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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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## 1   **Abstract**

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

18  
19   **Keywords:** *Lactobacillus*, bioactive peptides, valine-proline-proline (VPP), isoleucine-proline-  
20   proline (IPP), probiotic, ACE-inhibition

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## 22    **1. Introduction**

23    Biologically active peptides derived from food proteins are specific protein fragments that have a  
24    positive impact on body functions, going well beyond their nutritional value (Kamau et al., 2010).  
25    Milk proteins, especially caseins, are currently the main precursors of biologically active peptides  
26    (Silva and Malcata, 2005). Depending on size and amino acid sequence, milk-derived peptides may  
27    exert a number of different activities *in vitro* and/or *in vivo*, such as immuno-modulation, anticancer  
28    action, hypocholesteremic effect, as well as antimicrobial, mineral-binding, opioid, and peptidase  
29    inhibition activities (Fitzgerald and Murray, 2006; Mills et al., 2011). They can be released from  
30    milk proteins by gastro-intestinal (GI) digestion or by enzymatic hydrolysis during food processing  
31    and fermentation (Pihlanto, 2006). In particular, milk protein hydrolysates and fermented dairy  
32    products are enriched in antihypertensive and antioxidant peptides (Silva and Malcata, 2005;  
33    Pihlanto, 2006; Korhonen, 2009). Among these, lacto-tripeptides valine-proline-proline (VPP) and  
34    isoleucine-proline-proline (IPP) are resistant to GI digestion and can cross the mucosal barrier,  
35    resisting to digestion by serum peptidases (Foltz et al, 2008). VPP and IPP have been shown to  
36    reduce systolic blood pressure in hypertensive subjects (Boelsma and Kloek, 2010; Nakamura et al.,  
37    2011; Cicero et al., 2013), due to inhibition of angiotensin-converting enzyme (ACE), stimulation  
38    of vasodilator production, and modulation of sympathetic nervous activity (Usinger et al., 2010). In  
39    addition to antihypertensive effects, milk-derived peptidic fractions have been demonstrated to exert  
40    radical scavenging functionalities and prevent oxidative stresses associated with numerous  
41    degenerative chronic diseases, including cardiovascular ischemia, reperfusion, and atherosclerosis  
42    (Kudoh et al., 2001; Virtanen et al., 2007).

43    Proteolytic systems from lactic acid bacteria (LAB) are the main route to generate bioactive  
44    peptides during milk fermentation and cheese ripening (Gobbetti et al., 2004). LAB possesses  
45    variable patterns of proteinases, peptidases and peptide transport systems which affect release and  
46    intake of bioactive peptides in milk in a species- and strain-specific manner (Liu et al., 2010). These  
47    systems enable LAB to fulfill their amino acid requirements (Christensen et al., 1999) and

48 contribute to the formation of flavor and texture in dairy products (Settanni and Moschetti 2010).  
49 Cell-envelope proteases break down proteins in the growth media into peptides of about 5–30  
50 amino acids that are carried into the cell and further hydrolysed by endopeptidases into smaller  
51 peptides and amino acids for microbial protein synthesis. PepI, PepP, PepQ, PepR, and PepX  
52 endopeptidases have proline-specific hydrolytic activities which account for the main release of  
53 bioactive peptides from proline-rich  $\alpha$ -,  $\beta$ - and  $\kappa$ -caseins.

