Microencapsulation of new probiotic formulations for gastrointestinal delivery: *in vitro* study to assess viability and biological properties

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Abstract

The paper describes the preparation of new probiotic formulations based on chitosan-coated alginate microcapsules containing three different probiotic strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 taken individually and as a mixture of them. The effects of microencapsulation on the viability of the strains in conditions simulating the gastrointestinal tract and under industrial processes conditions were studied. In addition, an evaluation of their probiotic properties was also investigated by *in vitro* tests on the human intestinal cell line HT-29 to explore the effect of microencapsulation on health beneficial effect of the considered strains. Non-encapsulated cells were completely destroyed when exposed to simulated gastric juice and other stress conditions, while encapsulated cells exhibited a significantly higher resistance to artificial intestinal juice, and heat and osmotic treatment. Moreover, in this study the effect of the various microencapsulated probiotic strain formulations was compared with analogous formulations also containing the polysaccharide Pleuran. The microencapsulation effectively protected the considered probiotic bacteria from simulating the gastrointestinal tract and industrial processes conditions in delivering the viable cells to intestine, without any significant adverse effect on their functionalities.

Keywords: Probiotics, intestinal microbiota, microencapsulated bacteria, microencapsulation polymers

Introduction

Probiotics are defined as live microorganisms, which, when administered in sufficient amount, confer beneficial effects to the host including maintenance of health promoting gut microflora, inhibition of pathogens growth, stimulation of the immune system (Adhikari et al. 2000; WHO 2001; Robles Alonso and Guarner 2013). It is essential to maintain high cell viability of probiotic bacteria in the vast range of conditions encountered during their industrial applications. Indeed, probiotic-based functional foods have to provide live microorganisms in the amount of 10^6 CFU/g of food product, or assumed in sufficient amounts to achieve a daily engagement of 10^8 CFU (Foligne et al. 2013). Probiotics are highly stable in dairy products when compared to non-dairy products, depending on the selection of the durable strains in harsh environmental conditions and on the prebiotics used (Topping et al. 2003). Some of the probiotic bacteria do not have the capacity to survive under the adverse conditions such as the strong acidity and the presence of bile salts in the gastrointestinal tract (GI), or during industrial process for probiotic-based functional foods preparation (Charalampopoulos et al. 2002). Furthermore, probiotic bacteria viability must be preserved also during the overall storage time and shelf-life of probiotic-supplemented food products. In order to overcome the presented drawbacks concerning probiotic survival, a large number of attempts and solutions have been proposed, including strain selection in adverse environment or addition of prebiotics, non digestible food ingredient that can stimulate the growth and the activity of bacteria species (De Giulio et al. 2005; Topping et al. 2003).

An alternative strategy is based on the creation of a physical barrier surrounding the probiotic bacteria, and, in this contest, the microencapsulation strategy has received a remarkable interest in the last years (Godward and Kailasapathy, 2003). The development of a microencapsulation matrix that surrounds probiotic bacteria can provide a physical barrier against harsh conditions that probiotic can encounter during food preparation processes and storage, as well as in the conditions simulating the transit through the gastrointestinal tract. Among the biomaterials studied for the formation of a protecting capsule, alginate and chitosan are the most commonly used biopolymers

(Krasaekoopt et al. 2004; Lin et al. 2008). Alginate, a linear copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, provides many advantages when used as encapsulating material, including biodegradability, non-toxicity, mild condition gelation with bivalent cations (such as calcium ions), low cost and supply from natural sources (Martinsen et al. 1989). The alginate has to be degraded within the GI tract, allowing the release of probiotics in the intestinal compartment; very harsh conditions or calcium ion-chelating species can affect the stability of the alginate microcapsules and ultimately of the bacterial strains. To increase the chemical and/or mechanical stability of the microcapsules, as well as to permit a controlled release of the beads content, a coating process can be performed, by adding a layer of a poly-cationic material, such as chitosan, which strongly binds to the negative charged alginate capsules. Chitosan is a polymeric sugar composed of repeating units of β-1,4-D-glucosamine, obtained from the natural chitin (β-1,4-D-*N*-acetylglucosamine polymer) by a chemical de-acetylation process. Since the alginate polymer presents a relative instability at low pH values, the addition of a poly-cationic layer on the surface of the alginate beads can overcome the liability issues of the alginate structures during the passage in the gastric apparatus (Priya et al. 2011). Although the most recent studies exploit alginate-chitosan microcapsules for entrapping probiotic bacteria, as reported in Kanmani et al. 2011, few studies are reported concerning the effect of this kind of microencapsulation on the bacterial viability and activity on intestinal cell lines during *in vitro* tests.

The main body district in which probiotics exert their positive health effect is the intestine, due to their ability to colonize and repopulate the gut microbiota environment (Gueimonde et al. 2005; Nissen et al. 2009). The relevant relations they establish with the host lead to many beneficial effects, such as the prevention or restoration of the correct tissue permeability, or the mediation of the antioxidant and anti-inflammatory activity of the intestinal epithelial cells. The influence of the probiotic activity on the modulation of pro- and anti-inflammatory cytokines produced by the intestinal epithelial cells, as well as its modulating action on the oxidative stress, are examples of

the effects showed by the probiotic strains supplied individually or combined in different formulations.

In this paper, we describe the preparation of new probiotic formulations based on chitosan-coated alginate microcapsules containing three different probiotic strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 taken individually and as a mixture of them. After the determination of the viability of the encapsulated bacteria in conditions simulating the gastrointestinal tract and under industrial processes conditions, an evaluation of their probiotic properties was determined by *in vitro* tests on a human intestinal cell line. Moreover, in this study the effect of the various microencapsulated probiotic formulations was compared with analogous formulations also containing the polysaccharide Pleuran.

