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Peptide profiling of bovine kefir reveals 236 unique peptides released from caseins during its production by starter culture or kefir grains

Jennifer Ebner[a], *Ayşe Aşçı Arslan*[b], *Maria Fedorova*[c], *Ralf Hoffmann*[c], *Ahmet Küçükçetin*[b], *Monika Pischetsrieder*[a,*]

[a]*Food Chemistry Unit, Department of Chemistry and Pharmacy, Emil Fischer Center, Friedrich-Alexander Universität Erlangen-Nürnberg (FAU), Schuhstr. 19, 91052 Erlangen, Germany*
[b]*Food Engineering Department, Faculty of Engineering, Akdeniz Üniversitesi, Dumlupinar Boulevard, 07059 Antalya, Turkey*
[c]*Center for Biotechnology and Biomedicine, Universität Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany*

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ABSTRACT

Kefir has a long tradition in human nutrition due to its presupposed health promoting effects. To investigate the potential contribution of bioactive peptides to the physiological effects of kefir, comprehensive analysis of the peptide profile was performed by nano-ESI-LTQ-Orbitrap MS coupled to nano-ultrahigh-performance liquid chromatography. Thus, 257 peptides were identified, mainly released from β-casein, followed by α_{S1}-, κ-, and α_{S2}-casein. Most (236) peptides were uniquely detected in kefir, but not in raw milk indicating that the fermentation step does not only increase the proteolytic activity 1.7- to 2.4-fold compared to unfermented milk, but also alters the composition of the peptide fraction. The influence of the microflora was determined by analyzing kefir produced from traditional kefir grains or commercial starter culture. Kefir from starter culture featured 230 peptide sequences and showed a significantly, 1.4-fold higher proteolytic activity than kefir from kefir grains with 127 peptides. A match of 97 peptides in both varieties indicates the presence of a typical kefir peptide profile that is not influenced by the individual composition of the microflora. Sixteen of the newly identified peptides were previously described as bioactive, including angiotensin-converting enzyme (ACE)-inhibitory, antimicrobial, immunomodulating, opioid, mineral binding, antioxidant, and antithrombotic effects.

Biological significance
The present study describes a comprehensive peptide profile of kefir comprising 257 sequences. The peptide list was used to identify 16 bioactive peptides with ACE-inhibitory, antioxidant, antithrombotic, mineral binding, antimicrobial, immunomodulating and opioid activity in kefir. Furthermore, it was shown that a majority of the kefir peptides were not endogenously present in the raw material milk, but were released from milk caseins by proteases of the microbiota and are therefore specific for the product. Consequently, the proteolytic activity and the composition of the peptide profile can be controlled by the applied microflora (grains or starter culture). On

* *Corresponding author at:* Henriette Schmidt-Burkhardt Chair of Food Chemistry, Food Chemistry Unit, Department of Chemistry and Pharmacy, Emil Fischer Center, Friedrich-Alexander Universität Erlangen-Nürnberg (FAU), Schuhstr. 19, 91052 Erlangen, Germany. Tel.: +49 9131 8524102; fax: +49 9131 8522587.
E-mail address: monika.pischetsrieder@fau.de (M. Pischetsrieder).

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the other hand, a considerable portion of the peptide profile was identified to be typical for kefir in general and independent from production parameters.
In summary, the generated kefir peptide profile helped to reveal its origin and to identify bioactive peptides in kefir, which may advance the understanding of health benefits of this food product. The results further indicate that subsets of the kefir peptide list can be used as markers to control food authenticity, for example, to distinguish different types of kefir.

1. Introduction

Kefir, a refreshing, slightly carbonated and alcoholic fermented milk beverage with a sour and tart flavor, has a very long tradition in human nutrition. Originating in the Caucasian mountains, it has been consumed for its presupposed health benefits for thousands of years [1]. Some health promoting effects could indeed be scientifically confirmed in vivo over the last years, like immunological [2,3] and antioxidant [4,5] effects. Many of these are prescribed to probiotic functions, where the microorganisms themselves have the most important impact, but also the exopolysaccharide kefiran and other byproducts of the microbiological metabolism [6]. However, less is known about the role of bioactive peptides in kefir, which can be formed during fermentation. Peptides derived from milk proteins are a rich source of functional food ingredients. Among others, opioid, immunomodulating, antioxidant, and antimicrobial activities have been described [7]. Especially hypotensive and mineral-binding milk peptides are well investigated and have already been applied as bioactive ingredients in functional food products [8].

Untargeted profiling of endogenous milk peptides revealed that the composition is far more complex than expected [9]. Baum et al. identified 248 casein-derived peptides in raw milk including 22 peptides with an established physiological function [10]. Using a different mass spectrometric approach, Dallas et al. revealed the presence of 159 peptides in raw milk [11]. Most of the peptides in their study were derived from caseins, but also peptides from untypical or low abundant milk proteins were detected. It can be assumed that the number of bioactive peptides increases during the production of fermented milk products compared to raw milk and that the composition of the peptide fraction changes due to the proteolytic action of the employed microorganisms [12]. Some studies investigated bioactive peptides in traditional fermented milk products and identified, for example, the major angiotensin-converting enzyme (ACE)-inhibitory peptide of Manchego cheese or antimicrobial peptides in different cheese varieties [13,14]. Furthermore, Quiros et al. revealed two ACE-inhibitory peptides in caprine kefir [15]. Compared to some other fermented dairy products, kefir showed a higher rate of proteolysis [16] and could, therefore, be a good source of physiologically active peptides.

Different starter cultures for milk fermentation influence the peptide release, so that the choice of fermentation conditions may be expected to control the composition of bioactive peptides. Kefir is traditionally manufactured using kefir grains [6]. The microbial composition of the grains is very heterogeneous and depends on many factors, especially the origin of the primary grains [17]. Mainly strains from, *Lactobacilli*, *Lactococci*, *Leuconostoc* and acetic acid bacteria, but also different yeasts are present, all embedded in a polysaccharide matrix, but the overall microbiological composition has not been completely elaborated yet [1]. Milk from different species can be used for fermentation. It is inoculated either directly with kefir grains or with mother cultures prepared from grains to facilitate the production. In industrial production, however, mostly well-defined starter cultures are applied.

The purpose of the present study was the comprehensive analysis of the peptide profile of kefir from bovine milk. The resulting peptide database can be used to identify bioactive peptides in kefir. Because the peptide composition can be modulated by the applied microbiota used for fermentation, two typical kefir varieties were compared: a traditional product prepared with kefir grains and a kefir product prepared with industrially used starter cultures.

2. Material and methods

2.1. Kefir production

The kefir grains used in this research were obtained from the Department of Food Engineering, Akdeniz University, Turkey. Kefir grains were activated by inoculation into sterilized milk (4%) and subsequent incubation at 25 °C. After 24 h, the kefir grains were sieved out and placed in fresh sterilized milk. This step was repeated over three consecutive days. Commercial kefir starter culture was purchased from Danisco Biolacta (Olztyn, Poland) and consisted of *Lactococcus lactis* ssp., *Leuconostoc* ssp., *Streptococcus thermophilus*, *Lactobacillus* ssp., kefir yeast, and kefir grain microflora according to the product information.The raw bovine milk used for kefir production was skimmed to 0.1% fat. The standardized milk samples were heated at 90 °C for 5 min with a plate exchange heating system, subsequently cooled to 25 °C incubation temperature and inoculated with the above mentioned kefir grains or commercial starter culture. After inoculation, the milk samples were incubated at 25 °C until the pH decreased to 4.8. Experiments were performed twice, unless noted otherwise.

2.2. Microbiological analysis

Microbiological analyses were run in duplicates for each kefir sample. Ringer solution (1/4 strength) was used to prepare the dilutions for the microbiological analyses. Total numbers of viable microorganisms were evaluated on Plate Count Agar (PCA; Merck, Darmstadt, Germany). PCA plates were incubated aerobically at 30 °C for 2 days [18]. *Lactobacilli* counts were performed on MRS medium (pH 6.5 ± 0.2; Merck, Darmstadt, Germany) at an incubation temperature of 30 °C under anaerobic conditions (5%

CO_2) for 3 days. *Lactococci* enumerations were carried out on M17 medium (Merck, Darmstadt, Germany) at an incubation temperature of 30 °C under anaerobic conditions (5% CO_2) for 3 days [19]. Yeast counts were performed on Yeast Extract Glucose Chloramphenicol (YGC) agar being selective for yeasts with incubation at 25 °C under aerobic conditions for 3–5 days [20]. Mayeux, Sandline and Elliker (MSE) agar, a selective medium for *Leuconostoc*, was used for enumeration of *Leuconostoc* at an incubation temperature of 22 °C for 5 days und aerobic conditions [21]. Acetic acid bacterial counts were performed on *Acetobacter peroxydans* medium (APM) with aerobic incubation at 25 °C for 3–5 days [20].

2.3. Determination of the proteolytic activity

The proteolytic activities of microorganisms in two independent batches per kefir variety were assessed in duplicate by measuring the total amount of released amino acids and peptides by the o-phthaldialdehyde (OPA) method to quantify the increased content of amino groups [22]. For this purpose, 0.95 g of sodium tetraborate, 0.5 g of sodium dodecyl sulfate, and 0.1 mL of 2-mercaptoethanol were diluted with water to a final volume of 50 mL (solution A). Then, 40 mg of OPA was dissolved in 1 mL of methanol (solution B). Solutions A and B were mixed (solution C). Kefir samples were vortexed at 30 °C for 1 min (1 mL) and added to 2.2 mL of 0.68 N trichloroacetic acid. The mixture was kept at room temperature for 10 min and centrifuged for 10 min at 16,200 *g* and 4 °C. After centrifugation, an aliquot of 50 μL was taken from the supernatant and added directly to 1 mL of solution C in a 1.5 mL quartz cuvette. The solution was mixed briefly and the absorbance at 340 nm was measured. Four samples of raw milk and unfermented heat-treated skim milk, respectively, were analogously analyzed to monitor the proteolytic activity before fermentation.

2.4. Peptide extraction

Firstly, the samples were centrifuged at 1100 *g* and 4 °C for 30 min to remove coagulated caseins. The supernatant was filtered using sterile membrane filters with a diameter of 0.22 μm (PVDF, Carl Roth, Karlsruhe, Germany). The water-soluble kefir fraction of one representative sample per kefir variety was used for peptide extraction by means of C18-SPE (200 mg C18-E, 3 mL, Phenomenex, Aschaffenburg, Germany). For this purpose, 1 mL of sample was loaded onto a SPE cartridge preconditioned with 2 mL of ACN (LC–MS grade, VWR International, Darmstadt, Germany) followed by 2 mL of 0.1% TFA (HPLC grade, Sigma-Aldrich, Taufkirchen, Germany) in purified water. Peptide elution was achieved with 2 mL of a mixture of ACN and 0.1% TFA (60:40) after a washing step with 2 mL 0.1% TFA. This was repeated three times for both samples.

