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PII: S0963-9969(17)30166-7
DOI: doi: 10.1016/j.foodres.2017.04.020
Reference: FRIN 6679

To appear in: *Food Research International*

Received date: 30 January 2017
Revised date: 10 April 2017
Accepted date: 16 April 2017

Please cite this article as: Francesca Patrignani, Lorenzo Siroli, Diana I. Serrazanetti, Giacomo Braschi, Ester Betoret, Jorge A. Reinheimer, Rosalba Lanciotti , Microencapsulation of functional strains by high pressure homogenization for a potential use in fermented milk. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Frin(2017), doi: 10.1016/j.foodres.2017.04.020

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Microencapsulation of functional strains by high pressure homogenization for a potential use in fermented milk

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Abstract

This study was aimed to evaluate the potential of high pressure homogenization for the microencapsulation of two probiotic lactic acid bacteria, *Lactobacillus paracasei* A13 and *Lactobacillus salivarius* subsp. *salivarius* CET 4063 to produce functional fermented milks. Microcapsules of the considered functional microorganisms were obtained by HPH treatments at 50 MPa in the presence of sodium alginate and vegetable oil. The microencapsulated microorganisms were then inoculated as adjuncts to produce fermented milks. As controls were used fermented milks in which the two probiotic lactobacilli were inoculated without encapsulation. The viability of the strains was monitored during almost 2 months of refrigerated storage. The survival of lactic acid bacteria after the gastric-duodenal simulated test was determined. Fermented milk texture parameters, the presence of exo-polysaccharides and the production of volatile molecules were also evaluated over storage. The microcapsules, for both the considered probiotic strains, were homogeneous and with a size less than 100 μM and therefore did not adversely affect the sensory properties of the fermented milks. The encapsulation decreased the hyperacidity phenomena generally related to the inclusion of probiotic microorganisms in fermented milks. The lower acidity of the products due to the microencapsulation was fundamental for the improvement of the viability of the starter culture and the sensory characteristics of the products. The microencapsulation conditions increased the resistance to the simulated digestion processes, although the strain *Lb. paracasei* A13 generally showed a higher resistance to the gastric barrier respect to *Lb. salivarius* CECT 4063. By contrast, the data obtained showed a reduction of EPS production by the microencapsulation. The volatile profiles showed specific profiles in relation to the probiotic strain used and microencapsulation process. In conclusion, the results of this study underlined the applicative potential of HPH microencapsulation of probiotic microorganisms to produce fermented milk with improved functionality and with enhanced sensory properties.

Keywords: microencapsulation, high pressure homogenization, fermented milk, exopolysaccharydes, volatile molecule profiles

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1. Introduction

The development of a suitable technology for the maintenance of probiotic viability is a key step for the industrial production of functional foods, since, during the fermentation process or during the product storage, probiotic microorganisms could lose their viability (Sarao & Arora, 2017). In general, the viability of probiotic strains added to a food products, especially to a fermented milk, is affected by several factors such as strain sensitivity to process factors (low pH, oxygen, fermentation temperature), food matrix composition (water activity, pH, presence of natural antimicrobials, nutrient availability) and packaging and storage conditions (i.e. refrigeration temperature). Moreover, the gastrointestinal tract (GIT) conditions can affect the viability of the probiotic bacteria during the passage (Barbosa & Teixeira, 2017). In fact, the low pH of the stomach and the presence of bile salts in small intestine can further contribute the loss of viability. On the other hand, it is well established that to confer a functional effect within the body, a probiotic food should contain an adequate number of viable probiotic bacteria (>7 log CFU/g of food) (Espitia, Batista, Azeredo, & Otoni, 2016). Many attempts have been performed by many researchers to maintain high viability of probiotic strains in food products. For example, Patrignani et al. (2009) and Patrignani, Lanciotti, Mathara, Guerzoni, and Holzapfel (2006) increased the viability of functional strain isolated from Masai milk by the strain selection and the optimization of the milk formulation. Burns et al. (2008) and Patrignani et al. (2009) used high pressure homogenization (HPH), applied to milk (ranging between 60-100 MPa), to increase the availability of free amino acids end free fatty acids for the maintenance of *Lb. paracasei* A13 and *Lb. acidophilus* 08 viability in probiotic fermented milks and cheeses. Muramalla and Aryana (2011) and Tabanelli et al. (2013) demonstrated that sublethal HPH treatment (performed at 50 MPa) improved functional properties of probiotic bacteria (such as hydrophobicity, auto-aggregation and resistance to biological stresses) and preserved their viability during dairy product refrigerated storage. With the same purpose, some authors investigated other non-thermal technologies, applied to probiotic strains at sublethal level, such as Pulsed Electric Field (PEF) and High Hydrostatic

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Pressure (HPP) (Cueva, 2009; da Cruz et al., 2010; Francesca Patrignani, Lanciotti, & Guerzoni, 2011). Other evidences highlighted the use of growth factors for the preservation of high probiotic strain cell loads in dairy products during the storage (Prasanna, Bell, Grandison, & Charalampopoulos, 2012).

Recently, the literature data pointed out the use of polymers such as pectin, alginate, carrageenan, chitosan, whey, gelatin and lipids for microencapsulation of bacteria with positive effects in protection of probiotic cells during storage condition and GI environment (Ariful, Yun, Choi, & Cho, 2010). Although the most used entrapping techniques are the extrusion, freeze and spray drying, Ding and Shah (2009) proposed the use of a microfluidizer device with four different settings, based on all possible combinations between number of passes (10 or 20) and pressure (69 MPa or 138 MPa) for the encapsulation of probiotic bacteria starting from an emulsion of sodium alginate and vegetable oil. These process conditions gave microcapsules having a diameter <100 μm, contrarily to the emulsions obtained by some stirrer conditions (Ding and Shah, 2009). However, the final microcapsule dimensions are related to the pressure applied and to the cell surface properties of bacteria (Burgain et al., 2014).