Materials and Methods

Bacterial strains and culture conditions

This study comprised three probiotic strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 supplied from a private collection previously identified and characterized (Presti et al. 2015). *Lactobacillus* spp. strains were cultured in deMan, Rogosa and Sharpe (MRS) medium. For *B. animalis* subsp. *lactis* PBS075, MRS medium was supplemented with 0.3 g/l of L-cysteine hydrochloride monohydrate (cMRS) (Sigma-Aldrich). The cultures were incubated at 37 °C under microaerophilic or anaerobic conditions using anaerobic atmosphere generation bags (Anaerogen, Oxoid).

Microencapsulation of microbial strains

Alginate microencapsulation (core preparation)

For the microencapsulation of probiotic strains, the emulsion method was used, according to the procedure described by Sultana *et. al* (Sultana et al. 2000). The encapsulating material was constituted by an alginate solution (2% w/V, Sigma Aldrich), supplemented with D-glucose (2% w/V, Sigma Aldrich). For Pleuran-containing microcapsules, the initial alginate solution was

supplemented with a 2% V/V of Pleuran hydrogel (5% w/V, Principium Europe). The lyophilized bacteria cells were suspended in the alginate-based solution to a final concentration of 1% w/V. For the formulation composed by three probiotic strains, a 1:1:1 mixture of lyophilized bacteria was prepared, achieving a final concentration of 1% w/V in alginate solution. The suspension (5 mL) was gently poured in 25 mL of seed oil containing 0,02% V/V of Tween 80 (Sigma Aldrich), ensuring a vigorous stirring during the addition. Subsequently, 25 mL of 0.1 M calcium chloride (Sigma Aldrich) aqueous solution was added dropwise to the emulsion, maintaining a vigorous agitation of the mixture. After the addition, the stirring was stopped and the mixture was allowed to stand for 30 min at room temperature for the separation of calcium alginate beads on the bottom of the aqueous layer, and then maintained at 4°C overnight for a further hardening of the spheres. The oil phase was drained off and the microspheres were collected by centrifugation (7500 rpm, 20 min, 4°C), washed twice with a 0,9% saline solution containing 5% V/V glycerol (Sigma Aldrich) and once with deionized water. The collected beads were stored at 4°C or directly used for chitosan coating procedure.

Chitosan coating

The coating with chitosan was performed according to the procedure described by Krasaekoopt et al. (Krasaekoopt et al. 2004). Briefly, 0,4 g of high density chitosan (viscosity: 50 – 200 mPa AlfaChemiGroup) were suspended in 90 mL of deionized water, then acidified by adding 0,4 mL of acetic acid to obtain a clear solution. The pH was adjusted to 5.5 – 6.0 with 1 N NaOH and the final volume was brought to 100 mL. Then, 4 g of fresh alginate beads were immersed in 30 mL of the chitosan solution and the suspension was mixed at 200 rpm for 20 min. The suspension containing the microcapsules was centrifuged at 7500 rpm for 20 min, and the collected beads washed twice with deionized water. The chitosan-coated microcapsules were weighted and stored at 4°C.

Fluorescence Microscopy of microencapsulated bacteria

The determination of the entrapped probiotic strains inside the core of the microcapsule as well as the addition of Pleuran was evaluated by Fluorescence Microscopy analysis. Samples of

microencapsulated bacteria were stained by Calcofluor White Staning (Sigma-Aldrich) according to the description of the technical datasheet for polysaccharide Pleuran and SYBR Green for bacteria. The prepared samples were analysed by Fluorescence Microscopy with a Nikon Eclipse E600 microscope equipped with a Leica DC350F charge-coupled device camera and controlled by the Leica FW400 Software.

Viability of microbial strains

The determination of the number of entrapped bacteria cells in coated or non-coated microcapsules and then treated with different stress conditions (see after) was performed according to the method of Sheu et al. (Sheu et al. 1993). Briefly, 1 g of fresh beads (non-coated or chitosan-coated) were immersed in 10 mL of a phosphate buffer solution (0,1 M, pH 7.3) and mixed vigorously using a magnetic stirrer for 30 min, to ensure the solubilisation of the encapsulating matrix. The bacteria cells were enumerated by plating serial dilutions 1/10 of the suspension to obtain sensible dilutions for plating on MRS or cMRS agar and incubation at 37°C for 48 h.

Viability of microbial strains after treatment in simulated gastric juice (SGJ)

The resistance of free and alginate encapsulated, with and without chitosan coating, probiotic strains to the acidic conditions of gastric environment was established by evaluating the bacteria cell count upon treatment of free bacteria cells and the two different bacteria-containing capsules with a simulating gastric solution. The simulated gastric solution was constituted of a 0,9% saline acidified with 2 N HCl, achieving a final pH of 2.5. 1 g of fresh beads were suspended in 10 mL of simulated gastric juice (SGJ) and incubated at 37°C for 120 min. At regular time intervals (0, 30, 60, 120 min), the suspension was centrifuged (7500 rpm, 20 min, 4°C) and the supernatant removed. The collected beads were washed twice with deionized water and subsequently processed for the determination of the cell count by count plate method.

Viability of microbial strains after heat treatment

In order to determine the resistance upon heat treatment, both free and encapsulated bacteria were exposed to a temperature of 50°C for a prolonged time. Briefly, an aliquot of free lyophilized

microbial cells (2-3 mg) or 200 to 300 mg of fresh microcapsules loaded with probiotic bacteria, with and without chitosan coating, were re-suspended in 1 mL of water and incubated at 50°C for 5 hours. At regular time intervals (0, 15, 30, 60, 120, 180 and 300 min) the samples constituted by free bacteria (non-encapsulated) were collected and the viability was determined by count plate method. For encapsulated samples, the beads were collected at defined time intervals by centrifugation, and subjected to the solubilisation of the capsule matrix by dissolution in phosphate buffer, as described above. Serial dilutions of the final suspension were performed for the enumeration of the residual viability by count plate method.