For MALDI-TOF-MS, the peptide extract was applied directly. For analysis by nano-ultra-HPLC (UPLC) with hyphenated nano-ESI-MS/MS, the extracts were first frozen at −80 °C and then dried using a SpeedVac system (SPD 121P, Thermo Fisher Scientific, Bremen, Germany). The extracts were dissolved in 60% ACN containing 0.5% formic acid (FA) and diluted with 3% ACN containing 0.1% FA to a concentration of 25 nmol/mL (under the assumption of an average molecular weight of 1500 Da). Before injection, ultrafiltration was performed using centrifugal filter units with a 10 kDa cutoff (Merck, Darmstadt, Germany) for the removal of whey proteins, followed by a 1:5 dilution of the samples.

2.5. MALDI-TOF-MS

MALDI matrix consisted of 5 mg of α-cyano-4-hydroxycinnamic acid (HCCA, Sigma-Aldrich, Taufkirchen, Germany) in 1 mL of a 60:40 mixture of ACN and 0.1% TFA containing 5 mM ammonium dihydrogen phosphate. After mixing samples equally with matrix, aliquots of 0.7 μL each were spotted onto a ground steel target plate (Bruker Daltonik, Bremen, Germany) and air-dried. MALDI-TOF-MS was performed on a Bruker Autoflex mass spectrometer with a nitrogen laser in the positive reflector mode and after delayed extraction (140 ns). Acceleration voltage was set to 20 kV. For every sample, 300 single spectra were automatically generated from different spot positions in a mass range from 600 to 5000 Da and summed up. External calibration was carried out using Bruker peptide calibration standard II. Baseline subtraction and smoothing were carried out for the generated spectra by the *flexAnalysis* software from Bruker and peak picking was applied for signals with signal-to-noise ratios of 6 or higher.

2.6. Analysis by nano-UPLC-nano-ESI-MS/MS

Peptide separation was performed on a nanoAcquity UPLC (Waters, Eschborn, Germany) coupled to an LTQ Orbitrap XL ETD mass spectrometer with a nano-ESI source (Thermo Fisher Scientific, Bremen, Germany). Experiments were carried out in triplicate for both kefir varieties. A portion of 10 μL of each peptide sample was loaded onto a trap column (nanoAcquity symmetry C18, 180 μm × 20 mm, particle diameter 5 μm) with a flow rate of 10 μL/min and then separated using a nanoAcquity UPLC BEH 130 column (C18, 75 μm × 100 mm, and particle diameter 1.7 μm). The flow rate was set to 400 nL/min and separation was achieved by gradient elution as follows: 0–1.0 min 3% B, 1.0–1.1 min 3–9% B, 1.1–4.0 min 9–9.9% B, 4.0–14.0 min 9.9–17.1% B, 14.0–14.5 17.1–18% B, 14.5–14.6 min 18–20.7% B, 14.6–17.7 min 20.7–22.5% B, 17.7–20.7 min 22.5–25.6% B, 20.7–25,7 min 25.6–30.6% B, 25.7–28.5 min 30.6–37.8% B, 28.5–31.5 min 37.8–81% B, with eluent A as aqueous 0.1% FA and eluent B as ACN containing 0.1% FA. The transfer capillary temperature was 200 °C. Tube lens voltage was set to 120 V and ion spray voltage to 1.5 kV using online-coupled silica-coated nano-ESI emitter (New Objective, Berlin, Germany). Precursor ion survey scans were carried out in the positive mode in a mass range of *m/z* 400–2000 and with an orbitrap resolution of 60,000. For product ion scans, CID was applied for the six most abundant ions in each MS scan with a minimum charge state of +2 (isolation width 2.0 Da, activation Q 0.25, normalized collision energy 35%, activation time 30 ms).

The results from the product ion scans were subjected to a MASCOT search against SwissProt database (Release 2013_01) for peptide identification. Search parameters were: taxonomy mammals, no enzyme, no missed cleavages, no fixed modifications, oxidation (methionine) and phosphorylation (serine, threonine) as variable modifications, 10 ppm mass tolerance for precursor ions and 0.8 Da for product ions, charge minimum +2. Evaluation of the results was carried out by the Proteome

Discoverer software (Thermo Fisher Scientific, Dreieich, Germany). For reliable peptide identification, the following approach was applied: all peptides with a score >10 were preselected. Then it was checked if the rank of peptide assignment for these candidates was "1" and if the assigned peptide was from bovine origin. If these two conditions were fulfilled, the product ion spectra were verified manually by comparison with the calculated fragment ion masses.

2.7. Direct infusion ESI-MS/MS experiment for a selected HPLC fraction

RP-HPLC was performed on a JASCO HPLC (JASCO Deutschland, Groß-Umstadt, Germany) equipped with a Zorbax Eclipse XDB C8 column (4.6 × 150 mm, 5 μm particle diameter, 80 Å pore size, Agilent, Boeblingen, Germany) and a MD-1510 multiwavelength detector. For this purpose the water-soluble kefir fraction was used after an additional ultrafiltration step by centrifugal filter units with 10 kDa cutoff (Merck, Darmstadt, Germany). A portion of 50 μL of this peptide-containing solution was injected and the flow rate was set to 0.5 mL/min. The separation was carried out for 60 min using a linear gradient of 0–45% eluent B (95% ACN containing 0.1% TFA) after initial 5 min with eluent A (5% ACN containing 0.1% TFA). One fraction containing the peptides of interest was collected from 51.8 to 59.4 min for further analysis. This fraction was split in two parts of equal volume, frozen at –80 °C and dried by SpeedVac. One part of the fraction was analyzed by MALDI-TOF-MS to confirm the presence of the peptides of interest in this fraction. For this purpose, the dried samples were dissolved in 10 μL of MALDI matrix and analyzed as described above. To gain sequence information on the contained peptides, the other part of this fraction was used for direct infusion to the LTQ Orbitrap XL ETD mass spectrometer after dissolving the dried samples in 30 μL of 60% ACN, containing 0.5% FA and a 1:50 dilution with ESI buffer containing 50% ACN, 25% methanol and 1% FA. Data were recorded using gas phase fractionation data-dependent acquisition (DDA) methods as described previously. MS spectra were acquired in FT-MS scan mode with a target mass resolution of 100,000 at *m*/z 400. Consecutive DDA experiments (top 5) were performed within five *m*/z segments (*m*/z 400–551, 549–601, 599–801, 799–1001, and 999–2000) and specified offsets (*m*/z 0, 0.1, 0.2, 0.3, and 0.4 respectively) were used for the isolation of precursor ions in the linear ion trap [23]. The isolation width was set to *m*/z 1 in the first two segments and *m*/z 1.5 in the other three segments. CID normalized collision energy was set to 30% for all segments. Each segment was 3 min long and the dynamic exclusion lasted for 45 s, which formed a 15 min total run time method. Other MS parameters remained as mentioned above. For each sample, fractionation and following measurements were performed in triplicate. Peptide identification was carried out by a MASCOT search in the SwissProt database with the above mentioned search parameters. Assigned peptide sequences were verified manually for match confirmation.

2.8. Comparative peptide profile analysis by stage tip extraction and MALDI-TOF-MS

To investigate the reproducibility of the peptide profile of kefir samples produced under identical conditions, four samples per kefir type were produced independently in the course of 14 months and subjected to analysis. The peptides were extracted using stage tip microextraction according to Rappsilber et al. [24], modified by Baum et al. [25] with some minor changes. Three layers of a C18 Empore Disc (3 M, Neuss, Germany) were stamped out by a biopsy punch (1 mm diameter, KAI Medical, Solingen, Germany) and transferred to a 1–20 μL epTIP (Eppendorf, Hamburg, Germany) for stage tip assembly. As a conditioning step, 50 μL of ACN and 0.1 TFA were consecutively loaded and spun down by centrifugation at 1840 *g* and 25 °C for 1 min. Subsequently the procedure was repeated for sample loading with 50 μL of the water-soluble kefir fraction and with a centrifugation time of 5 min, and once again for the following washing step with 50 μL of 0.1% TFA and 3 min of centrifugation. Elution was achieved by 10 μL of a 60:40 mixture of ACN and 0.1% TFA and 3 min of centrifugation. Peptide extraction and the subsequent MALDI-TOF measurement, which was conducted as described above, were performed in triplicate for each sample.

3. Results

3.1. Microbiological analysis and proteolytic activity

The present study intended to evaluate the peptide profile of kefir by mass spectrometric methods. The peptide profile should then be used to reveal bioactive components and to obtain information about the proteolytic activity of the applied microorganisms. Because the starter culture may have considerable impact on the peptide formation, two different kefir varieties were produced and analyzed. For one product, heat-treated skim milk was fermented with a commercial kefir starter culture mixture, while kefir grains were used for the other type of sample. All other production parameters were identical for both varieties. The microbiological composition was characterized by monitoring the main components of the kefir microflora, namely the total viable mesophilic aerobic bacteria, *Lactobacilli*, *Lactococci*, *Leuconostoc*, acetic acid bacteria and yeast [1], using selective media for each group and counting the colony forming units (CFU). Fig. 1 displays the results of the microbiological analysis. The total number of mesophilic aerobic bacteria was slightly higher for kefir produced with commercial starter culture, but no significant differences were observed for *Lactobacilli*, *Lactococci*, *Leuconostoc*, and acetic acid bacteria. The yeast content showed a significantly higher number of CFU for milk fermented by kefir grains. The observed microbial composition is in good agreement with previous studies from Ender [26] and Kesenkaş et al. [27]. Freeze-drying, which exposes the cells to stress during the production of commercial starter culture, results in a loss of cell viability [28]. Thus, kefir produced with starter cultures containing freeze-dried lactic acid bacteria and yeasts from kefir grains has a lower number and variety of microorganisms than kefir produced directly with kefir grains [6], particularly with regard to the yeast content [29]. A reduced yeast population in kefir could consequently limit the amount of swelling in kefir packages, which is an important problem in dairy industry [30].

The proteolytic activity in the samples was assessed by the OPA method. The results revealed a 1.4-fold significantly higher proteolytic activity of kefir produced by commercial

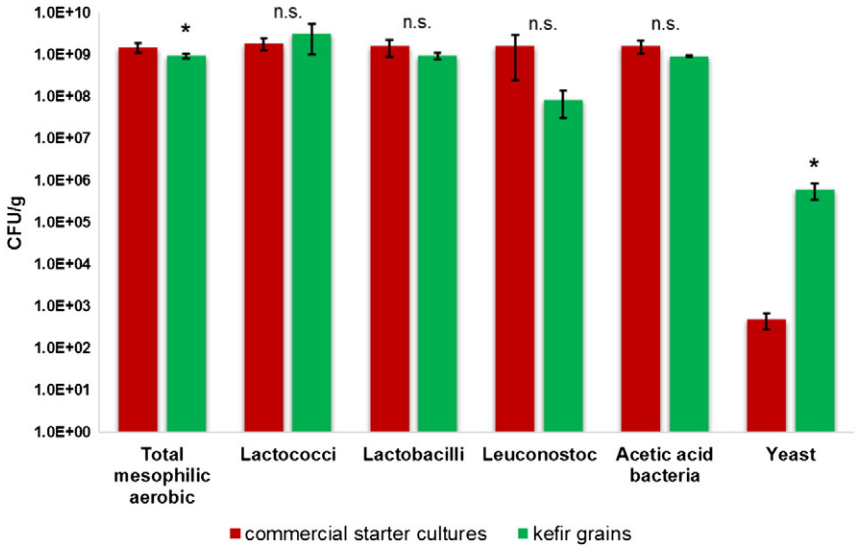

Fig. 1 – Colony forming units (CFU) for total mesophilic aerobic bacteria, *Lactobacilli*, *Lactococci*, *Leuconostoc*, acetic acid bacteria and yeasts in kefir produced by commercial starter culture or kefir grains, respectively. Mean value ± SD of CFU of two independent productions, each measured in duplicate, is shown. *p < 0.05; n.s. not significant (unpaired student's t-test).

starter culture compared to the samples produced by grains (Table 1). The proteolytic activity was also monitored for raw milk and unfermented skim milk after heat treatment. Both unfermented milk samples had significantly lower values indicating considerable peptide release during fermentation for both types of kefir. Although heat treatment of the raw milk resulted in a significant increase of proteolytic activity, the influence of fermentation on the release of peptides was substantially higher.

