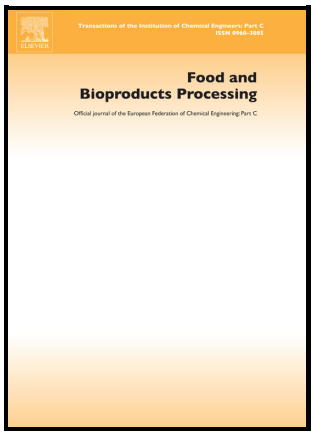

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PHYSICOCHEMICAL AND FUNCTIONAL CHARACTERISATION OF A FOOD INGREDIENT BASED ON OKARA CONTAINING PROBIOTICS

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ABSTRACT

A functional ingredient (OI) was prepared from fresh okara (ORM) by fermentation in solid state with *Lacticaseibacillus casei* (ATCC 393). The aim of the present work was to determine the physical, chemical, and functional characteristics of OI. The final product, OI, was a stable powder with aw: 0.485±0.003 and moisture content: 5.7±0.7% (w.b.), mainly composed by cell wall material (62%, d.b. alcohol insoluble residue, AIR) and protein (38.1%, d.b.). The stabilisation and fermentation processes reduced significantly ($p<0.05$) the AIR content of the ORM. Higher functional properties, swelling (SC), water holding (WHC) and oil holding (OHC) capacities (6.14±0.01g.cm-3; 5.4±0.2g.g-1; 3.4±0.6g.g-1, respectively), were observed in OI respect to the ORM. The probiotic initial count in OI was 10±1 log (CFU.g-1) and could

be safety stored at 25°C, showing 7.5±0.4 log (CFU.g-1) at 63 days storage. The cells resisted the simulated gastric and intestinal stress < 80% and < 65%, respectively. This property was maintained during the storage of OI. However, the adherence ability to Caco-2 cells decreased to ≈0.2% possibly due, in part, to the OI matrix interference. Overall, okara can be profit for developing functional ingredients containing L. casei cells, with the possibility of being stored safety at room temperature.

KEYWORDS

Okara, *Lacticaseibacillus casei,* Vegetable by-product, Physicochemical characterisation, Adhesion, Gastrointestinal resistance.

ABBREVIATIONS

Functional ingredient (FI), Dietary fibres (DF), Solid-state fermentation (SSF), Man, Rogosa, and Sharpe (MRS), Lactic acid bacteria (LAB), Okara Inoculated (OI), Okara control (OC), Okara raw material (ORM), Bulk density (ρ_b), Water activity (a_w), Alcohol insoluble residue (AIR), Water holding capacity (WHC), Swelling capacity (SC), Oil absorption capacity (OHC), Water-soluble fraction (WSF), standard deviation (SD), Fourier transform infrared spectroscopy (FTIR), Simulated gastrointestinal resistance (SGIR), Gastric digestion (GD), Intestinal digestion (ID), free cells (FC), Scanning Electron Microscopy (SEM).

1. INTRODUCTION

Nowadays, it is known that food Influences on people's health. Therefore, consumers require, in terms of the quality of their food, the existence of active components that have the potential to provide benefits to the body. So, the adoption of innovative foods must contain not only the concept of quality, but also the addition of so-called functional foods giving additional value (Palanivelu et al., 2022). The word "functional" is frequently used to refer to foods that are proved to have specific physiological benefits and to be useful to the human body in some way (Dinkçi et al. 2019). However, at the

moment there is no universally stipulated definition of what constitutes a functional food (Palanivelu et al., 2022). Functional foods can contain phytochemical agents, such as antioxidants, vitamins, fibre, minerals, fatty acids, and also probiotics. Probiotics are described as live bacteria that, when supplied in sufficient proportions, can provide health benefits by maintaining the balance of microorganisms in the human gastrointestinal tract, preventing various diseases, and stimulating the host's immune system (Frakolaki et al., 2020). In particular, *Lactobacillus casei (Lacticaseibacillus paracasei*, ISSAP 2020) has shown interesting probiotic functionalities such as, surviving under simulated gastric conditions (pH 3 at 37 °C); reducing inflammation and development of dental caries; and decreasing a phytic acid content in fermented food mixtures (Arepally et al., 2022). Previously, authors could corroborate some of these properties, and also other technological characteristics, for example ~~as~~ heating resistance in the strain here in used, *L. casei*, ATCC 393 (Genevois et al., 2018, Genevois et al., 2019).

The cell viability of probiotic microorganisms is important for designing functional foods. In addition, probiotic microorganisms supported on food matrices must tolerate the manufacturing processes and remain viable during the self-life of the product to guarantee the beneficial effect to the consumer (Tripathi et al., 2014).

Okara is the insoluble by-product of soymilk processing and it is rich in dietary fibre, protein, as well as phytochemical compounds like isoflavones and saponins, and other mineral elements, which are all related to health benefits (Swallah et al., 2021). Nevertheless, it is underutilized in the food industry as an ingredient because its deterioration begins soon after its production due to its high moisture content (Lazarin et al., 2020). Several processes based on nanocellulose technologies or based on ensiling were proposed for stabilising and improving its functional properties (Nagano et al., 2020; Rahman et al., 2021).

In a previous work, Castellanos-Fuentes et al. (2020) could demonstrate the adequate properties of the soy by-product to act as a substrate and carrier for *Lacticaseibacillus casei*. In that opportunity, dried okara was successfully used as raw material for designing a stable food ingredient (FI) containing probiotics through a sustainable process. To the best of our knowledge, no data related to the changes on physicochemical and functional properties of okara matrix due to *L. casei* fermentation in solid state conditions were reported till the moment. Besides, changes in the probiotic resistance to gastric-intestinal digestion, or their adhesion to the epithelium cells, due to okara matrix support; were not studied yet.

The objective of this research was to determine the physicochemical and functional characteristics of a novel okara-based food ingredient containing probiotic cells.

2. MATERIALS AND METHODS

2.1 Okara and systems preparation

Soybeans (*Glycine max*) were purchased at a local supermarket, and the preparation of okara and the systems assayed were performed based on the optimised method previously detailed (Castellanos-Fuentes et al., 2020) and summarised on **Figure 1**. Briefly, 50 g of fresh okara (moisture content ≈ 65±3 % wet basis (w.b.) were mixed with 5.4±0.1 g of cheese whey and 45.0±0.5 mL of distilled water in a 500 mL flask. The system was sterilised and after been cooled, it was inoculated with 2 mL of MRS broth containing ≈$8.5.10^3$ CFU/mL of *L casei* (ATCC®393TM) (Microbiologics, St Cloud, Minnesota, USA). This commercial strain was chosen due to the probiotic properties widely reported (Abd El Helim et al., 2016). The system inoculated was called Okara Inoculated (OI). Another batch was carried out in the same conditions but without inoculation, in order to be used as a control system (OC). In both cases, they were incubated for 24 h at 37 °C and after incubation they were submitted to centrifugation at 6000 rpm for 10 min and 6 °C (Eppendorf, HH, Germany), washing and vacuum (50

μmHg) dehydration for 36 h at 25 °C (Martin Christ Alpha 1-4 LSD, Osterode am Harz, NI, Germany). Subsequently, the pellets obtained were ground and separated into fractions according to the particle size. Those particles that were retained in the ASTM40 mesh, corresponded to an average size of ≈ 630 μm. Particles that passed through ASTM 40 mesh and were retained in ASTM 70 mesh, were considered with an average size of 393.76 μm. The okara raw material (ORM) was also analysed to study the effect of the process applied, independently of the inoculation, on the physicochemical properties of the plant matrix. Therefore, fresh okara was dried at 60 °C and milled without further treatment as described in (**Figure 1**). The stabilised ingredient was aseptically packed in low-density polyethylene bag (80 μm thickness) with a Ziploc®-type closure. The bag was stored in the dark at 25 °C.

