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Olive paste as vehicle for delivery of potential probiotic *Lactobacillus plantarum* 33

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Abstract

Use of probiotic bacteria and consumes in large − in novel foods to provide beneficial health effects has attracted an increasing interest by the food industry and fermented olives are an excellent example of a new generation of those foods from plant origin so as to assure maximum viability by the time of ingestion during processing and storage of food products, as well as during transit through the gastrointestinal tract.

Our study focused on production, characterization and assessment of efficacy of microencapsulation upon survival of probiotic strains and sensory properties of the final olive paste throughout refrigerated storage. Microencapsulation appears to be an effective technique for strain survival, depending on the operating temperature and experimental results on tolerance to gastrointestinal-like conditions, and ability to adhere to intestinal epithelium is thereby presented and discussed. The sensory panel rated all experienced matrices as good, including over- all acceptance without significant preference between them. However, the success of microencapsulation was more limited when incorporated into olive paste. Free cells of *Lactobacillus plantarum* 33 proved able to survive in olive paste during storage at refrigerated temperatures.

1. Introduction

An olive is a chief product consumed in fermented state, and is probably the most popular fermented food in the Mediterranean basin. The increasing consumer demand for foods bearing health benefits has led the food industry to diversify its portfolio. Therefore, efforts aimed at adding probiotic functionality to olives are in order – and likely to rein- force their health-favourable image – in unsaturated oil and antioxidant compounds (Peres, Peres, Hernández-Mendoza, & Malcata, 2012). Previous studies of probiotic strains in fermented olives have unfolded suitability of table olives as vehicle for probiotic strain (Lavermicocca et al., 2005) – and international patents covering these aspects have meanwhile been issued (Lavermicocca et al., 2007), under a new trend of plant matrices for delivery of probiotic traits.

An effective carrier system is mandatory so as to protect probiotics from the unfavorable conditions prevailing during preceding ingestion, and digestion afterwards; microencapsulation has arisen as a useful tool as it permits entrapment of live probiotic bacteria and consequent protection from external adverse environments (Susanna & Pirjo, 2010). Cells are accordingly retained within an encapsulating matrix or membrane, and this improves viability in food products and intestinal tract (Doleyres & Lacroix, 2005). Alginate added with starch has been found to protect for instance, plain probiotics against the low pH of gastric juice (Ding & Shah, 2007). Combination of calcium alginate with such prebiotic as starch not only increases microparticle stability, but also improves viability of probiotics – and allows integrated structures to be attained in capsules (Mirzaei, Pourjafar, & Homayouni, 2012) as a consequence of curbing diffusion of calcium ions outside of capsules, while protecting bacterial cells against adverse pH condition (Kailasapathy, 2006); corn starch offers indeed an ideal surface for adherence of probiotics through transit in the upper gastrointestinal tract (GIT), and promotes robustness and resilience to environmental stresses (Sultana et al., 2000). However, the microparticulate system must provide sufficient permeability for nutrients to pass through, while preventing the entry of hostile molecules that might destroy the encapsulated (live) bacterial cells (Rihova, 2000).

To be labelled as probiotic a food must carry specific probiotic strains at suitable viability levels (10^6-10^7 CFU/mL) through the whole shelf- life (Chávez & Ledeboer, 2007; Doleyres & Lacroix, 2005; Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). Daily, intake of probiotics at of 10^8-10^9 viable cells (Lopez-Rubio, Gavara, & Lagaron, 2006; Lourens-Hattingh & Viljoen, 2001), corresponding to 100 mL food portions at 10^6-10^7 CFU/mL (Doleyres & Lacroix, 2005) is recommended to maximize the beneficial health effects (Charalampopoulos, Pandiella, & Webb, 2002).

Besides yoghourts, probiotics have been inoculated to various food matrices, e.g., sausage (Muthukumarasamy & Holley, 2006), chocolate (Possemiers, Marzorati, Verstraete, & van de Wiele, 2010), ice cream (Homayouni, Ehsani, Azizi, Yarmand, & Razavi, 2009), juices (Luckow & Delahunty, 2004), cream-filled cake (Zanjani et al.,

2012), cheese (Kalavrouzioti, Hatzikamari, Litopoulou-Tzanetaki, & Tzanetakis, 2005; Madureira et al., 2008) and mayonnaise (Fahimdanesh et al., 2012); however, survival of free (or microencapsulated) probiotics in olive paste has not yet been reported. Hence, the aim of this study was to investigate the survival during storage at room and refrigerated temperatures of free and microencapsulated probiotic strains obtained from olive brines in the first place, but also to ascertain their tolerance to GIT conditions and ability to adhere to intestinal epithelium. This search ultimately envisages a better understanding of regulation of probiotic efficacy in olive matrices, in both free and microencapsulated forms.

2. Materials and methods

2.1. *Bacterial strains and cell and growth culture, media and conditions*

A potential probiotic strain, *Lactobacillus plantarum* 33 (LP33), previously isolated from Portuguese olive fermented brines, was obtained from Food Microbial Technology Laboratory (ITQB collection). The culture was activated and maintained in de Man, Rogosa and Sharpe (MRS) broth (pH 6.2, Merck, Darmstadt, Germany). Initiating the experiments, two subcultures in MRS broth with 1% (v/v) inoculum, and incubation at 37 °C for 24h and 16 h, were performed. For bacterial enumeration, plates with MRS agar medium and incubation at 37 °C for 48 h were employed. A probiotic strain, *Lactobacillus casei* Shirota (LCS, ACA-DC 6002), kindly provided by Laboratory of Microbiology and Biotechnology of Food (Agricultural University of Athens, Iera Odos, Greece) was used as reference.

The Caco-2 human colon adenocarcinoma cell line ACC169 (DSMZ collection, Germany) was used for adhesion assays, and was provided by the Animal Cell Technology Team (IBET, Oeiras, Portugal).

2.2. *Cell microencapsulation technique*

Bacterial strains were aseptically microencapsulated according to the extrusion method described by Jayalalitha, Balasundaram, and Palanidorai (2012) with modifications. Cell suspensions were accordingly prepared by centrifugating at 5000 ×*g* for 15 min 30 mL of 16 h-old culture, and then washed twice with phosphate-buffered saline solution (PBS). Finally, bacterial pellets were resuspended in 20 mL of sterile PBS. Cell suspension (ca. 10^9 CFU/mL) was then mixed with 60 mL of sodium alginate (2% w/v, VWR, Leuven, Belgium) plus Hi-maize® resistant starch (0.5% w/v, Laborspirit, Portugal), previously sterilized at 121 °C for 15 min. The mixture was dripped using a peristaltic pump (model 7518-00, Masterflex), set at flow rate 200 mL/min into a bath containing 100 mL of a sterile 0.1 M $CaCl_2$ solution (Sigma-Aldrich, Taufkirchen, Germany), under gentle stirring with a magnetic bar. The micro-beads were retained in the $CaCl_2$ solution for 30 min, and then rinsed with sterile distilled water.

