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Impact of non-starter lactobacilli on release of peptides with angiotensin-converting enzyme inhibitory and antioxidant activities during bovine milk fermentation

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ABSTRACT

This study aimed at evaluating non-starter lactobacilli (NSLAB) isolated from cheeses for their proteolytic activity and capability to produce fermented milk enriched in angiotensin-converting enzyme (ACE)-inhibitory and antioxidant peptides. Preliminarily, 34 NSLAB from Parmigiano Reggiano (PR) and 5 from Pecorino Siciliano cheeses were screened based on their capacity to hydrolyze milk proteins. Two NSLAB strains from PR, *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331, showed the most proteolytic phenotype and were positively selected to inoculate sterile cow milk. The fermentation process was monitored by measuring viable cell population, kinetic of acidification, consumption of lactose, and synthesis of lactic acid. Milk fermented with *Lb. casei* PRA205 exhibited higher radical scavenging (1184.83 ± 40.28 mmol/L trolox equivalents) and stronger ACE-inhibitory (IC_{50} = 54.57 μg/mL) activities than milk fermented with *Lb. rhamnosus* PRA331 (939.22 ± 82.68 mmol/L trolox equivalents; IC_{50} = 212.38 μg/mL). Similarly, *Lb. casei* PRA205 showed the highest production of ACE-inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro, which reached concentrations of 32.88 and 7.52 mg/L after 87 and 96 h of milk fermentation, respectively. This evidence supports *Lb. casei* PRA205, previously demonstrated to possess characteristics compatible with probiotic properties, as a promising functional culture able to promote health benefits in dairy foods.

1. Introduction

Biologically active peptides derived from food proteins are specific protein fragments that have a positive impact on body functions, going well beyond their nutritional value (Kamau et al., 2010). Milk proteins, especially caseins, are currently the main precursors of biologically active peptides (Silva and Mancada, 2005). Depending on size and amino acid sequence, milk-derived peptides may exert a number of different activities *in vitro* and/or *in vivo*, such as immuno-modulation, anticancer action, hypocholesteremic effect, as well as antimicrobial, mineral-binding, opioid, and peptidase inhibition activities (Fitzgerald and Murray, 2006; Mills et al., 2011). They can be released from milk proteins by gastro-intestinal (GI) digestion or by enzymatic hydrolysis during food processing and fermentation (Pihlanto, 2006). In particular, milk protein hydrolysates and fermented dairy products are enriched in antihypertensive and antioxidant peptides (Silva and Mancada, 2005; Pihlanto, 2006; Korhonen, 2009). Among these, lacto-tripeptides valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) are resistant to GI digestion and can cross the mucosal barrier, resisting to digestion by serum peptidases (Foltz et al., 2008). VPP and IPP have been shown to reduce systolic blood pressure in hypertensive subjects (Boelsma and Kloek, 2010; Nakamura et al., 2011; Cicero et al., 2013), due to inhibition of angiotensin-converting enzyme (ACE), stimulation of vasodilator production, and modulation of sympathetic nervous activity (Usinger et al., 2010). In addition to antihypertensive effects, milk-derived peptidic fractions have been demonstrated to exert radical scavenging functionalities and prevent oxidative stresses associated with numerous degenerative chronic diseases, including cardiovascular ischemia, reperfusion, and atherosclerosis (Kudoh et al., 2001; Virtanen et al., 2007).

Proteolytic systems from lactic acid bacteria (LAB) are the main route to generate bioactive peptides during milk fermentation and cheese ripening (Gobbetti et al., 2004). LAB possesses variable

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patterns of proteinases, peptidases and peptide transport systems which affect release and intake of bioactive peptides in milk in a species- and strain-specific manner (Liu et al., 2010). These systems enable LAB to fulfill their amino acid requirements (Christensen et al., 1999) and contribute to the formation of flavor and texture in dairy products (Settanni and Moschetti, 2010). Cell-envelope proteases break down proteins in the growth media into peptides of about 5–30 amino acids that are carried into the cell and further hydrolysed by endopeptidases into smaller peptides and amino acids for microbial protein synthesis. PepI, PepP, PepQ, PepR, and PepX endopetidases have proline-specific hydrolytic activities which account for the main release of bioactive peptides from proline-rich α-, β- and κ-caseins.

