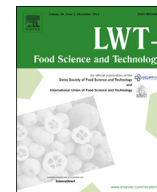




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## Microencapsulation by vibrating technology of the probiotic strain *Lactobacillus reuteri* DSM 17938 to enhance its survival in foods and in gastrointestinal environment

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## ABSTRACT

The probiotic strain *Lactobacillus reuteri* DSM 17938 was microencapsulated in alginate (MC) and in chitosan-alginate (CMC) matrix by vibrating technology with the main purpose to improve its tolerance to adverse environments. Microencapsulation procedure proved to not affect cell viability as indicated by a high encapsulation yield (97%) as well as further chitosan coating that minimally affected cell population. The viability of microencapsulated probiotic was evaluated under several stress conditions such as 28 days storage in different solutions at 4 and 20 °C, simulated gastrointestinal passage and osmotic stress conditions. Also reuterin production and diffusion through microcapsules were assessed. Results of survival assays showed that in most of tested conditions microencapsulation significantly ( $P < 0.05$ ) improved *L. reuteri* DSM 17938 resistance. Furthermore, microcapsules were freeze dried in skimmed milk and no significant variations in cell viability and microcapsules morphology were observed. Morphological analysis revealed some differences between MC and CMC although both microcapsules had a diameter of  $110 \pm 5 \mu\text{m}$ , a spherical shape and a uniform and continuous surface. This study proved that microencapsulation by n chitosan-alginate matrix could improve tolerance of *L. reuteri* DSM 17938 towards stress conditions encountered in food processing as well as preserve its functional properties.

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## 1. Introduction

Microencapsulation of living cells is becoming a very diffuse technique in several fields of biosciences including medicine (Blasi, Luca, & Mancuso, 2013), nutraceutical sciences (Burgain, Gaiani, Linder, & Scher, 2011) and food technology (Malmo, La Storia, & Mauriello, 2013; Rodrigues et al., 2012). In the field of food technology, De Vos, Faas, Spasojevic, and Sikkema (2010) reviewed literature on techniques and polymeric materials that have been applied to encapsulate bioactive food components. Despite of a wide choice of encapsulation technologies and materials, living cells require gentle techniques such as extrusion (De Vos et al., 2010). Microencapsulation of probiotic bacterial cells has been

described by some authors (Anal & Singh, 2007; O'Riordan, Andrews, Buckle, & Conway, 2001; Sohail, Turner, Prabawati, Coombes, & Bhandari, 2012). The primary objective of the aforementioned studies was mainly focused on protection of probiotic cells from gastrointestinal conditions (i.e. low pH and bile salts). However, an important aspect of relevance to microencapsulation of probiotics that has not been adequately demonstrated is the protection of living bacterial cells from technological hurdles (e.g. high or low temperature, high salt content, low water activity, antibacterial substances) normally applied in food processing and preservation. Therefore, substantial empirical evidence of the impact of food technology hurdles on microencapsulated probiotics would be necessary in designing modalities to ensure viability of bacterial cells in foods usually not considered as vehicle of living bacterial cells, such as the bakery products (Malmo et al., 2013). This should provide opportunity for expanding the range of foods that can be used for the delivery of probiotics to the human gut. De Vos et al. (2010) reported the different microencapsulation techniques at the time and one of them is the extrusion. This last is evolved in the vibrating technology, as recently described by

Abbreviations: MC, alginate microcapsules; CMC, chitosan-alginate microcapsules; FMC, freeze dried alginate microcapsules; FCMC, freeze dried chitosan-alginate microcapsules; EE, entrapment efficiency; GSS, gastric simulating solution; ISS, intestinal simulating solution; GI, gastrointestinal.

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Whelehan and Marison (2011). In 1882, the Nobel Prize in Physics Lord John William Strutt Rayleigh proposed that under specific condition, droplets of liquid could spout microscopic jets of fluid (Rayleigh, 1879). In particular, he theorised that when the wavelength of an asymmetric disturbance exceeds the jet circumference, the break-up occurs. Droplet size depends on nozzle (jet) diameter, viscosity of fluid, surface tension, jet velocity and frequency of disturbance. This is an almost new technology, developed before in the Inotech laboratories and then in the Buchi ones. Even though this technique was used for the first time in 1985 by Hulst, Tramper, Vantriet, and Westerbeek (1985) and some other authors used it in the last three decades (Brandenberger & Widmer, 1998; Dorati, Genta, Modena, & Conti, 2013; Mazzitelli et al., 2008; Serp, Cantana, Heinzen, Von Stockar, & Marison, 2000), only few works (Martoni, Bhatthana, Malgorzata, Urbanska, & Prakash, 2008; Shi et al., 2013) described the microencapsulation of bacterial cells by using the vibrating technology.

The aims of this work were to assess the effectiveness of bacterial cell microencapsulation using vibrating technology, to evaluate the cell viability during the storage of fresh and freeze-dried microcapsules and to investigate some of the probiotic and technological properties of probiotic strains after microencapsulation by vibrating technology.

## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

*Lactobacillus reuteri* DSM 17938 was isolated from Reuterin™ (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) and cultured in MRS Broth (OXOID Ltd., Basingstoke, Hampshire, England) at 37 °C, checked for purity and maintained on MRS Agar (Oxoid). Free and microencapsulated cells of *L. reuteri* DSM 17938 were routinely cultured and counted on MRS Agar at 37 °C for 48 h in aerobic conditions. *Pseudomonas fragi* 25P used in reuterin production test, belonging to microorganisms collection of Department of Agriculture, University of Naples Federico II, was previously isolated from fresh meat sample (Ercolini, Russo, Nasi, Ferranti, & Villani, 2009). It was cultured in Tryptone Soya Broth (TSB, Oxoid) supplemented with 5 g/L Yeast Extract Powder (Oxoid) at 20 °C.

### 2.2. Encapsulation of *L. reuteri* DSM 17938

Microencapsulation of bacterial cells was carried out by using the Encapsulator B-395 Pro equipped with an 80 µm nozzle and a syringe pump (BÜCHI Labortechnik, Flawil, Switzerland). A scheme of the Buchi encapsulator is reported in the Fig. 1. In detail, the cells of a defined volume of *L. reuteri* DSM 17938 culture in the early stationary phase were harvested by centrifugation at 5200 g for 15 min. The cell pellet was washed once in an equal volume of a sterile quarter-strength Ringer solution (Ringer), harvested by centrifugation and finally suspended in an equal volume of a 20 g/L alginate (Sigma, Milan, Italy, product n. A2033) solution, previously degassed and sterilized, to reach a concentration of about  $9.40 \pm 0.10$  Log. The syringe, used in the feeding system, was loaded with 50 ml of the alginate cell suspension and placed on the Encapsulator according to the instruction of supplier. The microencapsulation conditions used were: flow rate 2.91 ml/min, vibration frequency 1740 Hz, electrode voltage 950 mV. Alginate droplets containing bacterial cells were hardened in 200 ml of a 0.5 mol/L CaCl<sub>2</sub> solution (in ratio 4:1 with alginate cell suspension) for about 20 min in stirring to obtain monodisperse cross-linked microcapsules. Suspension was left 30 min at room temperature for the sedimentation of microcapsules and then a volume of 150 ml of the upper phase was gently sucked and discarded to

restore the initial cell concentration. Alginate microcapsules (MC) were routinely stored at 4 °C for further experiments.

