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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.

1. Introduction

Probiotics are live micro-organisms that, when administered in adequate amounts, confer health benefits on the host (FAO/WHO, 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

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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 *Lactobacillus 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 described 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×*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,