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Bioactive synbiotic coatings with lactobionic acid and *Lactobacillus plantarum* CECT 9567 in the production and characterization of a new functional dairy product

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ABSTRACT

Lactobionic acid (LBA), a prebiotic with beneficial health properties, can be mixed with a probiotic, *Lactobacillus plantarum* CECT 9567, to prepare novel synbiotic coatings. Coatings deposited on cottage cheese contained either probiotic (PRO, *L. plantarum* CECT 9567), prebiotic (PRE2 and PRE4; 20 and 40 g L^{-1} of LBA, respectively) or synbiotic compositions (SYN2 and SYN4). Coated cottage cheeses were analysed to determine probiotic and LBA concentration changes during storage, their textural properties and investigate the survival of bacteria during simulated digestion. Before the digestion test, PRO, SYN2 and SYN4 met minimal requirements to attain probiotic category and PRE2, PRE4, SYN2 and SYN4 contained adequate quantities of LBA throughout the experiment. Textural properties of cheese samples varied, with changes in the stickiness parameter. The digestion test showed that only SYN2 and SYN4 maintained acceptable probiotic values after simulated digestion, due to the presence of LBA, which increased probiotic survival.

1. Introduction

Currently, consumers demand food products that, in addition to satisfying their nutritional demands, have the ability to improve their health and/or reduce the risk of certain diseases. This demand has led to the development and production of functional foods which incorporate bioactive compounds such as prebiotics and probiotics (Ashwell, 2002; Batista et al., 2017).

Prebiotics comprise a wide range of compounds, within which, lactose derivatives such as lactobionic acid (LBA) have generated great interest. This compound is very useful in the food industry, as it is used as an antioxidant, moisturizer, chelating agent (Cardoso, Marques, Dagostin, & Masson, 2019) and as a prebiotic (Alonso, Rendueles, & Díaz, 2013). Furthermore, LBA is resistant to digestive enzymes and is badly absorbed in the small intestine, so it can be metabolised by gastrointestinal microflora (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003; Schaafsma, 2008). There are very few studies into commercial food products with LBA (García, Bautista, Rendueles, & Díaz, 2018; Sáez-Orviz, Camilleri, Marcet, Rendueles, & Díaz, 2019), although one of the most noteworthy investigates "Caspian Sea yogurt" (Kiryu et al., 2009), which was traditionally produced in the Caucasian region before its arrival in Japan, more than 30 years ago. The amount of LBA ingested when consuming this yogurt is between 0.5 and 1.0 g per year, and no harmful effects have been noted in the population (Kiryu et al., 2009). Nonetheless, at the moment, LBA has only been approved by the Food and Drug Administration (FDA) for its use in food in its salt form (Alonso et al., 2013; Cardoso et al., 2019; FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration, 2017), although future approval of LBA by the food authorities is expected.

Focussing now on probiotics, these can easily be added to a variety of food products, the most frequently chosen being those with a dairy matrix. Among the most commonly used probiotics are those belonging to the genera *Lactobacillus* and *Bifidobacterium* (Espitia, Batista, Azeredo, & Otoni, 2016). Furthermore, if probiotic strains and prebiotic compounds are mixed in the same product, the result is a synbiotic product. There are many dairy products in which probiotic and prebiotic are added together in milk before the cheese is made, in order to obtain a synbiotic product directly (Langa et al., 2019). However, in the case of LBA this process is impractical due to its high solubility in water (Alonso et al., 2013; Cardoso et al., 2019), which means that during the cheese curdling process this compound tends to be lost to the whey and does not get trapped in the curd. Therefore, an alternative means of obtaining a synbiotic functional food product through the incorporation of LBA in

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cheese is the use of bioactive coatings containing this prebiotic. Bioactive coatings allow different types of functional products to be developed, as they are very versatile (Espitia et al., 2016; Pavli et al., 2017). These types of coatings are an excellent option for maintaining the viability of the probiotic strain and also allowing the preservation of LBA in the food product.

In this regard, there are very few studies on food products with LBA (García et al., 2018; Sáez-Orviz et al., 2019), other than the few cases indicated previously. Therefore, the aim of this study is to develop and characterize a synbiotic coating, containing both a probiotic strain (*L. plantarum* CECT 9567) and a prebiotic compound (LBA), to take advantage of the properties of LBA. This coating is tested in cottage cheese, a real food model, with assessment of the LBA and probiotic concentrations in the coatings during storage time, the influence of the coating composition on the textural properties of the cheese and the effect of the LBA concentration in the coating on the protection offered to the probiotic bacteria as they pass through a simulated gastrointestinal tract (GIT).

2. Materials and methods

2.1. Probiotic microorganism and growth conditions

Lactobacillus plantarum CECT 9567 (from the Spanish Type Culture Collection, Valencia, Spain) was used as a probiotic strain and was maintained frozen (in 40% v/v glycerol solution at −20 °C). It was propagated on MRS (de Man, Rogosa and Sharpe, Steinheim, Germany, Sigma-Aldrich) agar plates, incubated for 48 h at 37 °C and then stored at 4 °C. As the microorganism is aerobic, its culture was carried out in liquid medium in a 500 mL Erlenmeyer flask containing 100 mL of MRS broth (medium volume to air ratio of 1:4), which was incubated in an orbital shaker at 200 rpm and at a temperature of 37 °C for 12 h.

