Survival and bioactivities of selected probiotic lactobacilli in yogurt fermentation and cold storage: new insights for developing a bi-functional dairy food

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Abstract

In previous work, we demonstrated that two probiotic strains, namely *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331, produce fermented milks with potent angiotensin-converting enzyme (ACE)-inhibitory and antioxidant activities. Here, we tested these strains for the survivability and the release of antihypertensive and antioxidant peptides in yogurt fermentation and cold storage. For these purposes three yogurt batches were compared: one prepared using yogurt starters alone (*Lactobacillus delbrueckii* subspecies *bulgaricus* 1932 and *Streptococcus thermophilus* 99), and the remaining two containing either PRA205 or PRA331 in addition to yogurt starters. Despite the lower viable counts at the fermentation end compared to PRA331, PRA205 overcame PRA331 in survivability during refrigerated storage for 28 days, leading to viable counts (>10^8 CFU/g) higher than the minimum therapeutic threshold (10^6 CFU/g). Analyses of *in vitro* ACE-inhibitory and antioxidant activities of peptide fractions revealed that yogurt supplemented with PRA205 displays higher amounts of antihypertensive and antioxidant peptides than that produced with PRA331 at the end of fermentation and over storage. Two ACE-inhibitory peptides, Valine-Proline-Proline (VPP) and Isoleucine-Proline-Proline (IPP), were identified and quantified. This study demonstrated that *L. casei* PRA205 could be used as adjunct culture for producing bi-functional yogurt enriched in bioactive peptides and in viable cells, which bring health benefits to the host as probiotics.

Keywords: *Lactobacillus*; antioxidant peptides; antihypertensive peptides; probiotics; yogurt.

1. Introduction

Probiotics are live micro-organisms that, when administered in adequate amounts, confer health benefits on the host (FAO 2006). Depending upon the strain and/or the species, probiotics survive transit through the gastro-intestinal tract (GIT) and provide measurable health benefits owing to their ability to modulate immune system of the host, balance intestinal microflora, and produce functionally valuable products (Kanmani et al., 2013). Recently, the traditional recommendation that probiotic strains for humans should come from humans (species-specificity criterion) (Vasiljevic and Shah, 2008) is becoming mitigated, because several starter and non-starter lactic acid bacteria (SLAB and NSLAB) isolated from fermented dairy products have been proven to possess healthy properties, beyond their technological functions (Milesi et al., 2009; Settanni and Moschetti, 2010). In particular, NSLAB belong to species from which probiotic strains were isolated and characterized (*Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus*) and, taken advantage from their proteolytic systems, they can release bioactive peptides primarily from αS1- and β-caseins, with proven anti-oxidant and anti-hypertensive activities (Pihlanto and Korhonen, 2014 and references therein).

In recent years, probiotics have increasingly entered the food supply as dietary adjuncts in variety of food system (Smug et al., 2014). One of the most popular fermented milk products for the delivery of probiotics cultures is yogurt (Lourens-Hattingh and Viljoen, 2001). According to the Codex standard for fermented milks (CODEX 2003), yogurt is strictly defined as milk fermented with symbiotic starter cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus*, which shall be in a viable state, active and still present in the product through the end of shelf life (FAO/WHO, 2011). Yogurt starter cultures may be considered probiotic since they may help to lessen the symptoms of lactose intolerance thanks to the release of lactose-hydrolyzing enzymes (Adolfsson et al., 2004; Guarner et al., 2005). However, yogurt starter cultures are not bile-resistant or acid-tolerant and thus cannot survive under the GIT conditions

(Vinderola and Reinheimer, 2003; del Campo et al., 2005). The terms 'yogurt-like product' or 'bio-yogurt' or 'functional yogurt' are used to define alternative culture yogurt (i.e. when *L. bulgaricus* is substituted by other *Lactobacillus* species for the fermentation of milk) or yogurt containing probiotic bacteria (Guarner et al., 2005). Saxelin et al. (2010) demonstrated that yogurts and fermented milks were as effective as capsules for the administration of probiotic bacteria, emphasizing the importance of such matrices as functional food matrices.

Obtaining desirable therapeutic effects in probiotic yogurts requires the viability of the starter and probiotic cultures to be maintained at a sufficient level throughout storage of the product. It has been suggested that probiotics should be present in the food product in minimal amounts of 10^6 colony forming units (CFU)/g. This amount could be translated into $\geq 10^6$ CFU/g/day of probiotics-containing yogurt given that 100 g is the daily serving portion. Such high dosage is required to compensate for the loss of cells during the passage through the upper and lower parts of the GIT (Tamime et al., 2005; Granato et al., 2010).

From a technological standpoint, yogurt supplementation with probiotic cultures is not easy, particularly with respect to maintaining the viability of the cultures (Corcoran et al., 2008). Many factors influence the viability of probiotics in yogurts: strain variation, acid accumulation, interaction with starter cultures, levels of dissolved oxygen and hydrogen peroxide (H_2O_2), and storage condition (Nighswonger et al., 1996; Donkor et al., 2006). Several studies reported that some commercially available dairy products contain insufficient number of viable probiotics (as defined by $< 10^6$ CFU/g or mL before the expiration date), thereby diminishing the potential health benefits conferred by these products (Coeuret et al., 2004, Huys et al., 2006, Lin et al., 2006).

In our previous works, we demonstrated that two NSLAB probiotic strains isolated from Parmigiano Reggiano cheese, namely *L. casei* PRA205 and *L. rhamnosus* PRA331, were able to resist GIT conditions (Solieri et al., 2014) and to release hypotensive casokinins Valine-Proline-Proline (VPP) and Isoleucine-Proline-Proline (IPP) during milk fermentation (Solieri et al., 2015). VPP and IPP resist GIT transit and cross the mucosal barrier, without being digested by serum

peptidases (Foltz et al., 2008). They *in vivo* reduce systolic blood pressure both in animal and human models owing to multiple anti-hypertensive actions, such as inhibition of angiotensin-converting enzyme (ACE), stimulation of vasodilator production, and modulation of sympathetic nervous activity (Boelsma and Kloek, 2010, Nakamura et al., 2011; Cicero et al., 2013).

The aim of the present work was to develop bi-functional yogurts, which deliver viable cells of potential probiotics *L. casei* PRA205 and *L. rhamnosus* PRA331 and their bioactive peptides VPP and IPP. Three set-type yogurts were prepared in order to evaluate the viability of probiotics during yogurt fermentation and 28 day (d) long refrigeration, as well as to investigate their effect on proteolytic, anti-hypertensive and antioxidant activities of TCA soluble peptide extracts from yogurts.

2. Materials and methods

2.1 Strains, media and culture conditions

Streptococcus thermophilus 99 and *L. delbrueckii* ssp. *bulgaricus* 1932 (*L. bulgaricus*) were kindly provided by Prof. Camilla Lazzi (University of Parma, Italy) and cultured for 24 h at 42°C under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) in M17 (Oxoid Basingstoke, UK) supplemented with 2% (w/v) lactose (LM17), and MRS (Oxoid Basingstoke, UK) media, respectively. *Lactobacillus rhamnosus* PRA331 and *Lactobacillus casei* PRA205 (deposited in the Unimore Culture Collection; www.umcc.unimore.it) were isolated from ripened Parmigiano Reggiano cheeses (Solieri et al., 2012) and routinely cultured in MRS medium for 24 h at 37°C. Prior to the experimental use, each culture was twice propagated in the corresponding growth medium. All LAB strains were maintained as frozen stock at -80°C in MRS or LM17 broth supplemented with glycerol at the final concentration of 25% (w/v).

2.2 Correlation curve between optical density and cell counts

To standardize the inoculum, exponentially growing cells of each bacterial strain with approximately 10^8-10^9 cells/mL were serially diluted (1, −2, −4, −8, −10 fold) with saline solution in duplicate. Then OD_{600} of the samples was measured spectrophotometrically. Sterile saline solution was used as blank. For counting cell numbers, the serially diluted bacterial cultures were further diluted with saline solution. Then the CFU measurements were typically obtained by spreading 100 μL of culture on 9-cm plates to obtain 100–400 colonies on the appropriate growth medium as reported above. The correlation between OD_{660} and cell count (CFU/mL) was established for each bacterial species by means of a standard curve. Correlation curves of OD_{600} *vs.* CFU/mL and conversion factors were listed in **Table S1**.

2.3 Determination of proteolytic activity in milk

The proteolytic activity of single cultures of starter and probiotic strains was determined in UHT cow skimmed milk over a fermentation time of 72 h, as previously described (Solieri et al., 2015).

