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Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins

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ABSTRACT

Scope: The microorganisms that make up kefir grains are well known for lactose fermentation, but the extent to which they hydrolyze and consume milk proteins remains poorly understood. Peptidomics technologies were used to examine the proteolytic activity of kefir grains on bovine milk proteins.
Methods and results: Gel electrophoresis revealed substantial digestion of milk proteins by kefir grains, with mass spectrometric analysis showing the release of 609 protein fragments and alteration of the abundance of >1500 peptides that derived from 27 milk proteins. Kefir contained 25 peptides identified from the literature as having biological activity, including those with antihypertensive, antimicrobial, immunomodulatory, opioid and anti-oxidative functions. 16S rRNA and shotgun metagenomic sequencing identified the principle taxa in the culture as *Lactobacillus* species.
Conclusion: The model kefir sample contained thousands of protein fragments released in part by kefir microorganisms and in part by native milk proteases.

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1. Introduction

Food fermentation is one of the oldest methods of extending shelf-life, and more than 3500 fermented foods are known (Farnworth, 2003). Kefir, an acidic, fermented milk beverage that originated thousands of years ago in the Caucasus Mountains, is still consumed worldwide. To produce it, kefir grains—a complex of polysaccharides, proteins, symbiotic lactic acid bacteria (*e.g. Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*) and yeast (*e.g. Saccharomyces*, *Candida*, *Kluyveromyces*, *Debaryomyces* and *Torulaspora*)—are incubated with heat-treated milk under aerobic conditions (Angulo, Lopez, & Lema, 1993; Leite et al., 2013; Wang et al., 2012). The fermentation of milk lactose by these microorganisms results in acidification of the product, which prevents the growth of spoilage organisms. Typically, kefir grains are inoculated at 2–8% concentration and allowed to incubate for 18–24 h at 20–25 °C (Otles & Cagindi, 2003). After incubation, the kefir is allowed to mature further at 4 °C for 20–24 h (Otles & Cagindi, 2003).

Though the lactic acid bacteria in kefir are known to hydrolyze casein, which is critical for texture and flavor development (Kunji, Mierau, Hagting, Poolman, & Konings, 1996), the extent to which caseins and other milk proteins are hydrolyzed by kefir microorganisms remains unclear. This study employed mass spectrometry-based peptidomics and gel electrophoresis to examine the peptides released from bovine milk proteins by the kefir microorganisms. To determine whether peptides in kefir were the result of kefir microorganism activity, were naturally occurring peptides, or were released by native milk proteases during incubation, this study compared peptides in heat- and kefir-treated raw milk with those in unincubated raw milk and in heat-treated incubated milk without kefir.

Milk peptides released during kefir fermentation were examined for homology with milk peptides known to have antimicrobial, antihypertensive, immunomodulatory, opioid and prebiotic properties (Clare & Swaisgood, 2000). Determining which functional peptides are released can lead to exploration of possible peptide-induced health benefits from kefir consumption.

Abbreviations: ANOVA, analysis of variance; HMc, heat-treated milk with closed vials during incubation; HMo, heat-treated milk with opened vials during incubation; HSD, honest significant difference; K, kefir; RM, raw milk; TCA, trichloroacetic acid.

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2. Materials and methods

2.1. Samples

Fresh milk was collected from a pool of six healthy Holstein cows at the University of California, Davis (USA) as described previously (Dallas et al., 2013b). Before attaching the milking pumps, all four teats were washed with water and then dipped in an antiseptic solution (Chlorhexidine Active Mastitis Prevention) with 0.5% chlorhexidine gluconate as the active ingredient. The milk was immediately frozen at −30 °C until use. Kefir grains were purchased from Fusion Teas (McKinney, Texas, USA) and were preserved in pasteurized milk at 4 °C.

2.2. Sample preparation

After thawing and gently mixing, the freshly collected, frozen raw milk was apportioned into twelve 1-mL subsamples (3 subsamples for each of the 4 study groups). Three subsamples were frozen at −20 °C to serve as the untreated control (raw milk, RM). The nine remaining subsamples were heated at 93 °C for 7 min using a thermomixer (Thermo Mixer C, Eppendorf, Hamburg, Germany), cooled in an ice bath for 20 min and brought to room temperature. This heat treatment was selected based on previous literature on kefir production (Otles & Cagindi, 2003). To have a concentration of 4.15% kefir grains, 41.5 mg of kefir grains were added to three of the nine samples (Kefir, group K). Prior to collection, the kefir grain supply was thoroughly mixed to ensure a representative sample was collected. The nine samples were incubated on a thermomixer at 23 °C for 24 h at 800 rpm and then matured at 4 °C for 24 h. During the incubation and maturation steps, the three sample vials with kefir grains (group K) were kept open to match the aerobic conditions typical for kefir production. To control for any environmental contamination by air-borne microorganisms, three of the six vials of heat-treated milk without kefir grains were closed (heat-treated milk with closed vials during incubation, group HMc), whereas the remaining three were open (heat-treated milk with opened vials during incubation, group HMo). The three vials of raw milk (group RM) were defrosted and all 12 subsamples were centrifuged at 16,000*g* at 4.5 °C for 10 min to separate and remove the milk fat and 500 μL of delipidated milk were collected from each vial.