2.2. Cottage cheese manufacture

Goat milk was supplied by a local farm from San Martín del Rey Aurelio (Asturias, Spain). Milk was pasteurised with a low temperature-long time (LTLT) procedure (at a temperature of 60 °C for 25 min). After pasteurization, milk was cooled to 34 °C and rennet was added (0.0025 g L^{-1}, Chy-Max®, CHR-Hansen, Denmark). No starter culture was added due to the soft pasteurization process that allows some lactic acid bacteria to survive. After incubating milk at 34 °C for 40 min, the curd was cut multiple times to stimulate syneresis. Finally, cottage cheese samples were made with 10 g of curd per piece.

2.3. Bioactive coating preparation

Cottage cheese was left for one hour at 4 °C and then given different coatings by a dipping process. The film-forming solution was prepared as follows. Sodium alginate (Sigma-Aldrich) was dissolved in distilled water to a concentration of 20 g L^{-1} and this solution was heated at 70 °C and kept in agitation at 700 rpm until a clear, homogeneous colour was obtained. Glycerol (Panreac S.A., Barcelona, Spain) was then added to reach a concentration of 15 g L^{-1} in the final volume and the coating-forming solution was cooled to room temperature. On the basis of previously performed preliminary experiments, for the prebiotic and synbiotic coatings LBA was added to the film-forming solution at a high concentration (40 g L^{-1}, PRE4 sample) and at a medium concentration (20 g L^{-1}, PRE2 sample), taking into consideration the amount of alginate in the film-forming solution. Then, this mixture was homogenized by stirring at 500 rpm at room temperature. In order to prepare the synbiotic coatings, after 12 h of growth, the *L. plantarum* culture was centrifuged at 13200 rpm for 10 min and the pellet was added to the coating-forming solutions PRE2 and PRE4 to reach a concentration of 10^9 CFU mL^{-1} in the final volume, thus forming the coatings SYN2 and SYN4. In addition, a probiotic alginate-based coating (PRO) with a concentration of *L. plantarum* of 10^9 CFU mL^{-1} but without LBA, and a negative control with only the alginate coating (NC2), were prepared. All the prepared coatings and their formulations are shown in Table 1.

Once all the solutions were prepared, the cheese was submerged in the coating solution for 2 min, and afterwards it was left at room temperature for 1 min to remove the excess solution. To harden the coating, the cottage cheese was immersed for 2 min in $CaCl_2$ solution (5% w/v, Merk KGaA, Darmstadt, Germany). Finally, the pieces were dried at room temperature for 30 min and then stored in a sterile container at 4 °C for subsequent analysis (Bambace, Alvarez, & Moreira, 2019). A negative control cottage cheese sample with no kind of coating was also prepared (NC1). All the experimentation was carried out under sterile conditions to avoid external contamination. A total of 18 pieces of cottage cheese of each type of coating were prepared.

2.4. Effect of storage time on probiotic counts and lactobionic acid concentration in the coatings

The growth of microorganisms inside the coatings (PRO, SYN2 and SYN4 cottage cheese samples) and the LBA concentration (PRE2, PRE4, SYN2 and SYN4 cottage cheese samples) were followed for 15 days, sampling taking place at day 0, and on days 1, 3, 6, 10 and 15. Three independent cottage cheese samples with each type of coating were tested at each time. The sampling was adapted from Pavli et al. (2017) and performed as follows. A piece of coating (1 cm^2) was peeled from the cottage cheese and washed with sterile distilled water in order to eliminate other lactic acid bacteria. The coatings were placed in a Stomacher™ bag (Seward, UK) with 1 mL sodium citrate 1% (pH 6.0) (Sigma-Aldrich) and heated for 30 min to 40 °C to break them down. The progress of microbial growth was analysed by making serial dilutions (1:10) (Pavli et al., 2017) employing NaCl 0.7% (w/v) (Sigma-Aldrich) and incubating on MRS agar plates for 48 h at 30 °C. The size and weight of the cottage cheeses samples were previously measured, which allowed the grams of cottage cheese per cm^2 of coating to be calculated and the expression of the results in CFU g^{-1} of cheese. The LBA content was measured according to Sáez-Orviz et al. (2019) using High Performance Liquid Chromatography equipment (HPLC) (Agilent 1200, Agilent Technologies Inc., Santa Clara, CA, USA). A Coregel ION 300 column (Teknocroma, Barcelona, Spain), coupled to a refractive index detector (at a temperature of 40 °C) was employed. Sulphuric acid solution (0.0450 mM L^{-1}, pH 3.1) was used with a flow rate of 0.3 mL min^{-1} and a column temperature of 75 °C. Data acquisition and analysis were performed with ChemStation software (Agilent). The microorganism and LBA concentrations were expressed per gram of coated cheese.

Table 1
The different cottage cheeses and their coatings.

