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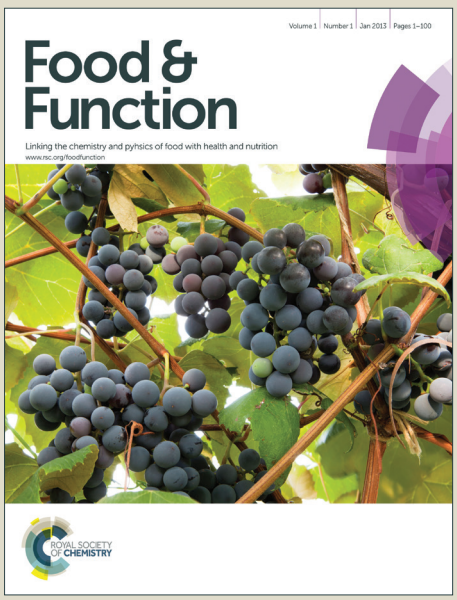

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In vitro Digestibility of Goat Milk Kefir with New Standardised Static Digestion Method (INFOGEST Cost Action) and Bioactivities of the Resultant Peptides

Sedef Nehir El*[1], Sibel Karakaya[1], Sebnem Simsek[1], Didier Dupont[2], Esra Menfaatli[3], Alper Tolga Eker[1]

[1]Department of Food Engineering, Nutrition Section, Engineering Faculty of Ege University, 35100, İzmir, Turkey

[2] INRA, UMR STLO 1253, 65 rue de St Brieuc 35042 Rennes, France

[3] Department of Biochemistry, Science Faculty of Ege University, 35100, İzmir, Turkey

*Corresponding author: sedef.el@ege.edu.tr Tel: + 90 232 3113005; Fax: + 90 232 3427592

Running Title: ***In vitro*** **Digestibility of Goat Milk Kefir with New Standardised Digestion Method**

ABSTRACT

Hydrolyses degree of goat milk and kefir during simulated gastrointestinal digestion system and some bioactivities of resultant peptides after fermentation and digestion were studied. Static *in vitro* digestion method by the COST FA1005 Action INFOGEST was used and goat milk and kefir were partially hydrolyzed during gastric phase and had above 80% hydrolysis after duodenal digestion. There were not any differences between digestibility of goat milk and kefir ($p>0.05$). Goat milk and kefir displayed about 7-fold antioxidant activity after digestion ($p<0.05$). Fermentation showed no effect on calcium-binding capacity of the samples ($p>0.05$), however after *in vitro* digestion calcium-binding capacity of the goat milk and kefir increased 2 and 5 fold, respectively ($p<0.05$). Digested goat milk and kefir showed a higher dose-dependent inhibitory effect on α-amylase comparing undigested samples ($p<0.05$). α-glucosidase inhibitory activities and *in vitro* bile acid-binding capacities of the samples were not determined at studied concentrations.

KEYWORDS: *Kefir, in vitro digestion, bioactive peptide, antioxidant activity, α-amylase inhibition, α-glucosidase inhibition, bile acid-binding, Ca-binding*

Introduction

A growing body of scientific evidence in the field of nutrition has revealed that many food proteins and peptides exhibit specific biological activities in addition to their established nutritional value. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health.[1-3] Peptides are inactive within the sequence of the parent protein and can be released by digestive enzymes during gastrointestinal transit or by fermentation or during food processing. The activity of these peptides is based on their

inherent amino acid composition and sequence.[1,4] Today nutritionists, biomedical scientists, food scientists, and technologists are working together to develop improved systems for discovery, testing, and validation of nutraceutical proteins and peptides with increased potency and therapeutic benefits. Bioactive peptides and proteins are being developed that positively impact on body function and human health by alleviating conditions such as coronary (ischemic) heart disease, stroke, hypertension, cancer, obesity, diabetes, and osteoporosis.[1,5-7] Especially milk proteins provide a major dietary source for humans, supplying amino acids for the synthesis of proteins and other nitrogen-containing compounds. In addition, some of these proteins contain bioactive peptides released by hydrolysis that may affect the human health. These effects include mineral binding, growth factors, blood pressure reduction, antioxidant activity and protective properties against different microorganisms and viruses. The generation of bioactive peptides during milk fermentation with dairy starter cultures is now well documented. Kefir has received considerable attention from scientists and recognized a healthy food product. Its chemical composition, microbiological characteristics, therapeutic effects, probiotic properties and rheological characteristics have been reported by several authors.[8-10] But there are few reported studies on the bioactive properties of kefir peptides. Especially the researches were focused on Angiotensin-Converting Enzyme Inhibitory Activity (ACE) of peptides derived from kefir.[11-13] Considering all the nutritional aspects more studies are needed about bioactive profile of kefir as traditional fermented milk derived beverage in many Eastern countries.

In this study, recently, proposed a standardised static *in vitro* digestion method by the COST FA1005 Action INFOGEST, as an international network joined by more than 200 scientists from 32 countries working in the field of digestion,[14] was applied to analyse proteins and some beneficial biological activities of the goat milk and kefir. The objectives are to identify hydrolyses degree and molecular

weight and to determine antidiabetic effect, cholesterol lowering effect, antioxidant activity and mineral binding capacity of the bioactive peptides produced during fermentation and released during simulated gastrointestinal digestion system of kefir derived from goat milk.