Thus, the main aim of this research was to evaluate the potential of microencapsulation, carried out by HPH, by using a lab scale equipment, of two probiotic bacteria, *Lactobacillus paracasei* A13 and *Lactobacillus salivarius* CET 4063, for the production of functional fermented milks characterized by high viability of the functional strains. More specifically, the two probiotic bacteria were encapsulated by HPH at 50 MPa using 5 passes, starting from a sodium alginate-vegetable oil emulsion, and used as adjuncts for the production of functional fermented milks. As controls, the products obtained by the same microorganisms without encapsulation were used. All the fermented milks were stored at 4 °C and the viability of fermentation starter and probiotic cells, encapsulated or not, was checked during the refrigerated storage and during a stomach duodenum passage simulation performed after 20 and 34 days of product storage. Moreover, the fermented

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milks were characterized 24 h after coagulation for their textural feature and EPS presence while their volatile molecule profiles were performed during the refrigerated storage up to 34 days.

2. Material and Methods

2.1 Bacterial strains

The strains used in this experimental work were *La*ctobacillus *paracasei* A13, originally isolated from Argentinean fermented milks, *Lactobacillus salivarius* CET 4063, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, obtained from Sacco srl (Italy). Stock cultures of lactobacilli *Lb. paracasei* and *Lb. salivarius* were maintained at -70 °C in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom), while for *Str. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were provided in lyophilized form in mix by Sacco. Fresh cultures of each strain were obtained from frozen stocks by two consecutive transfers in MRS broth (Oxoid, Basingstoke, United Kingdom) using a 1% (v/v) inoculum and incubated at 37 °C in aerobic conditions for overnight. The starter cultures were obtained in skim milk (Oxoid, Basingstoke, United Kingdom) inoculating the medium and incubating at 42 °C.

2.2 Production of microcapsules of Lactobacillus salivarius CET 4063 and Lactobacillus paracasei A13

Functional strains were cultivated in overnight at 37°C in MRS broth (1 L). In order to obtain a final concentration of 10^{10} CFU/mL, each strain was centrifuged and the pellet suspended in 100 mL of ringer solution. The strain cell loads were evaluated, after proper serial dilutions, onto MRS for *Lb salivarius* and MRS-LP (Litium Chlorine 0.2% and Sodium Propionate 0.3%) for *Lb. paracasei* A13. Twenty five millilitres of each functional strain, grown in MRS at 37 °C for 24 h, at a concentration of 10^{10} CFU/mL were added into 100 mL of 3% sodium alginate, previously sterilized in autoclave. The mixture was added of 1 mL di Tween 80 and then 200 mL of vegetable oil and gently mixed. Following, it was processed by high pressure homogenization using a Panda homogenizer (Gea, Parma, Italy), provided of a thermal exchanger, at 50 MPa for 5 cycles. This

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treatment was chosen after different previous trials performed to select the best parameters (in terms of level of pressure and number of passes) to guarantee a good capsule size and high viability of the functional strains. After the last homogenization cycle, sterile calcium chloride solution (0.1 M) was gently added to the emulsion until its breaking. The capsules were maintained at refrigerated conditions for overnight and then collected by centrifugation and let at 4 °C until their use.

2.3 Morphological analysis of the obtained microcapsules

Microcapsule morphology was investigated using scanning electron microscope (SEM) according to the method proposed by De Prisco, Maresca, Ongeng, and Mauriello (2015).

2.4 Enumeration of encapsulated functional strains and encapsulation yield

For each encapsulated strain, 1 g of microcapsules was diluted with 9 mL of phosphate buffer (pH 7) to permit the cell release from alginate. The tubes were left in agitation for 30 min and following serial dilutions were performed and plated onto MRS for *Lb salivarius* and MRS-LP (Litium Chlorine 0.2% and Sodium Propionate 0.3%) for *Lb. paracasei* A13. To calculate the encapsulation yield, the formula $(N/N_0)*100$ was used where N is the number of viable cells released from the microcapsules and N_0 is the number of viable cells in the cell concentrate for microencapsulation.

2.5 Preparation of fermented milks

The production of fermented milks was carried out in lab conditions. Milk, treated at 105°C for 7 min, was splitted in 100 mL containers and each one was inoculated with starter cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) at level of 6 log CFU/mL while the functional strains, encapsulated or not, were inoculated at level of at least 7 log CFU/mL. The inoculated milks were incubated at 42 °C until the reaching of pH 4.6. After that, the fermented milks were stored at refrigeration temperature (4°C) for 56 days. For each fermented milk, 30 repetitions were performed.

2.6 Reduction of pH

The reduction of pH during fermentation and its evolution in fermented milks during refrigerated storage was monitored using pH meter Basic 20 (Crison Instruments, Modena, Italy).

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2.7 Starter cultures and probiotic strain viability in fermented milks

Twenty grams of fermented milk was placed into 180 mL sodium citrate sterile solution (20 g/L) and homogenised in a stomacher (Lab-blender 80, Pbi International, Milan, Italy) for 3 min. Decimal dilutions of the homogenates were made in 0.9% of Ringer's solution, and 0.1 mL of appropriate dilutions was spread onto the surface of different agar media. *Str. thermophilus* was counted on M17 agar (Oxoid, Basingstoke, Hampshire, UK) (42 °C, 48 h), *Lb. delbrueckii* subsp. *bulgaricus* on MRS agar (Oxoid) acidified with glacial acetic acid (Merck, Darmstadt, Germany) at pH 5.4 (42 °C, 48 h) while *Lb. paracasei* A13 and *Lb. salivarius* CET 4063 were counted onto MRS-LP and MRS, respectively. The morphology of each strain on plates was checked. The fermented milks containing encapsulated probiotic strains were firstly diluted in Phosfate Buffer Solution at pH 7 to permit the release of the bacteria from alginate according to the procedure previously described.