Viability of microbial strains after isoglucose treatment

The resistance of both free and encapsulated bacteria to the osmotic stress associated to particular types of sugar-based food ingredients was determined by counting the residual viability of the samples incubated in a solution of High-Fructose Corn Syrup (HFCS), also called Glucose-Fructose Syrup or Isoglucose. This food ingredient is characterized by a minimum relative humidity (30% water, 70° Brix grade). Approximately 200 – 300 mg of fresh alginate beads, with or without chitosan coating, or 2-3 mg of lyophilized strains were immersed into 5 mL of isoglucose solution and incubated for 24 h at room temperature. At 0, 8, 16 and 24 h, free or microencacapsulated bacteria were collected from the isoglucose solution by centrifugation; the gathered cells or beads were successively washed twice with deionized water to remove the excess of isoglucose. Free microbial cells were directly re-suspended in MRS medium and plated after serial dilution; the encapsulated samples were subjected to treatment in phosphate buffer prior to enumeration of the residual viability by count plate method.

Resistance to the inflammatory stress

Inflammatory stress was induced by treating the human colon cell line HT-29 with Sodium Dodecyl Sulphate (SDS) as pro-inflammatory agent. SDS was added into the culture medium at a final concentration of 0.05 % (w/v). Cells were incubated in 5 % CO_2 at 37 °C. For these experiments, each individual microbial strain or the mixture of them, microencapsulated with chitosan coating,

and with or without Pleuran, was cultured in MRS, then the cells were re-suspended in DMEM at a final concentration of 10^7 CFU/mL. The inflammatory stress test on microencapsulated samples were performed on the microbial suspension obtained from the solubilisation of the microcapsules, which follows the incubation of the microsphere in SGJ, as described in the previous section, mimicking the passage through the gastric environment. For both free- and encapsulated bacteria, 100 µL of the final suspensions were added into each well containing the HT-29 cells. The residual cell viability at 24 hours and at 5 days was evaluated by MTT assay. The cell culture supernatants were removed and collected for cytokines determination. Cells were washed with Dulbecco's Phosphate Buffered Saline. Then, 200 µL of 0.05 % (w/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM was added into each well and incubated for 4 hours at 37 °C. At the end of the incubation, the MTT-medium was removed and replaced by an equal volume of solubilizing solution (10 % v/v Triton X-100, 0.1 N HCl in isopropanol) in order to dissolve the formazan produced by MTT reduction. After 30 min of incubation on a rotary shaker, the produced formazan was measured at 570 nm with a microplate reader. The results are expressed as A_{570} of treated cells – A_{570} untreated cells/A_{570} untreated cells × 100.

Cytokine production determination

The concentrations of interleukine 4 (IL-4), interleukine 10 (IL-10) and of tumor necrosis factor alpha (TNF-α) on cell culture supernatants, collected at 24 h and 5 days, were determined by enzyme-linked immune-sorbent assay (ELISA) commercial kits (Sun Red), according to manufacturer's instructions. The sensitivity of each cytokine assay was as follows: 7.8 pg/mL for IL-4 and IL-10, and 1 pg/mL for TNF-α. The results are expressed as pg/mL and compared with a negative control, not subjected to any treatment, and a positive control, subjected to the SDS treatment previously described.

Determination of the antioxidant potential

For FRAP assay, HT-29 cells were seeded in 12-well plates at a concentration of 10^5 cells/well and incubated in DMEM for 24 h at 37 °C in 5 % CO_2.

Bacterial suspensions from dissolved microcapsule (as described before) were added into each well at a final concentration of 10^7 CFU/ml. The negative control was carried out with an equal volume of DMEM without bacterial cultures. The improvement of the antioxidant ability of HT-29 cells in the presence of bacterial strains was evaluated by Ferric Reducing Antioxidant Parameter (FRAP) assay, as described by Benzie et al. 1996 (Benzie et al. 1996). Ferric to ferrous ion reduction at pH 3.6 causes a coloured ferrous-2,4,6-tripyridyl-s-triazine (TPTZ) complex. The absorbance at 595 nm of each sample was recorded after 30 min. The absorbance values are compared to a Fe(II) standard curve. Final results are expressed as Fe(II) μM.

Statistical analysis

Experiments were performed in triplicate and results elaborated as the mean ± standard error of the mean of three experiments. The statistical significance was assessed by Student's t-test. Differences were considered significant at p value < 0.05.

Results

Microencapsulation of microbial strains

The microencapsulation process was performed on three different strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 and on a 1:1:1 mixture of them. For each strain and for the mixture of the three strains, two types of encapsulated formulations were prepared, both containing a core structure constituted by an alginate matrix as entrapping agent, and by glucose, which was added to the formulation as a metabolic-aid additive. The first type of formulation contained only the core structure, the second type is constituted by the addition of β-glucan Pleuran at a final concentration of 0.1% w/V, supplemented to the glucose/sodium alginate initial solution. By the emulsion-gelation method used in this work, it was possible to obtain 2 to 3 grams of fresh alginate microcapsules, using the quantities described in the Materials and Methods. The coating procedure with the solution of chitosan increased the weight of the capsules, obtaining a final amount of fresh microcapsules ranging from 3 to 4 gr.

The shape and the size of the beads was determined by fluorescence microscopy analysis (Figure 1A, 1B, 1C). The emulsion methods used led to globular or drop-shaped alginate capsules, with a mean diameter in the order of 100 μm. The encapsulation efficiency of microbial cells and of polysaccharide Pleuran was qualitatively observed by carrying out staining procedures with suitable fluorophores, SYBR Green staining for microbial cells and Calcofluor White staining for Pleuran, respectively.