Materials and methods

Material

Porcine pepsin (P7012), pancreatin (P7545), bile extract porcine (B8631), porcine pancreatic lipase from porcine pancreas (L3126), hemoglobin from bovine blood (H2625), TAME (T4626), trybutirin (W222305), 4-Nitrophenyl α-D-glucopyranoside (PNPG) (N1377), Pefabloc® SC (76307), Bradford reagent (B6916), Na-dodecyl-sulfate (SDS) (L4390) Sigma marker molecular weight of 6,500-200,000 Da (S8445), o-phthaldialdehyde (OPA) (P1378), L-Serine (S4500), DL-Dithiothreitol (DTT) (S43819), bromophenol blue sodium salt (B 5525), 2-mercaptoetanol for electrophoresis (M 7154), Coomassie Brilliant Blue (27815), acetone powder from rat (I1630) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 12% Mini-PROTEAN® TGX™ Gel (456-1044) was purchased from Bio-Rad (USA). Bromophenol blue (L961422), sodium tetraborate decahidrate (6303) was purchased from Merck, and pancreatic α-amylase from Megazyme International Ireland Ltd. (Wicklow, Ireland). Bile-acid analysis kits and standards were purchased from Trinity Biotech (Bray Co., Wicklow, Ireland). Milli-Q water was used in all analyses (referred in text as water) and all other reagents were of analytical grade and all analyses were done in triplicate.

Goat Milk and Kefir Starter Culture

Saanen Goat's raw milk (produced according to the Regulation No. 2000/6 of Turkish Codex) was purchased from local market of Ege University, Agricultural Faculty, Dairy Technology Department and subjected to pasteurizing at 90 °C for 15 min and cooling down to about 23 °C. A commercial

freeze-dried Kefir starter culture was purchased from (DC, Danisco, Poland). According to the supplier information, this starter culture contains a mixture of *Lactococcus lactis* subsp., *Leuconostoc* sp., *Streptococcus thermophilus*, *Lactobacillus* sp.

Kefir manufacturing

Kefir working-culture was prepared by inoculating the pasteurized goat milk with the commercial freeze-dried culture at 3% (w/v), incubating at 23 °C for 16 h. The pasteurized and cooled goat milk was inoculated with the working-culture at 5% (v/v) and then incubated in a thermostatically controlled chamber at 23 °C for 14 h and maturing at 4 °C for at least one day before further use.

Chemical analyses

Ash, total solids, pH, titratable acidity, fat, protein, and casein contents of goat milk and goat's milk kefir were determined in triplicate according to the AOAC standard method.[15]

***In vitro* digestion assay**

In vitro digestion analyses were performed according to the procedure recently described by Minekus and coworkers (2014 and Electronic supplementary information, ESI).[14] In this consensus protocol, within the COST FA1005 INFOGEST Network, practical static digestion method is based on human gastrointestinal physiologically relevant conditions. They concluded that this protocol should be tested by different research groups for a variety of application on different food samples. Also, we are as participants of INFOGEST, we modified this method without salivary amylase but include simulated salivary fluid, and intestinal phase was performed with pancreatin instead of individual enzymes such as trypsin, chymotrypsin.

Enzyme activity assays

Pepsin activity assay: A stock solution of porcine pepsin was prepared just before analyses by dissolving 10 mg in 10 mL (150 mM) NaCl and adjusted to pH 6.5 using 100 mM NaOH. In this stage, inversion was used instead of vortex. The stock solution has to be kept on ice or refrigerated at 4 °C during analyses. Before the assay, stock was diluted at 5, 10, 15, 20, 25, 30, and 35 μg mL^{-1}. 0.5 ml hemoglobin solution (2% w/v, 0.5 g was solubilized in 20 mL of water and adjusted to pH 2 with 300 mM HCl and make up the volume 25 mL) was pipetted into 2 mL Eppendorf and incubated in shaking incubator or thermo block at 37 °C for 3-4 minutes to achieve temperature equilibration. 0.1 mL enzyme solution (5-35 μg mL^{-1}) was added to eppendorfs (except the blanks) and incubated for exactly 10 minutes. To stop reaction, 1 mL TCA (5% w/v Trichloroacetic acid) was added to every tubes. After this step, 0.1 mL enzyme solution was added to only blanks. The mix was centrifuged 6000 × g at 30 minutes to precipitate hemoglobin. Meanwhile, new eppendorf tubes for each enzyme concentration and blank were prepared. Supernatants were taken to each new tube and incubated at 20 °C or room temperature until temperature equilibration about 5 min if spectrophotometer in use lacks a temperature controller. Absorbance of samples in a quartz cuvette was read at 280 nm and enzyme activity was calculated by using Equation 1.[14]

$$Units\ mg^{-1} = \frac{(A280\ Sample - A280\ Blank)}{\Delta t \times X} \qquad (1)$$

Δt: duration of the reaction, *i.e.* 10 minutes; X: concentration of pepsin in the final reaction mixture in cuvette (mg mL^{-1}).

Trypsin activity of pancreatin assay**:** To measure the trypsin activity of pancreatin, a stock solution of pancreatin was prepared as 1 mg mL^{-1} in cold 1 mM HCI. During preparation, centrifugation step was not used to prevent degradation; only inversion was used for mixing. At least 3 dilutions of the enzyme solution in a range of concentrations of 10-20 μg mL^{-1} were prepared. 10 mM TAME in water was prepared as substrate of the analysis. For each concentration two cuvettes (one for enzyme

and one for blank) were prepared.1.3 mL 0.046 Tris-HCl buffer at pH 8.1 (including 0.0115 M $CaCl_2$) and 150 µL of TAME was added to each cuvette and mixed by inversion. The change in absorbance at 247 nm was started to record immediately following the addition of 50 µL enzyme solution during 10 minutes. The absorption changes (y-axis) versus the time (x-axis) in minutes were plotted and the slope (ΔA_{247}) from the initial linear part of the curve was determined and used for the calculation of enzyme activity with Equation 2[14].

$$Units\ mg^{-1} = \frac{(\Delta A247\ Sample - \Delta A247\ Blank)x\ 1000x1.5}{540\ x\ X} \qquad (2)$$

