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Encapsulation and subsequent freeze-drying of *Lactobacillus reuteri* CRL 1324 for its potential inclusion in vaginal probiotic formulations

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ABSTRACT

Probiotic formulations must include a high number of viable and active microorganisms. In this work, the survival of human vaginal *Lactobacillus reuteri* CRL 1324 during encapsulation, lyophilization and storage, and the activity of encapsulated and/or freeze-dried bacterial cells were evaluated. Extrusion-ionic gelation technique was applied to encapsulate *L. reuteri* CRL 1324, using xanthan and gellan. Encapsulated and free bacterial cells were freeze-dried with or without lactose and skim milk as lyoprotectors. The different systems obtained were stored at room temperature and at 4°C for 150 days. The following determinations were performed: *L. reuteri* CRL 1324 viability, microorganism released from capsules, survival in a medium simulating the vaginal fluid and maintenance of beneficial properties (growth inhibition of opportunistic pathogenic *Streptococcus agalactiae* NH 17 and biofilm formation). *L. reuteri* CRL 1324 encapsulation was efficient, allowing the recovery of a high number of entrapped lactobacilli. The survival of encapsulated *L. reuteri* during lyophilization and storage was significantly higher in the presence of lyoprotectors. At the end of storage, highest numbers of viable cells were obtained in free or encapsulated cells freeze-dried with lyoprotectors, stored at 4°C. Encapsulated and/or liophilized *L. reuteri* cells maintained their viability in simulated vaginal fluid as well as the ability to inhibit *S. agalactiae* NH 17 growth and to form biofilm. Encapsulated and freeze-dried *L. reuteri* CRL 1324 can be included in a suitable pharmaceutical form for vaginal application to prevent or treat urogenital infections in women.

Key words: Vaginal probiotic, encapsulation, freeze-drying, storage, activity maintenance, simulated vaginal fluid survival

1. Introduction

Female urogenital tract infections affect one billion people worldwide each year (Reid, 2014). Conventional treatments with different drugs have failed to restore the normal vaginal microbiome, which is dominated by lactobacilli (Human Microbiome Project Consortium, 2012; Witkin, 2015). Therefore, the use of probiotics containing beneficial lactobacilli is a promising alternative to prevent or treat urogenital tract infections (Reid, 2014). Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001; Hill et al., 2014).

The effectiveness of probiotics depends on the number of viable cells, being 10^7 and 10^9 colony forming units (CFU) the recommended amount per dose of formulation (Ding and Shah, 2009; Mastromarino et al., 2013; Hill et al., 2014). Therefore, during the design of probiotic products, the main technological requirement is the microbial stability during the processes of biomass production (to include the required number of viable and active microorganisms in the final product) and during the shelf life period (du Toit et al., 2013; Domig et al., 2014; Hill et al., 2014).

Freeze-drying and encapsulation are methods widely applied to preserve and protect microbial viability (Pliszczak et al., 2011; Muller et al., 2013). Encapsulation protects the active ingredients against stressing physical-chemical factors such as heat, humidity, extreme pH, harmful substances, etc. (Islam et al., 2010; Solanki et al., 2013). This process could favor an increase in microbial stability in the conditions of the ecological niche in which the microorganisms will be applied. On the other hand, this technology can support a controlled release and a reduction in the irritant effect of certain active pharmaceutical ingredients (Remuñán López and Alonso Fernández, 1997).

In vaginal probiotic products, the microorganisms must be included in matrixes with low water activity (Pliszczak et al., 2011). However, the preservation processes applied to

microbial cells can cause structural and physiological cell damage by several mechanisms, leading to decrease of viability (Weinbreck et al., 2010; du Toit et al., 2013). Freeze-drying of beneficial lactic acid bacteria strains has been proved successful to preserve bacterial stability during the elaboration of various pharmaceutical forms for vaginal application and during storage for different time periods (Borges et al., 2013; Muller et al., 2013, 2014; Verdenelli et al., 2014). However, to the best of our knowledge, the stability (viability and activity) of vaginal probiotic strains during encapsulation and freeze-drying and their subsequent storage has not yet been reported.

In previous studies, vaginal *Lactobacillus reuteri* CRL 1324 was selected as a candidate probiotic for its potential beneficial properties. This microorganism inhibits the growth of urogenital pathogenic *Streptococcus agalactiae*, *Enterococcus faecalis*, *Enterococcus* sp., *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* sp., *Neisseria gonorrhoeae* and *Garnerella vaginalis* strains, shows high auto-aggregative and mucin-adhesive capacity, forms biofilm under different culture conditions, co-aggregates with *S. agalactiae* and *S. aureus*, exerts a preventive effect against vaginal colonization by *S. agalactiae* in a murine experimental model and survives in conditions simulating the vaginal fluid (Juárez Tomás et al., 2003, 2011; Leccese Terraf et al., 2012, 2014; De Gregorio et al., 2014, 2015). The aim of this work was to evaluate the stability (viability and maintenance of beneficial properties) of *L. reuteri* CRL 1324 during encapsulation, freeze-drying and storage under different conditions.