The number of entrapped bacteria for each encapsulated sample was determined quantitatively by liquefying an aliquot of fresh microcapsules in phosphate buffer solution, and counting the viability of the resulting suspension by the count plate method. The results are shown in Table 1 for all the prepared samples. Sample 9 represents a control sample of the 1:1:1 mixture of the three not encapsulated microbial strains. Samples 10 and 11 are empty microcapsules, in which no bacteria were encapsulated, and prepared to test the anti-inflammatory efficacy of the single components of the capsules. For all the encapsulated samples, a significant grade of bacterial entrapment was observed, ranging from 8.5 to 10 log CFU/g bead, starting from an initial cell load of the alginate solution between 9 – 10 log CFU/mL, both for chitosan-coated and not-coated capsules. In addition, the presence of glucose and of the β-glucan Pleuran during the encapsulation process did not affect the final viability. The achieved grade of bacterial encapsulation proved to be suitable for their addition to food ingredient as functional supplement, since the target level of probiotics in food must be 6 log CFU/g of food product.

Viability of the microencapsulated microbial strains in Simulated Gastric and Intestinal Juice

In order to simulate the passage through the gastric tract, the microencapsulated and non microencapsulated probiotic strain *L. plantarum* PBS067 was subjected to the treatment in SGJ (saline solution, pH 2.5) for 2 h. Both chitosan-coated, not-coated, Pleuran-supplemented, and not-supplemented microcapsules were tested to evaluate the effect of the additives and of the coating material. In a previous work (Presti et al., 2015), an evaluation of the resistance of non-encapsulated lactobacilli and bifidobacteria strains in simulated intestinal juice (SIJ, bile salts and pancreatine

solution) was performed. The three examined strains showed a significant resistance to bile salts, with negligible loss of viability upon exposure to SIJ at 37°C for 3 h (data not shown), while a consistent loss of viability was observed at low pH values. The exposure of the microcapsules to SGJ for 2 h did not affect the integrity of the material and the persistence of the chitosan layer on the capsules was assayed with a qualitative analysis by fluorescence microscopy after Calcofluor staining. The addition of the β-glucan Pleuran did not influence the resistance of the encapsulated probiotic strain to the acid condition, whereas only the formulation constituted by alginate-glucose and chitosan coating allowed a total maintenance of the initial viability of the encapsulated bacteria after exposure to SGJ. The absence of chitosan coating led to a decrease in total viability of two order of magnitude. The absence of glucose and of chitosan coating led to a decline of viability to uncountable levels after 2 h of treatment with SGJ (Figure 2).

Effect of heat and isoglucose treatment on microencapsulated bacteria

The resistance profile of the examined strains to some simulating food processing conditions, both in not-encapsulated (free cells) and in microencapsulated samples, was determined. We tested the effect of the microencapsulation on the viability of *L. plantarum* cells when exposed to high temperatures for a prolonged time. Both microencapsulated (with chitosan coating) and not-encapsulated bacteria were incubated at 50°C for 5 hours and, at established time intervals, the residual viability was determined by the count plate method. The results (Figure 3) indicated that the microencapsulating formulation constituted by alginate beads containing glucose (2%), Pleuran (2% of 5% Hydrogel) and an external layer of chitosan (coating) had a significant beneficial effect on the viability of the encapsulated probiotic strain. In particular, a very small decrease in viability (less than one order of magnitude) of *L. plantarum* was observed within the first hour of treatment. On the contrary, free cells of *L. plantarum* underwent a rapid decline of viability within the first hour of incubation, similarly to the microencapsulated form of *L. plantarum* which did not have the external coating of chitosan (data not-shown). The chitosan-coated *L. plantarum* microcapsules showed a high viability even after 5 h of incubation, although the final measured viability did not

exceed 5 log CFU/g. A slight, but significant, improvement of heat resistance was observed also for the encapsulated not chitosan -coated samples during the treatment (data not shown).

Furthermore, the new prepared encapsulating formulation composed of alginate, glucose and external chitosan coating allowed to contrast in a significant and effective manner the osmotic stress condition to which *L. plantarum* free-cells were subjected (Figure 4). In this food processing simulation, samples were immersed in a solution of isoglucose or glucose-fructose syrup, that has the characteristic to exert a strong osmotic stress condition, since the overall water content of this food ingredient is 30%. The complete formulation of the *L. planarum* microcapsules, containing glucose and alginate and a chitosan layer led to a small decrease in viability (less than one order of magnitude), compared to not-coated beads, in which the loss of viability after 24 h of incubation in isoglucose is much more accentuated.

Characterization of anti-inflammatory properties of microencapsulated probiotic formulations *in vitro*

The microencapsulated formulations of probiotic strains developed in this work were characterized and screened for their capacity to exert a protection against inflammatory stress and for their antioxidant potential on the human intestinal cell line HT-29. To this aim, microcapsules with *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 and a mixture in a 1:1:1 combination of them, in two different formulations, one constituted by alginate-glucose-chitosan coating (C1) and the second constituted by alginate-glucose-Pleuran-chitosan coating (C2), were prepared and tested *in vitro* experiments. The microencapsulated bacteria were first subjected to acid treatment (incubation in SGJ) and to disaggregation in phosphate solution (incubation in SIJ), in order to simulate gastric and intestinal conditions that these preparations have to encounter after their intake, and then supplied to the HT-29 cells. The viability of HT-29 cells, treated with SDS (Sodium Dodecyl Sulfate) and in presence or in absence of probiotic digested suspension was determined (Fig. 5). SDS is an irritating agent and the cell line was subjected to its pro-inflammatory activity for 24 hours and 5 days. The results were compared to the negative control

(without pro-inflammatory treatment) and to positive control (cells treated exclusively with SDS, with no probiotic samples added). The 100% of viability was associated with the negative control, and results were expressed as relative percentage of residual viability. The results indicated a loss in viability for all the tested formulations, higher after 5 days of treatment. The higher levels of residual viability (24 hours and 5 days) was determined for samples containing the mixture of the three probiotic strains. Samples of single microencapsulated bacteria exerted a lower ability to contrast the loss in viability compared to the mixed formulation. Encapsulating materials (without bacteria) did not show particular cytotoxic effect at 24 h and 5 days, compared to the other formulations.