ΔA247: slope of the initial linear portion of the curve, (unit absorbance/minute) for the sample (with enzyme) and blank; 540: molar extinction coefficient of TAME at 247 nm; 1.5: Volume (in mL) of the reaction mixture (Tris-HCl + TAME + Enzyme); X: quantity of pancreatin in the final reaction mixture (cuvette)[mg]

Pancreatic lipase activity assay: Porcine pancreatic lipase activity was based on tributyrin as a substrate: one unit liberated 1 µmol butyric acid per minute at 37 °C and at pH 8.0; specific activity of pure human pancreatic lipase was 8000 U mg^{-1} protein on tributyrin. Assay solution was prepared as 200 mL solution containing 7.2 mg of Tris-(hydroxymethyl) aminomethane, 1800 mg of NaCl, 40 mg of $CaCl_2$ and 420 mg of sodium taurodeoxycholate and kept at 4 °C. Pancreatin/lipase was dissolved in 1 mL water (1 mg mL^{-1}). 14.5 mL of assay solution and 0.5 mL tributyrin were mixed in a titration vessel containing a mechanical stirrer and equilibrated at 37 °C. Then pH was adjusted to 8 with 0.1 M NaOH for 10 min and checked for the pH change (this was blank). Then 1-100 µL of the pancreatin/lipase was added and the amount of NaOH required to compensate the liberations of fatty acids keeping the pH at 8, 37 °C was monitored for 10 minutes by pH-stat (KEM AT 510, Japan). The enzyme activity was calculated using Equation 3[14].

$$Units\ mg^{-1} = \frac{R(NaOH) \times 1000}{v \times [E]} \qquad (3)$$

R(NaOH): rate of NaOH delivery in µmol NaOH per minute, i.e., µmol free fatty acid release per minute. Only the linear region of the curve was used. v: volume (µL) of enzyme solution added in the pH-stat vessels; E: concentration of the enzyme solution (mg powder mL^{-1})

Analysis of bile concentration: The molecular weight of bile acid was determined with Bile Acid Diagnostic Kits (No. 450-11 and 450-100).

Preparation of simulated digestive fluids

The solutions of simulated digestion fluids (SSF: simulated salivary fluid, SGF: simulated gastric fluid, SIF: Simulated intestinal fluid) were prepared as seen Table 1. The stock solutions were prepared and warmed up at 37 °C before the digestion procedure. They could also be prepared and stored -20 °C with a small headspace.

Digestion procedure

Oral Phase (final ratio of food to SSF of 50:50 (w/v)). The 5 mL of goat milk or kefir were mixed with 4 mL of warmed up SSF electrolyte stock solution. In our study, salivary α-amylase was not added due to the low carbohydrate content of the products. Then 25 µL of 0.3 M $CaCl_2 (H_2O)_2$ and 975 µL of water were added and thoroughly mixed. The sample was incubated for 2 minutes at 37 °C at 150 rpm in orbital shaker (Stuart S 1500, UK).

Gastric phase (final ratio of food to SGF of 50:50 (v/v)). Oral bolus was mixed with 8 mL of previously warmed up SGF electrolyte stock solution and 5 µL of 0.3 M $CaCl_2 (H_2O)_2$. pH was adjusted to 3.0 with 3 M HCl. 1 mL porcine pepsin stock solution prepared in water (2000 U mL^{-1} in

the final mixture) was added and the volume was adjusted to 20 mL with water. The sample was placed into the shaker and incubated for 2 h at 37 °C at 150 rpm.

Duodenal phase (final ratio of gastric chyme to SIF of 50:50 (v/v)). Gastric chyme was mixed with 11 mL previously warmed up SIF electrolyte stock solution. pH was adjusted to 7.0 with 3 M 1 M NaOH. 40 µL of 0.3 M $CaCl_2$ and bile solution (prepared in water in order to reach the concentration of 10 mM in the final mixture and solubilized on a rotating wheel at 37 °C for 30 min) was added to mixture. Pancreatin solution was prepared in SIF in order to reach 100 U mL^{-1} of trypsin activity in the final mixture and additional pancreatic lipase (to obtain 2000 U mL^{-1} in the final mixture) was added to the mixture according to the calculated lipase activity of the pancreatin. Final volume was made up with water to 40 mL. The sample was incubated on orbital shaker for 2 h at 37 ºC, 150 rpm. After 2h, 8 µl of 5 mM Pefabloc was added to the samples to stop the enzyme reaction. Then samples were ultrafiltrated with 10 kDa cut-off membranes (Sartorius Stedim Biotech GmbH, Germany). All samples were freeze-dried and stored at -18 ºC until analysis. Protein content of samples were determined by Bradford method.[16]

SDS–PAGE

Undigested and digested samples of goat milk and kefir were separated according to their molecular weights on precasted 12% Mini-PROTEAN® TGX™ gel in accordance with Laemmli (1970)[17] buffer system. Samples were diluted with water to have 1 mg mL^{-1} as final concentration and mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol) in the ratio of 1:1. After that samples were incubated at 95 °C for 10 minutes for denaturation of proteins. 20 µL of denatured samples loaded into wells and electrophoresis was performed using a Mini Protean Tetra Cell unit (Bio-Rad, USA) at constant

voltage (100 V). The gels were stained with Coomassie Brilliant Blue and destained with water by heating for short periods.