2. Materials and methods

2.1. Microorganisms and culture conditions

Biofilm-forming *L. reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 was originally isolated from human vagina in Tucumán, Argentina. *L.*

reuteri CRL 1324 was obtained from subcultures of a single colony and its purity was checked by Gram staining and catalase test. *Lactobacillus* strain was stored in a milk-yeast extract (13% non-fat milk, 0.5% yeast extract and 1% glucose) at -20°C (Ocaña et al., 1999). Before experimental use, stored *L. reuteri* CRL 1324 was grown in De Man-Rogosa-Sharpe (MRS) broth (Merck, Germany) (De Man et al., 1960) at 37°C for 24 h under static conditions and subcultured in the same medium at 37°C for 12 h (second subculture) and then for 16 h (third subculture).

For inhibition assays, opportunist pathogenic vaginal *S. agalactiae* NH 17 (previously isolated at the Nuevo Hospital 'El Milagro', Salta, Argentina) was used as a sensible strain to *L. reuteri* CRL 1324 (De Gregorio et al., 2014). *S. agalactiae* NH 17 was cultured in LAPTg (yeast extract/peptone/tryptone/Tween 80/glucose) broth (Raibaud et al., 1973) at 37°C for 12 h and then subcultured at 37°C in the same medium for 9 h up to 0.8 optical density at 600 nm ($OD_{600\ nm}$) (Spectronic 20, Bausch and Lomb, Rochester NY).

2.2. Encapsulation of L. reuteri CRL 1324

Bacterial cells from the third *L. reuteri* CRL 1324 broth subculture were centrifuged (6000 *g* for 10 min at 4°C), washed twice and resuspended in distilled water (to decrease the amount of ions interacting with encapsulating polymers), and a concentrated culture of around 10^{10}-10^{11} CFU/ml was obtained.

Extrusion-ionic gelation was the encapsulation technique applied, according to the methodology described by Sun and Griffiths (2000) and Jiménez-Pranteda et al. (2012), with modifications. Before the encapsulation assay, 1% xanthan gum-0.75% gellan gum polymeric mixture (polymers from Sigma-Aldrich, St. Louis, Missouri, USA) was prepared. Adequate amounts of each polymer powder were dispersed in 15 ml of deionized preheated water

(80°C) by magnetic stirring and kept at 80°C until complete hydration of the polymeric mixture, which was autoclaved (for 15 min at 121°C) and stored at 4°C.

Concentrated cultures of *L. reuteri* CRL 1324 were homogeneously resuspended in the xanthan-gellan polymeric mixture [1:15 (vol/vol) ratio]. The bacterial cells and polymers mix was extruded at a flow rate of 90 ml/h through a syringe pump (KDS Model 100 Series, KD Scientific, Inc., USA) equipped with a 10 ml syringe (BD, Belgium) connected to a needle (TIP 27 G x ½ in., BD, Belgium). The capsules were recovered and hardened in 0.1 M $CaCl_2$ for 30 min at 4°C (under gently magnetic stirring), washed with distilled water and separated by filtration (with a sterile funnel and filter paper). The material weight was determined and the encapsulation yield, defined as the percentage of capsule weight obtained with respect to the total weight of the material used (active ingredient -bacterial cells- and polymeric mixture) was calculated (Alli, 2011).

2.3. Lyophilization of encapsulated cells and cell suspensions of L. reuteri CRL 1324

Capsule aliquots containing *L. reuteri* were subjected to a subsequent freeze-drying process, resuspended or not in a lyoprotector solution containing 12% (wt/vol) lactose and 6% (wt/vol) reconstituted skim milk (lactose+skim milk) (Juárez Tomás et al., 2009). On the other hand, *L. reuteri* CRL 1324 free cells (concentrated cultures) were resuspended in lactose-skim milk [1:15 (vol/vol) ratio] and freeze-dried. The samples of capsules and *L. reuteri* CRL 1324 free cells were distributed in Petri dishes, frozen at 70°C for 24 h and dried in a chamber type freeze-drier (Lyovac GT2; Leybold, Köln, Germany) for 16 h at 0.3 mbar (1 bar = 100 kPa), which yielded products with <1% residual moisture.

2.4. Storage conditions

The different systems (nonlyophilized capsules, freeze-dried capsules and freeze-dried free cells of *L. reuteri* CRL 1324) were dispensed aseptically into screw-capped vials. The vials were placed in sealed plastic containers (Parafilm® M, Wertheim, Germany) containing silica-gel desiccants and stored at room temperature and at refrigeration temperature (4°C) under dark conditions for 150 days. Fig. 1 summarizes the conditions of preparation and storage of the different systems assayed to preserve the stability of *L. reuteri* CRL 1324.

