysis, using
144 methodologies published elsewhere being easily available: pH, TTA, the bacterial
145 enumerations were carried out once a week for a total of 4 weeks (1, 7, 14, 21, and 28 days) in
146 triplicate for each batch at different dilutions (four serial dilutions of 1/10). From each dilution,
147 a 100 µl aliquot was plated on MRS agar (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada)
148 as described previously (Espírito Santo et al., 2010), incubated (37 °C, 24 h), and colony counts
149 converted to log cfu/ml.

150 *2.5.2. Determination of proteolytic activity*

151 Proteolytics activitis of starter culture of Kefir with differentes formulation assessed by
152 measuring liberated amino acids and peptides using the O- phthaldialfdehyde (OPA) methode
153 (Donkar, Nilmini, Stolic, Vasiljevic & Shah, 2007). The protelytic activity was expressed as
154 the absorbance of OPA derivatives at 340nm.

155 *2.5.3. Determination of organic acids*

Determination of lactic, acetic, butyric and propionic acids was carried out using the method described previously (Sarmiento-Rubiano, Zúñiga, Pérez-Martínez, & Yebra, 2007). Briefly, 1 ml of kefir samples from different days of storage (1, 7, 14, 21, and 28 days) were resuspended in 9 ml of phosphoric acid (0.1%, pH2.4), vortexed and centrifuged at 9000g for 20 min at 2°C. Supernatants were filtred throught Einmalfilter 0.45µm filter unit (chromafil, Germany) and injected in HPLC (Jasco Corporation, Japan) with a UV detector at 210 nm. Samples were loaded in a Rezex ROA Organic Acid column (Phenomenex,USA) placed at 30°C and phosphoric acid 0.1%, pH 2.4, was used as the mobile phase in isocratic conditions.

156 At least three determinations were made for all assays. Analysis of variance by the
157 general linear models (GLM) procedure, means comparison by Duncan's test, Pearson

158 correlation, and variance component analysis (VARCOMP) were performed according to
159 Statistical Analysis System, SAS 9.1 for Windows (SAS Institute Inc, 1990).

160 ## *3.* **Results and Discussion**

161 ### *3.1. Phenolics and antioxidant activity*

162 The hull and cotyledon represented 14.2% and 85.1% (w/w) of Vicia faba minor,
163 respectively in accordance with our previous study(Boudjou, Oomah, Zaidi, & Hosseinian,
164 2013). Faba bean hulls and cotyledons had the highest and lowest concentration, respectively
165 of total phenolics and flavonoids (Table 1) exhibiting the highest antioxidant activity among
166 the faba bean fractions. Total phenolic content of faba bean hulls were four times those in the
167 whole seeds and nine-fold their concentration in cotyledons and within the range reported for
168 Tunisian faba bean hulls (Chaieb, González, López-Mesas, Bouslama, & Valiente, 2011).
169 Flavonoids also concentrated in the hulls with over fivefold the content in whole faba beans.
170 These trends were similar to those reported earlier for acetone extracts of faba bean fractions
171 (Boudjou et al., 2013). Antioxidant activity of the whole faba bean was within the range of
172 those reported for Canadian genotypes(Oomah et al., 2011). Whole chickpea displayed the
173 lowest total phenolic content, although its flavonoid and ORAC values were not significantly
174 different from those of faba bean cotyledons. However, our results for chickpea phenolics and
175 antioxidants were higher than those reported previously (Xu & Chang, 2007). The yield of
176 aqueous extract from chickpea-hereby considered as mucilage was 28.4%. It had twice the
177 flavonoid content of whole chickpea and exhibited the lowest antioxidant activity (Table 1).
178 The antioxidant activity of the samples correlated significantly ($r2 = 0.899$ and 0.874; $P <$
179 0.0001) with total phenolic and flavonoid contents, respectively.
180 The phenolic HPLC profile of whole faba beans and cotyledon was closely related with the
181 latter devoid of three flavonoids: epicatechin, quercetin and quercetin 3β-glucoside (Table 2).
182 This is similar to closely associated phenolic pattern of faba bean cotyledon and whole seeds