54 *Lactobacillus* genus is predominant in dairy food and encompasses the main acid-producing starter  
55 cultures (SLAB), as well as the mesophilic species most responsible for cheese ripening (indicated  
56 as non-starter LAB, NSLAB). The role of SLAB lactobacilli in bioactive peptide release is well  
57 documented during milk fermentation (reviewed by Gobbetti et al., 2004; Fitzgerald and Murray,  
58 2006). Among SLAB, thermophilic *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp.  
59 *bulgaricus* strains have been extensively studied for their strong and specific proteolytic activity in  
60 milk which results in release of higher amount of active peptides compared to other lactobacilli  
61 (Sadat-Mekmene et al., 2011 and references herein). In contrast, a few of works deals with the  
62 ability of mesophilic NSLAB strains to hydrolyze caseins into bioactive peptides (Fuglsang et al.,  
63 2003; Ramchandran and Shah, 2008; Wang et al., 2010). Within NSLAB, facultatively  
64 heterofermentative *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are  
65 extensively used both as probiotics and adjunct cultures in different dairy products (Settanni and  
66 Moschetti, 2010; De Vos, 2011). Potential health benefits associated with the consumption of these  
67 bacteria rely on their ability to interact with the intestinal epithelial cells directly, but also indirectly,  
68 through the production of biogenic compounds (Lebeer et al., 2010 and references herein). When  
69 probiotics are delivered with fermented dairy products, milk-derived peptides with different  
70 biological activities can be produced (Fitzgerald and Murray, 2006; Hayes et al., 2007).

71 In our previous works, we established a de-replicated set of *Lb. casei*, *Lb. paracasei*, and *Lb.*  
72 *rhamnosus* NSLAB strains isolated from Parmigiano Reggiano cheese (Solieri et al., 2012) and  
73 assessed their probiotic aptitude (Solieri et al., 2014). Aim of this work was to explore the potential

74 of these strains to release bioactive peptides with radical scavenging and ACE-inhibitory activities  
75 during cow milk fermentation, with a special focus on their ability to release VPP and IPP from  
76 milk caseins.

77

## 78 **2. Materials and methods**

### 79 *2.1 Materials*

80 All MS/MS reagents were from Biorad (Hercules CA, U.S.A.), whereas the remaining chemicals  
81 were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise stated. De Man, Rogosa and  
82 Sharpe (MRS) medium was provided by Oxoid (Basingstoke, Hampshire, England). Amicon Ultra-  
83 0.5 regenerated cellulose filters with a molecular weight (MW) cut-off of 3 kDa were supplied by  
84 Millipore (Milan, Italy). Ultra-high-temperature-treated (UHT) skimmed bovine milk was obtained  
85 from a local producer (protein, 3.15%; fat, 0.3%; lactose, 4.95%). VPP and IPP peptides (95%  
86 purity) were synthesized by DBA (Milan, Italy). The absorbance was read using a Jasco V-550  
87 UV/Vis spectrophotometer (Orlando FL, U.S.A.), with the exception of DNA samples that were  
88 quantified by Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA). Taq DNA  
89 polymerase was from Takara (Kyoto, Japan), while primers were provided by MWG (Heidelberg,  
90 Germany).

91

### 92 *2.2 Bacteria and growth conditions*

93 The bacterial strains used in this study are listed in **Table 1**. Thirty-four strains were isolated from  
94 ripened Parmigiano Reggiano cheeses (Solieri et al., 2012) and deposited in the Unimore Culture  
95 Collection (UMCC) ([www.unimore.umcc.it](http://www.unimore.umcc.it)). Nine *Lb. rhamnosus* strains isolated from Sicilian  
96 Pecorino cheese and human vaginal samples, were kindly provided by Prof. C. Randazzo  
97 (University of Catania, Italy). All LAB strains were maintained as frozen stock at -80°C in MRS  
98 broth supplemented with 25% glycerol. Prior to the experimental use, the cultures were twice  
99 propagated in MRS medium and incubated at 37°C for 24 h under anaerobic conditions. For

100 preliminary screening of NSLAB proteolytic activity, bacterial cells grown until late exponential  
101 phase in MRS medium, were harvested by centrifugation, and washed twice with 50 mmol/L Tris-  
102 HCl buffer (pH 6.5) and inoculated, in triplicates, in 10 mL of ultra-high temperature-treated (UHT)  
103 skimmed milk (2% v/v). Negative control was realized by replacing the cell suspension with Tris-  
104 HCl buffer. After incubation at 37°C for 72 h in shaking conditions (10 rpm), samples were treated  
105 with 1% trichloroacetic acid (TCA) for 10 min and centrifuged (10,000g, 20 min, 4°C) for  
106 proteolytic activity determination and relative quantification of VPP and IPP.