2.5. Sampling and analytical procedures

The amount of active ingredient (number of *L. reuteri* CRL 1324 viable cells) before and after encapsulation and freeze-drying processes and during storage (0, 21, 60 and 150 days) was determined in samples of all the systems assayed. Survival rate during encapsulation or lyophilization was expressed as N_{AE}/N_{BE} or N_{AL}/N_{BL}, where N_{AE} and N_{BE} are the log CFU/g after encapsulation and before encapsulation, respectively, and N_{AL} and N_{BL} are the log CFU/g after lyophilization and before lyophilization, respectively (Martos et al., 2007; Juárez Tomás et al., 2009).

2.5.1. Determination of viable cells

Nonlyophilized and lyophilized encapsulated bacterial cells were released by dissolving a weighed quantity of xanthan-gellan capsules in 0.05 M sodium phosphate buffer (g/l: NaH_2PO_4, 5.30; $Na_2HPO_4.2H_2O$, 13.32; pH 7.0) and disaggregated with a Teflon pestle homogenizer for 1 min. Lyophilized capsules were previously rehydrated in 0.05 M sodium phosphate buffer for 10 min at room temperature. In the case of freeze-dried free cells, they were rehydrated in saline for 10 min at room temperature before viable cell counting. The number of *L. reuteri* CRL 1324 viable cells was determined from aliquots of each sample by the successive dilution method, using saline as dilution medium and MRS agar (MRS-1.5%

agar, pH 6.5) as culture medium. Plates were incubated under aerobic conditions at 37°C for 48 h, and the number of CFU registered.

2.5.2. Optical microscopy and scanning electronic microscopy of freeze-dried capsules

The morphological characteristics and internal structure of some capsules (i.e. xanthan-gellan capsules lyophilized in presence of lactose-skim milk) containing *L. reuteri* CRL 1324 were analyzed by scanning electronic microscopy. Freeze-dried capsules were previously rehydrated in a medium simulating the vaginal fluid (MSVF, g/l composition: glucose, 10; lactic acid, 2; acetic acid, 1; albumin, 2; mucin, 0.25; urea, 0.50; NaCl, 3.5; KCl, 1.5; cysteine, 0.50; 1 ml/l Tween 80; pH 4.25 ± 0.05) (Juárez Tomás and Nader-Macías, 2007) for 10 min at room temperature.

Samples were fixed with Karnovsky fixative [8% (wt/vol) paraformaldehyde in phosphate buffer; 16% (wt/vol) glutaraldehyde] overnight at 4°C. Fixed samples were washed three times with phosphate buffer for 10 min and treated overnight with 1:1 osmium/phosphate buffer solution (2% OsO_4). The osmium solution was later removed. The samples were washed twice with 30% ethanol for 10 min and then dehydrated through a series of increasing alcohol concentrations: 50, 70, 80, 90 and 100% ethanol three times, 10 min each time. 100% ethanol was eliminated and 100% acetone added for 1 h. Later, the samples were mounted in an aluminum holder, covered with a gold layer by using a metallizer and observed with high vacuum in a Zeiss Supra 55VP (Carl Zeiss microscope, Oberkochen, Germany) scanning electron microscope. The size of freeze-dried capsules was determined from images acquired by optical microscopy (Axio A1 Carl Zeiss microscope, Oberkochen, Germany). The images were processed using the Axio-Vision Release 4.8 software.

2.5.3. Release of L. reuteri CRL 1324 from xanthan-gellan capsules. Survival of freeze-dried and encapsulated cells in genital tract conditions

A weighed quantity of xanthan-gellan capsules (lyophilized in lactose-skim milk) was inoculated into tubes containing 2.5 ml MSVF (a tube for each time point) and incubated at 37°C without agitation. Samples were taken at 3, 6, 9 and 24 h of incubation. Each sample was filtered to recover only cells in suspension (i.e. cells released into MSVF from capsules). The number of viable *L. reuteri* CRL 1324 cells released was determined by the successive dilution method in MRS agar plates, as described previously (item *2.5.1.*). For each sampling time, the **release rate** was expressed as N_t/N_i, where N_t is the log CFU/g in incubation time "t" and N_i is the log CFU/g in the initial incubation time.

On the other hand, for each sampling time, capsules retained in the filter were later transferred to 0.05 M sodium phosphate buffer (pH 7.0) and disaggregated with a homogenizer to release encapsulated cells mechanically, as described above. The number of *L. reuteri* CRL 1324 viable cells in the different samples was determined by the successive dilution method in MRS agar plates, as described previously (item *2.5.1.*). The survival of encapsulated cells in MSVF was calculated considering the total number of viable cells (cells released into MSVF from capsules plus encapsulated cells that were mechanically released). The **survival rate in MSVF** was expressed as N_t/N_i, where N_t is the log CFU/g in incubation time "t" and N_i is the log CFU/g in the initial incubation time.

