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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.

Keywords: *Lactobacillus*, bioactive peptides, valine-proline-proline (VPP), isoleucine-proline-proline (IPP), probiotic, ACE-inhibition

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 Malcata, 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 Malcata, 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 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 amount 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 triplicates, in 10 mL of ultra-high temperature-treated (UHT) skimmed milk (2% v/v). Negative control was realized by replacing the cell suspension with Tris-HCl buffer. After incubation at 37°C for 72 h in shaking conditions (10 rpm), samples were treated with 1% trichloroacetic acid (TCA) for 10 min and centrifuged (10,000g, 20 min, 4°C) for proteolytic activity determination and relative quantification of VPP and IPP.

2.3 Cow milk fermentation

Fermented milk was produced with the best strains previously chosen in preliminary screening, by using UHT skimmed milk under sterile conditions in order to exclude enzyme interference by contaminant microorganisms. Fifty mL of UHT skimmed milk were inoculated with single-strain cultures of selected LAB strains [2% v/v corresponding to ca. 10^8 colony forming units (cfu)/mL final concentration], as reported above, and incubated at 37°C for 120 h in shaking conditions (10 rpm). Three biological replicates were performed for each strain. For each replicate, two aliquots were taken at 11 time points (0, 15, 24, 39, 48, 63, 72, 87, 96, 111, and 120 h). One aliquot was immediately used to determine the number of viable cells and the pH, whereas the other one was frozen for subsequent determinations of sugars, lactic acid, amount of VPP and IPP, as well as ACE-inhibitory, proteolytic and radical scavenging activities.

2.4 Microbiological analysis

The number of presumptive viable LAB cells was determined at different time points (0, 15, 24, 39, 63, 87, and 111 h) by plating 10-fold diluted samples of fermented milks on MRS agar. After incubation at 37°C for 48 h under anaerobic conditions, the number of cfu per mL was counted and expressed as Log cfu/mL.

125 At 63 h of fermentation (corresponding to pH lower than 4.4), 9 colonies for each trial were picked
126 from the highest dilutions of MRS plates, cultured in MRS broth at 37 °C for 24 h, and subjected to
127 DNA extraction as described by Ulrich and Hughes (2001), with a few modifications. Briefly, the
128 complete cellular lysis was obtained by increasing the number of freeze-thaw steps from three to
129 six. A final treatment with 1.5 µL RNase (10 mg/mL) at 37°C for 30 min was added to assure
130 complete RNA degradation. DNA was quantified spectrophotometrically and properly diluted. The
131 V1 region of the *Lb. casei* 16S rRNA gene and the *Lb. rhamnosus* 16S–23S rRNA intergenic spacer
132 region (ISR) were amplified using the species-specific primer pairs Casei/Y2 and PrI/RhaII,
133 respectively (Solieri et al., 2012). PCR conditions were according to Solieri et al. (2012) with the
134 following exceptions: annealing temperature was shifted from 55 to 58°C and the final
135 concentration of template DNA was increased from 2 to 4 ng/µL. Strain genotyping was carried out
136 through microsatellite primed-PCR (MSP-PCR) using the arbitrarily chosen sequence $(GTG)_5$ (5'-
137 GTG GTG GTG GTG GTG-3'), as previously reported (Solieri e al., 2012). Amplified-fragment
138 profile comparison in disposable databases (Bionumerics 5.10, Applied Maths, Belgium) was
139 performed as described by Solieri et al. (2012), using a similarity threshold of 90%. Only
140 reproducible well-marked amplified fragments were included in gel analysis, faint bands being
141 ignored.
142
143 *2.5 Determination of proteolytic and radical scavenging activities*
144 Aliquots of fermented milk were treated with 1% TCA for 10 min and centrifuged (10,000*g*, 20
145 min, 4°C) to obtain TCA-soluble supernatants containing peptide fractions. Proteolytic activity was
146 quantified by measuring the amount of released amino groups in TCA-soluble supernatants using
147 TNBS method (Adler-Nissen, 1979). A calibration curve was prepared using leucine as standard
148 (range 0.1–2.0 mmol/L). The results were expressed as mmol/L of leucine equivalents.
149 The radical scavenging activity of TCA-soluble supernatants was measured using the ABTS method
150 (Re et al., 1999) and the results were expressed as µmol/L of Trolox.

151

152 *2.6 Chemical analysis*

153 Amounts of lactose, galactose, glucose, and lactic acid were enzymatically determined according to
154 the manufacturer's instructions (Megazyme International, Wicklow, Ireland) and the results were
155 expressed as mmol/L. Samples were centrifuged (10,000*g*, 20 min, 4°C) and then used for the
156 analysis.

157

158 *2.7 Angiotensin I-converting enzyme (ACE) inhibitory activity*

159 For the ACE-inhibitory activity assay, the peptide fractions were obtained by ultrafiltration to avoid
160 interference in the assay caused by TCA. Samples (500 µL) collected at 0, 87, 96, 111 and 120 h
161 were centrifuged (10,000*g*, 20 min, 4°C) and the supernatant added to an Amicon Ultra-0.5
162 centrifugal filter with a molecular weight cut-off of 3kDa and centrifuged at 14,000*g* for 120 min at
163 4°C. ACE-inhibitory activity of the resulted filtrates was determined spectrophotometrically as
164 reported by Ronca-Testoni (1983) using the tripeptide, 2-furanacryloyl--phenylalanylglycylglycine
165 (FAPGG) as substrate, with some modifications. Briefly, 600 µL of FAPGG solution (1.33 mM in
166 reaction buffer containing 100 mmol/L Tris-HCl, 0.6 mol/L NaCl, pH 8.2), were mixed directly in
167 cuvette with 100 µL of the same reaction buffer or 100 µL of ultrafiltrated samples. The solution
168 was kept at 37°C for 3 min before adding 21 µL of ACE solution in order to reach the final enzyme
169 activity of 50 mU/mL. The reaction was monitored at 345 nm for 10 min. The ACE-inhibitory
170 activity was calculated as percent of inhibition (ACEi%). The samples with the highest ACE-
171 inhibitory activity were used to calculate the inhibitory power as IC_{50} (defined as the concentration
172 of peptides required to inhibit 50% of the enzymatic activity). The IC_{50} values were determined
173 using nonlinear regression analysis and fitting the data with the log (inhibitor) *vs*. response model.
174 Peptide concentrations correspond to the amount of free amino groups measured with the TNBS
175 asssay on ultrafiltrated samples as reported above.