2.4 Yogurt preparation and storage

Yogurt preparation and experimental strategy are summarized in **Fig. 1**. Briefly, yogurt was prepared by heat-treating reconstituted skimmed milk (14 % w/v) at 85°C for 30 min followed by cooling to 45°C, and aseptically inoculating with 10^7 CFU/mL of each of *L. bulgaricus* and *S. thermophilus*. The inoculated milk was divided into equal portions; one portion was used as a control (referred to as yogurt 1), while the other portions were further inoculated with 10^7 CFU/mL of probiotic culture either of *L. casei* (referred to as yogurt 2) or *L. rhamnosus* (referred to as yogurt 3), separately. Non-inoculated milk was used as negative control. The mixes were poured into polystyrene cups aseptically and incubated at 42°C. Decrease of pH was monitored every 1.5-3 h until the required pH value of 4.5 ± 0.5 was reached (approx. 8 h). Cooling to 4°C was done to halt

further acidification. Yogurts were stored at 4°C for 28 days (d), the typical shelf life of commercial yogurts. Aliquots of samples were removed after 8 h of fermentation at 42°C and at 3, 14 e 28 d of cold storage for subsequent microbiological and biochemical characterizations. Aliquots of yogurt were treated with 1% TCA for 10 min and centrifuged (10,000 x *g*, 20 min, 4°C) to obtain TCA-soluble supernatants containing peptide fractions. The experiments were carried out in triplicate.

2.5 Selective enumeration of LAB species

Cell populations of yogurt starters *S. thermophilus* and *L. bulgaricus* were selectively enumerated using LM17 medium under aerobic incubation at 45°C for 24 h and MRS agar medium (pH adjusted to 5.2 using 1 mol/L HCl) at 45°C for 72 h anaerobically, respectively. Probiotic cultures of *L. casei* and *L. rhamnosus* were selectively enumerated from yogurt cultures using MRS vancomycin agar (pH 6.2; 1 ppm vancomycin) at 37°C for 72 h anaerobically (Sah et al., 2014). Plates containing 25–250 colonies were considered for enumeration and the results were reported as log CFU/g.

2.6 Determination of proteolytic and antioxidant activities from yogurts

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.

2.7 Angiotensin I-converting enzyme (ACE) inhibitory activity

ACE inhibitory (ACEi) activity of TCA-soluble supernatants 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 mmol/L in reaction buffer containing 100 mmol/L TrisHCl, 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 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%).

2.8 Determination of VPP and IPP

The identification and quantification of IPP and VPP were carried out on 2 µL of suitably diluted TCA-soluble supernatant through nanoLC-MS/MS experiments performed on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Milano, Italy). Chromatographic separation was performed on a ProtID-Chip- 43(II) including a 4 mm 40 nL enrichment column and a 43 mm x 75 mm analytical column, both packed with a C18 phase (Agilent Technologies). The mobile phase composition and the gradient were the same as reported by Solieri et al. (2015). The mass spectrometer was tuned, calibrated and set with the same parameters as reported by Dei Più et al. (2014). VPP and IPP were selectively fragmented using a mass to charge ratio of 312.18 and 326.21 (charge +1), respectively and identified by comparing the retention time and fragmentation spectra of a synthetic standard tripeptide. VPP and IPP was quantified according to Solieri et al. (2015). The assignment process was complemented and validated by the manual inspection of MS/MS.

2.9 Statistical analysis

All experiments were carried out in triplicate and data are shown as means ± standard deviation (SD). We used two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test to determine significant differences ($P < 0.05$) among means. Correlations between variables were assessed using Pearson's method. All analyses were performed with GraphPad Prism version 6.00 (GraphPad software, San Diego, CA).

3. Results and Discussion

In our previous work, we demonstrated that probiotic strains PRA205 and PRA331 release significant amounts of VPP and IPP from caseins starting from 48 h of milk fermentation (Solieri et al., 2015). Furthermore, *L. casei* PRA205 and *L. rhamnosus* PRA331 have been proven to possess good GIT-resistance and other functional properties, which make these strains promising probiotic candidates (Solieri et al., 2014). In this work, we investigated the possibility to develop yogurt carriers for delivering VPP and IPP-producers PRA205 and PRA331. However, probiotic bacteria grow slowly in milk and the rate of acidification is usually too slow to support an adequate fermentation process in yogurt (Tamime et al., 2005). Standard yogurt cultures, on the other hand, cause an accelerated and efficient lactic acid production during fermentation process of yogurt due to a proto-cooperation between *L. bulgaricus* and *S. thermophilus*. In order to develop yogurts with acceptable sensorial properties and to enrich yogurts in bioactive peptides and probiotic cells, we decided to test probiotic cultures as adjuncts in combination with starter yogurt cultures *L. bulgaricus* 1932 and *S. thermophilus* 99.

3.1 Characterization of proteolytic activity of starter yogurt cultures and probiotic strains

Conventionally, one of the proto-cooperative interactions between *L. bulgaricus* and *S. thermophilus* relies on the metabolite exchange of amino acids and peptides from the proteolytic *L. bulgaricus* to the non-proteolytic (Prt-) *S. thermophilus* (Sieuwerts et al., 2008). However, several *S. thermophilus* Prt+ strains have been recently documented in industrial bioprocesses, which were able to grow independently from *L. bulgaricus* in milk leading to substantial acidification (Delorme et al., 2010 and references therein). To evaluate whether *S. thermophilus* strain 99 has a Prt+ phenotype and to better understand how yogurt starter cultures affect proteolytic profiles of yogurt during co-fermentation with VPP and IPP-producer probiotic strains, we determined proteolytic activity of *S. thermophilus* 99 and *L. bulgaricus* 1932 during 72 h of milk fermentation. As shown

in **Fig. S1**, the milk fermented by *S. thermophilus* strain 99 exhibited higher concentrations of leucine equivalents than milk batches fermented by other strains during all the fermentation time. Therefore, *S. thermophilus* strain 99 displayed a strong Prt+ phenotype and overcame both *L. bulgaricus* starter strain 1932 and probiotic strains PRA205 and PRA331 in proteolytic activity. This Prt+ phenotype suggests that this *S. thermophilus* strain may possess the cell-envelope proteinase PrtS which initiates casein breakdown into oligopeptides (Delorme et al., 2010).

3.2 Yogurt fermentation and viability of starter and probiotic cultures

Yogurt acidification was monitored for each fermentation batch, as shown in **Fig. S2**. Supplementation of milk with only the starter cultures (yogurt 1) resulted in a substantially slower lowering of pH compared to co-fermentation trials with probiotic strains ($P < 0.05$). In particular, the milk acidification was enhanced when the yogurt starter strains 99 and 1932 were cultivated together with *L. casei* PRA205 compared to *L. rhamnosus* PRA331. This result disagrees with those obtained with mono-cultures, where milk acidification was higher with *L. rhamnosus* PRA331 than with *L. casei* PRA205 (Solieri et al., 2015).

The first and most critical aspect in the development of a probiotic yogurt is the coexistence between starter and probiotic cultures, since a negative interaction can interplay between them. Factors such as dissolved oxygen, post-fermentation acidification and the consequent acid injuries may adversely influence the viability of probiotic strains (Vasiljevic and Shah, 2008). *L. rhamnosus*, *L. acidophilus*, and *Bifidobacterium lactis* appear to be inhibited when co-cultured with the fast-acidifying strain *S. thermophilus*, resulting in a low probiotic content of the final product (de Souza Oliveira et al., 2009). Vinderola et al. (2002) found that the cell-free supernatant obtained from skim milk cultures fermented by *S. thermophilus* and *L. bulgaricus* had no positive effect on probiotic *L. casei*, indicating that the extracellular products secreted by these yogurt bacteria were not conducive to the growth of *L. casei*. As the viability of probiotic organisms is considered a key parameter for developing probiotic yogurt, we determined the viable counts of probiotic and starter

organisms at the end of acidification and monitored their survival during a period of 28 d of refrigerated storage (**Fig. 2**). In yogurt 1 *S. thermophilus* and *L. bulgaricus* reached comparable viable counts at the end of acidification, without any strain dominating over the other (**Fig. 2**, panels A and B). In yogurts 2 and 3 starter culture concentrations were slightly affected by the presence of probiotic cultures after 8 h of acidification. In particular *L. bulgaricus* 1932 significantly increased viable cell counts from 8.52 ± 0.16 (yogurt 1) to 8.96 ± 0.19 log CFU/g ($P < 0.05$) in co-culturing with probiotic strain PRA205 (yogurt 2) (**Fig. 2**, panel B). At the end of fermentation, probiotic strain PRA331 exhibited higher viable counts ($P < 0.05$) than PRA205 (**Fig. 2**, panel C).