The inflammatory response of HT-29 cell culture, treated with the pro-inflammatory agent SDS for 24 hours and 5 days in presence or in absence of probiotic-based samples was further evaluated by monitoring the release of TNF-α, as pro-inflammatory cytokine, and of IL-4 and IL-10 as anti-inflammatory cytokines. Results, showed in Figure 6, indicated that the releasing of TNF-α was comparable to the negative control, except for microencapsulating formulations with *B. animalis* subsp. *lactis* PBS075, for which, however, a significant reduction of released TNF-α was observed, compared to positive control. The formulations containing the three mixed strains showed the best level of TNF-α release reduction. No relevant changes in TNF-α release were observed between 24 h and 5 days after the treatment. Regarding the samples of encapsulating material, a partial decrement of the TNF-α concentration was observed, with a more accentuated effect of the C2 sample after 5 days of treatment. The sample contained the beta-glucan Pleuran, that could have exerted its anti-inflammatory activity on the cell culture.

The analysis of the release of IL-4 (Figure 7) from cell culture treated with the pro-inflammatory agent SDS revealed a significant increase in the production of the cytokine for all the examined samples after a prolonged exposure (5 days). Results showed a higher production of IL-4 after 5 days of treatment for *L. plantarum* PBS067, *L. rhamnosus* PBS070 and for the encapsulated mixture of the three strains, in particular for the sample constituted by the three strains and C2

material. Encapsulating material as such led to small increments in IL-4 release after 24 h and 5 days. The diagram of the production and release of IL-10 (Figure 8) anti-inflammatory cytokine by HT-29 cells has a comparable profile to that of IL-4 production, with the best results associated to the anti-inflammatory action of the encapsulating formulation containing the 1:1:1 mixed probiotic strains.

Antioxidant potential of the microencapsulated probiotic formulations *in vitro*

The activity of the probiotic encapsulated formulations on a cell culture model of inflammatory state was further investigated and characterized by evaluating the antioxidant potential of treated cell cultures. The evaluation was based on the FRAP assay, in which the capacity of a cell culture to transform the trivalent iron species (Fe (III), oxidized form) into bivalent iron (Fe (II), reduced) is measured. In this experimental system, the iron atom represents a suitable oxidising agent due to its nature of transition metal atom, and therefore able to induce oxidative stress. The results, showed in Figure 9, were compared to the negative control, in which cells have not been treated. For all the samples used in this test, an increase of the Fe(II) concentration was observed, much higher after 5 days of treatment. Encapsulated samples of *L. plantarum* PBS067, *L. rhamnosus* PBS070 and the 1:1:1 mixed composition showed the highest grade of Iron(III) conversion. Samples derived from encapsulated materials (C1 and C2) also exerted a significant antioxidant capacity on the cell line.

Discussion

Probiotic-supplemented products represent a valid response to an emerging demand of functional foods able to promote health benefits and to reduce the risk of diseases following their dietary intake (Gbassi et al. 2009). Among probiotics, bacteria mainly belonging to *Lactobacillus* and *Bifidobacterium* genera, show the best health properties by means of the ability to colonize the GI tract of humans and animals (Walter 2008), and to support the intestinal and immune system functions (Floch 2005). The introduction of probiotic strains to food products must be associated to methods and techniques capable to guarantee the efficient survival of the probiotics both in the food

products processing and storage, and during the transit within the gastrointestinal tract. Microencapsulation of bacteria in polymeric and biodegradable matrix represents a valid strategy not only for the efficient delivery of probiotic strains to the intestine, where they can exert the beneficial functions, but also for the maintenance of the viability of the probiotic strains under the harsh conditions of food product preparations and processes (Serna-Cock and Vallejo-Castillo, 2013).

In this contest, the present paper describes the study of new formulations of alginate-chitosan coating microencapsulation of three probiotic strains and a mixture of them and illustrates the protective effects of such formulations on viability in conditions that simulates the gastrointestinal environment, as well as in two adverse food processing conditions, heat and isoglucose treatments. Moreover, an evaluation of the probiotic properties on a human intestinal cell line *in vitro*, was performed. The strains used in this work, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS, were characterized in a previous work for their microbiological features and for their probiotic properties (Presti et al. 2015). In order to exert their beneficial effect on the intestine they have to reach this district with a high percentage of viability. To achieve this goal, we propose the microencapsulation of these bacteria based on the emulsion-gelification method, generating a gel matrix constituted of polymeric microcapsules that can be further coated with different layers. The encapsulation of bacteria in the calcium alginate matrix is a simple and low-cost method (Sheu et al. 1993; Krasaekoopt et al. 2003); the emulsion-gelification technique does not involve expensive instrumentations and processes (Cook et al. 2012), differently from other types of encapsulation methods, like extrusion, spray-drying or aerosol technique (Gbassi et al. 2009; Sohail et al. 2011). Neverthless, the emulsion method provided samples characterized by small-sized alginate beads, in the order of few micrometers in diameter. This result represents an advantage for the potential use of the microcapsules as food additives, since the smaller sizes reduce the sensory detection, limiting the negative impact on the organoleptic properties of food ingredients.