2.2 Physicochemical and functional properties of the ingredient based on okara and containing *Lacticaseibacillus casei*

2.2.1 Physicochemical properties

The physicochemical properties of the okara-based ingredient containing *L. casei* (OI) were characterised according to Genevois et al. (2019). The water activity (a_w) was measured using an AquaLab Series 3 meter (Decagon Devices Inc, Pullman, WA, USA) at 20 °C, and moisture was determined with a halogen moisture analyser (Ohaus MB-45, NJ, USA) until a constant value was reached. The lipid and protein contents were determined with 960.39 (Soxhlet) and 920.152 (Kjeldahl) methods respectively (AOAC, 2006). The DF composition (uronic acid, total non-cellulosic carbohydrate, cellulose, and lignin contents) was analysed on the alcohol insoluble residue (AIR) after been hydrolysed by sulfuric acid in three different conditions. Lignin and cellulose were determined gravimetrically following the procedure detailed in de Escalada Pla et al. (2012). The non-cellulosic carbohydrates and the uronic acids were quantified on the corresponding neutralised hydrolysates by spectrophotometric methods according to Dubois et al. (1956), and Filisetti-Cozzi and Carpita (1991) respectively. The used

reagents were analytical grade from Biopack (BA, Argentina). All determinations were performed in triplicate.

2.2.2 Functional characterization

Functional characterization such as hydration properties; water holding capacity (WHC) and swelling capacity (SC) and oil absorption capacity (OHC) were determined following the methodology described in de Escalada Pla, et al. (2012). Briefly, a weighed quantity of dried sample was mixed with water or oil and kept for 18 hours at 25 °C. The WHC and SC, were determined respectively by measuring the amount of water absorbed and the volume of the swollen particles. The OHC were determined gravimetrically after centrifugation. The water-soluble fraction (WSF) was determined on the supernatant obtained from the WHC. This was frozen and lyophilised, determining the final weight of the solids. At least three replicates were made for each determination from independent samples. Results are reported as the mean value ± standard deviation (SD).

2.2.3 Chemical structure

Chemical structure was determined performing Fourier transform infrared spectroscopy (FTIR). An FTIR spectrometer (V5.3.1 Spectrum, Perking Elmer, Inc., USA) provided with a diffuse reflectance accessory (DRIFTS) was used. The solid-state samples were mixture with KBr (Merck, USA) prior to analysis. The spectra were obtained between 400 and 4000 cm^{-1} with a resolution of 2 cm^{-1}. All determinations were made at least in duplicate.

2.2.4 Determination of carbohydrates

Raffinose, stachyose, lactose, and glucose content were analysed by HPLC using a silica-based packing bonded with polyamine, Polyamine II YMC-Pack column (250 x 4.6 mm, YMC Co., Kyoto, Japan), according to Genevois et al., (2018), with some

modifications. The dried sample (≈ 0.5000 g) was re-suspended in 10 mL of 20 %(v/v) ethanol in a heat bath at 35 °C for 60 min with constant shaking. Then, they were centrifuged at 6 °C at 9000 rpm for 20 min. The supernatant was collected, filtered twice, through a 0.45 µm Nylon® syringe filter and using a Sep-Pak® Plus NH_2 cartridge (Waters, Milford, MA, USA) and freeze dried (Martin Christ Alpha 1-4, Osterode am Harz, NI, Germany) for 24 h. Before the determination, it was suspended in ultrapure water, filtered through a 0.45 µm and a 0.25 µm Nylon® filter. Finally, 20 µL was injected into the HPLC equipment connected to an in-line degasser (Model AF, Waters, Milford, MA, USA), a mobile phase solvent delivery pump (Model 1525, Waters, Milford, MA, USA) and a refractive index detector (Model 2414, Waters, Milford, MA, USA). Operating conditions were as follows: thermostatic column at 35 °C, detector at 39 °C, mobile phase was acetonitrile: water (70:30, v/v) Biopack (BA, Argentina), flow rate of 1.0 $mL.s^{-1}$. Calibration curves were performed with HPLC-grade glucose, raffinose and lactose external standards Sigma-Aldrich (MO, USA). Results were expressed as $mg.g^{-1}$ of sample (dry basis, d.b.). Determinations were performed in duplicate, from independent samples, and the mean value ± SD is given in the results.

2.2.5 Phytic acid content

Phytic acid content was evaluated using the methodology described by Genevois et al., 2018) with slight modifications. Briefly, ≈0.2500 g of sample was defatted with petroleum ether and subsequently, it was submitted to extraction with 5 mL of 2.4 %(v/v) HCl, with constant stirring for 16 h. Next, ≈0.5 g NaCl was added with stirring for 10 min, and allowed to stand at -20 °C for 20 min, followed by centrifugation at 10 °C at 3000 rpm for 20 min. A 750 µL aliquot of the supernatant dilution (1:25) was mixed with modified Wade reagent (0.03 % $FeCl_3.6H_2O$ + 0.3 % sulfosalicylic acid.$2H_2O$) Biopack (BA, Argentina) in distilled water. The calibration curve was performed with a phytic acid sodium standard salt Sigma-Aldrich (MO, USA) and the absorbance was measured using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at a

wavelength of 500 nm. The phytic acid content was expressed in mg of phytic acid per g of sample ($g.g^{-1}$d.b.). The determinations were made in duplicate for each system, from independent samples, and the mean value ± SD is reported in the results.

2.3 Probiotic resistance to gastrointestinal stress and adhesion ability

2.3.1 Simulated gastrointestinal resistance (SGIR)

The SGIR was performed as reported by Genevois et al. (2016). The assay was performed periodically during the 63 days storage time of the samples at 25 ºC. The results were expressed as percentage of survival *L. casei* under simulated gastrointestinal digestion conditions, [(log Nt / log N_0) x 100]. Were N tis the probiotic viable count ($CFU.g^{-1}$) in the FI after each digestion step at "t" time of storage and N_0 is the cell count ($CFU.g^{-1}$), in the FI before digestion (Genevois et al., 2016). The reagents were analytical grade from Biopack (BA, Argentina), pepsin Merck (BA, Argentina) and bile salts Parafarm (BA, Argentina).

2.3.2 Adhesion to epithelial cells

The adhesion assays, were analysed according to Golowczyc, et al. (2007). Briefly, a Caco-2 / TC-7 monolayer, previously prepared, was incubated with 0.5 mL of suspension of the FI containing the probiotic (2×10^8 $CFU.mL^{-1}$ of PBS) and 0.5 mL of adhesion medium, Dulbecco modified Eagle's minimal essential medium, without antibiotics or supplements, for 1 h at 37 °C in atmosphere of 5% CO_2-95% air. Then, the monolayer was washed and lysed (40-50 min) by adding sterile distilled water. To determine the number of viable microorganisms adhered to Caco-2 / TC-7 cells, appropriate dilutions in 0.1 % (w/v) tryptone Biokar Diagnostics (París, France) were seeded on MRS agar and colony counts were performed after 48 h of incubation at 37 °C, expressing the microbial population as Log $CFU.well^{-1}$.

For comparison purposes, a fresh inoculum was prepared in MRS broth (24 h incubation) of free *L. casei* cells (FC), used as control for SGIR and adhesion tests,

containing approximately the same amount ($p>0.05$) of the 7.5±1.1 log probiotic ($CFU.g^{-1}$). All experiments were done in triplicate.

2.4 Microstructure and location of main components

2.4.1 Scanning Electron Microscopy (SEM)

The dried sample was mounted on the sample holder of the equipment with the help of double-sided adhesive tape and metallized bysputtering with a layer of gold (Cressington Scientific Instruments 108 Sputter, UKA). The micrographs were obtained using a scanning electron microscope (Zeiss Supra 40, Germany) operated at an accelerating voltage of 3 kV.

2.4.2 Confocal laser electron microscopy

The microstructures of the samples were visualized using an Olympus FV1000 confocal microscope, equipped with three detection channels: channel 1 (505-525 nm), channel 2 (655-755 nm) and channel 3 (640-670 nm). The fluorochromes were selected based on their affinity to associate them with the different components within the samples. According to Preece et al., (2015), nile blue and rhodamine B suspended in 96% ethanol were preselected. The dried sample was suspended in double distilled water in 2 mL Eppendorf tubes, a drop of stock stain solution was added to all samples and homogenized prior to slide preparation. Two lasers were used for excitation: an argon laser (λ excitation 543 nm), and a helium-neon laser (λ excitation 635 nm). The images were recorded by sweeping fields at different depths in the sample. For the visualisation of all the materials, a 60X objective with immersion oil was used.