During cold storage, species-specific bacterial counts differed between the yogurt 1 and the two probiotic yogurts 2 and 3, as well as between the two probiotic yogurts. There were minimal differences over time in the viability of *S. thermophilus* 99 during refrigeration of yogurt 1 (**Fig. 2**, panel A), whereas *L. bulgaricus* 1932 cell counts declined from 8.52 ± 0.16 to 6.50 log CFU/g (**Fig. 2**, panel B). These results are congruent with previous findings, which indicated that *S. thermophilus* is more resistant to cold stress than *L. bulgaricus* (Donkor et al., 2007). Addition of *L. casei* PRA205 positively affected the *S. thermophilus* viable counts at the early cold storage ($P < 0.05$) compared to control yogurt, but not those of *L. bulgaricus* which decreased of 100-fold. Compared to yogurt 1, the co-culturing of starter bacteria with *L. rhamnosus* determined an opposite trend, with the reduction of *S. thermophilus* survival at the early cold storage ($P < 0.05$), and the increase of *L. bulgaricus* viable counts.

Although *L. rhamnosus* displayed higher viable counts at the beginning of refrigeration, cold storage resulted in a 100-fold decrease of its viable population (from 9.08 ± 0.09 to 7.29 ± 0.13 log CFU/g) (**Fig. 2**, panel C). In contrast, viable counts of *L. casei* PRA205 slightly increased during early storage ($P < 0.05$), which could be due to residual activity during this period; after that they remained relatively constant (approximately $>10^8$ CFU/g) over the late storage time (**Fig. 2**, panel C). Significantly, *L. casei* exhibited viable population at 4°C higher than *S. thermophilus* strain 99 ($P < 0.05$). These results indicated that yogurt provided an environment that was favorable to

maintain high numbers of added *L. casei* cells during refrigerated storage. Remarkably, viable counts of probiotic strain PRA205 and, to a lesser extent, PRA331 after 28 d of refrigeration, were sufficient to exhibit probiotic effect, as the reported minimum therapeutic count of 10^6 CFU/g is below that obtained in the present work.

3.3 Proteolytic activity

Lactic acid bacteria produce proteolytic enzymes during yogurt manufacturing, which cleave peptide bonds of milk proteins leading to generation of bioactive peptides and free amino acids (Donkor et al., 2007). The extent of protein hydrolysis in yogurt samples was estimated by determining free amino groups, as reported in **Fig. 3**. Yogurt 2 displayed the highest amount of free amino groups at the end of fermentation. During refrigerated storage, the amount of free amino groups (expressed as mmol/L leucine equivalents) increased significantly ($P < 0.05$) in all samples, suggesting a continuation of proteolytic activities even at low temperature. In particular, the free amino group content remained significantly higher in yogurt 2 than yogurts 1 and 3 after 72 h of refrigeration, whereas a significant increase ($P < 0.05$) in proteolytic activity was observed in yogurt 1 compared to yogurt 2 and 3 starting from 14 d of cold storage. A similar reduction of free amino group content in co-culturing between starter and probiotic strains compared to starter yogurt control was also observed by Ramchandran et al. (2007), and it could be due to the higher demand for free amino acids and peptides required to sustain the survival of both starter and probiotic LABs compared to starter yogurt alone.

When probiotic yogurts were compared each other, yogurt 2 (with *L. casei* PRA205) overcame ($P < 0.05$) yogurt 3 (with *L. rhamnosus* PRA331) in free amino group content at every time point of cold storage (**Fig. 3**). This result is congruent with the high proteolytic activities previously observed for *L. casei* strain PRA205 (Solieri et al., 2015) and with its higher stability in cold conditions compared to PRA331 (**Fig. 2**). Since *S. thermophilus* viable counts were similar in

yogurts 2 and 3, we supposed that the decline of *L. bulgaricus* viability observed in yogurt 2 at low temperature did not negatively affect the proteolytic potential of this probiotic yogurt.

3.4 Antioxidant activity

Protein hydrolysates can contain antioxidant peptides that can protect the human body by scavenging free radicals, such as reactive oxygen species, and also increase shelf life of foodstuffs by retarding the process of lipid peroxidation through hydrogen atom or electron transfer mechanisms (Pihlanto and Korhonen, 2014). To analyze the scavenging capacity of TCA-soluble extracts of yogurts, we used the ABTS assay and compared the antioxidant activities after yogurt manufacturing and during cold storage with that of the positive control, trolox, a vitamin E analog and known antioxidant. In order to confirm that the measured antioxidant activity was generated during the fermentation, the absorbance of TCA-soluble extract of skimmed milk immediately after inoculum was subtracted from that of the TCA-soluble extract from yogurts. The resulting values, expressed as μmol/L Trolox equivalents, are shown in **Fig. 4**. Generally, the antioxidant activities increased significantly ($P < 0.05$) during storage in all yogurt batches with respect to fermentation end. A positive correlation between proteolytic and ABTS scavenging activities was observed only for probiotic yogurts 2 ($P < 0.05$, Pearson r = 0. 93) and 3 ($P < 0.05$, Pearson r = 0.94). Furthermore, all yogurt samples exhibited varying degrees of scavenging capacities for ABTS radicals, indicating differences in generated TCA-soluble fractions of the yogurts. While yogurt 2 exhibited antioxidant activity similar to yogurt 1 only at the end of fermentation, yogurt 1 overcame the probiotic yogurts in scavenging capacities for ABTS radicals during refrigeration, suggesting that the combination of starter and probiotic LABs lead to lower scavenger activity than starter cultures alone at low temperature (**Fig. 4**). However, yogurt co-fermented with PRA205 exhibited higher antioxidant values than yogurt co-fermented with PRA331, mainly at the end of refrigeration (**Fig. 4**). Previous works found that fermented milks and yogurts produced using mixed cultures showed a higher radical scavenging activity than milks fermented using a single strain (Virtanen et

al., 2006; Sah et al., 2014). In our case, we can speculate that competition between starter and probiotic cultures for free amino acids and peptides may reduce the antioxidant peptide fraction in yogurts 2 and 3. Moreover, the bioactivities of protein hydrolysates depend upon a number of factors, such as amino acid sequence, size and structure of the peptides, as well as on the types of enzyme from the LAB, as specific proteases are involved in the hydrolysis of specific peptide bonds (Sarmadi and Ismail, 2010).

3.5 Angiotensin-converting enzyme inhibitory activity

The search for ACEi activity is the most common method for the selection of antihypertensive peptides derived from milk proteins. Therefore, ACEi activity of TCA-soluble fractions was determined for all yogurt batches at end of fermentation and during storage at 4 °C (**Fig. 5**).

Differently from those reported by previous authors (Donkor et al., 2005; Sah et al., 2014), the degree of ACE inhibition by yogurt produced supplemented with probiotic organisms was not always greater than that of the control yogurt consisting solely of the yogurt cultures. As expected from the increase of proteolytic activities starting from the middle stage of refrigeration, control yogurt overcame probiotic yogurts in ACEi percentage during cold storage, but not at the end of fermentation (**Fig. 5**). A similar difference between starter and probiotic batches was reported by Donkor et al. (2005) which found that ACEi activity increased from 70% to 90% in control yogurt and decreased from 100% to 70% in probiotic yogurt over 28 day refrigerated storage. Statistical analysis revealed a slight positive and statistically not significant correlation (Pearson $r = 0.73$; $P > 0.05$) between the TNBS results and ACE inhibition, indicating that the extent of ACE inhibition might only partially depends on the extent of the proteolytic activity. In particular, despite enhance of proteolytic activity during cold storage (**Fig. 3**), in yogurt 1 ACEi activity declined ($P < 0.05$) from 14 to 28 d of refrigeration (**Fig. 5**).

Overall, addition of *L. casei* PRA205 in yogurt formulation led to enrichment in ACEi peptides higher than *L. rhamnosus* PRA331. According to previous work (Solieri et al., 2015),

strain PRA205 exhibited a basal ACEi activity immediately after inoculum, which significantly increased after 8 h of fermentation at 42°C. Remarkably, this ACEi activity did not significantly change during cold storage ($P > 0.05$). This result indicated that, despite the declining number of live *L. bulgaricus* bacteria during refrigeration (**Fig. 3**), the proteinase-dependent activities of viable *St. thermophilus* and *L. casei* PRA205 cells were enough to counterbalance the breakdown of peptides into free amino acids by proteolytic peptidases released from lysing cells of *L. bulgaricus*. Remarkably, yogurt 1, which displayed higher proteolytic activity than yogurt 2 in the late stage of storage (**Fig. 3**), showed ACEi percentages similar ($P > 0.05$) to yogurt 2 after 28 d of refrigeration (**Fig. 6**). Similarly, Donkor et al. (2007) and Papadimitriou et al. (2007) found no significant differences in ACEi activity between yogurt containing starter cultures and yogurt containing both starter and probiotic *L. paracasei* cultures. Rojas-Ronquillo et al. (2012) observed that *L. casei* Shirota and *S. thermophilus* released ACEi peptides during milk fermentation.