2.5.4. Growth inhibition of opportunist pathogenic S. agalactiae NH 17 by L. reuteri CRL 1324

Associated cultures between *S. agalactiae* NH 17 and *L. reuteri* CRL 1324 [aliquots of disaggregated lyophilized in lactose-skim milk capsules, rehydrated freeze-dried free cells or fresh free cells (from the third subculture in MRS broth)] were performed in LAPTg broth

at 37°C under static conditions. A 1:100 ratio of initial viable cells between NH 17 and CRL 1324 was assayed (De Gregorio et al., 2014). Pure cultures of each microorganism were grown under the same culture conditions as control. At 24 h of incubation, the number of viable cells was determined by the successive dilution method, using selective culture media: MRS agar (pH 5.5) for lactobacilli and Bacto Todd-Hewitt agar (pH 7.8) (Becton Dickinson) for streptococci (De Gregorio et al., 2014). The plates were incubated at 37°C for 48 h

2.5.5. Formation of biofilm

The ability of *L. reuteri* CRL 1324 to form biofilm in MRS without Tween 80 was previously demonstrated (Leccese Terraf et al., 2012). Aliquots of disaggregated capsules (lyophilized in lactose-skim milk) containing *L. reuteri* CRL 1324, rehydrated freeze-dried free cells or fresh free cells (from the third subculture in MRS broth) were incubated in 5 ml MRS broth without Tween 80 for 24 h at 37°C. After this incubation period, the inoculum for biofilm formation assay was prepared with bacterial pellets from each culture, washed and resuspended in saline up to an OD_{540nm} of 1.5. The microplate assay was carried out according to the technique previously described (Leccese Terraf et al., 2012, 2014), always including sterile culture medium as negative control, and using crystal violet as stain and 30% acetic acid as stain eluent. OD_{570nm} values (in Microplate Spectrophotometer, VERSAmax, Molecular Devices, Sunnyvale, CA, USA) were used to express biofilm formation quantitatively (Leccese Terraf et al., 2012).

2.6. Statistical analysis

Analysis of variance (ANOVA) using a general linear model was applied to determine the main and interaction effects of factors evaluated in each of the following processes: a) encapsulation: time of the encapsulation process (pre- and post-encapsulation); b)

lyophilization: time of the lyophilization process (pre- and post-lyophilization) and lyophilization condition (freeze-dried capsules in the presence or absence of lactose-skim milk and lyophilized free cells in lactose-skim milk); c) storage: system in which *L. reuteri* was stored (freeze-dried capsules in the presence or absence of lactose-skim milk, nonlyophilized capsules and lyophilized free cells in lactose-skim milk), temperature (room and refrigerated temperatures) and time (0, 21, 60 and 150 days). The number of *L. reuteri* CRL 1324 viable cells (log CFU/g) was the response of interest analyzed in the different processes.

ANOVA using a general linear model was also applied to analyze: a) the effects of the condition in which *L. reuteri* was inoculated (cells included in lyophilized capsules in the presence of lactose-skim milk, lyophilized free cells in lactose-skim milk and fresh free cells) and incubation time (0, 3, 6, 9 and 24 h) on the survival rate in MSVF; b) the effects of the culture type (pure and associated) and condition in which *L. reuteri* was inoculated on the viability of *S. agalactiae* NH 17 and lactobacilli (log CFU/ml), in *S. agalactiae* NH 17 inhibition assay; c) the effects of the condition in which *L. reuteri* was inoculated on biofilm formation ($OD_{570\ nm}$).

In each analysis, significant differences ($P < 0.05$) between mean values were determined by Tukey's test, using MINITAB statistical software (version 16 for Windows).

3. Results

3.1. Encapsulation of L. reuteri CRL 1324

When applying the extrusion-ionic gelation technique to encapsulate *L. reuteri* CRL 1324 cells using xanthan-gellan, high encapsulation yield of capsules (72.5 ± 2.0 %) and high *L. reuteri* CRL 1324 survival rate during encapsulation (0.89 ± 0.02) were obtained. The number of viable cells before and after the encapsulation process was statistically different

(viability decrease = 1.15 ± 0.27 log units). However, a high number of encapsulated cells was obtained (log CFU/g capsules = 9.06 ± 0.27).

