Profiling of Multiphosphorylated Peptides in Kefir and Their Release During Simulated Gastrointestinal Digestion

Maria Luisa Savastano,†,‡,§ Yufang Liu,†,§ Jennifer Mels,† Daniel Dittrich,† Sabrina Haus,† Sabrina Gensberger-Reigl,† and Monika Pischetsrieder*†,‡

†Department of Chemistry and Pharmacy, Food Chemistry, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Nikolaus-Fiebiger-Straße 10, 91058 Erlangen, Germany
‡Department of Agricultural, Food and Environmental Sciences (SAFE), University of Foggia, Via Napoli 25, 71100 Foggia, Italy

ABSTRACT: Casein phosphopeptides are multiphosphorylated milk peptides, which can have anticariogenic activity and improve mineral absorption by binding bivalent metal ions. The present study investigated phosphopeptides in kefir because fermentation may lead to their enhanced release from milk proteins. After selective enrichment by hydroxyapatite extraction, phosphopeptides and their phosphorylation degree were identified by matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) before and after enzymatic dephosphorylation. Peptide structures were determined by ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC–ESI–MS/MS) revealing 27 phosphopeptides in kefir, including nine peptides containing the motif pSpSpSEE, which binds minerals most efficiently. The majority (18) of phosphopeptides were derived from β-casein, but only three were derived from the most abundant milk protein αs1-casein. After simulated gastrointestinal digestion, MALDI-TOF-MS analysis detected eight putative phosphopeptides in kefir, four of which were assigned by UHPLC–ESI–MS/MS to αs2-casein124–133, αs2-casein137–146, β-casein30–40r and κ-casein147–161. These results indicate that kefir is a good dietary source of multiphosphorylated peptides.

INTRODUCTION

Kefir is produced by milk fermentation using either traditional kefir grains, which are a complex mixture of microorganisms, mainly yeasts, lactic acid and acetic bacteria, casein and polysaccharides, or industrial mother cultures prepared from grains, or commercial freeze-dried kefir starter cultures. Traditionally, kefir consumption has been linked with health-promoting effects such as anticarcinogenic, antimicrobial, and immunomodulatory activities, mainly attributed to the probiotic microflora and to its metabolites. Recently, peptide profiling in kefir revealed sixteen peptides originating from milk proteins with established bioactivity.

Casein phosphopeptides (CPPs) are a subgroup of bioactive milk peptides consisting of multiphosphorylated peptides, which can be endogenously present in milk, or released from precursor proteins by digestive enzymes, or by microbial enzymes during fermentation. CPPs can efficiently bind and solubilize bivalent metal ions such as Ca\(^{2+}\), thus enhancing the absorption of minerals in the gastrointestinal tract, improving tooth enamel remineralization in the oral cavity, and buffering the plaque pH. Indeed, it was reported that β-casein125–135P (P = phosphate group) and α-casein199–205P can bind 24 and 17 mol of Ca\(^{2+}\) and phosphate, respectively. Besides, CPPs have even higher affinity to zinc, iron, and copper. Calcium binding strongly correlates with the presence of phosphorylated serine residues, which are necessary to enhance the calcium absorption from the small intestine. Dephosphorylated peptides, in contrast, do not bind calcium. Calcium dissolution by phosphopeptides is due to the calcium-binding capacity of the phosphoserine (pS) residues, which form amorphous calcium-phosphate nanoclusters. Furthermore, the phosphorylated sequences make CPPs more resistant to gastrointestinal enzymatic digestion so that they are suitable carriers for metal ions.

Fermentation is expected to promote the formation of CPPs during kefir production compared to unfermented milk. It is therefore important to include CPPs with their potential effects on remineralization and calcium bioavailability in the evaluation of health-promoting effects of kefir. Based on these considerations, our goal was to identify multiphosphorylated peptides in kefir. Although liquid chromatography coupled to tandem mass spectrometry analysis (LC–MS/MS) or matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can efficiently achieve peptide profiling in milk and milk products, multiphosphorylated peptides are negatively discriminated in a

Received: November 20, 2018
Accepted: March 18, 2019
Published: May 1, 2019
complex peptide matrix. During LC–MS/MS analysis, the negative charge of the phosphate group leads to low ionization efficiency\(^{17,18}\) and ion suppression by nonphosphorylated peptides.\(^{19}\) For example, the list of 257 peptides previously identified in kefir included only mono- or diphosphorylated peptides besides nonphosphorylated sequences,\(^8\) although multiphosphorylated peptides were also expected to occur. Among the latter species, those containing the motif pSpSpSEE (pS = phosphoserine) are the most capable of calcium binding and have, therefore, particular biofunctional relevance.\(^9\) Enrichment of phosphopeptides has been achieved, for example, by immobilized metal-affinity chromatography, metal oxide-affinity chromatography with TiO\(_2\), ZrO\(_2\), Fe\(_2\)O\(_3\), or Al\(_2\)O\(_3\),\(^{20}\) or by hydroxyapatite.\(^{21}\) The present study used hydroxyapatite enrichment prior to MS-based phosphopeptide profiling to improve the coverage of multiphosphorylated peptides.

Although CPPs mainly act as anticariogenic agents in the oral cavity, they could also improve mineral resorption, if CPP structures are actually present in the gastrointestinal tract. The extensive degradation of bioactive milk peptides during digestion is well known, but gastrointestinal enzymes can also form novel bioactive sequences.\(^{22-25}\) Even though CPPs are more resistant to enzymatic hydrolysis than unphosphorylated peptides, subsequent formation and degradation of CPPs during pancreatin treatment has been observed.\(^9\) Therefore, our second purpose was to monitor the formation and degradation of kefir phosphorylated peptides during simulated gastrointestinal digestion.

