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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

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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

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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%