3.2. Peptide profile analysis of kefir

A more detailed peptide analysis was then performed by direct MALDI-TOF measurement in the *m/z* range from 600 to 5000 Da. Fig. 2 displays typical MALDI-TOF-MS spectra for both kefir varieties. Most of the signals were detected in a range from 800 to 2200 Da, (Fig. 2, inserted windows), i.e. corresponding to peptide lengths of approximately 7–20 amino acids. In the samples produced with commercial starter culture 49 peptides could be detected, whereas 34 signals were observed in the kefir grain sample. Both kefir types shared more than half of the signals including the two most abundant peptides at *m/z* 1718.0 and *m/z* 1881.1 suggesting major similarities, but also differences in the peptide release dependent on the type of starter culture. Because MALDI-TOF-MS was performed directly, i.e. without further peptide fractionation, negative discrimination of peptides may occur during the ionization process due to ion suppression. Nevertheless, peptide profiles recorded by MALDI-TOF-MS give a good overview of predominant peptides.

To detect more kefir peptides and to reveal their identity, ESI-LTQ-Orbitrap-MS after nano-UPLC separation was combined with a MASCOT database search in the SwissProt database for interpretation of the fragment spectra. All resulting assigned peptide sequences of the main bovine proteins were manually checked to achieve unambiguous identification. Thus, 253 different peptide sequences were confirmed, all representing casein fragments. Most peptides originated from β-casein (107), followed by α_{S1}-casein (56), κ-casein (53), and α_{S2}-casein (37), with 62 peptides being mono- or diphosphorylated. The sequence coverage was 98% for β-casein, 83% for κ-casein, 72% for α_{S1}-casein, and about 60% for α_{S2}-casein. Due to the insufficient quality of the product ion spectra, however, sequence assignment failed for the two most intense signals of the MALDI-TOF mass spectra (peptides at *m/z* 1718.0 and 1881.1). Therefore, the kefir peptides were fractionated by HPLC. The fraction containing these two major peptides was subjected to an additional direct infusion measurement by ESI-LTQ-Orbitrap MS. The peptide sequences of the signals could be confirmed as

Table 1 – Proteolytic activity measured by the o-phthaldialdehyde method and expressed as absorption units at 340 nm. Mean values and SD of four samples of raw milk and unfermented, heated skim milk and two samples of milk fermented with commercial kefir starter culture or kefir grains, respectively, are displayed. Each sample was analyzed in duplicate.

Proteolytic activity	Milk samples		Kefir samples	
	Raw milk	Unfermented, heated skim milk	Milk fermented with commercial kefir starter culture	Milk fermented with kefir grains
Mean ± SD	0.10 ± 0.01[a]	0.12 ± 0.00[b]	0.28 ± 0.01[c]	0.20 ± 0.01[d]

Values with different superscript letters are significantly different (p < 0.001) as calculated by an unpaired student's t-test.

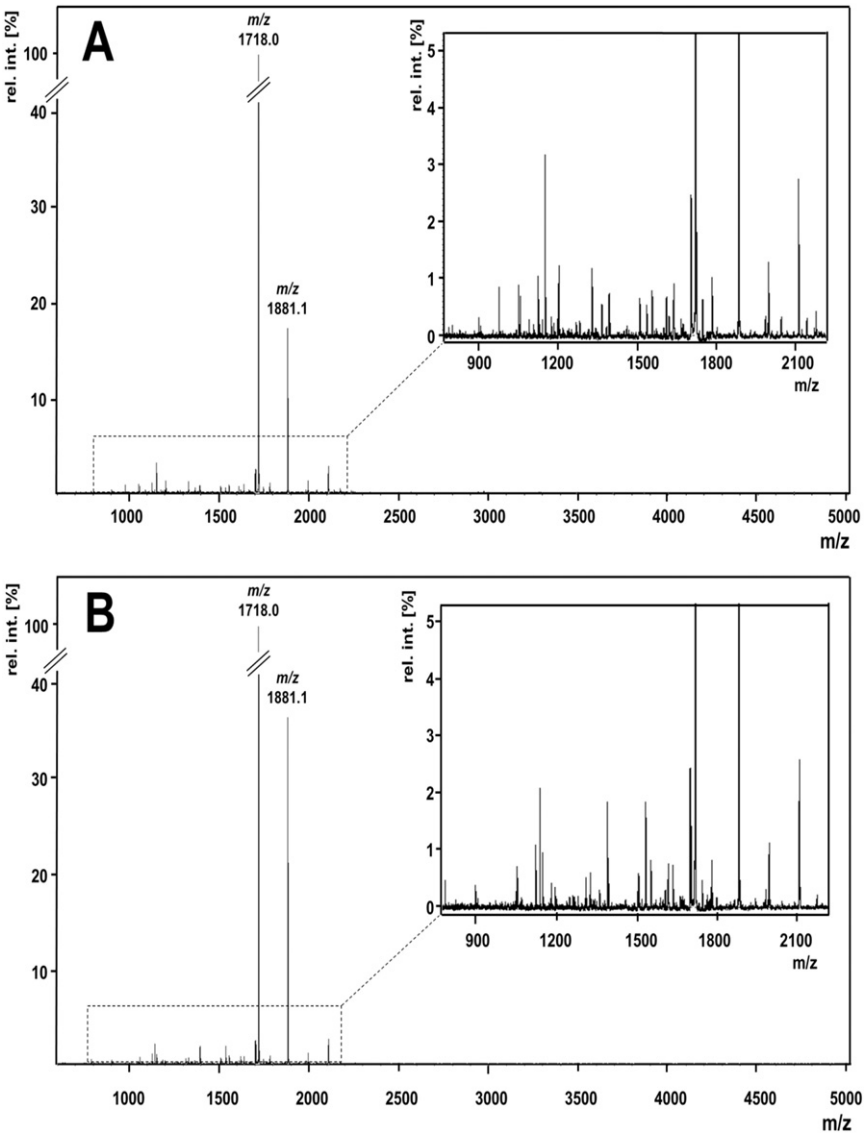

Fig. 2 – MALDI-TOF mass spectra of milk fermented with commercial kefir starter culture (A) and milk fermented with kefir grains (B) in a mass range from *m/z* 600 to 5000. The most intense signal of each spectrum was used for spectra normalization. Inserted windows magnify the signals in a range from 800 to 2200 Da. The two most intense signals *m/z* 1718.0 and *m/z* 1881.1 are marked in both spectra.

QEPVLGPVRGPFPIIV and YQEPVLGPVRGPFPIIV, both fragments from the C-terminal region of β-casein f194–209 and f193–209, respectively. Two additional peptides could further be identified by this experiment, namely f192–209 and f191–209, both deriving from the same region. Including these four peptides from the direct infusion experiment, 257 peptides were identified as displayed in Table 2 including peptide sequence, precursor protein, position, and origin.

Fig. 3 summarizes the detected cleavage sites of the parent proteins indicating that the released peptides are not distributed homogenously among the protein sequences. Instead, there are distinct parts in every protein where more peptides are released than in other parts. In β-casein, K_{28}–K_{29}, K_{29}–I_{30}, Q_{175}–K_{176}, and Q_{182}–R_{183} seem to be especially prone to degradation by the kefir microflora. In α_{S1}-casein, L_{40}–S_{41} and Q_{130}–Q_{131} show a high susceptibility to proteolysis. In κ-casein, N_{53}–Q_{54}, A_{65}–A_{66}, and E_{151}–V_{152} are favored cleavage sites, whereas E_{12}–S_{13}, K_{21}–Q_{22}, and E_{133}–N_{134} are especially affected in α_{S2}-casein. A comparison with the cleavage sites targeted by the majority of lactococcal proteinases (black triangles in Fig. 3) shows that about 17% of all observed cleavage sites for the kefir peptides could be explained by the activity of these enzymes. Proteinases of other microorganisms involved in casein degradation in kefir are probably responsible for the activity at the remaining cleavage sites.

3.3. *Effect of starter cultures on the kefir peptide profile*

Peptide matches between both analyzed kefir types are shown in total and individually for each of the four parent proteins in Fig. 4. A high number of peptides was present in both samples (97 out of 257) indicating the presence of kefir-typical peptides.

Table 2 – Peptide profiles of kefir. Sequences are shown in single letter code and peptide masses are given in Da. Identified peptides in milk fermented with commercial starter culture and kefir grains are presented as well as matches to the peptide profile of raw milk [10,11].