183 reported earlier (Bekkara, Jay, Viricel, & Rome, 1998). The cotyledon generally had
184 significantly ($P < 0.05$) higher phenolic acids, except gallic acid, but lower flavonoids than the
185 whole faba bean seeds. Faba bean hull was the richest flavonoid source due to its high catechin,
186 epicatechin and rutin content; it also had the highest gallic and protocatechuic acids and
187 pyrogallol in addition to being the only sample containing coumaric and hydroxybenzoic acids
188 and kaempferol. The highest flavonoid concentration of TF (Table 2) corresponded with the
189 highest content observed among all samples (Table 1). High concentrations of chlorogenic acids
190 were present in faba bean cotyledon and seed, representing almost 50% of the total phenolic
191 acids. The presence of catechin, epicatechin, and quercetin in faba bean is consistent with
192 previous report (Baginsky et al., 2013). Flavonoid concentration was over two and half times
193 higher than those of phenolic acids in chickpea due to the high quercetin and myricetin contents.
194 Pyrogallol and epicatechin were the only phenolic compounds identified in chickpea mucilage.
195 Quercetin, myricetin, gallic and vanillic acids representing major chickpea phenolic
196 constituents have also been previously reported (Sreerama, Sashikala, & Pratape, 2010).

197 *3.2. Physicochemical and Microbial analysis*

198 Two factors: supplement (samples) and storage time were studied to further elucidate the
199 variability in kefir storage parameters. Variance component analysis revealed that storage time
200 predominantly contributed to the variation in microbial growth (73%), TTA (73%) and pH
201 (97%) (fig.2). The optimal (8.1 – 8.7 log cfu/ml) and minimal (7.3 – 7.7 log cfu/ml) microbial
202 count occurred on storage days 14 and 1, respectively for all kefirs (Fig. 1). Titratable acidity
203 (Fig. 2a) was found to be highly inversely correlated ($r = -0.789$ to -0.868; $P < 0.0005$) with pH
204 (Fig. 2b) for all faba bean supplemented kefirs and chickpea supplemented kefir suggesting that
205 pH reduction favored acidification probably resulting from amino acid release through protein
206 hydrolysis (Baik & Han, 2012; Coda et al., 2015).

207 This study confirmed our earlier report(Boudjou et al., 2014) that faba bean supplementation
208 maintains cell viability during extended kefir storage. The rapid TTA decline and simultaneous
209 microbial growth during the first week of storage (d1 –d7) corresponds to the behavior of
210 different microbial community, particularly the lactic acid and acetic acid bacteria found in
211 Brazilian kefir (Leite et al., 2013). Faba bean hull and cotyledons, in particular, maintained
212 microbial stability during 7 – 21 days kefir storage. Furthermore, the highest TTA increase
213 during faba bean cotyledon supplemented kefir storage infers that its highly fermentable
214 raffinose family oligosaccharides (Quemener, 1988) were readily available to kefir
215 microorganisms. The higher microbial counts of legume-supplemented kefir demonstrate their
216 superior prebiotic effect compared to inulin during refrigerated storage. The superior
217 performance of faba bean and chickpea compared to inulin supplementation during kefir storage
218 suggests that these legumes provide an opportunity for their development and use as prebiotic
219 s similar to those of the well-established inulin.
220 The high microbial count (≥ 7.4 log cfu/ml) after 28 days kefir storage was above the
221 recommended level (≥ 6 – 7 log cfu/ml) required for probiotic food suggesting that the
222 supplemented kefirs can exert the probiotic health benefits to the host and therefore applicable
223 for health claim (Matejčeková, Liptáková, & Valík, 2017)
224 *3.3. Proteolytic activity of starter culture of Kefir*
225 Proteolytic activity varied significantly ($P < 0.0001$) among kefir samples, storage time and
226 their interactions. However, storage time accounted for the highest total variation (57%)
227 compared with kefir treatments and their interactions (17 and 18%, respectively). The mean
228 (average) proteolytic activity of kefirs increased linearly ($r = 0.99$) with storage time (1-21 days)
229 (Table 3). This linear increase ($r \geq 0.96$) was also observed for whole faba bean, faba bean hull,
230 chickpea and chickpea mucilage supplemented kefirs during storage (1-14 days). Proteolytic
231 activity of chickpea mucilage supplemented kefir was not significantly different from the

control kefir during storage, except at the 14th day. Similarly, whole faba bean, faba bean cotyledon and chickpea flour supplemented kefir did not differ significantly in proteolytic activity during storage with few exceptions. Faba bean hull supplemented kefir had proteolytic activity not significantly different than those with inulin during 1-14 days storage suggesting similar prebiotic effect in releasing peptides and amino acids associated with proteolytic activity (Ramchandran & Shah, 2010).

3.4.Production of organic acids

The analysis of variance for organic acids showed that kefir samples (treatments), storage time and their interactions were all highly significant ($P < 0.0001$). The variance in lactic acid was predominantly associated with kefir treatment (49% of the total variation), higher than that of storage time (29.7%) and their interaction (15.7%). The variance associated with kefir treatment, storage time and their interactions were 14.3, 58.9 and 18.1% (of the total variation), respectively for butyric acid; and 39.6, 38.2, and 21.8% (of total variation), respectively for acetic acid. Kefir treatment, storage and their interaction had no significant effect on the variability of propionic acid, since their variance (25, 20 and 10% of the overall variation, respectively) was smaller than that of the experimental error (44.4%).