3.2. Lyophilization of encapsulated and free cells of L. reuteri CRL 1324

The survival of encapsulated and free cells of *L. reuteri* CRL 1324 during lyophilization was compared. Significant differences between *L. reuteri* CRL 1324 viability before and after the lyophilization process and between the different systems assayed were observed (Fig. 2). When encapsulated *L. reuteri* CRL 1324 was freeze-dried, survival was significantly higher in the presence of the lactose-skim milk protective mixture than in its absence. However, the viability decrease after the lyophilization of free bacterial cells with lactose-skim milk (0.75 ± 0.01 log CFU/g units) was significantly lower than that of encapsulated cells lyophilized either with or without lactose-skim milk (decrease of 3.17 ± 0.12 or 4.07 ± 0.09 log CFU/g units, respectively). Therefore, the survival rate of free cells (0.92 ± 0.001) during lyophilization was higher than encapsulated cells (0.70 ± 0.01 or 0.62 ± 0.01 with or without lactose-skim milk, respectively).

3.3. Viability during storage of encapsulated and lyophilized L. reuteri CRL 1324

The different factors assayed (system in which bacterial cells were stored, temperature and time) significantly affected the viability of *L. reuteri* CRL 1324 during storage (Fig. 3). System-temperature, system-time and temperature-time interaction effects were also significant ($P < 0.05$), indicating that the effect of each factor was dependent on the other factors considered. In general, the highest survival of *L. reuteri* CRL 1324 during storage was obtained from the systems (free and encapsulated cells) that were lyophilized with lactose-skim milk. In all the systems assayed, bacterial viability was significantly higher after storage

at 4°C than at room temperature. On the other hand, a significant decrease in viability was observed at different storage times (Fig. 3).

Based on the differences observed in *L. reuteri* CRL 1324 viability at the two storage temperatures, bacterial survival in different systems and storage times were analyzed for each temperature (Fig. 4a). At room temperature, highest *L. reuteri* survival was evidenced in lyophilized free cells. After 21 days of storage, no viable cells were recovered from nonlyophilized capsules or from freeze-dried capsules without lactose-skim milk. At day 60, no viable cells were obtained in freeze-dried capsules with lactose-skim milk.

At refrigerated temperature, significant differences in *L. reuteri* CRL 1324 viability were evidenced between the different systems and between the storage times assayed (Fig. 4b). After 150 storage days, the highest numbers of viable cells were obtained in freeze-dried, nonencapsulated cultures (4×10^8 CFU/g) and in freeze-dried capsules with lactose-skim milk (1×10^6 CFU/g). In these systems, bacterial viability did not decrease significantly during the storage period assayed. However, in lyophilized capsules without lyoprotectors, viability at day 150 was significantly lower than at days 0, 21 and 60. In this system, no viable cells were detected at day 21 of storage, in a similar way to the results obtained at room temperature.

3.4. Characterization of xanthan-gellan capsules. Functional and beneficial properties of freeze-dried and/or encapsulated L. reuteri CRL 1324

On the basis of the viability results obtained from the different encapsulated systems and storage conditions assayed, the xanthan-gellan capsules lyophilized with lactose-skim milk and stored at 4°C were selected for its characterization. In this system, a high number of viable cells was evidenced at the end of the storage period. Moreover, lyophilized free cells dried with lactose-skim milk and stored at 4°C were evaluated in most of the following

assays, since the highest recovery of *L. reuteri* CRL 1324 viable cells during storage was evidenced in this system.

3.4.1. Morphology, size and internal structure of selected xanthan-gellan capsules

Xanthan-gellan capsules lyophilized in the presence of lactose-skim milk were observed as imperfect spheres with an average diameter of 2.42 ± 0.47 mm. Scanning electronic microscopy analysis showed that the capsule surface was heterogeneous, with no evident pores or fractures (Fig. 5a-b). When analyzing the capsules inside, individual or aggregated bacterial cells included in the polymeric matrix were observed (Fig. 5c-d).

3.4.2. Release of L. reuteri CRL 1324 from xanthan-gellan capsules into a medium simulating vaginal fluid

L. reuteri CRL 1324 was markedly released in MSVF when included in xanthan-gellan capsules during the first 3 h of incubation (Fig. 6a). At that time, the release rate was 0.78 ± 0.01, corresponding to a difference of around one log unit between the total number of encapsulated microorganisms and the released number. The viable cells in suspension were similar between 3 and 24 h of incubation.

3.4.3. Survival of freeze-dried and encapsulated cells in genital tract conditions

For each system in which *L. reuteri* CRL 1324 was inoculated in MSVF, survival rates were not significantly different during 24 h of incubation at 37°C. On the other hand, at the end of incubation, the survival rate of bacteria from fresh cultures was significantly lower than that from lyophilized free cells (data not shown). However, no significant differences were observed in the survival rates of freeze-dried encapsulated bacteria and freeze-dried or control cultures.