Degree of hydrolysis (OPA)

Degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. Several methods exist for determining DH; the most commonly used of these include the pH-stat, trinitrobenzenesulfonic acid (TNBS), *o*-phthaldialdehyde (OPA) and formol titration methods. The principle of the reaction is based on the reaction of OPA with primary amino groups and a SH-compound (dithiothreitol, DTT) to form a compound that will absorb light at 340 nm. OPA reagent was prepared according to Nielsen, Petersen and Dambamann (2001)[18] as follows: 7.620 g di-Na-tetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) were dissolved in 150 mL water. 160 mg o-phthaldialdehyde (OPA) was dissolved in 4 mL ethanol. The OPA solution was then transferred to the above-mentioned solution. 176 mg dithiothreitol 99% (DTT) was added to the solution and volume was made up to 200 mL with water. The serine standard was prepared as follows: 50 mg serine was diluted in 500 mL water (0.9516 meqv L^{-1}) and blanks were prepared from water. 20 µL samples were taken at regular time intervals from *in vitro* digestion medium and 150 µL of OPA reagent were added to all tubes. The tubes stood for exactly 2 min before measuring the optimal density (OD) at 340 nm in the Microplate reader (Thermo Scientific Varioskan Flash, Finland). The number of amino groups was determined with reference to a L-serine standard curve and the proteolytic activity was calculated according to the equation 4,5 to have value for h (cleaved peptide bonds), and the degree of hydrolysis (DH) was calculated according to the Equation 6.

$$\mathbf{Serine} - NH_2 = OD_{sample} - OD_{blank} \Big/ OD_{standard} - OD_{blank} \times 0.\frac{916\mathrm{meqv}}{\mathrm{L}} \times 0.1 \times \frac{100}{\mathrm{X}} \times P \quad (4)$$

$$h = (serine - NH_2 - ß) / \alpha \frac{\text{meqv}}{\square} \text{g protein} \qquad (5)$$

Where, serine-NH_2= meqv serine NH_2/g protein; X= g sample; P= protein % in sample; ß and α are 0.383 and 1.039 for casein, respectively.

$$DH = \frac{h}{\text{htot}} x\, 100 \qquad (6)$$

Where, h_{tot} is the total number of peptide bonds in the casein as protein substrate and assumed to be 8.2 meq g^{-1}

***In vitro* calcium binding capacity**

Calcium-binding activities of samples were determined by measurement of free Ca^{2+} with a Ca-selective electrode. Lyophilized samples were demineralized by medium pressure chromatography (ÄKTA prime PLUS, Sweeden) using HiTrap column. Samples were buffered with PBS (pH 7.0) and 100 µL 0.1 M $CaCl_2$ was added to 10 mL of samples. After mixing for 2 min the Ca^{2+} concentration was measured by means of Ca^{2+} selective electrode (Orion 93-20, Boston MA) connected to ion analyzer Orion 290A. The calibration curve (mV/C^{2+}) with standard Ca^{2+} solutions was built, and the calcium bonded was determined after subtraction of the measured free calcium from the total calcium.[19]

***In vitro* phosphomolybdenum reducing capacity**

Phosphomolybdenum (Mo) assay according to Prieto, Pineda and Aguilar (1999)[20] was used to estimate the capability of the samples to reduce transition-metal ions. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) complex at acidic pH. An aliquot of 0.2 mL extract (10 µg mL^{-1}) was combined with 2 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95 °C for 90 min.

After the samples had cooled to room temperature, the absorbance of the green phosphomolybdenum complex was measured at 695 nm against blank. A typical blank contained 2 mL of reagent solution and an appropriate volume of the same solvent used for the sample and were incubated under the same conditions as the rest of the samples. The appropriate solutions of ascorbic acid were used as reference and reducing capacity was expressed as the ascorbic acid equivalents (AAE) (μM ascorbic acid mg^{-1} protein of sample).

In vitro antidiabetic activity

Desalting of samples

Samples were desalted prior to antidiabetic activity analysis to prevent the interferation of salts coming from the digestive fluids. The lyophilized samples were dissolved in 1.5 mL water containing 0.1% TFA. The samples were loaded on equilibrated OASIS HLB cartridge (3 cc, 60 mg). For removal of salts cartridge was washed with 3 mL 0.1% TFA and 2 mL water, respectively. The peptides were eluted with 1.5 mL 70% acetonitrile. The solvent was removed under nitrogen, lyophilized and stored -18 °C until the analysis.

α-Amylase inhibitory activity assay

α-amylase inhibitory assay was performed according to the method described by Koh, Wog, Loo, Kasapis and Huang (2010)[21] and Yang, Huang, Jin, Sun, Song and Chen (2012)[22] with some modifications. Pancreatic α-amylase solution was prepared to the final concentration of 2 mg mL^{-1} in phosphate buffer (pH 6.9, 50 mM, 6.85 mM NaCl) in ice bath. Lyophilized samples were diluted with water in different concentrations. Then 82 μL of the sample were incubated with 10 μL α-amylase. Following incubation for 5 min at 37 °C, 8 μL potato starch solution (1%, in phosphate buffer) was added to start the reaction. The reaction mixture was further incubated for 12 min, at

37 °C and followed by addition of 50 µL HCl (10%) to stop the reaction. To determine the amount of starch in the medium, 15 µL iodine solution (0.0025 M I_2/0.0065 M KI) and 50 µL water were added and absorbance at 620 nm was measured by Microplate reader. A control was prepared by replacing the sample with phosphate buffer. The experiment was repeated by substituting the active enzyme with inactive (denatured) enzyme treated for 10 min in water bath at 100 °C. Acarbose was used as positive control. Percentage of α-amylase inhibition was calculated by using Equation 7. A curve of percentage inhibition against sample concentration was plotted with means. The concentration of sample required to produce a 50% inhibition of the initial rate of reaction (IC_{50}) was determined by GraphPad Prism 6.0.

$$Inhibition\ (\%) = \frac{(\text{Acontrol} - \text{Acontrol blank}) - (\text{Asample} - \text{Asample blank})}{\text{Acontrol} - \text{Acontrol blank}} \times 100 \qquad (7)$$

Where; $A_{control}$, $A_{control\ blank}$, A_{sample}, $A_{sample\ blank}$ refer to absorbance reading of reaction mixture containing active enzyme and buffer, inactive enzyme and buffer, active enzyme and sample (inhibitor) and inactive enzyme and sample (inhibitor), respectively. Substrate was present in all mixtures.