Microencapsulated samples are coded by P – protected and free cells by NP – non-protected.

2.3. *Capsule morphology and particle size*

An inverted light microscope (Motik AE31, Wetzlar, Germany), coupled with a digital colour camera (Moticam® 5-mP), was used to de- termine surface morphology and particle size of microcapsules. Image acquisition, annotation and data analysis were performed using the Motic Images Plus 2.0.23 capture software, and objective 10 ×/0.25 (CCIS® Plan Achromatic) as hardware. Size distribution and mean size were calculated by sampling yield and stability of entrapment 30 beads.

2.4. *Releasing and cell quantification*

For quantification of entrapped cells, beads (1 g) were liquefied in 99 mL of 1% (w/v) sterile sodium citrate solution (Merck, KGaA, Germany), at pH 6.0 and room temperature, for 15 min. For capsule depolymerisation a blender homogenizer (Stomacher Circulator 400, Seward Medical, London, UK) set at 360 rpm and operated for 3 min, was used. After disintegration, viable probiotic bacteria were released and quantified. Enumeration was conducted on each duplicate sample of three independent assays, at the start of the assay and during the storage period. Both free and entrapped cells were plated on MRS.

2.5. *Survival of encapsulated cells during storage*

To determine stability of microencapsulated cells during storage, 1 g of microparticles was placed into polyethylene tubes and stored at 4 and 22 °C for 30 d. Additionally, a blank assay (control) consisting of bacteria suspended into alginate and corn starch solution was performed. At specified 3 d intervals, samples were withdrawn, and cell survival rates were determined using a standard plating technique.

2.6. *Resistance to heat shock*

Resistance to heat stress was assessed as proposed by Sabikhi, Babu, Thompkinson, and Kapila (2010). Hence, 1 g of microencapsulated culture was transferred to 10 mL of sterile distilled water (pH 6.4 ± 0.2) in thin walled test tubes. The contents were subjected to three different thermal conditions (72, 85, and 90 °C) for 30 s, and immediately cooled by immersing in ice for 10 min. The lysates were centrifuged at 2200 ×*g* for 10 min, and free and encapsulated cells were enumerated by plating on MRS.

2.7. *Effect of relative humidity upon survival*

The moisture retention of microcapsules was determined via the static method,

based on saturated salt solutions of various concentrations and able to generate specific equilibrium relative humidity (RH) in a closed chamber (Li, Chen, Cha, Park, & Liu, 2009). Four different salts [$MgCl_2$, $Mg(NO_3)_2$, NaCl and $BaCl_2$] (Merck KGaA, Darmstadt, Germany) were accordingly used to provide relative humidities of 32.9, 53.6, 76.5 and 97%, respectively. Before starting assays, saturated salt solutions were maintained in vacuum desiccators for 24 h at 25 °C, allowing water vapour to reach equilibrium. Then, 1 g of *Lactobacillus*-loaded microcapsules, at different RHs, was held in the appropriate chamber for 15 d. Cells were enumerated on MRS after bead depolymerisation.

2.8. *Effect of simulated gastrointestinal conditions upon survival*

Free (NP) and microencapsulated (P) *Lactobacillus* cells, previously stored at refrigeration conditions for 30 d, were exposed to a simulated gastrointestinal tract. A continuous model was used to simulate the whole digestion path, including peristaltic movements (Peres et al., 2014). Briefly, gastric-stress was stimulated by haring cells (at an initial inoculum of 10^8 CFU/g) exposed to lysozyme and pepsin at pH 5.0 at initiation, which was gradually decreased to 4.1, 3.0, 2.1 and 1.8 by adding 1 M HCl (Merck, Germany) to the cell suspension. To simulate intestinal stress, recovered cells were adjusted to pH 6.5, and then treated with porcine bile salts and pancreatin (Sigma-Aldrich, USA). To mechanically mimic the cyclic movements, cyclic suction was applied to tubing connected to the controlled vacuum chambers, thus facilitating study of mechanoregulation of intestinal function. A cyclic stretching regimen (0.85 cm/min) was used to approximate the mechanical microenvironment experienced by human epithelial cells in the gut. After 160 min of exposure to dynamic stress, bacteria were enumerated by pour-plating method on MRS following depolymerization.

2.9. *Ability to adhere to intestinal epithelium*

Adhesion assays of the microencapsulated and free bacteria, previously submitted to simulated digestion, were carried out according to Peres et al. (2014). Briefly, for adhesion of entrapped cells, beads (1 g) were released as described in Section 2.4. After simulated digestion, free and encapsulated cells were harvested by centrifugation, and washed twice with 5 mL PBS buffer. The final inoculum concentration was 10^8 CFU/mL (after simulated digestion). An aliquot of adjusted suspension (2 mL) was centrifuged, and recovered cells were then re-suspended in 1.0 mL RPMI-1640 medium (without serum and antibiotics) and added to each well containing Caco-2 monolayer; co-incubation was performed under 5% CO_2/95% air at 37 °C for 60 min. After incubation, the medium was removed and the monolayer washed three times with sterile PBS (1 mL). The cells were detached by adding 2 mL of 0.05% (w/v) Trypsin–EDTA solution to each well, and then incubated at 37 °C for 10 min. The bacterial cell suspension obtained was plated on MRS agar, by serial decimal

dilution, to assess the number of adherent bacterial cells; the plates were incubated at 37 °C for 24–48 h, and colonies were counted thereafter. The bacterial cells initially added to each well of the 6-well plates (9.5 cm^2; Falcon Microtest™, Becton Dickinson, Franklin Lakes NJ, EUA) were also counted. The efficiency (%) of adhesion was expressed as ratio of viable cells remaining adherent to Caco-2 enterocytes to bacteria added per well (Candela et al., 2008).

2.10. *Design and characterization of probiotic olive paste*

The olive paste was prepared by resorting the following recipe: green natural fermented pitted olives from Portuguese 'Azeiteira' variety; extra virgin olive oil 15% (w/v); wine vinegar 5% (w/v); and minced garlic 5% (w/v) were used. All ingredients were homogenized in a homemade blender for a few seconds until smooth (the paste might be slightly granular). After blended, paste was heating to boil during 20 min using a temperature-controlled water bath. Then mixture was cooled quickly in an ice bath. After thermal treatment, selected strain

L. plantarum 33 or *L. casei* Shirota were aseptically added as free or encapsulated cells (10% w/w) followed by homogenisation and storage at 4 °C for 4 weeks. Three samples for chemical characterization were collected.