No.	Observed $[M + H]^+$	Calculated $[M + H]^+$	Charge of precursor ion	Parent protein	Amino acid	Sequence	Starter culture kefir	Kefir grain kefir	Raw milk [10]/[11]
1	806.40	806.41	+2	α_{s1}-Casein	24–30	FVAPFPE	●		
2	876.46	876.46	+2	α_{s1}-Casein	193–199	KTTMPLW	●	●	
3	905.48	905.48	+2	α_{s1}-Casein	17–23	NENLLRF		●	●/–
4	916.51	916.51	+2	α_{s1}-Casein	33–40	GKEKVNEL	●		
5	920.48	920.48	+2	α_{s1}-Casein	86–93	VPSERYLG		●	
6	940.47	940.47	+2	α_{s1}-Casein	182–190	IPNPIGSEN		●	
7	963.44	963.45	+2	α_{s1}-Casein	56–63	DIKQMEAE	●		
8	970.56	970.57	+2	α_{s1}-Casein	15–22	VLNENLLR	●		●/–
9	991.52	991.52	+2	α_{s1}-Casein	26–34	APFPEVFGK		●	●/–
10	1015.52	1015.52	+2	α_{s1}-Casein	131–139	QKEPMIGVN	●	●	
11	1052.55	1052.55	+2	α_{s1}-Casein	17–24	NENLLRFF	●	●	●/–
12	1055.49	1055.50	+2	α_{s1}-Casein	181–190	DIPNPIGSEN	●	●	
13	1063.57	1063.58	+2	α_{s1}-Casein	32–40	FGKEKVNEL	●	●	
14	1092.48	1092.49	+2	α_{s1}-Casein	55–63	EDIKQMEAE	●	●	
15	1092.54	1092.54	+2	α_{s1}-Casein	191–199	SEKTTMPLW		●	●/–
16	1093.50	1093.50	+2	α_{s1}-Casein	52–60	QAMEDIKQM	●		
17	1100.57	1100.58	+2	α_{s1}-Casein	129–138	AQQKEPMIGV	●		
18	1108.53	1108.53	+2	α_{s1}-Casein	191–199	SEKTTMPLW M6 (oxidation)		●	
19	1120.56	1120.57	+2	α_{s1}-Casein	26–35	APFPEVFGKE	●	●	●/●
20	1132.47	1132.48	+2	α_{s1}-Casein	113–121	PNSAEERLH S3 (phospho)	●		
21	1136.66	1136.67	+2	α_{s1}-Casein	105–114	KVPQLEIVPN	●	●	
22	1139.45	1139.46	+2	α_{s1}-Casein	115–123	SAEERLHSM S1 (phospho)	●	●	
23	1142.53	1142.53	+2	α_{s1}-Casein	180–190	SDIPNPIGSEN		●	
24	1142.60	1142.61	+2	α_{s1}-Casein	70–79	EIVPNSVEQK	●		
25	1143.58	1143.58	+2	α_{s1}-Casein	131–140	QKEPMIGVNQ	●	●	
26	1162.64	1162.65	+2	α_{s1}-Casein	31–40	VFGKEKVNEL	●	●	
27	1198.57	1198.57	+2	α_{s1}-Casein	85–94	DVPSERYLGY	●	●	
28	1206.58	1206.58	+2	α_{s1}-Casein	190–199	NSEKTTMPLW	●	●	
29	1207.57	1207.57	+2	α_{s1}-Casein	111–120	IVPNSAEERL S5 (phospho)	●		
30	1208.52	1208.53	+2	α_{s1}-Casein	51–60	DQAMEDIKQM	●	●	
31	1214.62	1214.62	+2	α_{s1}-Casein	129–139	AQQKEPMIGVN	●	●	
32	1219.63	1219.64	+2	α_{s1}-Casein	25–35	VAPFPEVFGKE	●		●/–
33	1222.57	1222.57	+2	α_{s1}-Casein	70–79	EIVPNSVEQK S6 (phospho)	●		
34	1223.53	1223.53	+2	α_{s1}-Casein	110–119	EIVPNSAEER S6 (phospho)		●	
35	1230.61	1230.61	+2	α_{s1}-Casein	129–139	AQQKEPMIGVN M7 (oxidation)	●	●	
36	1235.62	1235.63	+2	α_{s1}-Casein	83–92	KEDVPSERYL	●	●	
37	1249.68	1249.68	+2	α_{s1}-Casein	31–41	VFGKEKVNELS	●		
38	1271.57	1271.58	+2	α_{s1}-Casein	181–192	DIPNPIGSENSE	●	●	
39	1272.62	1272.63	+2	α_{s1}-Casein	131–141	QKEPMIGVNQE	●	●	
40	1288.62	1288.62	+2	α_{s1}-Casein	131–141	QKEPMIGVNQE M5 (oxidation)	●		
41	1344.63	1344.63	+2	α_{s1}-Casein	111–121	IVPNSAEERLH S5 (phospho)	●	●	
42	1385.70	1385.71	+2	α_{s1}-Casein	131–142	QKEPMIGVNQEL	●	●	
43	1401.70	1401.70	+2	α_{s1}-Casein	131–142	QKEPMIGVNQEL M5 (oxidation)	●	●	
44	1478.61	1478.62	+2	α_{s1}-Casein	36–47	KVNELSKDIGSE S6 (phospho) S11 (phospho)	●		
45	1577.60	1577.60	+2	α_{s1}-Casein	41–54	SKDIGSESTEDQAM S8 (phospho)	●		
46	1619.81	1619.81	+2	α_{s1}-Casein	131–144	QKEPMIGVNQELAY	●		
47	1657.57	1657.57	+2	α_{s1}-Casein	41–54	SKDIGSESTEDQAM S6 (phospho) S8 (phospho)	●	●	
48	1673.56	1673.56	+2	α_{s1}-Casein	41–54	SKDIGSESTEDQAM S6 (phospho) S8 (phospho) M14 (oxidation)	●		
49	1706.64	1706.65	+2	α_{s1}-Casein	41–55	SKDIGSESTEDQAME S8 (phospho)	●		
50	1786.61	1786.61	+2	α_{s1}-Casein	41–55	SKDIGSESTEDQAME S6 (phospho) S8 (phospho)	●		
51	1795.74	1795.74	+2	α_{s1}-Casein	36–50	KVNELSKDIGSESTE S11 (phospho) S13 (phospho)	●		
52	1802.61	1802.61	+2	α_{s1}-Casein	41–55	SKDIGSESTEDQAME S6 (phospho) S8 (phospho) M14 (oxidation)	●		
53	1832.84	1832.84	+2	α_{s1}-Casein	75–89	SVEQKHIQKEDVPSE S1 (phospho)	●		
54	1877.08	1877.09	+3	α_{s1}-Casein	1–16	RPKHPIKHQGLPQEVL	●		
55	2120.17	2120.17	+3	α_{s1}-Casein	1–18	RPKHPIKHQGLPQEVLNE	●		
56	2234.21	2234.22	+3	α_{s1}-Casein	1–19	RPKHPIKHQGLPQEVLNEN	●		●/●
57	932.49	932.49	+2	α_{s2}-Casein	67–74	EEVKITVD	●	●	
58	933.54	933.54	+2	α_{s2}-Casein	175–182	ALPQYLKT	●		

(continued on next page)

Table 2 (*continued*)

No.	Observed $[M + H]^+$	Calculated $[M + H]^+$	Charge of precursor ion	Parent protein	Amino acid	Sequence	Starter culture kefir	Kefir grain kefir	Raw milk [10]/[11]
59	973.47	973.48	+2	α_{s2}-Casein	89–95	YQKFPQY	●		
60	975.60	975.60	+2	α_{s2}-Casein	198–205	TKVIPYVR		●	●/–
61	989.55	989.55	+2	α_{s2}-Casein	64–72	VATEEVKIT		●	
62	1020.42	1020.43	+2	α_{s2}-Casein	13–20	SIISQETY S4 (phospho)	●		
63	1061.49	1061.49	+2	α_{s2}-Casein	14–21	IISQETYK S3 (phospho)	●	●	
64	1068.55	1068.56	+2	α_{s2}-Casein	13–21	SIISQETYK	●		
65	1076.57	1076.57	+2	α_{s2}-Casein	80–88	KALNEINQF	●		
66	1083.45	1083.45	+2	α_{s2}-Casein	25–33	NMAINPSKE S7 (phospho)	●		
67	1128.62	1128.62	+2	α_{s2}-Casein	190–198	MKPWIQPKT	●		
68	1148.52	1148.52	+2	α_{s2}-Casein	13–21	SIISQETYK S4 (phospho)	●	●	
69	1151.46	1151.47	+2	α_{s2}-Casein	141–149	MESTEVFTK S3 (phospho)	●	●	
70	1167.46	1167.46	+2	α_{s2}-Casein	141–149	MESTEVFTK M1 (oxidation) S3 (phospho)	●	●	
71	1186.71	1186.72	+2	α_{s2}-Casein	194–203	IQPKTKVIPY	●	●	
72	1195.67	1195.67	+2	α_{s2}-Casein	175–184	ALPQYLKTVY	●		
73	1195.67	1195.68	+2	α_{s2}-Casein	114–124	RNAVPITPTLN	●	●	
74	1210.67	1210.68	+2	α_{s2}-Casein	116–126	AVPITPTLNRE	●		
75	1238.43	1238.43	+2	α_{s2}-Casein	125–133	REQLSTSEE S5 (phospho) S7 (phospho)	●	●	
76	1251.74	1251.75	+2	α_{s2}-Casein	198–207	TKVIPYVRYL	●		●/●
77	1276.58	1276.58	+2	α_{s2}-Casein	13–22	SIISQETYKQ S4 (phospho)	●		
78	1277.56	1277.57	+2	α_{s2}-Casein	12–21	ESIISQETYK S5 (phospho)	●	●	
79	1279.56	1279.56	+2	α_{s2}-Casein	141–150	MESTEVFTKK S3 (phospho)	●	●	
80	1318.59	1318.59	+2	α_{s2}-Casein	14–23	IISQETYKQE S3 (phospho)		●	●/–
81	1324.72	1324.72	+2	α_{s2}-Casein	115–126	NAVPITPTLNRE	●	●	
82	1352.47	1352.48	+2	α_{s2}-Casein	124–133	NREQLSTSEE S6 (phospho) S8 (phospho)		●	
83	1385.59	1385.59	+2	α_{s2}-Casein	123–133	LNREQLSTSEE S9 (phospho)	●	●	
84	1405.62	1405.62	+2	α_{s2}-Casein	13–23	SIISQETYKQE S4 (phospho)	●		
85	1406.60	1406.61	+2	α_{s2}-Casein	11–21	EESIISQETYK S6 (phospho)		●	
86	1465.56	1465.56	+2	α_{s2}-Casein	123–133	LNREQLSTSEE S7 (phospho) S9 (phospho)	●	●	
87	1474.75	1474.75	+3	α_{s2}-Casein	68–79	EVKITVDDKHYQ		●	
88	1533.71	1533.72	+2	α_{s2}-Casein	13–24	SIISQETYKQEK S4 (phospho)		●	
89	1565.86	1565.86	+2	α_{s2}-Casein	115–128	NAVPITPTLNREQL		●	
90	1580.69	1580.69	+2	α_{s2}-Casein	135–147	SKKTVDMESTEVF S9 (phospho)	●		
91	1694.73	1694.73	+2	α_{s2}-Casein	134–147	NSKKTVDMESTEVF S10 (phospho)	●	●	
92	1710.72	1710.73	+2	α_{s2}-Casein	134–147	NSKKTVDMESTEVF M8 (oxidation) S10 (phospho)	●	●	
93	1718.03	1718.04	+3	α_{s2}-Casein	194–207	IQPKTKVIPYVRYL	●		
94	802.44	802.45	+2	β-Casein	176–182	KAVPYPQ	●	●	
95	803.47	803.48	+2	β-Casein	132–138	NLHLPLP	●		
96	820.44	820.44	+2	β-Casein	161–168	SVLSLSQS	●	●	
97	830.42	830.42	+2	β-Casein	183–189	RDMPIQA		●	
98	867.53	867.53	+2	β-Casein	168–175	SKVLPVPQ	●	●	
99	930.57	930.58	+2	β-Casein	134–141	HLPLPLLQ		●	
100	948.53	948.54	+2	β-Casein	161–169	SVLSLSQSK		●	
101	969.52	969.53	+2	β-Casein	6–14	LNVPGEIVE	●		
102	977.48	977.49	+2	β-Casein	183–190	RDMPIQAF	●		
103	979.49	979.49	+2	β-Casein	120–128	TESQSLTLT	●		
104	991.49	991.49	+2	β-Casein	124–132	SLTLTDVEN	●	●	
105	993.48	993.48	+2	β-Casein	183–190	RDMPIQAF M3 (oxidation)		●	
106	995.58	995.59	+2	β-Casein	167–175	QSKVLPVPQ	●	●	
107	997.54	997.55	+2	β-Casein	45–52	LQDKIHPF	●		
108	1017.54	1017.54	+2	β-Casein	155–163	VMFPPQSVL	●		
109	1017.60	1017.61	+2	β-Casein	134–142	HLPLPLLQS	●		
110	1025.56	1025.57	+2	β-Casein	83–91	VVPPFLQPE	●		
111	1028.51	1028.52	+2	β-Casein	49–57	IHPFAQTQS	●		
112	1043.66	1043.66	+2	β-Casein	133–141	LHLPLPLLQ	●		
113	1056.54	1056.55	+2	β-Casein	47–55	DKIHPFAQT		●	
114	1079.60	1079.61	+2	β-Casein	73–82	NIPPLTQTPV	●	●	
115	1082.62	1082.62	+2	β-Casein	166–175	SQSKVLPVPQ	●	●	
116	1088.50	1088.50	+2	β-Casein	29–36	KIEKFQSE S7 (phospho)	●		
117	1089.45	1089.45	+2	β-Casein	30–37	IEKFQSEE S6 (phospho)	●	●	
118	1090.57	1090.57	+2	β-Casein	183–191	RDMPIQAFL	●		
119	1094.54	1094.55	+2	β-Casein	43–51	DELQDKIHP	●		
120	1100.57	1100.58	+2	β-Casein	59–68	VYPFPGPIPN	●	●	
121	1118.49	1118.50	+2	β-Casein	38–46	QQQTEDELQ	●	●	