2.8 Resistance of starters and functional strains to simulated gastrointestinal digestion in fermented milks

The resistance of starter cultures and probiotic strains to simulated gastrointestinal digestion (RSGD) was performed after 1, 20 and 34 days of refrigerated storage. This time sampling was chosen to verify the strain resistance in the first month of storage of the product. The analyses were performed according to the method proposed by Vinderola, Binetti, Burns, and Reinheimer (2011).

2.9 Texture analyses

Texture analyses were performed on fermented milks after 24 h from coagulation. Firmness, consistency, cohesiveness and viscosity indexes were evaluated using a back extrusion cell (A/AB) on a Texture Analyser TA DHI (Stable Micro System, UK) according to the manufacturer's instructions. A solid rod (35 mm diameter) was thrust into the sterile container holding 100 mL sample using a 5-kg load cell.

2.10 Quantification of exopolysaccharides

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The determination of exopolyssacharides in fermented milks was performed after 24 h from coagulation according to the method described by Patrignani et al. (2016). Briefly, 10 mL of fermented milk was adjusted to pH 7 with NaOH and was added 100 lL of filter-sterilised flavourzyme (10% w/w) (Sigma- Aldrich, Milan, Italy). The sample was incubated at 50 °C in a shaker for 4 h and then subjected to vortexing for 15 s. The sample (100 L) was mixed with 2.9 mL of distilled water and 7 mL of chilled absolute ethanol and left overnight at 4 °C. The sample was centrifuged at 27 000 g, 4 °C for 40 min. After centrifugation, the supernatant was removed and 3 mL of distilled water was used to re-suspend the pellet. Seven millilitres of chilled 99.7% ETOH was added into the sample, left overnight and centrifuged similarly to the conditions above. The pellet was then re-suspended in 1 mL of distilled water. One millilitre of 5% (w/v) phenol solution was added to the sample and mixed using a vortexing (15s) and then 5 mL of concentrated sulphuric acid was added directly to the sample. The sample was left at room temperature for 30 min prior to measuring absorbance at 485 nm using a spectrophotometer (Ultrospec 2000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The total amount of exopolysaccharides was determined based on a standard calibration curve prepared using glucose or dextran (Sigma-Aldrich, Milan, Italy).

2.11 Aroma profiles

Volatile compounds of fermented milks were monitored after 24 h from coagulation and after 13 and 22 days of refrigerated storage using a GC-MS coupled with a solid phase micro extraction (SPME). The method used was described by Patrignani et al. (2016).

2.12 Statistical analysis

The analyses are the mean of three independent repetitions. Significant differences in microbiological data, exopolysaccharides and texture parameters, were tested using a two-tailed paired t-test (Statistics for Windows, Statsoft Inc, Tulsa, UK).

3. **Results & Discussion**

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3.1 Production of microcapsules of Lactobacillus salivarius CET 4063 and Lactobacillus paracasei A13

In the present research, the use of high pressure homogenization performed at 50 MPa for 5 cycles, using sodium alginate in emulsion with vegetable oil, permitted to obtain an encapsulation yield of viable *Lactobacillus paracasei A13* and *Lactobacillus salivarius CET 4063* of 87% and 83%, respectively. These data, if compared with the literature ones, can be considered as positive results. In fact, Ding and Shah (2009), testing the encapsulation of several probiotic bacteria, by microfluidizer, found an encapsulation average of 77.72%, by using the same carrier but different process conditions (i.e. combination of pressures of 69 MPa or 138 MPa and 10 or 20 cycles). The Figure 1 reports a micrograph obtained by scanning electronic microscope (SEM) of encapsulated *Lb. paracasei* A13, where it is showed that the diameters of the capsule obtained, sphere-like and quite rough, was less than 100 µm. This is fundamental because it was demonstrated that large capsule sizes can negatively influence the texture and mouthfeel of dairy products (Ding & Shah, 2009; Godward & Kailasapathy, 2003; Hansen, Allan-Wojtas, Jin, & Paulson, 2002; Iravani, Korbekandi, & Mirmohammadi, 2015). In fact, when capsules are generally rounded and small in size, they are not perceived by the consumers giving positive results in sensory evaluation. In addition, it is reported that the application of pressure of 50 MPa are not able to induce stresses to the microbial cells (Lanciotti, Patrignani, Iucci, Saracino & Guerzoni, 2007), contrarily to the use of atomizers that are able to reduce capsule size but impart physical stresses to the cells, reducing the viability of probiotic bacteria (Lee & Heo, 2000). In addition, Burns et al. (2015) demonstrated that this level of pressure of 50 MPa, when applied to *Lb. paracasei* A13, is suitable to maintain its high viability in Caciotta cheese during the ripening while Tabanelli et al. (2012) founded that when sub-lethal HPH treated cells of *Lb. paracasei* A13 were used to feed BALB/c mice, a higher IgA response was observed.

Moreover, the type of equipment used in this research permitted to obtain microcapsules similar to those reported in the literature for dimension and characteristics (mean size and shape) by using

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more sustainable process condition in terms of level of pressure and number of cycles if compared to the literature data (Ding & Shah, 2009).