### 2.3. Coating of MC with chitosan

A 7 g/L chitosan solution was prepared dissolving Chitosan (Sigma, product no. 448869) in a 0.14 mol/L acetic acid solution (pH 3.2) under stirring at 8000 rpm for 20 min at 50 °C. The solution was sterilized at 121 °C for 15 min. MC were added 1:10 (w/v) and stirred at 4000 rpm for 15 min to obtain, after sedimentation and gentle draining of supernatant, chitosan-alginate microcapsules (CMC). They were suspended in Ringer to reach the same volume of MC sample and routinely stored at 4 °C.

### 2.4. Freeze drying of MC

Pellets from MC and CMC after discarding excess of CaCl<sub>2</sub> solution were diluted in a volume of sterile skimmed milk (Oxoid) to restore the cell concentration of alginate-cell suspension. It was frozen at -18 °C overnight and then subjected to lyophilization process (Thermo Scientific Heto PowerDry PL6000 Freeze Dryer). For scanning electron microscopy (SEM) analysis skimmed milk was replaced with 50 g/L trehalose and 50 g/L sucrose solution (Chen & Mustapha, 2012). Freeze dried MC and CMC are below referred as FMC and FCMC, respectively. For cell counting of freeze-dried microcapsules they were before resuspended in Ringer in order to make the initial solid content.

### 2.5. Entrapment efficiency (EE) of microencapsulation process

Cell loading was determined on integer and disrupted fresh and lyophilized MC and CMC. Microcapsules were serially diluted in Ringer or 0.5 mol/L phosphate buffer solution pH 7.0 (Gombotz & Wee, 1998) and plate-counted. Entrapment efficiency (EE) was calculated by dividing the viable count of disrupted MC by the cell load before the microencapsulation (Graff et al., 2007). Furthermore, we calculated for all samples the average number of CFU for each microcapsule with the formula:

$$N = N_d / N_i$$

where N is the average number of CFU for each microcapsule; N<sub>d</sub> is cell count (CFU/ml) of disrupted microcapsules; N<sub>i</sub> is cell count of integer microcapsules. We supposed that each colony is derived from a single integer microcapsule and therefore the measure unit of N<sub>i</sub> (CFU/ml) may be replaced from microcapsules/ml.

### 2.6. Sizing of microcapsules by light microscopy

Samples were analysed by using a Zeiss Axiovert 135 light microscope (Carl Zeiss S.p.A., Aresa, MI, Italy) at × 320 and × 200 magnification and a calibrated micrometre. About 20 microcapsules from each encapsulation trial were analysed immediately after the process, during the storage at different conditions and after each treatment described below.

### 2.7. Scanning electron microscopy (SEM) analysis of microcapsules

Samples were analysed with a scanning electron microscopy (SEM-Evo 40, Carl Zeiss, Oberkochen, Germany) to examine the surface morphology of microcapsules. MC and CMC were rinsed three times with MilliQ water (Lichrosolv Water for Chromatography) and then 10 µl of the sample was placed on a pin type SEM specimen mount and maintained at 42 °C for 1 h in order to reach a gentle dehydration of the microcapsules and their fixing. FMC and

The vibration nozzle technology is based on the principle that a laminar flowing liquid jet breaks up into equal sized droplets by a superimposed vibration. The selectable vibration frequency determines the quantity of droplets produced, for example a vibration frequency of 700 Hz generates 700 droplets per second.

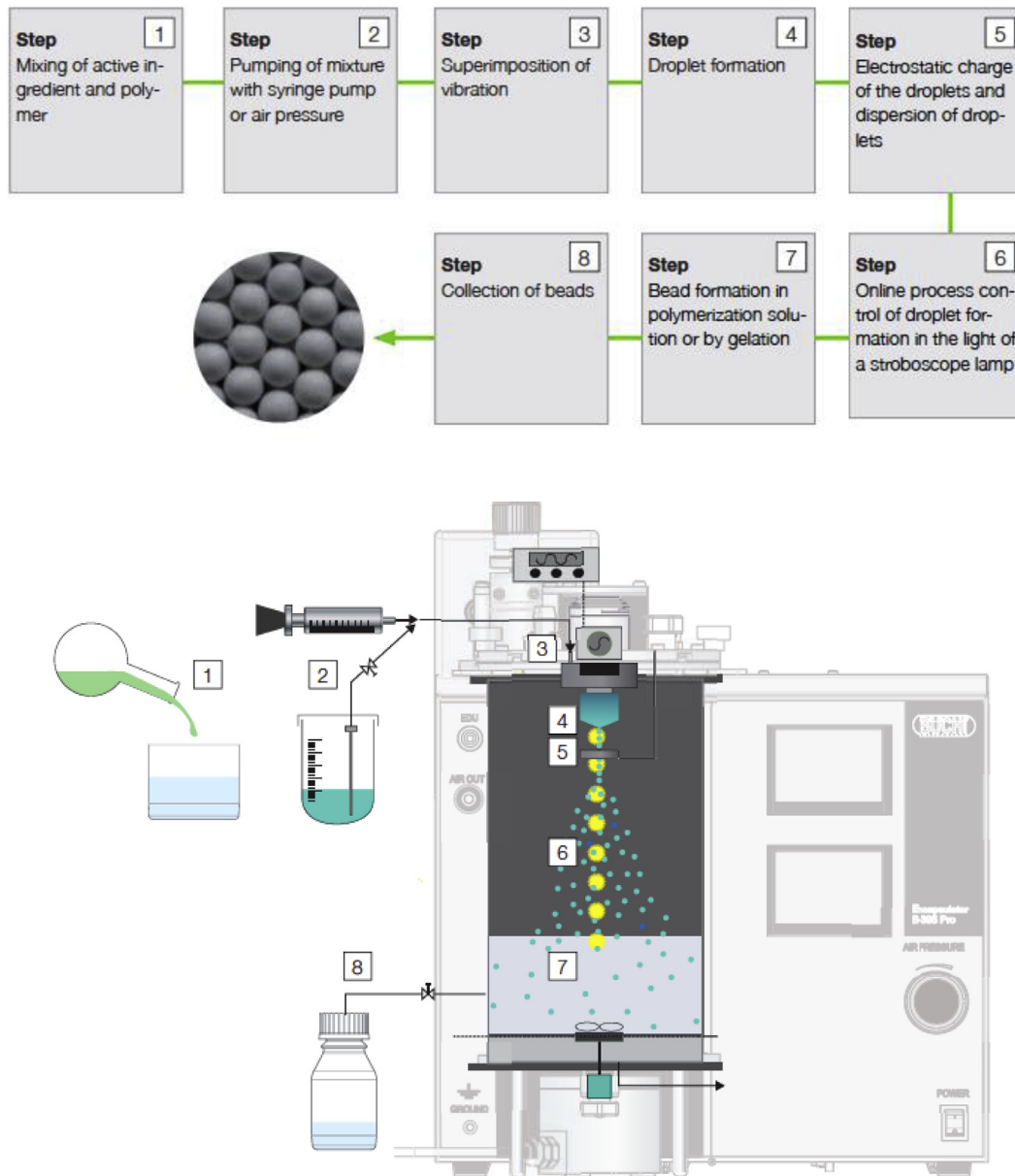


Fig. 1. Steps of vibrating technology and scheme of the Encapsulator B-395 Pro (BUCHI, Switzerland).

FCMC were directly placed on the SEM specimen mount previously coated with a double sided tape to fix the microcapsules. All samples were sputter-treated in a metallizer (Agar Sputter Coater) with gold–palladium to reach a thickness of coating of 100 Å and then observed in high vacuum mode.