2.10.1. *Survival of entrapped and free cells in olive paste storage*

During the 30 d-storage period, samples were aseptically removed every 3 d from each flask, and analysed for viable counts of *L. plantarum* 33 and *L. casei* Shirota. For enumeration, 1 g of paste was resuspended in 99 mL 1% (w/v) sterile sodium citrate (pH 6), and incubated at 37 °C for 10 min – followed by homogenization step using a stomacher at room temperature, at 230 rpm for 3 min. The counts (CFU/g) were obtained by standard plating techniques.

2.10.2. *Changes of acidity in olive paste during storage*

The pH value was determined using a Microph 2002 potentiometer (Crison, Barcelona, Spain), coupled with an electrode for solid products. Total acidity was determined by titration with a solution of 0.1 M NaOH (Merck, Germany), using phenolphthalein as pH indicator. Briefly, samples (10 g) of olive paste were mixed with 50 mL of ethanol/ ether solution (1:1), kept at room temperature for 30 min, and filtered; 10 mL (V) of filtrate was then titrated. Acidity is expressed as lactic acid percent, and was calculated as follows (NP-1421 (1977):

$$\%\text{Acidity} = [(0.1 \times V \times 0.9)/20] \times 100.$$

2.10.3. *Changes of colour in olive paste during storage*

The Commission Internationale de l´Eclairage (CIE) L*a*b* system for colour analysis was used to determine colour changes of olive paste along storage time (Afshari-Jouybari & Farahnaky, 2011; Girolami, Napolitano, Faraone, & Braghieri, 2013; Yam

& Papadakis, 2004); a wooden box with internal black opaque walls was accordingly designed. Image acquisition was performed under controlled conditions,i.e. illumination, distance between camera and sample, colour cards, camera angle and light source. The lightning system consisted of low voltage halogen lamps (60 cm in length), with a reflector that provided uniform illumination of an area with a colour temperature of 4000 K. Lamps were located 50 cm above the sample, at a 45° angle; photo- graphs were taken at a shutter speed of ISO-800 – and a maximum aperture of 3.61328125 using a high-resolution digital camera (Olympus Digital Camera model E-510) with 30 cm distance between sample (olive paste) and camera lens. The camera angle was adjusted to minimise the reflective surface. Additionally, the camera was calibrated with a colour - and - white balance card (Prodisk 2/mini Model PD202). Pictures were analysed for L*a*b* values using an image editor software (Adobe Photoshop CS6).

To assess proper lighting within each image, white squares were used. The total colour difference between the samples from start and end storage was evaluated using the formula:

$$\Delta E\,Lab = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

2.10.4. *Sensory assessment of probiotic olive paste*

For sensory assessment, the sensory quantitative descriptive analysis adapted from IOOC method (IOOC, 2011) was used, with an unstructured scale; 100 mm was converted to numerical values (0-8 conventional units – c.u.). Descriptors were chosen from the IOOC method of table olives, and defined previously taking into account the new product characteristics. Sensory analysis was done in coded samples at the end of storage time (4 weeks). A volunteer panel, consisting of 9 experienced panellists for scoring table olives, assessed the (coded) olive paste samples in random order, and scored general appearance, colour, taste, texture, and granularity on a 9-point scale – where (9) is the best and (1) is the worst. On the basis of the aforementioned description, the sensory panel also indicated an overall acceptability (low-very high) for each sample on a separate scale. Three replicates of each sample were submitted to sensory analysis, and results were statistically analysed.

2.11. Statistical analyses

Statistical analysis of the experimental data was performed via one-way analysis of variance (ANOVA), using Statistical Package for Social Sciences software (v. 20: SPSS Chicago IL, USA). Experiments were conducted under sterile conditions, and performed in triplicate; three independent cultures of each bacterium were analysed as described; to search for significant differences between experiments, Tukey's test (at 5% level of significance) was employed.

3. Results and discussion

3.1. *Survival of encapsulated cells during storage*

The viability of probiotic bacteria is of paramount importance during shelf life of our food product, since minimum threshold living cells need to exist by the time of consumption to guarantee a probiotic effect. Microencapsulation of probiotic bacteria is an alternative to provide extra protection to living cells exposed to adverse environments (Burgain, Gaiani, Linder, & Scher, 2011), thus allowing bacteria to resist processing and packaging conditions, while preserving most of their metabolic activity (Serna-Cock & Vallejo-Castillo, 2013). Maintenance of probiotic characteristics throughout the product whole shelf-life is critical for its success in the market, with storage temperature being one of the most important parameters that regulates the activities of microorganisms in food systems (Doleyres & Lacroix, 2005).

To establish the effect of storage temperature upon survival, LP33 and LCS (positive control) cells were stored at two distinct temperatures, under refrigeration (4 °C) and room temperature (22 °C), and their viability was determined over a 30-day period (Fig. 1). At 22 °C, storage time data after 12 d showed that survival rates of coated cells (P) declined by as much as (log) 0.5 orders of magnitude, while free cells (NP) underwent a reduction of ca. 1–2 log cycle of their initial counts for LP33 and LCS, respectively. By the end of the 30-day storage period, 2–3 and 4 (log) orders of magnitude reductions were observed, for P and NP of LP33 and LCS, respectively. On the contrary, survival rates of both P and NP, at 4 °C, remained stable during the first 12 d for both strains. However, by 30 d of storage at 4 °C, an important reduction on viability of LCS cells was observed; viable cell remained above 10^7 CFU/g, the minimum recommended for a therapeutic effect.

In general, lactic acid bacteria (LAB) cell survival depended on the storage temperature and, as expected, the low temperature storage proved beneficial for microencapsulated cells; our results agree with those of previous researchers (Champagne, Mondou, Raymond, & Roy, 1996; Eun et al., 2007; Mortazavian et al., 2008; Teixeira, Castro, Malcata, & Kirby, 1995). The extended viability of the preserved samples was eventually associated with storage temperature close to 4 °C, where metabolic activity nearly ceases. At temperatures close to 22 °C, metabolic activity still exists, thus resulting in cell death and loss of cell viability (Soto et al., 2011). Compared with the NP cells, the P cells can be protected by the microcapsules during the process of storage at both temperatures.

3.2. *Capsule morphology and size particle size*

Particle size plays an important role upon activity (and thus food applications) of probiotics, and it may affect the textural and sensory properties of the food itself (Burgain et al., 2011). Microcapsules must be small enough to prevent an unfavourable effects upon the sensory properties of the product (Champagne & Fustier, 2007); a minimum diameter of 100 μm has been suggested to offer the best

protection under gastric conditions (Hansen, Allan-Wojtas, Jin, & Paulson, 2002), with an allowable range of 100–200 μm (McMaster, Kokott, Reid, & Abratt, 2005).