3.4.4. Growth inhibition of S. agalactiae NH 17 by L. reuteri CRL 1324

L. reuteri CRL 1324 cells from encapsulated and/or freeze-dried samples inhibited the growth of *S. agalactiae* NH 17 after 24 h of co-culture at 37°C in LAPTg broth. At this time, the number of NH 17 viable cells was significantly lower in CRL 1324-NH 17 associative cultures than in NH 17 pure cultures (Fig. 6b). There were no statistical differences in *S. agalactiae* NH 17 viability decrease (2.89 ± 0.13 log CFU/ml) between associative cultures that were inoculated with encapsulated, freeze-dried or control *L. reuteri* cells. The viability of *L. reuteri* CRL 1324 was similar in pure and mixed cultures under the different conditions assayed.

3.4.5. Formation of biofilm

Encapsulated and/or freeze-dried *L. reuteri* maintained the capability to form biofilm in MRS without Tween 80. No significant differences were observed in the biofilm formed (expressed as $OD_{570\ nm}$ values) from freeze-dried encapsulated cells, freeze-dried free cells or control cells (Fig. 6c).

4. Discussion

Freeze-drying and microencapsulation are methods frequently applied in food and pharmaceutical industries to protect various compounds and microorganisms against different stressing physical-chemical factors (Solanki et al. 2013; Endo et al. 2014). Several studies on encapsulation of microorganisms with potential food application were carried out to protect them from adverse conditions in the gastrointestinal tract (Ding and Shah, 2009; Islam et al., 2010; Weinbreck et al., 2010; Jiménez-Pranteda et al., 2012). Only a few works reported the encapsulation of potential probiotic microorganisms for the human urogenital tract (Martín

Villena et al., 2010; Pliszczak et al., 2011). These authors applied the emulsification-gelation method (using bioadhesive polymers) and assayed the survival of microorganisms during the encapsulation process but not during storage.

Polymers of different chemical nature are commonly used to encapsulate microorganisms, e.g. alginate, pectin, hyaluronic acid (natural polysaccharides), xanthan, gellan and pullulan gums (microbial natural exopolysaccharides), hydroxypropyl methylcellulose (a semisynthetic cellulose derivative mucoadhesive) and polyethylene glycol (a synthetic polyether), among others (Ding and Shah, 2009; Martín Villena et al., 2009; Alli, 2011, Pliszczak et al., 2011). In the pharmaceutical area, several polymers with mucoadhesive properties are applied to improve or increase the permanence time of the systems in the mucosa of interest (Pliszczak et al., 2011), of main importance by the localization of the vaginal tract in the human body.

In the present work, encapsulation and freeze-drying methods were applied to preserve *L. reuteri* CRL 1324, a urogenital probiotic candidate strain selected for its beneficial properties. Xanthan and gellan gums were employed to encapsulate *L. reuteri* CRL 1324 on the basis of the results obtained in other studies, in which different *Lactobacillus* and *Bifidobacterium* strains (for products administered orally) were successfully encapsulated with the aforementioned polymers (Sun and Griffiths, 2000; Jiménez-Pranteda et al., 2012). Moreover, microbial natural exopolysaccharides, widely applied in foods, are safe for human use (Jiménez-Pranteda et al., 2012). *L. reuteri* CRL 1324 was efficiently encapsulated applying the extrusion-ionic gelation technique. A high number of viable cells were recovered (around 10^9 CFU/g of capsules), indicating that this microorganism mantained its viability during the encapsulation process.

The lyoprotective effect of several substances against stress by freeze-drying was widely reported (Santivarangkna et al., 2008; Savini et al., 2010). In previous studies

performed on the lyophilization of vaginal *Lactobacillus* free cells, the effects of lactose and sucrose suspended in water or reconstituted skim milk on the stability during freeze-drying and subsequent storage of vaginal lactobacilli strains were evaluated (Juárez Tomás et al., 2009). Optimal stability results were obtained in the presence of lactose+skim milk (Juárez Tomás et al., 2009). Supported by the results obtained in previous studies, the lactose+skim milk protective mixture was used for the freeze-drying of free or encapsulated *L. reuteri* CRL 1324 cells.

When evaluating the survival of encapsulated *L. reuteri* CRL 1324 during the freeze-drying process, bacterial viability was higher when the capsules were suspended previously in lactose+skim milk. In the case of encapsulated *L. reuteri* CRL 1324, the effect of lyoprotectors during drying could be explained, for example, by the formation of a protective cover with milk proteins over the xanthan-gellan capsules, thus providing a more compact physical barrier for the encapsulated cells (Gerez et al., 2012).

On the other hand, the survival of encapsulated *L. reuteri* CRL 1324 cells during freeze-drying was significantly lower than cell suspensions with lyoprotectors. These results suggest that the xanthan-gellan polymeric mixture was efficient in the process of encapsulating microorganism, but failed to provide adequate protection against the stress caused by freeze-drying process. When comparing the survival during lyophilization of free and encapsulated *L. reuteri* cells, the effect of lyoprotectors decreased when the cells were encapsulated. The polymeric matrix probably caused a decrease in the interaction between lyoprotectors and bacterial cells included into capsules, compared to free microorganisms. In the protectors-cells interaction, milk proteins could form a protective cover directly over the cells, and carbohydrates could help to prevent or reduce the lethal effect of intracellular ice formation during freezing, through hydrogen bonds with water and cellular structures (stabilizing the cell membrane and proteins) (Santivarangkna et al., 2008).