107

### 108 *2.3 Cow milk fermentation*

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

119

### 120 *2.4 Microbiological analysis*

121 The number of presumptive viable LAB cells was determined at different time points (0, 15, 24, 39,  
122 63, 87, and 111 h) by plating 10-fold diluted samples of fermented milks on MRS agar. After  
123 incubation at 37°C for 48 h under anaerobic conditions, the number of cfu per mL was counted and  
124 expressed as Log cfu/mL.

At 63 h of fermentation (corresponding to pH lower than 4.4), 9 colonies for each trial were picked from the highest dilutions of MRS plates, cultured in MRS broth at 37 °C for 24 h, and subjected to DNA extraction as described by Ulrich and Hughes (2001), with a few modifications. Briefly, the complete cellular lysis was obtained by increasing the number of freeze-thaw steps from three to six. A final treatment with 1.5 µL RNase (10 mg/mL) at 37°C for 30 min was added to assure complete RNA degradation. DNA was quantified spectrophotometrically and properly diluted. The V1 region of the *Lb. casei* 16S rRNA gene and the *Lb. rhamnosus* 16S–23S rRNA intergenic spacer region (ISR) were amplified using the species-specific primer pairs Casei/Y2 and PrI/RhaII, respectively (Solieri et al., 2012). PCR conditions were according to Solieri et al. (2012) with the following exceptions: annealing temperature was shifted from 55 to 58°C and the final concentration of template DNA was increased from 2 to 4 ng/µL. Strain genotyping was carried out through microsatellite primed-PCR (MSP-PCR) using the arbitrarily chosen sequence (GTG)<sub>5</sub> (5'-GTG GTG GTG GTG GTG-3'), as previously reported (Solieri et al., 2012). Amplified-fragment profile comparison in disposable databases (Bionumerics 5.10, Applied Maths, Belgium) was performed as described by Solieri et al. (2012), using a similarity threshold of 90%. Only reproducible well-marked amplified fragments were included in gel analysis, faint bands being ignored.

#### 2.5 Determination of proteolytic and radical scavenging activities

Aliquots of fermented milk were treated with 1% TCA for 10 min and centrifuged (10,000g, 20 min, 4°C) to obtain TCA-soluble supernatants containing peptide fractions. Proteolytic activity was quantified by measuring the amount of released amino groups in TCA-soluble supernatants using TNBS method (Adler-Nissen, 1979). A calibration curve was prepared using leucine as standard (range 0.1–2.0 mmol/L). The results were expressed as mmol/L of leucine equivalents. The radical scavenging activity of TCA-soluble supernatants was measured using the ABTS method (Re et al., 1999) and the results were expressed as µmol/L of Trolox.



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2.6 Chemical analysis

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

2.7 Angiotensin I-converting enzyme (ACE) inhibitory activity

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

## 177 2.8 Identification and quantification of IPP and VPP

178 The identification and quantification of IPP and VPP were carried out on 2  $\mu$ L 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  $\mu$ m analytical column,  
183 both packed with a C18 phase (Agilent Technologies). The mobile phase consisted of (A)  
184 H<sub>2</sub>O/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B) acetonitrile/H<sub>2</sub>O/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  $\mu$ g/L. Each solution contained the internal standard EGVNDNEEGFFSAR at the  
200 concentration of 50  $\mu$ g/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  $\pm$  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.*

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

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

237

### 238 3.2 Fermentation process

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

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

relationship between the decrease in pH and the increase in lactic acid concentrations for both the fermentation trials (PRA331:  $r = -0.9553$ ,  $R^2 = 0.9127$ ,  $P = 0.0008$ ; PRA205:  $r = -0.9276$ ;  $R^2 = 0.8605$ ;  $P = 0.0009$ ).

Lactose content decreased during fermentation from  $124.22 \pm 9.36$  mmol/L to  $82.53 \pm 8.23$  mmol/L for strain PRA205 and from  $123.72 \pm 7.58$  mmol/L to  $60.97 \pm 10.46$  mmol/L for strain PRA331. Glucose content (about 0.4 mmol/L in both samples immediately after the inoculum) halved in the first 15 h and remained constant until 120 h after inoculum, whereas galactose content was below the detection limit of the assay at each sampling time.