Lactobacillus genus is predominant in dairy food and encompasses the main acid-producing starter cultures (SLAB), as well as the mesophilic species most responsible for cheese ripening (indicated as non-starter LAB, NSLAB). The role of SLAB lactobacilli in bioactive peptide release is well documented during milk fermentation (reviewed by Gobbetti et al., 2004; Fitzgerald and Murray, 2006). Among SLAB, thermophilic *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* strains have been extensively studied for their strong and specific proteolytic activity in milk which results in release of higher amounts of active peptides compared to other lactobacilli (Sadat-Mekmene et al., 2011 and references herein). In contrast, a few of works deals with the ability of mesophilic NSLAB strains to hydrolyze caseins into bioactive peptides (Fuglsang et al., 2003; Ramchandran and Shah, 2008; Wang et al., 2010). Within NSLAB, facultatively heterofermentative *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are extensively used both as probiotics and adjunct cultures in different dairy products (Settanni and Moschetti, 2010; De Vos, 2011). Potential health benefits associated with the consumption of these bacteria rely on their ability to interact with the intestinal epithelial cells directly, but also indirectly, through the production of biogenic compounds (Lebeer et al., 2010 and references herein). When probiotics are delivered with fermented dairy products, milk-derived peptides with different biological activities can be produced (Fitzgerald and Murray, 2006; Hayes et al., 2007).

In our previous works, we established a de-replicated set of *Lb. casei*, *Lb. paracasei*, and *Lb. rhamnosus* NSLAB strains isolated from Parmigiano Reggiano cheese (Solieri et al., 2012) and assessed their probiotic aptitude (Solieri et al., 2014). Aim of this work was to explore the potential of these strains to release bioactive peptides with radical scavenging and ACE-inhibitory activities during cow milk fermentation, with a special focus on their ability to release VPP and IPP from milk caseins.

2. Materials and methods

2.1. Materials

All MS/MS reagents were from Biorad (Hercules CA, U.S.A.), whereas the remaining chemicals were purchased from Sigma–Aldrich (Milan, Italy) unless otherwise stated. De Man, Rogosa and Sharpe (MRS) medium was provided by Oxoid (Basingstoke, Hampshire, England). Amicon Ultra-0.5 regenerated cellulose filters with a molecular weight (MW) cut-off of 3 kDa were supplied by Millipore (Milan, Italy). Ultra-high-temperature-treated (UHT) skimmed bovine milk was obtained from a local producer (protein, 3.15%; fat, 0.3%; lactose, 4.95%). VPP and IPP peptides (95% purity) were synthesized by DBA (Milan, Italy). The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer (Orlando FL, U.S.A.), with the exception of DNA samples that were quantified by Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA). Taq DNA polymerase was from Takara (Kyoto, Japan), while primers were provided by MWG (Heidelberg, Germany).

2.2. Bacteria and growth conditions

The bacterial strains used in this study are listed in Table 1. Thirty-four strains were isolated from ripened Parmigiano Reggiano cheeses (Solieri et al., 2012) and deposited in the Unimore Culture Collection (UMCC) (www.unimore.umcc.it). Nine *Lb. rhamnosus* strains isolated from Sicilian Pecorino cheese and human vaginal samples, were kindly provided by Prof. C. Randazzo (University of Catania, Italy). All LAB strains were maintained as frozen stock at −80 °C in MRS broth supplemented with 25% glycerol. Prior to the experimental use, the cultures were twice propagated in MRS medium and incubated at 37 °C for 24 h under anaerobic conditions. For preliminary screening of NSLAB proteolytic activity, bacterial cells grown until late exponential phase in MRS medium, were harvested by centrifugation, and washed twice with 50 mmol/L Tris–HCl buffer (pH 6.5) and inoculated, in

Table 1
Strains used in the present work.

Species	Strains	Isolation source	References
Lb. rhamnosus	GG (ATCC 53103)	faecal human sample	Goldin et al., 1992
	PRA101	6 month long ripened PR	Solieri et al., 2012
	PRA251, PRA321	7 month long ripened PR	
	PRA202, PRA211, PRA222	10 month long ripened PR	
	PRA231, PRA331	11 month long ripened PR	
	PRA011, PRA141, PRA152, PRA161, PRA172	12 month long ripened PR	
	PRA091	14 month long ripened PR	
	PRA272	18 month long ripened PR	
	PRA291	23 month long ripened PR	
	LOC5, LOC6, LOC12, LOC46	human vagina samples	provided by C. Randazzo
	E24, E31, E33, D21, D55	Sicilian Pecorino cheeses	Randazzo et al., 2006
Lb. paracasei	PRA104	6 month long ripened PR	Solieri et al., 2012
	PRA191, PRA213, PRA221	10 month long ripened PR	
	PRA181	11 month long ripened PR	
	PRA021, PRA071, PRA081, PRA171, PRA313, PRA111, PRA121, PRA131, PRA142	12 month long ripened PR	
	PRA241	13 month long ripened PR	
Lb. casei	PRA322	7 month long ripened PR	Solieri et al., 2012
	PRA205	10 month long ripened PR	
	PRA041	12 month long ripened PR	

Abbreviations: ATCC, American Type Culture Collection; PR, Parmigiano Reggiano cheese.