Stability studies of *L. reuteri* CRL 1324 in xanthan-gellan capsules evidenced that no viable cells were obtained after a short time of storage (21-60 days) at room temperature (in lyophilized or nonlyophilized capsules) or at refrigerated temperature (only in nonlyophilized capsules). However, viability loss was minimal in freeze-dried free cells and freeze-dried encapsulated cells with lactose+skim milk. Carbohydrates could protect against free radicals produced during the storage and rehydration of freeze-dried cultures (Santivarangkna et al., 2008). On the other hand, moisture content is critical to microbial viability during storage (Weinbreck et al., 2010). However, in this work the water content of the different systems under study was not determined during the stability testing period by technical limitations; in further studies, this parameter will be evaluated as a quality parameter.

Systems constituted by xanthan-gellan capsules and free cell powders containing *L. reuteri* CRL 1324 (both freeze-dried with lactose+skim milk) were the optimal systems for storage at refrigerated temperature. Since *L. reuteri* CRL 1324 in xanthan-gellan capsules could be included in the design of a product for vaginal application, the release of bacterial cells in a medium similar to genital secretions was assayed. Results evidenced a marked release of microorganism in a short incubation time (3 h) at 37°C followed by a stable number of viable cells. Pliszczak et al. (2011) demonstrated that different commercial probiotic lactobacilli, which were included in microparticles of pectin and hyaluronic acid sodium salt, were released into MSVF in a sustained way during the first 10 h of incubation at 37°C, up to total release after 16 h. In contrast to our results, Pliszczak et al. (2011) observed bacterial proliferation after the complete release of encapsulated lactobacilli.

The ability of microorganisms to maintain their viability and expression of beneficial characteristics during storage processes as well as stability in the conditions of the tract where will be applied are relevant properties that should be evaluated in each potential probiotic strain (du Toit et al., 2013; Domig et al., 2014). Free or encapsulated *L. reuteri* CRL 1324

cells, freeze-dried and stored at refrigerated temperature, maintained both their ability to form biofilm and their antagonist activity against opportunist pathogenic *S. agalactiae* NH 17, two characteristics previously evidenced (Leccese et al., 2012; De Gregorio et al., 2014). On the other hand, *L. reuteri* CRL 1324 viability did not decrease significantly during 24 h of incubation in MSVF at 37°C and at a low pH (4.5). The survival of lyophilized cells in MSVF was higher than that of nonlyophilized fresh cultures, suggesting that the lactose+skim milk protective mixture or the xanthan-gellan polymeric cover of capsules could favor the survival of *L. reuteri* CRL 1324 under nonstandard conditions. In this work, the pH of MSVF assayed was similar to normal vaginal pH. Further experiments need to be performed to evaluate bacterial stability under different genital tract conditions (e.g. different pH conditions in the vaginal lumen in the case of genital infections and in the presence of semen) (Borges et al., 2013).

The systems selected (lyophilized powders and capsules) in this work could be used in appropriate pharmaceutical forms for vaginal application for the prevention and/or treatment of human female urogenital tract infections. The main advantage of these systems is the inclusion of dried bacterial cells, which is a critical point to preserve the stability of probiotic microorganisms (Weinbreck et al., 2010). Therefore, future incorporation of powders and capsules into vaginal solid forms with low water activity (e.g. capsules, tablets and ovules) will be carried out. Vaginal tablets and Witepsol® ovules including freeze-dried vaginal probiotic strains have been proved effective to preserve bacterial viability during storage for different periods (Borges et al., 2013; Muller et al., 2014; Verdenelli et al., 2014). On the contrary, in potential probiotic liquid formulations, marked viability losses were evidenced (Borges and Teixeira, 2014; Verdenelli et al., 2014).

On the other hand, vaginal solid and semi-solid delivery forms allow obtaining a continuous release of active principles for several hours, unlike liquid formulations that

present a short residence time in vagina (Pliszczak et al., 2011). In this sense, mucoadhesive anionic polymers as xanthan and gellan used to encapsulate *L. reuteri* CRL 1324 could help and promote the permanence of the active ingredients in the vaginal mucosa.

In conclusion, encapsulation (by extrusion-ionic gelation) and freeze-drying in the presence of lyoprotectors (lactose+skim milk) and their subsequent storage in refrigeration conditions favored the maintenance of vaginal *L. reuteri* CRL 1324 viability and ability to survive in MSVF, form biofilm and inhibit pathogenic *S. agalactiae*. Further studies should be performed to improve the survival of encapsulated *L. reuteri* CRL 1324 under adverse conditions during production industrial processes and subsequent storage and to evaluate the interaction of formulated systems with mucosa vaginal components.