176

177 *2.8 Identification and quantification of IPP and VPP*

178 The identification and quantification of IPP and VPP were carried out on 2 µL of suitably diluted
179 TCA-soluble supernatant through nanoLC-MS/MS experiments performed on a 1200 Series Liquid
180 Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS
181 (Agilent Technologies, Milano, Italy). Chromatographic separation was performed on a ProtID-
182 Chip-43(II) including a 4 mm 40 nL enrichment column and a 43 mm x 75 µm analytical column,
183 both packed with a C18 phase (Agilent Technologies). The mobile phase consisted of (A)
184 H_2O/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B) acetonitrile/H_2O/formic acid (94.9:5:0.1,
185 v/v/v). The gradient started at 3% B for 0.5 min then linearly ramped up to 11% B in 10 min. The
186 mobile phase composition was raised up to 40% B in 3 min, then 95% B in 1 min and maintained
187 for 4 min in order to wash both enrichment and analytical columns. The flow rate was set at 500
188 nL/min. The mass spectrometer was tuned, calibrated and set with the same parameters as reported
189 by Dei Più et al. (2014). VPP and IPP were selectively fragmented using a mass to charge ratio of
190 312.18 and 326.21 (charge +1), respectively. The assignment process was complemented and
191 validated by the manual inspection of MS/MS spectra. The relative amount of the tri-peptides was
192 estimated by integrating the area under the peak (AUP) for samples collected during pre-screening.
193 AUP was measured from the extracted ion chromatograms (EIC) obtained for each peptide.
194 Absolute quantification of VPP and IPP was carried out in fermented milk samples collected at 0,
195 39, 48, 63, 72, 87, 96, 111, and 120 h. Calibration curves were constructed as follows. Synthetic
196 tripeptides IPP and VPP were solubilized at 5 g/L in 0.1 mmol/L potassium phosphate buffer (pH
197 7.0), and then diluted 1:1000 with solvent A, to obtain the 5 mg/L solution. Subsequent dilutions
198 were made in the same solvent A to yield, for both the peptides, the following concentrations: 1, 5,
199 10, 20, 50 and 100 µg/L. Each solution contained the internal standard EGVNDNEEGFFSAR at the
200 concentration of 50 µg/L. The calibration curves were constructed from the peak area of the peptide
201 relative to the peak area of the internal standard *versus* concentration. The concentrations of
202 tripeptides in fermented milk samples were calculated using the following linear equations:

203 $y = 6282.4x - 109.2$ ($R^2 = 0.9938$)

204 and

205 $y = 12585x - 3945$ ($R^2 = 0.9940$)

206 for VPP and IPP, respectively.

207

208 *2.9 Statistical analysis*

209 All data were presented as mean ± SD for three replicates for each prepared sample. ANOVA with
210 Tukey *post-hoc* test and two way ANOVA with Bonferroni post test were performed using Graph
211 Pad Prism (GraphPad Software, San Diego, CA). The differences were considered significant with
212 $P < 0.05$. Correlation and non-linear regression analysis was also performed with Graph Pad Prism.
213 The correlation, expressed as Pearson *r*-value, was considered significant when $P < 0.05$.

214

215 **3. Results**

216 *3.1 Assessment of proteolytic activity*

217 As preliminary screening, the proteolytic potential of 39 NSLAB strains isolated from dairy sources
218 (34 from PR and 5 from Sicilian Pecorino cheeses, respectively) and 5 strains isolated from human
219 samples was tested on milk proteins after 72 h of incubation (**Figure 1**). Values of leucine
220 equivalents (mmol/L) were normalized with respect to negative control, which showed a value of
221 1.22 mmol/L of leucine equivalents at time 0 and, as expected, remained constant until the end of
222 the experiment (72 h).

223 As depicted in **Figure 1**, the extent of proteolysis varied significantly among the strains. Out of 44
224 analyzed strains, 32 displayed low or absent proteolytic activity. A set of 12 strains, including 11
225 *Lb. rhamnosus* and 1 *Lb. casei*, showed concentrations of released amino groups after 72 h of
226 incubation in a range from 3.89 to 9.01 mmol/L of leucine equivalents. Milk samples fermented
227 with these strains underwent coagulation after 24-39 h of fermentation (data not shown). *Lb. casei*
228 PRA205 showed the best proteolytic activity (9.01 mmol/L of leucine equivalents) followed by *Lb.*

rhamnosus LOC12 (5.42 mmol/L of leucine equivalents) and *Lb. rhamnosus* PRA331 (5.25 mmol/L of leucine equivalents).

Twelve strains showing the best proteolytic activity were further evaluated for their ability to release VPP and IPP. The relative amount of VPP and IPP was quantified through nanoLC/MS and tandem MS experiments and expressed as AUC. As shown in **Figure 2,** *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 overcame the other strains in releasing IPP and VPP. On the basis of their highest proteolytic activity and ability to release IPP and VPP, *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 were chosen for further characterization.

3.2 Fermentation process

The selected lactobacilli PRA205 and PRA331 were inoculated into sterile cow milk. Their fermentative performance was assessed at regular intervals up to 120 h after the inoculum based on the production of lactic acid as primary metabolite, the consumption of sugars, and the pH decline. The growth was also assessed by determining viable cell counts.