2.5 Statistical analysis of the results

Statistical analysis to determine significant differences between the systems was performed by analysis of variance with a significance level (α) of 0.05, followed by Fisher's least significant difference method for discriminating between means. The correlation study was carried out through Pearson's product moment coefficients. For

the statistical analysis, the study of the correlations, as well as the linear regressions, the Statgraphics Centurion XV program (V 2, 15.06, 2007, USA) was used.

3. RESULTS AND DISCUSSION

3.1 Physicochemical and functional properties of the ingredient based on okara and containing *Lacticaseibacillus casei*

3.1.1 Chemical composition

It can be observed in **Table 1** that okara systems had, in general, a similar chemical composition being cellulosic and non-cellulosic polysaccharides of the cell wall (AIR >62%) the main components, followed by protein content (~38.7%). Other authors have reported okara as an important source of DF and good quality protein (Guimarães et al.,2019; Préstamo et al., 2007). In addition, uronic acid was around half of the non-cellulosic fraction, in agreement with previous reports (Porfiri et al., 2016). It was determined that the chemical composition of okara depended on the variety of soybeans, the climatic conditions (Vong et al., 2016) and the amount of water added and extracted in the obtaining process (Kamble, 2020). In addition, cellulose and hemicellulose residues are related as part of the cotyledon cells (O'Toole, 2004; Redondo-Cuenca et al., 2008). On the other hand, the stabilisation and fermentation processes significantly ($p<0.05$) affected the chemical composition in both OC and OI systems which presented 55% and 35% lower non-cellulosic carbohydrates content than ORM, respectively; at the same time, insoluble compounds like cellulose were concentrated, showing higher ($p<0.05$) values than ORM. A decrease in pH (4.37±0.07) during fermentation, evidenced that some carbohydrates were solubilised during thermal process and metabolized during *L. casei* cells growth and / or probably lost during washings (Castellanos-Fuentes et al., 2020).

3.1.2 Physical and Functional Characterisation

At the end of the process and at the end of storage (63 days), the systems OI and OC present the same a_w and moisture content averaging values of 0.485±0.003 and 5.7±0.7 % (w.b.) respectively, ensuring microbiological stability at room temperature (Bonazzi et al., 2011). The hydration properties (SC and WHC) as well as the OHC, WSF and ρb were studied for two fractions of different size, 630 µm and 393.75 µm, of each system (**Table 2**). Density values ranged from 0.254 to 0.5006 $g.cm^{-3}$, being all the systems significantly different ($p<0.05$). The process of washing and vacuum dehydration applied for obtaining OC and OI reduced the density of the raw material (ORM) between ≈ 32 to 49%, suggesting the generation of a more porous structure. On the other hand, the inoculation of the material, as well as the reduction in size in the vacuum dehydrated systems, increased slight but significantly ($p<0.05$) the density value. Hydration properties represent the ability of the vegetal matrix to retain water through weak intermolecular interactions such as hydrogen bonding or among dipoles. The studied systems presented values between 4.8 and 6.14 $cm^3.g^{-1}$ for SC and 3.8 and 5.7 $g.g^{-1}$ for WHC. The treatment applied for OC and OI, regardless of inoculation, tends to improve these properties, although it was only observed significantly ($p<0.05$) for WHC in the 393 µm fractions. The probiotic presence did not significantly affect the hydration properties, while the reduction in the particles size would lead to higher WHC ($p<0.05$). Other authors have reported that although the smaller particle size generates a higher density; the composition and structure also contribute to the distribution of water, observing, in certain dietary fibres, better water absorption by reducing the particle size (Garcia-Amezquita et al., 2018, Robertson et al., 2000). According to dos Santos et al. (2019), WHC of okara flour after centrifugation was around 3.9 $g.g^{-1}$ okara flour. While for okara dried by different methods, Ostermann-Porcel, et al. (2016) determined similar SC values (7.9-7.6 $mL.g^{-1}$).

Regarding OHC, it represents the material capacity to associate hydrophobic compound on the surface, the systems presented values from 1.7 to 4.3 $g.g^{-1}$. In general, each gram of material in the analysed fractions of the different systems

absorbed a greater quantity of water than of oil (**Table 2**). On the other hand, a significant effect of the applied process on the raw material was observed. The sterilisation, washing and subsequent vacuum drying process allowed an important increase ($p<0.05$) around 2 and 2.5 times on the OHC value in relation to ORM. Furthermore, it was observed that neither the presence of the probiotic nor the particle size tested affected this property. The values of the oil absorption properties for the studied systems are higher than those reported by Espinosa et al. (2009), 0.20 $g.g^{-1}$ for okara, but are in the order of those reported by dos Santos et al. (2019), 3.9 $g.g^{-1}$ for okara flour.

Respect to the WSF, the samples presented percentages between 6.7% - 15%. Neither the presence of the probiotic nor the particle size affected this parameter. It is important to remark that the raw material comes from the wet milling of the grain. Therefore, part of the water-soluble proteins and carbohydrates are lost in ORM. In contrast, the control and inoculated systems were added with cheese whey and summited to a later processed with sterilisation and washing. Regarding these treatments, the WSF of the systems affected in a different way according to the matrix, which would explain the differences observed in **Table 2** between ORM and OC or OI. Ma et al. (1984) indicated that there is an increase in solubility due to the thermal denaturation of soluble proteins.

According to the statistical analysis, three correlations were observed and are showed in **Table 3**, indicating that systems with lower densities have higher OHC and WSF. Accordingly, vacuum-dried systems (OC and OI) increased the OHC values and WSF since the material structure was less dense than raw material (ORM) (de Escalada Pla et al., 2012, Genevois et al., 2019). Many authors have reported a relationship between OHC and apparent density (ρ_b) or specific volume (Femenia et al., 1997; Guillon et al., 2000, de Escalada Pla et al., 2010). The apparent density, or the specific volume, can be used as an index of structural differences (Cadden, 1987) and the highest values of the specific volume were associated with the ability to absorb more oil. In the present

research, the ORM system presented OHC values significantly lower than OC and OI ($p<0.05$), which would indicate that the process applied, regardless inoculation, improved this property. On the other hand, a non-significant trend to lower OHC and WHC were observed in OI when compared with OC, possibly related to the higher density registered (**Table 2**). Therefore *L. casei* fermentation produced denser matrix ($p<0.05$) but not enough for significantly affecting the functional properties OHC and WHC. These results have relevance from nutritional and also from technological point of view. The application of OI as ingredient and its incorporation in different food formulation could reduce oil and water loses and therefore could improve food properties associated to these effects, for instance, in emulsion stability, in increasing viscosity and stability of functional beverages, etc.

3.1.3 Microstructure

For better understanding the functional properties results, the microstructure of the studied systems was determined by SEM (**Figure 2**). It can be observed a honeycomb like porous matrix with the presence of smooth and thin walls, in both OC and OI systems (**Figure 2, panel A and B**). On the contrary, ORM resulted holey, dense, and compact (**Figure 2, panel C**). Such differences could be attributed to different stabilisation processes applied: air forced drying for ORM and vacuum drying for OC and OI. Moreover, it was possible to observe that OC microstructure presents porous with higher size than OI that is consistent with de lower bulk density determined for OC (**Table 2**). Such results could be associated with the heating and vacuum drying processes applied on OC and OI systems, increasing their porosity and surface, and affecting the functional properties (Guillon et al., 2000), in comparison with the fraction of ORM from a wet milling of the grain**.** Similar structures were observed by Ostermann-Porcel et al. (2016) for okara dried using a rotary dryer or by freeze-drying.