3.2 Viability of starter cultures and probiotic adjuncts in fermented milk during the refrigerated storage

During the manufacturing of the fermented milks, all the samples, containing the starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbreueckii* subsp. *bulgaricus*) and the probiotic adjuncts (encapsulated or not), reached the pH 4.6 in five hours at 42 °C (data not showed). The microbial cell loads of the starter cultures detected in fermented milks during the refrigerated storage are reported in Table 1. The microbial cell loads were monitored for 56 days since in general the shelf-life of this kind of product is of almost 2 months. The data clearly showed that the cell loads of the starter cultures decreased overall the storage in relation to the presence of the probiotic bacteria and their encapsulation. *Lb. delbrueckii* subsp. *bulgaricus* decreased during the storage its viability particularly in presence of *Lb. paracasei* A13, encapsulated or not. In particular, *Lb. delbrueckii* subsp. *bulgaricus* cell load was found under the detection limit after 50 days of storage in the samples supplemented with *Lb. paracasei* A13 not encapsulated while, in products containing the encapsulated adjuncts it was not determined after 56 days of storage. Differently, in the presence of *Lb. salivarius*, its cell load decrease was slower compared to the other conditions and, particularly, in the presence of the encapsulated strain and up to 40 days of storage. After 40 days of storage *Lb. delbrueckii* subsp. *bulgaricus* dramatically decrease also in the presence of encapsulated *Lb. salivarius* cells. By contrast *St. thermophilus* viability was less affected than the other starter culture by the presence of the probiotic strains independently on their encapsulation. However, after 56 d of product refrigerated storage, the cell load of *St. thermophilus* was higher in fermented milks where the probiotic strains were added in encapsulated form. The decreasing of the commercial starter culture viability can be due to the interaction with probiotic strains (particularly when they are used in planktonic form) and to the hyper acidification that they are able to induce in the product. In fact, Patrignani et al (2009) showed that *Lb. paracasei* A13 used as adjunct was able

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to significantly decrease the pH of fermented milk obtained with traditional yoghurt starter cultures. Also the ability to produce high quantities of organic acid of *Lb. salivarius* CECT 4063, with proved effect against *Helicobacter pylori,* was confirmed (Betoret et al., 2012; Betoret, Calabuig-Jiménez, Patrignani, Lanciotti, & Dalla Rosa, 2016). In this context, the encapsulation can be considered a valid strategy for protecting probiotic bacteria from the stringent condition during process and storage and for limiting the exchange of metabolite, produced by the probiotic cultures, with the external environment (Okuro, Thomazini, Balieiro, Liberal, & Fávaro-Trindade, 2013). In fact, the presence of the microcapsules limited also the pH decrease of the products independently on the adjunct strain considered. At the end of the storage (56 days), the fermented milks obtained with the not encapsulated probiotic cultures were characterized by lower pH values (pH 4.27±0.02, in the presence of *Lb. paracasei* A13 and pH 4.40 ±0.03, in the presence of *Lb. salivarius*) than those of the samples supplemented with encapsulated cells (pH 4.35±0.03, in the presence of *Lb. paracasei* and pH 4.55±0.02, in the presence of *Lb. salivarius*). The prevention of hyper acidification phenomena can also result in a highest viability of the probiotic bacteria, that is still a prerequisite to confer a functional effect on the host (Barbosa & Teixeira, 2017). In fact, as showed by Table 2, the microencapsulation permitted to maintain a higher viability both for *Lb. paracasei A13,* after 34 days of product refrigerated storage, and *Lb. salivarius CET 4063*. However, this last strain was more sensitive to the adopted process conditions than *Lb. paracasei* A13 whose resistance to acid environment is well documented (Burns et al., 2008; Patrignani et al., 2009).

3.3 Adjunct cultures cell load reduction after simulated stomach duodenum passage

The performances of microencapsulation in maintaining strain viability are also demonstrated during the simulated stomach duodenum passage and especially after 20 days of product storage (Table 3). In fact, the encapsulation, as expected, increased the protection for the strains against the natural physiological barriers. *Lb. salivarius* totally decreased in the product when used in its planktonic form since the application of acid condition, while when encapsulated it was reduced under the detection limit only after the combined stress from bile and pancreatine. However, this

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strain is generally used in functional fields for its antimicrobial activity at stomach level, and in particularly against *H. pylori* and consequently the resistance to low pH is a key selection factor to exert its antagonistic effect (Betoret et al., 2012). On the contrary *Lb. paracasei* A13, when encapsulated, was able to survive also after the entire simulated duodenum conditions. After 34 days of product storage, the ability to survive in the product after stomach-duodedum simulated stress conditions is reduced for *Lb. salivarius*, independently on the encapsulation. On the contrary, *Lb. paracasei* A13, when encapsulated, confirmed its ability to survive following all the stress applied.

3.4 Evaluation of exopolysaccharydes and textural parameters of fermented milk

Also the presence of exopolysaccharydes (EPS) and textural parameters were evaluated in the fermented milks in relation to the probiotic strain used and their encapsulation after 24 h from the coagulation (Table 4). The encapsulation of functional bacteria reduced the release of EPS from both the strains in the products analogously to the other metabolic products. However, the EPS are very important both for the textural properties of the product and for their functional role (Goh, Haisman, Archer, & Singh, 2005; Mende, Rohm, & Jaros, 2016). They were found able to affect both the probiotic cell immuno-modulatory abilities and their capacity to adhere to epithelial cells (Wang, Zhao, Yang, Zhao, & Yang, 2015). This last features is very interesting because an increased adhesion permit to probiotic strains to better colonize the epithelial cell and to be a barrier against pathogenic species colonization (Wang et al., 2015). In the fermented milk obtained with *Lb. paracasei* not encapsulated 289 µg/mL of EPS were detected while in the fermented milks containing *Lb. salivarius,* encapsulated or not, the detected levels were lower (113.97 µg/mL). On the other hand, the ability to produce EPS is species and strain dependent but significantly affected by medium composition and particularly by the concentration and type of sugar present (Torino, Font de Valdez, & Mozzi, 2015). Between the considered probiotic species, the higher concentration of EPS produced by not encapsulated *Lb. paracasei* A13 resulted in higher values of firmness and consistency of the products. However, the texture parameters of a fermented milks are

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generally affected by several physico-chemical, process and compositional parameters (Patrignani et al., 2007). Among the compositional variables also the type of EPS, in its turns depending on the species and strain, is reported to affect the textural parameters affecting the interaction among the macromolecules of the system. Also the acidification ability and metabolic activities (e.g. proteolytic activities) of the strains are reported to be key factors affecting the micro and macrostructural features (dimension, charge, water binding activity) of the dairy products and their interactions (Vannini et al., 2008). The effect of the encapsulation on the metabolism of the tested strains (reducing the nutrient and the exchange with environment) can explain the differenced detected among the products. In fact, also in product containing *Lb. salivarius* where the same amount of EPS was detected, independently on the encapsulation, significant differences in consistency, cohesiveness and viscosity index were found (Table 4).