The initial value of viable lactobacilli population was about Log 8 cfu/mL for both fermentation trials. Starting from 63 h, the values of viable cell counts remained constant, with final Log values of 11.43 cfu/mL and 9.47 cfu/mL for trials inoculated with PRA205 and PRA331, respectively. At the end of fermentation (corresponding to  $\text{pH} \leq 4.2$  after app. 63 h), colonies grown on the highest dilution MRS plates were randomly picked and submitted to *Lb. rhamnosus*- and *Lb. casei*-specific PCR assays, as well as to MSP-PCR genotyping. As expected, PCR targeting *Lb. rhamnosus* 16-23S rRNA intergenic region was positive only for the isolates retrieved from milk inoculated with PRA331, whereas PCR targeted V1 region of the *Lb. casei* 16S rRNA gene was positive for those recovered from milk inoculated with PRA205 (supplementary **Table S1**). MSP-PCR with primer (GTG)<sub>5</sub> showed that each set of isolates displayed the same MSP-fingerprints of the corresponding inoculated strain (supplementary **Table S1**). Both data sets were congruent to confirm the identity of each inoculated strain during fermentation.

275

### 276 3.3 Proteolytic and radical scavenging activities

The TCA-soluble peptide fractions of milk samples fermented with either PRA205 or PRA331 were collected at different times to assess both proteolytic and radical scavenging activities. During milk fermentation, the proteolysis degree increased reaching the maximum value after 96 h for both trials (**Figure 4A**). In particular, strain PRA205 increased significantly ( $P < 0.05$ ) the proteolytic activity

281 between 24 and 39 h of incubation, rising from a value of  $1.88 \pm 0.12$  mmol/L to a value of  $6.67 \pm$   
 282  $0.75$  mmol/L of leucine equivalents. After 39 h the proteolytic activity of strain PRA205 increased  
 283 more gradually, reaching a peak corresponding to  $13.55 \pm 1.41$  mmol/L of leucine equivalents at 96  
 284 h after inoculum (**Figure 4A**). Starting from 111 h of incubation, the amount of free amino groups  
 285 decreased of 22% ( $10.64 \pm 0.75$  mmol/L of leucine equivalents). The value after 120 h of  
 286 incubation was not significantly different from the value at 111 h. Strain PRA331 showed a more  
 287 gradual increase in proteolytic activity over time than PRA205, reaching the maximum value of  
 288  $8.75 \pm 0.25$  mmol/L of leucine equivalents after 96 h of incubation. Then, a significant decrease of  
 289 26% in the amount of free amino groups was observed (**Figure 4A**). After 111 h of monitoring  
 290 there were no significant changes in proteolytic activity in milk fermented with PRA331.  
 291 Development of radical scavenging activity during milk fermentation was determined through  
 292 ABTS assay. As shown in **Figure 4B**, we found significant differences between strains PRA205  
 293 and PRA331 for the radical scavenging activity. Milk fermented with strain PRA205 showed  
 294 greater ( $P < 0.05$ ) radical scavenging activity than that fermented with PRA331 starting from 39 h.  
 295 The radical scavenging activity increased in both trials getting to maximum values after 111 h,  
 296 when milk fermented by PRA205 exhibited  $1184.83 \pm 40.28$  mmol/L of trolox equivalents, while  
 297 that fermented by PRA331 exhibited  $939.22 \pm 82.68$  mmol/L of trolox equivalents.

298

### 299 *3.4 ACE-inhibitory activity*

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

ACE-inhibitory peptides during the fermentation in pre-culture. The highest ACEi% was observed after 120 h of incubation both for *Lb. casei* PRA205 (100ACEi%) and *Lb. rhamnosus* PRA331 (48ACEi%). Therefore, ACE-inhibitory power was calculated as IC<sub>50</sub> on the samples after 120 h of incubation. Strain PRA205 displayed higher activity (IC<sub>50</sub> 54.57 µg/mL) than PRA331 (IC<sub>50</sub> 212.38 µg/mL).