3.1.4 Chemical structure

The analysis of FTIR spectrum allows the study of the chemical groups present in the sample, where characteristic and distinctive bands can be observed. In **Figure 3**, the FTIR spectra of okara systems are shown. In general, all profiles were similar: a broad band in the region of 3300 cm^{-1} corresponding to the stretching of O-H groups (H-bonds), a weak band at 2920 cm^{-1} attributable to the C-H group of polysaccharides from cellulose and hemicellulose (Ma and Mu, 2016) and C-H_2 bands corresponding to the lipid chains of fatty acids at 2930 - 2860 cm^{-1}(Quintana et al., 2017), a sharp band at 1740 cm^{-1} associated to carbonyl groups (C=O) of the fatty acids or esters (Quintana et al., 2017) and the -COOH stretching in uronic acid (Ullah et al., 2017), a signal in the region of 1510 to 1500 cm^{-1} due to -CH_2 stretching in cellulose, a peak in the 880 cm^{-1} region associated with the β-glycosidic bond in cellulose (Ullah et al., 2017) and finally, bands between 1200-800 cm^{-1} attributed to the absorption of the glycosidic bond C-O-C, present in the polysaccharides that constitute dietary fibre (Romano et al., 2014; Soni and Mahmoud, 2015; Wen et al., 2017). Particularly, differences in the intensity of 2920 and 1720 cm^{-1} bands, and in the range ≈1500-1000 cm^{-1} of OI and OC in comparison with the raw material (ORM), could be due to the lipid and cellulose contents (**Table 1**), in agreement with Quintana et al. (2017). Nevertheless, there were no detectable specific peaks related to probiotics presence.

3.1.5 Sugars and phytic acid content

Mono and oligosaccharide sugars could be extracted from okara samples, observing significant differences in all systems (**Table 3**). Although ORM contained sucrose (1.7±0.9 $mg.g^{-1}$) and raffinose (0.34±0.03 $mg.g^{-1}$), not significant amounts of stachyose and glucose were detected. It must be highlighted that okara is the residue from the soy wet milling, and therefore free sugars remain soluble mainly in the liquid phase consequently only residual sugar content is determined in the insoluble phase, ORM. It was observed that such residual sugars were reduced in the treated systems, OC and OI, since raffinose was not detected and sucrose was only quantified in OI samples

(0.5±0.2 mg.g^{-1}), tending to lower content than ORM ($p>0.05$). During fermentation process, a reduction in pH and an increasing in lactic acid content had been registered (Castellanos-Fuentes et al., 2020), Therefore, fermentation process and/or possibly washing step reduced free sugars content. In addition, the presence of probiotic could contribute to the sucrose and raffinose reduction (Wang et al., 2003). Regarding stachyose, it could not be detected in any sample, while glucose was only found in OI (6.1±0.2 mg.g^{-1}). Probably, lactic bacteria (LAB) metabolism may have hydrolysed some oligosaccharides, increasing the amount of free glucose that remained in OI samples despite the washing process. Besides, some enzyme activity could occur even during storage in dried conditions (Brogan et al., 2014). In a previous work, the presence α-galactosidase activity was reported for the same LAB strain here in used (Genevois et al., 2018). On the other hand, similar ($p<0.05$) amounts of lactose were detected in OC and OI respectively (**Table 3**), due to the addition of cheese whey in these systems.

It is known that phytic acid may act as an antinutrient factor since it is a sequestrant (chelator) of some minerals (Masum Akond et al., 2011). The phytic acid content in the systems did not show significant differences ($p>0.05$), being the mean value 1.26±0.29 mg.g^{-1}. Such level is in the order of the data reported by Al-Wahsh et al., (2005) who compared concentrations of phytic acid from different soy-based foods, including soy milk (0.8 - 1.33 mg.g^{-1}). Previously, phytase activity could be verified for this strain (Genevois et al., 2018) in a fermented soy desert based on soy beverage. Possibly, in solid phase like OI matrix, the activity of phytase was affected.

3.2 Location of main components

The distribution and structure of main components of the okara tissue were analysed using confocal microscopy. In **Figure 4**, fluorescence micrographs for OC (**panels A,B, C**) and OI (**panels D, E, F**) suspensions are shown. Rhodamine B and nile bluewere used in the micrographs with emission wavelengths between (560-620 nm) and (655-

755 nm) respectively. The prepared samples (aqueous medium) emitted fluorescence, allowing the visualization of proteins and lipids. Parts of the cotyledon cells were present within the suspension of the ingredients containing the intracellular material. The cell wall was observed broken for both OC and OI systems. Campbelland Glatz (2009) and Preece et al., (2015) also found similar structures in soybean meal by transmission microscopy and confocal microscopy respectively. The agglomerated material was visualized outside the cotyledon cells, being in a range of4-7 μm approximately. The areas that emitted a yellowish colour were a combination of red and green. Lipids appeared in red and protein bodies in green. The protein bodies within the cotyledon cells remained mainly intact. The probiotic cells could be observed in (OI) with an approximate size of 1.02 ± 0.05 μm. Using confocal microscopy, it was possible to observe the location of lipids, proteins, and *L. casei* using dyes selected for this purpose. In addition, the agglomerated protein bodies caused by heat treatment were found inside and outside the cotyledon in both systems.

3.3 Survival and probiotic properties of *L. casei* during storage

The data obtained from the viable cell count allows evaluating cell loss during storage, being important information for the food industry. In order to fit with the current regulation, a minimum viable cell count must be guaranteed at the end of the self-life (Makinen et al., 2012). It must be highlighted that the FI, showed a cell count higher than 7.5 log $CFU.g^{-1}$ along 63 storage days (Castellanos-Fuentes et al., 2020). Otherwise, resistance to simulated gastrointestinal stress condition is required for a probiotic strain (Buriti et al., 2010; Reale et al., 2015). **Figure 5** shows the survival of *L.casei* after gastric and intestinal digestion under simulated conditions during the storage at 25 °C. *L. casei* supported in the FI, presented an initial count of 10±1 log ($CFU.g^{-1}$) and 7.5±0.4 log ($CFU.g^{-1}$) at 63 days storage. The supported cells resisted gastric stress being the percentage of survival more than 80 % and this property was maintained during the storage at 25 °C; while the FC from a fresh inoculum with an

initial count of 8.3±0.6 log ($CFU.g^{-1}$) survived gastric conditions just in ≈30%. It can be inferred that *L. casei* adhered to the okara matrix, was protected against DGI media (low pH, gastric pepsin, bile salts). The same strain was previously assayed by the authors who demonstrated that pumpkin matrix could protect the cells against stress factor (Genevois et al., 2018). This agrees with the observations of Wang et al., (2009), who reported that the gastric survival rate of an isolated strain from koumiss, *L. casei* Zhang, was higher when this microorganism was present in fermented soy juice than in a pure culture. In relation to intestinal conditions, the resistance of the probiotic when it was supported in the FI remained between 66-77%, while significantly lower resistance ($p<0.05$) ≈40% was recorded with the FC (**Figure 5**). Vinderola et al., (2011) have also reported changes in the tolerance of the probiotic to the acidic medium and to the presence of bile during refrigerated storage in commercial fermented milk, despite not observing changes in cell viability. In the case of OI, it showed good stability throughout storage, and better survival to gastric conditions than intestinal ones. In addition, the count of viable probiotic microorganisms was > 6 log ($CFU.g^{-1}$) at the end of 63 days of storage at 25 °C and more than 65 % of them reached viable the intestinal tract according to the results in the *in vitro* ID assay herein tested.