Kinetics of decrease in pH and increase in lactic acid content are shown in **Figure 3** for both fermentation trials. After inoculum, the values of pH were 6.61 ± 0.02 and 6.57 ± 0.02 in milk with PRA205 and PRA331, respectively. Strain PRA205 reached pH value of about 4.0 after 72 h of growth, while PRA331 needed 48 h to get the same value of pH. With the exception of the initial time, pH values were significantly lower ($P < 0.05$) for the milk fermented with PRA331 compared to the milk fermented with PRA205. In both trials the lactic acid concentration reached a plateau after 63 h (**Figure 3**) and, starting from the time 39 h, the amount of lactic acid produced by PRA331 was significantly greater ($P < 0.05$) than that produced by PRA205. The highest lactic acid content was reached at the end of fermentation with PRA331 (262.82 ± 8.49 mmol/L) which showed faster and more intensive acidification than PRA205 (183.29 ± 18.14 mmol/L). According to pH decline, coagulation started after 24 h in milk inoculated with strain PRA331 and after 39 h in milk inoculated with strain PRA205. As expected, correlation analysis showed an inverse

255 relationship between the decrease in pH and the increase in lactic acid concentrations for both the
256 fermentation trials (PRA331: r = -0.9553, R^2 = 0.9127, P = 0.0008; PRA205: r = -0.9276; R^2 =
257 0.8605; P = 0.0009).
258 Lactose content decreased during fermentation from 124.22 ± 9.36 mmol/L to 82.53 ± 8.23 mmol/L
259 for strain PRA205 and from 123.72 ± 7.58 mmol/L to 60.97 ± 10.46 mmol/L for strain PRA331.
260 Glucose content (about 0.4 mmol/L in both samples immediately after the inoculum) halved in the
261 first 15 h and remained constant until 120 h after inoculum, whereas galactose content was below
262 the detection limit of the assay at each sampling time.
263 The initial value of viable lactobacilli population was about Log 8 cfu/mL for both fermentation
264 trials. Starting from 63 h, the values of viable cell counts remained constant, with final Log values
265 of 11.43 cfu/mL and 9.47 cfu/mL for trials inoculated with PRA205 and PRA331, respectively. At
266 the end of fermentation (corresponding to pH ≤ 4.2 after app. 63 h), colonies grown on the highest
267 dilution MRS plates were randomly picked and submitted to *Lb. rhamnosus*- and *Lb. casei*-specific
268 PCR assays, as well as to MSP-PCR genotyping. As expected, PCR targeting *Lb. rhamnosus* 16-
269 23S rRNA intergenic region was positive only for the isolates retrieved from milk inoculated with
270 PRA331, whereas PCR targeted V1 region of the *Lb. casei* 16S rRNA gene was positive for those
271 recovered from milk inoculated with PRA205 (supplementary **Table S1**). MSP-PCR with primer
272 $(GTG)_5$ showed that each set of isolates displayed the same MSP-fingerprints of the corresponding
273 inoculated strain (supplementary **Table S1**). Both data sets were congruent to confirm the identity
274 of each inoculated strain during fermentation.
275
276 *3.3 Proteolytic and radical scavenging activities*
277 The TCA-soluble peptide fractions of milk samples fermented with either PRA205 or PRA331 were
278 collected at different times to assess both proteolytic and radical scavenging activities. During milk
279 fermentation, the proteolysis degree increased reaching the maximum value after 96 h for both trials
280 (**Figure 4A**). In particular, strain PRA205 increased significantly (P < 0.05) the proteolytic activity

between 24 and 39 h of incubation, rising from a value of 1.88 ± 0.12 mmol/L to a value of 6.67 ± 0.75 mmol/L of leucine equivalents. After 39 h the proteolytic activity of strain PRA205 increased more gradually, reaching a peak corresponding to 13.55 ± 1.41 mmol/L of leucine equivalents at 96 h after inoculum (**Figure 4A**). Starting from 111 h of incubation, the amount of free amino groups decreased of 22% (10.64 ± 0.75 mmol/L of leucine equivalents). The value after 120 h of incubation was not significantly different from the value at 111 h. Strain PRA331 showed a more gradual increase in proteolytic activity over time than PRA205, reaching the maximum value of 8.75 ± 0.25 mmol/L of leucine equivalents after 96 h of incubation. Then, a significant decrease of 26% in the amount of free amino groups was observed (**Figure 4A**). After 111 h of monitoring there were no significant changes in proteolytic activity in milk fermented with PRA331.

Development of radical scavenging activity during milk fermentation was determined through ABTS assay. As shown in **Figure 4B**, we found significant differences between strains PRA205 and PRA331 for the radical scavenging activity. Milk fermented with strain PRA205 showed greater ($P < 0.05$) radical scavenging activity than that fermented with PRA331 starting from 39 h. The radical scavenging activity increased in both trials getting to maximum values after 111 h, when milk fermented by PRA205 exhibited 1184.83 ± 40.28 mmol/L of trolox equivalents, while that fermented by PRA331 exhibited 939.22 ± 82.68 mmol/L of trolox equivalents.