## RESULTS AND DISCUSSION

### Profiling of Phosphopeptides in Kefir by MALDI-TOF-MS

To screen for phosphopeptides in kefir, peptides were isolated and phosphopeptides were enriched using hydroxyapatite extraction,\(^{26}\) which is based on the interaction of the phosphate groups with Ca\(^{2+}\) ions of the hydroxyapatite.\(^{27}\) This method is particularly suitable to cover multiphosphorylated peptides because the binding affinity increases with the number of phosphorylation sites.\(^{21}\) The phosphopeptide fraction was then analyzed by MALDI-TOF-MS, which has been successfully applied before to identify (multi-)phosphorylated peptides in milk.\(^6\)

To confirm that the detected peptides actually were phosphopeptides, MALDI-TOF-MS analysis of the samples was repeated after enzymatic dephosphorylation.\(^{17}\) Figure 1 shows a representative MALDI-TOF-MS spectrum after hydroxyapatite enrichment before (a) and after (b) dephosphorylation. Signals that were no longer detectable after the hydrolysis step were assigned to a phosphopeptide structure and subjected to further analysis. The disappearance of the phosphopeptide signal was accompanied by a new signal with a mass shift of \(-80\) Da or a multiple thereof corresponding to an unphosphorylated peptide formed by the loss of one or more HPO\(_{4}^{-}\) groups. The resulting signal pairs are marked by boxes of identical color in Figure 1. In total, 27 signal pairs were detected (please note that Figure 1 highlights predominant signals only).

#### Identification of Phosphopeptides by UHPLC–ESI–MS/MS

In the next step, the amino acid sequences of the detected phosphopeptides were identified by ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC–ESI–MS/MS). For this purpose, enhanced product ion mass spectra were recorded using the \(m/z\) values of the dephosphorylated peptides detected by MALDI-TOF-MS as the parent ion (Table 1). LC–ESI–QTrap-MS/MS analysis determined 18 dephosphopeptide structures. Because the quality of the LC–ESI–QTrap-MS/MS product-ion spectra of nine dephosphopeptides was not sufficient, corresponding structures were definitely identified by LC–ESI–QTOF-MS/MS analysis (Table 1, peptides no. 7, 12, 15, 19, 23, 24, 25, 26, and 27).

Figure 2 displays the resulting MS/MS spectrum of the multiphosphopeptide \(\beta\)-casein\(_{1-37}\) (Table 1, no. 26), with the relevant sequence scheme showing the amino acid bond cleavage leading to the experimentally detected fragments. As expected, y-ion residues predominated in the LC–ESI–QTOF-MS/MS spectrum compared to b-ions.\(^{28}\) The multiphosphopeptide \(\beta\)-casein\(_{1-37}\) is a breakdown product of \(\beta\)-casein, which contains five phosphorylated serine units and the sequence motif pSpSpSEE, which is most active in binding minerals.\(^8\)

Sequence structures could thus be attributed to 26 phosphopeptides detected by MALDI-TOF-MS (Table 1). Additionally, chromatographic separation of the dephosphorylated peptides revealed two different dephosphopeptides with \(m/z\) 1461 eluting at different retention times, which had not been distinguished by MALDI-TOF-MS. Subsequently, enhanced product-ion mass spectra identified \(\beta\)-casein\(_{1-21}\) (Table 1, peptide no. 13) and glycosylation-dependent adhesion molecule (GlyCAM)-1\(_{22-33}\) (Table 1, no. 14). In total, 27 phosphorylated peptides could be identified (Table 1). Finally, MALDI-TOF-MS detected one peptide with \(m/z\) 3161.6 and the putative phosphorylation degree of 4, but the quality of UHPLC–ESI–MS and MS/MS spectra was not sufficient for structural elucidation.

The mass of the detected phosphorylated peptides ranged between 1040 and 4636 Da; 14 out of 27 identified peptides were multiphosphorylated, with two to five pS residues. The present results confirmed that hydroxyapatite enrichment combined with the use of a suitable buffer is highly efficient for the analysis of multiphosphorylated peptides.\(^8\) As shown in Table 1, also partially phosphorylated peptides were identified in kefir, such as peptide GlyCAM-1\(_{12-41}\)P (no. 8) and \(\alpha\)-casein\(_{41-55}\)P (no. 17), both containing three possible phosphorylation sites. Additionally, \(\beta\)-casein\(_{10-21}\)IP and 3P,
Phosphopeptide profiling was achieved by MALDI-TOF-MS using two complementary methods (A and B) after hydroxypaptite enrichment, which were performed before and after dephosphorylation to assess the phosphorylation degree. The sequences identified by subsequent UHPLC–ESI–MS/MS analysis after enrichment with hydroxyapatite and enzymatic dephosphorylation are shown in the single-letter code and peptide masses are given in Da. Phosphorylated serine residues reported in the literature are underlined (S) and cleavage sites are indicated by dots.