The adhesion to the intestinal epithelium is a prerequisite for bacterial colonisation and has been considered one of the main criteria for the characterisation of probiotic bacteria (Tuomola et al., 2000). Due to the difficulty of investigating bacterial adhesion in vivo, an assay was performed using intestinal cell lines as in vitro model. One of these cell lines is Caco-2 /TC-7 (Minnaard et al., 2007; Bengoa et al., 2018) and it was used to study if the probiotic supported in the okara could adhere to the intestinal epithelium. Figure 6 shows the *L. casei* cell initial count inoculated into wells containing Caco 2 cells and the count after adhesion assay, for OI and for FC of the probiotic as control system. Free *L. casei* was able to adhere to Caco 2 cells being the percentage of adhesion about 2 % agreeing this value with that reported for other Lactobacillus strains (Bengoa et al., 2018; Golowczyc et al., 2007). Nevertheless, when the probiotic

cells were supported into the OI the adhesion ability decreases to ≈0.2 % indicating that the component of the matrix interferes with the adhesion ability. Tuomola and Salminen (1998) studied the adhesion of 12 Lactobacillus strains and reported that it was dependent on the number of bacteria inoculated initially. In this sense, it must be highlighted that FC came from a fresh 24 h inoculum in MRS broth, while OI system presented 20 days of storage, showing a trend to a lower initial cell count (**Figure 6**). A high stability of OI during storage, mainly between days 10-40, had been observed by the authors in previous studies (Castellanos-Fuentes et al., 2020). Therefore, for this study 20 days of storage was selected as a representative storage time. Szekér et al. (2007) observed that cell aggregate formation reduced the plate count enumeration of *L. casei* subsp. Pseudo plantarum 2750. These aggregates could be disintegrated through gastrointestinal tract (Bengoa et al., 2018). In the present study OI matrix interfered in the adhesion results possibly due to agglomeration mechanism. Therefore, more studies are needed to know the ability to adhere to Caco 2 cells in *in vivo* intestinal environment where the digestion could modify the structure of the OI and release the probiotic allowing interaction to epithelial cells.

4. Conclusions

In the present research, fresh okara (ORM) was successfully used to prepare a functional ingredient (OI) by solid state fermentation (SSF) with *Lacticaseibacillus casei* and subsequent washing and vacuum drying. It was possible to characterise the obtained OI and the effects of the process applied, performing a study of the physical, chemical, and functional properties.

The SSF and drying reduced the amount of cell wall material (AIR) originally present in ORM, while better oil and water holding capacities were obtained in OI, mainly due to microstructure modifications (higher porosity). The OI was useful to maintain the viability of *L. casei* at required levels by regulation (>6 Log ($UFC.g^{-1}$)), during 63 days

of storage at room temperature. In addition, OI protected the probiotics against simulated gastrointestinal conditions along the storage and partially reduced the adhesion to Caco-2 cells. Therefore, the developed OI constitute in a highly stable powdered product which can be incorporated into food, beverages, and nutraceuticals as an ingredient rich in probiotics and as a source of dietary fibre and protein. Such applications and consumer acceptability are extremely important and will be studied in future research.

The findings obtained are relevant for food industry since they allow to consider the re-use of a by-product of soy based food production, like okara, to obtain a new ingredients. Particularly, it was demonstrated that okara can be used as a substrate to support probiotics, using simple and sustainable processes. In addition, the developed functional ingredient offers news possibilities to incorporate active components to different foods, improving nutritional quality. The OI, also present important water and oil retention capacities which could be technologically useful to modulate physical stability of foods.

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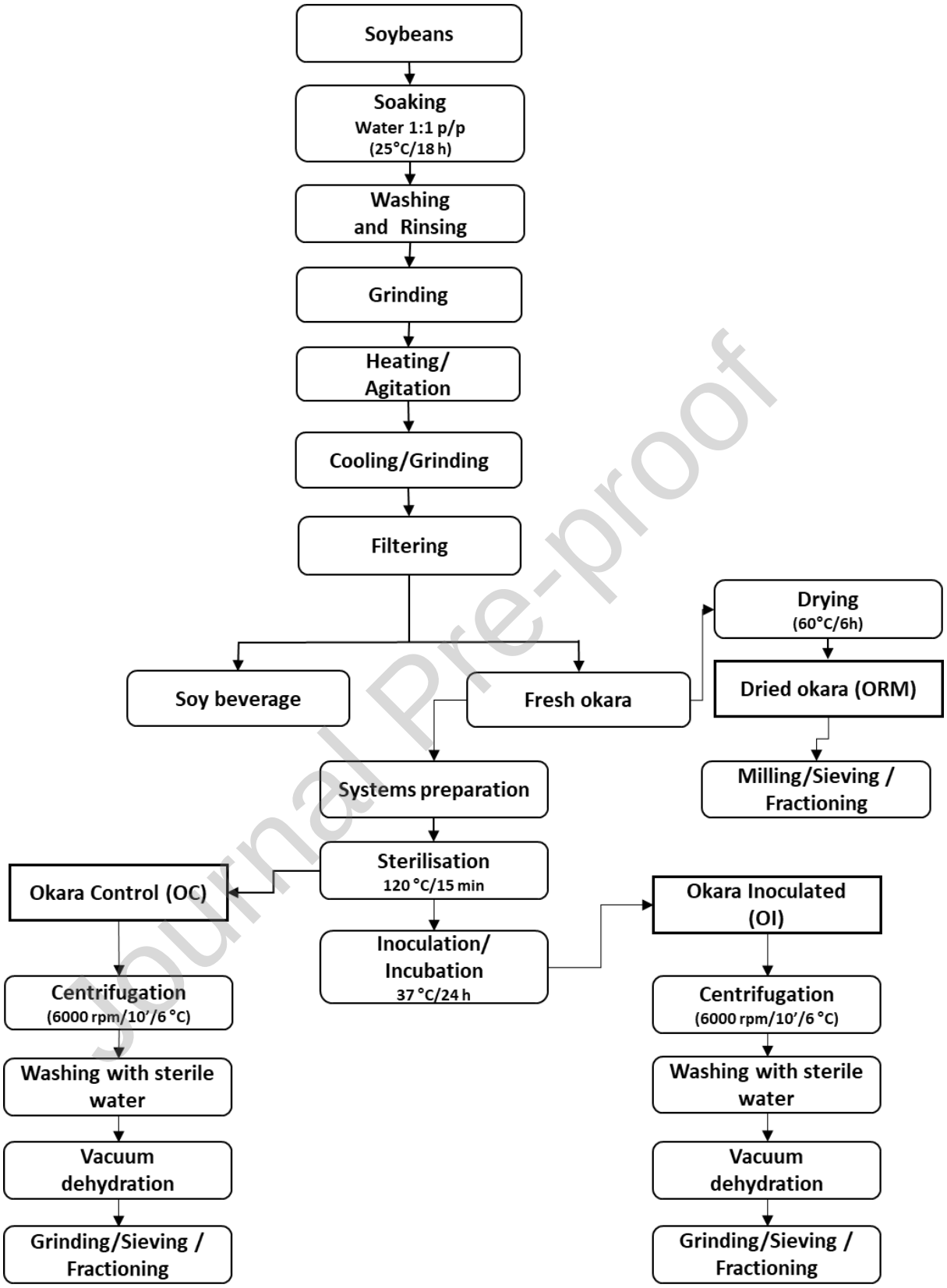


Figure 1. Flow sheet for obtaining okara raw material (ORM), okara control (OC) and okara inoculated (OI).