3.4 ACE-inhibitory activity

We evaluated the dynamics of ACE-inhibitory activity (ACEi%) in ultrafiltrated peptide fractions of samples with the highest proteolytic activity (namely samples collected after 87, 96, 111, 120 h of incubation). In both fermentation trials ACE inhibition activity was observed at each sampling time (**Figure 5**). ACEi% values increased with the fermentation time apart from slight fluctuations at time 111 h for strain PRA205 and 96 h for strain PRA331. Interestingly, milk inoculated with *Lb. casei* PRA205 already had approximately 20% ACEi at 0 h. This may be due to the production of

306 ACE-inhibitory peptides during the fermentation in pre-culture. The highest ACEi% was observed
307 after 120 h of incubation both for *Lb. casei* PRA205 (100ACEi%) and *Lb. rhamnosus* PRA331
308 (48ACEi%). Therefore, ACE-inhibitory power was calculated as IC_{50} on the samples after 120 h of
309 incubation. Strain PRA205 displayed higher activity (IC_{50} 54.57 µg/mL) than PRA331 (IC_{50} 212.38
310 µg/mL).
311
312 *3.5 VPP and IPP quantification*
313 The results described above indicate that strains PRA205 and PRA331 release peptides with ACE-
314 inhibitory activity from milk. Therefore we determined the concentrations of the antihypertensive
315 peptides IPP and VPP during fermentation by nano-flow LC with Q-TOF mass spectrometry.
316 **Figure 6** shows the release of VPP (panel A) and IPP (panel B) over time. VPP started to be
317 detectable after 39 h in both trials and reached a maximum value after 111 h in milk inoculated with
318 PRA205 (32.88 mg/L) and after 87 h in that fermented with PRA331 (11.87 mg/L). There was a
319 significant difference between strains in the ability to release VPP, with PRA205 being more able
320 than PRA331 to produce VPP (**Figure 6A**). In milk fermented with PRA331, VPP concentrations
321 decreased strongly starting from 96 h such that, after 120 h of fermentation, the amount of VPP was
322 about the 40% of the maximum value observed after 87 h.
323 The trend in IPP release was similar to that observed for VPP (**Figure 6B**). In milk fermented with
324 *Lb. casei* PRA205 the highest IPP concentration was reached after 96 h (7.52 mg/L), thereafter it
325 remained constant until 111 h of monitoring and then slightly decreased. In milk fermented with
326 PRA331, the maximum IPP content (3.62 mg/L) was lower compared to PRA205 and was reached
327 after 87 h of monitoring. After this time, IPP values decreased and, at the end of the fermentation,
328 IPP amount was about 30% of the maximum value. Based on these results, *Lb. casei* PRA205 was
329 able to release higher amount of the antihypertensive tripeptides VPP and IPP than *Lb. rhamnosus*
330 PRA331.
331

4. Discussion

Bioactive peptides present in dairy fermented products have attracted increasing attention as natural compounds with antihypertensive (mainly ACE-inhibitory) and antioxidant properties (Usinger et al., 2009). To assure their survival in cheese, NSLAB possess sets of hydrolytic enzymes which contribute to cheese maturation and could have the potential to release bioactive peptides. To test this potential, the proteolytic activity and the ability to release the ACE-inhibitory tripeptides VPP and IPP during milk fermentation have been determined in a pool of strains belonging to the most common facultatively hetero-fermentative species dominant in ripened cheese varieties. Based on our results, *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 are the most proteolytic strains and so they are the best candidates to produce fermented milk with antioxidant activity and enriched in VPP and IPP.

The rate of pH decrease is a parameter indicative of the fermentative performance of a microbial culture and of the effectiveness of its proteolytic and glycolytic systems to sustain the microbial growth (Kunji et al., 1996). Under the experimental conditions of this study, both strains PRA205 and PRA331 ended fermentation within 24-39 h, reaching final viable cell populations of 11.4 and 9.5 Log cfu/mL, respectively. Although there is no general agreement in the minimum concentration of functional cells to achieve therapeutic benefits, these values greatly exceed the thresholds of 6.0-8-0 Log cfu/mL recommended by several authors (Kurmann and Rasic, 1991; Lourens-Hattingh and Vilijeon, 2001). Interestingly, strain PRA205 has been proved to possess antibiotic susceptibility profile and tolerances to different conditions miming single and multiple stresses occurring during GI transit (Solieri et al., 2014). *Lb. casei* PRA205 overcomes *Lb. rhamnosus* PRA331 in survival after 3h-treatments with both synthetic pancreatic and gastric juices, as well as in viability after acidic, salt bile, and lysozyme stresses, respectively (Solieri et al., 2014). The high number of viable PRA205 cells in combination with their *in vitro* GI resistance suggests that fermented milk could be effective for *in vivo* delivering potentially probiotic PRA205 cells.

357 The rates of lactose consumption/lactic acid production are parameters closely strain- and species-
358 dependent (Elfahri et al., 2014). Accordingly, the ability to use lactose was different between the
359 two strains, which in turn influences the amount of lactic acid. *Lb. rhamnosus* PRA331 exhibits a
360 more vigorous fermentation than *Lb. casei* PRA205, resulting in a more pronounced acidification,
361 production of lactic acid, and reduction in lactose content. The slower trend of acidification in milk
362 inoculated with PRA205 may represent a technological advantage, allowing caseins to mostly
363 remain in solution and increasing the release of bioactive peptides.
364 Proteolytic activity is a desirable feature for LAB exploited in functional foods (De Vuyst and
365 Leroy, 2007). In both fermented milks, the progressive increasing in the amount of free amino
366 groups is indicative of proteolytic activity and confirms the ability of strains PRA205 and PRA331
367 to efficiently utilize milk proteins and to produce peptides which can be accumulated in the
368 surrounding medium (Donkor, 2007). The extent of proteolysis varied between strains, with
369 PRA205 showing higher activity than PRA331. Differences in proteolysis could reflect differences
370 in proteases and peptidases patterns between *Lb. casei* and *Lb. rhamnosus* or in their different
371 transcriptional regulation (Liu et al., 2010).
372 Several evidences are accumulating that demonstrate the ability of proteolytic *Lactobacillus* strains
373 to produce high concentrations of antioxidant peptides in dairy products (López-Fandiño et al.,
374 2006). A radical scavenging peptide derived from κ-casein was isolated in milk fermented by *Lb.*
375 *delbrueckii* ssp. *bulgaricus* (Kudoh et al., 2001). In addition, Hernandez-Ledesma et al. (2005)
376 detected moderate anti-radical activity in different fermented milks, but without any direct
377 relationship between degree of hydrolysis and antioxidant activity, as *Lb. helveticus* exhibited
378 highest proteolytic activity but intermediate antioxidant activity. The development of radical
379 scavenging activity appears to be more strictly dependent on strain and their specific pattern of
380 proteolytic enzymes (Virtaren et al., 2006). Our data show that peptide fractions of both fermented
381 milks possessed radical scavenging activity which increased during fermentation in agreement with
382 the proteolytic trend. At the end of incubation, antioxidant activities in milks fermented with