The size distribution of calcium alginate–starch particles was analysed by inverted optical microscopy, at 10 × magnification, and data was produced using Motic Images Plus software. Images showed that the shape of microcapsules was in general spherical and uniform with a mean diameter of ca. 416 ± 37 μm. Ainsley-Reid et al. (2005) explained the advantage of such a spherical shape by the Ca^{2+}-driven effect in extrusion, aimed at producing a much smoother and compact surface, and providing extra resistance to environmental conditions. Despite that the micron size capsules raised some barrier effect, it assures less variation in structure of food product and further inhibition of sandy texture development (Fahimdanesh et al., 2012; Mokarram, Mortazavi, Najafi, & Shahidi, 2009; Sultana et al., 2000).

3.3. *Resistance to heat shock*

Heat shock is the most studied technological stress with regard to the *Bifidobacterium* genus. In response to this challenge, several heat- shock proteins, e.g. chaperons (proteins related to DNA and RNA synthesis and cell division), increase in concentration and this mechanism appears to be conserved in several species (Ruiz et al., 2011). Control of tolerance of probiotic bacteria to temperature stress holds potential practical benefits in industrial fermentation processes, when strains are to be exposed to thermal processing and thus require enhanced thermo-tolerance (Ross, Desmond, Fitzgerald, & Stanton, 2005). It has been found that heat adaptation increases the thermal tolerance of lactobacilli. A nonlethal heat shock induces thermal tolerance, thus allowing bacteria to tolerate a second heat stress higher in intensity (Gouesbet, Jan, & Boyaval, 2002). This also supports adaptation strategies during subsequent stress events, and adapts bacteria to adverse conditions during drying, storage and other processes (Kim, Perl, Park, Tandianus, & Dunn, 2001).

The free and encapsulated cells of LP33 and LCS exposed to high temperatures of 72, 85, and 90 °C were examined for survival (Fig. 2). Statistical analysis indicated that cell survival was significantly affected ($P < 0.05$) by all heat treatments relative to the initial counts (~ 10^9 CFU/g). The NP of LP33 was more sensitive to heat shock than P cells, reducing their counts by 2 and 4 log cycles at 72 and 85 °C, respectively; survivors were not detected at 90 °C. Protected cells in microcapsules failed to show significant differences at all temperature tested ($P > 0.05$), and this effect was more apparent in LP33 strain. For this strain, microencapsulation effectively protected cells from heat shock.

In general, the reduction in survival of coated-LCS cells was higher ($P < 0.05$) than for coated-LP33, but no significant differences ($P > 0.05$) were observed among NP and P LCS cells after increasing the stress condition from of 72 to 90 °C.

3.4. *Effect of relative humidity upon survival*

Water activity is well known to significantly influence viability of probiotics during storage, and to influence the microbial and physicochemical stability of foods (Jixian & Mittal, 2013). The intended relative humidity (RH) was attained by saturated salt solutions in a closed chamber. As expected, higher relative humidity produced lower numbers of viable cells (Fig. 3) of both strains; 32.9% RH approved to be the best conditions for survival of both strains, with no significant (P N 0.05) differences between them by 15 d storage. LCS strain revealed a low susceptibility to RH% increases, and there were no significant (P N 0.05) differences above 10^4 CFU/g viable cells upon 15 d storage. Storage at high RH 97.0% strongly decreased survival of bacterial, likely by increasing the water content of microcapsules themselves; this may be detrimental to likely bacterial survival due to acceleration of oxidation and, according to Heidebach, Först, and Kulozik (2012), low values of RH may actually extend the storage time. Therefore, it appears that performance in terms of viability is affected by RH% prevailing along storage, in agreement with Zhang, Li, Park, and Zhao (2013). Higher relative humidities unfolded an unfavorable effect upon both probiotic bacteria (Hoobin et al., 2013).

3.5. *Effect of simulated gastrointestinal condition upon survival*

Viability of probiotic bacteria is the most critical parameter for probiotic products because it determines their eventual impact upon consumer health. Such benefits can be anticipated only when viable cells survive their translocation through the stomach and the remaining GIT and subsequently reach the small intestine for colonisation. Therefore, acceptable criteria for in vitro selection of probiotic bacteria include effective performance in the GIT (Kaisapathy & Chin, 2000).

Microencapsulation of probiotics has been examined for its ability to increase viability of probiotics in food products, and all the way through the colon. The objective of this part of the study was to evaluate whether coating of LP33 and LCS, previously stored at typical refrigeration conditions (4 °C) for 30 d, affects viability of those strains when exposed to conditions simulating GIT passage. Total viable counts of probiotics through each digestion step were monitored, and results are depicted in Fig. 4. Cell survival after exposure to stomach gastric juice was 1.36×10^7, 1.09×10^7, 2.37×10^7, and 1.09×10^7 CFU/g, for P and NP LP33, and P and NP LCS strains, respectively. The best survival of protected and free cells in simulated gastric fluid is probably the result of natural resistance to pH, consubstantiated on cell enzyme inventory and cell wall composition.

Cell survival, after exposure to simulated small intestine, was 7.9×10^6, 1.02×10^6, 6.95×10^6 and 1.01×10^6 CFU/g for P and NP cells of LP33 and LCS strains, respectively. Under intestinal stress, there were significant (P b 0.05) differences in both strains between treatments, but such a deed was more evident for the control

strain (LCS). Data showed that encapsulation by the extrusion method with sodium alginate and starch conferred additional protection to LP33, when exposed to simulated stress environment – thus potentially preventing cell loss. Results agree with data by other authors (Ayama, Sumpavapol, & Chanthachum, 2014) that there is some protective effect of microencapsulation upon cells. Heidebach, Först, and Kulozik (2009) also concluded that microencapsulation greatly improves survival rate of *Bifidobacterium lactis*, when compared to free cells under similar conditions of (low) pH.

The encapsulated cells of both probiotics were able to survive throughout the simulated small intestine, with a gradual reduction by ca. 2 and 3 log cycles relative to original number. Viable cells remained above $10^6/10^7$ CFU/g for all microparticles by the end of the complete treatment. Remember that the recommended number of viable cells by the time of consumption is 10^7 CFU/mL (or CFU/g; equivalent to 10^9 CFU per 100 g or 100 mL portions) (Chaikham et al., 2013; Lourens-Hattingh & Viljoen, 2001).