311

### 3.5 VPP and IPP quantification

The results described above indicate that strains PRA205 and PRA331 release peptides with ACE-inhibitory activity from milk. Therefore we determined the concentrations of the antihypertensive peptides IPP and VPP during fermentation by nano-flow LC with Q-TOF mass spectrometry.

**Figure 6** shows the release of VPP (panel A) and IPP (panel B) over time. VPP started to be detectable after 39 h in both trials and reached a maximum value after 111 h in milk inoculated with PRA205 (32.88 mg/L) and after 87 h in that fermented with PRA331 (11.87 mg/L). There was a significant difference between strains in the ability to release VPP, with PRA205 being more able than PRA331 to produce VPP (**Figure 6A**). In milk fermented with PRA331, VPP concentrations decreased strongly starting from 96 h such that, after 120 h of fermentation, the amount of VPP was about the 40% of the maximum value observed after 87 h.

The trend in IPP release was similar to that observed for VPP (**Figure 6B**). In milk fermented with *Lb. casei* PRA205 the highest IPP concentration was reached after 96 h (7.52 mg/L), thereafter it remained constant until 111 h of monitoring and then slightly decreased. In milk fermented with PRA331, the maximum IPP content (3.62 mg/L) was lower compared to PRA205 and was reached after 87 h of monitoring. After this time, IPP values decreased and, at the end of the fermentation, IPP amount was about 30% of the maximum value. Based on these results, *Lb. casei* PRA205 was able to release higher amount of the antihypertensive tripeptides VPP and IPP than *Lb. rhamnosus* PRA331.

331

#### 332 4. Discussion

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

343 The rate of pH decrease is a parameter indicative of the fermentative performance of a microbial  
344 culture and of the effectiveness of its proteolytic and glycolytic systems to sustain the microbial  
345 growth (Kunji et al., 1996). Under the experimental conditions of this study, both strains PRA205  
346 and PRA331 ended fermentation within 24-39 h, reaching final viable cell populations of 11.4 and  
347 9.5 Log cfu/mL, respectively. Although there is no general agreement in the minimum  
348 concentration of functional cells to achieve therapeutic benefits, these values greatly exceed the  
349 thresholds of 6.0-8.0 Log cfu/mL recommended by several authors (Kurmann and Rasic, 1991;  
350 Lourens-Hattingh and Vilijeon, 2001). Interestingly, strain PRA205 has been proved to possess  
351 antibiotic susceptibility profile and tolerances to different conditions miming single and multiple  
352 stresses occurring during GI transit (Solieri et al., 2014). *Lb. casei* PRA205 overcomes *Lb.*  
353 *rhamnosus* PRA331 in survival after 3h-treatments with both synthetic pancreatic and gastric juices,  
354 as well as in viability after acidic, salt bile, and lysozyme stresses, respectively (Solieri et al., 2014).  
355 The high number of viable PRA205 cells in combination with their *in vitro* GI resistance suggests  
356 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  $\kappa$ -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 (Virtanen 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