Cottage cheese	Coating-forming solution	Lactobionic acid (LBA)	*L. plantarum* CECT 9567
Negative control 1 (NC1)	No	–	–
Negative control 2 (NC2)	Yes	–	–
Prebiotic cheese (PRE2)	Yes	20 g L^{-1}	–
Prebiotic cheese (PRE4)	Yes	40 g L^{-1}	–
Probiotic cheese (PRO)	Yes	–	10^9 CFU mL^{-1}
Synbiotic cheese (SYN2)	Yes	20 g L^{-1}	10^9 CFU mL^{-1}
Synbiotic cheese (SYN4)	Yes	40 g L^{-1}	10^9 CFU mL^{-1}

2.5. Mechanical properties of the coated cheese

The texture was analysed with a TA.XTplus Texture Analyzer (Stable Systems, Godalming, Surrey, UK). Cottage cheese samples were subjected to a penetration test, at room temperature. The spherical probe employed was SMS P/0.5S with a test speed of 2.0 mm s^{-1} and a 5 kg load cell. Results are expressed in terms of firmness and stickiness values (grams). Three independent cottage cheese samples with each type of coating and NC1 were analysed in triplicate. The reported results correspond to the mean value.

2.6. Distribution of Lactobacillus plantarum CECT 9567 in the coatings

In order to determine the distribution of the probiotic organisms inside the bioactive coatings, they were analysed using fluorescence microscopy. For sampling purposes, a fragment of each of the different coatings (PRO, SYN2 and SYN4) was taken, as well as another from a control cheese covered exclusively with the coating-forming solution with no pro- or prebiotic (NC2). These fragments were washed with distilled water twice and then placed for 3 min in a solution containing 0.1% acridine orange (Sigma-Aldrich), previously dissolved in 67 mM phosphate buffer (pH 6.0). Samples were washed with phosphate buffer for 1 min and then placed for 30 s in 100 mM $CaCl_2$. Finally, the samples were dried, mounted on a slide and sealed and observed with a Leica TCS-SP-AOBS spectral confocal laser microscope ($\lambda_{excitation}$ 480 nm, $\lambda_{emitting}$ 508–603 nm). Photographs were taken at day 0 and day 7 (from day of cheese making), after which point no further differences were observed.

2.7. Simulated digestion test of the bioactive coatings

A simulated digestion test of the coatings of the PRO, SYN2 and SYN4 cheese samples was performed only to check the survival of the probiotic, as LBA is a non-digestible fibre, resistant to human digestive enzymes (Cardoso et al., 2019). The concentration of microorganisms in the coatings was determined after each digestive fluid test and calculated per gram of coated cheese. In addition, to determine the survival of the free bacteria in the digestive fluids, an MRS broth culture with a concentration of probiotic of 10^9 CFU mL^{-1} was also tested (control sample).

Gastric and intestinal conditions were simulated according to Minekus et al. (2014) with some modifications. The composition of the simulated gastric fluid (SGF) employed was 0.517 g L^{-1} KCl, 0.123 g L^{-1} KH_2PO_4, 2.106 g L^{-1} $NaHCO_3$ and 2.75 g L^{-1} NaCl. Simulated intestinal fluid (SIF) was prepared by mixing 0.509 g L^{-1} KCl, 0.110 g L^{-1} KH_2PO_4, 11.68 g L^{-1} $NaHCO_3$ and 2.24 g L^{-1} NaCl (all from Sigma-Aldrich).

Firstly, the concentration of microorganisms in the coatings was checked at time zero, before the gastric and intestinal digestion were carried out. For that purpose, an area of 10 cm^2 of coating was peeled from the cottage cheese samples and analysed as described below. Three independent samples with each type of coating were analysed.

In order to simulate the gastric digestion of the coatings, coating samples peeled from the surface of coated cheeses (~10 cm^2) were mixed with 18.4 mL SGF, 1.6 mL pepsin (863 U mg^{-1} protein, CAS 9001-75-6, Sigma-Aldrich) with a concentration of 15.15 g L^{-1}, using SGF as solvent, 5 μL of $CaCl_2$ 0.3 M (Sigma-Aldrich) and 0.696 μL of distilled water. The pH was adjusted to 3.0 with 1 M HCl (Sigma-Aldrich). Then, the mixtures were shaken at 300 rpm and 37 °C for 90 min and coating samples (measuring 1 cm^2) were collected at the end of the gastric phase. In the case of the control sample, 1 mL of the bacterial suspension was added directly to the gastric fluid prepared as explained above and was incubated at the same conditions of agitation and temperature. After the simulated gastric digestion 1 mL of fluid was collected.

After the gastric simulation, intestinal simulation was carried out. For that purpose, 12.5 mL of SIF, bovine chymotrypsin (0.3% w/v) (60 U mg^{-1} protein, EC 232-671-2, Sigma-Aldrich), porcine pancreatin (0.1% w/v) (Sigma-Aldrich), 40 μL of 0.3 M $CaCl_2$ and 1.3 mL of distilled water were added to the fluids resulting from the gastric digestion. The pH was adjusted to 7.0 using 1 M NaOH (Sigma-Aldrich) and the mixtures were shaken at 300 rpm and 37 °C for 2 h. At the end of the intestinal digestion, coating samples (1 cm^2) were collected and analysed. In the case of the control, 1 mL of the intestinal fluid was collected at the end of this treatment.