2.2.1. Trichloroacetic acid protein precipitation

Skim milk proteins in each of the twelve subsamples were precipitated by addition of trichloroacetic acid (200 g/L TCA, EMD Millipore, Darmstadt, Germany) in a 1:1 v/v ratio. After centrifugation at 4000g, at 20 °C for 10 min, 850 μL of the supernatant from each of the twelve subsamples were collected for extraction of peptides.

2.2.2. Extraction of peptides with C18 microplate

Sugars, salt and TCA were removed from the supernatants using a C18 solid-phase extraction microplate procedure (Dallas et al., 2015) with no modifications. Peptide fractions were dried by centrifugal evaporation (miVac Quattro, Genevac, Ipswich, UK) at 44 °C and preserved at −20 °C.

2.3. Sample analysis

2.3.1. Mass spectrometry-based peptide analysis

LC separation was performed on a Waters Nano Acquity UHPLC (Waters Corporation) with a Proxeon nanospray source. The peptides were reconstituted in 2% ACN, 0.1% TFA. Two micrograms of each sample were loaded onto the column based on measured absorbance at 280 nm. Peptides were first loaded onto the trap column (a 100 μm × 25 mm Magic C18 100 Å 5 U reverse-phase column) for online desalting and then onto a 75 μm × 150 mm Magic C18 200 Å 3 U reverse-phase column (Waters, Milford, MA) for analytical separation. Peptides were eluted using a gradient of 0.1% formic acid (A) and 100% acetonitrile (B) with a flow rate of 300 nL/min. The 60-min gradient was designed as follows: 5–35% B over 50 min, 35–80% B over 3 min, 80% B for 1 min, 80–5% B over 1 min and then held at 5% B for 5 min. Each sample injection was followed by a 30 min column wash.

Mass spectra were collected on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) in a data-dependent mode with one MS precursor scan followed by 15 MS/MS scans. A dynamic exclusion of 20 s was used. MS spectra were acquired with a resolution of 70,000 and a target of 1×10^6 ions or a maximum injection time of 30 ms. MS/MS spectra were acquired with a resolution of 17,500 and a target of 5×10^4 ions or a maximum injection time of 50 ms. Peptide fragmentation was performed using higher-energy collision dissociation with a normalized collision energy value of 27. Unassigned charge states as well as ions >+6 were excluded from MS/MS fragmentation.

2.3.2. Spectral analysis and peptide identification

Spectra were analyzed by database searching in X!Tandem as described previously (Dallas, Guerrero, Khaldi, et al., 2013; Dallas, Guerrero, Parker, et al., 2013), with minor modifications. No complete (required) modifications or potential modifications were allowed. Spectra were searched against a bovine milk library compiled from previous bovine milk proteome literature. The data were deposited to the ProteomeXchange with identifier PXD001826.

2.3.3. Peptide peak area determination

An in-house curated bovine milk protein library was imported in .fasta file format into Skyline (Schilling et al., 2012). A library of identified peptides was uploaded from the .xml outputs of the X!Tandem program for each sample to create the spectral library. After applying all settings, the spectral library was searched against the raw data files (.raw) to extract the peaks for each peptide in each sample.

The settings for the extraction were as follows. Precursor mass was calculated based on the monoisotopic ion. Allowed precursor charges were 1–6. Ion types were set as precursor only. The ion match tolerance was set to 0.5 m/z. The instrument acquisition window was set between 300 and 1600 m/z with a tolerance of 0.055 m/z. For MS1 filtering, isotope peaks included by count were employed. The precursor mass analyzer was set to "Orbitrap." The resolving power was set to 60,000 at 400 m/z. The precursor isotopic import filter was set to a count of 3 (M, $M+1$, $M+2$). MS/MS filtering was set to none. Retention time filtering was set to 1 min of the MS/MS identifications.

After data import, all peaks were manually inspected for proper peak picking of the MS1-filtered peptides. Only peaks with $\leqslant$3 ppm mass error and an idotp score of $\geqslant$80 were retained. Peaks were selected based on mass error and retention time proximity to the identified peptide retention time. Peaks that did not match these criteria were deleted. Peaks too close to the noise level to be visually discernable were excluded.

After manual inspection, the data were exported to a .csv file. To reassign the protein name to peptide sequence and collapse multiple charge states into a single compound, the file was processed through an in-house script.

2.3.4. Functional peptide search

Identified peptide sequences from the samples were searched against a library of known functional milk peptides from the literature (Hayes, Stanton, Fitzgerald, & Ross, 2007; Maruyama, Nakagomi, Tomizuka, & Suzuki, 1985; Minervini et al., 2003;

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