PRA205 and PRA331 were approximately 1.2 and 0.9 mmol/L of Trolox equivalents, respectively. These values were equal or superior to most of the fermented milk samples analysed by Hernandez-Ledesma et al. (2005).

Various types of milk fermented with *Lb. helveticus, Lb. bulgaricus* ssp. *bulgaricus, Lb. rhamnosus, Lb. acidophilus,* and *Lc. lactis* ssp. *cremoris* have been shown to contain peptides with variable ACE-inhibitory activity (Gobbetti et al., 2004). In our study both strains PRA205 and PRA331 were able to release ACE-inhibitory peptides during milk fermentation. Milk fermented with *Lb. casei* PRA205 displayed IC_{50} value 3.9 times lower than milk fermented by *Lb. rhamnosus* PRA331. This difference could reflect different type, quality or concentration of peptides produced by PRA205 and PRA331, which in turn could be related to cell envelope proteinase/peptidases with different specificity (Savijoki et al., 2006).

Nejati et al. (2013) obtained IC_{50} values ranging from 220 and 1750 μg/mL in pH 4.6-soluble fractions from milk fermented by different lactobacilli. In our experiments, *Lb. casei* PRA205 showed an IC_{50} value 4 and 5 time lower than the highest IC_{50} values described for *Lc. lactis* DIBCA2 and *Lb. casei* FC113, respectively (Nejati et al., 2013). In according to our data, Nejati et al. (2013) also found *Lb. rhamnosus* strains less efficient than *Lb. casei* in release of ACE-inhibitory peptides with IC_{50} values ranging from 700 to 1500 μg/mL. Anyway, these values are 3 to 7 times higher than the values obtained with *Lb. rhamnosus* PRA331. Our results are similar than that found by Muguerza et al. (2006) using *Lb. rhamnosus*. On the other hand, milk fermented with *Lb. delbrueckii* subsp. *bulgaricus* or *Lc. lactis* subsp. *cremoris* showed IC_{50} values ranging from 8 to 11.2 μg/mL (Gobbetti et al., 2000).

It is difficult to establish a direct link between IC_{50} values and *in vivo* antihypertensive effects, as the digestive stability and bioavailability of peptides are often uncertain. Although a large number of peptides with ACE-inhibiting *in vitro* effects are released during milk fermentation, the present work focused only on tripeptides VPP and IPP, whose antihypertensive effects have been well demonstrated in several human studies (Cicero et al., 2013). *Lb. helveticus* releases IPP and VPP

409 from milk proteins at IC_{50} values of 5 μmol/L and 9 μmol/L, respectively (Seppo et al., 2003;
410 Tuomilehto et al., 2004). However, Butifoker et al. (2007) found high concentrations of IPP and
411 VPP in cheeses which did not include *Lb. helveticus* as starter culture, suggesting that other
412 lactobacilli are capable to produce these tripeptides during cheesemaking. IPP and VPP were also
413 detected in milk fermented with *Lb. acidophilus* and *Lb. rhamnosus* although at lower
414 concentrations than those found in milk fermented with *Lb. helveticus* (Muguerza et al., 2006). Our
415 results showed that *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 are able to release VPP and IPP
416 from milk caseins, and that strain PRA205 overtakes PRA331 in VPP and IPP production.
417 Although IPP is present both in κ-casein and in β-casein, the amount of released IPP was generally
418 lower than VPP (Butifoker et al., 2007). Accordingly, we found the ratio between VPP and IPP of
419 4.37 and 3.27 after fermentation with PRA205 and PRA331, respectively. This could be related
420 either to the specificity of the cell envelope proteinases and/or endopeptidases on caseins or to the
421 higher intake of IPP compared to VPP through the peptide transport system in lactobacilli.
422 Several clinical studies on hypertensive subjects showed that the administration of VPP and IPP via
423 fermented milk has positive effects on blood pressure (Boelsma and Kloek, 2010; Nakamura et al.,
424 2011, Cicero et al., 2013). Daily doses (150 ml portion) of VPP/IPP in the range of 2-6 mg were
425 associated with a decrease of the blood pressure between 1.5 and 10 mmHg (Seppo et al., 2003;
426 Cicero et al., 2013). After 111 h of incubation, milk fermented with strain PRA205 contained
427 concentrations of VPP/IPP equal to about 40.2 mg/L, corresponding to about 6 mg in 150 mL. In
428 milk fermented with strain PRA331, the maximum VPP/IPP concentration (15.5 mg/L after 87 h of
429 fermentation) corresponds to about 2.3 mg in 150 mL. Therefore, 150 mL portions of milk
430 fermented with PRA331 or, especially, with PRA205, could correspond to doses which have *in vivo*
431 hypotensive effect.
432 In conclusion, the strains selected in the present study, and in particular *Lb. casei* PRA205, are able
433 to produce, during milk fermentation, amounts of antihypertensive peptides (VPP and IPP) at doses
434 able to reduce blood pressure in hypertensive subjects *in vivo*. To the best of our knowledge, this is