Chickpea and whole faba bean flour supplementation produced the highest amount of lactic and butyric acids at 14 days storage. Acetic acid was highest with chickpea flour (1-14 days storage), although consistently high concentration occurred with faba bean hull supplementation. Chickpea mucilage generally produced lower amounts of organic acids than the chickpea flour. Acetic acid production was induced by faba bean hulls since the cotyledons had the lowest concentration (days 7 and 14) or levels similar to the control kefir (days 1 and 21). Kefir containing faba bean hull and whole faba bean flour had the highest and lowest propionic acid content (21 day storage), respectively; although differences among kefir

257 treatments were generally not significant. Lactic acid production decreased linearly (r = ≥ 0.91)
258 with storage time (7-28 days) for the control kefir and kefir supplemented with inulin, faba bean
259 cotyledons and chickpea mucilage; increased linearly (r = 0.997) with storage time (1-14 days)
260 for whole faba bean flour and remained unchanged during storage (1-21 days) for faba bean
261 hulls (Table 4). Acetic acid content increased linearly (r = ≥ 0.94) with storage (1-14 days) for
262 kefir supplemented with chickpea and faba bean cotyledon flours and decreased linearly ((r =
263 ≥ 0.95) with storage for inulin (7-28 days) and whole faba bean flour (14-28 days). Butyric acid
264 also increased linearly (r = ≥ 0.94) with storage (1-14 days) for the control kefir and the
265 chickpea and faba bean hull supplemented kefirs.
266 The molar ratios of the SCFAs, acetic, propionic and butyric acids changed during storage
267 reflecting differences in kefir microbiota; the change was highly dependent on the prebiotic
268 supplement and occurred often on the 21st day. The molar ratio of acetate decreased (64 to 50%)
269 with concomitant increase in butyrate (27 to 38%) and propionate (9 to 12%) during storage (1-
270 28 days) of the control kefir. These changes may be due to the significant decrease in
271 *L.acidophilus* and *Bifidobacterium* sp. reported during cold storage of kefir for 21 days (Kök-
272 Taş, Seydim, Ozer, & Guzel-Seydim, 2013). Whole faba bean flour supplementation induced
273 the greatest changes in molar ratios of acetate: propionate: butyrate (from 70:7:23 [day 1] to
274 45:8:47 day 28]) during storage, whereas minimal changes in SCFAs molar ratio occurred with
275 chickpea flour supplementation. Changes in SCFAs molar ratio were similar for inulin (from
276 72:6:22 to 63:7:30) and chickpea mucilage (from 70:8:22 to 59:12:28z) supplemented kefir
277 during storage suggesting the significant beneficial effect on the viability of *bifidobacteria*
278 observed after 28 days of refrigerated storage of fermented milk (Varga, Szigeti, & Gyenis,
279 2006). Faba bean hull was the only treatment that increased acetic acid and concomitantly
280 decreased propionic and butyric acids ratios (from 50:13:37 to 77:5:18; difference between the
281 1st and 28th storage days), although the molar ratios remained almost constant (7 to 21 days

storage). However, butyrate yield from faba bean hull was significantly higher than the control kefir during storage (1-14 days) indicating the potential beneficial effects of this natural fiber. In fact, faba bean hull supplemented kefir displayed the overall highest SCFAs production among all samples.

3.5. Kefir (ORAC) antioxidant activity

Storage time had minimal effects on kefir antioxidant activity, except for inulin supplemented kefir where the variation among storage days was highly significant ($P < 0.001$). Peak antioxidant activity was generally reached on days 14 or 21 of storage (Fig. 3). Antioxidant activity of kefir decreased in the following order: TF > WF > CF ≥ CP ≥ IN > MCP > K during the storage period. The high antioxidant activity of the faba bean hull supplemented kefir probably originates from its high phenolic content. Differences between faba bean cotyledon, chickpea and inulin supplemented kefir were not significant, particularly between 1 – 14 days storage and may therefore be considered to exhibit similar behavior.