TCA-soluble peptides extracted from yogurt supplemented with PRA331 displayed a trend of ACE inhibition markedly different from that observed in probiotic strain PRA205 (**Fig. 5**). Differently from PRA205, strain PRA331 did not show any ACEi activity after inoculum. The maximum ACEi percentage was recorded after 72 h of refrigeration, thereafter the values drop out below the detection limits of the assay. These results are in agreement with previous observations that ACEi activity decreased when proteolysis exceeded a certain level (Pihlanto et al., 2010). The content of ACEi peptides appears to rely on a balance between their formation and further breakdown into inactive peptides and amino acids, in turn depending upon storage time and conditions (López-Fandiño et al., 2006). The decline in *L. bulgaricus* and *L. rhamnosus* viable counts at low temperature (**Fig. 2, panels b and c**) can contribute to the unbalance of formation/degradation rate of ACEi peptides towards their breakdown. The impact of aminopeptidase (PepN) and X-prolyl dipeptidyl aminopeptidase (PepX) activities of NSLAB has been poorly explored, but we speculate that some peptidases of *L. rhamnosus* may hydrolyze oligopeptides, decreasing the content of ACEi peptides. Accordingly, in *L. helveticus* and *Lc. lactis*

the depletion of peptidases PepX and PepN increases the ACEi activity during milk fermentation due to the hindering of peptide degradation (Algaron et al., 2004; Kilpi et al. 2007).

3.6 Characterization of VPP and IPP contents

During cold storage, yogurts 1 and 2 displayed higher ACEi activity than yogurt 3. Furthermore, probiotic PRA205 is a higher VPP and IPP- producer than PRA331 (Solieri et al., 2015). In order to get further insights into the nature of bioactive peptides contributing to these observed ACEi activities, we determined VPP an IPP contents on crude peptide fractions extracted from yogurts 1, 2 and 3 after 3, 14 and 28 d of refrigeration. As shown in **Fig.6** (panel A), in control yogurt 1 VPP amount was 334.3 ± 26.1 µg/L after 3 d of cold storage and increased ($P < 0.05$) during cold storage reaching a concentration of 1373.4 ± 463.0 µg/L at the end of refrigeration times. IPP concentration was much lower than VPP and followed the same trend of increase as VPP over storage (**Fig. 6**, panel B). A clear correlation was found between the amount of VPP and IPP and the proteolysis (Pearson r=0.7748; P=0.0142) whereas no correlation was observed between the amount of these lactrotripeptides and the ACEi values, suggesting that, in addition to VPP and IPP, other peptides with ACEi activity are released during yogurt manufacturing and cold storage.

In yogurts supplemented with either *L. casei* PRA205 or *L. rhamnosus* PRA331 amounts of VPP and IPP were similar ($P > 0.05$) to that observed in starter yogurt after 3 d of cold storage (**Fig. 6**, panels A and B). Similarly, Donkor et al. (2007) found VPP and IPP in yogurts prepared either using yogurt cultures *L. bulgaricus* and *S. thermophilus*, or *L. acidophilus*, *L. casei* and *Bifidobacterium lactis* in addition to yogurt culture. However, in our probiotic yogurt 2, VPP content increased from 3 to 28 d of refrigeration to a lesser extent ($P < 0.05$) respect to the yogurt 1, whereas IPP content did not change over storage (**Fig. 6**, panels A and B). Differently from yogurts 1 and 2, the amount of VPP and IPP in yogurt 3 remained stable during the cold storage period.

At the end of the cold storage, the sum of VPP and IPP in yogurt 1 (1684.3 ± 477.7 µg/L) was significantly ($P < 0.05$) higher than the sum of VPP and IPP found in yogurt 2 (734.4 ± 15.2 µg/L)

and 3 (505.0 ± 36.4 µg/L). However, yogurt 2, containing the strain PRA205, showed significantly higher amount ($P < 0.05$) of the lactotripeptides respect to the yogurt 3, containing PRA331, after 28 d of cold storage.

Results suggested that, despite all three yogurts containing VPP and IPP lactotripeptides, their amount resulted from a complex and dynamic interplay between formation and breakdown, which strongly depends on proteinase and/or peptidase specificity in the various microorganisms. In yogurts containing multiple probiotic and starter cultures, both the major requirements for amino acids and peptides and the lower viability of *L. bulgaricus* can explain the observed lower values of VPP and IPP.

4. Conclusions

From the results presented in this work, probiotic *L. casei* PRA205 was able to remain viable in cow milk yogurt after 28 d of storage at 4 °C with improved proteolysis, enhanced antioxidant properties, and increased ACEi activity compared to probiotic *L. rhamnosus* PRA331. The results of this study indicated that addition of probiotic *L. casei* PRA205 into cow milk yogurt manufacturing is an effective and scalable strategy to develop a bi-functional yogurt, which delivers viable probiotic cells at concentrations higher than the probiotic threshold and displays health status through increased antihypertensive and radical-scavenging capabilities. Furthermore, our work highlighted how combination of starter and probiotic cultures in dairy food fermentation resulted in complex interactions, which are beyond the additive properties of single mono-cultures. Microbial dynamics and competition for nutrients affect bacteria survivability, and shape metabolic bio-functionalities, such as the release of antihypertensive and antioxidant peptides. Further studies are required to understand routes of formation and degradation of bioactive peptides in yogurt fermentation carried out by multiple microbial cultures.

5. References

Adler-Nissen, J., 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzene sulfonic acid. J. Agric. Food Chem. 27, 1256–1262.

Adolfsson, O., Meydani, S.N., Russell, R.M., 2004.Yoghurt and gut function. Am. J. Clin. Nutr. 80, 245-256.

Algaron, F., Miranda, G., Le Bars, D., Monnet, V., 2004. Milk fermentation by *Lactococcus lactis* with modified proteolytic systems to accumulate potentially bio-active peptides. Lait. 84, 115-123.

Boelsma, E., Kloek, J., 2010. IPP-rich milk protein hydrolysate lowers blood pressure in subjects with stage 1 hypertension, a randomized controlled trial. Nutr. J. 9, 52-58.

CODEX STAN 243–2003: standard for fermented milks. Available at http://www.fao.org/docrep/015/i2085e/i2085e00.pdf (2003).

Cicero, A.F.G., Aubin, F., Azais-Braesco, V., Borghi , C., 2013. Do the lactotripeptides isoleucine–proline–proline and valine–proline–proline reduce systolic blood pressure in European subjects? A meta-analysis of randomized controlled trials. Am. J. Hypertens. 26, 442-449.

Corcoran, B.M., Stanton, C., Fitzgerald, G., and Ross, R.P., 2008. Life under stress: The probiotic stress response and how it may be manipulated. Curr. Pharm. Des. 14, 1382-1399.

Coeuret, V., Gueguen, M., Vernoux, J.P., 2004.Numbers and strains of lactobacilli in some probiotic products. Int. J. Food Microbiol. 97, 147-156.

de Souza Oliveira, R.P., Perego, P., Converti, A., de Oliveira, M.N., 2009. Effect of inulin on growth and acidification performance of different probiotic bacteria in co-cultures and mixed culture with *Streptococcus thermophiles*. J. Food Eng. 91, 133-139.

del Campo, R., Bravo, D., Canton, R., Ruiz-Garbajosa, P., Garcia-Albiach, R., Montesi-Libois, A., Yuste, F.J., Abraira, V., Baquero, F., 2005. Scarce evidence of yogurt lactic acid bacteria in human feces after daily yogurt consumption by healthy volunteers. Appl. Environ. Microbiol. 71, 547-549.

Delorme, C., Bartholini, C., Bolotine, A., Ehrlich, S.D., Renault, P., 2010. Emergence of a cell wall protease in the *Streptococcus thermophilus* population. Appl. Environ. Microbiol. 76, 451–460.

Dei Più, L., Tassoni, A., Serrazanetti, D. I., Ferri, M., Babini, E., Tagliazucchi, D., Gianotti, E., 2014. Exploitation of starch industry liquid by-product to produce bioactive peptides from rice hydrolyzed proteins. Food Chem. 155, 199-206.

Donkor, O.N., Henriksson, A., Vasiljevic, T., Shah, N. P., 2005. Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yogurt. J. Food Sci. 70, m375–m381.

Donkor, O.N., Henriksson, A., Vasiljevic, T., Shah, N.P., 2006. Effect of acidification on the activity of probiotics in yoghurt during cold storage. Int. Dairy J. 16, 1181-1189.

Donkor, O.N., Henriksson, A., Singh, T.K., Vasiljevic, T., Shah, N.P., 2007. ACE-inhibitory activity of probiotic yoghurt. Int. Dairy J. 17, 1321-1331.