3.5 Volatile molecule profiles of fermented milk

The functional fermented milks obtained with the probiotic bacteria, encapsulated or not, and their controls were subjected to SPME/GC/MS analysis 24 h after coagulation and during the product refrigerated storage (13 and 22 days). Although the fermented milks were characterized by a specific “aromagramme”, in relation to the presence of the probiotic bacteria and encapsulation, molecules belonging to alcohols, aldehydes, ketones, organic acid and esters were found in all the samples. However, the most representative compounds were acids and ketones, while esters and aldehydes were scarce (data not showed). To better understand the effects of the variables adopted (presence of probiotic, encapsulation, storage time), the compounds detected were analyzed using a principal component analysis (PCA). In Figures 2 a and b, the projection of samples and detected molecules is reported, in relation to the variables considered. The PCA was able to explain 63% of the total variance among the samples. As evidenced by the Figure 2a, the encapsulation affected the distribution and clustering of the samples on the space after 24 h of product storage (day1). On the contrary, the used probiotic strains did not affect, at this storage time, the sample grouping. In particular, the fermented milks obtained with probiotic strains in their planktonic form clustered

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together independently on the species (cluster 1), while fermented milks from encapsulated bacteria grouped as one (cluster 2). These data showed that the encapsulation plays a significant and "selective" role on the release of volatile compounds in the first time of the refrigerated storage. Also the material used for encapsulation can affect these parameters affecting both the metabolism of the strains but also the release of compound against the external environment (Inguva, Ooi, Desai, & Heng, 2015). The results clearly showed that the clusterization of the samples overtime is more affected by the probiotic species used as adjunct than the encapsulation. As evidenced by Figure 2b, the classes of compounds affecting the grouping for cluster 1 and 2 were aldehydes and alcohol, respectively while ketones and acids affected more the clusterization in the products over refrigerated storage. On the other hand, molecules such as 2-propanone, diacetyl, acetoin and 2,3-butanediol with acetaldehyde are recognized as main aroma compounds of yoghurt-type products (Janiaski et al., 2016; Lanciotti, Vannini, Pittia, & Guerzoni, 2004; Patrignani et al., 2007, 2009, 2016) and the presence and the strength of the microcapsules modified their release or the interaction with the system (Inguva et al., 2015).

4. Conclusion

The results obtained in this research pointed out the potential of high pressure homogenization for the microencapsulation of the used functional strains. In fact, using process conditions more sustainable with respect to those proposed by some Authors (69 MPa or 138 MPa for 10 or 20 passes), microcapsules with a good yield of encapsulation and acceptable size were obtained. Moreover, the microspheres presented a high yield in terms of entrapped viable cells. The data highlighted that, especially for *Lb. paracasei*, the obtained microcapsules were also functional to maintain high probiotic viability during the fermented milk refrigerated storage satisfying, also at the end of the storage, the rules requested for functional foods (at least 7 log cfu/g product). Furthermore, the type of microcapsules produced affected the metabolism of probiotic strain avoiding hyper acidification in the products and modulating the release of volatile molecules, that are, in their turn, able to affect the product sensory profile. Also fermented product texture

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parameters were influenced by the encapsulation of probiotic strains since it modulated the release of exopolysaccharides. Although further investigations are necessary to understand in deep the dynamics of release of probiotic bacteria after the ingestion of the product and the interaction of the microcapsules with the system, this approach can be a useful tool for the production of innovative products with specific functionality.

Figure caption

Figure 1. Scanning electron microscope micrograph of the obtained microcapsule containing *Lb. paracase*i A13.

Figure 2. Plot of cases (a) and variables (b) obtained by PCA analysis of the total significant volatile molecules characterising the fermented milks in relation to the presence of probiotic strains, their encapsulation and refrigerated storage conditions. T1: 24 h after coagulation, T13: after 13 days of storage, T22: after 22 days of storage

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Table 1. Viability (log cfu/g) of starter cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) in fermented milks in relation to the presence of probiotic strains, microencapsulation and refrigerated storage time

Lactobacillus delbrueckii* subsp. *bulgaricus

	Time (d)									
	0	**1**	**7**	**13**	**20**	**28**	**34**	**41**	**50**	**56**
FM1**+ *Lb. salivarius*	7.2 ± 0.1^{A}	7.2 ± 0.2^{A}	6.8 ± 0.1^{A}	6.5 ± 0.2^{A}	4.0 ± 0.2^{A}	3.7 ± 0.1^{A}	3.7 ± 0.2^{A}	3.7 ± 0.3^{A}	3.7 ± 0.5^{A}	3.0 ± 0.3^{A}
FM 2+ encapsulated *Lb. salivarius*	7.4 ± 0.1^{A}	7.9 ± 0.1^{B}	6.4 ± 0.1^{B}	5.9 ± 0.1^{B}	4.3 ± 0.2^{A}	4.1 ± 0.2^{A}	4.7 ± 0.3^{B}	5.4 ± 0.3^{B}	3.6 ± 0.1^{B}	3.5 ± 0.2^{A}
FM3+ *Lb. paracasei*	7.7 ± 0.2^{a}	7.3 ± 0.2^{a}	5.9 ± 0.2^{a}	3.9 ± 0.2^{a}	3.7 ± 0.1^{a}	3.4 ± 0.1^{a}	3.0 ± 0.1^{a}	2.2 ± 0.1^{a}	-*^{a}	-
FM4+ encapsulated *Lb. paracasei*	8.2 ± 0.1^{b}	8.0 ± 0.1^{b}	6.0 ± 0.1^{a}	4.1 ± 0.3^{a}	4.0 ± 0.1^{b}	3.7 ± 0.1^{b}	3.9 ± 0.2^{b}	4.0 ± 0.2^{b}	4.0 ± 0.2^{b}	-