Table 1. Identification of Phosphopeptides in Kefir

| no.  | [M + H]+ phosphorylated | [M + H]+ dephosphorylated | parent protein | position | sequence | phosphorylation degree |
|------|-------------------------|---------------------------|---------------|----------|----------|------------------------|
| 1    | 1040.4*                 | 960.4                     | k-casein      | 143–151  | T.VATLEDQPE.V   | 1                      |
| 2    | 1089.5*                 | 1009.5                    | β-casein      | 30–37    | K.IEKFOSEE.Q   | 1                      |
| 3    | 1139.5*                 | 1059.5                    | αs-casein     | 115–123  | N.SAEERLSMK    | 1                      |
| 4    | 1216.8*                 | 1136.7                    | β-casein      | 28–36    | N.KKIEKFOSEE.E | 2                      |
| 5    | 1217.8*                 | 1137.6                    | β-casein      | 29–37    | K.IEKFOSEE.Q   | 1                      |
| 6    | 1228.6*                 | 1148.5                    | β-casein      | 141–151  | E.STVATLEDQPE.V | 1                     |
| 7    | 1296.6*                 | 1136.5                    | β-casein      | 10–20    | P.GIEVEIS.SSE.E | 2                      |
| 8    | 1340.6*                 | 1260.5                    | GlyCAM-1      | 32–42    | E.DLIKQIEQIR.S | 1                      |
| 9    | 1345.6*                 | 1265.6                    | β-casein      | 10–21    | P.GIEVEIS.SSE.E | 1                      |
| 10   | 1393.8*                 | 1313.8                    | GlyCAM-1      | 43–53    | E.DLIKQIEQIR.S | 1                      |
| 11   | 1505.9*                 | 1265.8                    | β-casein      | 10–21    | P.GIEVEIS.SSE.E | 1                      |
| 12   | 1684.7*                 | 1604.7                    | αs-casein     | 64–78    | E.PSSSEIEFPSVEQ.K | 3                     |
| 13   | 1702.1*                 | 1461.8                    | β-casein      | 8–21     | N.VPGEVEIS.SSE.E.S | 3                      |
| 14   | 1541.8*                 | 1461.8                    | GlyCAM-1      | 22–33    | Q.FIRNLQINSNL.S | 1                      |
| 15   | 1728.8*                 | 1648.6                    | β-casein      | 25–37    | T.RINNKIEKFOSEE.Q | 2                     |
| 16   | 1781.9*                 | 1461.5                    | β-casein      | 8–21     | N.VPGEVEIS.SSE.E.S | 1                      |
| 17   | 1786.6*                 | 1626.7                    | αs-casein     | 41–55    | L.KDGQPEQEDQAME.D | 2                     |
| 18   | 1895.6*                 | 1575.8                    | β-casein      | 7–21     | L.NVGEVEIS.SSE.E.S | 4                      |
| 19   | 2029.9*                 | 1950.0                    | β-casein      | 22–37    | E.SITRINKKIEKFOSEE.Q | 1                     |
| 20   | 2196.8*                 | 1876.9                    | β-casein      | 7–24     | L.NVGEVEIS.SSE.E.SIT.R | 4                     |
| 21   | 2579.7*                 | 2260.0                    | β-casein      | 7–27     | L.NVGEVEIS.SSE.E.SITRIN.K | 4                     |
| 22   | 2665.1*                 | 2345.2                    | β-casein      | 1–21     | RELEELNVGEVEIS.SSE.E.S | 4                      |
| 23   | 2989.1*                 | 2669.4                    | β-casein      | 15–37    | E.SI.SSE.E.SITRINKKIEKFOSEE.Q | 4                     |
| 24   | 3068.9*                 | 2669.2                    | β-casein      | 15–37    | E.SI.SSE.E.SITRINKKIEKFOSEE.Q | 5                      |
| 25   | 3808.4*                 | 3488.7                    | αs-casein     | 49–79    | N.EEYSGSSE.EAETVEFKITVDDKHYQ.K | 4                     |
| 26   | 3906.5*                 | 3506.6                    | β-casein      | 7–37     | L.NVGEVEIS.SSE.E.SITRINKKIEKFOSEE.Q | 5                     |
| 27   | 4635.9*                 | 4236.1                    | β-casein      | 7–43     | L.NVGEVEIS.SSE.E.SITRINKKIEKFOSEEQ.OTED.E | 5                     |

aPhosphopeptide profiling was achieved by MALDI-TOF-MS using two complementary methods (A and B) after hydroxypaptite enrichment, which were performed before and after dephosphorylation to assess the phosphorylation degree. The sequences identified by subsequent UHPLC–ESI–MS/MS analysis after enrichment with hydroxyapatite and enzymatic dephosphorylation are shown in the single-letter code and peptide masses are given in Da. Phosphorylated serine residues reported in the literature are underlined (S) and cleavage sites are indicated by dots.

bDetected using method A. cDetected using method B.

Figure 2. Tandem mass spectrum of peptide β-casein26 (Table 1, no. 26) after dephosphorylation applying a precursor mass of m/z 877.4 (quadruple charge). The b- and y-ion series are single- to triple-charged. Potential phosphoserine residues reported in the literature are underlined.

β-casein10–20, β-casein21–33, and αs-casein64–78 were present, all with four possible phosphorylation sites, and the β-casein15–24P with five possible phosphorylation sites. Non-phosphorylated or partially phosphorylated caseins have been detected in the mammary gland.30 In the presence of cations, such as Mg2+, Ca2+, or Mn2+, casein kinase catalyzes the phosphorylation of partially phosphorylated caseins in the lactating mammary gland using adenosine triphosphate as a phosphate donor.30 Furthermore, caseins could be partially dephosphorylated due to the activity of alkaline phosphatase, which is associated with a high somatic cell count.31

The identified phosphopeptides are breakdown products of the main milk proteins, such as αs-casein (34% of the peptide fraction in milk), β-casein (25%), κ-casein (9%), αs-casein (8%), and GlyCAM-1 (Figure 3). The latter protein is a small phosphoglycoprotein, also known as proteose peptone component 3 (PP3) or lactophorin, which is the main component of the proteose peptone fraction of bovine milk (approximately 25%).32 GlyCAM-1 contains 135 amino acids and five pSSs (S25, S34, S38, S40, and S46), as shown in Figure 3. Phosphopeptides found in kefir derived mainly from β-casein (64% of the identified phosphopeptides), followed by αs-casein (11%) and GlyCAM-1 (11%), κ-casein (7%), and lastly, αs-casein with one fragment (Table 1). These results confirmed β-casein as the preferential substrate for microbial protein degradation during the transformation of milk to kefir,37 even though αs-casein is the most abundant milk protein.