#### 2.8. Evaluation of cell viability during storage of MC

Pellet from MC after discarding excess of  $\text{CaCl}_2$  solution was diluted in a volume of different sterile solutions to restore the cell concentration of alginate–cell suspension. Solutions were: i) Ringer, ii) 9 g/L sodium chloride (NaCl) and iii) 0.5 mol/L calcium chloride ( $\text{CaCl}_2$ ). Samples were stored for 28 days at 4 and 20 °C and viable counting was performed on MRS agar at 0, 7, 14, 21 and 28 days. Cell

load was determined after disruption of microcapsules. Free cells were managed in the same way and used as control sample.

#### 2.9. Simulated gastrointestinal (GI) conditions

A 5 g/L sodium chloride saline solution added with 3 g/L pepsine (Sigma, product no. P7000) at pH 2.5 was prepared (gastric simulating solution, GSS). Intestinal condition was simulated with a 5 g/L bile (Sigma, product no. B8381) solution (intestinal simulating solution, ISS). Both solutions were sterilized by filtration (0.22 µm). One ml of MC and CMC was suspended in 9 ml of GSS and ISS and incubated at 37 °C with shaking by hand each 30 min for 3 and for 4 h, respectively. After treatment samples were plate counted after their disruption. Free cells were used as control.

### 2.10. Osmotic stress conditions

The resistance of free and microencapsulated *L. reuteri* DSM 17938 cells in osmotic stress condition was evaluated by using a medium with a high sugar concentration. In particular, 100 g of apricot jam were inoculated with 1 g of MC or CMC. Samples were stored at 4 °C and analysed for viable count immediately after the adding and after 3 h. Free cells, used as control, were decimally serially diluted in Ringer, while MC and CMC in 0.5 mol/L phosphate buffer pH 7.0, both supplemented with 100 g/L sucrose (Sigma) and plate counted.

### 2.11. Reuterin production assay

An antagonistic deferred agar spot test was carried out to detect reuterin production against *P. fragi* 25P. Briefly, 10 µl of free cells and microencapsulated cells were spotted on MRS Agar supplemented with glycerol (250 mmol/L) and incubated at 37 °C for 24 h. After cell growth, the plates were covered with 10 ml of TSB supplemented with 7.5 g/L agar and 5 g/L yeast extract seeded with 2% of an overnight culture of the *P. fragi* 25P. After the incubation at 20 °C for 24 h, the antimicrobial activity was observed as a growth inhibition zone of the indicator strain.

### 2.12. Monitoring of cell damage by a fluorescence microscopy test

Free and microencapsulated *L. reuteri* DSM 17938 cells were dyed by using a LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA) to investigate cell membrane damage at different steps of the microencapsulation process and after the exposure to previously described stress conditions. Samples were stained and visualized by epifluorescence microscope according to the procedure followed by Malmö et al. (2013). It was possible to distinguish between damaged and non damaged cells, stained in red and green, respectively. Images were captured by a Nikon Coolpix 4500 Digital Camera equipped with a microscope adapter.

### 2.13. Data analysis

Analysis was carried out in triplicate and the cells viability after microencapsulation was calculated as average of five independent experiments. A Two-way Anova test and a *t*-test analysis (Microsoft Excel for Mac version 11.5) were performed to ascertain significant differences between averages; significance was declared at  $P < 0.05$ .

## 3. Results

### 3.1. Entrapment efficiency (EE) and bacterial survival

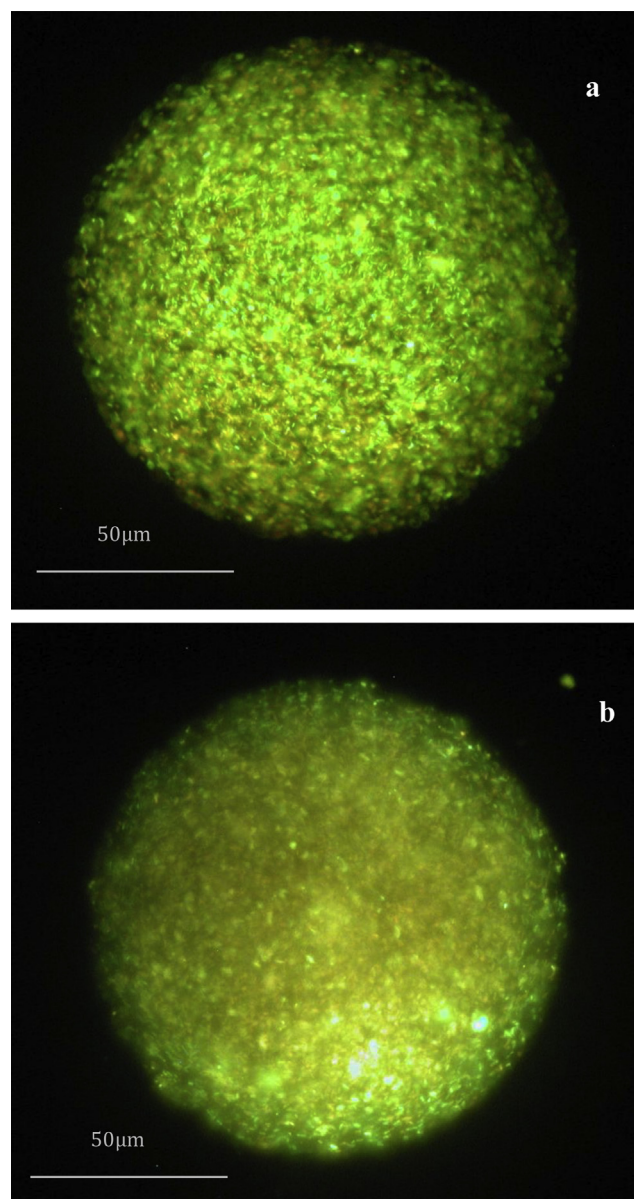
The bacterial load before and after microencapsulation was evaluated to assess the efficiency of microencapsulation experiments (Table 1). Results of viable counts of disrupted MC revealed a reduction in the cell load with respect to the alginate-cell suspension of only 0.35 Log cycles suggesting a high EE value (Table 1). This is corroborated by a cell count of about  $10^3$  CFU/ml which was recovered in the CaCl<sub>2</sub> MC supernatant (data not shown). Viable cell counts from integer and disrupted MC were significantly higher ( $P < 0.05$ ) compared to counts from CMC samples (Table 1). CFU for each microcapsule was estimated to be 99 for MC and 92 for CMC (Table 1). In addition, the bacterial load of lyophilized microcapsules showed 100% survival immediately after the freeze drying process (Table 1). Verification of the integrity of cell membrane of the microencapsulated cells following the staining reaction by fluorescence microscope (Fig. 2) show that all the cells in both MC and CMC were green thus indicating lack of damage.

**Table 1**

Bacterial load (Log CFU/ml) of microcapsules before (MC and CMC) and after (FMC and FCMC) freeze drying obtained by using a cell/alginate suspension of  $9.40 \pm 0.10$  Log CFU/ml and parameters of microencapsulation yield.

Microcapsule samples	Integer	Disrupted	Parameters		
			EE (%)	N (CFU/microcapsule)	Survival after drying (%)
MC	$7.14 \pm 0.20^A$	$9.15 \pm 0.14^A$	97	99	–
CMC	$6.89 \pm 0.15^B$	$8.82 \pm 0.22^B$	–	92	–
FMC	$7.27 \pm 0.23^A$	$9.19 \pm 0.13^A$	–	99	100
FCMC	$6.81 \pm 0.18^B$	$8.92 \pm 0.20^B$	–	92	100

Mean results of five independent trials  $\pm$  standard deviation. Different indices within the same column indicate a significant difference ( $P < 0.05$ ).