Streptococcus thermophilus

	Time (d)									
	0	**1**	**7**	**13**	**20**	**28**	**34**	**41**	**50**	**56**
FM1 + *Lb. salivarius*	7.0 ± 0.2^{A}	7.3 ± 0.3^{A}	7.7 ± 0.2^{A}	6.6 ± 0.3^{A}	7.2 ± 0.1^{A}	6.3 ± 0.1^{A}	7.0 ± 0.3^{A}	6.1 ± 0.2^{A}	5.8 ± 0.2^{A}	6.0 ± 0.1^{A}
FM2 + encapsulated *Lb. salivarius*	7.9 ± 0.1^{B}	8.1 ± 0.1^{B}	7.2 ± 0.2^{B}	7.4 ± 0.1^{B}	7.2 ± 0.2^{A}	6.9 ± 0.2^{B}	7.1 ± 0.2^{B}	6.5 ± 0.1^{B}	6.5 ± 0.2^{B}	6.4 ± 0.2^{B}
FM3+ *Lb. paracasei*	7.0 ± 0.1^{a}	7.7 ± 0.1^{a}	7.6 ± 0.2^{a}	7.0 ± 0.1^{a}	7.4 ± 0.3^{a}	6.7 ± 0.2^{a}	7.0 ± 0.3^{a}	6.0 ± 0.1^{a}	6.0 ± 0.1^{a}	5.8 ± 0.3^{a}
FM4+ encapsulated *Lb. paracasei*	7.7 ± 0.1^{b}	8.0 ± 0.2^{a}	7.9 ± 0.1^{a}	7.3 ± 0.2^{a}	7.6 ± 0.2^{a}	6.9 ± 0.3^{a}	6.6 ± 0.4^{a}	6.2 ± 0.2^{a}	6.7 ± 0.4^{b}	6.7 ± 0.2^{b}

*under the detection limit

**fermented milk 1 was obtained by the traditional starter cultures and not encapsulated *Lb.salivarius*; fermented milk 2 was obtained by the traditional starter cultures and the encapsulated *Lb.salivarius*; fermented milk 3 was obtained by the traditional starter cultures and not encapsulated *Lb.paracasei;* fermented milk 4 was obtained by the traditional starter cultures and the encapsulated *Lb.paracasei.*

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In the same column, FM 1 data must be compared to FM2 ones while FM3 data must be compared to FM4 ones. Within the same group, data with the same superscript letter must be considered not statistically different ($p>0.05$)

Table 2. Viability (log cfu/g) of probiotic cultures (*Lactobacillus paracasei* A13 and *Lactobacillus salivarius* CET 4063) in fermented milks in relation to the microencapsulation and refrigerated storage time.

	Time (d)									
	0	**1**	**7**	**13**	**20**	**27**	**34**	**41**	**50**	**56**
FM1* + *Lb. salivarius*	7.2 ± 0.1^{A}	7.0 ± 0.2^{A}	6.8 ± 0.1^{A}	6.1 ± 0.2^{A}	6.3 ± 0.1^{A}	6.8 ± 0.2^{A}	6.9 ± 0.1^{A}	6.7 ± 0.2^{A}	6.0 ± 0.3^{A}	5.8 ± 0.1^{A}
FM 2 + encapsulated *Lb. salivarius*	7.6 ± 0.2^{B}	7.7 ± 0.3^{B}	7.0 ± 0.3^{A}	7.2 ± 0.4^{B}	6.5 ± 0.3^{A}	7.3 ± 0.2^{B}	7.3 ± 0.2^{B}	7.1 ± 0.1^{B}	6.9 ± 0.2^{B}	6.7 ± 0.3^{B}
FM 3+ *Lb. paracasei*	7.0 ± 0.3^{a}	7.0 ± 0.1^{a}	7.2 ± 0.2^{b}	7.4 ± 0.2^{a}	7.6 ± 0.3^{a}	7.3 ± 0.1^{a}	7.4 ± 0.2^{a}	7.4 ± 0.3^{a}	7.2 ± 0.3^{a}	6.9 ± 0.1^{a}
FM 4+ encapsulated *Lb. paracasei*	7.9 ± 0.1^{b}	8.2 ± 0.3^{b}	8.4 ± 0.4^{c}	8.3 ± 0.3^{b}	7.9 ± 0.1^{a}	8.3 ± 0.3^{b}	8.1 ± 0.2^{b}	8.0 ± 0.1^{b}	8.0 ± 0.2^{b}	7.5 ± 0.2^{b}

* fermented milk 1 was obtained by the traditional starter cultures and not encapsulated *Lb.salivarius*; fermented milk 2 was obtained by the traditional starter cultures and the encapsulated *Lb.salivarius*; fermented milk 3 was obtained by the traditional starter cultures and not encapsulated *Lb.paracasei;* fermented milk 4 was obtained by the traditional starter cultures and the encapsulated *Lb.paracasei.*

In the same column, FM 1 data must be compared to FM2 ones while FM3 data must be compared to FM4 ones. Within the same group, data with the same superscript letter must be considered not statistically different ($p>0.05$)

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Table 3. Cell load reductions (log cfu/ml) of *Lb. paracasei* A13 and *Lb. salivarius* CECT 4063 to simulated gastrointestinal digestion in fermented milks in relation to microencapsulation and refrigerated storage time