Table 2 (*continued*)

No.	Observed $[M + H]^+$	Calculated $[M + H]^+$	Charge of precursor ion	Parent protein	Amino acid	Sequence	Starter culture kefir	Kefir grain kefir	Raw milk [10]/[11]
122	1119.48	1119.48	+2	β-Casein	37–45	EQQQTEDEL	●	●	
123	1119.55	1119.55	+2	β-Casein	123–132	QSLTLTDVEN	●	●	
124	1126.58	1126.59	+2	β-Casein	44–52	ELQDKIHPF	●	●	
125	1126.62	1126.63	+2	β-Casein	173–182	VPQKAVPYPQ	●		
126	1130.69	1130.69	+2	β-Casein	133–142	LHLPLPLLQS	●		
127	1135.56	1135.56	+2	β-Casein	18–27	SSEESITRIN		●	
128	1151.69	1151.69	+2	β-Casein	199–209	GPVRGPFPIIV	●		●/–
129	1155.59	1155.60	+2	β-Casein	1–10	RELEELNVPG	●	●	
130	1163.54	1163.54	+2	β-Casein	140–148	LQSWMHQPH	●		
131	1178.62	1178.63	+2	β-Casein	92–102	VMGVSKVKEAM	●		
132	1184.60	1184.61	+2	β-Casein	47–56	DKIHPFAQTQ	●	●	
133	1195.70	1195.70	+2	β-Casein	165–175	LSQSKVLPVPQ	●	●	
134	1201.72	1201.73	+3	β-Casein	22–31	SITRINKKIE	●		
135	1203.65	1203.66	+2	β-Casein	183–192	RDMPIQAFLL	●		
136	1216.60	1216.60	+2	β-Casein	28–36	KKIEKFQSE S8 (phospho)	●		
137	1217.54	1217.55	+2	β-Casein	29–37	KIEKFQSEE S7 (phospho)	●	●	
138	1219.65	1219.65	+2	β-Casein	183–192	RDMPIQAFLL M3 (oxidation)	●		
139	1241.61	1241.62	+2	β-Casein	43–52	DELQDKIHPF	●	●	
140	1244.73	1244.74	+2	β-Casein	132–142	NLHLPLPLLQS	●		
141	1247.53	1247.54	+2	β-Casein	37–46	EQQQTEDELQ	●	●	
142	1271.64	1271.64	+2	β-Casein	47–57	DKIHPFAQTQS	●	●	
143	1273.64	1273.64	+3	β-Casein	106–115	HKEMPFPKYP	●		
144	1276.77	1276.77	+3	β-Casein	24–33	TRINKKIEKF	●		
145	1282.63	1282.64	+2	β-Casein	144–154	MHQPHQPLPPT	●	●	
146	1282.73	1282.74	+2	β-Casein	164–175	SLSQSKVLPVPQ	●	●	
147	1284.64	1284.64	+2	β-Casein	1–11	RELEELNVPGE	●	●	●/–
148	1300.69	1300.69	+2	β-Casein	57–68	SLVYPFPGPIPN	●		
149	1301.66	1301.67	+2	β-Casein	176–186	KAVPYPQRDMP	●		
150	1312.66	1312.66	+2	β-Casein	46–56	QDKIHPFAQTQ		●	
151	1322.63	1322.63	+2	β-Casein	120–131	TESQSLTLTDVE	●	●	
152	1325.68	1325.68	+2	β-Casein	44–54	ELQDKIHPFAQ	●	●	
153	1345.56	1345.57	+2	β-Casein	30–39	IEKFQSEEQQ S6 (phospho)	●	●	
154	1345.64	1345.64	+2	β-Casein	28–37	KKIEKFQSEE S8 (phospho)	●		
155	1355.51	1355.51	+2	β-Casein	15–25	SLSSSEESITR 2× phosphorylated		●	
156	1362.56	1362.57	+2	β-Casein	37–47	EQQQTEDELQD	●		
157	1370.65	1370.66	+2	β-Casein	42–52	EDELQDKIHPF	●		
158	1384.72	1384.72	+2	β-Casein	47–58	DKIHPFAQTQSL	●	●	
159	1392.76	1392.76	+2	β-Casein	194–206	QEPVLGPVRGPFP	●	●	●/–
160	1399.69	1399.70	+2	β-Casein	46–57	QDKIHPFAQTQS	●	●	
161	1410.83	1410.83	+2	β-Casein	164–176	SLSQSKVLPVPQK	●		●/–
162	1412.61	1412.62	+2	β-Casein	8–20	VPGEIVESLSSSE S11 (phospho)	●		
163	1426.73	1426.73	+2	β-Casein	44–55	ELQDKIHPFAQT	●	●	
164	1436.67	1436.68	+2	β-Casein	120–132	TESQSLTLTDVEN	●	●	
165	1448.52	1448.52	+2	β-Casein	33–43	FQSEEQQQTED S3 (phospho)		●	
166	1461.59	1461.59	+2	β-Casein	32–42	KFQSEEQQQTE S4 (phospho)	●	●	
167	1468.71	1468.72	+2	β-Casein	143–154	WMHQPHQPLPPT	●		
168	1473.62	1473.63	+2	β-Casein	30–40	IEKFQSEEQQQ S6 (phospho)	●		
169	1473.66	1473.66	+2	β-Casein	29–39	KIEKFQSEEQQ S7 (phospho)	●		
170	1476.89	1476.89	+2	β-Casein	22–33	SITRINKKIEKF	●		
171	1543.58	1543.58	+2	β-Casein	35–46	SEEQQQTEDELQ S1 (phospho)		●	
172	1554.79	1554.79	+2	β-Casein	44–56	ELQDKIHPFAQTQ	●	●	
173	1555.74	1555.75	+2	β-Casein	142–154	SWMHQPHQPLPPT	●		
174	1555.82	1555.83	+2	β-Casein	193–206	YQEPVLGPVRGPFP	●		●/–
175	1563.92	1563.93	+2	β-Casein	169–182	KVLPVPQKAVPYPQ	●		
176	1574.67	1574.67	+2	β-Casein	30–41	IEKFQSEEQQQT S6 (phospho)	●		
177	1576.61	1576.62	+2	β-Casein	32–43	KFQSEEQQQTED S4 (phospho)		●	
178	1601.72	1601.72	+2	β-Casein	29–40	KIEKFQSEEQQQ S7 (phospho)	●		
179	1625.83	1625.84	+2	β-Casein	1–14	RELEELNVPGEIVE	●		
180	1641.82	1641.82	+2	β-Casein	44–57	ELQDKIHPFAQTQS	●	●	
181	1650.95	1650.96	+2	β-Casein	168–182	SKVLPVPQKAVPYPQ	●		
182	1669.81	1669.82	+2	β-Casein	43–56	DELQDKIHPFAQTQ	●		
183	1702.77	1702.77	+2	β-Casein	29–41	KIEKFQSEEQQQT S7 (phospho)	●		

(*continued on next page*)

Table 2 (*continued*)