383 PRA205 and PRA331 were approximately 1.2 and 0.9 mmol/L of Trolox equivalents, respectively.  
 384 These values were equal or superior to most of the fermented milk samples analysed by Hernandez-  
 385 Ledesma et al. (2005).  
 386 Various types of milk fermented with *Lb. helveticus*, *Lb. bulgaricus* ssp. *bulgaricus*, *Lb.*  
 387 *rhamnosus*, *Lb. acidophilus*, and *Lc. lactis* ssp. *cremoris* have been shown to contain peptides with  
 388 variable ACE-inhibitory activity (Gobbetti et al., 2004). In our study both strains PRA205 and  
 389 PRA331 were able to release ACE-inhibitory peptides during milk fermentation. Milk fermented  
 390 with *Lb. casei* PRA205 displayed IC<sub>50</sub> value 3.9 times lower than milk fermented by *Lb. rhamnosus*  
 391 PRA331. This difference could reflect different type, quality or concentration of peptides produced  
 392 by PRA205 and PRA331, which in turn could be related to cell envelope proteinase/peptidases with  
 393 different specificity (Savijoki et al., 2006).  
 394 Nejati et al. (2013) obtained IC<sub>50</sub> values ranging from 220 and 1750 µg/mL in pH 4.6-soluble  
 395 fractions from milk fermented by different lactobacilli. In our experiments, *Lb. casei* PRA205  
 396 showed an IC<sub>50</sub> value 4 and 5 time lower than the highest IC<sub>50</sub> values described for *Lc. lactis*  
 397 DIBCA2 and *Lb. casei* FC113, respectively (Nejati et al., 2013). In according to our data, Nejati et  
 398 al. (2013) also found *Lb. rhamnosus* strains less efficient than *Lb. casei* in release of ACE-  
 399 inhibitory peptides with IC<sub>50</sub> values ranging from 700 to 1500 µg/mL. Anyway, these values are 3  
 400 to 7 times higher than the values obtained with *Lb. rhamnosus* PRA331. Our results are similar than  
 401 that found by Muguerza et al. (2006) using *Lb. rhamnosus*. On the other hand, milk fermented with  
 402 *Lb. delbrueckii* subsp. *bulgaricus* or *Lc. lactis* subsp. *cremoris* showed IC<sub>50</sub> values ranging from 8  
 403 to 11.2 µg/mL (Gobbetti et al., 2000).  
 404 It is difficult to establish a direct link between IC<sub>50</sub> values and *in vivo* antihypertensive effects, as  
 405 the digestive stability and bioavailability of peptides are often uncertain. Although a large number  
 406 of peptides with ACE-inhibiting *in vitro* effects are released during milk fermentation, the present  
 407 work focused only on tripeptides VPP and IPP, whose antihypertensive effects have been well  
 408 demonstrated in several human studies (Cicero et al., 2013). *Lb. helveticus* releases IPP and VPP

409 from milk proteins at IC<sub>50</sub> 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 Kloeck, 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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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  $\mu\text{mol/L}$  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
<i>Lb. rhamnosus</i>	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
<i>Lb. paracasei</i>	E24, E31, E33, D21, D55	Sicilian Pecorino cheeses	Randazzo et al., 2006
	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	
<i>Lb. casei</i>	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