**Fig. 2.** Fluorescence microscopy images at  $\times 400$  magnification of stained *Lactobacillus reuteri* DSM 17938 in alginate (a) and chitosan-alginate microcapsules (b).

### 3.2. Morphology of microcapsules

Size of MC and CMC was evaluated immediately after their production and during the storage in different conditions (only for MC). Optical microscopy images are reported in Fig. 3. They show microcapsules with an average diameter ( $\pm$  standard deviation) of  $110 \pm 5 \mu\text{m}$  in both samples, suggesting that chitosan coating did not lead to a significant variation in microcapsule bead diameter. Moreover, size of MC remained unchanged during their storage in different conditions (data not shown). Images show microcapsules regular in shape and without agglomeration among them and the border-line of CMC less regular compared to that of MC. SEM images show that MC were perfectly spherical in shape, rough and continuous in surface without hollow zones or cracks (Fig. 4a). Occasionally, collapsed microcapsules were visualized (Fig. 4b). As shown in Fig. 5a, chitosan coating led to a little modification of microcapsules shape that appeared wrinkled and rougher compared to MC. The surfaces of CMC samples were also found to be continuous without any observable hollow or cracks. On the other hand, increased roughness and more marked spongy-like features were observed on the surface of CMC (Fig. 5b) compared to that of MC (Fig. 4c).

Both microcapsules were found to retain their shape after freeze-drying (Figs. 6a and 7a). However, the surface of FMC (Fig. 6b) appeared more plain and skinny than the case before freeze-drying (Fig. 4c) while FCMC surface roughness remained fairly the same after freeze drying (Fig. 7b).

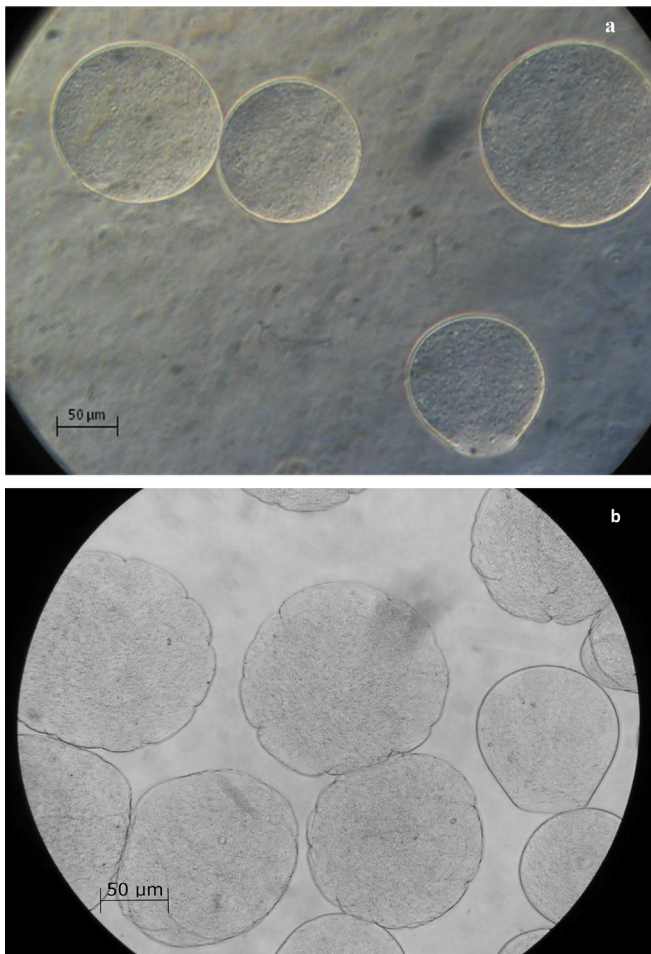


Fig. 3. Optical microscopy images of MC at  $\times 200$  magnification (a) and of CMC at  $\times 320$  magnification (b).

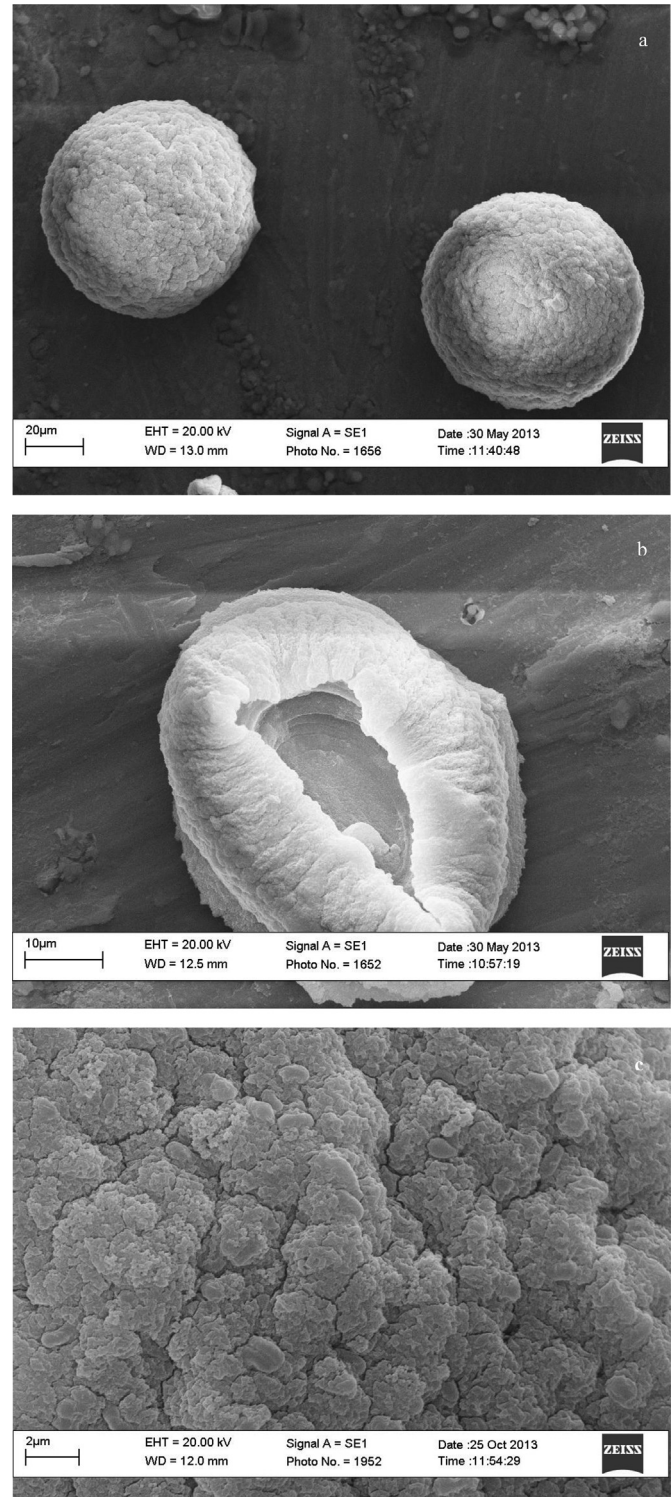
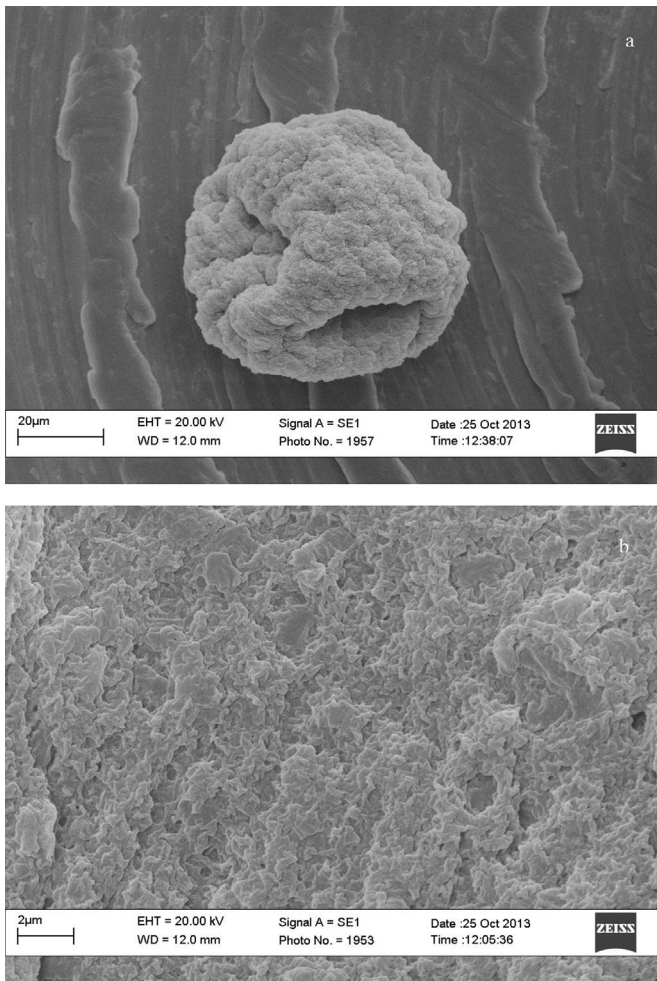