4. Conclusion

Kefir storage has several components, some of which were investigated in this study relative to pulse supplementation. Storage time contributed the most to variability in microbial growth, TTA and pH, production of organic acid and proteolytic activity, whereas the type/source of pulse supplementation determined kefir antioxidant activity. In this context, faba bean hull supplementation would be preferred for its rich total phenolics, phenolic compounds (gallic and protocatechuic acids), flavonoids (catechin, epicatechin and rutin) and antioxidant activity. Moreover, increase in microbial count during kefir storage (day 1 – 28) favored supplementation with faba bean cotyledon, chickpea flour and chickpea mucilage that may be considered as efficient prebiotics. Substantially influenced the production of SCFA and

307 improved proteolytic activity. Therefore, proper selection of pulse based ingredients is pertinent
308 to kefir storage and shelf-life. Pulse supplemented kefir may combine the probiotic and
309 antioxidant activities to offer synergistic efficacy in blocking cellular oxidation mechanisms
310 and their harmful effects on human health. Thus, pulse ingredients are very potent prebiotics,
311 stimulating the growth of beneficial bacteria and moreover exert strong antioxidant activity due
312 to the presence of pulse-bound polyphenols.

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Figure Captions

Fig 1. Bacterial enumeration of kefir (K, control) supplemented with whole faba bean flour (K+WF), faba bean cotyledon (K+CF), faba bean hull (K+TF), chickpea flour (K+CP), chickpea mucilage (K+MCP), and inulin (K+IN) during refrigerated storage (4 °C, 28 days).

Fig 2. a) Titratable acidity and b) pH of kefir (K, control) supplemented with whole faba bean flour (K+WF), faba bean cotyledon (K+CF), faba bean hull (K+TF), chickpea flour (K+CP), chickpea mucilage (K+MCP), and inulin (K+IN) during refrigerated storage (4 °C, 28 days).

Fig 3. Antioxidant activity (ORAC value) of kefir (K, control) supplemented with whole faba bean flour (K+WF), faba bean cotyledon (K+CF), faba bean hull (K+TF), chickpea flour (K+CP), chickpea mucilage (K+MCP), and inulin (K+IN) during refrigerated storage (4 °C, 28 days).

Fig1.

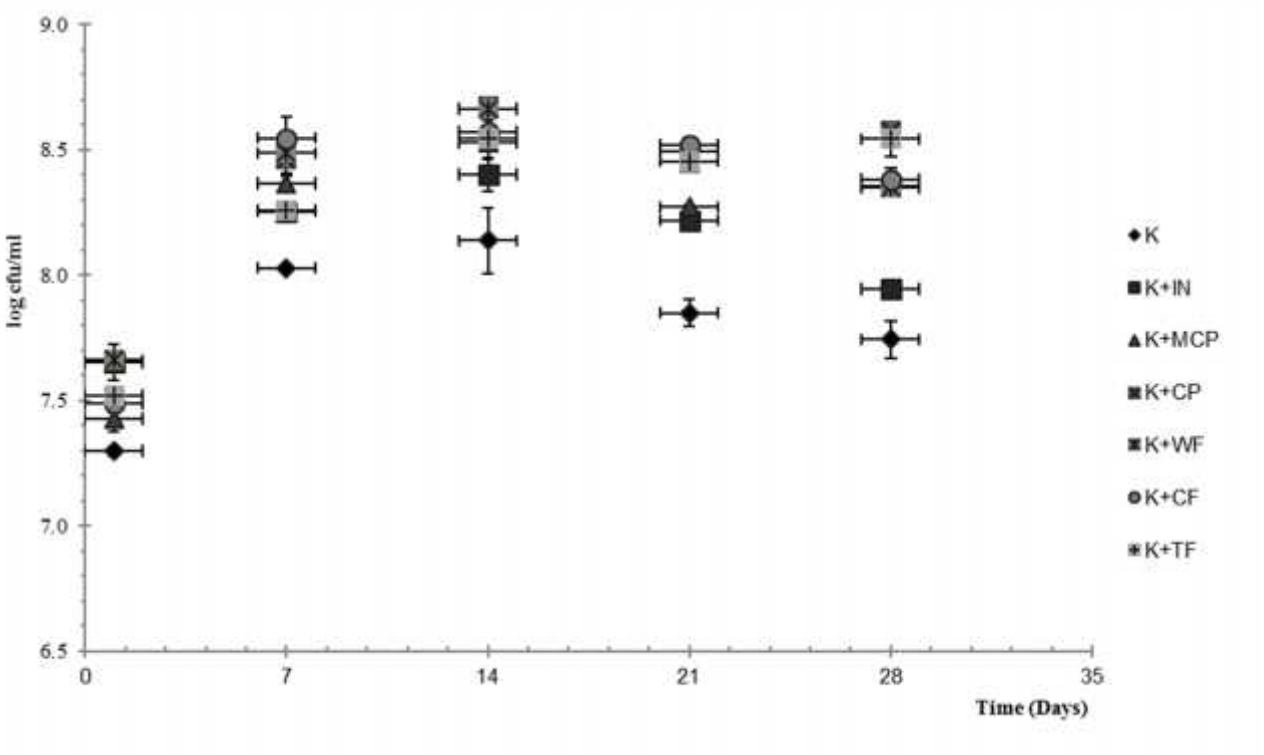
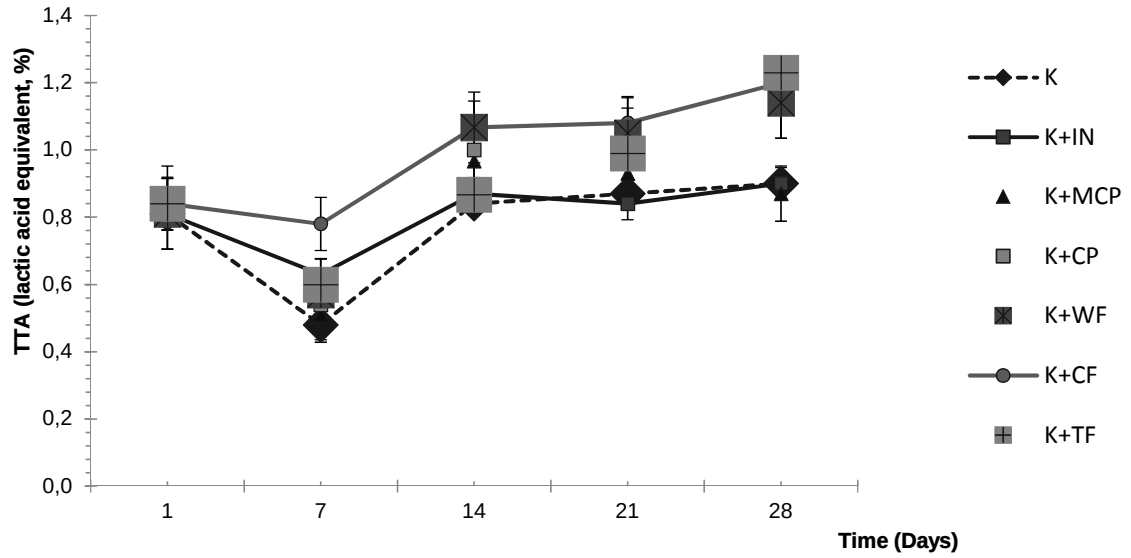