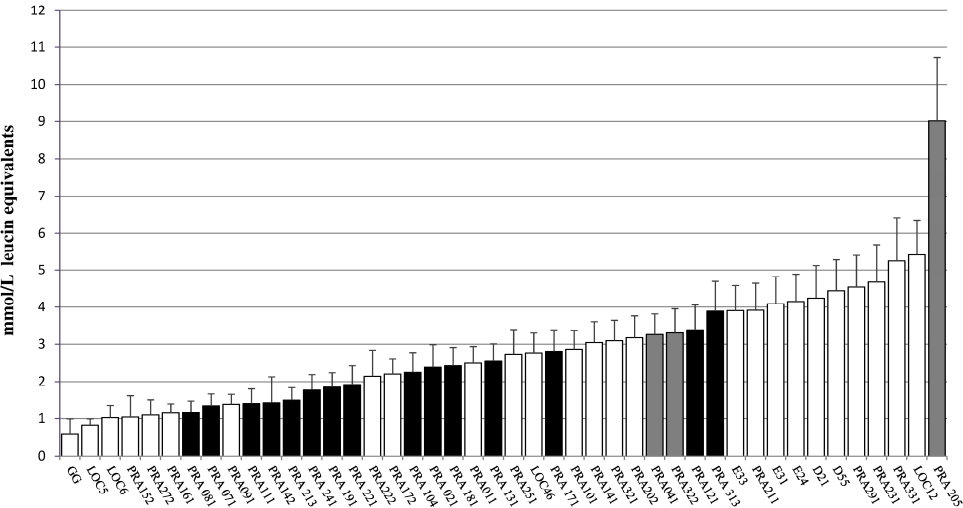


Figure 2

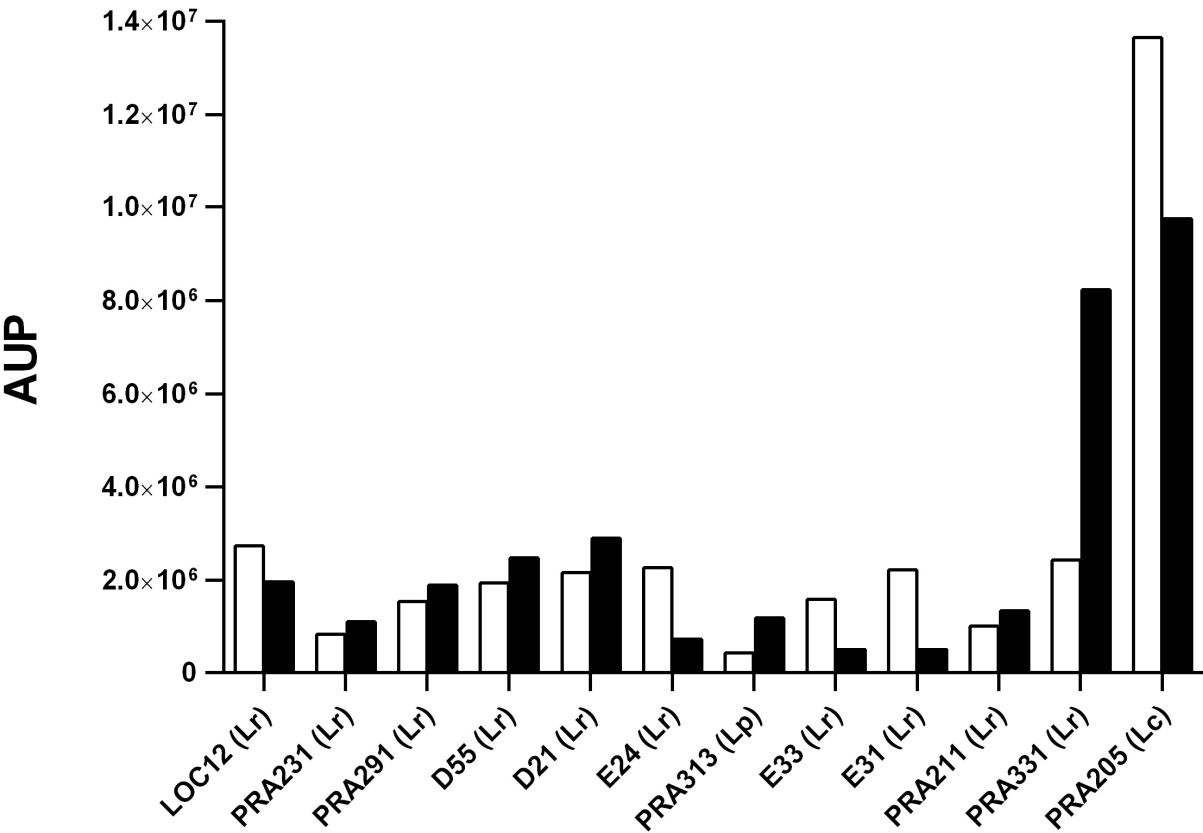


Figure 3

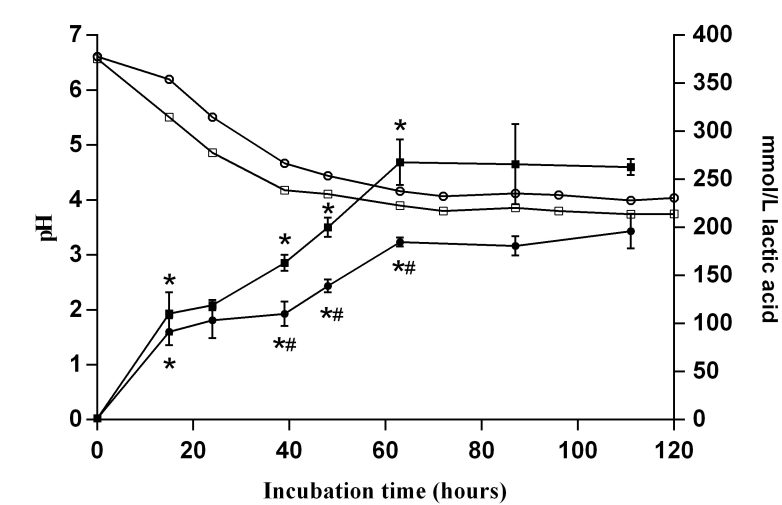