Potential Bioactivity of the Identified Kefir Phosphopeptides. Caseins feature several possible phosphorylation
Followed by three tide, even though the released phosphopeptides (see Table 1). Phosphoserine residues are marked in red.

12 peptide sequences deriving from the breakdown of α-casein, which often occur in clusters (Figure 3). In particular, the high-polar acidic motif pSpSpSEE of CPPs is responsible for the remineralization of tooth enamel, calcium shows predominant bioactivity such as mineral binding, which particularly the high-polar acidic motif pSpSpSEE of CPPs in the literature are underlined (S) and cleavage sites are indicated by dots.

Besides the typical cluster sequence, other peptide factors are also important for the intestinal uptake of minerals, such as the total negative charge, the total number of amino acids, the phosphorylation degree, and the amino acid composition next to the phosphorylated region. In fact, a positive effect on bone mineralization has been shown for β-casein125 in vitro, although the peptide carries only one phosphorylated serine. Three phosphopeptides (Table 1, no. 2, 4, and 5) of α1-casein contained the mineral-binding motif E61 to pS67 and β-turn of residue E61 to pS67, which often occur in clusters (Figure 3). In our experiments, phosphopeptides after simulated gastrointestinal digestion and tested for phosphorylated and one double-phosphorylated peptides. Because the phosphopeptide β-casein125 found in kefir would effectively enhance the absorption of calcium in the intestine. Nevertheless, studies on the enhancement of mineral absorption by CPPs are controversial.

Identification of Phosphopeptides after Gastrointestinal Digestion. During gastrointestinal digestion, CPPs could not only be degraded but also newly released from their parent proteins. Thus, the present study subjected kefir to simulated gastrointestinal digestion and tested for phosphopeptides as described above. After the simulated gastrointestinal digestion of kefir, MALDI-TOF-MS analysis identified eight peptide pairs before and after enzymatic dephosphorylation that indicated the presence of phosphopeptides, including seven single-phosphorylated and one double-phosphorylated peptides. Because of the poor quality of the enhanced product-ion spectra of putative phosphopeptides generated by UHPLC–ESI–QTOF-MS, only four phosphopeptides were identified (Table 2), including two α1-casein breakdown sites, namely 13 in α1-casein, 9 in α1-casein, 5 in β-casein, and 2 in κ-casein, which often occur in clusters (Figure 3). In particular, the high-polar acidic motif pSpSpSEE of CPPs shows predominant bioactivity such as mineral binding, which is responsible for the remineralization of tooth enamel, calcium absorption, and calcium bioavailability. In our experiments, 12 peptide sequences deriving from the breakdown of β-casein (8) and α1-casein (1) contained the mineral-binding motif (Figure 3).

The phosphopeptide profile was obtained after hydroxyapatite enrichment by MALDI-TOF-MS using method B. Peptide sequences determined by UHPLC–ESI–QToF-MS/MS are shown in the single-letter code and peptides masses are given in Da. Phosphorylated serine residues reported in the literature are underlined (S) and cleavage sites are indicated by dots.

Table 2. Identification of Kefir Phosphopeptides after Simulated Gastrointestinal Digestion

| no. | [M + H]+ phosphorylated Da | [M + H]+ dephosphorylated Da | parent protein | position | phosphorylation degree |
|-----|---------------------------|-----------------------------|---------------|---------|------------------------|
| 1   | 1218.6                    | 1138.5                      | α1-casein     | 137–146 | K.KTVDMSTEV.F          | 1 |
| 2   | 1352.6                    | 1192.5                      | α1-casein     | 124–133 | L.NREKSTSEEN           | 2 |
| 3   | 1473.7                    | 1393.6                      | β-casein      | 30–40   | KIEKFOSEQQKT          | 1 |
| 4   | 1735.7                    | 1655.7                      | κ-casein      | 147–161 | LEDSPFVEQPIEENTV       | 1 |

*The phosphopeptide profile was obtained after hydroxyapatite enrichment by MALDI-TOF-MS using method B. Peptide sequences determined by UHPLC–ESI–QToF-MS/MS are shown in the single-letter code and peptides masses are given in Da. Phosphorylated serine residues reported in the literature are underlined (S) and cleavage sites are indicated by dots.

