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Impact of microbial cultures on proteolysis and release of bioactive peptides in fermented milk

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ABSTRACT

This study aimed at evaluating co-cultures of selected microorganisms for their proteolytic activity and capability to produce fermented milk enriched with ACE-inhibitory (ACEI) peptides. Selected yeasts (*Torulaspora delbruekii* KL66A, *Galactomyces geotrichum* KL20B, *Pichia kudriavzevii* KL84A and *Kluyveromyces marxianus* KL26A) and lactic acid bacteria strains (*Lactobacillus plantarum* LAT03, *Lb. plantarum* KLAT01 and the not virulent *Enterococcus faecalis* KE06) were screened as single cultures for their capacity of releasing ACEI peptides without producing bitter taste. Three strains cultures (yeast, *Lb. plantarum* and *E. faecalis*) were performed to evaluate the combined impact on microbial growth, lactic acid production, citric acid consumption, proteolysis, ACEI activity, and bitter taste after 36 h of fermentation at 28 °C. While *G. geotrichum* KL20B showed a strong stimulating effect on *Lb. plantarum* strains and the production of peptides with ACEI activity, the presence of *T. delbruekii* KL26A in the cultures was deleterious both to ACEI activity and product taste. The most effective combination was *P. kudriavzevii* KL84A, *Lb. plantarum* LAT3, *E. faecalis* KL06, which showed the highest ACEI activity ($IC_{50} = 30.63 \pm 1.11$ μg ml^{-1}) and gave no bitter taste for 7 days at 6 °C. Our results highlight the importance of choosing the strains combination carefully, to obtain a high yield of ACEI activity without bitter taste.

1. Introduction

Kumis is a natural fermented cow milk, widely consumed in rural and urban areas in south west Colombia. The fermentation is carried out by lactic acid bacteria (LAB) and yeasts at room temperature for about two days (Chaves-López et al., 2012).

Spontaneous milk fermentation involves a number of metabolic pathways, in which metabolites contribute to confer chemical, biochemical and nutritional attributes to fermented milk. In particular, proteolysis leads to the production of bioactive peptides that have a positive impact on body conditions or functions, and may ultimately influence human health (Kitts and Weiler, 2003). The proteolytic system of LAB is a very complex system that consists of three major components: a cell-wall bound proteinase that promotes extracellular casein degradation into oligopeptides, then peptide transporters move peptides into the cytoplasm, where finally various intracellular peptidases degrade peptides into smaller molecules and amino acids (Liu et al., 2010). On the other hand, yeasts species also possess both intracellular and extracellular proteases. In particular, extracellular enzymes are known for the genera *Candida, Cryptococcus, Rhodotorula, Pichia* and *Yarrowia.* Yeasts peptidases like aminopeptidases and carboxypeptidases play an important role in the proteolysis of milk proteins (Ferreira and Viljoen, 2003).

Several anti-hypertensive peptides produced during milk fermentation have a strong activity against Angiotensin I-Converting Enzyme (ACE), a dipeptidyl carboxypeptidase that plays a major role in the regulation of blood pressure within the renin–angiotensin system (Riordan, 2003), inducing blood pressure increase. *In vivo* studies evidenced a reduction of blood pressure after consumption of fermented milks (Pina and Roque, 2008; Hernández-Ledesma et al., 2004). Moreover, in vitro ACE-inhibitory (ACEI) activity of different traditional fermented milks has been reported in the literature (Chaves-López et al., 2011; Sun et al., 2009). Thus, selection of microorganisms to be used in fermented products is gaining importance, due to the inherent variations in their ability to produce bioactive peptides, particularly those with specific health claims (Ramchandran and Shan, 2008). Interactions between different microorganisms in fermented milks may contribute to product quality, therefore, the positive or negative effect of the interaction becomes very important (Viljoen,

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2001) in fermented milk production. In previous studies, we selected bacteria and yeasts strains able to produce high ACEI activity in vitro during milk fermentation (Chaves-López et al., 2011, 2012); in this study we evaluate co-cultures of selected microorganisms for their proteolytic activity and capability to produce fermented milk enriched with ACEI peptides. We also determined product bitterness, which might hamper consumer acceptance of milks containing bioactive peptides as functional ingredients.

2. Materials and methods

2.1. Strains

The strains used in this study, belonging to the Culture Collection of the Faculty of Bioscience and Technology for Food, Agriculture and Environment of the University of Teramo, were isolated from Colombian Kumis and selected for their capability to produce fermented milk with high ACEI activity. Pure strains of not virulent *Enterococcus faecalis* KE06 (Chaves-López et al., 2011) *Lactobacillus plantarum* KLAT1, *L. plantarum* LAT03, *Torulaspora delbrueckii* KL66A, *Galactomyces geotrichum* KL20B, *Pichia kudriavzevii* KL84A, and *Kluyveromyces marxianus* KL26A, were activated from their frozen forms (stored in 40% glycerol at −80 °C) by transferring once in MRS broth (*Lb. plantarum*) or in M17 broth (*E. faecalis*), and YPD broth for yeasts. After activation, two successive transfers into the same medium were performed. During this procedure, all microorganisms were incubated at 30 °C.