3.6. *Ability to adhere to intestinal epithelium*

The microencapsulation techniques produce major enhancement in viability of these microorganisms in food products, as well as in the GIT. In order to exert positive health effects, LAB have to resist gastric juice and bile salts – and should eventually attach to the epithelium of the intestine and grow therein. The mechanisms underlying probiotic functionality, such as adhesion and related competitive exclusion of pathogens, are in fact considered important probiotic traits (Kaushik et al., 2009). Therefore, adhesion features are an important issue, especially, their ability to adhere to the intestinal mucosa – which is one of the essential selection criteria for probiotics, as long as it represents the first step in microbial colonisation (Tuomola, Crittenden, Playne, Isolauri, & Salminen, 2001).

The main objective of our studies was to evaluate the degree of adherence and survival of probiotic bacteria in a simulated gastrointestinal environment, following microencapsulation (for the samples taken after storage). After exposure to GIT stress, both P and NP cells of LP33 and LCS similarly adhered to Caco-2 cells without significant ($P > 0.05$) cell loss in both treatments (Fig. 5). Further to the protection capacity of coating materials preventing bacterial degradation along the GIT, selection of materials with convenient biochemical features is highly recommended; excellent muco-adhesive properties are typical of hydrophilic polymers (e.g. alginate), and this property is useful to enhance the in situ delivery of bacteria along the GIT (Chen, Cao, Ferguson, Shu, & Garg, 2013). Strains microencapsulated in alginate have been included in such food formulations as ice cream, frozen yogurt and mayonnaise (Corona-Hernandez et al., 2013). Compared to the LCS strain, the adhesion levels of LP33 were significantly ($P < 0.05$) higher. Data were consistent with those by Moussavi and Adams (2010) and Piątek et al. (2012)). The microencapsulation procedure, after simulated digestion, did not adversely affect the bacterial function that is essential for adhesion.

3.7. *Design and characterization of probiotic olive paste during storage*

The final matrix used in the commercial product, defined as the delivery environment of the probiotic culture, can play a role upon probiotic cell survival in the final product, as well as throughout the GIT. A single trial of olive paste was set-up to test the incorporation of probiotic strains, under NP and P conditions.

3.7.1. *Survival of probiotic strains*

Only the refrigerated temperature (4 °C) was used for paste storage, owing to the results obtained pertaining microcapsules along storage. The resulting data of survival of LP33 and LCS during storage, in an olive paste model containing NP and P cells, are shown in Fig. 6. No significant difference (P N 0.05) in survival decrease was observed in cell viability of both strains during storage compared to day 0 with 1 log cycle decrease over a period of 6 days for protected cells (up to 10^6 CFU/g). Conversely, the free (NP) strain cells underwent a reduction in viability after 15 days of storage of ca. 7 log cycles (below 10^2 CFU/g) by the end storage. These results are in agreement with other studies (Sharp, McMahon, & Broadbent, 2008) pertaining to the matrices, e.g. ice-cream and yogurt (Deepika, Rastall, & Charalampopoulos, 2011). In general, the food industry has applied the recommended level of 10^6 CFU/g at the time of consumption (Doleyres & Lacroix, 2005).

In free cells, the behaviour could be explained by the olive matrix composition – which includes an acidic pH (0.74, Table 1) and an important level of fatty acids, namely oleic acid. Fatty acids proved to possess a protective role upon the survival of *Lactobacillus rhamnosus* GG when exposed to the acidic environments; previous data suggest that probiotic lactobacilli can use an exogenous oleic acid source to increase survival – and the underlying mechanism most likely involves the ability to increase to oleic acid content of their membrane. This could be part of a survival mechanism – reduction of increased proton concentration within the cell during acid stress (Corcoran, Stanton, Fitzgerald, & Ross, 2007; Muller, Ross, Sybesma, Fitzgerald, & Stanton, 2011). On the other hand, the low pH of olive paste (3.4 – somewhat similar to pH in the stomach) and low storage temperature (4 °C) did not negatively affect survival of LP33 (Holzapfel, Haberer, Snel, Schillinger, & Huis in´t Veld, 1998). Reported by Chandran, Grover, and Batish (2014) unfolded indigenous probiotic *L. plantarum* 91 to be quite robust due to atpD gene expression that is essential for survival under acidic environments. In cheeses solid matrix and fat content protect probiotics from harsh conditions (Escobar et al., 2012); note that strain was picked from an olive fermentation both under acidic conditions, and was screened for its tolerance to acidic pH (Peres et al., 2014).

According to our results, it can be assumed that microcapsules did not play a significant role upon cell protection in olive paste model since free cells are stable per se.

3.7.2. *Acidity changes of olive paste*

The inoculated olive paste was evaluated through storage of 30 d at 4 °C, in attempts to monitor pH and acidity changes of NP and P probiotic bacteria (Table 2). The pH of olive paste was significantly affected (P b 0.05) during storage time (except per *L. plantarum* LP33, NP treatment), although at low levels – but still compatible with product quality (3.57 to 4.42-3.50 for pH, and from 0.74 to 0.64% acidity, between days 0 and 30, respectively). This may be due to the good conditions of survival of free probiotics (see Fig. 6) at low pH of olive paste (3.57).

In agreement with the trends entertained by pH and titratable acidity values, no statistically significant (P b 0.05) differences were found for among the two treatments performed (P and NP). Similar results were reported by Brinques and Ayub (2011) and Su et al. (2011) in other acidic matrices.

The stability of strain growth and accompanying features can be related to the absorption of nutrients and release of metabolites through the calcium alginate-starch matrix, as well as lack of cell growth (Homayouni et al., 2009).

3.7.3. *Colour changes of olive paste*

Colour is one of the important attributes of foods; it is often considered as quality indicator, and determines consumer's acceptance. In food research, colour is frequently represented using the *L*a*b** colour space – and is described in terms of lightness (L value), ranging from 0 (darkness) to 100 (brightness); redness (a* value), where a positive a* value represents redness, while a negative a* represents greenness; and yellowness (b* value), where a positive b* represents yellowness, while a negative b* represents blueness. In order to study the total colour differences between control and to storage and paste models, the values of ΔE were calculated.

For pastes incorporated with free cells (NP), our results indicated that the L* value of all treatments decreased significantly (P b 0.05) over time. For LP33 P sample, no significant difference (P N 0.05) in the L* value was observed, thus indicating that the encapsulated sample was more efficient in preserving the lightness of the olive paste through shelf-life (Table 3). A different behaviour was recorded for olive paste incorporated with LCS (positive control). On the contrary, a* (green- ness) and b* (yellowness) values increased significantly over storage time (P b 0.05) in all treatments for both strains. This is probably due to the decrease in dark green colour, loss in green colour and increase in yellowing, respectively. In terms of total colour, changes were higher in paste incorporated with free cells than in paste with protected cells–thus indicating a better efficiency in the case of incorporation of protected cells. With regard to ΔE, samples from P strain LP33 presented the lowest and significant (P b 0.05) level relative to LCS strain, in both treatments (P and NP).