Figure 3. Amino acid sequences of milk proteins identified as parent proteins of phosphopeptides in kefir in the single letter code. Arrows indicate the released phosphopeptides (see Table 1). Phosphoserine residues are marked in red.
products (\(\alpha_2\)-casein,124–133 and \(\alpha_2\)-casein,137–146), as well as one \(\beta\)-casein- and one \(\kappa\)-casein fragment (\(\beta\)-casein,30–40 and \(\kappa\)-casein,147–161). The low number of identified peptides may reflect either the low concentration of CPPs in the digest and/or their low ionization efficiency. \(\kappa\)-Casein,147–161 had been detected before in a casein-derived byproduct after tryptic hydrolysis before and after simulated gastrointestinal digestion.\(^{42}\) Additionally, Picariello et al. found \(\beta\)-casein,30–40 in a peptide mixture, which survived the gastrointestinal digestion of casein in vitro.\(^{43}\) Although the list of phosphopeptides after simulated gastrointestinal digest is not comprehensive, these results indicate that phosphopeptides can be released from precursor proteins and peptides in the gastrointestinal tract after the ingestion of kefir. It has to be considered, however, that brush border enzymes at the intestinal microvilli including proteases and phosphatases are not covered by the applied model of gastrointestinal digestion and may further modify the phosphopeptide profile after digestion in vivo. Still, a certain stability of CPPs during digestion has been recently demonstrated when phosphorylated casein fragments were detected in the human plasma after the consumption of cheese.\(^{44}\) Furthermore, increased calcium-binding capacity compared to goat milk was observed for goat kefir in vitro digestion but not directly after production, indicating that CPPs were released during the simulated digest.\(^{45}\)

**CONCLUSIONS**

The present study demonstrated that multiphosphorylated casein peptides are present in kefir. Because CPPs are strong chelators for bivalent metal ions, the detected phosphopeptides may thus exert anticariogenic activity. Moreover, kefir-derived phosphorylated peptides were detected after digestion in vitro, indicating that they may also influence mineral absorption. Thus, the discussion of various health-promoting effects of kefir should also consider CPPs. Further studies are required, however, to determine the role of kefir-derived phosphopeptides in the oral cavity or in the gastrointestinal tract for mineral absorption in vivo.

**MATERIALS AND METHODS**

**Materials and Reagents.** Hydroxyapatite (CHT Ceramic Type I, 40 \(\mu\)m) was purchased from Bio-Rad (Munich, Germany). Formic acid (LC–MS grade), potassium chloride, 85% \(\alpha\)-phosphoric acid, ammonium hydrogen carbonate, 4-chloro-\(\alpha\)-cyanocinnamic acid, and 2,5-dihydroxybenzoic acid were obtained from Sigma-Aldrich (Taufkirchen, Germany). Acetonitrile (LC–MS grade) was purchased from Honeywell Fluka (Seelze, Germany) and 1,4-dithiothreitol was purchased from Carl Roth (Karlsruhe, Germany). Tris(hydroxymethyl)-aminomethane hydrochloride and ammonia (25%) were purchased from Acros Organics (Geel, Belgium) and urea was purchased from Merck Millipore (Darmstadt, Germany). Alkaline phosphatase (1500 U, grade I) from calf intestine was purchased from Roche Diagnostics (Mannheim, Germany). Tris(hydroxymethyl)aminomethane hydrochloride and ammonia (25%) were purchased from Acros Organics (Geel, Belgium) and urea was purchased from Merck Millipore (Darmstadt, Germany). The present study demonstrated that multiphosphorylated casein peptides are present in kefir. Because CPPs are strong chelators for bivalent metal ions, the detected phosphopeptides may thus exert anticariogenic activity. Moreover, kefir-derived phosphorylated peptides were detected after digestion in vitro, indicating that they may also influence mineral absorption. Thus, the discussion of various health-promoting effects of kefir should also consider CPPs. Further studies are required, however, to determine the role of kefir-derived phosphopeptides in the oral cavity or in the gastrointestinal tract for mineral absorption in vivo.

**Sample Preparation.** After centrifuging the kefir sample at 3850 rpm and 4 °C, for 30 min, to separate caseins and other insoluble components, the supernatant was passed through a sterile membrane filter (0.22 \(\mu\)m; Roth, Karlsruhe, Germany). The water-soluble fraction was divided into aliquots of 5 mL and frozen at −80 °C until use. For UHPLC–ESI–MS/MS, the aliquots were filtered once more using centrifugal filter units with 10 kDa molecular weight cutoff (Merck, Darmstadt, Germany) at 12 000 rpm and 4 °C, for 90 min, and stored at −80 °C until use.

**Phosphopeptide Enrichment by Hydroxyapatite Extraction.** Phosphopeptide enrichment was carried out according to an established protocol\(^{20}\) with some modifications. Briefly, the sample was incubated with hydroxyapatite for 15 min at room temperature in a thermomixer (Eppendorf, Hamburg, Germany) at 1000 rpm. Then, it was centrifuged at 7500 rpm for 3 min. Identical centrifugation conditions were applied for the washing steps. Then, 4 mg of the hydroxyapatite–phosphopeptide mixture was dissolved in 240 \(\mu\)L of 5% phosphoric acid solution to solubilize the phosphopeptides. Stage Tip extraction using C18 Empore Disk with 2–200 \(\mu\)L pipet tips (Eppendorf, Hamburg, Germany) was performed as described before.\(^{23}\) The peptide fraction was eluted with 10 \(\mu\)L of acetonitrile/0.1% formic acid in aqueous solution (60:40). The samples were extracted in quadruplicate, and the eluates were combined to a final volume of 40 \(\mu\)L and analyzed by MALDI-TOF-MS either directly or after dephosphorylation.

**Dephosphorylation of the Phosphopeptide Fraction.** Prior to dephosphorylation, samples were vacuum-dried (SpeedVac, Thermo Electron, Dreieich, Germany) and reconstituted with 50 \(\mu\)L of 0.4% ammonium bicarbonate solution (pH 9.0, adjusted with 25% ammonia).\(^{26}\) After adding 1 \(\mu\)L of alkaline phosphatase, the sample was incubated at 37 °C, for 1 h, in a thermomixer at 550 rpm. Following enzymatic hydrolysis, the sample was cooled on ice, purified once again by Stage Tip as described above, and analyzed by MALDI-TOF-MS and UHPLC–ESI–MS/MS.