No.	Observed $[M + H]^+$	Calculated $[M + H]^+$	Charge of precursor ion	Parent protein	Amino acid	Sequence	Starter culture kefir	Kefir grain kefir	Raw milk [10]/[11]
184	1703.71	1703.72	+2	β-Casein	30–42	IEKFQSEEQQQTE S6 (phospho)	●		
185	1708.81	1708.81	+3	β-Casein	38–51	QQQTEDELQDKIHP	●	●	
186	1718.00	1718.00	+2	β-Casein	194–209	QEPVLGPVRGPFPIIV	●	●	●/–
187	1728.86	1728.87	+3	β-Casein	25–37	RINKKIEKFQSEE S11 (phospho)	●		
188	1745.87	1745.87	+2	β-Casein	106–119	HKEMPFPKYPVEPF	●		
189	1818.74	1818.74	+2	β-Casein	30–43	IEKFQSEEQQQTED S6 (phospho)	●	●	
190	1831.81	1831.81	+2	β-Casein	29–42	KIEKFQSEEQQQTE S7 (phospho)	●		
191	1866.04	1866.05	+3	β-Casein	166–182	SQSKVLPVPQKAVPYPQ	●		
192	1881.06	1881.06	+2	β-Casein	193–209	YQEPVLGPVRGPFPIIV	●	●	●/–
193	1946.84	1946.84	+2	β-Casein	29–43	KIEKFQSEEQQQTED S7 (phospho)	●	●	
194	1975.95	1975.96	+3	β-Casein	106–121	HKEMPFPKYPVEPFTE	●		
195	1981.97	1981.98	+3	β-Casein	144–160	MHQPHQPLPPTVMFPPQ	●		
196	1994.15	1994.15	+2	β-Casein	192–209	LYQEPVLGPVRGPFPIIV	●	●	●/–
197	2042.05	2042.06	+3	β-Casein	103–119	APKHKEMPFPKYPVEPF	●		
198	2062.99	2062.99	+3	β-Casein	106–122	HKEMPFPKYPVEPFTES	●		
199	2066.16	2066.16	+2	β-Casein	164–182	SLSQSKVLPVPQKAVPYPQ	●		
200	2107.24	2107.23	+2	β-Casein	191–209	LLYQEPVLGPVRGPFPIIV	●	●	●/–
201	2173.09	2173.10	+3	β-Casein	102–119	MAPKHKEMPFPKYPVEPF	●		
202	2191.05	2191.05	+3	β-Casein	106–123	HKEMPFPKYPVEPFTESQ	●		
203	2286.14	2286.15	+3	β-Casein	22–39	SITRINKKIEKFQSEEQQ S14 (phospho)	●		
204	2359.17	2359.18	+3	β-Casein	103–122	APKHKEMPFPKYPVEPFTES	●		
205	815.44	815.44	+2	κ-Casein	72–77	QILQWQ	●		
206	898.45	898.45	+2	κ-Casein	153–160	IESPPEIN	●	●	
207	905.49	905.49	+2	κ-Casein	161–169	TVQVTSTAV	●	●	–/●
208	915.51	915.51	+2	κ-Casein	116–123	KTEIPTIN	●		
209	924.51	924.52	+2	κ-Casein	24–30	KYIPIQY	●		
210	946.53	946.54	+2	κ-Casein	43–50	YQQKPVAL	●	●	
211	954.57	954.57	+2	κ-Casein	66–74	AVRSPAQIL	●		
212	984.50	984.50	+2	κ-Casein	155–163	SPPEINTVQ	●		
213	984.51	984.51	+2	κ-Casein	31–38	VLSRYPSY	●		
214	996.58	996.58	+2	κ-Casein	45–53	QKPVALINN	●	●	
215	997.52	997.52	+2	κ-Casein	152–160	VIESPPEIN	●	●	
216	1002.51	1002.51	+2	κ-Casein	122–131	INTIASGEPT	●		
217	1010.60	1010.60	+2	κ-Casein	44–52	QQKPVALIN	●	●	
218	1027.59	1027.59	+2	κ-Casein	72–79	QILQWQVL	●		
219	1030.54	1030.54	+2	κ-Casein	115–123	DKTEIPTIN	●		
220	1039.52	1039.52	+2	κ-Casein	41–48	NYYQQKPV	●		
221	1040.42	1040.42	+2	κ-Casein	143–151	VATLEDSPE S7 (phospho)	●	●	
222	1041.53	1041.54	+2	κ-Casein	31–39	VLSRYPSYG	●		
223	1058.57	1058.57	+2	κ-Casein	118–127	EIPTINTIAS	●		
224	1082.63	1082.63	+2	κ-Casein	66–75	AVRSPAQILQ	●	●	
225	1098.56	1098.57	+2	κ-Casein	152–161	VIESPPEINT	●	●	
226	1108.64	1108.64	+2	κ-Casein	22–30	IAKYIPIQY	●		
227	1109.59	1109.60	+2	κ-Casein	42–50	YYQQKPVAL	●	●	
228	1122.51	1122.52	+2	κ-Casein	132–142	STPTTEAVEST	●		
229	1124.64	1124.64	+2	κ-Casein	45–54	QKPVALINNQ	●		
230	1124.64	1124.64	+2	κ-Casein	44–53	QQKPVALINN	●		
231	1130.63	1130.63	+2	κ-Casein	61–71	YAKPAAVRSPA	●		
232	1136.67	1136.67	+2	κ-Casein	24–32	KYIPIQYVL	●		
233	1141.46	1141.46	+2	κ-Casein	142–151	TVATLEDSPE S8 (phospho)	●	●	
234	1158.60	1158.60	+2	κ-Casein	114–123	QDKTEIPTIN	●		
235	1172.52	1172.52	+2	κ-Casein	12–20	EKDERFFSD	●		
236	1173.66	1173.66	+2	κ-Casein	43–52	YQQKPVALIN	●	●	
237	1182.61	1182.62	+2	κ-Casein	56–65	LPYPYYAKPA	●	●	
238	1228.49	1228.50	+2	κ-Casein	141–151	STVATLEDSPE S9 (phospho)	●	●	
239	1236.73	1236.74	+2	κ-Casein	21–30	KIAKYIPIQY	●		
240	1252.70	1252.70	+2	κ-Casein	44–54	QQKPVALINNQ	●		
241	1268.71	1268.71	+2	κ-Casein	66–76	AVRSPAQILQW	●		
242	1287.70	1287.71	+2	κ-Casein	43–53	YQQKPVALINN	●	●	
243	1290.60	1290.61	+2	κ-Casein	125–137	IASGEPTSTPTTE	●		
244	1318.60	1318.60	+2	κ-Casein	128–140	GEPTSTPTTEAVE	●		
245	1325.69	1325.69	+2	κ-Casein	152–163	VIESPPEINTVQ	●		
246	1329.66	1329.66	+2	κ-Casein	96–106	ARHPHPHLSFM	●		

Table 2 (*continued*)

No.	Observed $[M + H]^+$	Calculated $[M + H]^+$	Charge of precursor ion	Parent protein	Amino acid	Sequence	Starter culture kefir	Kefir grain kefir	Raw milk [10]/[11]
247	1336.72	1336.73	+2	κ-Casein	42–52	YYQQKPVALIN	●	●	
248	1391.65	1391.65	+2	κ-Casein	124–137	TIASGEPTSTPTTE	●		
249	1396.76	1396.77	+2	κ-Casein	66–77	AVRSPAQILQWQ	●		
250	1448.88	1448.89	+2	κ-Casein	21–32	KIAKYIPIQYVL	●		
251	1450.76	1450.77	+2	κ-Casein	42–53	YYQQKPVALINN	●	●	
252	1457.74	1457.75	+2	κ-Casein	54–65	QFLPYPYYAKPA	●	●	
253	1527.64	1527.65	+2	κ-Casein	138–151	AVESTVATLEDSPE S12 (phospho)	●		
254	1535.92	1535.92	+2	κ-Casein	21–33	KIAKYIPIQYVLS	●		
255	1564.81	1564.81	+2	κ-Casein	41–53	NYYQQKPVALINN	●		
256	1578.82	1578.83	+2	κ-Casein	42–54	YYQQKPVALINNQ	●		
257	2663.42	2663.43	+3	κ-Casein	107–131	AIPPKKNQDKTEIPTINTIASGEPT	●		

The recorded peptide profile of kefir produced by commercial starter culture consisted of 230 different peptides, whereas 124 peptide sequences were identified in kefir from kefir grains. The general pattern is similar for all four caseins, i.e. most peptides were exclusively identified in the peptidome of commercial starter culture kefir (133/257), whereas a great number were shared by both kefir types (97/257), and only few peptide sequences were unique for the kefir grain sample (27/257).

The heat maps in Fig. 5 illustrate the origin of the peptides in the amino acid sequence of the corresponding parent proteins for both kefir varieties. Red color indicates preferential sites of proteolysis in the respective region, while a dark green color means that none of the identified peptides derived from this part of the protein. In both kefir types, the highest number of peptides is found to be released from the region of β-casein amino acid 28–56. Other common hot spots are amino acids 164–175 and the C-terminal region of β-casein, 129–142 in the sequence of α_{S1}-casein, 13–21 and 123–147 in the α_{S2}-casein sequence and 42–53 regarding κ-casein (red and yellow parts in Fig. 5). The biggest differences between both samples were found in β-casein at position 106–119, at amino acids 41–55 of the α_{S1}-casein sequence and at 21–33 and 114–140 for κ-casein. While a relative high number of peptides were released from these regions in kefir produced by commercial starter culture, none or only a few peptides were found in milk fermented with kefir grains. In both kefir varieties no peptides were found to be released from the following regions: amino acids 64–69, 95–104, 124–128, and 145–179 in α_{S1}-casein, the N-terminus as well as amino acids 34–63, 96–113, 161–174, and 185–189 in α_{S2}-casein, amino acids 69–72 in β-casein and the N-terminus as well as amino acids 80–95 in κ-casein (dark green parts in Fig. 5). In summary, the peptide profiles of both kefir varieties are largely identical, but more peptides could be identified in kefir after fermentation by commercial starter culture.

3.4. Comparison of the peptide profile of kefir and raw bovine milk

Table 1 indicates 1.7- to 2.4-fold higher proteolytic activity after the fermentation of unfermented milk. Recent studies, however, showed that even the initial peptide profile of raw bovine milk is very complex [10,11]. Therefore, it was also investigated to which extent the composition of the peptide fraction is altered by fermentation. Baum et al. identified 129 endogenous peptides of raw milk from α_{S1}-casein, 63 peptides from β-casein, 54 derived from α_{S2}-casein and 1 peptide each from κ-casein and β-lactoglobulin [10]. The results from Dallas et al. showed a similar distribution for different raw milk samples with most peptides deriving from α_{S1}-casein (about 40%), followed by β-casein and α_{S2}-casein and some peptides from minor milk proteins [11]. However, only 21 of the peptides identified in the kefir samples have previously been identified as components of raw milk, whereas 236 were unique for kefir (Table 2, Fig. 4). Fig. 4 depicts an almost entire shift of the whole peptide profile during fermentation. Moreover, a shift in the mass range of identified peptides was observed: whereas the milk peptides have masses between approximately 800 and 4400 Da, the masses of peptides identified in kefir are less or equal to about 2700 Da. The number of identified peptides in kefir from commercial starter culture is similar to the number of peptides in the milk peptidome. In the case of kefir from kefir grains it is even lower. Hence, no increase of complexity could be observed, although the high proteolytic activity suggests a higher concentration of peptides after fermentation.

3.5. Interbatch variations of the kefir peptide profiles

To investigate the reproducibility of the peptide formation during fermentation, the peptide profiles of four independently manufactured samples of both kefir types, namely from commercial starter culture or kefir grains, were comparatively analyzed by MALDI-TOF-MS. Before mass spectrometric analysis, peptides were extracted by stage tips, because this method has been proven to be simple, fast and ideally suitable for MALDI-TOF-MS, allowing for reproducible results and parallel workup of many samples [31].