Figure 4

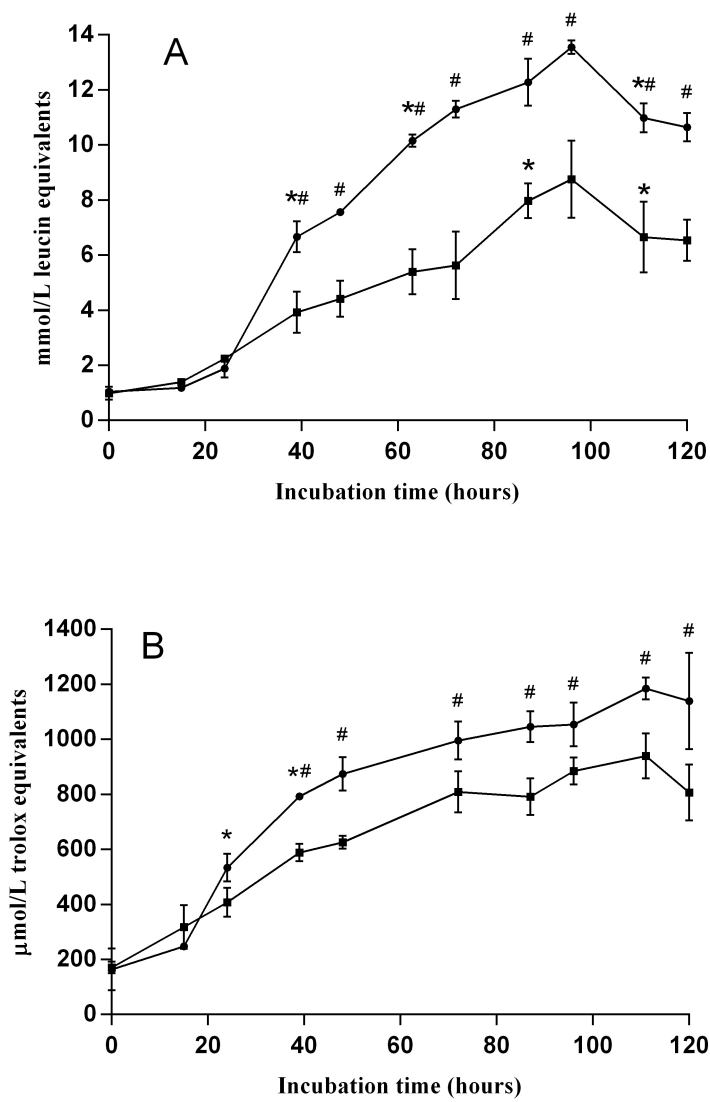


Figure 5

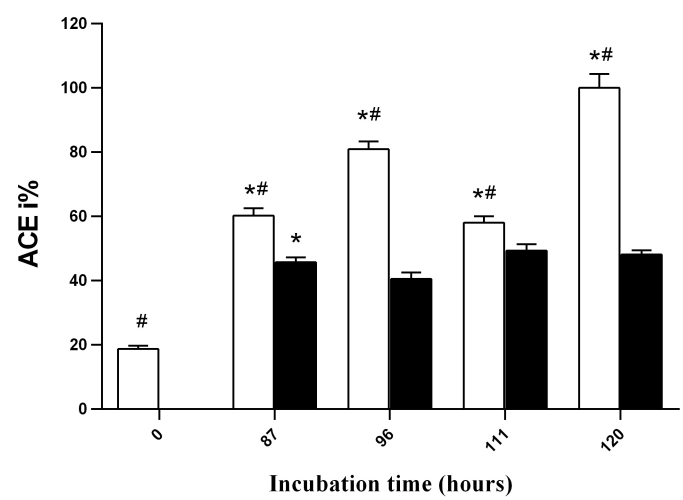


Figure 6