2.2. Preparation of inoculum

Inoculum of single cultures was obtained as follows: purified colonies were transferred into 1 ml of MRS, M17 or YPD media at 28 °C for 24 h. Cells were centrifuged at 5000 rpm × 10 min in a refrigerated centrifuge, the pellet was then suspended in 100 ml of sterile reconstituted (10%) skimmed milk (Biolife, Milan, Italy), and incubated at 28 °C for 18 h for LAB and 48 h for yeasts. Each preculture sample was used to inoculate (1% v/v) 400 ml pasteurized whole cow milk in single culture and in co-cultures (Table 1), to obtain approximately 7.0 Log CFU ml^{-1} of LAB and 6 Log CFU ml^{-1} of yeasts. Un-inoculated milk used as control and inoculated samples were incubated at 28 °C for 36 h (period used to make traditional fermented Colombian Kumis). Fermentation was stopped by heating at 75 °C for 1 min. Subsequently, samples inoculated with culture 5, selected for the best performance, were refrigerated at 6 °C for 7 days to evaluate the stability of ACEI activity and the taste.

At the end of fermentation and during the refrigerated storage, samples were analysed to determine pH, microbial growth, lactic and citric acid, proteolysis, ACEI activity and sensory characteristics.

2.3. Microbial counts

Before and after fermentation and refrigerated storage, aliquots of 1 ml of fermented milks were diluted with sterile saline solution (0.85% NaCl), and 100 μl of the samples were plated onto MRS agar for *Lb. plantarum*, Kanamicine Azide Agar for *E. faecalis* and YPD agar with chloramphenicol (150 ppm) for yeasts. All the samples were incubated at 30 °C (24 h for bacteria and 48 for yeast).

Table 1
Microorganisms used as starters to produce fermented milks.

Co-culture	Yeasts	*Enterococcus faecalis*	*Lactobacillus plantarum*
1	*T. delbrueckii* KL66A	KE06	LAT3
2	*T. delbrueckii* KL66A	KE06	KLAT1
3	*G. geotrichum* KL20B	KE06	LAT3
4	*G. geotrichum* KL20B	KE06	KLAT1
5	*P. kudriavzevii* KL84A	KE06	LAT3
6	*P. kudriavzevii* KL84A	KE06	KLAT1
7	*K. marxianus* KL26A	KE06	LAT3
8	*K. marxianus* KL26A	KE06	KLAT1

2.4. Determination of lactic and citric acid

Organic acids were quantified by high-performance liquid chromatography (HPLC) analysis (ThermoFinnigan Italia Spa, Rodano, Italy), as described by Sarantinopoulos et al., 2001, using a chromatographic system consisted of a Spectra System P4000 pump and a Spectra System AS3000 autosampler (ThermoFinnigan Italia Spa, Rodano, Italy).

2.5. Assay for ACEI activity and peptides profiles and quantification

The antihypertensive activity of peptides was determined based on ACEI activity and IC_{50} value. The IC_{50} value was defined as the concentration of peptide (mg ml^{-1}) required to reduce 50% of absorbance peak height of the hippuric acid, which was determined by regression analysis of ACE inhibition (%) versus protein concentration.

The measurement of ACEI activity was carried out spectrophotometrically using the pH 4.6 soluble fraction of milk obtained by centrifugation (10 000 g × 10 min at 4 °C), as previously reported (Pan et al., 2005).

The determination of IC_{50}, proteins, peptides and amino acids content as well as the determination of the stability and formation of new ACEI peptides after hydrolysis with pepsin and pancreatin was performed as reported by Chaves-López et al. (2011; 2012).

2.6. Sensory analysis

Samples for sensory analysis were tasted at room temperature and analysis was performed according to Chaves-López et al. (2012).

2.7. Statistic analysis

All the experiments were carried out in triplicate, and the analyses in duplicate. Experimental data were subjected to analysis of variance (ANOVA), and pair-comparison of treatment means was achieved applying Tukey's procedure at $p < 0.05$, using Statistica for Windows.

3. Results

3.1. Co-cultures growth and milk acidification

In co-culture trials (Table 2), yeasts growth was lower than in single culture: the lactose fermenting *K. marxianus* KL26A (mix 7 and 8) increased only by about 0.5 Log CFU ml^{-1}, *P. kudriavzevii* KL84A did not grow in mix 5, whereas *T. delbrueckii* KL66A and *G. geotrichum* KL20B decreased in all the mixes. *E. faecalis* KE06 counts were similar in all the mixes with an increase of approximately 1.5 Log CFU ml^{-1} and with slightly significant differences with respect to single cultures. On the contrary, both *Lb. plantarum* strains grew significantly ($p < 0.05$) in association with *E. faecalis* KE06 and *G. geotrichum* KL20B (Mixes 3 and 4) compared to single cultures, with an increase of about 1 and 1.5 Log CFU ml^{-1}.

Growth of the co-cultures was associated to accumulation of organic acids, leading to medium acidification from pH 6.6 to 4.1 after 36 h of fermentation. Acidification was highly correlated with lactic acid production, which was higher in co-cultures 3 and 4 with *G. geotrichum* KL20B (from 11.4 to 13.1 mg ml^{-1}) probably due to an