3.7.4. *Sensory properties of olive paste*

Remember once again that probiotics must remain viable in food products above a threshold level (e.g., 10^6 CFU/g) until the time of consumption, to be considered to

offer probiotic health benefits−but without adversely altering sensory attributes. Encapsulated cells should indeed not affect the sensory characteristics of the food in any perceptible way, remain stable throughout processing and storage of the product, and be resistant to the gastrointestinal environment.

Flavour is normally the first indicator of food choice, followed by considerations regarding health benefits. The sensory data produced to ascertain the effects of probiotic microcapsules into olive paste upon the potential response by the consumer are depicted in Fig. 7. Probiotic olive paste can induce off-flavours when compared with conventional one − triggered by capsule components, e.g. Na-alginate and corn starch can influence sensory profile and final acceptability of the olive paste. The average sensory scores by all panellists produced to ascertain the effects of technological factors are displayed, as radar graphs, in Table 3 and Fig. 7.

Data for appearance, colour, taste, grainy feel, and odour showed that use of NP or P probiotics had no significant effect (P N 0.05) on sensory properties of olive paste model. Any marked off-flavour and grainy texture were noticed in both samples; a bitter taste was detected in both samples and seems to play a critical role upon acceptability. Compatibility of microcapsule incorporation depends chiefly on physical characteristics of the supporting food matrix (Heidebach et al., 2012). Olive paste structure naturally associated with coarseness seems to be suitable for their application.

The sensory panel rated all matrices, in general, as good − with overall acceptance of all samples (5.7-6.2), without a significant (P N 0.05) preference between them. Regarding the effect of microcapsules incorporated in paste upon textural parameters, their lower size presented detection of granules; this unfolded an application of microencapsulated probiotic cells, since this characteristic (LP33 and LCS) does not significantly (P N 0.05) affect overall sensory parameters. Such microcapsules were small enough to avoid a grainy structure in olive paste, and no defects in two types (free or non-protected and protected cells) were noted. In terms of firmness, no significant differences were found between NP and P treatments. The scores for general acceptability were similar. The panellists could not pinpoint differences between olive pastes prepared with encapsulated probiotics and containing free cells.

4. Conclusions

To be considered as true probiotic strains must remain viable in food above the threshold level of 10^6 CFU/g until the time of consumption, without compromising sensory attributes. Incorporation of probiotic bacteria, either as encapsulated or free cells did not influence the sensory and textural properties of olive paste. Hence, olive paste has a good potential as carriers of *L. plantarum* 33 free cells, since microencapsulation in alginate supplemented with maize starch did not significantly improve viability in the olive paste during refrigerated storage.

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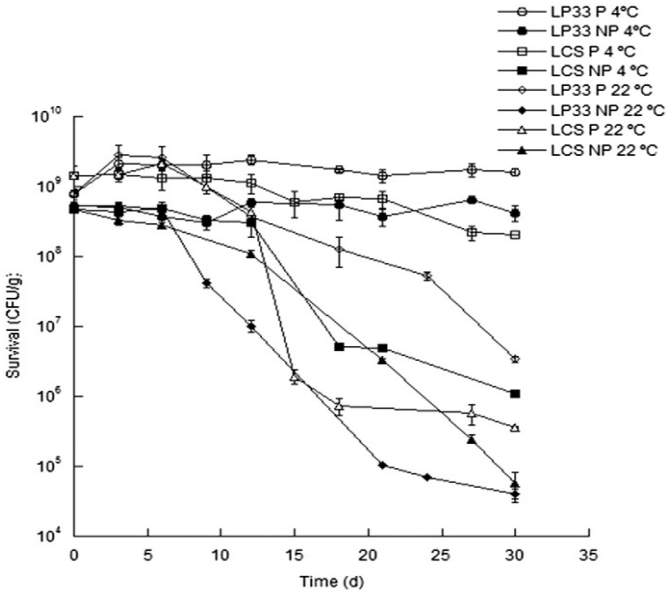

Fig. 1. Survival of free (NP) and microencapsulated (P) lactobacilli through storage at 4 °C and 22 °C. Sodium alginate polymer containing corn starch was used as filler. LCS = control; LP33 = *Lactobacillus plantarum* 33. An initial inoculum of 10^9 CFU/mL was used; enumeration was conducted at samples collected every 3 days for 30 d storage. Data are average of duplicate samples, obtained in three independent assays.

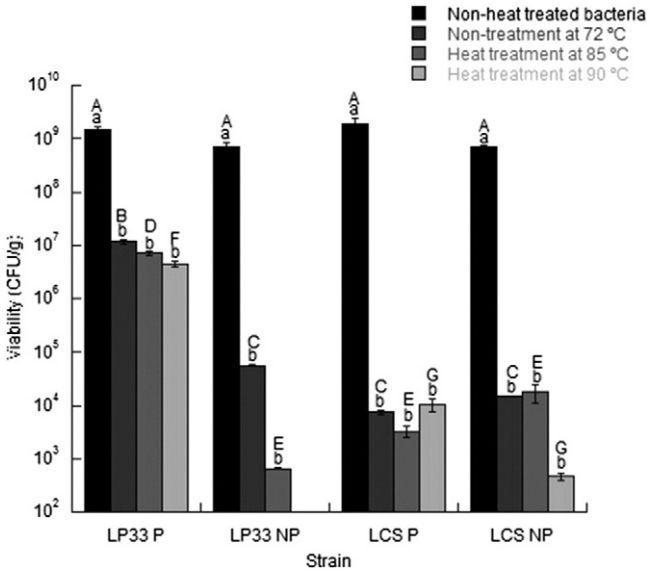

Fig. 2. Viability of free (NP) and microencapsulated (P) lactobacilli, after different heat treatments; an initial inoculum of 10^9 CFU/mL was used. LCS = control; LP33 = *Lactoba- cillus plantarum* 33. Data are average of duplicate samples, obtained in three independent assays. Bars with no common letter differ significantly ($P < 0.05$) from each other; lower case letters pertain to differences between temperature for each strain, and capital letters pertain to differences between temperatures for each strain.