All coating samples (taken at time zero, and then after the gastric digestion and after the intestinal digestion) were broken down by immersion for 30 min at 40 °C in 20 mL of a 1% (pH 6.0) sodium citrate solution. An aliquot of 1 mL was taken from every sample tested and serial dilutions (1:10) with NaCl 0.7% (w/v) were incubated on MRS agar plates (30 °C for 48 h). Regarding the control, the 1 mL of sample of fluid collected after the gastric and intestinal digestion was centrifuged (10000 rpm, 10 min), the pellet resuspended in 20 mL of NaCl 0.7% (w/v) and incubated on MRS agar plates. Each sample was carried out in triplicate. Plates were incubated at 30 °C for 48 h. Results were expressed in CFU g^{-1}.

2.8. Statistical analysis

All experiments were carried out in triplicate with three independent batches of coated cottage cheese and the reported results correspond to the mean value. Analysis of variance (ANOVA) was applied. The method used to determine significant differences between the data is Fischer's Least Significant Difference (LSD) procedure. The analysis was performed using Statgraphics 18® Centurion statistical software.

3. Results and discussion

3.1. Growth and viability of Lactobacillus plantarum CECT 9567 in the bioactive coating during storage

The number of viable bacteria in the coating during storage is of the greatest importance for the development of a functional product, because if their level of survival is not sufficiently high, the food product cannot be considered probiotic. There is no consensus on the specific number of microorganisms that must be consumed to obtain a beneficial effect, but the minimum estimated quantity is 10^6 CFU mL^{-1} or g^{-1} of product (Angiolillo, Conte, Faccia, Zambrini, & Del Nobile, 2014; Aureli et al., 2011; Kechagia et al., 2013; Pavli et al., 2017).

Results for probiotic growth during storage are shown in Fig. 1, which indicates the initial microbial growth observed in every sample tested, followed by a decrease stage and a final stabilisation stage at the end of the experiment. PRO, SYN2 and SYN4 cheese samples reached a final concentration of 6.53, 6.72 and 7.23 log CFU g^{-1}, respectively. The

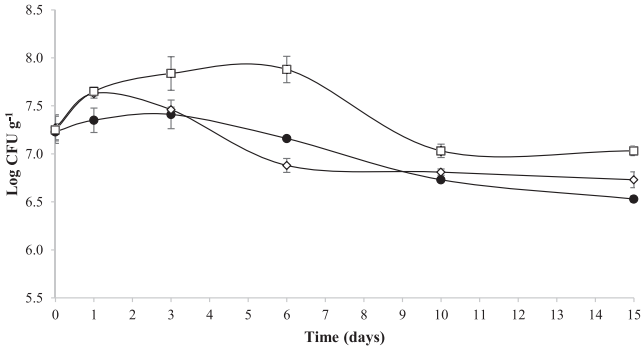

Fig. 1. Growth of *L. plantarum* CECT 9567 in the coating during 15 days of storage. The concentration of microorganisms is represented as log CFU per gram of cheese. (●) probiotic cottage cheese (PRO), (◇) synbiotic cottage cheese with 2% of LBA (SYN2), (□) synbiotic cottage cheese with 4% LBA (SYN4). Experiments were carried out in triplicate and reported results correspond to the mean value.

best result was obtained with SYN4, showing significant differences with PRO and SYN4 cheeses ($P < 0.05$). In addition, as they reach the established minimum required concentration, all these coated cheeses would attain the category of "probiotic" during the storage period analysed.

Furthermore, it must be borne in mind that sodium alginate is a good matrix for maintaining the viability of the probiotic. There are a number of studies in which this polysaccharide has been used as a coating in different types of food products using different species of probiotic microorganisms. For example, different species of *Lactobacillus*, such as *L. plantarum* B282, *L. plantarum* L125 and *L. pentosus* L33, were added to an alginate-based film-forming solution to produce coatings that were used to cover ham, obtaining values over 6 log CFU g^{-1} after 70 days of sampling (Pavli et al., 2017).

The concentration of LBA in the coatings was analysed during 15 days of storage and the results are shown in Fig. 2, where it can be observed that there is a rapid decline in the LBA concentration during the first three days of the experiment with the PRE2 and PRE4 samples, which may be due to the lactic acid bacteria present in the cottage cheese. If some of the LBA is accessible to these bacteria, they may be using it as a substrate. However, from day 4, the concentration remains constant in both these cases (0.95 mg LBA g^{-1} of cheese for PRE2 and 1.13 mg LBA g^{-1} of cheese for PRE4). In the synbiotic cheeses (SYN2 and SYN4), the concentration remains constant from the beginning of the experiment and throughout it in both coatings (0.912 mg LBA g^{-1} of cheese for SYN2 and 1.07 mg LBA g^{-1} of cheese for SYN4 at the end of this experiment). In any case, the amount of LBA that would be ingested with the coated cheese samples PRE2, PRE4, SYN2 and SYN4 at the end of the storage time would fall within the usual intake values measured in Japan with the "Caspian Sea yogurt" (Kiryu et al., 2009) and are far lower than the values that would cause lactose intolerance-like effects (24 g LBA day^{-1}) (Cardoso et al., 2019).