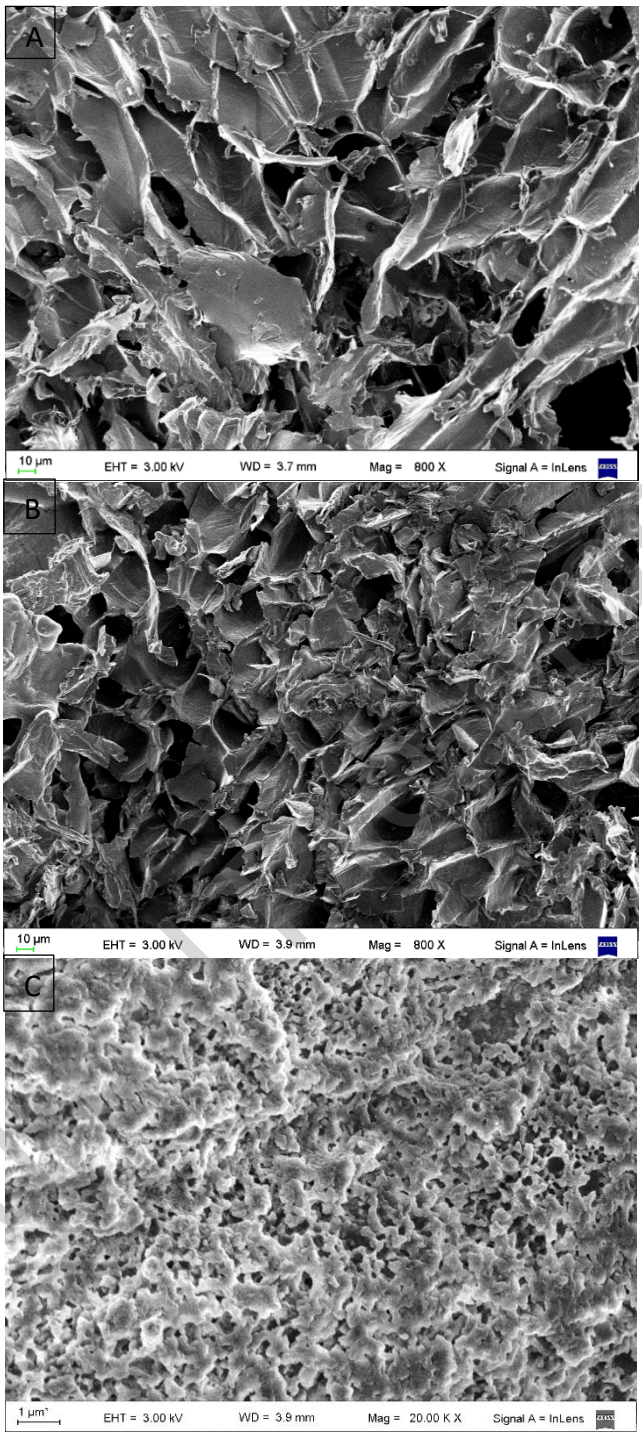


Figure 2. Scanning electron microscopy: panel A) okara control (OC), panel B) okara inoculated (OI) and panel C) okara raw material (ORM). Magnifications: 800 X for A) and B); 20.00 kX for C).

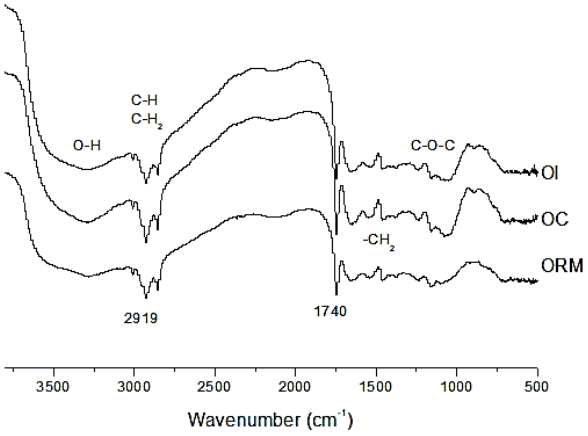
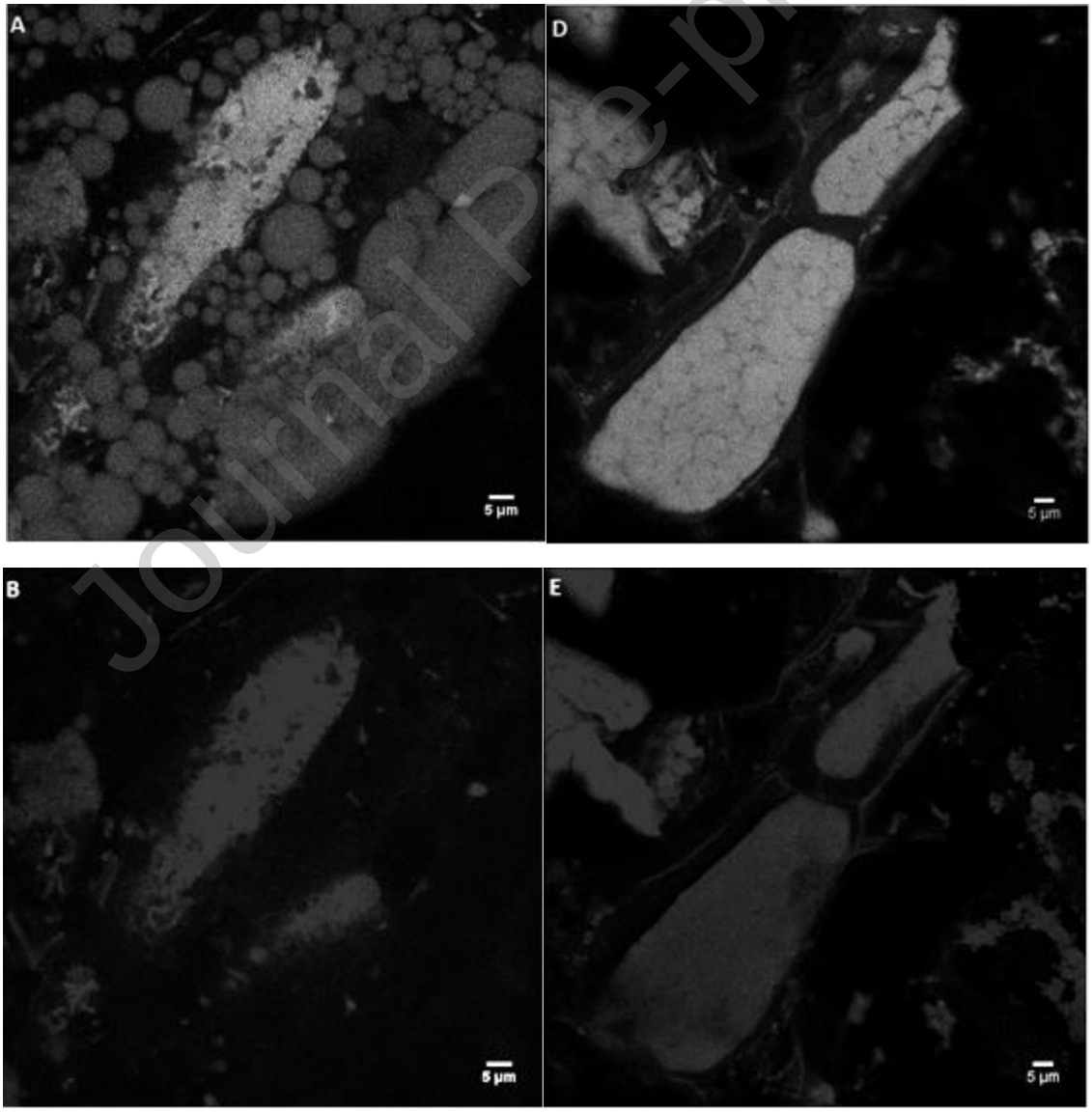


Figure 3. Fourier transform infrared spectroscopy (FTIR) of different systems obtained from okara.