**In Vitro Digestion of Kefir.** Kefir was digested in vitro as described previously.\(^{25}\) Briefly, the samples were treated with \(\alpha\)-amylase and mucin for 5 min, at 37 °C (oral phase). After adjusting the pH to 2–3, pepsin was added and incubated for 2 h, at 37 °C (gastric phase). Finally, pancreatin and bile extract were added, the pH was adjusted to 7 and digestion was continued for 2 h, at 37 °C (duodenal phase). As discussed before,\(^{23}\) this protocol differs only minimally from the standardized protocol,\(^{46}\) mainly by the concentration of the inorganic salts and the absence of organic additives. The standardized protocol had been applied before to analyze the peptide release from low-fat caprine kefir. After enrichment by hydroxyapatite extraction as described above, phosphopeptides were identified by MALDI-TOF-MS before and after dephosphorylation.

**MALDI-TOF-MS Analysis.** For MALDI-TOF-MS analysis, samples were mixed 1:1 with a matrix. The matrix consisted either of (A) 10 mg of 2,5-dihydroxybenzoic acid in 1 mL of acetonitrile/2.4% \(\alpha\)-phosphoric acid aqueous solution (50:50) or (B) 5 mg 2,5-dihydroxybenzoic acid in 1 mL of acetonitrile/0.1% trifluoroacetic acid (60:40). Method B detected some additional peptides, mainly in the lower molecular range. Samples (0.7 \(\mu\)L) were spotted onto a MALDI target (Bruker Daltonik, Bremen, Germany) and air-dried. Mass calibration was achieved by a mixture (1:5) of Bruker peptide standard...
solution II and the matrix. Analysis was performed on a Bruker Autoflex III MALDI-TOF mass spectrometer equipped with nitrogen laser in the positive reflector mode measuring a mass range of 600–5000 Da. The ions were time-delayed (140 ns) and accelerated at a voltage of 20 kV. Each measurement summed up 200–250 individual spectra generated manually from five different spots.

**UHPLC–ESI–QTrap-MS/MS Analysis.** UHPLC–ESI–QTrap-MS/MS analyses were performed on a Dionex Ultimate 3000 RS system (ThermoFisher Scientific, Idstein, Germany) coupled to a 4000 QTrap mass spectrometer (AB Sciex, Darmstadt, Germany) with an ESI source. Chromatographic separation was achieved with an Acquity BEH 300 C18 column (100 mm × 2.1 mm, 1.7 μm; Waters, Eschborn, Germany) at 30 °C. The mobile phase consisted of 0.1% formic acid aqueous solution (eluent A) and acetonitrile (eluent B) and the separation was conducted applying the gradient 6.0–5.0 min 2% B, 5.0–25.0 min 5–42.5% B, 25.0–25.5 min 42.5–95% B, and 25.5–30.0 min 95% B with a flow rate of 0.3 mL/min. The sample volume was 20 μL. To determine the retention time and charge state of the phosphopeptides previously detected by MALDI-TOF-MS, measurements were carried out in the positive enhanced mass spectra mode with the m/z 400–1200 and 1100–2000 ranges. The ion source voltage was set to 5000 V and declustering potential to 50 V. To obtain fragmentation spectra of the phosphopeptides, tandem mass spectra were acquired in the enhanced product-ion mode, with collision energies in the range of 20–30 V and collision energy spread of 10 V. Nitrogen was used for collision-induced dissociation. Acquisition of spectra with insufficient fragmentation was repeated with 40 V collision energy and 10 V collision energy spread. Analyst software version 1.6.3 was used for data acquisition and processing.

**UHPLC–ESI–QTOF-MS Analysis.** Phosphopeptides were analyzed by microUHPLC (Ultimate 3200 RS, ThermoFisher Scientific, Idstein, Germany) interfaced with an ESI–QTOF mass spectrometer (6600 TripleTOF, AB Sciex, Darmstadt, Germany). A Triart C18 column (100 × 0.5 mm, 3 μm; YMC Europe, Dinslaken, Germany) equipped with a precolumn containing the same material, operating with a flow rate of 30 μL/min and 35 °C column temperature was chosen for chromatographic separation. The mobile phase consisted of 0.1% formic acid solution (eluent A) and acetonitrile containing 0.1% formic acid (eluent B) and the separation was conducted applying the gradient 15.0 min 2% B, 5.0 min 2% B, 5.0 min 42.5% B, 55.5 min 95% B, and 65.0 min 95% B. The sample volume was 4 μL. Ion spray voltage was set to 5200 V and declustering potential to 80 V. Nitrogen was used as the collision gas in the MS/MS experiments for peptide sequencing. Raw data were processed using PeakView (version 2.2, AB Sciex, Darmstadt, Germany) and Protein Pilot (version 5.0, AB Sciex, Darmstadt, Germany).