α-Glucosidase inhibitory activity assay

The α-glucosidase inhibitory assay was performed according to previously described method by Koh and coworkers (2010).[21] Reaction substrate PNPG (30 mM) and α-glucosidase enzyme (25 mg mL^{-1}) solutions were prepared in phosphate buffer, pH 6.9. After mixing for 15 min in the ice bath, the enzyme solution was centrifuged at 4 °C and 10000 × g for 30 min. The supernatant was utilized in the assay. First 102 µL of sample of different concentrations was pipetted into separate wells of micro plate reader. Next, 6 µL of enzyme solution was added into each well, and incubated for 10 min, at 37 °C. Then 12 µL PNPG as substrate was added to the mixture to start the reaction. The mixture was incubated at 37 °C for 15 min, followed by addition of 60 µL Na_2CO_3 (1M) solution to

terminate the reaction. The amount of released product (p-nitrophenol) was measured at 400 nm using a Microplate reader to estimate the enzymatic inhibition activity. A control was prepared by replacing the sample with phosphate buffer. The experiment was repeated by substituting the active enzyme with inactive (denatured) enzyme treated for 10 min in water bath at 100 °C. The IC_{50} value was defined as the concentration of an inhibitor required to inhibit 50% of the α-glucosidase activity under the assay conditions. Inhibitory activity was calculated by using the Equation 7.

In vitro bile acid binding capacity

Cholesterol-lowering effect of the samples can be predicted by measuring their *in vitro* bile acid binding capacity based on positive correlations found between *in vitro* and *in vivo* studies. The *in vitro* bile acid binding procedure was performed as described by Kahlon and Smith (2007)[23] with slight modifications. The stock bile acid solution was prepared in 0.1 M PBS (1.8 mM) and diluted to the working solution (0.72 µM) just before the assay. 100 mg goat milk/kefir was weighed in tube and digested in1 mL 0.01 N HCl for 1 h in a 37 °C shake bath. After incubation, pH of the mixture was adjusted to 6.3 with 0.1 mL of 0.1 N NaOH. Bile acid working solution (4 mL) was added to each test sample. After addition of porcine pancreatin (5 mL), tubes were incubated for 1 h in a 37 °C shaker bath. Mixtures were centrifuged at 10000 x g for 10 min. Cellulose (a non-bile acid binding fiber) and cholestyramine (a bile acid binding anionic resin) were used as negative and positive controls, respectively. The free bile acids in the supernatant were analyzed using Trinity Biotech bile acids procedure No. 450 using Microplate reader. Values were determined from a standard curve obtained by analyzing Trinity Biotech bile acid calibrators at 5, 25, 50, 100 and 200 µM concentrations.

Statistical Analysis

In order to study significant differences between the variables, a variance analysis (ANOVA) was performed, with a confidence interval of 95% ($p<0.05$). Duncan and the Least Squares Difference (LSD) tests were applied to compare means tests using the program Statistica for Windows (Version 21.0, SPSS Inc., Chicago, IL, USA).

Results and discussion

Chemical composition

Table 1 shows some chemical components of goat milk and kefir. The changes during kefir production from goat milk in total solids; ash, protein, fat and casein were not significant ($p > 0.05$). The titratable acidity increased during fermentation from a mean value of 0.17% (w/w) lactic acid to 0.96% (w/w), which could be explained by the presence of other minor organic acids in the kefir ($p < 0.05$). The values observed are similar to those reported by authors for goat milk.[9, 24-26] Mestawet, Girma, Ådnøyc, Devolda, Narvhus and Vegarud (2012)[27] studied chemical composition of four goat breeds in Ethiopia and reported that the variation from the reported results could be accounted to the difference in the environmental factors, management including feeding and lactation stage at which samples were taken.

SDS-PAGE protein profile

Undigested and digested goat milk and kefir samples were analyzed by SDS-PAGE electrophoresis to estimate their molecular weight and to characterize the proteins. The SDS-PAGE gel of protein patterns from all samples, and MW markers is shown in Fig. 1. The SDS-PAGE analysis of goat milk and kefir showed similar protein profile that the applied fermentation has no significant impact on milk proteins. Comparing the undigested and the digested samples of goat milk and kefir showed that there were no visible bands of milk proteins after *in vitro* digestion. Also protein profiles of

digested samples and enzyme blank found to be same. The protein bands between 45-55 kDa and 20-25 kDa were corresponded for lipases, amylases and proteases from intestinal pancreatine. Gastric pepsin with 35 kDa molecular weight can be seen less intensive than other bands due to probable hydrolysis by the other enzymes during intestinal phase. Most high-MW components (>26.6 kDa) were relatively susceptible to pepsin degradation and were completely degraded within the first 1 h of hydrolysis. In addition, there were peptide stacks of digested samples, which cannot be seen at undigested samples. All of these data supported a complete hydrolysis of all milk proteins to smaller fragments, which is the lower separation limit of SDS-PAGE after *in vitro* digestion.

Hydrolyses degree

Proteolysis is the breakdown of large and complex proteins into smaller, simpler peptides. The average proteolytic activity of kefir culture in goat milk during kefir production was estimated by determination of free amino groups and results are presented in Fig. 2. The significant difference between the goat milk and kefir were observed in the beginning of hydrolyses (at 0 min) ($p< 0.05$). This could be attributable to differences in size of protein and the amount of free amino acids due to the microbial proteolysis during kefir production. The values obtained for gastric phase simulation at 120 min were 27.3% and 26.1% for goat milk and kefir, respectively. Hydrolyses degrees of goat milk and kefir in the beginning of intestinal phase (135 min) were determined as 59.4 % and 60.3%, respectively. At the end of simulated *in vitro* digestion degree of hydrolysis of goat milk and kefir were determined as 80.4% and 83.6%, respectively, indicating no significant difference ($p>0,05$).