9.0
8.5
8.0
7.5
7.0
6.5
log cfu/ml
0
7
14
21
28
35
Time (Days)
K
K+IN
K+MCP
K+CP
K+WF
K+CF
K+TF

Fig 2. a. b.


TTA (lactic acid equivalent, %)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
1
7
14
21
28
Time (Days)
K
K+IN
K+MCP
K+CP
K+WF
K+CF
K+TF

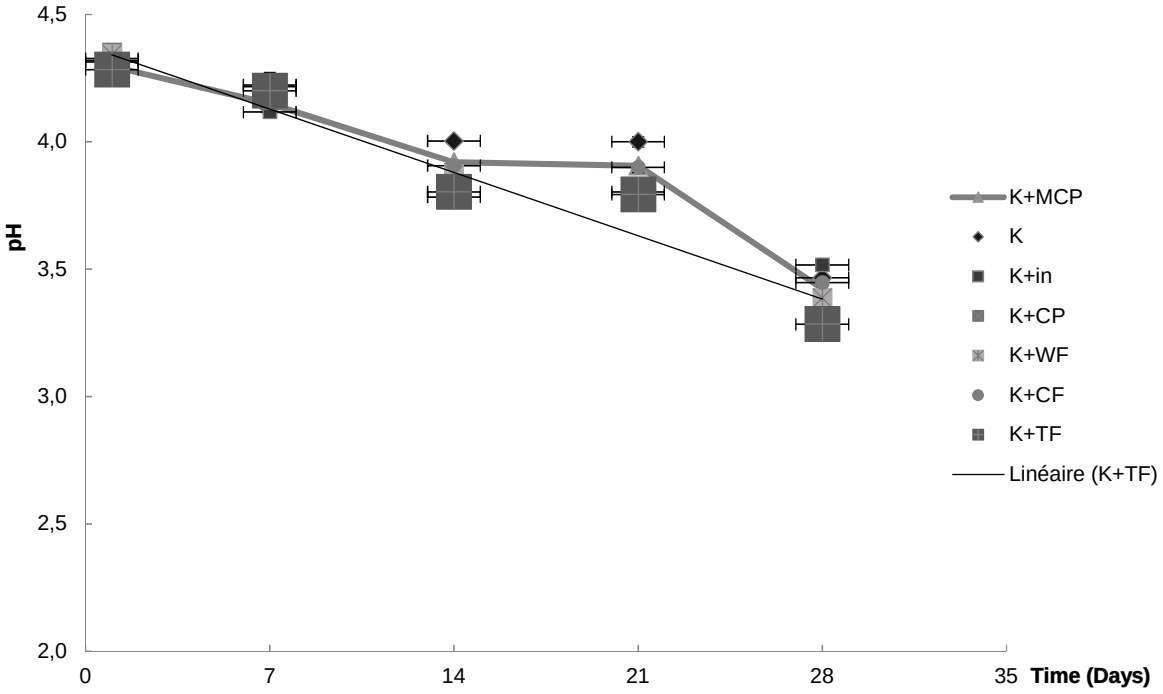

pH
4,5
4,0
3,5
3,0
2,5
2,0
0
7
14
21
28
35
Time (Days)
K+MCP
K
K+in
K+CP
K+WF
K+CF
K+TF
Linéaire (K+TF)

Fig 3.

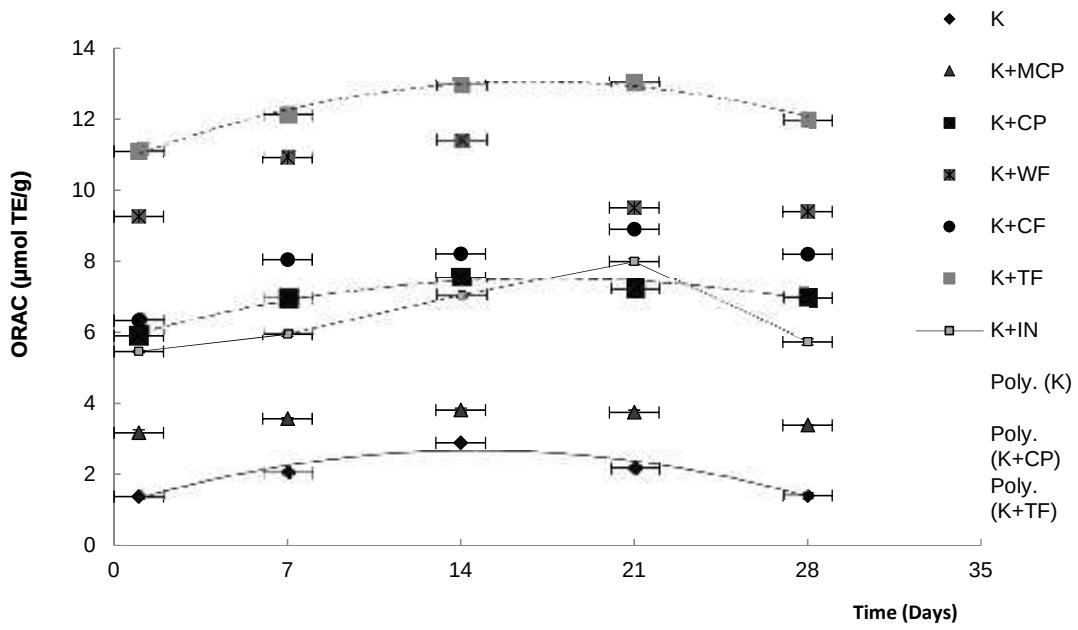

ORAC (µmol TE/g)
0
2
4
6
8
10
12
14
0
7
14
21
28
35
Time (Days)
K
K+MCP
K+CP
K+WF
K+CF
K+TF
K+IN
Poly. (K)
Poly. (K+CP)
Poly. (K+TF)

Table 1. Total phenolic, flavonoid contents and antioxidant activities of samples

Each value represents (means ±standard deviation n=3). Means in a column with different letters are significantly different ($P < 0.05$). Total phenolics and flavonoids are expressed in mg gallic acid and quercetin equivalents/g sample, respectively. ORAC – oxygen radical absorbance capacity values are expressed in μmol trolox equivalent/g sample.

Table 2. Phenolic compounds (mg/g)

Each value represents (means ±standard deviation n=3). Means in a row with different letters are significantly different ($P < 0.05$). n.d: not detected. WFB, CF and TF denote whole, cotyledon and hull of faba bean, respectively; CP, chickpea; MCP, chickpea mucilage.

Tabel 3. Proteolytic activities of Kefir samples during storage time at 4°C

kefir (K, control) supplemented with whole faba bean flour (K+WF), faba bean cotyledon (K+CF), faba bean hull (K+TF), chickpea flour (K+CP), chickpea mucilage (K+MCP), and inulin (K+IN) during refrigerated storage (4 °C, 28 days). Each value represents (means ±standard deviation n=3). Means in a same column with different letters are significantly different ($P < 0.05$).