It must also be pointed out that the concentration of LBA is lower for SYN samples than for PRE samples at the beginning of storage time, with significant differences at zero time ($P < 0.05$) (Fig. 2). These observed differences may occur due to the consumption of LBA by *L. plantarum* during the preparation of the coated cheese samples. The initial concentration of this bacteria in the coating solution could be considered high (9 log CFU mL^{-1}) and the LBA is the only carbon source that can be consumed by the microorganisms in the coat-forming solution. Although Fig. 1 shows no difference in the initial concentration of the probiotic in PRO, SYN2 and SYN4 samples, it should be noted that only viable cultivable bacteria were being counted, and the improvement in the growth caused by the presence of LBA is particularly noticeable during the first day of storage. The following days, the concentration of LBA in the SYN2 and SYN4 coatings remains constant, which is likely to be because the remaining LBA is not accessible to the bacteria, or because the bacteria cannot continue to grow because of space limitation in the coatings.

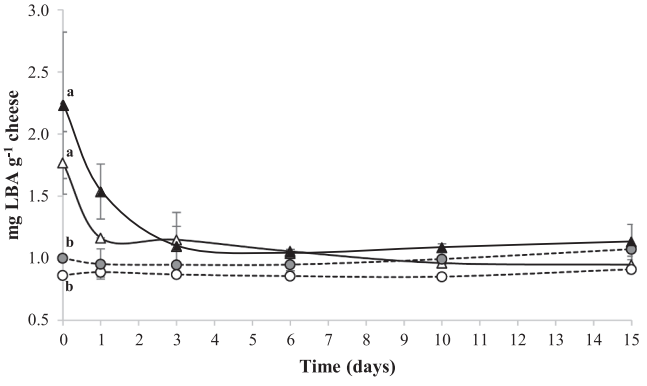

Fig. 2. Changes in the concentration of LBA in the coating during 15 days of storage. LBA concentration is represented as mg of LBA per g of cheese. (△) prebiotic cottage cheese with 2% of LBA (PRE2), (▲) prebiotic cottage cheese with 4% LBA (PRE4), (○) synbiotic cottage cheese with 2% LBA (SYN2), (●) synbiotic cottage cheese with 4% LBA (SYN4). There are only significant differences ($P < 0.05$) between PRE2/PRE4 and SYN2/SYN4 at time 0. Different letters indicate significant differences at the same storage time. Experiments were carried out in triplicate and reported results correspond to the mean value.

These results confirm that prebiotics can be incorporated into the coatings to improve the stability and viability of the probiotic strains. In the case of the higher concentration (SYN4), the presence of LBA exerted a positive effect, since at the last sampling time of the experiment an increase was observed in the concentration of probiotic in SYN4 samples of 80.11%, as compared with the probiotic concentration in PRO cottage cheeses.

The increase in the viability of probiotics in coatings when prebiotics are present has been observed by other authors. In particular, similar results were obtained with cheeses coated with sodium alginate and FOS (fructo-oligosaccharides) as a prebiotic and using lactic acid bacteria as the probiotic bacteria. The presence of FOS made it possible to improve the survival of the probiotic, which is capable of using this compound as a substrate, maintaining a concentration of 10^9 CFU 100 g^{-1} of cheese when employing *Lactobacillus rhamnosus* GG as the probiotic (Angiolillo et al., 2014). There are also examples of the beneficial effect of prebiotics in sodium alginate bioactive coatings employed in non-dairy products, specifically, in products such as blueberries. In this case, the sodium alginate coatings were enriched with inulin and oligofructose as FOS, which led to an increase in the viability of *L. rhamnosus* CECT 8361 compared to the results in coatings that lacked prebiotics (Bambace et al., 2019). In other food products, such as fresh-cut apples, sodium alginate bioactive coatings had inulin and oligofructose as prebiotics and *L. rhamnosus* GG as the probiotic. Using these coatings, pieces of apple with a bacterial concentration of 10^8 CFU g^{-1} were obtained, which is enough to attain a probiotic effect (Rößle, Brunton, Gormley, Ross, & Butler, 2010). Therefore, the mixture of prebiotics and probiotics in coatings makes it possible to obtain improved products that are beneficial to consumers because prebiotics enable higher persistence of probiotics in the foodstuffs. There are several studies on the preparation of synbiotic goat's milk products. Most of them investigate yoghurt and ice cream, with very little research having been devoted to cheese (Verruck, Dantas, & Schwinden, 2019), the subject of the work described here.

3.2. Textural characterization

To develop a new foodstuff, it is essential to study and analyse its structure in order to understand and improve its texture, which is an important factor in the sensory perception of consumers (Wilkinson, Dijksterhuis, & Minekus, 2001; Zhong, Cavender, & Zhao, 2014). Texturometry allows different parameters, like firmness and stickiness, to be measured and in this case, the results of the textural characterization of the coated cheeses are shown in Fig. 3.

Although there is variability in terms of firmness between the different types of coatings, the highest average values were found in NC1 coated cheeses (Fig. 3-A). The mean value for this parameter for uncoated cottage cheese (NC1) increased after three days, and then remained constant over time, which suggests a higher loss of water during the first three days of the experiment for this uncoated sample in comparison with the coated cheese tested. This evidence corroborates the positive influence of such coatings on the preservation of moisture, and their role in delaying the hardening process when they are used to coat pieces of cheese (Costa, Maciel, Teixeira, Vicente, & Cerqueira, 2018; Ramos et al., 2012; Zhong et al., 2014). In the other coated samples, although there are statistically significant differences between some of the samples tested for this parameter, the similar average values obtained suggest that the presence of LBA or *L. plantarum* did not produce any clear differential effect on the firmness of the samples assessed (Fig. 3-A).