Fig. 4. SEM images of alginate microcapsules (MC). a) MC with a characteristic morphology at  $\times 1600$  magnification; b) collapsed MC at  $\times 4300$  magnification; c) particular of MC surface at  $\times 15,000$  magnification.

### 3.3. Survival of probiotic cells during storage of MC

Survival kinetics of free and microencapsulated *L. reuteri* in alginate matrix stored in different conditions are reported in Fig. 8. The storage at  $20^\circ\text{C}$  led to a dramatic decrease in viability for both free and microencapsulated probiotic cells. Total reduction in the

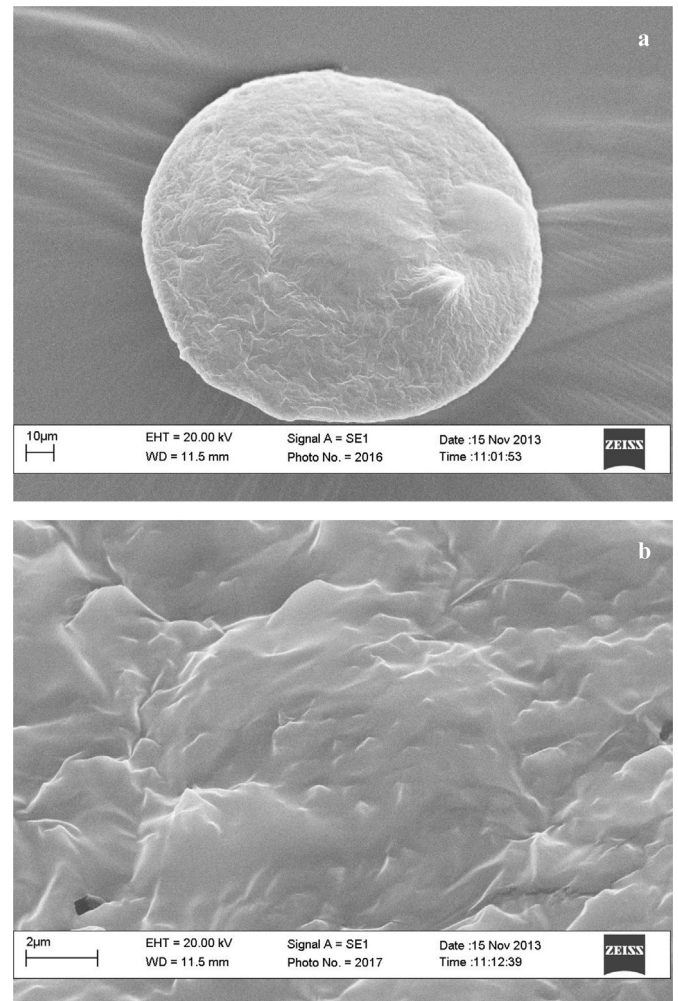


**Fig. 5.** SEM images of chitosan-alginate microcapsules (CMC). a) CMC with a characteristic morphology at  $\times 240$  magnification; b) particular of CMC surface at  $\times 15,000$  magnification.

cell load was observed on the 14th day of storage for free cells in  $\text{CaCl}_2$  and on the 21st day of storage for all other samples except for MC in NaCl, where total cell reduction was observed at the endpoint of the storage period (Day 28: Fig. 8a). Different results were obtained for samples stored at  $4^\circ\text{C}$  (Fig. 8b). Best microcapsule preservation was achieved for MC stored in Ringer and in NaCl for which only 1 Log reduction in viable cell counts ( $P < 0.05$ ) was observed after 28 days in both cases. On the contrary, viable count of free cells in the same solutions decreased by about 3 Log cycles at the end of storage. Furthermore, a reduction of 3 Log cycles in viable cell count was registered for MC in  $\text{CaCl}_2$  while about 4.5 Log reduction was observed in the case of free cells instead (Fig. 8b).

### 3.4. Gastrointestinal (GI) stress conditions

The results of viable counts and survival percentage of free and microencapsulated *L. reuteri* cells after exposure to simulated GI conditions are shown in Fig. 9. Results show that free cells were very sensitive to GSS. A significant ( $P < 0.05$ ) reduction of 2.09 Log cycles was observed after 3 h, resulting in a percentage survival of less than 1%. On the contrary, treatment of free cells for 4 h in ISS led to a reduction of only 0.82 Log cycles which translates to about 16% of cell survival. Both CM and CMC conferred resistance to cells in GI conditions ( $P < 0.05$ ) with exception of MC in GSS where a