FAO/WHO, 2006. Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation. FAO Food and Nutrition Paper World Health Organization and Food and Agriculture Organization of the United Nations.

FAO/WHO, 2011. Codex Alimentarius: Codex standards for fermented milks 243-2003, in World Health Organization, Food and Agriculture Organization of the United Nations (eds), Milk and Milk products, 2nd Edition Rome, pp. 6-16.

Foltz, M., Cerstiaens, A., van Meensel, A., Mols, R., van der Pijl, P.C., Duchateau, G.S.M.J.E., Augustijin, P., 2008. The angiotensin converting enzyme inhibitory tripeptides Ile-Pro-Pro and Val-Pro-Pro show increasing permeabilities with increasing physiological relevance of absorption models. Peptides 29, 1312–1320.

Granato, D., Branco, G.F., Cruz, A.G., Faria, J.A.F., and Shah, N.P., 2010. Probiotic dairy products as functional foods. Comp. Rev. Food Sci. Food Saf. 9, 455-470.

Guarner, F., Perdigon G., Corthier, G., Salminen, S., Koletzko, B., Morelli L., 2005. Should yoghurt cultures be considered probiotic? Br J. Nutr. 93, 783–786.

Huys, M. Vancanneyt, K. D'Haene, V. Vankerckhoven, H. Goossens, Swings, J., 2006. Accuracy of species identity of commercial bacterial cultures intended for probiotic or nutritional use. Res. Microbiol. 157, 803-810.

Kanmani, P., Satish Kumar, R., Yuvaraja, N., Paaria, K.A., Pattukumara, V., Arula, V., 2013. Probiotics and its functionally valuable products—a Review. Crit Rev Food Sci Nutr. 53, 641-658.

Kilpi, E., Kahala, M., Steele, J.M., Pihlanto, A., Joutsjoki, V., 2007. Angiotensin I-converting enzyme inhibitory activity in milk fermented by wild-type and peptidase-deletion derivatives of *Lactobacillus helveticus* CNRZ32. Int. Dairy J. 17, 976-984.

Lin, W.H., Hwang, C.F., Chen, L.W., Tsen, H.Y., 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microbiol. 23, 74-81.

Lòpez-Fandiño, R., Otte, J., Van Camp, J., 2006. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. Int. Dairy J. 16, 1277-1293.

Lourens-Hattingh, A., Viljoen, B.C., 2001. Yogurt as probiotic carrier food. Int. Dairy J. 11, 1-11.

Milesi, M.M., Vinderola, G., Sabbag, N., Meinardi, C.A., Hynes, E., 2009. Influence on cheese proteolysis and sensory characteristics of non-starter lactobacilli strains with probiotic potential. Food Res. Int. 42, 1186-1196.

Nakamura, T., Mizutani, J., Ohki, K., Yamada, K., Yamamoto, K., Takeshi, M., Takazawa, M., 2011. Casein hydrolysate containing Val-Pro-Pro and Ile-Pro-Pro improves central blood pressure and arterial stiffness in hypertensive subjects: A randomized, double-blind, placebo-controlled trial. Atherosclerosis 219, 298–303.

Nighswonger, B.D., Brashears, M.M., Gilliland, S.E., 1996. Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage. J. Dairy Sci. 79, 212-219.

Papadimitriou, C.G., Vafopoulou-Mastrojiannaki, A., Silva, S.V., Gomes, A.M., Malcata, F.X., Alichanidis E., 2007. Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity. Food Chem. 105, 647–656.

Pihlanto, A., Virtanen, T., Korhonen, H., 2010. Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. Int. Dairy J. 20, 3–10.

Pihlanto, A., Korhonen, H., 2014. Bioactive peptides from fermented food and health promotion, in Holzapfel, W., Advances in fermented foods and beverages: improving quality, technologies and health benefits. Elsevier, Cambridge, pp 39-74.

Ramchandran, L., Shah, N.P, 2007. Characterization of functional, biochemical and textural properties of synbiotic low-fat yogurts during refrigerated storage. LWT-Food Sci. Technol. 43, 819-827.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical action decolorization assay. Free Rad. Biol. Med. 26, 1231–1237.

Rojas-Ronquillo, R., Cruz-Guerrero, A., Flores-Nájera, A., Rodríguez-Serrano, G., Gómez-Ruis, L., Reyes-Grajeda, J.P., et al., 2012. Antithrombotic and angiotensin-converting enzyme inhibitory properties of peptides released from bovine casein by *Lactobacillus casei* Shirota. Int. Dairy J. 26, 147–154.

Ronca-Testoni, S. 1983. Direct spectrophotometric assay for angiotensin-converting enzyme in serum. Clin. Chem. 29, 1093–1096.

Sah, B.N.P., Vasiljevic, T., McKechnie, S., Donkor, O.N., 2014. Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. Food Chem. 156, 264-270.

Sah, B.N.P., Vasiljevic, T., McKechnie, S., Donkor, O.N., 2015. Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. J. Dairy Sci. 98, 5905-5916.

Sarmadi, B. H., Ismail, A., 2010. Antioxidative peptides from food proteins: A review. Peptides, 31, 1949–1956.

Saxelin, M:, Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., et al., 2010. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. Int. J. Food Microbiol. 144, 293-300.

Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol. 27, 691–697.

Sieuwerts, S., de Bok, F.A., Hugenholtz, J., van Hylckama Vlieg, J.E., 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. Appl. Environ Microbiol. 74, 4997-5007.

Smug, L.N., Salaminen, S., Snaders, Ebner, M.E., 2014. Yoghurt and probiotic bacteria in dietary guidelines of the members states of the European Union. Benef. Microbes 5, 61-66.

Solieri, L., Bianchi, A., Giudici, P., 2012. Inventory of non starter lactic acid bacteria from ripened Parmigiano Reggiano cheese as assessed by a culture dependent multiphasic approach. Syst Appl Microbiol. 35, 270-277.

Solieri, L., Bianchi, A., Mottolese, G., Lemmetti, F., Giudici, P., 2014. Tailoring the probiotic potential of non-starter Lactobacillus strains from ripened Parmigiano Reggiano cheese by in vitro screening and principal component analysis. Food Microbiol. 38, 240-249.

Solieri, L., Rutella, G.S., Tagliazucch,i D., 2015. Impact of non-starter lactobacilli on release of peptides with angiotensin-converting enzyme inhibitory and antioxidant activities during bovine milk fermentation. Food Microbiol. 51, 108-16.

Tamime, A.Y., Saarela, M., Søndergaard, A.K., Mistry, V.V., Shah N.P., 2005. Production and maintenance of viability of probiotic microorganisms in dairy products, in: Tamime, A.Y. (Ed.), Probiotic Dairy Products. Blackwell Publishing Ltd., Oxford, UK, pp. 39-72.

Vasiljevic, T., Shah, N.P., 2008. Probiotics – from Metchnikoff to bioactives. Int. Dairy J. 18, 714-728.

Vinderola, C.G., Mocchiutti, P., Reinheimer, J.A., 2002. Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. J. Dairy Sci. 85, 721-729.

Vinderola, C.G., Reinheimer, J.A., 2003. Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance. Food Res. Int.36, 895-904.

Virtanen, T., Pihlanto, A., Akkanen, S., Korhonen, H., 2006. Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. J. Appl. Microbiol. 102, 106-115.

Figure captions

Fig. 1. Experimental strategy for the preparation and characterization of bi-functional yogurts. Panel A represents the rationale underlined the development of bi-functional yogurt, which is enriched in VPP and IPP (stars) and, at the same time, is carrier for potential probiotic cells (circles) capable to survive under GI stress. Panel B details the experimental steps performed for preparing and characterizing bi-functional yogurts. Yogurt starter cultures *S. thermophilus* strain 99 and *L. bulgaricus* strain 1932 are generally represented as triangles, while *L. casei* PRA205 and *L. rhamnosus* PRA331 as circle and square, respectively. Abbreviation: NC, negative control.

Fig. 2. Changes in bacterial viable counts during set-yogurt fermentation and cold storage. Panels A and B represent *S. thermophilus* (triangles) and *L. bulgaricus* (diamonds) viable populations, expressed as CFU/g, in yogurts 1 (black line), 2 (gray line) and 3 (dotted black line) after 8 h of fermentation and during cold storage, respectively. Panel C represents *L. casei* PRA205 (circles) and *L. rhamnosus* PRA331 (squares) viable populations, expressed as CFU/g, in yogurts 2 (gray line) and 3 (dotted black line) after 8 h of fermentation and during cold storage. # means $P < 0.05$ respect to the previous time in each set-type yogurt; * means $P < 0.05$ respect to set-type yogurt 1 (control yogurt without probiotics).