The new formulation presented in this work was based on the introduction of several ingredients with the aim to modulate and increase the probiotic potential and viability of the encapsulated strains. A 2% of D-glucose was added to the starting sodium alginate solution, since Corcoran and co-workers (Corcoran et al. 2005) have demonstrated an enhancement of the survival of lactobacilli in acid environment, that represents one of the main stress condition in the gastrointestinal tract. Moreover, the sugar supplemented in the alginate micro-particle preparation could improve the long-term viability of encapsulated probiotic, by acting as cryo-preserving agent during storage under refrigerated-conditions, as demonstrated by Priya et al. (Priya et al. 2011; Kanmani et al. 2011). The formulation was completed by the addition of the β-glucan Pleuran, as additive (or polysaccharide with immunomodulating effect) inside the microcapsule, and by an external coating of chitosan. Pleuran is an unsoluble glycan polymer (β-1,3-D-glucan) isolated from *Pleurotus ostreatus*, well-characterized for its capacity to exert modulating effect on intestinal bowel diseases, such as colitis (Bobek et al. 2001), and to enhance antioxidant potential of the colon wall against inflammatory state (Majtan et al. 2009; Rop et al. 2009). The importance of an external chitosan coating layer on microspheres of alginate is well documented (Cook et al. 2013; Nazzaro et al. 2012). The addition of multiple coating layers leads to improved chemical and mechanical properties of the microcapsules, as well as the possibility to tune and control the release of the beads content.

In order to verify the efficacy of the microencapsulation in protecting the bacterial strains in harsh environmental conditions, we initially prepared and tested different formulations using *L. plantarum* PBS067 as bacterial strain, that had previously shown to be the less resistant to harsh conditions (Presti et. al 2015). Our results show the efficacy of the microencapsulating formulation developed in this work on the capacity to protect the probiotic bacteria against the harsh conditions of a simulated gastrointestinal environment and of simulating industrial processes. In both cases (with or without Pleuran), the formulation composed of alginate core supplemented with glucose and the chitosan coating allowed a significant enhancement of viability in the simulated conditions,

compared to the viability of free probiotic cells and of encapsulated cells without chitosan coating. This result is in agreement with the data reported in literature, for other probiotic-based microcapsule with a chitosan external coating as protecting material (Chavarri et al. 2010; Cook et al. 2013). The encapsulated formulation resulted to improve the resistance of the model strain *L. plantarum* PBS067 also in experiments that simulated typical food industrial conditions as prolonged heat treatment and osmotic stress condition caused by food products with high concentration of sugars. The heat treatment can simulate a typical pasteurization process or general heat treatment which are at the basis of food preparation processes; the stress condition caused an inevitable decline in viability of not-encapsulated strains, whereas a significant improvement of the resistance was measured for the microencapsulated-chitosan coated strain with our formulation, even at prolonged time intervals (up to 5 h, compared to free cells). The osmotic stress, caused by an exposure of probiotics to a sugar-rich environment and, as a consequence, to a very low water content, affects the final viability of these bacteria. Many food products and semi-prepared ingredients are constituted by sugar-rich preparations, such as the high glucose-fructose corn syrup (HFCS). The microcapsules developed in this paper, allowed a good protection for encapsulated probiotics, minimizing the impact of the stress on the final viability, after a treatment of 48 h in such hyperosmotic environment. The chitosan layer and the presence of glucose in the formulation seem to play a central role in the enhanced resistance against the harsh condition. These results are in agreement with what described by Kim et al., whose microencapsulation process offered an effective protection for the probiotic strain *Lactobacillus acidophilus* ATCC 43121 against acid and heat stress treatment (Kim et al. 2008).

Proved the efficacy of alginate-chitosan coated microencapsulation to guarantee viability on our strain in harsh conditions, we examined the probiotic/protective properties of the three selected bacterial strains on a human intestinal cell line by *in vitro* tests. In order to investigate the influence of microencapsulation material and additives on the probiotic biological properties, the selected bacteria were tested as single strains and as a mixture of the three strains in the two formulations

with the Pleuran (C1) and without Pleuran (C2), in experimental conditions simulating the passage through the gastrointestinal tract. The examined strains were able to modulate the release of the interleukins IL-4 and IL-10, and the TNF-α by the HT-29 cells *in vitro*. Furthermore, it is demonstrated that probiotic bacteria contribute in the reduction of oxidative damage in humans (Amaretti et al. 2013; Lin and Yen 1999; Spyropoulos et al. 2011). Results showed that these strains seemed to have a stronger effect on the antioxidative potential of the cell line. The comparative study of the immunomodulatory properties of each single strain and the mixture of the three strains revealed a synergic effect of the probiotic mixture, but no appreciable difference between the two kind of formulations could be detected. One possible explanation could reside in the fact that the effect of Pleuran is covered by the higher potential of the probiotic strains, nevertheless this effect is evident in the control formulation C1 without bacteria. Our results are in agreement with the data, previously observed for the not-microencapsulated strains (Presti et al. 2015), concerning the *in vitro* effects, but, to our knowledge, no data are available concerning a synergic effect of the strains with the polysaccharide additive and with the coating material. Moreover, although the effect of some prebiotic on the viability of probiotic bacteria is known, no indications are available from literature concerning the use of substance as Pleuran, supporting the novelty of this paper. It is noteworthy that the response profile obtained in co-culture assays with bacteria and intestinal cell lines was demonstrated to be predictive of their *in vivo* probiotic activities.

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References

Adhikari K, Mustapha A, Grun IU, Fernando L (2000) Viability of microincapsulated bifidobacteria in set yogurt during refrigerated storage. J Diary Sci 83: 1946-1951.

Amaretti A, di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A (2013) Antioxidant properties of potentially probiotic bacteria: *in vitro* and *in vivo* activities. Appl Microbiol Biotechnol 97: 809–17.

Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal Biochem 239: 70-6.