Antioxidant activity

The antioxidant power of the samples has been assessed with the phosphomolybdenum reduction assay. The measurements and the ascorbic acid equivalents of reducing power were reported in Table

3. The digested kefir and goat milk has a significantly higher antioxidant activity than undigested samples ($p<0.05$). After digestion, the antioxidant activities of goat milk and kefir were increased 7 and 6 times, respectively. This increase could be resulted from the release of peptides and other bioactive compounds from the food matrix due to hydrolyses by proteolytic enzymes, which have potential antioxidant activity.[28,29] We didn't found significant differences between antioxidant activities of goat milk and kefir ($p>0.05$). Unlike, Turker, Kizilkaya and Arifoglu (2014)[30] and Li, Jiang, Yue, Wang, Wang and Su (2013)[31] demonstrated that kefir produced from goat milk exhibited higher antioxidant activity against to DPPH radical. The differential response of the foods in various antioxidant tests may be explained by the fact that the transfer of electrons/hydrogen from antioxidants occur at different redox potential in various assay systems and the transfer also depends on the structure of the antioxidants.[28] Also, we determined higher antioxidant activity for a replicate of goat milk and kefir, which were harvested in winter season. It can be explained by the changes of contents and composition of nutrient and bioactive compounds in lactation period. As lactation advances after freshening, fat and protein levels decrease with increasing milk yield, and when production declines in mid to late lactation, fat and protein concentrations increase.[32]

Calcium-binding capacity

Ca-binding capacities of goat milk, kefir, digested goat milk and digested kefir are presented in Table 2. The fermentation process did not have any effect on Ca-binding capacity of goat milk ($p>0.05$). However after *in vitro* digestion milk and kefir had an increase in Ca-binding capacities, also increasing in kefir was much more significant than milk ($p<0.05$). Milk proteins interact specifically or chelate strongly with minerals and trace elements. There are two different groups of proteins according to the type of interaction with metals. The first group includes proteins with specific binding site(s) for minerals such as lactoferrin and α-lactalbumin from milk. The other group

includes proteins rich in acidic clusters, that is, phosphoproteins such as phosvitin from caseins from milk. These proteins are able to sequester a high amount of divalent cations, in particular, through electrostatic interactions between highly negative clusters and positive charges of metals. These phosphate groups are important to the structure of the casein micelle. Calcium binding by the individual caseins is proportional to the phosphate content. Caseinophosphopeptides (CPPs) are bioactive phosphorylated casein- derived peptides latent until they are released from αs1-, αs2-, and β-casein during gastrointestinal digestion of milk. It has already been consistently proven that phosphorylated fragments of casein, caseinophosphopeptides (CPPs) can form organophosphate salts with minerals, such as iron, calcium, magnesium, manganese, copper, and selenium. Such sequestration prevents precipitation of minerals because it improves their solubility and stability under different physicochemical conditions in particular pH.[33,34] The phosphorylated sequences can be changed throughout enzymatic digestion of a casein mixture. Four structured regions have been identified in the calcium-bound β-casein-(1-25): residues Arg1 to Glu4 were found to be involved in a loop- type structure and residues Val8 to Glu11, Ser(P)17 to Glu20, and Glu21 to Thr24 were found to be implicated in β-turn conformations. Other factors are also important for calcium binding, in particular, number of negative charge, number of aminoacids, and sequence of phosphopeptide. It could be noted that *in vitro* gastrointestinal digestion was the predominant factor considering these structural and conformation differences. This phenomenon highly affects the biological responses of peptides.[33-35] Dimitrov (2009)[19] reported that the expected peptides with Ca-binding effect are mainly phospho-peptides and four amino acid residues could be phosphorylated: such as threonine, methionine, tyrosine and serine. The release of such small bioactive peptides as these with Ca-binding effect is in parallel with a strong proteolytic activity of the strains. They determined that casein fractions were subjected to proteolytic attacks during ripening of the cheeses. Other, Ca-binding proteins typically contain higher concentrations of aspartic acid and glutamic acid. The

presence of these amino acids, especially densely distributed in peptides or protein could create a favorable environment for divalent metal ions to coordinate, most likely due to the modification of the local charge density by the carboxylate groups in the side chains. Chen, Mu, Huang, Ruiyan, Zunying and Mingyong (2014)[35] studied on a Ca-binding peptide purified from protein hydrolysate of tilapia (*Oreochromis niloticus*) and determined that there were serum and femur calcium content in the rats fed with TSPH-Ca (tilapia scale protein hydrolysate calcium complex), were significantly higher than those of rats in control and $CaCO_3$ groups. In our study, the technological conditions as the temperature and the duration of kefir production could not be sufficient for the necessary break-down of the casein fractions by the enzymes and for release of sufficient quantity of bioactive peptides. However, the increasing Ca-binding activities of samples after *in vitro* digestion had been attributed to the peptides known to enhance the ability of calcium solubilization. These peptides were supposed to be formed *in vitro* following digestion of samples by gastric pepsin and intestinal pancreatin, relatively resistant to further proteolytic degradation by kefir culture during fermentation. However inconsistent and conflicting results have been reported in published studies.[33,34] Zhao and coworkers (2015)[36] purified a novel tripeptide from whey protein. The oxygen atom of the carbonyl group and nitrogen of amino group or imino group in phosphoamino acid tyrosine, acidic aspartic acid and branched chain threonine played important roles to the calcium-chelating capacity. The Ca-binding capacity of tripeptide purified hydrolysate complex was 134% compared with the protein hydrolysate. In another study, they purified a dipeptide to be phenyalanine-aspargine that the Ca-binding capacity increased by 116% when compared to the whey protein hydrolysate complex. Casein phosphopeptides and purified tripeptides or dipeptides have been observed by some investigators to enhance the absorption of calcium and the main reason may be the increased solubility of calcium in the intestine by the formation of calcium complexes with divalent minerals. In this respect, further studies may needed in the future. However, the relatively high price and

complexity of producing these peptides prevents their universal application as a calcium supplement. Consequently, exploiting novel calcium compositions based on other protein hydrolysates is an ideal alternative.[35] Our data showed that kefir provided Ca-binding peptides as a natural and cheap drink.