	GIT challenge			
		Cell load reduction (log cfu/ml)		
	Initial cell loads	Acid (pH 2.5)	Bile (1%)	Bile-Pancreatin (0.3-0.1%)
20 days				
FM1* +*Lb salivarius*	6.2±0.1	6.2^{A}	6.2^{A}	6.2^{A}
FM 2 +*Encapsulated Lb salivarius*	6.7±0.1	4.9 ± 0.3^{B}	5.5 ± 0.2^{B}	6.7^{A}
FM 3+*Lb paracasei*	7.4±0.2	7.4^{a}	7.4^{a}	7.4^{a}
FM 4+*Encapsulated Lb paracasei*	7.7±0.1	4.8 ± 0.3^{b}	5.3 ± 0.3^{b}	6.3 ± 0.1^{b}
34 days				
FM1* +*Lb salivarius*	6.7±0.3	6.7^{A}	6.7^{A}	6.7^{A}
FM 2 +*Encapsulated Lb salivarius*	7.0±0.2	7.0^{A}	7.0^{A}	7.0^{A}
FM 3+*Lb paracasei*	7.4±0.2	4.4 ± 0.2^{a}	7.4^{a}	7.4^{a}
FM 4+*Encapsulated Lb paracasei*	7.8±0.2	4.5 ± 0.2^{a}	6.0 ± 0.3^{b}	6.8 ± 0.3^{b}

* fermented milk 1 was obtained by the traditional starter cultures and not encapsulated *Lb.salivarius*; fermented milk 2 was obtained by the traditional starter cultures and the encapsulated *Lb.salivarius*; fermented milk 3 was obtained by the traditional starter cultures and not encapsulated *Lb.paracasei;* fermented milk 4 was obtained by the traditional starter cultures and the encapsulated *Lb.paracasei.*

In the same column, FM 1 data must be compared to FM2 ones while FM3 data must be compared to FM4 ones. Within the same group, data with the same superscript letter must be considered not statistically different (p>0.05)

Table 4. Exopolysaccharides (µg/mL) and textural parameters (g, g s) detected in fermented milks in relation to the presence of probiotic and their encapsulation

		Textural parameters			
	EPS	Firmness (g)	Consistency (g s)	Cohesiveness (g)	Viscosity index (g s)
FM1* +*Lb. salivarius*	113.97 ± 8.24^{A}	20.71 ± 2.45^{A}	286.59 ± 12.39^{A}	15.27 ± 0.98^{A}	30.21 ± 1.76^{A}

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FM2 + encapsulated *Lb. salivarius*	114.93±5.20^{A}	17.18±1.50^{A}	227.73±9.26^{B}	9.66±1.02^{B}	14.67±2.73^{B}
FM3 + *Lb. paracasei*	289.65±11.76^{a}	22.47±2.73^{a}	287.50±13.20^{a}	11.67±1.29^{a}	27.63±1.40^{a}
FM4 + encapsulated *Lb paracasei*	115.06±7.65^{b}	21.64±3.10^{a}	278.13±8.38^{a}	13.28±1.15^{a}	23.95±2.10^{b}

* fermented milk 1 was obtained by the traditional starter cultures and not encapsulated *Lb.salivarius*; fermented milk 2 was obtained by the traditional starter cultures and the encapsulated *Lb.salivarius*; fermented milk 3 was obtained by the traditional starter cultures and not encapsulated *Lb.paracasei;* fermented milk 4 was obtained by the traditional starter cultures and the encapsulated *Lb.paracasei.*

In the same column, FM 1 data must be compared to FM2 ones while FM3 data must be compared to FM4 ones. Within the same group, data with the same superscript letter must be considered not statistically different ($p>0.05$)

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Figure 1.

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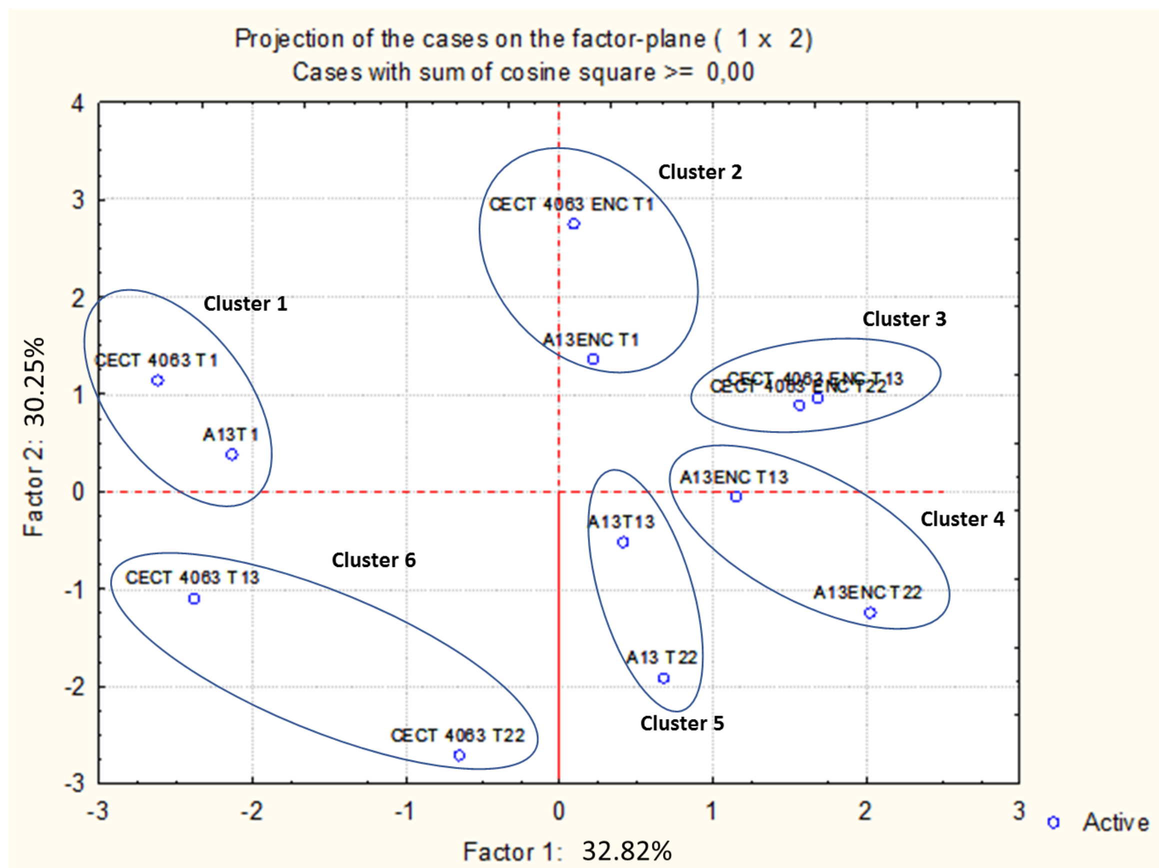
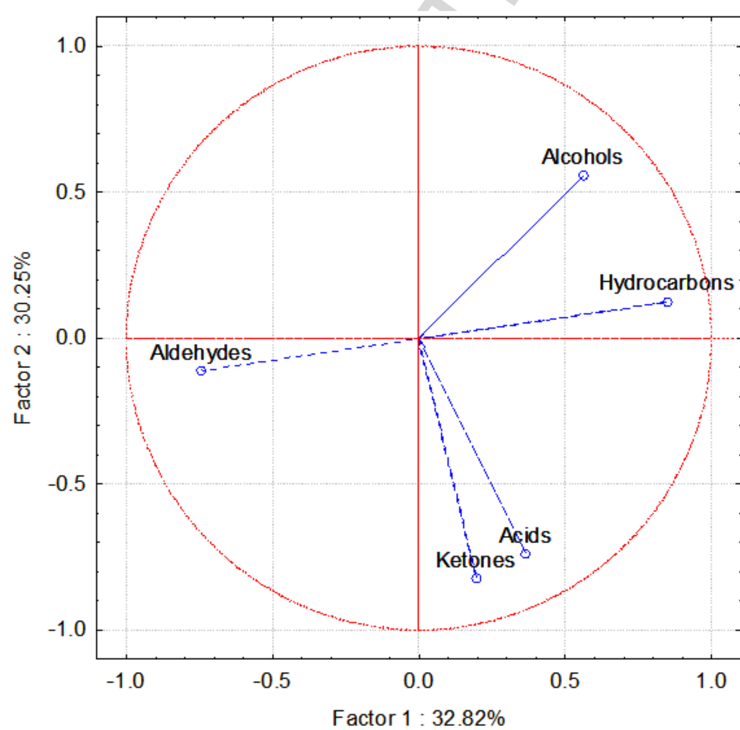
Figure 2.