Tabel 4. Concentration of Lactic, acetic, propionic and butyric acid in Kefir samples produced by starter cultures in presence of different supplementations

kefir (K, control) supplemented with whole faba bean flour (K+WF), faba bean cotyledon (K+CF), faba bean hull (K+TF), chickpea flour (K+CP), chickpea mucilage (K+MCP), and inulin (K+IN) during refrigerated storage (4 °C, 28 days). Each value represents (means ±standard deviation n=3). Means in a same column with different letters are significantly different ($P < 0.05$).

Table 1.

Sample	ID	Phenolics	Flavonoids	ORAC
Faba bean				
Whole	WF	14.65±0.4b	0.148±0.003b	175.29±6.4b
Hull	TF	57.53±1.7a	0.79±0.02a	265.97±5.4a
Cotyledon	CF	6.09±0.1c	0.022±0.0005d	90.63±1.2c
Chickpea				
Whole	CP	3.84±0.2d	0.027±0.003d	75.74±4.1c
Mucilage	MCP	5.28±0.6cd	0.054±0.005c	29.30±1.4d

Table 2.

Phenolic compounds	**WFB**	**CF**	**TF**	**CP**	**MCP**
Phenolic acids					
Caffeic	0.340±0.02b	0.346±0.03a	0.297±0.1c	n.d	n.d
Chlorogenic	2.013±0.2b	2.016±0.4a	n.d	n.d	n.d
Gallic	0.381±0.03b	0.367±0.02c	0.389±0.3a	0.349±0.4d	0.001±0.0003e
O-Coumaric	n.d	n.d	0.045±0.03a	n.d	n.d
P-Coumaric	n.d	n.d	0.040±0.001a	n.d	n.d
p-Hydroxybenzoic	n.d	n.d	0.327±0.3a	n.d	n.d
Protocatechuic	0.682±0.6b	0.747±0.5a	0.554±0.02c	n.d	n.d
Pyrogallol	0.383±0.02d	0.551±0.02b	0.705±0.03a	0.094±0.001e	0.482±0.05c
Synapic	0.207±0.02b	0.210±0.002a	n.d	n.d	n.d
Vanillic	0.0155±0.001b	0.0164±0.004a	n.d	0.0124±0.01c	0.00001±0.00002d
∑Phenolic acids	4.022b	4.252a	2.242c	0.456d	0.483d
Flavonoids					
Apigenin	n.d	n.d	0.048±0.001a	0.031±0.002b	n.d
Catechin	0.239±0.003b	0.076±0.009d	0.955±0.04a	0.222±0.06c	n.d
Epicatechin	0.157±0.002c	n.d	0.408±0.2a	n.d	0.189±0.1b
Epicatechine gallate	0.154±0.002a	0.109±0.09c	0.115±0.01b	n.d	n.d
Kaempferol	n.d	n.d	0.142±0.001a	n.d	n.d
Myricetin	n.d	n.d	n.d	0.390±0.02a	n.d
Quercetin	0.509±0.05b	n.d	n.d	0.523±0.02a	n.d
Quercetin 3β-glucoside	0.208±0.08a	n.d	n.d	n.d	n.d
Rutin	0.238±0.06c	0.261±0.01b	0.444±0.05a	n.d	n.d
∑Flavonoids	1.504b	0.446d	2.112a	1.165c	0.188e
Ratio Phenolics/Flavonoids	2.67b	9.54a	1.06c	0.39d	2.56b

Tabel 3.

Samples (Abs 340)	1	7	14	21	28	**Mean**
K	0.61±0.06c	1.08±0.2bc	0.94±0.03e	1.30±0.3c	1.17±0.01c	1.02Z
K+in	0.95±0.05b	0.98±0.08c	1.28±0.01cd	1.35±0.03b	1.17±0.02c	1.15X
K+wf	1.13±0.03a	1.20±0.3ab	1.31±0.08bc	1.42±0.03a	1.23±0.01b	1.26W
K+cf	1.19±0.07a	1.09±0.08bc	1.37±0.02ab	1.44±0.03a	1.23±0.02b	1.26W
K+tf	0.94±0.1b	1.04±0.04c	1.28±0.02cd	1.29±0.03c	1.28±0.03a	1.17X
K+cp	1.07±0.05ab	1.25±0.05a	1.42±0.009a	1.44±0.02a	1.19±0.008c	1.27W
K+mcp	0.74±0.01c	0.99±0.008c	1.23±0.02d	1.31±0.01bc	1.18±0.001c	1.09Y
Mean	0.95E	1.09D	1.26B	1.37A	1.21C	1.17

Tabel 4.