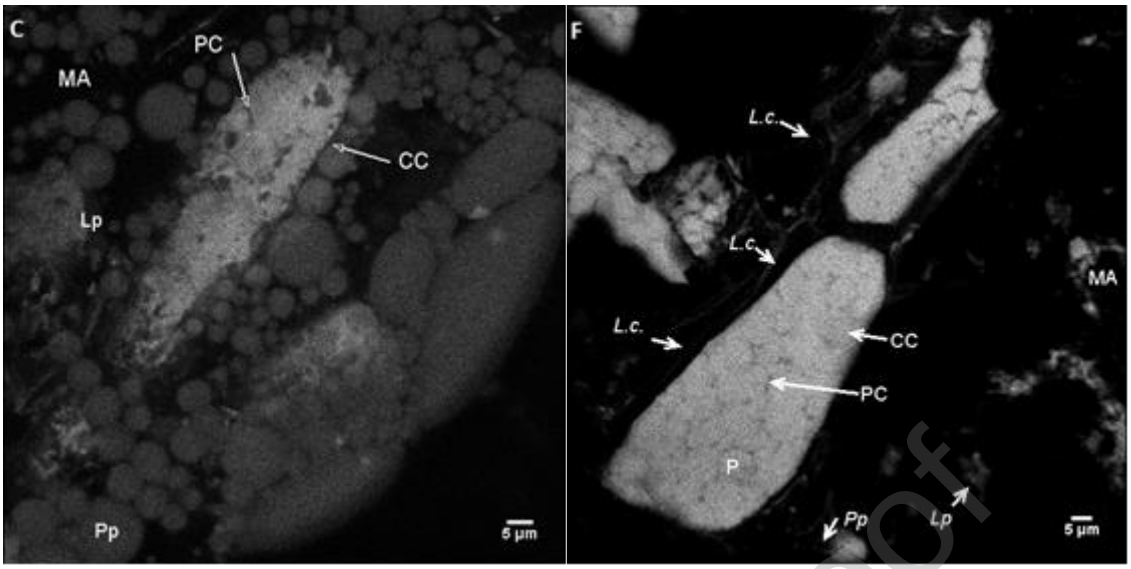
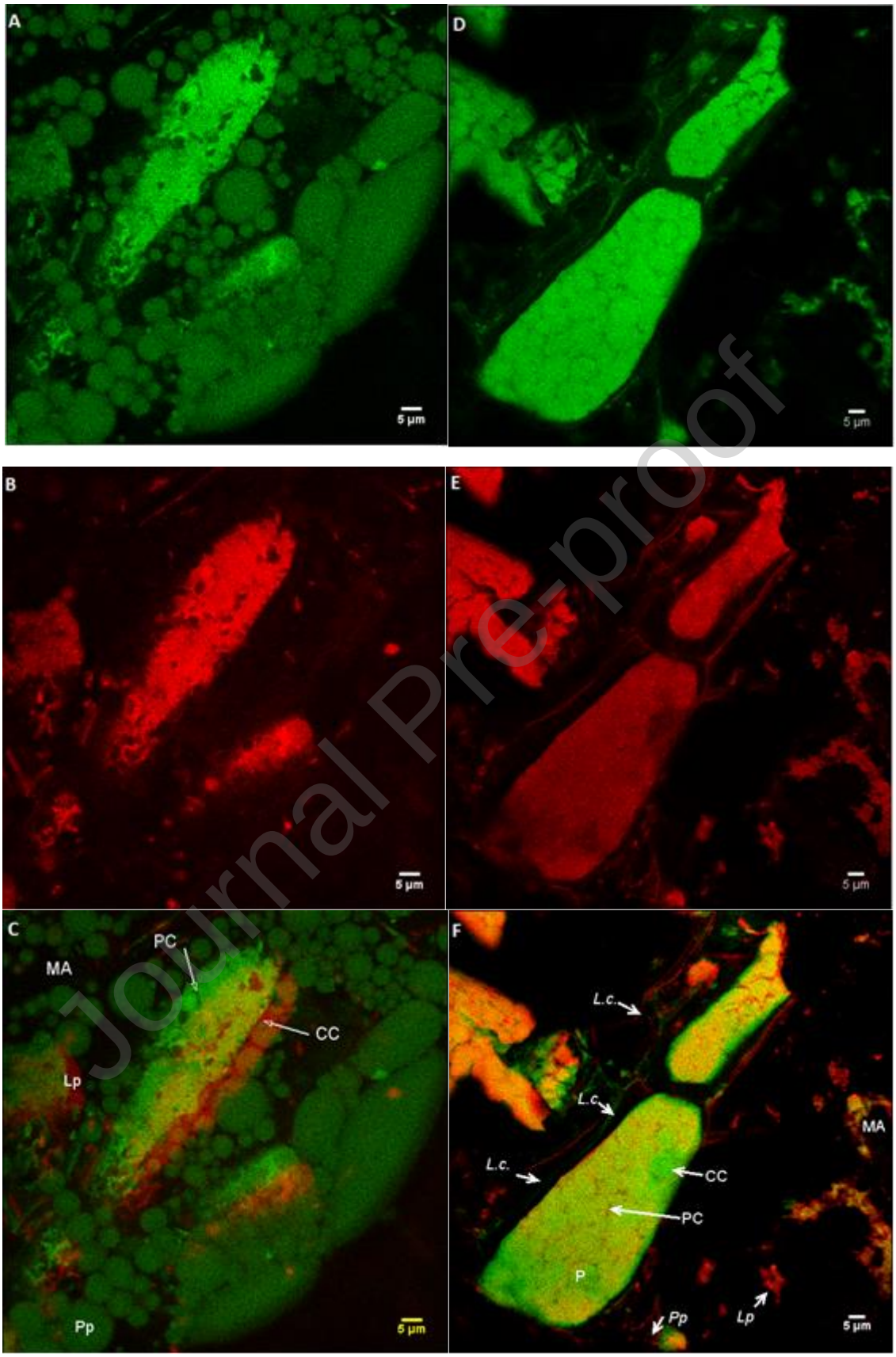


Figure 4. Confocal micrograph of suspensions of: Okara Control (OC) (Panels A, Band C) and Okara Inoculated (OI) (Panels D, E and F). From above to below: image in Rhodamine B (A, D), Nile Blue (B, E) and the superposition of both (C, F). Cotyledon cell wall (PC, dark lines), agglomerated material (MA), cotyledon cells (CC), protein bodies within the cotyledon cells (P), lipids (Lp), protein (Pp) and *Lacticaseibacillus casei* (*L.c.).* For the interpretation of the colour references in this figure, the reader is referred to the web version of this article. Different levels of the observations can be also found in supplementary data.



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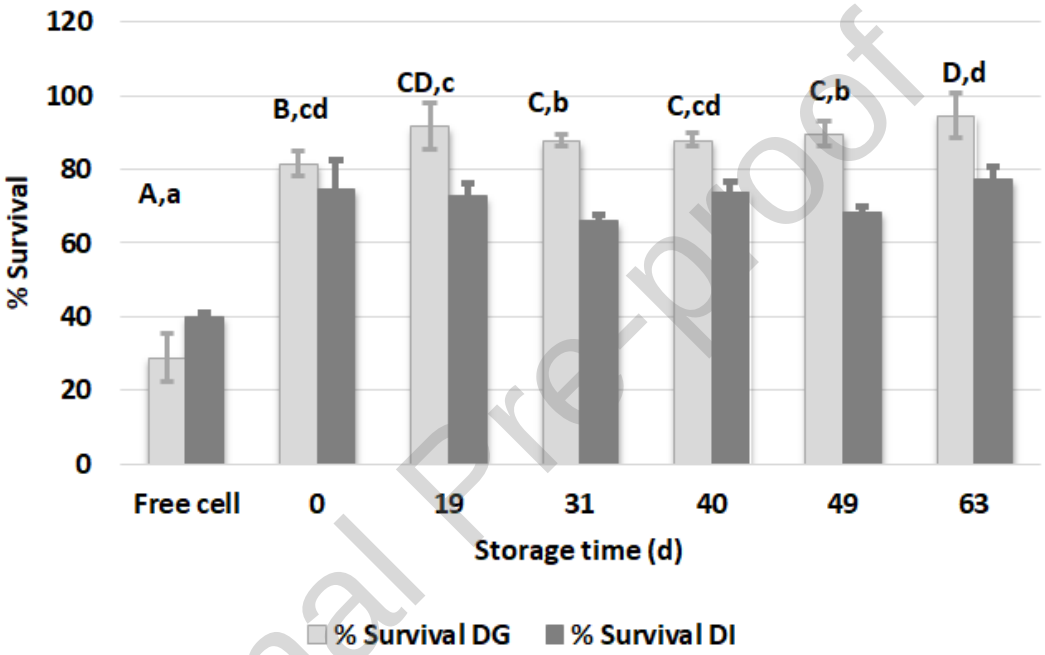

Figure 5. Percentage of survival of *Lacticaseibacillus casei* after gastric (■ DG) and intestinal (■ DI) digestion under simulated conditions for the FI, based on inoculated okara, throughout storage at 25 °C. Free cells correspond to a culture of *L. casei* in MRS broth for 24 hours, before digestion.