Bobek P, Nosalova V, Cerna S (2001) Effect of Pleuran (beta-glucan from Pleurotus ostreatus) in diet or drinking fluid on colitis in rats. Nahrung-Food 45 (5): 360-363.

Charalampopoulos D, Wang R, Pandiella SS, Webb C (2002) Application of cereals and cereal components in functional foods: a review. Intern J Food Microbiol 79: 1-2.

Chavarri M, Maranon I, Ares R, Ibanez FC, Marzo F, del Carmen Villaran M (2010) Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Intern J Food Microbiol 142 (1-2): 185-189.

Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV (2012) Microencapsulation of probiotics for gastrointestinal delivery. J Control Release 162 (1): 56-67.

Cook MT, Tzortzis G, Khutoryanskiy VV, Charalampopoulos D (2013) Layer-by-layer coating of alginate matrices with chitosan-alginate for the improved survival and targeted delivery of probiotic bacteria after oral administration. J Material Chem B 1 (1): 52-60.

Corcoran BM, Stanton C, Fitzgerald GF, Ross RP (2005) Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Appl Environ Microbiol 71 (6): 3060-3067.

De Gulio B, Orlando P, Barba G, Coppola R, De Rosa M, Sada A, De Prisco PP, Nazaro F (2005) Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. World J Microbiol and Biotechnol 21 (5): 739-746.

Floch MH (2005) Use of diet and probiotic therapy in the irritable bowel syndrome - Analysis of the literature. J Clin Gastroenterol 39 (4):S243-S246.

Foligne B, Daniel C, Pot B (2013) Probiotics from research to market: the possibilities, risks and challenges. Curr Opinion Microbiol 16 (3): 284-292.

Food and Agriculture Organization/World Health Organization (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.

Gbassi GK, Vandamme T, Ennahar S, Marchioni E (2009) Microencapsulation of *Lactobacillus plantarum* in an alginate matrix coated with whey proteins. Intern J Food Microbiol 129 (1): 103-105.

Godward SE, Kailasapathy K (2003) Viability and survival of free, encapsulated and co-encapsulated probiotic bacteria in yogurt. Milk Science Int 58: 396-399.

Gueimonde M, Salminen S (2005) Microbiota of intestine. Probiotics. In Encyclopedia of Human Nutrition (Second Edition) Caballero B Ed. Elsevier, Oxford. Pages: 244-251.

Kanmani P, Kumar RS, Yuvaraj N, Paari KA, Pattukumar V, Arul V (2011) Cryopreservation and microencapsulation of a probiotic in alginate-chitosan capsules improves survival in simulated gastrointestinal conditions. Biotechnol Bioprocess Eng 16 (6): 1106-1114.

Kim SJ, Cho SY, Kim SH, Song OJ, Shin IS, Cha DS, Park HJ (2008) Effect of microencapsulation on viability and other characteristics in *Lactobacillus acidophilus* ATCC 43121. Food Science and Technol 41: 493-500.

Krasaekoopt W, Bhandari B, Deeth H (2003) Evaluation of encapsulation techniques of probiotics for yogurt. Intern Dairy J 13 (1): 3-13.

Krasaekoopt W, Bhandari B, Deeth H (2004) The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. Intern Dairy 14 (8): 737-743.

Lin MY, Yen CL (1999) Antioxidative ability of lactic acid bacteria. J Agricultural Food Chem 47: 1460–66.

Lin J, Yu W, Liu X, Xie H, Wang W, Ma X (2008) *In vitro* and *in vivo* characterization of alginate-chitosan-alginate-artificial microcapsules for therapeutic oral delivery of live bacterial cells. J Biosci Bioengi 105: 660-665.

Majtan J, Kumar P, Koller J, Dragunova J, Gabriz J (2009) Induction of Metalloproteinase 9 Secretion from Human Keratinocytes by Pleuran (beta-Glucan from *Pleurotus ostreatus*). Zeitschrift Fur Naturforschung Section C-a . J Biosci 64 (7-8): 597-600.

Martinsen A, Skjakbraek G, Smidsrod O(1989) Alginate as immobilization material. Correlation between chemical and physical-propreties of alginate gel beads. Biotechnol and Bioeng 33 (1): 79-89.

Nazzaro F, Orlando P, Fratianni F, Coppola R (2012) Microencapsulation in food science and biotechnology. Curr Opin Biotechnol 23 (2): 182-186.

Nissen L, Chingwaru W, Sgorbati B, Biavati B, Cencic A (2009) Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. strains: A functional study in the small intestinal cell model. Intern J Food Microbiol 135 (3): 288-294.

Nosal'ova V, Bobek P, Cerna S, Galbavy S, Stvrtina S (2001) Effects of Pleuran (beta-glucan isolated from *Pleurotus ostreatus*) on experimental colitis in rats. Physiol Res 50 (6): 575-581.

Presti I, D'Orazio G, Labra M, La Ferla B, Mezzasalma V, Bizzaro G, Giardina S, Michelotti A, Tursi F, Vassallo M, Di Gennaro P (2015) Evaluation of the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains and their *in vitro* effect. Appl Microbiol Biotecnol DOI: 10.1007/s00253-015-6482-8.

Robles Alonso V, Guarner F (2013) Linking the gut microbiota to human health. J Nutr 109 Suppl: S21–6.

Rop O, Mlcek J, Jurikova T (2009) Beta-glucans in higher fungi and their health effects. Nut Rev 67 (11): 624-631.

Priya AJ, Vijayalakshmi SP, Raichui AM (2011) Enhanced Survival of Probiotic *Lactobacillus acidophilus* by Encapsulation with Nanostructured Polyelectrolyte Layers through Layer-by-Layer Approach. J Agricultural Food Chem 59 (21): 11838-11845.

Serna-Cock L, Vallejo-Castillo V, (2013) Probiotic encapsulation. African J Microbiol Res 7 (40): 4743-4753.