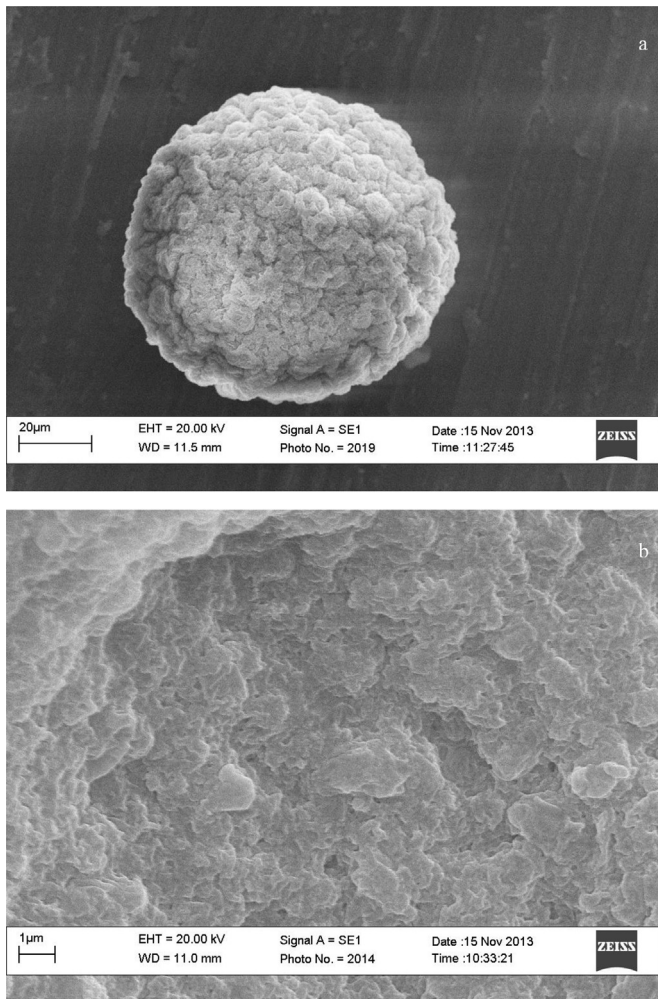


**Fig. 6.** SEM images of freeze dried alginate microcapsules (FMC). a) FMC with a characteristic morphology at  $\times 2400$  magnification; b) particular of FMC surface at  $\times 15,000$  magnification.

significant ( $P < 0.05$ ) but moderate cell count reduction of about 0.35 Log cycles was observed. Results of viable staining of both microcapsule types after GSS (Fig. 10a and b) and ISS treatments (Fig. 10c and d) are depicted in Fig. 10. Generally, microcapsules maintained their shape and size features. However, after GSS treatment a modest but clear reduction in size of CMC, as well as their aggregation was observed (Fig. 10b). Epifluorescence microscopic images showed that most of the cells were green-stained for all samples although in a few cases, zones of some capsules appeared light red. However, this red staining was much less marked than observed in the case of the free damaged cells (data not shown). The presence of free cells indicates a slight release of cells from both microcapsules when they were exposed to the GSS (Fig. 10a and b). Finally, green stained debris were clearly visible after 4 h of exposure of CMC to ISS (Fig. 10d).

### 3.5. Osmotic stress condition

Results of this experiment are reported in Fig. 11. It was observed that both microcapsules were able to protect probiotic cells from osmotic stress. In fact, at time zero ( $T_0$ ), a reduction of 1.2 Log cycles was observed for free cells while only 0.6 Log reduction was registered in the case of microcapsules. A further significant



**Fig. 7.** SEM images of freeze dried chitosan-alginate microcapsules (FCMC). a) FCMC with a characteristic morphology at  $\times 2400$  magnification; b) particular of FCMC surface at  $\times 15,000$  magnification.

( $P < 0.05$ ) reduction in cell viability was registered at T3 for free cells (0.7 Log cycles) and CMC (0.4 Log cycles) while no variation was found in the case of the MC. Hereafter, subjecting the microcapsules and free cells to osmotic stress beyond 3 h led to a significant ( $P < 0.05$ ) over reduction of viable cell count by about 2 Log cycles for free cells and by about 0.67 and 1 Log cycle in the case of MC and CMC, respectively.

### 3.6. Reuterin production

Results of reuterin test showed that neither alginate microencapsulation nor further chitosan-coating of MC affected the antimicrobial substance production by *L. reuteri*. The MC and CMC showed an inhibition halo of the indicator strain by 38 and 31 mm, respectively, compared to 42 mm produced by free cells.

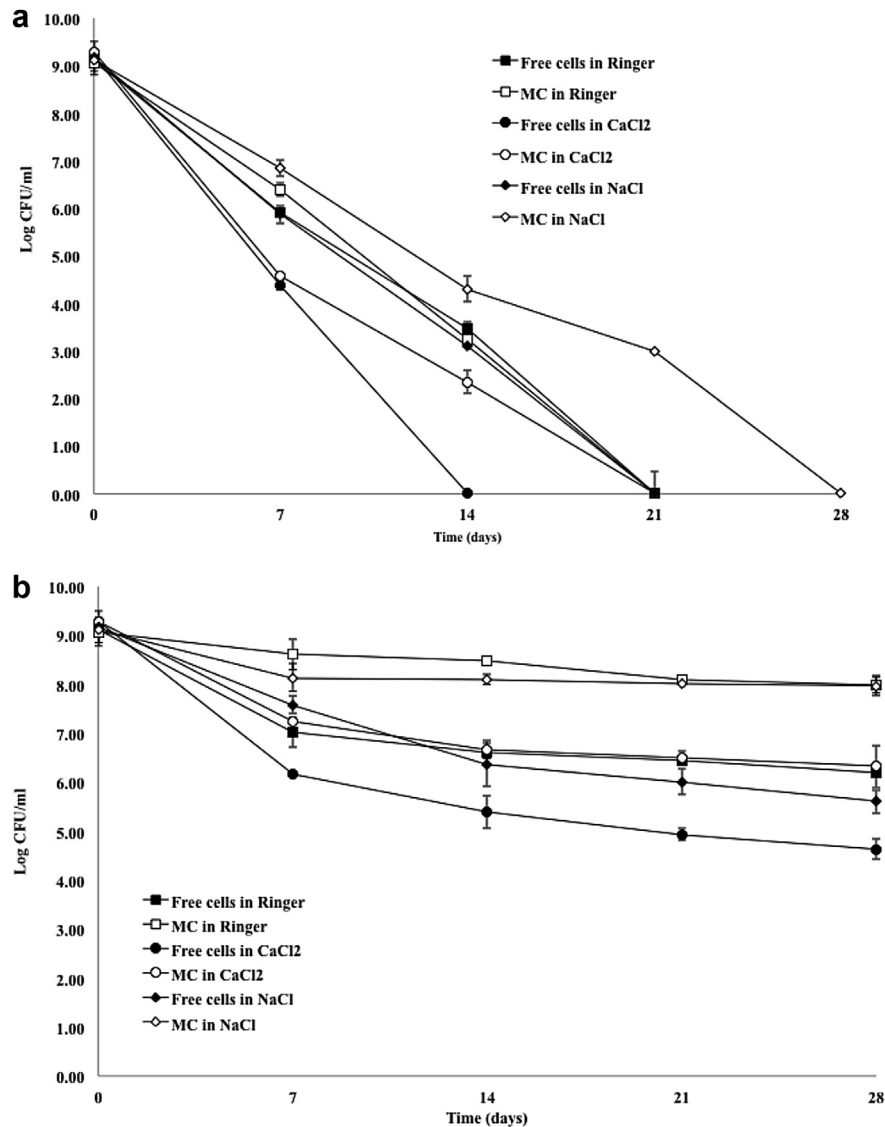
## 4. Discussion

Extrusion, emulsion and spray drying are the main techniques reported in literature for the microencapsulation of probiotic bacteria. Emulsion technique is easy to scale up but the main disadvantages are the high cost and varying size and shape of microcapsules (Burgain et al., 2011). Extrusion, instead, is a simple and cheap method producing homogeneous-shaped capsules with