Antidiabetic activity

Kefir, digested goat milk and digested kefir were found to inhibit α-amylase (IC_{50}: 139±12, 27±5, 32±7 μg protein mL^{-1}, respectively) (Table 2). However, none of the samples showed higher than 50% inactivation on α-glucosidase enzyme within the concentration range studied, therefore IC_{50} value for α-glucosidase inhibition could not be estimated. The high α-amylase inhibition could cause many side effects depend on the excessive inhibition of pancreatic α-amylase results in abnormal bacterial fermentation of undigested carbohydrates in the colon.[37] Because of this, a moderate level of α-amylase inhibitors from kefir could be considered as a practical dietary approach to manage hyperglycemia and diabetes. There are many studies on the potential antidiabetic activity on the α-glucosidase and α-amylase of the peptides derived from the egg white, soya bean, legumes and fish.[37-40] In this study, microbial activity during fermentation and proteolytic activity of digestive enzymes during *in vitro* simulated digestion could be resulted with the release bioactive peptides. Huang and Wu (2010)[41] purified and characterized a small, including 17 amino acids, antidiabetic peptide from shark livers. They reported that this peptide could markedly decrease the level of fasting plasma glucose in alloxan-diabetic mice. Nakaoka and coworkers (2010)[42] found that Leu-Ser-Glu-Leu (LSEL) was the main active ingredient of globin digest that had an antidiabetic effect in induced diabetic mice. Although the inhibitory effects of the peptides derived from the goat milk have been demonstrated in our *in vitro* digestion system, further *in vivo* studies are suggested in explanation of the binding mechanisms between the peptides and active site of the enzyme.

Bile binding capacity

In vitro bile binding capacities of all samples were below 50%, so their IC_{50} values could not be estimated. Bile acids are acidic steroids that are biosynthesized from cholesterol in the liver and are reabsorbed by the terminal ileum. Thus, disruption of the reabsorption of bile acids through their binding to bile acid sequestrates leads to further degradation of cholesterol in the liver, reducing its level in the blood.[43] Different bile acid binding capacities obtained for the vegetable and fruit juices can be due to the fermentation process that leads to release of bioactive peptides from intact protein, or anionic or cationic nature of metabolites produced during digestion.[23,44] Barbana and coworkers (2011)[43] reported that lentil protein concentrate showed a greater bile binding capacity than that bound by reference standard cholestryramine. However, their values were expressed as μmol 100 mg^{-1} sample on dry basis and higher than that of our sample amount that considered in analyses. Ahmed and coworkers (2013)[10] reviewed the kefir and health from a contemporary perspective. They reported that Mann and Spoerry provided the earlier record of hypocholesterolemic activity on fermented dairy products in 1974 and that later on, a number of studies confirmed these facts, but some studies showed contradictory results on hypocholesterolemic activity of fermented dairy products, yet the majority of results indicated that fermented dairy products, especially kefir, possessed good hypocholesterolemic activity.

Conclusion

In this study, a standardized static *in vitro* digestion method, as an international consensus, recommended by the COST action INFOGEST, was applied on milk and kefir without salivary amylase but include simulated salivary fluids, and intestinal phase was performed with pancreatin instead of individual enzymes such as trypsin, chymotrypsin. In conclusion, there is an increasing trend to consume probiotic foods besides having positive bioactivities on mechanisms in the body, in

population. The health benefits of kefir are attributed to bioactive peptides derived parent protein. However, further works using animal models will be needed to conclude if any of these bioactivities may have a positive physiological role in metabolic regulation. However, the variations in qualities of traditional fermented beverages were affected from several factors such as utilization of different raw materials, manufacturing methods, natural microbiota and fermentation. A further investigation is needed to maintain standardized and efficient production for kefir processing.

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CAPTIONS

Table 1. Composition of stock solutions of simulated digestion fluids

Table 2. Proximate analyses of goat milk and kefir

Table 3. Antioxidant activity, Ca-binding capacity and α-amylase inhibition of samples[a]

Table 1. Composition of stock solutions of simulated digestion fluids

		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt solution added	Stock conc.	mL of Stock added to prepare 0.4 L	Final salt conc. In juice	mL of Stock added to prepare 0.4 L	Final salt conc. in juice	mL of Stock added to prepare 0.4 L	Final salt conc. in juice
	mol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}
KCl	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	0.5	3.7	1.35	0.9	0.9	0.8	0.8
$NaHCO_3$	1	6.8	13.68	12.5	25	42.5	85
NaCl	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$NH_4(CO_3)_2$	0.5	0.06	0.06	0.5	0.5	-	-
$CaCl_2(H_2O)_2$	0.3	-	1.5	-	0.15	-	0.6

[a]SSF, Simulated Salivary Fluid; SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid.

[b]The concentrations correspond to 400 mL and final volume was up to 500 mL after addition of the enzymes, bile and $CaCl_2(H_2O)_2$ during in vitro digestion.

[c]The necessary volume of $CaCl_2(H_2O)_2$ was added to the final mixture of the digestion medium to prevent precipitation.