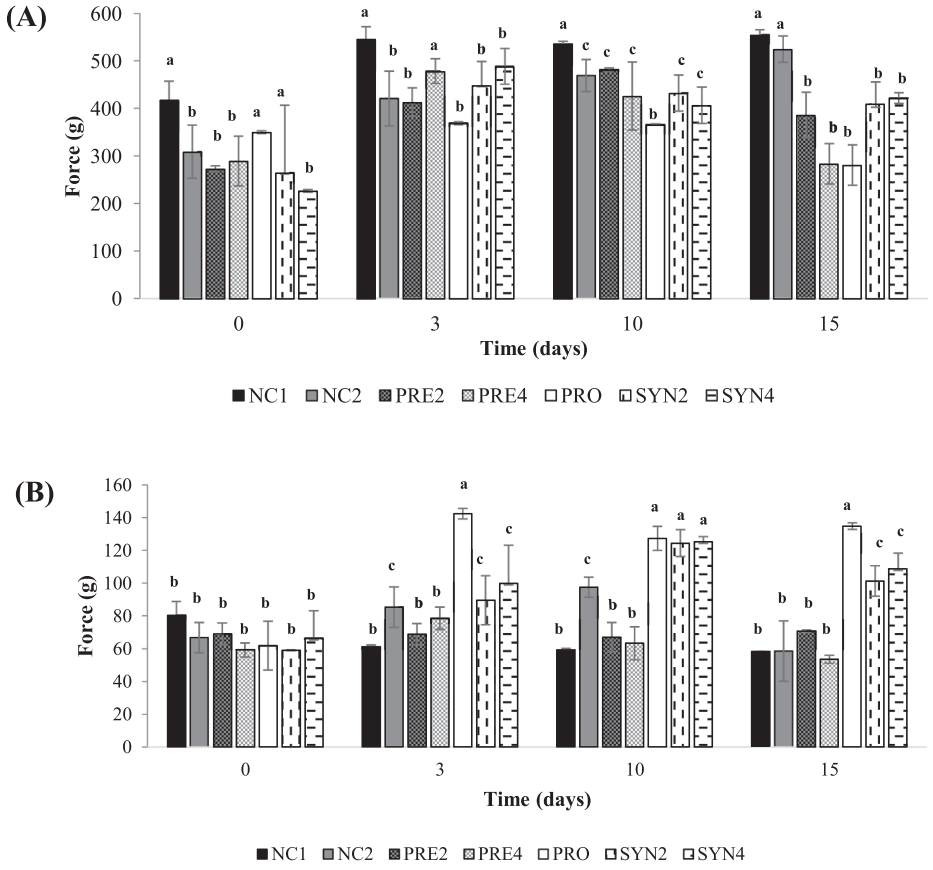

Fig. 3. Textural analysis of coated cottage cheeses; (A) firmness analysis and (B) stickiness analysis (data represented as absolute value) with the standard deviation. Both are expressed in force units (g). Different letters indicate significant differences at the same storage time ($P < 0.05$). Experiments were carried out in triplicate and reported results correspond to the mean value.

Regarding the stickiness, there are differences depending on the sample and on the storage time (Fig. 3-B). At zero-time there are no significant differences between the samples. As the period of storage time increased, the loss of water due to the ripening of the cheese caused changes in the stickiness and it was observed that LBA and the probiotic noticeably affected this parameter. PRO showed the highest stickiness values obtained after 3 and 15 days of assay. When LBA was incorporated together with *L. plantarum*, the pieces of cheese assessed (SYN2 and SYN4) showed higher values for this parameter than PRE2 and PRE4 samples, but not as high as those values for the PRO coating. It suggests