Different letters indicate significant differences ($p \leq 0.05$): uppercase between the percentages of resistance to DG along the storage and lowercase between the percentages of resistance to DI along the storage.

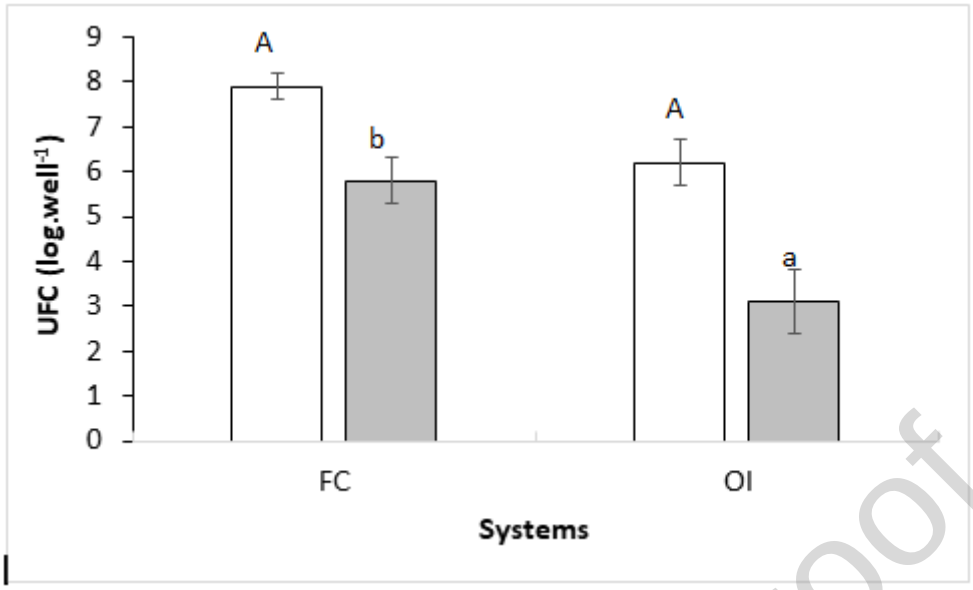

Figure 6. *L. casei* bacteria associated to Caco-2/TC-7 cells in the FI based on okara Inoculated (OI) and free cells (FC) as a control. White bars show initial count. Grey bars shown *L. casei* count remained in the well after adhesion assay.

Table 1. Chemical composition of okara systems.

Systems	Protein[1]	Lipids[1]	Moisture[2]	Cellulose[3]	Lignin[3]	HC no cellulosic[3]	Uronic Acids[3]	AIR[1]
OC	38±1^{a}	23±5^{a}	6.2±0.6^{b}	24±3^{ab}	2±1^{a}	18±2^{a}	11±3^{a}	62±3^{a}
OI	38.1±0.1^{a}	24±5^{a}	5.7±0.7$^{a}_{b}$	30±3^{b}	0.7±0.1^{a}	26±2^{b}	13±3^{a}	62±3^{a}
ORM[4]	39.9±0.5^{a}	16±2^{a}	4.1±0.6^{a}	18.14±0.04^{a}	1.9±0.8^{a}	31±5^{c}	13±4^{a}	79±4^{b}

[1]$g.100^{-1}g^{-1}$ sample d.b.
[2]$g.100^{-1}g^{-1}$ sample w.b.
[3]$g.100^{-1}g^{-1}$ of AIR.
[4]ORM from Castellanos-Fuentes et al., 2020
AIR: alcohol insoluble residue.
HC no cellulosic: total non-cellulosic carbohydrates
Different letters in the same column, indicate significant differences ($p < 0.05$) among systems. OC (Okara Control), OI (Okara Inoculated), ORM (Okara Raw Material).

Table 2. Functional properties of okara inoculated (OI), okara control (OC) and okara raw material (ORM) with different particle sizes.

Systems	Size (μm)	Functional Properties of Okara				
		ρ_b ($g.cm^{-3}$)	SC[1] ($cm^3.g^{-1}$)	WHC[2] ($g.g^{-1}$)	OHC[3] ($g.g^{-1}$)	WSF[4] (%)
OC	630	0.254 ± 0.004^{a}	6.13 ± 0.01^{c}	4.7 ± 0.3^{bc}	3.8 ± 0.3^{b}	16 ± 1^{b}
	393.75	0.2644 ± 0.0002^{b}	5.36 ± 0.01^{ab}	5.70 ± 0.5^{d}	4.3 ± 0.7^{b}	15.0 ± 0.3^{b}
OI	630	0.2782 ± 0.0001^{c}	5.8 ± 0.5^{bc}	4.4 ± 0.4^{ab}	3.6 ± 0.3^{b}	14 ± 1^{b}
	393.75	0.29 ± 0.01^{d}	6.14 ± 0.01^{c}	5.4 ± 0.2^{cd}	3.4 ± 0.6^{b}	14 ± 3^{b}
ORM	630	0.5006 ± 0.0002^{f}	5.36 ± 0.02^{ab}	3.8 ± 0.1^{a}	$1.9\pm0.3_{a}$	6.7 ± 0.4^{a}
	393.75	0.4363 ± 0.0001^{e}	4.8 ± 0.3^{a}	4.5 ± 0.3^{ab}	$1.7\pm0.3_{a}$	7.23 ± 0.04^{a}

Different letters in the same column, indicate significant differences ($p < 0.05$). The mean and standard deviation of n = 3 are shown.

[1] swelling capacity: cm^3 of hydrated and swollen fibre per gram of dry mass.

[2] water absorption capacity: gram of water per gram of dry mass.

[3] oil retention capacity: gram of sunflower oil retained per gram of dry mass.

[4] water soluble fraction.

Table 3. Pearson moment coefficients for the correlation of functional properties of soy okara systems.

Functional Properties	ρ_b ($g.cm^{-3}$)	SC ($cm^3.g^{-1}$)	WHC ($g.g^{-1}$)	OHC ($g.g^{-1}$)	FSA (%)
ρ_b ($g.cm^{-3}$)	**1**	-0.7169	-0.1262	**-0.9137 (p<0.01)**	**-0.9654 (p<0.001)**
SC ($cm^3.g^{-1}$)	-0.7169	**1**	0.5428	0.5062	0.6852

WHC ($g.g^{-1}$)	-0.1262	0.5428	**1**	-0.0654	0.1506
OHC ($g.g^{-1}$)	**-0.9137 (p<0.01)**	0.5062	-0.0654	**1**	**0.8164 (p<0.04)**
FSA (%)	**-0.9654 (p<0.0019)**	0.6852	0.1506	**0.8164 (P<0.04)**	**1**

Significant correlations are highlighted in gray. The statistical significance of the estimated correlations is indicated between brackets.

Table 4. Content of phytic acid, glucose, sucrose, lactose and raffinose in the systems obtained from okara.

System	Sucrose	Raffinose	Lactose	Glucose	Phytic acid
OC	0.5 ± 0.2 [a]	N.D.	11.4 ± 0.3 [b]	N.D.	1.25 ± 0.24 [a]
OI	N.D.	N.D.	12 ± 1 [b]	6.1 ± 0.2	1.27 ± 0.4 [a]
ORM	1.7 ± 0.9 [a]	0.34 ± 0.03	N.A.	N.D.	1.27 ± 0.24 [a]

$mg.g^{-1}$ sample (d.b.)

Different letters in the same column, indicate significant differences (p <0.05) among okara systems.

N.D.: Not Detected.

N.A.: Not Applicable.

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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Soy by-product, okara, was profited to obtain a functional ingredient (FI).
- Okara can be a favourable substrate for developing FI supporting *L. casei* cells.
- Stabilisation processes applied improved functional properties of dehydrated IF.
- Immobilised probiotic in FI survived to *in vitro* gastrointestinal digestion.
- Probiotics supported in FI were able to adhere to epithelium cells.