Fig. 3. Proteolytic activity of TCA-soluble peptide fractions extracted from yogurts after 8 h of fermentation and during 28-day long cold storage. Proteolytic activity values over time are mean ($n \geq 2$) ± standard deviation (SD) and expressed as mmol/L leucine equivalents. Yogurts 1 (only yogurt starters), 2 (yogurt starters and probiotic PRA205) and 3 (yogurt starters and probiotic PRA331) are colored in black, white and gray, respectively. Bars with different letters are different from one another ($P < 0.05$) based on two-way ANOVA analysis of variance and subsequent Tukey's *post hoc* test; upper case letters, yogurt 1 (control without probiotics); lower case letters, yogurts 2 and 3 with probiotics. ** means $P < 0.01$, *** means $P < 0.005$ while **** $P < 0.0001$. # means $P < 0.05$ respect to the previous time in each set-type yogurt

Fig. 4. Antioxidant activity of TCA-soluble peptide fractions extracted from yogurts. Radical scavenging activity values over time are mean (n ≥ 2) ± standard deviation (SD) and expressed as μmol/L Trolox equivalents. Yogurts 1 (only yogurt starters), 2 (yogurt starter and probiotic PRA205) and 3 (yogurt starter and probiotic PRA205) are colored in black, white and gray, respectively. Bars with different letters are different from one another ($P < 0.05$) based on two-way ANOVA analysis of variance and subsequent Tukey's *post hoc* test; upper case letters, yogurt 1 (control without probiotics); lower case letters, yogurts 2 and 3 with probiotics. * means $P \leq 0.05$, while **** $P < 0.0001$. # means $P < 0.05$ respect to the previous time in each set-type yogurt.

Fig. 5. Angiotensin-converting enzyme inhibitory activity (ACEi) of TCA-soluble peptide fractions extracted from yogurts. The extent of *in vitro* ACE inhibitory activity was calculated over time as a percentage mean (n ≥ 2). Yogurts 1 (only yogurt starters), 2 (yogurt starters and probiotic PRA205) and 3 (yogurt starters and probiotic PRA331) are colored in black, white and gray, respectively. Bars with different letters are different from one another ($P < 0.05$) based on two-way ANOVA analysis of variance and subsequent Tukey's *post hoc* test; upper case letters, yogurt 1 (control without probiotics); lower case letters, yogurts 2 and 3 with probiotics. *** means $P \leq 0.005$, while **** $P < 0.0001$. # means $P < 0.05$ respect to the previous time in each set-type yogurt.

Fig.6. Concentrations of lactotripeptides VPP (panel A) and IPP (panel B) in refrigerated yogurts. TCA soluble fractions were extracted from starter yogurt 1 (black bars) and probiotic yogurts 2 (white bars) and 3 (gray bars) after 3, 14 and 28 days of cold storage. Values (μg/L) are means of at least two independent replicates ± standard deviation (SD). Bars with different letters are different from one another ($P < 0.05$) based on two-way ANOVA analysis of variance and subsequent Tukey's *post hoc* test; upper case letters, yogurt 1 (control without probiotics); lower case letters, probiotic yogurts 2 and 3. *** means $P < 0.005$, while **** $P < 0.0001$.

Supporting Information

Table S1. Correlation curves of optical density (OD) *versus* cell count (CFU/mL) and conversion factors for each bacterial strain.

Fig. S1. Proteolytic activities of mono-cultures during milk fermentation. Proteolytic activity (expressed as mmol/L of leucine equivalents) is measured during milk fermentation with *S. thermophilus* 99 (black triangle), *L. bulgaricus* 1932 (empty triangle), *L. casei* PRA205 (black circles) and *L. rhamnosus* PRA331 (black squares).

Fig. S2. Yogurt acidification kinetics. Triangles represent yogurt 1 (starter cultures *S. thermophilus* 99 and *L. bulgaricus* 1932), whereas circles and squares yogurt 2 (starter cultures *S. thermophilus* 99 and *L. bulgaricus* 1932 and probiotic strain *L. casei* PRA205) and yogurts 3 (starter cultures *S. thermophilus* 99 and *L. bulgaricus* 1932 and probiotic strain *L. rhamnosus* PRA331), respectively. Data are represented by the mean (n = 3); error bars (when visible) show standard deviation. * means $P < 0.05$ respect to the yogurt 1 used as control. Non-inoculated yogurt maintained pH of 6.53 ± 0.2 for all the fermentation time and was omitted from the figure.

Table S1.

Strain	**Correlation curve**	**Conversion factor OD_{600}=1 (CFU/mL)**
St. thermophilus 99	$y=6*10^8x + 5\cdot10^6$	$6.05\cdot10^8$
L. delbrueckii subsp. *bulgaricus* 1932	$y=3*10^7x-6\cdot10^6$	$2.4\cdot10^7$
L. casei PRA205	$y=8*10^7x-6\cdot10^6$	$7.4\cdot10^7$
L. rhamnosus PRA331	$y=5*10^7x - 2\cdot10^6$	$4.8\cdot10^7$

Figure 1

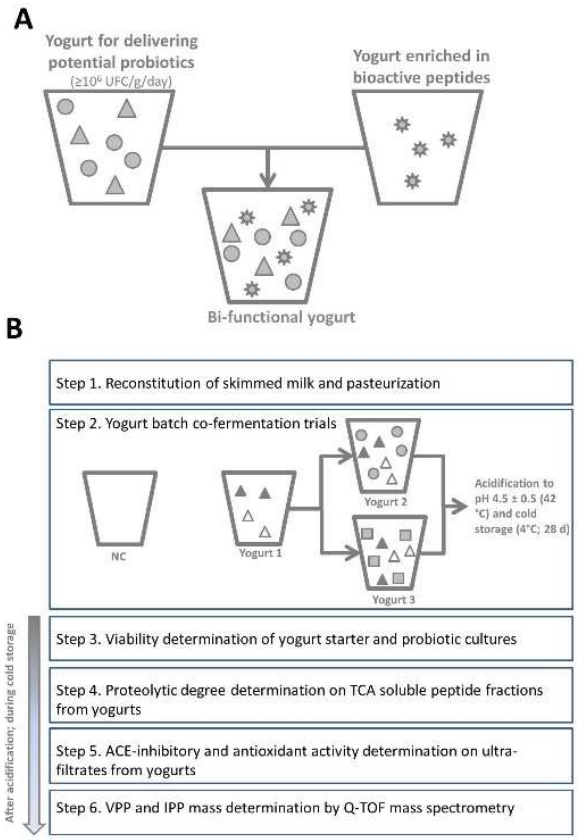

A
Yogurt for delivering potential probiotics
(≥10⁶ UFC/g/day)
Yogurt enriched in bioactive peptides
Bi-functional yogurt
B
Step 1. Reconstitution of skimmed milk and pasteurization
Step 2. Yogurt batch co-fermentation trials
NC
Yogurt 1
Yogurt 2
Yogurt 3
Acidification to pH 4.5 ± 0.5 (42 °C) and cold storage (4°C; 28 d)
After acidification; during cold storage
Step 3. Viability determination of yogurt starter and probiotic cultures
Step 4. Proteolytic degree determination on TCA soluble peptide fractions from yogurts
Step 5. ACE-inhibitory and antioxidant activity determination on ultra-filtrates from yogurts
Step 6. VPP and IPP mass determination by Q-TOF mass spectrometry