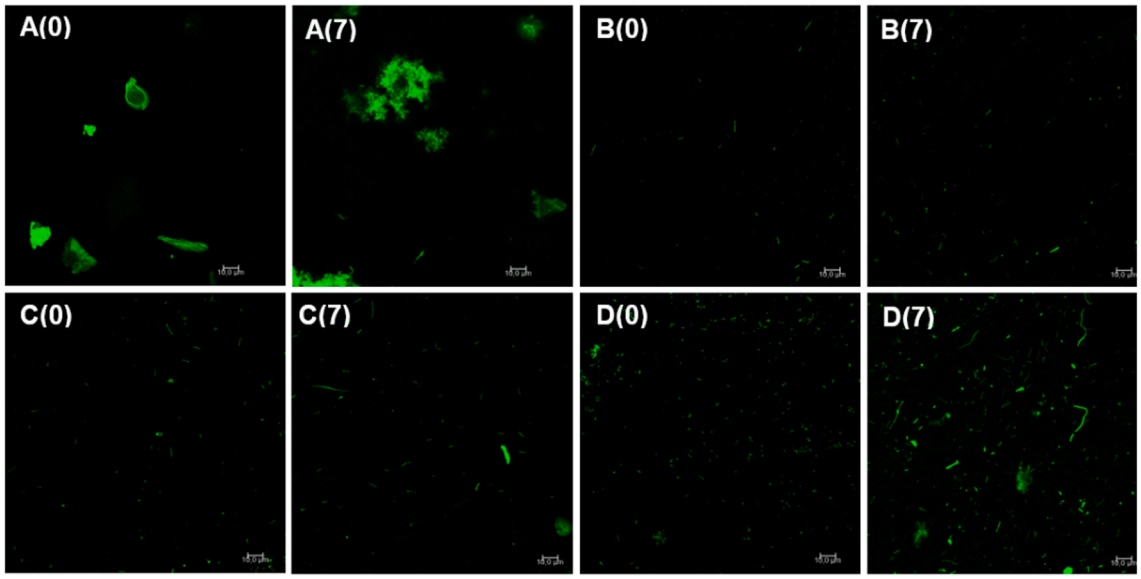

Fig. 4. Fluorescence microscopy images of the coatings at zero time and after seven days of storage at 4 °C. (A) Negative control. (B) PRO, (C) SYN2 and (D) SYN4 coatings. Numbers 0 and 7 in the images refer to each of the sampling times.

that the presence of the probiotic bacteria produced an increase in the sample's stickiness, maybe owing to the excretion of cellular metabolites or the growth of the microorganism population embedded within the coating, but this trend was at least partially limited by the incorporation of LBA in the coating composition.

3.3. Status of Lactobacillus plantarum CECT 9567 in the bioactive coatings

A fluorescence microscopy technique was used to observe *L. plantarum* CECT 9567 inside the probiotic and synbiotic coatings. A coating without any bacteria was also analysed as a negative control. Results at time 0 and after 7 days of storage at 4 °C are shown in Fig. 4.

The visual appearance of the coatings was transparent and similar in all cases. As expected, no bacteria were observed in the negative control, but some large, amorphous structures were found. This is because acridine orange is capable of staining certain biological compounds, such as proteins and fat molecules (Yiu, 1985). Specifically, in the case of matrices such as cheese, acridine orange is used especially for dyeing proteins (Heilig, Göggerle, & Hinrichs, 2009). Therefore, the voluminous structures observed could be caseins that may remain attached to the film as these structures were also observed in the images of the probiotic and synbiotic coatings.

Fluorescent bacillus-shaped structures were differentiated, indicating the presence of *L. plantarum* inside the coatings. At time 0, the amount of *L. plantarum* observed in the PRO and SYN coating samples was quite similar. After 7 days of storage, a higher number of microorganisms were observed in the films. More bacteria were observed in the SYN coatings compared to the probiotic one, illustrating the synergic effect existing between *L. plantarum* CECT 9567 and LBA. Comparing the SYN2 and SYN4 coatings, the one with 4% LBA showed a higher number of microorganisms, a result that agrees with the data in Fig. 1.

3.4. Simulated digestion of the bioactive coatings

Probiotics and prebiotics have the capacity to modify the GIT microflora by enhancing the growth of beneficial bacteria. Probiotics act mostly in the small intestine, while prebiotics usually act in the colon (Lopez-Rubio, Gavara, & Lagaron, 2006). In order to be effective as a probiotic, bacteria must withstand gastric juices and be able to proliferate in the intestine. In this case, to test the protective effect on *L. plantarum* CECT 9567 of the alginate-based cheese coatings, the cottage cheese samples PRO, SYN2 and SYN4 were assessed in a simulated digestion test. *L. plantarum* CECT 9567 without coating (9 log CFU mL^{-1}) was also used as control (Fig. 5).

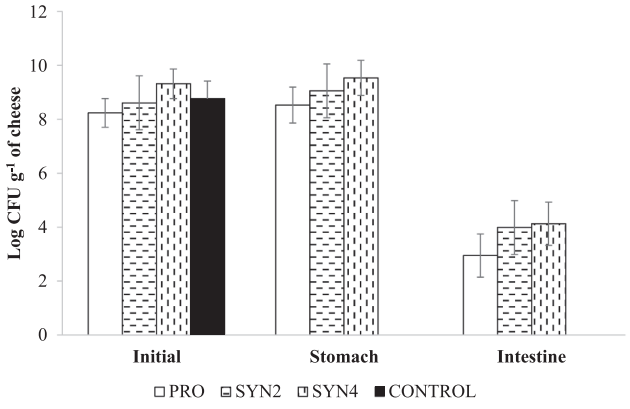

Fig. 5. Concentration of probiotic (log CFU g^{-1} of cheese) throughout the digestion test. "Initial" refers the concentration of microorganism before the digestion trial. Experiments were carried out in triplicate and reported results correspond to the mean value.

At the initial time, the microbial concentration was similar for every coating, in the range of 8–9 log CFU g^{-1}. After the digestive simulation of the stomach, no survival of the microorganism without coating was observed. It is known that in the gastric phase, the long exposure to an acid pH, together with the presence of pepsin, cause the death of microorganisms (Chan & Zhang, 2005). However, the bacteria inside PRO, SYN2 and SYN4 coatings did survive, with a mean value of 8.53, 9.06 and 9.54 log CFU g^{-1}, respectively, showing that the coating exerted a protective effect. Sodium alginate is a water-soluble polymer but with a limited solubility at low pH values (Parreidt, Müller, & Schmid, 2018). The use of $CaCl_2$ could increase the insolubility of sodium alginate, due to the ability of calcium to bind cell wall polymers, making them stronger and allowing microorganisms to remain alive embedded in the coating during digestion in the stomach.