Sheu TY, Marshall RT (1993) Microentrapment of lactobacilli in calcium alginate gels. J Food Science 58 (3): 557-561.

Sohail A, Turner MS, Coombes A, Bostrom T, Bhandari B (2011) Survivability of probiotics encapsulated in alginate gel microbeads using a novel impinging aerosols method. Intern J Food Microbiol 145 (1): 162-168.

Spyropoulos BG, Misiakos EP, Fotiadis C, Stoidis CN (2011) Antioxidant properties of probiotics and their protective effects in the pathogenesis of radiation-induced enteritis and colitis. Dig Dis Sci 56: 285–94.

Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K (2000) Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yogurt. Intern J Food Microbiol 62 (1-2): 47-55.

Topping DL, Fukushima, Bird AR (2003) Resistant starch as a prebiotic and synbiotic: state of the art. Proc Nut Soc 62(01): 171-176.

Walter J (2008) Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. Appl Environ Microbiol 74 (16): 4985-4996.

LEGENDS

Figure 1. Fluorescence Microscopy analysis of microcapsules containing *L. plantarum* and Pleuran: staining with Calcofluor White for Pleuran and SYBR Green for bacteria. a) size of the microcapsules containing probiotic bacteria (Green light); (b) Green light, staining with SYBR Green; c) Blue light, staining with Calcofluor White.

Figure 2. Residual viability and kinetic of resistance of microencapsulated (♦) and not-encapsulated (▲)samples of *L. plantarum* in SGJ over 2 hours.

Figure 3. Residual viability and kinetic of resistance of microencapsulated (♦) and not-encapsulated (▲) samples of *L. plantarum* in heat conditions (50°C, 5 hours).

Figure 4. Residual viability and kinetic of resistance of chitosan-coated alginate microcapsules (♦) and not-coated (▲) containing *L. plantarum* subjected to osmotic stress conditions (20°C, 24 hours).

Figure 5. Viability of the HT-29 cell culture exposed to SDS for 24 hours (■) and 5 days (□), in presence or in absence of probiotic-based samples and encapsulating materials. Each treatment was performed as triplicate and the error bars indicate the men standard deviation for each test group. The negative control was represented by not-treated cells; C1 represented microencapsulating formulation composed of alginate-glucose and chitosan coating; C2 represented microencapsulating formulation composed of alginate-glucose-Pleuran and chitosan coating; Mix strains represented the mixture of the three not-encapsulated .and encapsulated examined strains.

Figure 6. TNF-α release of HT-29 cell culture upon exposure to SDS for 24 hours (■) and 5 days (□), in presence or in absence of probiotic-based samples and encapsulating materials. Each treatment was performed as triplicate and the error bars indicate the men standard deviation for each test group. The negative control was represented by not-treated cells; C1 represented microencapsulating formulation composed of alginate-glucose and chitosan coating; C2 represented microencapsulating formulation composed of alginate-glucose-Pleuran and chitosan coating; Mix strains represented the mixture of the three not-encapsulated .and encapsulated examined strains.

Figure 7. IL-4 production levels of HT-29 cell culture upon exposure to SDS for 24 hours (■) and 5 days (□), in presence or in absence of probiotic-based samples and encapsulating materials. Each treatment was performed as triplicate and the error bars indicate the men standard deviation for each test group. The negative control was represented by not-treated cells; C1 represented microencapsulating formulation composed of alginate-glucose and chitosan coating; C2 represented microencapsulating formulation composed of alginate-glucose-Pleuran and chitosan coating; Mix strains represented the mixture of the three not-encapsulated .and encapsulated examined strains.

Figure 8. IL-10 release of HT-29 cell culture upon exposure to SDS for 24 hours (■) and 5 days (□), in presence or in absence of probiotic-based samples and encapsulating materials. Each treatment was performed as triplicate and the error bars indicate the men standard deviation for each test group. The negative control was represented by not-treated cells; C1 represented microencapsulating formulation composed of alginate-glucose and chitosan coating; C2 represented microencapsulating formulation composed of alginate-glucose-Pleuran and chitosan coating; Mix strains represented the mixture of the three not-encapsulated .and encapsulated examined strains.

Figure 9. Effect of microencapsulated and not-microencapsulated probiotic strains on the potential antioxidant of HT-29 cells. The cell culture at 24 hours (■) and 5 days (□) was monitored by FRAP assay. The results, expressed as μM of Fe(II), are the means ± standard deviations, * p-value < 0.05, ** p-value < 0.01 as compared to the negative control.

Table 1. Samples of microencapsulated probiotic strains prepared in this paper.

Sample	Strain	Content of the microcapsule	mass of microcapsule (g)	Viability (number of entrapped cells, CFU/g fresh beads)
1	*L. plantarum*	Alginate – Glucose	3.33	3.9*10^8
2	*L. plantarum*	Alginate – Glucose Pleuran	3.99	1.3*10^9
3	*L. rhamnosus*	Alginate – Glucose	3.28	5.8*10^8
4	*L. rhamnosus*	Alginate – Glucose Pleuran	2.99	8.8*10^9
5	*B. lactis*	Alginate – Glucose	2.90	1.0*10^8
6	*B. lactis*	Alginate – Glucose Pleuran	3.2	2.0*10^8
7	*L. plantarum* *L. rhamnosus* *B. lactis*	Alginate – Glucose	3.00	8*10^7
8	*L. plantarum* *L. rhamnosus* *B. lactis*	Alginate – Glucose Pleuran	2.80	1.0*10^8
9	*L. plantarum* *L. rhamnosus* *B. lactis*	/	/	1.0*10^8
10	/	Alginate – Glucose	2.90	/
11	/	Alginate – Glucose Pleuran	2.28	/