a narrow size distribution, which could be designed for mass production of microcapsules (Whelehan & Marison, 2011). In both systems, due to the gentle operation conditions, a slight damage is usually registered on the bacterial cells (Burgain et al., 2011). On the contrary, spray drying, which is the most commonly used microencapsulation method in the food industry, causes cell envelope damage owing to the high temperature applied during the process (Anal & Singh, 2007). In this study we applied the vibrating technology, a special system based on the extrusion technique, to microencapsulate cells of the well-known probiotic strain *L. reuteri* DSM 17938. Two important outcomes of the microencapsulation process of living cells are the structural quality of the microcapsules and the microencapsulation yield. We studied the former by microscopic investigations and the latter by viable counting of cells released following the disruption of microcapsules. In agreement with Whelehan and Marison (2011) we produced, by using the vibrating technology, microcapsules with perfect spherical shape and a diameter of  $110 \pm 5 \mu\text{m}$  (Fig. 3). SEM image (Fig. 5) showed smaller diameter but it could be a consequence of the preliminary treatment of samples for the microscopic analysis. Solanki et al. (2013) recently reviewed literature on microencapsulation of probiotic cells and reported  $356 \mu\text{m}$  as smallest diameter of beads that could be obtained by extrusion technique at that time. On the other hand, microcapsules loaded with fluorescein and produced by vibrating technology at high frequency had a diameter of  $158 \mu\text{m}$  although a wide range of other sizes were also reported (Dorati et al., 2013). Our opinion is that the dimension of microcapsules can affect the average number of cells that each microcapsule can hold. Thus a smaller microcapsule containing fewer cells is likely to ensure a more homogeneous behaviour of the bacterial population in it both in terms of resistance to stress conditions and metabolic activity. Furthermore, some authors have alluded to the impact of microcapsule dimension on organoleptic characteristics of some foods. For example, Kailasapathy et al. (2006) found that microspheres of about  $300 \mu\text{m}$  containing probiotic bacteria cause a significant increase in the smoothness of yogurt compared to that with free cells. Similar results were reported by Krasaekoopt and Kitsawad (2010), who found some modifications in the texture characteristics of fruit juice supplemented with microcapsules containing probiotics. Finally, Truelstrup-Hansen, Allan-Wojotas, Jin, and Paulson (2002) suggested that microcapsules with a size below  $100 \mu\text{m}$  could avoid negative sensory impact in food products.

In this study, we investigated the morphology of microcapsules by SEM analysis and our finding indicates that the conditions of microencapsulation applied (i.e. technique, alginate concentration and  $\text{CaCl}_2$  solutions, combination of alginate with bacterial cells) promote formation of capsules with reduced diameter, perfectly spherical and continuous in surface dimension. SEM images (Figs. 4–7) show that cells are not visible on the surface of microcapsules. Furthermore, the absence of cracks ensures the protection of bacterial cells from adverse environment conditions. Also a typical microcapsules (Fig. 4b) did not show the presence of cells on the surface, sustaining the hypothesis that they take origin from “collapsing and not breaking phenomena”. Our results on the effect of chitosan coating are also in agreement with those reported by Lee, Cha, and Park (2004).

The results from this study on the encapsulation yield (Table 1) are in agreement with the current literature describing the application of extrusion technique for living cells microencapsulation. Shi et al. (2013) reported an encapsulation yield close to 100% when cells of *Lactobacillus bulgaricus* were microencapsulated in alginate-milk microspheres through vibrating technology. Graff, Hussain, Chaumeil, and Charrueau (2007) reported an encapsulation rate of 53% using the same technology to encapsulate *Saccharomyces boulardii* in chitosan-alginate. We report a parameter that is not



**Fig. 8.** Survival kinetics of free and microencapsulated *Lactobacillus reuteri* DSM 17938 in alginate matrix (MC) stored in Ringer, NaCl and CaCl<sub>2</sub> at 20 °C (a) and 4 °C (b) along 28 days. Differences between mean values were analysed by two-way ANOVA. Significance was declared at  $P < 0.05$ . The error bars represent standard deviations.

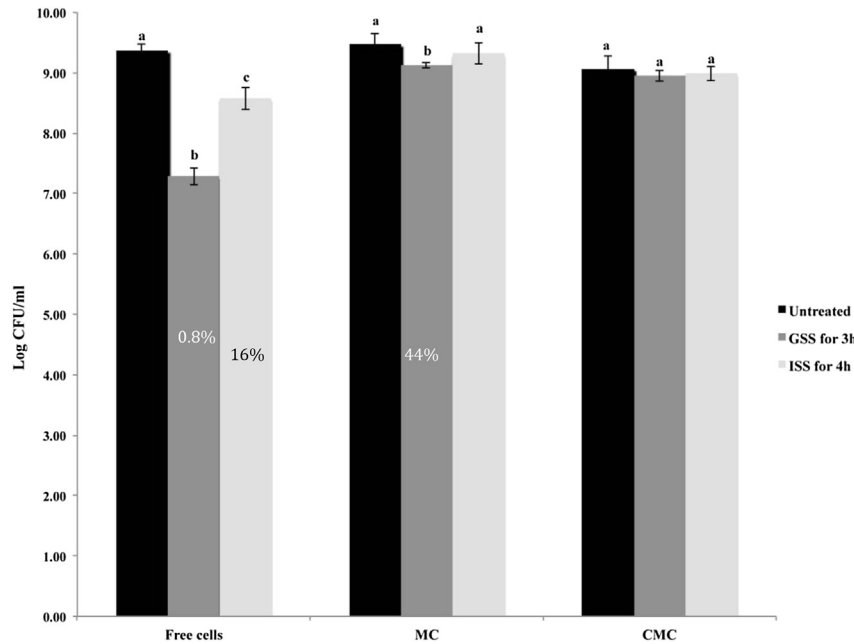
usually calculated by other authors, that is the estimated number of CFU for each microcapsule (N). In fact, we reasonably assumed that 1 bacterial colony originates from 1 microcapsule, so by counting cells from intact microcapsule samples we produced data for the number of microcapsules. We registered a little difference of N value between MC and CMC (Table 1), probably due to a release of cells during chitosan coating. Also Graff et al. (2007) did not register reduction of cell load after chitosan coating, while in a previous work (Malmo et al., 2013) we reported a more marked reduction of cell load when chitosan coating was carried out on microcapsules obtained by spray drying. However, our opinion is that spray drying produces microcapsules with features too much different from that obtained by vibrating technology to permit a suitable comparison.

Freeze-drying of microcapsules was performed to confer further viable cell stabilization. Solanki et al. (2013) reported that combining encapsulation with freeze-drying could reduce cell membrane damage from freezing. Our results showed that in addition to having high survival (Table 1), microcapsules maintained their morphological characteristics after lyophilization (Figs. 6 and 7). High cell survival was reported also by Chen and

Mustapha (2012), who lyophilized *Lactobacillus acidophilus* microcapsules produced by extrusion/emulsion technique.

Our results showed that cells of *L. reuteri* DSM 17938 were well preserved during storage at 4 °C when microencapsulated in alginate matrix (Fig. 8). However, better probiotic protection systems were described by other authors (Rodrigues et al., 2012; Shi et al., 2013), whose results could be related to the efficiency of a double layer system in preserving the viability of different probiotic strains during a long-term storage. This provides credence to our findings from this study, which show that greater stability could be obtained by coating alginate microcapsules with chitosan or other different matrices. On the other hand, it should be highlighted that different results could be due to different resistance of the microencapsulated strains.