After the intestinal simulation, the probiotic load decreased abruptly. The change of the pH (~pH 7.0) and the presence of different enzymes may have affected the integrity of the coating. After 2 h, the PRO coating showed a mean concentration of microorganisms of 2.95 log CFU g^{-1}, SYN2 of 3.99 log CFU g^{-1} and SYN4 of 4.13 log CFU g^{-1} (Fig. 5). As the initial bacterial concentration in the PRO, SYN2 and SYN 4 coatings was different, the microbial reduction (in log CFU g^{-1}) between the gastric phase and the intestinal phase was statistically analysed. The average microbial reduction was 5.60 ± 0.12, 5.01 ± 0.23 and 5.21 ± 0.17 log CFU g^{-1} in PRO, SYN2 and SYN4, respectively. It was found that there were significant differences between the SYN2-SYN4 and PRO samples ($P < 0.05$). Therefore, the viability was higher for the coatings that included LBA in their composition (SYN2 and SYN4), which may be because the addition of prebiotics increases the resistance of microorganisms in the GIT environment (Lopez-Rubio et al., 2006). In support of this idea, there are many studies describing the improvement in the viability and in the tolerance of probiotics to simulated *in vitro* GIT conditions when prebiotics such as FOS and GOS (galacto-oligosaccharides) are included in the food composition (Krasaekoopt & Watcharapoka, 2014; Langa et al., 2019; Orozco-Parra, Mejía, & Villa, 2020; Padilha, Morales, Vieira, Costa, & Saad, 2016; Ranadheera, Baines, & Adams, 2010). In the experiments carried out here, LBA seems to have the same effect as the other more frequently studied prebiotics.

Furthermore, it is important to bear in mind that probiotics are only effective if the dosage is sufficiently high. Although there is no consensus within the international scientific community about a specific concentration necessary to obtain beneficial health effects, the minimum dosage is between 10^6-10^9 CFU per day (Espitia et al., 2016; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013), but these bacteria must resist passage through the digestive tract in order to modify the GIT microflora. The recommended daily fresh cheese intake is between 80 and 125 g (Carcamo Vargas & Mena Bastías, 2006), and therefore, taking into consideration the digestion testing results for the coatings and a portion of 125 g, the only coated cheeses tested here that meet the conditions to qualify as probiotic after *in vitro* digestion are SYN2 and SYN4, with values of 9.24 ± 0.56 and 9.56 ± 0.55 log CFU $portion^{-1}$ (before digestion) and 6.89 ± 0.81 and 7.03 ± 0.80 log CFU $portion^{-1}$ (after *in vitro* digestion), respectively (Table 2). Regarding LBA, as it is a prebiotic, it is a non-digestible fibre and is resistant to human digestive enzymes (Cardoso et al., 2019), reaching the large intestine intact (Sáez-Orviz et al., 2019).

Table 2
Concentration of probiotic in a portion (125 g) of coated cottage cheese after and before *in vitro* digestion.

	Concentration of probiotic before *in vitro* digestion (log CFU $portion^{-1}$)	Concentration of probiotic after *in vitro* digestion (log CFU $portion^{-1}$)
PRO	8.95 ± 0.53	5.84 ± 0.80
SYN2	9.24 ± 0.56	6.89 ± 0.81
SYN4	9.56 ± 0.55	7.03 ± 0.80

As can be seen from the results obtained, PRO, SYN2 and SYN4 would meet the minimum legal requirements to attain the probiotic category but only the synbiotic ones (SYN2 and SYN4) would also have the capacity to deliver the adequate probiotic dose to the GIT.

4. Conclusions

The development of bioactive coatings to prepare synbiotic functional products with LBA and *L. plantarum* CECT 9567 was carried out successfully. PRO, SYN2 and SYN4 coated cottage cheeses meet the minimum legal requirements (10^6 CFU g^{-1} cheese) to attain the category of probiotic. PRE2, PRE4, SYN2 and SYN4 cheeses contain an adequate amount of LBA, equivalent to the regular intake of the only product containing LBA on the market ("Caspian Sea yogurt"). Texturometry experiments showed that the uncoated cottage cheeses were firmer and those with a probiotic coating showed slightly higher stickiness, although no great variations between samples were detected. After the simulated digestion tests, it was found that SYN2 and SYN4 cheeses were the only ones that would have the capacity to deliver an adequate number of probiotic organisms to the lower GIT, which leads to the conclusion that LBA increased the viability and tolerance of the probiotic as it passed through the GIT. With a view to further investigation, it is important to state that organoleptic properties, such as colour, taste, smell and texture are some of the most important parameters for new products to be accepted by consumers, so it would be of great interest to carry out a sensory study on these cheeses in the future. Therefore, the development of bioactive coatings, in the case of SYN2 and SYN4, makes it possible to obtain synbiotic, functional and innovative cottage cheeses that could, from a nutritional point of view, have a high level of acceptance by consumers.

5. Ethics statement file

This research did not include any human subjects and/or animal experiments.

CRediT authorship contribution statement

S. Sáez-Orviz: Methodology, Formal analysis, Investigation, Writing - original draft. **C. Puertas:** Investigation. **I. Marcet:** Conceptualization, Writing - review & editing. **M. Rendueles:** Conceptualization, Writing - review & editing. **M. Díaz:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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