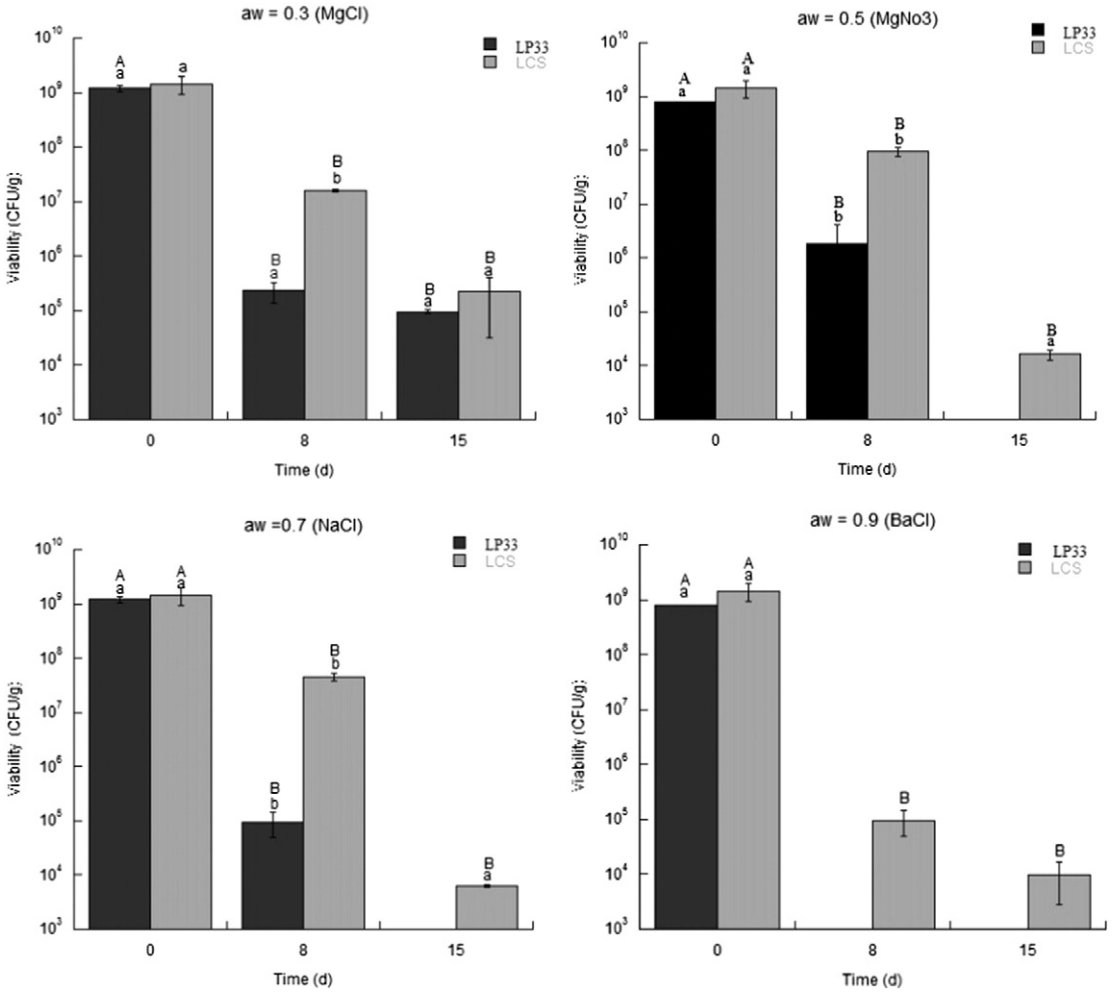

Fig. 3. Viability of microencapsulated lactobacilli, after storage at 22 °C under different relative humidities (32.9, 53.6, 76.5, and 97.0%). LCS = control; LP33 = *Lactobacillus plantarum* 33. An initial inoculum of 10^9 CFU/mL was used. Data are average of duplicate samples, obtained in three independent assays. Bars with no common letter differ significantly ($P < 0.05$) from each other; lower case letters pertain to differences between temperature for each strain, and capital letters pertain to differences between temperatures for each strain.

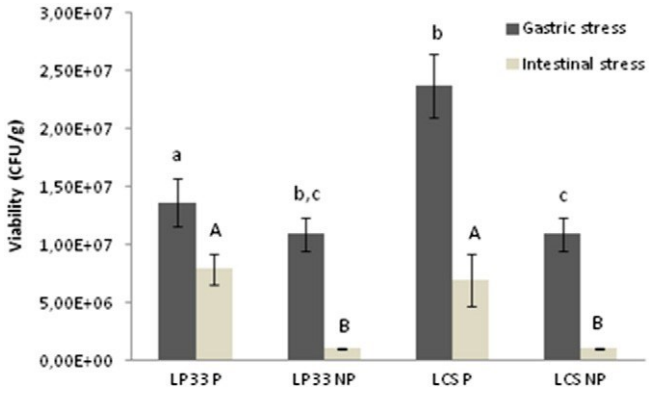

Fig. 4. Viability of free (NP) and microencapsulated (P) lactobacilli, after continuous simulated gastrointestinal conditions. LCS = control; LP33 = *Lactobacillus plantarum* 33. Inoculum 10^8 CFU/g (after simulated digestion). Data are average of duplicate samples, obtained in three independent assays. Bars with no common letter differ significantly ($P < 0.05$) from each other; lower case letters pertain to differences between strains in each gastric stress, and capital letters pertain to differences between strains in intestinal stress.

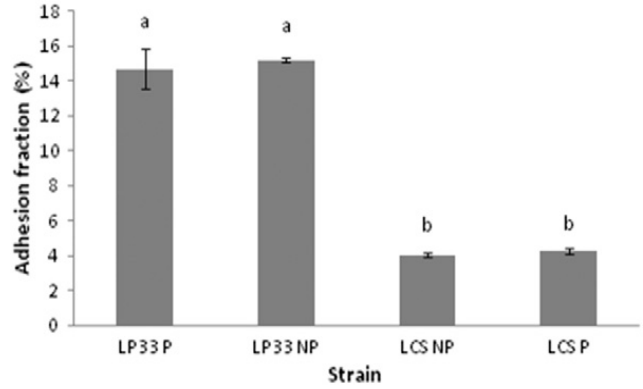

Fig. 5. Efficiency of free (NP) and microencapsulated (P) lactobacilli adhesion-expressed as ratio (%) of lactobacilli viable cells that remained adhered to the Caco-2 enterocytes. LCS: positive control; LP33 = *Lactobacillus plantarum* 33. Data are average of duplicate samples, obtained in three independent assays. Bars with no common letter differ significantly ($P < 0.05$) from each other.