**Database Search.** For structure identification, MS/MS spectra were searched against the UniProt database by mMass (Open Source Mass Spectrometry Tool, version 5.5) and Protein Pilot software. Because peptide sequences randomly generated by kefir microflora were scarce in the online databases, we manually searched the phosphopeptide sequences of known bovine phosphorylated proteins to achieve an unambiguous identification. The amino acid sequences of phosphopeptides were obtained by de novo peptide sequencing. First, a bovine milk peptide database was generated by an ion fragment calculator in mMass software including precursor, position, and mass of all possible peptides derived from αs1- (P02662), αs2- (P02663), β- (P02666), and κ-casein (P02668), serum albumin (P02769), GlyCAM-1 (P80195), and osteopontin (P31096) without enzyme restriction. Peak picking was applied for signals with signal-to-noise ratios of four or higher. The peptides were manually identified by comparing the m/z values of product ions determined in the experimental spectra with the theoretical m/z values of product ions of all possible sequences in the database with the same m/z value of the precursor ion. Mass tolerance was set to 0.2 Da for precursor-ion spectra and 0.2 and 0.02 Da for product-ion spectra. Only peptides identified in all three kefir batches were included.

## Author Information

**Corresponding Author**

*e-mail: monika.pischetsrieder@fau.de. Phone: +49-9131-8565592.

**ORCID**

Monika Pischetsrieder: 0000-0003-3465-4179

### Author Contributions

M.L.S. and Y.L. contributed equally to the work.

### Funding

Financial support by the China Scholarship Council (CSC) to Y.L. is gratefully acknowledged (no. 201206790009).

### Notes

The authors declare no competing financial interest.

### Acknowledgments

We thank Dr. Xiang from the Institute of Biochemistry, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), for providing the MALDI-TOF mass spectrometer and Christine Meissner for proofreading the manuscript.

### Abbreviations

CPP, casein-phosphopeptides; ESI, electrospray ionization; GlyCAM, glycosylation-dependent adhesion molecule; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS/MS, tandem mass spectrometry; P, phosphate group; pS, phosphoserine; UHPLC, ultrahigh-performance liquid chromatography.

### References

1. Otles, S.; Cagindi, O. Kefir: A probiotic dairy-composition, nutritional and therapeutic aspects. *Pak. J. Nutr.* 2003, 2, 54–59.

2. Farnsworth, E. R. Kefir—a complex probiotic. In *Food Science and Technology Bulletin: Functional Foods*; Gibson, G. R., Ed.; IFIS Publishing: Reading, 2005; Vol. 2, pp 1–17.

3. Leite, A. M. d. O.; Miguel, M. A. L.; Peixoto, R. S.; Rosado, A. S.; Silva, J. T.; Paschoalin, V. M. F. Microbiological technological and nutritional and therapeutic aspects. *Braz. J. Microbiol.* 2013, 44, 341–349.

4. Ebner, J.; Asi Aslan, A.; Fedorova, M.; Hoffmann, R.; Kögłe, A.; Pischetsrieder, M. Peptide profiling of bovine kefir reveals 236 unique peptides released from caseins during its production by starter culture or kefir grains. *J. Proteomics* 2015, 117, 41–57.

5. Baum, F.; Ebner, J.; Pischetsrieder, M. Identification of multiphosphorylated peptides in milk. *J. Agric. Food Chem.* 2013, 61, 9110–9117.