Figure 2

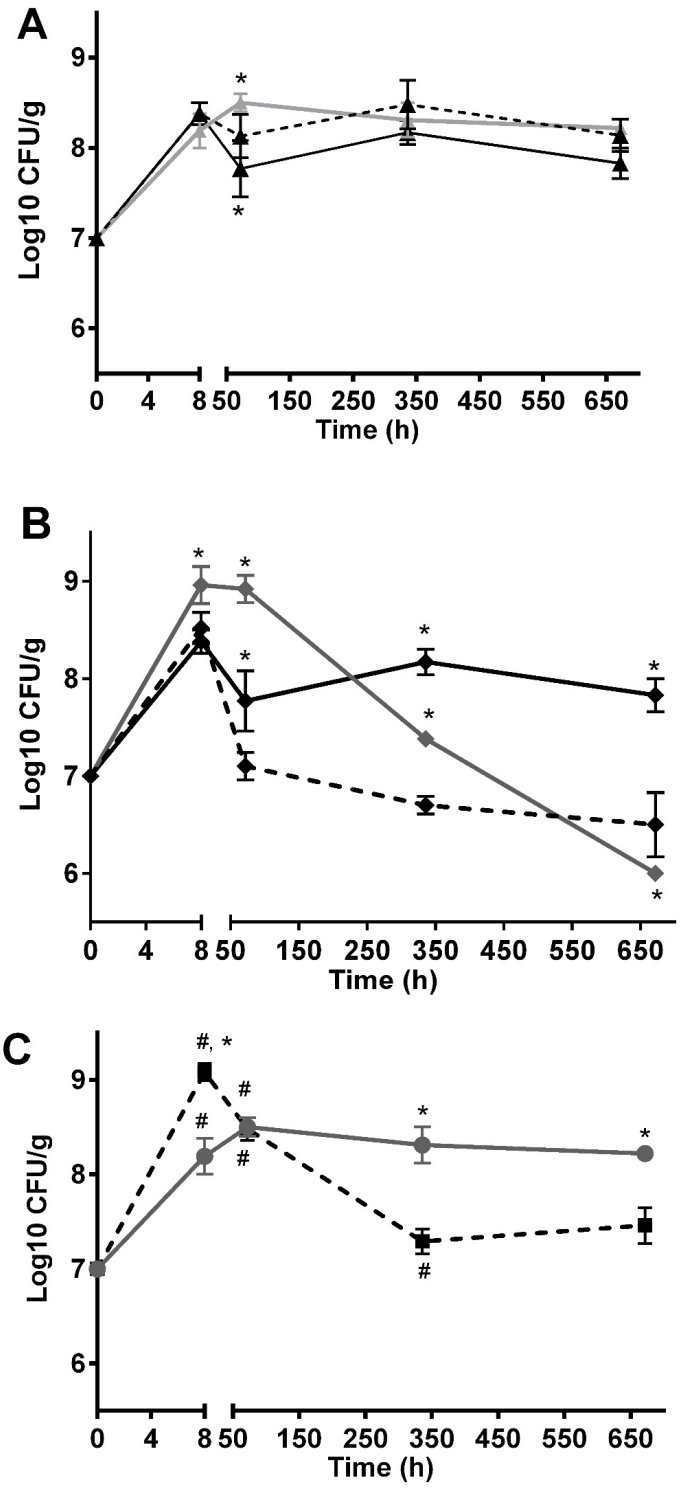
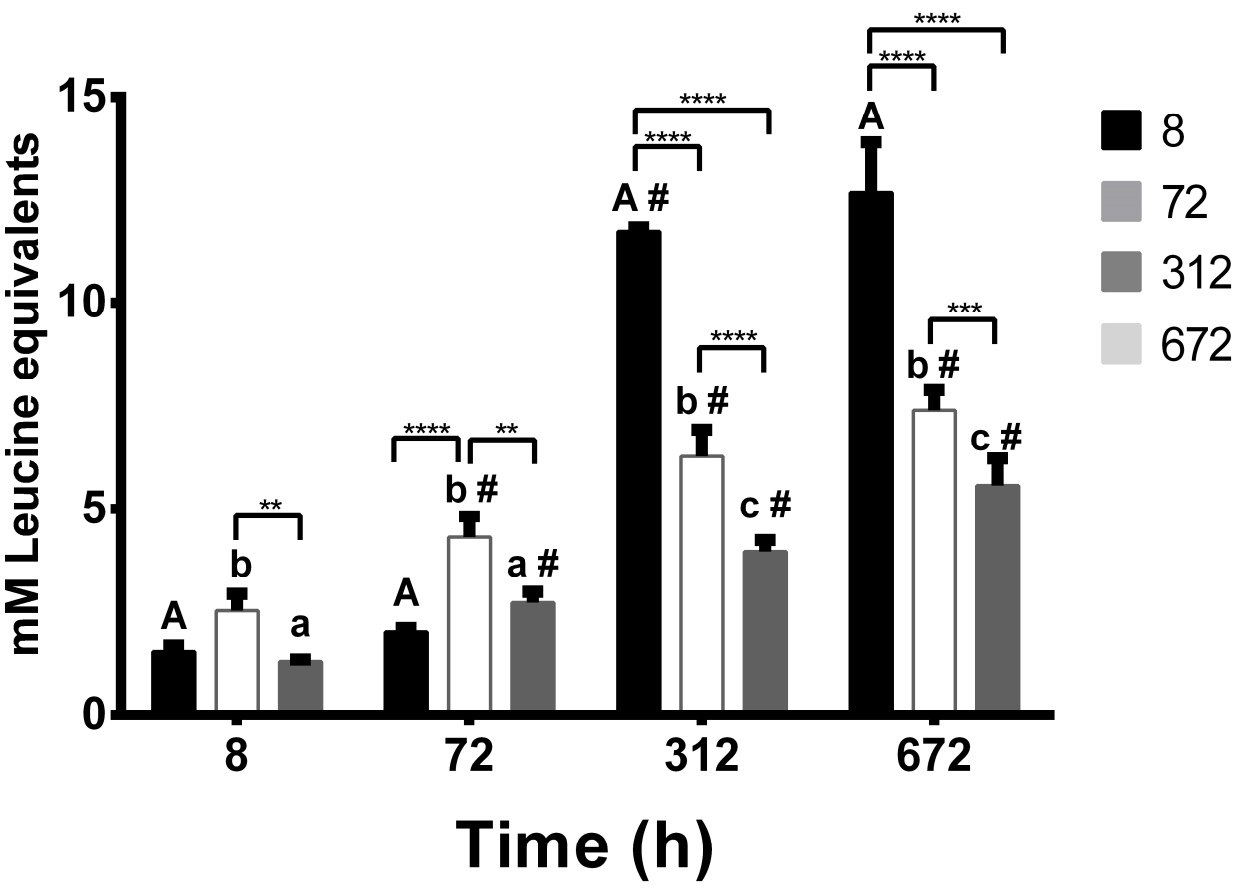

A
Log10 CFU/g
9
8
7
6
0 4 8 50 150 250 350 450 550 650
Time (h)
B
Log10 CFU/g
9
8
7
6
0 4 8 50 150 250 350 450 550 650
Time (h)
C
Log10 CFU/g
9
8
7
6
0 4 8 50 150 250 350 450 550 650
Time (h)

Figure 3


mM Leucine equivalents
15
10
5
0
8
72
312
672
Time (h)
A
b
a
A
b #
a #
A #
b #
c #
A
b #
c #
**
****
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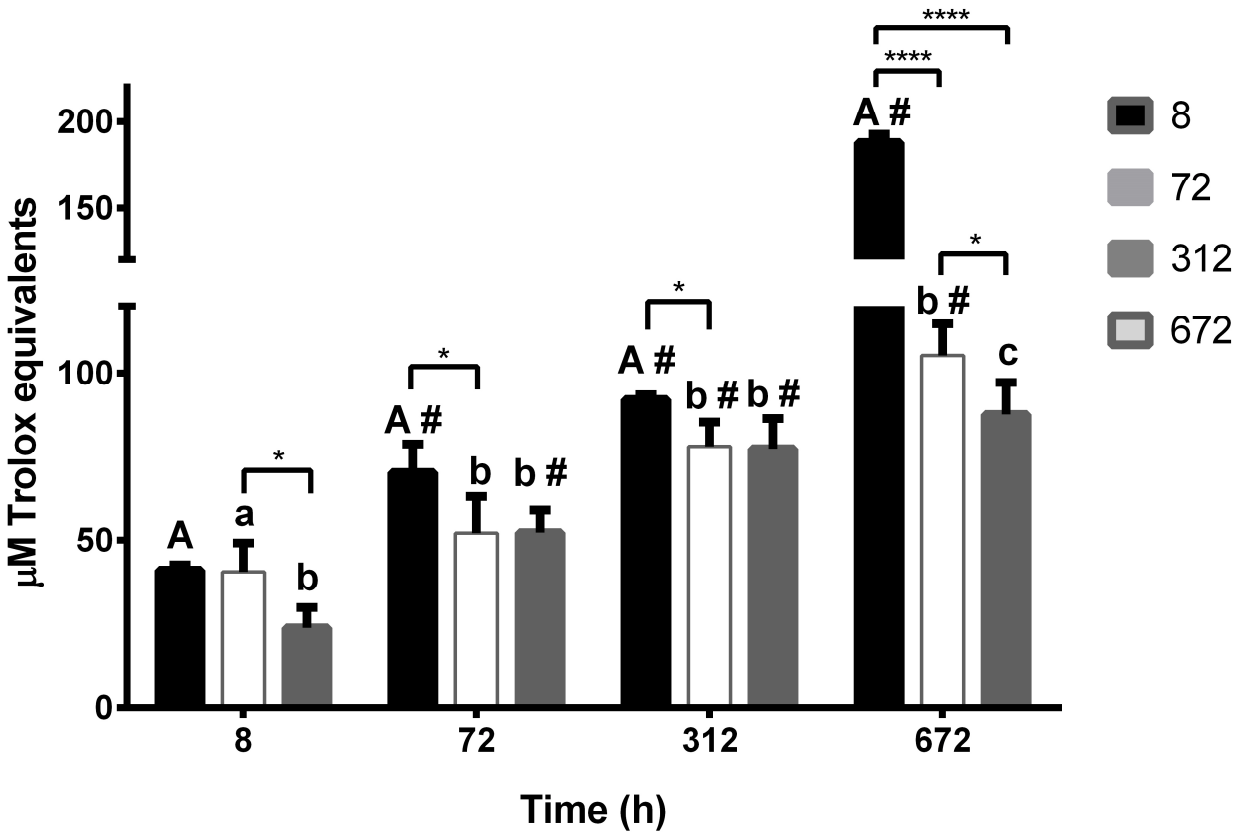
***
8
72
312
672