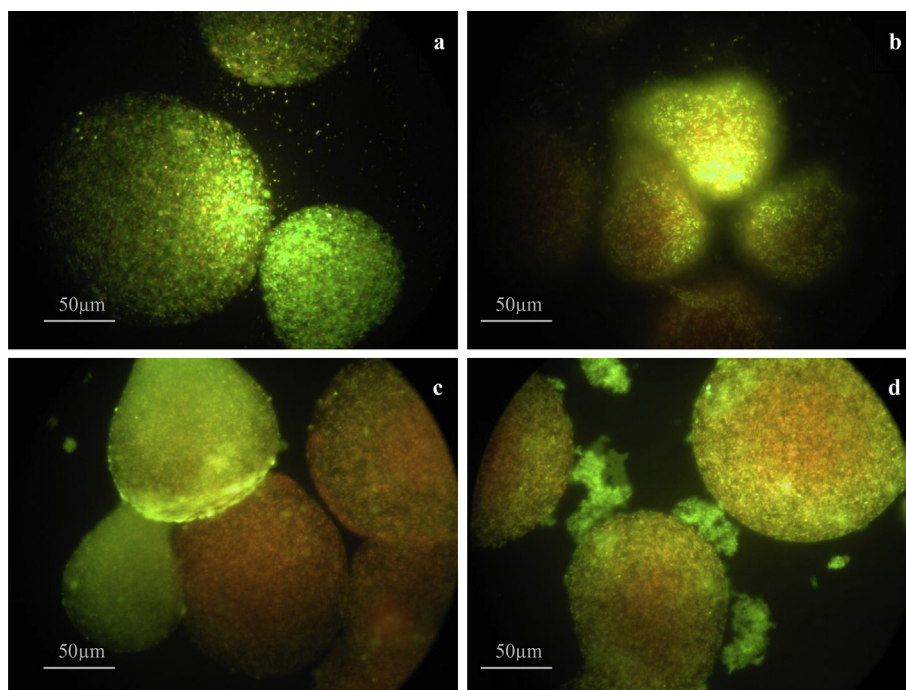
In order to extrapolate their benefit effects on the human or animal hosts, probiotic strains have to resist stressful conditions in the gastro-intestinal tract (Expert Committee FAO/WHO, 2002). *L. reuteri* DSM 17938 showed to be very sensitive in gastric-simulated condition (Fig. 9). This was also reported in a previous study (Malmo et al., 2013). The results of the current study strengthen the



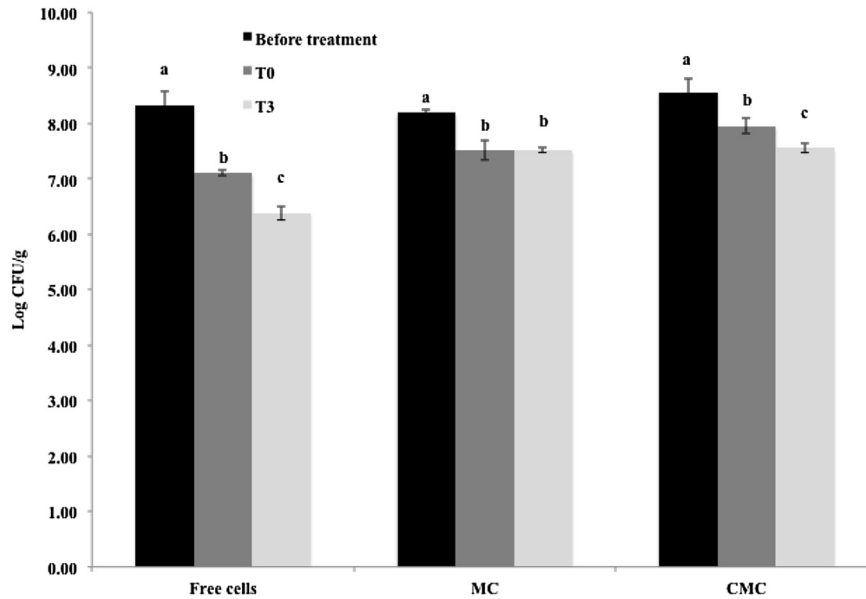
**Fig. 9.** Viable counts and survival percentage of free and microencapsulated *Lactobacillus reuteri* DSM 17938 before and after incubation in GSS for 3 h and in ISS for 4 h. The error bars represent standard deviations. Different letters labelling bar graphs of the sample categories indicate that mean values are significantly different ( $P < 0.05$ ) as determined by *t*-test.

findings from other studies which show that microencapsulation in alginate matrix improve cell resistance to gastric pH, which is further increased when alginate microcapsules are coated with other matrices (Li, Chen, Sun, & Cha, 2010; Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012). In contrast with the previous work (Malmo et al., 2013) the microcapsules obtained with alginate alone (MC) did not show any evidence of the effect of bile salts (Fig. 9). Again, the difference in

findings between this study and the work of Malmo et al. (2013) could be attributed to differences in microencapsulation technologies applied in the different study. Although with little difference, we observed that CMC performed better than MC in protecting probiotic cells from bile salts. This finding is in agreement with results reported earlier by other authors (Li et al., 2010; Malmo et al., 2013). The better protective effect of the CMC could be due to the combination of the physical barrier offered by encapsulating



**Fig. 10.** Fluorescence microscopy images at  $\times 400$  magnification of viable staining of microencapsulated *Lactobacillus reuteri* DSM 17938 after treatment in gastrointestinal conditions. MC (a) and CMC (b) after 3 h in GSS, MC (c) and CMC (d) after 4 h in ISS.



**Fig. 11.** Viable counts of free and microencapsulated *Lactobacillus reuteri* DSM 17938 before and after incubation at 4 °C in apricot jam for 0 (T0) and 3 (T3) hours. The error bars represent standard deviations. Different letters labelling bar graph of the same sample category indicate that mean values are significantly different ( $P < 0.05$ ) as determined by *t*-test.

matrix and the reaction between chitosan and bile salts that resulted in the formation of an insoluble complex (green stained debris in Fig. 10d) and in the limited diffusion of bile salts into the matrix core (Corona-Hernandez et al., 2013).

During food processing and storage, probiotic bacteria if added to the food matrix inevitably get exposed to several stressful conditions (oxidative stress, temperature, pH variations and osmotic stress), which ultimately affect their survival. In considering the effects of the aforementioned stress factors, it is important to recognise the requirement that any probiotic food product must contain at least  $10^6$ – $10^7$  CFU/g of the viable probiotic bacteria at the time of consumption in order to have beneficial effects of the probiotic exerted on human health (Expert Committee FAO/WHO, 2002). High sugar content as found in foods such as confectionaries, jams, fruit nectars, etc., confers high osmotic condition likely to affect survival of probiotic strains in such foods. In this study therefore, we tested the survival of free and microencapsulated cells in apricot jam. Apricot jam was selected to represent foods with high osmotic pressure. Surprisingly, MC exhibited a higher efficacy than CMC in protecting probiotic cells from osmotic stress in apricot jam. The low performance of CMC observed could be attributed to the mechanical injury on probiotic cells brought about by increased pressure within microcapsules due to the stronger alginate-chitosan wall following the efflux of alginate interstitial water into the jam matrix.

## 5. Conclusions

This study demonstrated that microencapsulation by vibrating technology can produce stable probiotic microcapsules with dimension and morphological characteristics suitable for food-based applications. In particular, we demonstrated that: (i) alginate-based microcapsules improved *L. reuteri* DSM 17938 survival during storage and exposure to gastrointestinal and osmotic stress conditions and that immobilisation of the organism in the alginate-based microcapsules did not affect reuterin production and diffusion of the antimicrobial substance out of the microcapsules; (ii) Co-encapsulation of the probiotic in chitosan-alginate

matrix enhanced survival of the probiotic. These findings indicate that microencapsulation by vibrating technology is a promising tool that can be exploited for protection of probiotic bacteria during food processing, storage and their delivery in foods not usually considered as a vehicle of probiotics.

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