Table 2. Proximate analyses of goat milk and kefir

	Goat Milk	Kefir
Total solids (%)	11.7 ±0.26	11.5 ± 0.13
Ash (%)	0.78 ± 0.01	0.72 ± 0.03
Protein (%)	3.01 ± 0.04	3.04 ± 0.05
Fat (%)	3.82 ± 0.26	3.80 ± 0.39
Casein (%)	2.66 ± 0.02	2.68 ± 0.04
Titratable acidity*	0.17 ± 0.01[a]	0.96 ± 0.04[b]

[a] Values are mean ± SD of the number (n = 6) of samples

[b] Superscripted letters after the mean within a row indicate significant difference ($p<0.05$).

*Titratable acidity is equivalent to % lactic acid

72 Table 3. Antioxidant activity, Ca-binding capacity and α-amylase inhibition of samples[a,b]

Samples	Antioxidant activity (μM ascorbic acid eqv mg^{-1} protein)	Ca-binding capacity (mg Ca mg^{-1} protein)	α-amylase inhibition* IC_{50} (μg protein mL^{-1})
Goat milk	352 ± 357[a]	0.086 ± 0.086[a]	2429 ± 182[a]
Kefir	437 ± 402[a]	0.062 ± 0.008[a]	139 ± 12[b]
Digested goat milk	2469 ± 647[b]	0.188 ± 0.016[b]	27 ± 5[c]
Digested kefir	2867 ± 633[b]	0.327 ± 0.006[c]	32 ± 7[c]

73 a Values are mean ± SD of the number (n = 6) of samples

74 b Means ± SD within a column with different superscripts differ significantly (p<

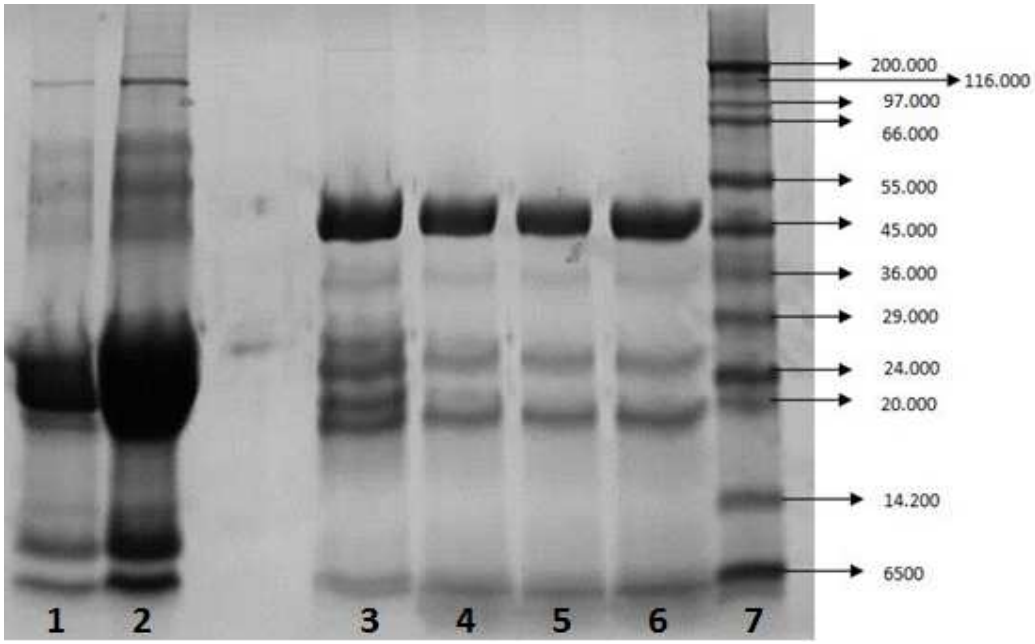
75 0.05)

76 * Equivalent Acarbose concentration is 6.36 μM.

CAPTIONS

Figure 1. SDS PAGE analysis of goat milk and kefir before and after in vitro digestion. Lanes: (1) Undigested goat milk, (2) Undigested kefir, (3) Enzyme blank, (4) Digested goat milk, (5) Digested kefir, (6) Digested kefir, (7) Protein molecular weight standard.

Figure 2. Hydrolyses degree (DH) of goat milk and kefir



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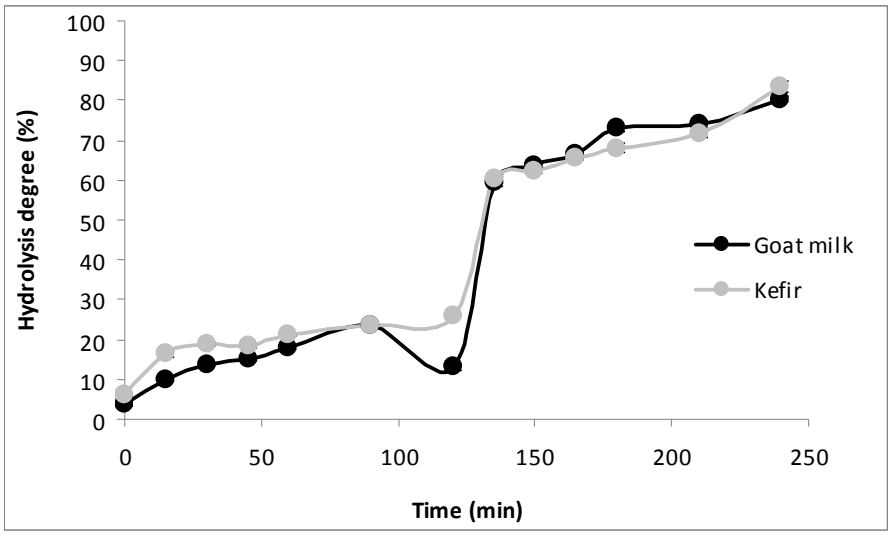

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