(a)



(b)



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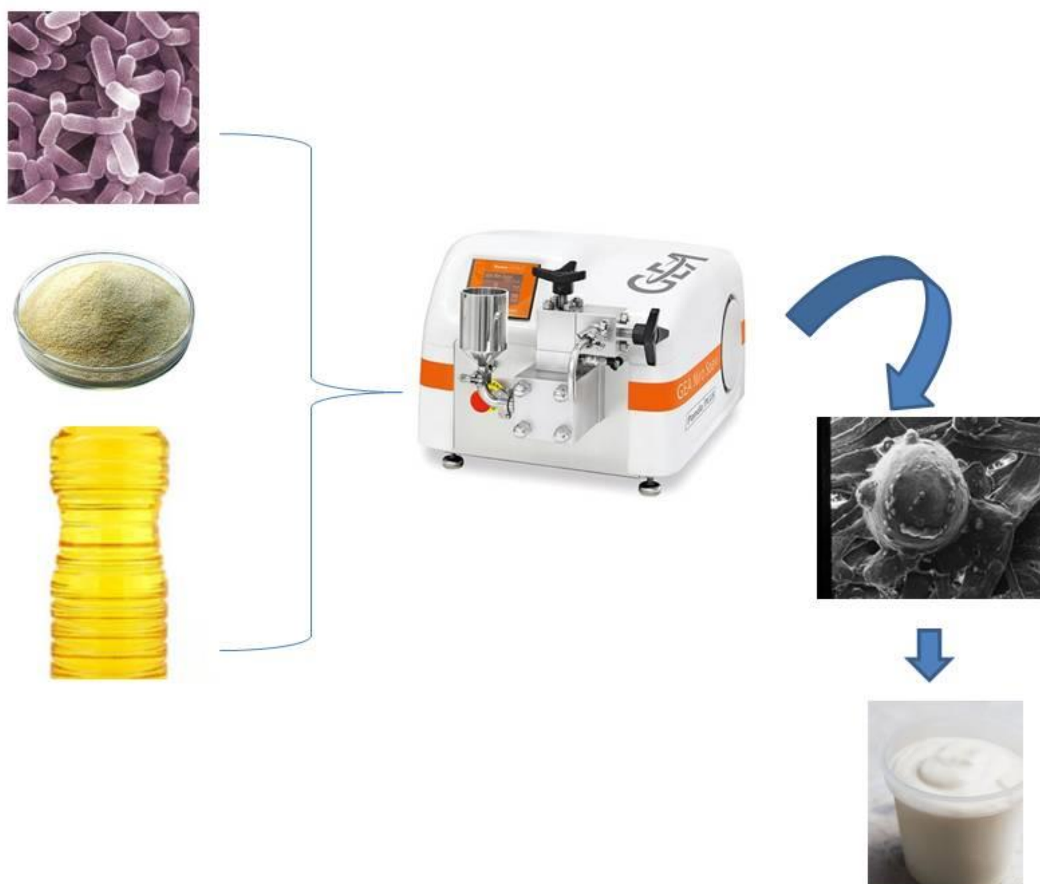
Graphical abstract



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Highlights

A treatment of 50 MPa for 5 passes resulted in stable microcapsules of Lactobacilli

Microcapsules presented a high yield in terms of entrapped Lactobacilli viable cells

Microcapsules maintained high lactobacilli viability in fermented milk during storage

Microencapsulation of adjunct bacteria reduced the acidity of the fermented milk

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