435 the first evidence that *Lb. casei* and *Lb. rhamnosus* strains are able to synthesize amounts of VPP
436 and IPP comparable to those released by *Lb. helveticus* from milk proteins. *Lb. casei* PRA205 has
437 been previously demonstrated to have high resistance to GI tract, antibiotic susceptibility, and
438 adhesive phenotype (Solieri et al., 2014). These evidences overall suggest that *Lb. casei* PRA205
439 may be a promising culture to produce functional milk with high amounts of VPP and IPP,
440 antioxidant activities, and high content in health-promoting viable cells.
441

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444 Prof. A. Conte for useful comments to manuscript, as well as Diego Pinetti and Filippo Genovese
445 for technical support in MS experiments.
446

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Table Caption

Table 1. Strains used in the present work

Figure Captions

Figure 1. Screening of *Lactobacillus casei* (grey bars), *Lactobacillus rhamnosus* (white bars) and *Lactobacillus paracasei* (black bars) strains based on proteolytic activity after 72 h of inoculation in UHT skimmed milk. The extent of proteolysis is expressed as mmol/L leucin equivalents. Values of leucine equivalents (mmol/L) are normalized with respect to negative control (1.22 mmol/L). Data are represented by the mean (n = 3); error bars show standard deviation.

Figure 2. Relative quantification (expressed as area under the peak, AUP) of Val-Pro-Pro (VPP) (white) and Ile-Pro-Pro (IPP) (black) released by 12 pre-selected lactobacilli after 72 h of inoculation in UHT skimmed milk. Lr, *Lactobacillus rhamnosus*; Lp, *Lactobacillus paracasei*; Lc, *Lactobacillus casei*.

Figure 3. Kinetics of acidification (open symbols) and lactic acid production (solid symbols) in milk fermented with *Lactobacillus casei* PRA205 (circles) and *Lactobacillus rhamnosus* PRA331 (squares). Data are represented by the mean (n = 3); error bars show standard deviation. * means $P<0.05$ respect to the previous time in the same strain; $^{\#}$ means $P<0.05$ respect to the strain PRA331.

Figure 4. Proteolytic (A) and radical scavenging (B) activities measured during fermentation of milk with *Lactobacillus casei* PRA205 (circles) and *Lactobacillus rhamnosus* PRA331 (squares). Proteolysis activity is expressed as mmol/L of leucine equivalents, whereas radical scavenging activity as µmol/L of Trolox. Data are represented by the mean (n = 3); error bars show standard deviation. * means $P<0.05$ respect to the previous time in the same strain; $^{\#}$ means $P<0.05$ respect to the strain PRA331.

Figure 5. Angiotensin converting enzyme (ACE) inhibition activity (ACEi%) of milk samples fermented with *Lactobacillus casei* PRA205 (white) and *Lactobacillus rhamnosus* PRA331 (black) at different sampling times. Data are represented by the mean (n = 3); error bars show standard

deviation. * means $P<0.05$ respect to the previous time in the same strain; $^{\#}$ means $P<0.05$ respect to the strain PRA331.

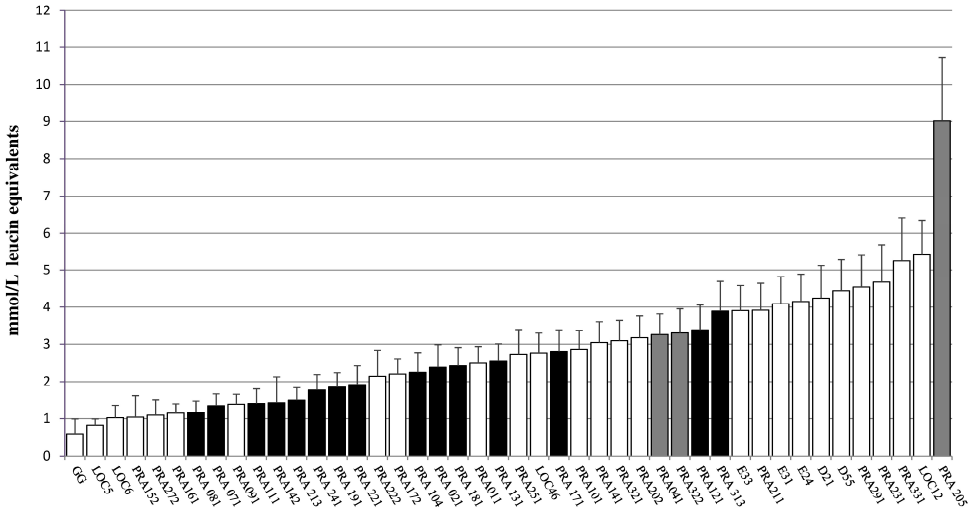
Figure 6. Release of Val-Pro-Pro (VPP; panel A) and Ile-Pro-Pro (IPP; panel B) by *Lactobacillus casei* PRA205 (circles) and *Lactobacillus rhamnosus* PRA331 (squares) during milk fermentation. Data are represented by the mean (n = 3); error bars show standard deviation. * means $P<0.05$ respect to the previous time in the same strain; $^{\#}$ means $P<0.05$ respect to the strain PRA331.

Table 1.

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.