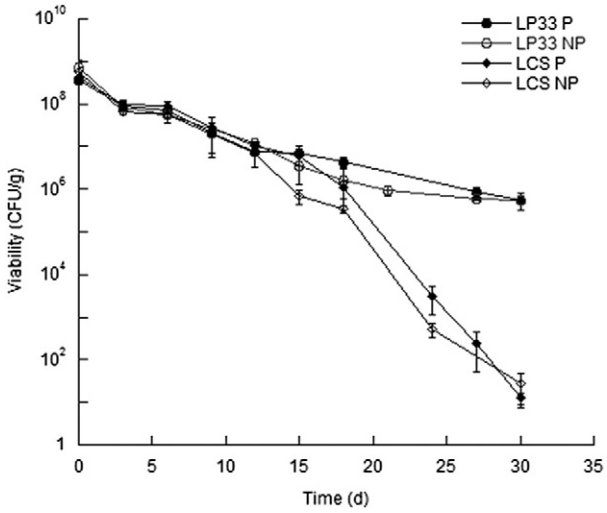

Fig. 6. Viability of free (NP) and microencapsulated (P). Lactobacilli in olive paste, after storage at 4 °C for different periods. LCS: positive control; LP33 = *Lactobacillus plantarum* 33. Data are average of duplicate samples, obtained in three independent assays. Bars with no common letter differ significantly ($P < 0.05$) from each other.

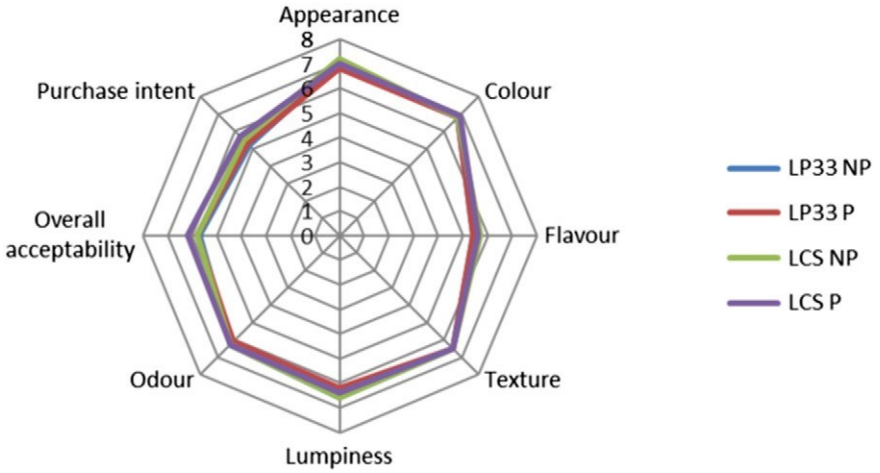

Fig. 7. Radar plots (8 corners) of sensory parameters (appearance, colour, flavour, texture, lumpiness, odour, overall linking, purchase intent), ranging from 2 (minimum) to 8 (maximum) level, of olive pastes containing encapsulated (P) and free (NP) lactobacilli after 30 d of storage at 4 °C. LCS: positive control; LP33 = *Lactobacillus plantarum* 33.

Table 1

Changes in pH and acidity of olive paste containing free (NP) and encapsulated (P) lactobacilli before and after 30 d storage. LCS: positive control; LP33 = *Lactobacillus plantarum* 33. Data are average of duplicate samples, obtained in three independent as- says. Columns with no common letter differ significantly (P b 0.05) from each other be- tween strains and treatments.

Strain	pH		Acidity (%)	
	0 d	30 d	0 d	30 d
LP33 NP	3.57 ± 0.01^{a}	$3.50 \pm 0.05^{a,b}$	0.74 ± 0.03^{a}	0.64 ± 0.03^{b}
LP33 P	3.57 ± 0.01^{a}	$3.49 \pm 0.04^{b,c}$	0.74 ± 0.03^{a}	$0.68 \pm 0.01^{a,b}$
LCS NP	3.57 ± 0.01^{a}	$3.46 \pm 0.05^{b,c}$	0.74 ± 0.03^{a}	$0.69 \pm 0.01^{a,b}$
LCS P	3.57 ± 0.01^{a}	3.42 ± 0.02^{c}	0.74 ± 0.03^{a}	$0.69 \pm 0.02^{a,b}$

Table 2

Changes in colour parameters (ΔE, L*, a*, b*) of olive paste containing free (NP) and encapsulated lactobacilli (P) before and after storage at 4 °C. LCS: positive control; LP33 = *Lactobacillus plantarum* 33. Data are average of duplicate samples, obtained in three independent assays. Columns with no common letter differ significantly (P < 0.05) from each other between strains and treatments.

Time (d)	Strain	L*	a*	b*	ΔE
0	–	84.60 ± 1.66^{a}	9.66 ± 1.15^{a}	57.8 ± 0.23^{a}	–
30	LP33 P	$78.60 \pm 0.70^{a,c}$	11.00 ± 1.25^{a}	$61.90 \pm 7.18^{a,c}$	8.75 ± 3.62^{a}
	LP33 NP	69.00 ± 1.29^{b}	$17.40 \pm 1.06^{b,c}$	78.30 ± 2.35^{b}	26.90 ± 0.73^{b}
	LCS P	61.30 ± 4.00^{b}	12.00 ± 2.94^{c}	$63.60 \pm 4.00^{a,b,c}$	21.60 ± 1.46^{b}
	LCS NP	$70.50 \pm 2.00^{b,c}$	14.50 ± 0.23^{b}	$74.50 \pm 0.35^{b,c}$	22.40 ± 1.04^{b}

Table 3

Sensory features of olive paste containing free (NP) and encapsulated lactobacilli (P) after storage at 4 °C. LCS: positive control; LP33 = *Lactobacillus plantarum* 33. Data are average of duplicate samples, obtained in three independent assays. Columns with no common letter differ significantly ($P < 0.05$) from each other between strains and treatments

Parameters	*Lactobacillus* strain			
	LP33 P	LP33 NP	LCS P	LCS NP
Appearance	7.00 ± 0.06^{a}	6.80 ± 0.33^{a}	7.20 ± 0.24^{a}	7.00 ± 0.16^{a}
Colour	6.80 ± 0.16^{a}	6.80 ± 0.12^{a}	6.80 ± 0.18^{a}	6.90 ± 0.16^{a}
Flavour	5.40 ± 0.23^{a}	5.40 ± 0.38^{a}	5.70 ± 0.27^{a}	5.60 ± 0.06^{a}
Texture	6.50 ± 0.27^{a}	6.50 ± 0.27^{a}	6.50 ± 0.24^{a}	6.50 ± 0.29^{a}
Lumpiness	6.60 ± 0.11^{a}	6.20 ± 0.39^{a}	6.60 ± 0.10^{a}	6.40 ± 0.06^{a}
Odour	6.30 ± 0.57^{a}	6.10 ± 0.99^{a}	6.30 ± 0.48^{a}	6.30 ± 0.57^{a}
Overall acceptability	5.70 ± 0.19^{a}	5.80 ± 0.51^{a}	5.80 ± 0.32^{a}	6.20 ± 0.16^{a}
Purchase intent	5.20 ± 0.23^{a}	5.30 ± 0.11^{a}	5.50 ± 0.39^{a}	5.70 ± 0.40^{a}