Figure 4


µM Trolox equivalents
200
150
100
50
0
A
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b
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A #
b #
c
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****
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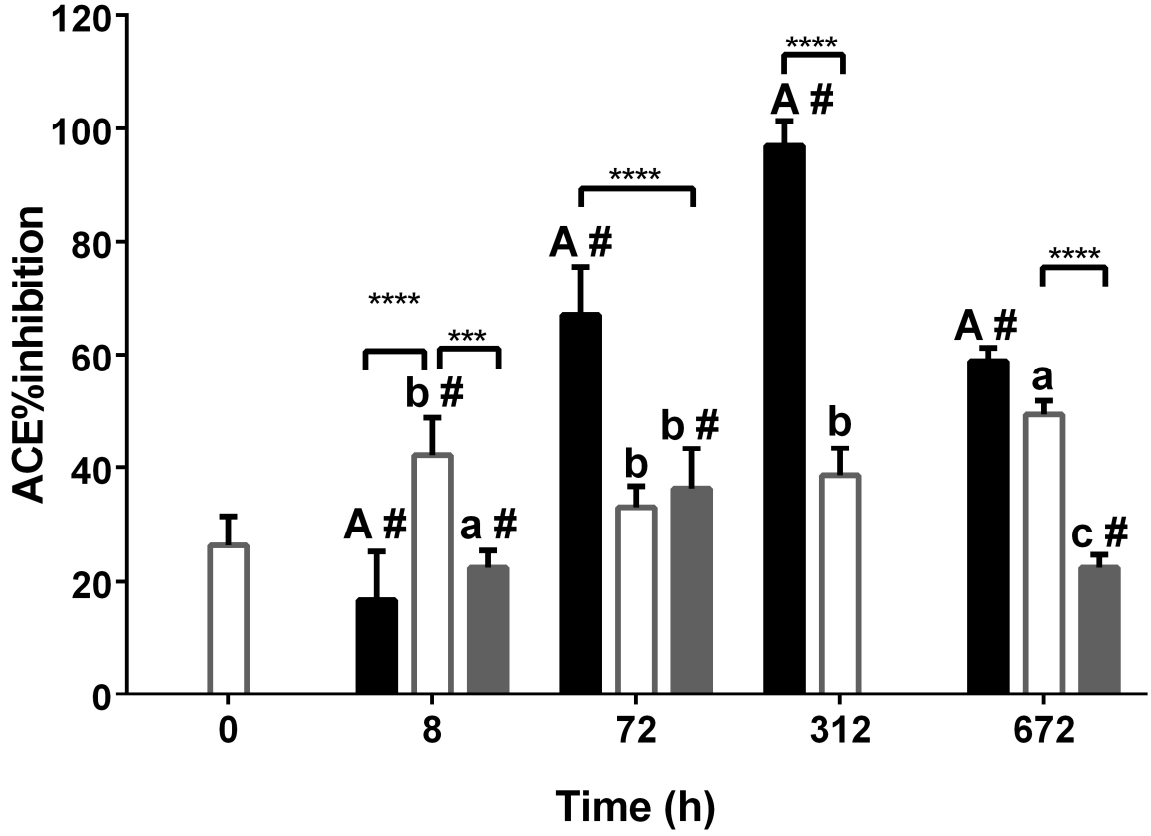
8
72
312
672
Time (h)

Figure 5


ACE%inhibition
120
100
80
60
40
20
0
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A #
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b #
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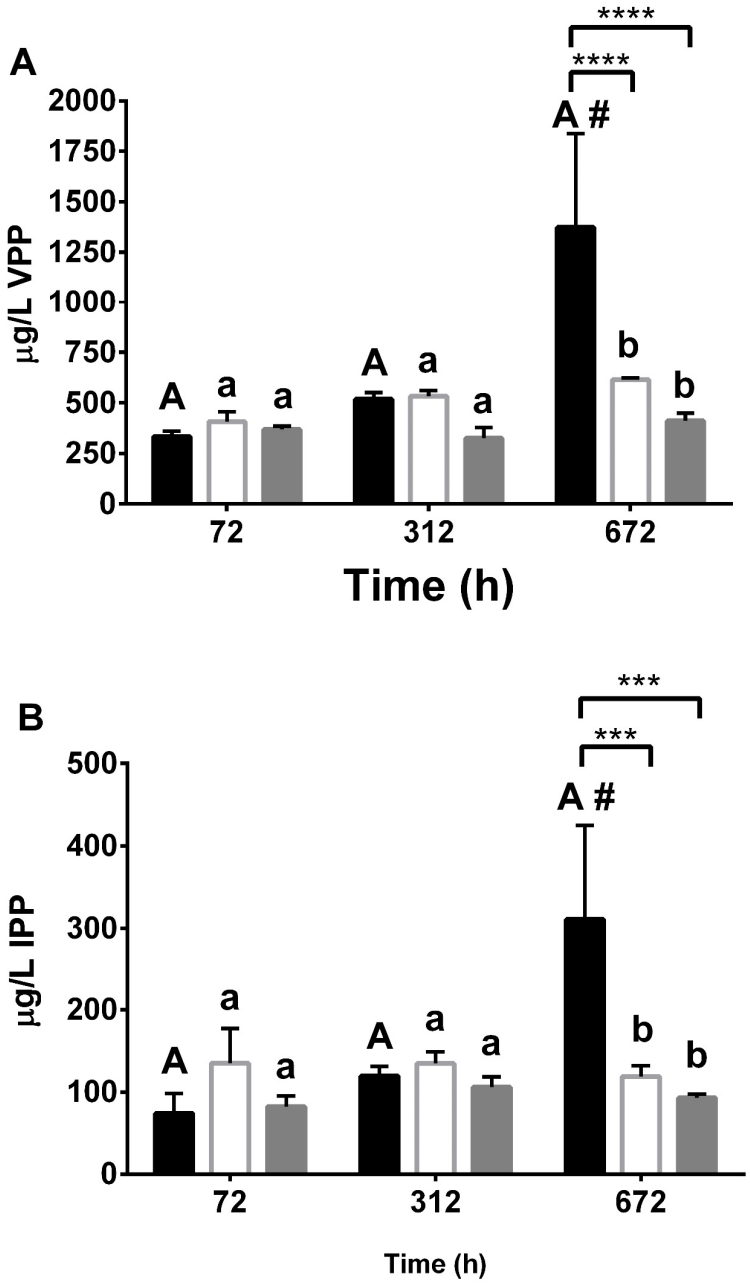
a
c #
0
8
72
312
672
Time (h)

Figure 6


A
μg/L VPP
2000
1750
1500
1250
1000
750
500
250
0
****
****
A #
b
b
A
a
a
A
a
a
72
312
672
Time (h)
B
μg/L IPP
500
400
300
200
100
0
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A #
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A
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672
Time (h)

Figure S1

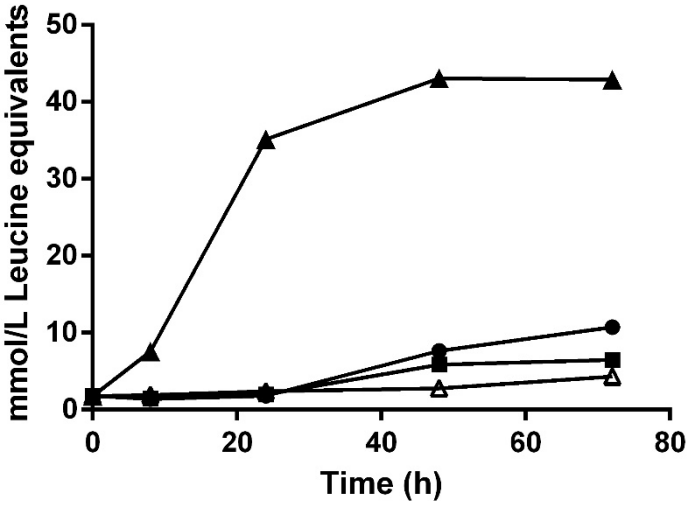

Figure S2