Figure 1


mmol/L leucin equivalents
0
1
2
3
4
5
6
7
8
9
10
11
12
GG
LOC5
LOC6
PRA152
PRA272
PRA161
PRA081
PRA071
PRA091
PRA111
PRA142
PRA213
PRA241
PRA191
PRA221
PRA222
PRA172
PRA104
PRA182
PRA181
PRA011
PRA131
PRA251
LOC96
PRA177
PRA101
PRA141
PRA321
PRA202
PRA041
PRA322
PRA121
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PRA211
E31
E24
D21
D5
PRA291
PRA231
PRA331
LOC12
PRA205

Figure 2

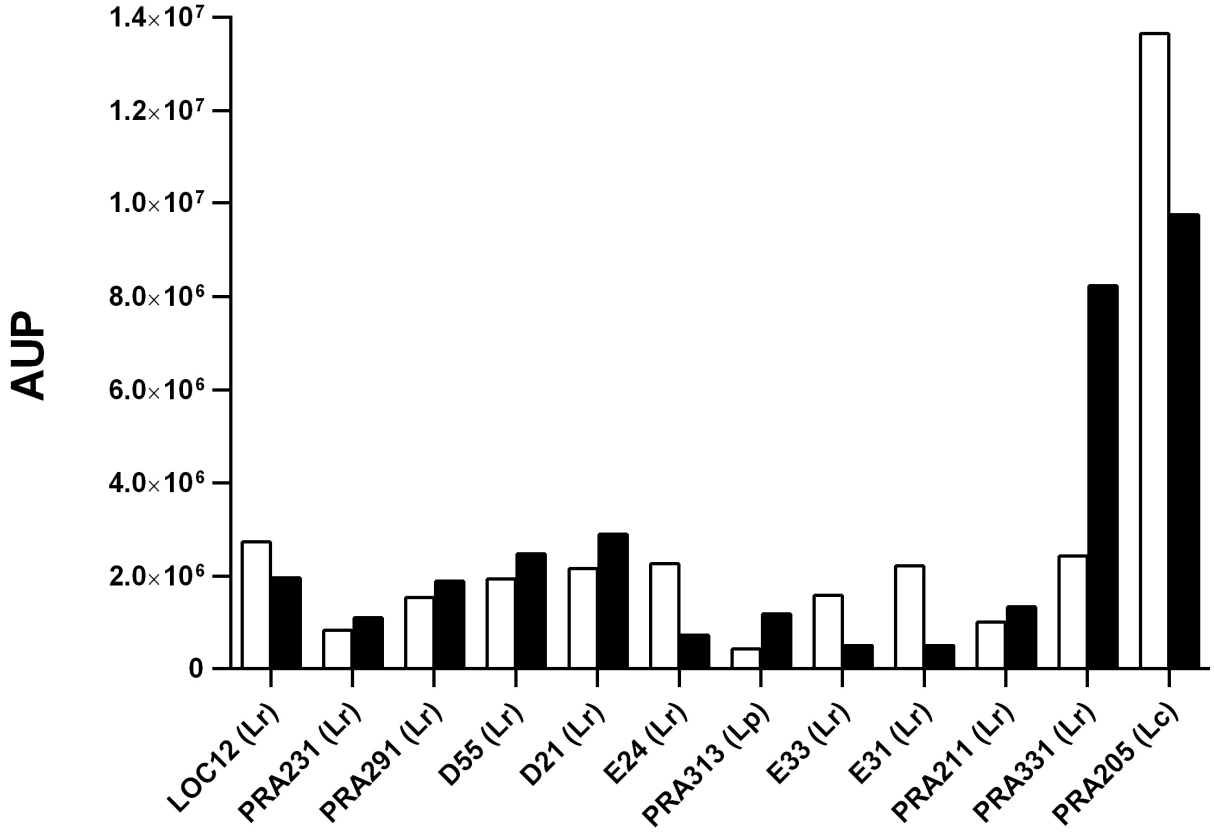

AUP
1.4×10⁷
1.2×10⁷
1.0×10⁷
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
LOC12 (Lr)
PRA231 (Lr)
PRA291 (Lr)
D55 (Lr)
D21 (Lr)
E24 (Lr)
PRA313 (Lp)
E33 (Lr)
E31 (Lr)
PRA211 (Lr)
PRA331 (Lr)
PRA205 (Lc)

Figure 3

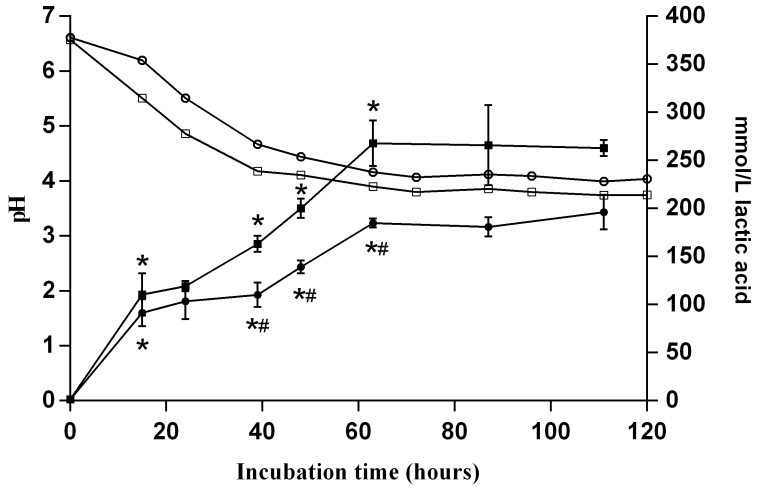

pH
mmol/L lactic acid
Incubation time (hours)
0
20
40
60
80
100
120
1
2
3
4
5
6
7
50
150
200
250
300
350
400
*
*#