The lists of detected masses were compared to identify common features as well as differences between the different batches. In case of commercial starter culture the percentage of overlapping peptides per sample, i.e. peptides

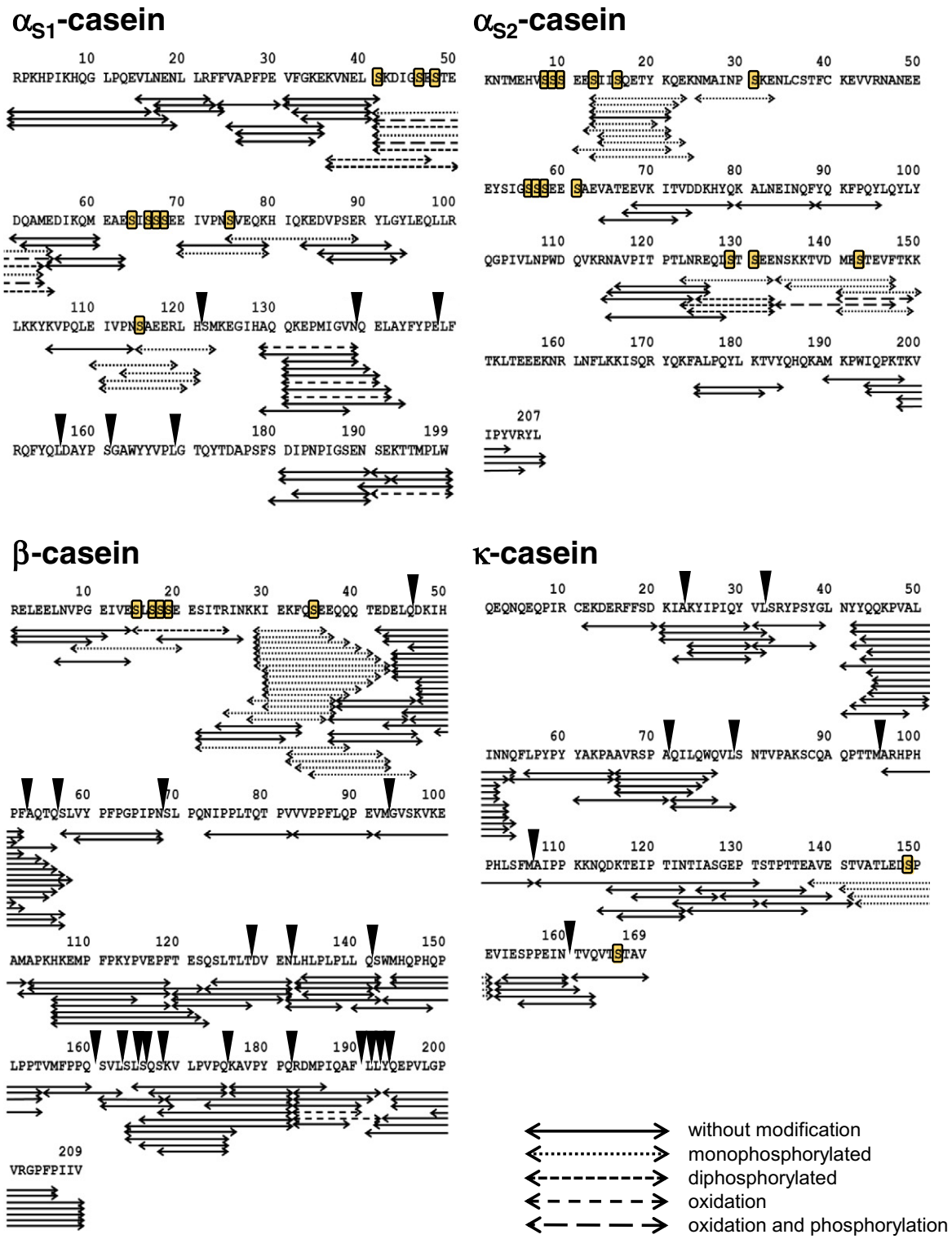

Fig. 3 – Amino acid sequences of the parent proteins α_{S1}-, α_{S2}-, β-, and κ-casein in single letter code. Arrows represent the identified released peptides. Marked serine residues are possible phosphorylation sites [33,35]. Black triangles indicate cleavage sites common for the majority of lactococcal proteinases [36].

which were detected in two or more samples, was 83 ± 15%. Hence, only a small part of 17 ± 15% was sample-specific. The kefir grain samples showed a similar picture: on average, 83 ± 7% of the signals per sample were detected in two or more samples, while 17 ± 7% were sample-specific and variable. These findings indicate a high reproducibility of the peptide profile of kefir and relatively low interbatch variations for both kefir varieties.

4. Discussion

4.1. Analysis of the kefir peptide profile

The present study performed comprehensive analysis of the peptide profile of kefir. To date, only a few studies analyzed the peptides released during kefir production, although the presence of microbiota with proteolytic activity has been previously identified in kefir [32]. Initial studies by Kahala et al. compared the rate of proteolysis and the generated peptide profile by HPLC-UV analysis of different fermented milk products. Their investigation revealed a high proteolysis rate in kefir and enabled the identification of seven peptides, all of which being β-casein fragments [16]. Moreover, studies by Farnworth showed that most of the peptides in kefir have a molecular weight ≤ 5000 Da (unpublished data cited in [6]). Quirós et al. focused on the ACE-inhibitory properties of caprine kefir and identified 16 peptides by RP-HPLC–MS/MS [15]. To our knowledge, no comprehensive peptidome analysis of kefir has been carried out yet. The present study is, therefore, the first to analyze the peptides released during kefir production in their entirety in an untargeted approach. The results shall improve the understanding of proteolytic processes and serve as a basis for the discovery of bioactive components. Among the 257 identified peptides, about 43% derived from β-casein, 22% from α_{S1}-casein, 21% from κ-casein, and 14% from α_{S2}-casein. Compared to other milk proteins, caseins show a clear prevalence with about 80% of the whole milk proteome, which are divided into 34% α_{S1}-caseins, 25% β-caseins, 9% κ-caseins, and 8% α_{S2}-caseins. The rest are β-casein breakdown products. The main components of whey proteins, which represent 20% of the total milk proteins, are β-lactoglobulin and α-lactalbumin with a content of 9% and 4%, respectively [33]. If the microbiological protein degradation would take place randomly to the same extent in all proteins, the origin of the kefir peptides would reflect the protein composition of milk, but this is clearly not the case. Especially β-casein must, therefore, be more prone to microbiological degradation by the kefir microflora. A surprising fact is that no peptides from the major whey proteins were found in the

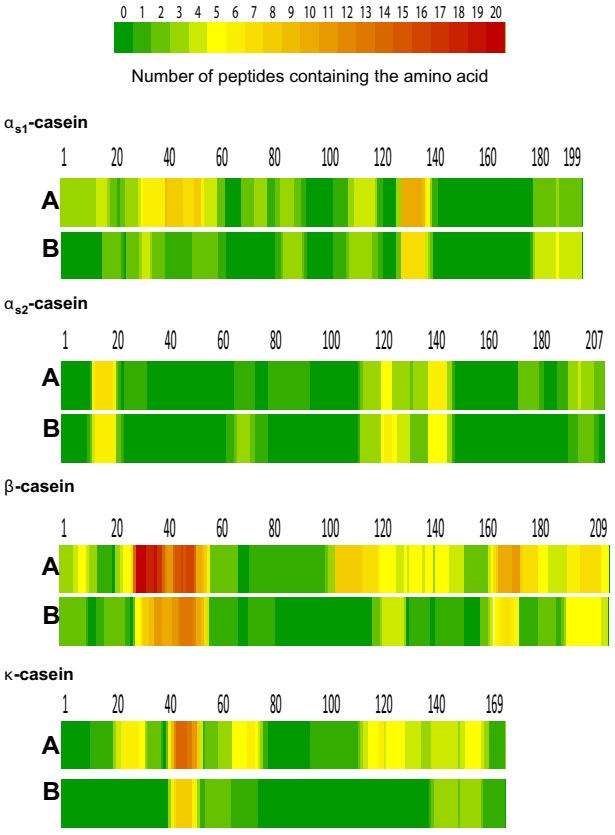

Fig. 5 – Origin of identified peptides in the sequences of the parent proteins α_{S1}-, α_{S2}-, β-, and κ-casein for milk fermented with commercial kefir starter culture (A) or kefir grains (B). The color depicts the number of peptides that include the amino acid.

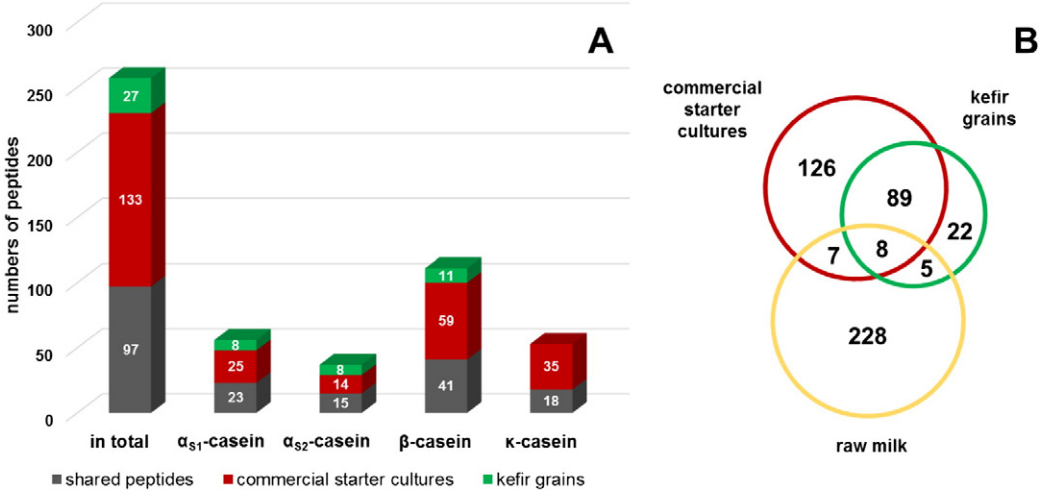

Fig. 4 – Distribution of identified peptides between the different sample types. Numbers show the total quantity of peptides identified either in milk fermented with commercial kefir starter culture or in milk fermented with kefir grains in total and for all parent proteins, i.e. α_{S1}-, α_{S2}, β- and κ-casein (A), and the comparison to the peptide profile of raw milk [10] in a Venn diagram (B).

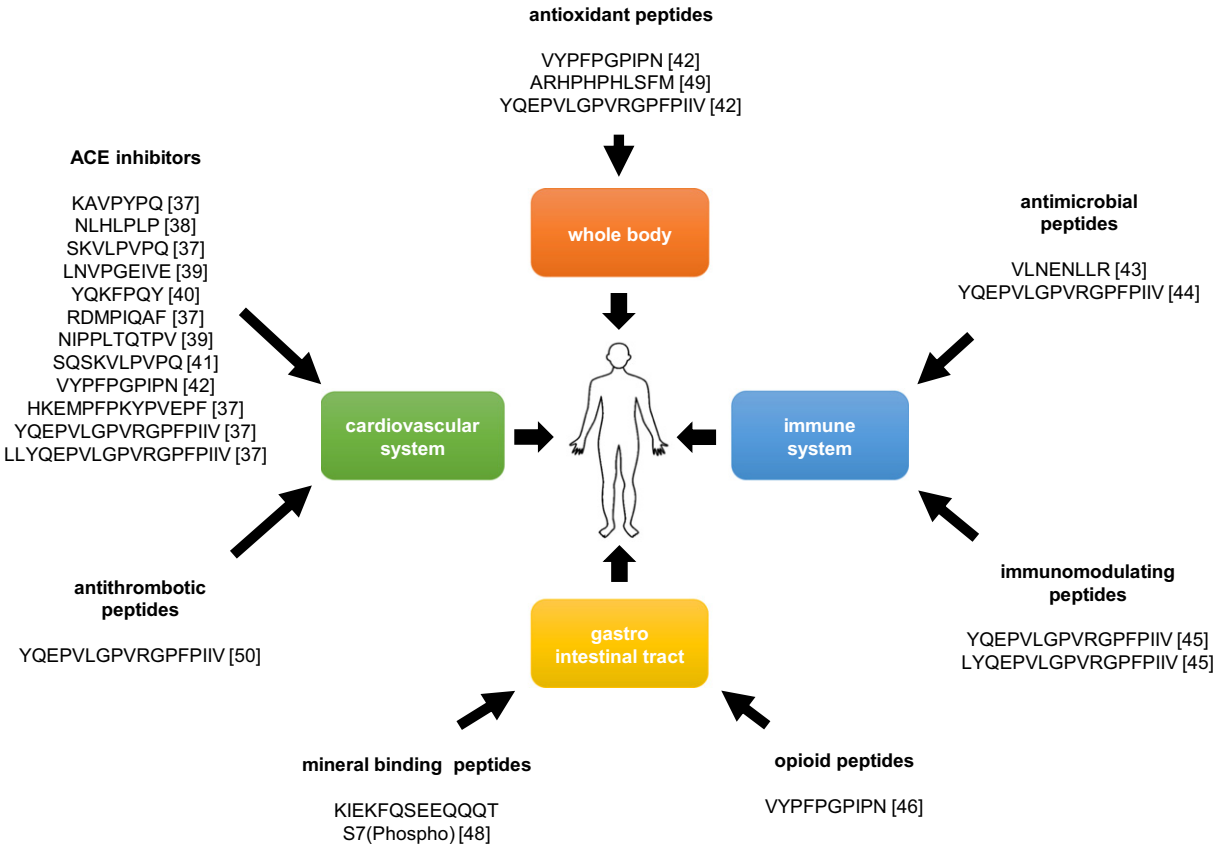

Fig. 6 – Known bioactive peptides found in kefir and their possible target sites in the human body. Peptide sequences are shown in single letter code.

samples, which could be explained by their better resistance to proteases because of their globular structure. Caseins, in contrast, are probably more accessible for the microorganisms due to their flexibility, which is partially caused by the high proline content [34].