Sample (µmol/ g of Kefir)	Storage time (days)					
	1	7	14	21	28	Mean
			Lactic acid			
K	5.26±0.07g	8.27±0.3c	7.18±0.4c	6.63±0.5d	5.00±0.4e	6.47Z
K+in	7.24±0.1e	8.35±0.3c	8.25±0.06bc	7.26±0.2d	6.94±0.3cd	7.61Y
K+wf	8.25±0.01c	9.72±0.2ab	11.12±0.2a	9.14±0.1b	7.41±0.6bc	9.13W
K+cf	6.44±0.2f	9.41±0.3b	8.23±0.9bc	6.72±0.2d	6.66±0.3d	7.49Y
K+tf	8.07±0.07d	8.64±0.4c	8.47±0.8b	8.88±0.3bc	7.62±0.2b	8.34X
K+cp	9.00±0.09b	10.22±0.4a	11.32±0.8a	10.88±0.3a	8.57±0.02a	10.00V
K+mcp	9.21±0.01a	9.63±0.2ab	8.72±0.6b	8.35±0.2c	7.10±0.2bcd	8.60X
Mean	7.64C	9.18A	9.04A	8.27B	7.04D	
			Acetic acid			
K	1.22±0.02d	1.77±0.01d	1.83±0.04e	0.81±0.02e	0.60±0.04e	1.24Y
K+in	1.63±0.01c	2.05±0.03c	1.84±0.03e	1.44±0.05c	0.82±0.05d	1.56W
K+wf	1.60±0.02c	1.79±0.03d	2.01±0.03d	0.99±0.03d	0.57±0.03e	1.39X
K+cf	1.22±0.03d	1.36±0.03e	1.63±0.03f	0.81±0.04e	0.82±0.04d	1.17Z
K+tf	1.01±0.05e	2.43±0.04a	2.66±0.04b	2.06±0.05a	2.06±0.05a	2.04U
K+cp	2.31±0.02a	2.43±0.03a	2.82±0.03a	1.84±0.05b	1.63±0.04b	2.21T
K+mcp	1.92±0.02b	2.17±0.04b	2.17±0.03c	2.04±0.06a	1.43±0.05c	1.95V
Mean	1.56C	2.00B	2.14A	1.43D	1.13E	
			Propionic acid			
K	0.18±0.01b	0.17±0.03b	0.20±0.02ab	0.20±0.02b	0.14±0.02ab	0.18YZ
K+in	0.20±0.05ab	0.21±0.05ab	0.20±0.05ab	0.25±0.05ab	0.17±0.04ab	0.21XY
K+wf	0.17±0.02b	0.20±0.01ab	0.19±0.02b	0.11±0.02c	0.10±0.02b	0.15Z
K+cf	0.20±0.02ab	0.22±0.01ab	0.26±0.02a	0.26±0.03ab	0.18±0.02b	0.22WX
K+tf	0.26±0.02a	0.26±0.04a	0.26±0.05a	0.31±0.06a	0.14±0.07ab	0.25W
K+cp	0.20±0.04ab	0.21±0.03ab	0.21±0.04ab	0.20±0.03b	0.16±0.05b	0.20XY
K+mcp	0.17±0.03b	0.17±0.03b	0.22±0.02ab	0.20±0.04b	0.17±0.03b	0.19Y
Mean	0.20A	0.21A	0.22A	0.22A	0.15B	
			Butyric acid			
K	0.52±0.03c	0.60±0.08c	0.71±0.03d	0.76±0.02a	0.45±0.02cd	0.61Y
K+in	0.50±0.05c	0.68±0.05bc	0.76±0.03d	0.57±0.04b	0.39±0.05d	0.58YZ
K+wf	0.52±0.02c	0.69±0.03b	0.99±0.3a	0.71±0.02a	0.58±0.05b	0.70X
K+cf	0.39±0.03d	0.71±0.04b	0.71±0.01d	0.54±0.03b	0.49±0.04bcd	0.57Z
K+tf	0.73±0.04a	0.81±0.02a	0.89±0.06bc	0.59±0.09b	0.48±0.03bcd	0.70X
K+cp	0.61±0.04b	0.82±0.04a	0.92±0.04b	0.79±0.05a	0.54±0.07bc	0.74X
K+mcp	0.57±0.03bc	0.83±0.03a	0.85±0.01c	0.72±0.05a	0.69±0.05a	0.73X
Mean	0.55D	0.73B	0.83A	0.67C	0.52E	