6. Meisel, H.; Meisel, H.; Fairweather-Tait, S.; FitzGerald, R. J.; Hartmann, R.; Lane, C. N.; McDonagh, D.; Teucher, B.; Wal, J. M.
Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects. Br. J. Nutr. 2003, 89, 351–359.
(7) Dallas, D. C.; Citerne, F.; Tian, T.; Silva, V. L. M.; Kalanetra, K. M.; Frese, S. A.; Robinson, R. C.; Mills, D. A.; Barile, D. Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins. Food Chem. 2016, 197, 273–284.
(8) FitzGerald, R. J. Potential use of caseinophosphopeptides. Int. Dairy J. 1998, 8, 451–457.
(9) Nongonierma, A. B.; Fitzgerald, R. J. Biofunctional properties of caseinophosphopeptides in the oral cavity. Caries Res. 2012, 46, 234–267.
(10) Sato, R.; Noguchi, T.; Naito, H. The necessity for the phosphate portion of casein molecules to enhance Ca absorption from the small-intestine. Agric. Biol. Chem. 1983, 47, 2415–2417.
(11) Berrocal, R.; Chanton, S.; Juillerat, M. A.; Favillare, B.; Scherz, J.-C.; Jost, R. Tryptic phosphopeptides from whole casein. II. J. Agric. Food Chem. 1998, 46, 3147–3152.
(12) Holt, C.; Wahlgren, M. N.; Drakenberg, T. Ability of a β-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous decalcium phosphate nanoclusters. J. Agric. Food Chem. 2005, 53, 979–985.
(13) Bhandari, A.; Lebrilla, C. B.; Barile, D.; German, J. B. Peptidomic profiling and the bioactivity character of yogurt in the simulated gastrointestinal digestion. J. Proteome Res. 2003, 2, 1599–1607.
(14) Marsset-Baglieri, A.; Benamouzig, R.; Tome, D.; Leonil, J. Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. Am. J. Clin. Nutr. 2013, 97, 1314–1323.
(15) Sánchez-Rivera, L.; Diezhandino, I.; Gómez-Ruiz, J. Á.; Fresno, J. M.; Miralles, B.; Recio, I. Peptidomic study of Spanish blue cheese (Valdeon) and changes after simulated gastrointestinal digestion. Electrophoresis 2014, 35, 1627–1636.
(26) Pinto, G.; Caira, S.; Cuollo, M.; Fierro, O.; Nicolai, M. A.; Chianese, L.; Addeo, F. Lactosylated casein phosphopeptides as specific indicators of heated milks. Anal. Bioanal. Chem. 2012, 402, 1961–1972.
(27) Bernardi, G.; Cook, W. H. Separation and characterization of the two high density lipoproteins of egg yolk, alpha and beta-lipoprotein. Biochim. Biophys. Acta 1960, 44, 96–105.
(28) Steen, H.; Mann, M. The ABC’s (and XYZ’s) of peptide sequencing. Nat. Rev. Mol. Cell Biol. 2004, 5, 699–711.
(29) Singh, V. N.; Dave, S. S.; Venkatasubramanian, T. A. Effect of puromycin in vitro on the phosphorylation of precursor protein in lactating rat mammary-gland slices. Biochim. J. 1967, 104, 48C–50C.
(30) Bingham, E. W.; Farrel, H. M., Jr. Casein kinase from the Golgi apparatus of lactating mammary gland. J. Biol. Chem. 1974, 249, 3647–3651.
(31) Pinto, G.; Caira, S.; Nicolai, M. A.; Mauriello, R.; Cuollo, M.; Pirisi, A.; Piredda, G.; Chianese, L.; Addeo, F. Proteolytic and partial dephosphorylation of casein are affected by high somatic cell counts in sheep milk. Food Res. Int. 2013, 53, 510–521.
(32) Paquet, D. Revue bibliographique : la fraction protéose-peptones du lait. Lait 1989, 69, 1–21.
(33) Tsuchita, H.; Suzuki, T.; Kuwata, T. The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. Br. J. Nutr. 2001, 85, 5–10.
(34) Cross, K. J.; Huq, N. L.; Palamara, J. E.; Perich, J. W.; Reynolds, E. C. Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. J. Biol. Chem. 2005, 280, 15362–15369.
(35) Zong, H.; Peng, L.; Zhang, S.; Lin, Y.; Feng, F. Effects of molecular structure on the calcium-binding properties of phosphopeptides. Eur. Food Res. Technol. 2012, 235, 811–816.
(36) Ferraretto, A.; Gravaghi, C.; Fiorilli, A.; Tettamanti, G. Casein-derived bioactive phosphopeptides: role of phosphorylation and primary structure in promoting calcium uptake by HT-29 tumor cells. FEBS Lett. 2003, 551, 92–98.
(37) Pihlanto, A.; Korhonen, H. Bioactive peptides and proteins. In Advances in Food and Nutrition Research; Taylor, S. D., Ed.; Elsevier Inc.: San Diego, 2003; Vol. 47, pp 175–276.
(38) Cross, K. J.; Huq, N. L.; Bicknell, W.; Reynolds, E. C. Cation-dependent structural features of beta-casein-(1-25). Biochem. J. 2001, 356, 277–286.
(39) Huq, N. L.; Cross, K. J.; Reynolds, E. C. A 1H-NMR study of the casein phosphopeptide alpha s1-casein(59-79). Biochim. Biophys. Acta. 1995, 1247, 201–208.
(40) Korhonen, H.; Pihlanto, A. Bioactive peptides: Production and functionality. Int. Dairy J. 2006, 16, 945–960.
(41) Caroli, A.; Bulgari, B.; Chessa, S.; Cocchi, D.; Tulipano, G. In vitro evaluation of caseinophosphopeptides from different genetic variants on bone mineralization. Ital. J. Anim. Sci. 2009, 8, 42–44.
(42) Cruz-Huerta, E.; García-Nebot, M. J.; Miralles, B.; Recio, I.; Amigo, L. Caseinophosphopeptides released after tryptic hydrolysis versus simulated gastrointestinal digestion of a casein-derived by product. Food Chem. 2015, 166, 648–655.
(43) Picariello, G.; Ferranti, P.; Fierro, O.; Mamone, G.; Caira, S.; Di Luccia, A.; Monica, S.; Addeo, F. Peptides surviving the simulated gastrointestinal digestion of milk proteins: biological and toxicological implications. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2010, 876, 295–308.
(44) Caira, S.; Pinto, G.; Vitaglione, P.; Dal Piaz, F.; Ferranti, P.; Addeo, F. Identification of casein peptides in plasma of subjects after a cheese-enriched diet. Food Res. Int. 2016, 84, 108–112.
(45) Nehir, E. L.; Karakaya, S.; Simsek, S.; Dupont, D.; Menafati, E.; Eker, A. T. In vitro digestibility of goat milk and kefir with a new standardised static digestion method (INFOSTG cost action) and bioactivities of the resultant peptides. Food Funct. 2015, 6, 2322–2330.
(46) Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; Dufour, C.; Egger, L.; Golding, M.; Karakaya, S.; Kirkhus, B.; Le Feunteun, S.; Lesmes, U.; Macierzanka, A.; Mackie, A.; Marze, S.; McClements, D. J.; Ménard, O.; Recio, I.; Santos, C. N.; Singh, R. P.; Vegarud, G. E.; Wickham, M. S. J.; Weitschies, W.; Brodkorb, A. A standardised static in vitro digestion method suitable for food—an international consensus. *Food Funct.* **2014**, *5*, 1113−1124.

(47) Simsek, S.; Sánchez-Rivera, L.; El, S. N.; Karakaya, S.; Recio, I. Characterisation of in vitro gastrointestinal digests from low fat caprine kefir enriched with inulin. *Int. Dairy J.* **2017**, *75*, 68−74.

(48) The UniProt Consortium. UniProt: A hub for protein information. *Nucleic Acids Res.* **2015**, *43*, D204−D212.

(49) Niedermeier, T. H. J.; Strohalm, M. mMAs a software tool for the annotation of cyclic peptide tandem mass spectra. *PLoS One* **2012**, *7*, No. e44913.