All caseins are phosphoproteins. The major variant of α_{S1}-casein has eight to nine phosphorylation sites. The α_{S2}-casein sequence carries eleven phosphate groups, although some members of the α_{S2}-casein family differ in the number of phosphoserine residues. β-Casein usually contains five serine-bound phosphates, while κ-casein is mainly monophosphorylated [33]. In the present study, 62 phosphopeptides were identified: 52 of them were monophosphorylated and 10 peptides carried two phosphate groups. All phosphorylation sites were in agreement with current knowledge and the described phosphorylation sites in the UniProt database (http://www.uniprot.org) [33,35]. Loss of the phosphate group from a previously phosphorylated peptide during kefir production was observed only in a few cases.

Our results clearly demonstrate that fermentation caused the 1.7- to- 2.4-fold additional release of peptides compared to unfermented milk. Furthermore, a small, but significant contribution of the heat treatment to the peptide formation was detected. Thus, it can be concluded that the fermentation process improves the health promoting effects of milk that arise from the presence of bioactive peptides.

Both processes, heating and fermentation, led to a shift in the peptide profile from mainly α_{S1}-casein peptides to more β- and κ-casein fragments, most likely due to a combination of the generation of new peptides and the degradation of others. Comparing the peptide profiles of raw milk [10,11] and kefir, there are only few common components, whereas 236 of the identified peptides were unique for kefir. Consequently, not only the concentration of bioactive peptides may increase during the fermentation process, but the bioactivity profile may also be completely different in kefir compared to milk. Except for 21 peptides, the entire peptide profile changed during fermentation. Therefore, the process of peptide formation must be substantially different. While the milk peptides are generated mainly by the action of endogenous proteinases like plasmin, cathepsins B, D and G, microbiological degradation plays a key role in fermented products. Two general specificity classes of proteinases are reported for *Lactococci*. Whereas the primary substrates of P_I are β- and κ-casein, P_{III} additionally shows activity for α_{S1}-casein [36]. This would explain the particularly high release of β- and κ-casein fragments during the fermentation by lactic acid bacteria.

A closer look at the cleavage sites of the released peptides allows an insight into the formation mechanisms. The fact that some cleavage sites appear more often than others could be related to the specificity of some microbiological proteinases. The cleavage sites E–S, L–S, Q–S, E–V, E–D, E–N, Q–Q, Q–R, E–K, N–S, E–E, and Q–K (listed in descending order of frequency) were observed ten times or more indicating a preference of the active enzymes for C-terminal cleavages of the acidic residue glutamic

acid or of its amide glutamine. There are also indications of the action of exopeptidases due to the appearance of homologous series of peptides, e.g. f29–36, f29–37, f29–39, f29–40, f29–41, f29–42, and f29–43 from β-casein. As shown in Fig. 3, some of the cleavage sites are in accordance with previous reports and typical for the majority of lactococcal proteinases (black triangles) [36]. However, these protease effects explain the cleavage pattern of kefir only incompletely. *Lactococci* are merely one part of the complex kefir microflora. Other microorganisms in kefir also clearly demonstrate proteolytic behavior, although the specificity of their proteinases is not equally well investigated. Yüksekdag et al. analyzed the proteolytic properties of selected kefir grain *Lactobacilli* and showed that several strains have potent activity [32].

4.2. Influence of starter cultures

Because kefir varies greatly according to the microorganisms used for fermentation, two different product types were analyzed and compared. One kefir was produced using traditional kefir grain from Turkey, while another type of kefir was manufactured using a commercial starter culture mixture, thus addressing the two most common products. The microbiological composition of both fermented products was investigated to elucidate the differences. The overall numbers of CFU/g were comparable with only a slight difference in the number of total aerobic mesophilic bacteria and a bigger discrepancy in the yeast content, with more yeast CFU in the kefir grain product. The proteolytic activity of both products, however, varied considerably, being about 1.4-fold higher in the kefir produced by commercial starter. This difference cannot be explained solely by the slightly lower number of total mesophilic aerobic microorganisms, which were detected in the grain kefir. Possibly, the composition of species and subspecies of the bacteria varies between both products leading to different proteolytic behaviors. A larger share of bacterial strains with potent proteolytic properties in the commercial starter mixture compared to kefir grains could explain these findings. Fermentation with commercial starter cultures did not only result in a larger quantity of released amino acids and peptides, but even to a different composition of the peptide profile compared to the kefir grain variety. Both the MALDI-TOF peptide screening and the extended peptide profile generated by nano-UPLC-ESI-MS/MS revealed more peptides in kefir from commercial starter mixture.

Nevertheless, the major part of the peptides could be detected in both product types with matches accounting for 42% of the detected peptides in starter culture kefir and 78% in the grain kefir. Thus, it can be concluded that a typical kefir peptide profile does exist, which is relatively independent from the individual microflora used for manufacturing. It can be hypothesized that components of the common part of the kefir peptide profile may be used as markers for kefir products in general, whereas components of the variable part of the profile may serve as markers to differentiate kefir varieties and manufacturing parameters.

To investigate batch variations of the peptide profiles, four kefir samples per kefir type were independently produced in the course of 14 months and compared by means of stage tip microextraction and subsequent MALDI-TOF screening. Although MALDI-TOF screening does not cover the complete peptide profile of the samples, it is a quick method to reveal the variability of the peptide composition of a considerable number of samples in general. The four kefir samples from commercial starter culture as well as the four kefir grain samples showed a high percentage of peptides that were detected in more than one sample (83%), which demonstrates a high level of reproducibility.

4.3. Bioactive potential of kefir peptides

A detailed literature survey was performed for the identification of known bioactive sequences in the generated kefir peptide list. The major part of bioactive milk protein-derived peptides are ACE-inhibitors, influencing the blood pressure by inhibiting the conversion of angiotensin I to the vasoconstrictive angiotensin II and the degradation of the vasodilator bradykinin to its inactive fragments [12]. Twelve sequences among the kefir peptides have already been identified as ACE-inhibitors (Fig. 6) [37–42]. Additionally, two antimicrobial peptides, caseicin B [43] and casecidin 17 [44] with the sequences VLNENLLR (α_{S1}-casein$_{15–22}$) and YQEPVLGPVRGPFPIIV (β-casein$_{193–209}$) were identified. Caseicin B showed activity against *Escherichia (E.) coli* DPC6053 with minimal inhibitory concentration (MIC) values of 0.22 mM and casecidin 17 was active against the same strain and against *E. coli* DH5a with MICs of 0.4 mg/mL and 0.5 mg/mL, respectively. Two peptides with immunomodulating activity were identified among the kefir peptides [45]. Additionally, a relation of ACE inhibition and immunomodulation has been discussed, because the inhibition of ACE also hampers the inactivation of the immunostimulating bradykinin [7]. Thus, the ACE-inhibitory peptides detected in the present study could also be potential immunomodulators. Some milk-derived peptides are capable of binding to opioid receptors. Agonistic and antagonistic mechanisms are described resulting, e.g., in antidiarrheal effects or the modulation of amino acid transport [12]. One peptide VYPFPGPIPN (β-casein$_{59–68}$) was identified in the kefir peptide profile with opioid activity [46]. Another important part of biologically active milk peptides are caseinophosphopeptides, which bind minerals and thus have a beneficial effect on dental remineralization and possibly also on calcium resorption [8,47]. For one of the kefir peptides, KIEKFQSEEQQQT (β-casein$_{29–41}$, phosphoserin at S7), calcium binding was confirmed [48], but other sequences from the 62 identified phosphopeptides might have mineral binding properties as well. Finally, three kefir peptides with established antioxidant activity and one antithrombotic peptide were also identified [42,49,50].

Two of the identified bioactive peptides are of particular importance. Casecidin 17 is a multifunctional peptide exerting antimicrobial, ACE-inhibitory, immunomodulating, antioxidant, and antithrombotic activities [37,42,44,45,50]. Interestingly, this peptide is the signal with the second highest intensity in the MALDI-TOF mass screen (Fig. 2, peptide mass 1881.1 Da) indicating that it is highly abundant. Considering its interesting biological functions and high abundance, the peptide is expected to be of particular relevance for the health promoting effects of kefir. The peptide VYPFPGPIPN (β-casein$_{59–68}$, also called V-β-casomorphin-9) is another multifunctional peptide showing ACE-inhibitory, antioxidant, and opioid activity [42,46].

Additionally to these peptides, which were reported before to exert biological activity, many other identified sequences show a high homology to previously described physiologically active peptides and, thus, may have similar biofunctions. Moreover, it may be assumed that the bioactivity of most kefir peptides has not been investigated yet. Thus, the newly generated kefir peptide database can be used to identify further bioactive components in this product.

In summary, the present study established a kefir peptide database that includes 257 sequences: 236 of these peptides were unique for kefir and were not detected in milk previously. Thus, it can be concluded that the fermentation process considerably modifies the bioactivity profile of the peptide fraction. The presence of 97 peptides, which are common in kefir samples prepared either by commercial starter culture or traditional kefir grains, indicates a typical kefir peptide profile, which is relatively independent from the specific microflora used for manufacturing. Components of the variable part of the peptide profile may be used as indicators for different production parameters. Products prepared by starter culture show a higher proteolytic activity, which may have a beneficial influence on its health promoting effects.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

ACE	angiotensin-converting enzyme
PCA	plate count agar
OPA	o-phtaldialdehyde
UPLC	ultra-HPLC
FA	formic acid
DDA	data-dependent acquisition
CFU	colony-forming units
MIC	minimal inhibitory concentration

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