Figure 4

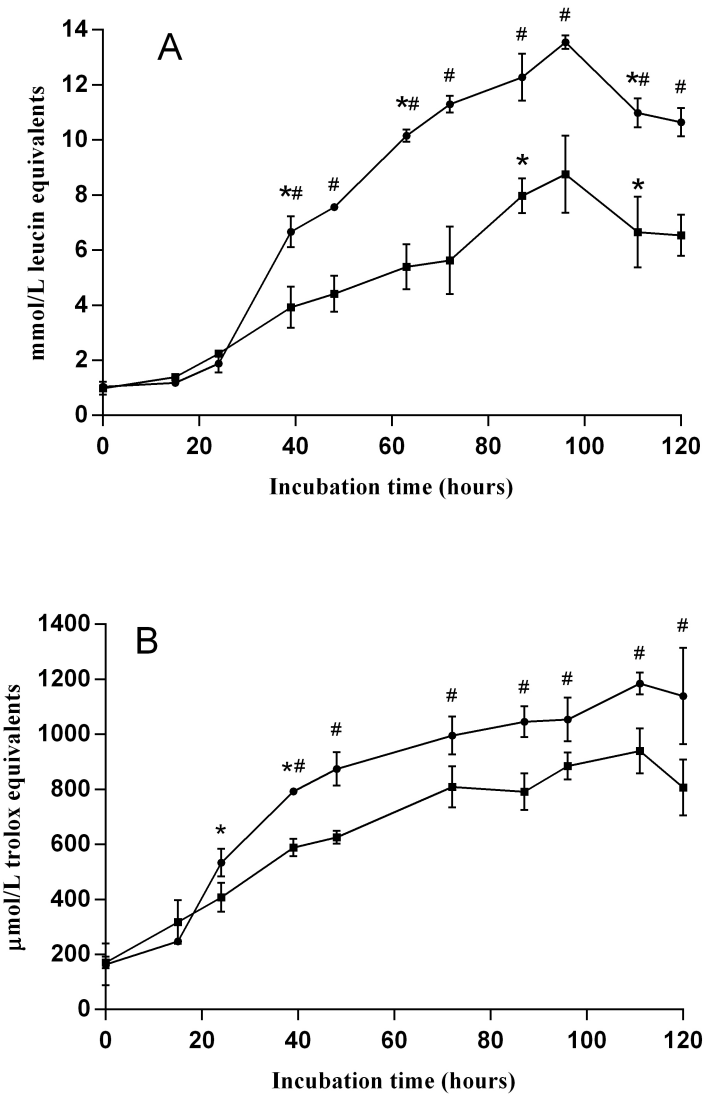

A
mmol/L leucin equivalents
Incubation time (hours)
B
µmol/L trolox equivalents
Incubation time (hours)

Figure 5

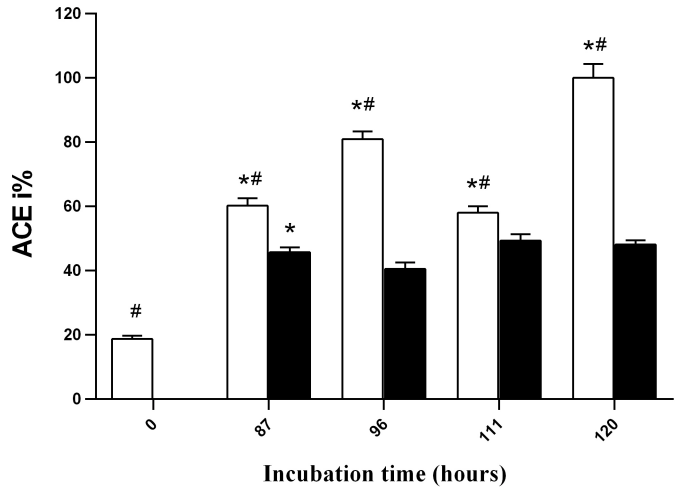
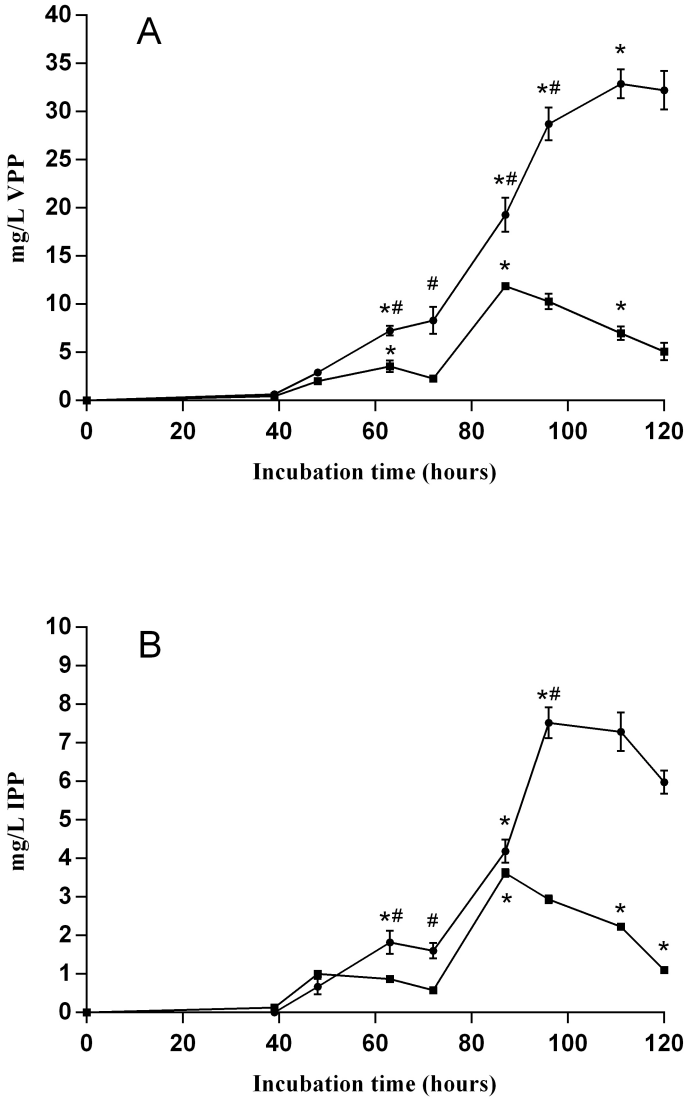


Figure 6


A
mg/L VPP
Incubation time (hours)
B
mg/L IPP
Incubation time (hours)