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None of the authors have a conflict of interests for the publication of the manuscript.

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Figure legends

Fig. 1. Scheme summarizing the preparation and storage conditions of the different systems assayed in this work to preserve the stability of *Lactobacillus reuteri* CRL 1324.

Fig. 2. Viability of *Lactobacillus reuteri* CRL 1324 during the lyophilization process. Different systems were freeze-dried: capsules in the presence of a lactose+skim milk protective mixture (Capsules with L-SM), capsules in the absence of protectors (Capsules without L-SM) and free cells in the presence of protectors (Free cells with L-SM). The data are plotted as the mean values of viable cells numbers (log CFU/g ± SEM). Different letters indicate statistically significant differences ($P < 0.05$) in the number of viable cells between the different systems subjected to lyophilization on the different times of process (Pre-FD, before freeze-drying; Post-FD, after freeze-drying), according to Tukey's test.

Fig. 3. Main effects of **condition, temperature** and **time** on *Lactobacillus reuteri* CRL 1324 survival during the storage process for 150 days. In the "**Condition"** panel, each point indicates the mean value of log CFU/ml for each condition at all storage times and temperatures. In the "**Temperature"** panel, each point indicates the mean value of log CFU/ml for each temperature, at all storage times and conditions. In the **"Time"** panel, each point indicates the mean value of log CFU/g for each time at all conditions and temperatures. Conditions (systems) in which bacterial cells were stored: freeze-dried capsules in the presence of lactose-skim milk protective mixture (FD cap. with L-SM); freeze-dried capsules in the absence of protectors (FD cap. without L-SM), nonlyophilized capsules (Non FD cap.) and freeze-dried free cells in the presence of protectors (FD free cells with L-SM). In each panel, different letters indicate statistically significant differences ($P < 0.05$) in the mean

values of log CFU/g between the levels of the factor assayed (condition, temperature or storage time), according to Tukey's test.

Fig. 4. Viability of *Lactobacillus reuteri* CRL 1324 during storage for 150 days at room temperature (**a**) and refrigeration (4°C) temperature (**b**). Conditions (systems) in which bacterial cells were stored: (-■-) freeze-dried capsules in the presence of lactose+skim milk protective mixture; (-□-) freeze-dried capsules in the absence of protectors, (-♦-) nonlyophilized capsules; (-▲-) freeze-dried free cells in the presence of protectors. The data are plotted as the mean values of viable cell numbers (log CFU/g ± SEM). Different letters indicate statistically significant differences ($P < 0.05$) in the number of viable cells between the different conditions and storage times at a same temperature, according to Tukey's test.

Fig. 5. Scanning electronic microscopy of xanthan-gellan capsules containing *Lactobacillus reuteri* CRL 1324 that were freeze-dried in the presence of lactose+skim milk protective mixture. **a-b)** Whole capsules observed at 90x. **c-d)** Capsule section with microorganisms included in a polymeric matrix, observed at 3000x and 10000x.

Fig. 6. Release rate in a medium similar to vaginal fluid and potential beneficial properties of *Lactobacillus reuteri* CRL 1324. **a)** Release rate of *L. reuteri* CRL 1324 from freeze-dried capsules in the presence of lactose+skim milk protective mixture, incubated in a medium similar to vaginal fluid (MSVF) at 37°C for 24 h. Release rate was calculated as N_t/N_i, where N_t is the log CFU/g at incubation time "t" and N_i is the log CFU/g at initial incubation time. Data are plotted as the mean values of release rates ± SEM. **b)** Mixed and pure cultures of *L. reuteri* CRL 1324 (CRL 1324) and *S. agalactiae* NH 17 (NH 17). Data are plotted as the mean values of viable cell numbers (log CFU/ml ± SEM) of CRL 1324 and NH 17 at 24 h of

incubation at 37°C in LAPTg broth. Different letters indicate statistically significant differences ($P < 0.05$) in the number of viable cells for each microorganism in pure and mixed cultures, according to Tukey's test. **c)** Biofilm formation from freeze-dried encapsulated cells (FD capsules), freeze-dried free cells (FD free cells) and fresh free cells (Fresh culture) of *L. reuteri* CRL 1324 after 72 h of incubation at 37°C in MRS without Tween 80. The data express the mean $OD_{570\ nm}$ values ± SEM of acetic acid-solubilized crystal violet-stained cultures from polystyrene microplates. Different letters indicate significant differences ($P < 0.05$) in biofilm formation between the different conditions assayed, according to Tukey's test. The results of panels **a)**, **b)** and **c)** correspond to systems stored for 150 days; similar results were obtained during all storage periods.

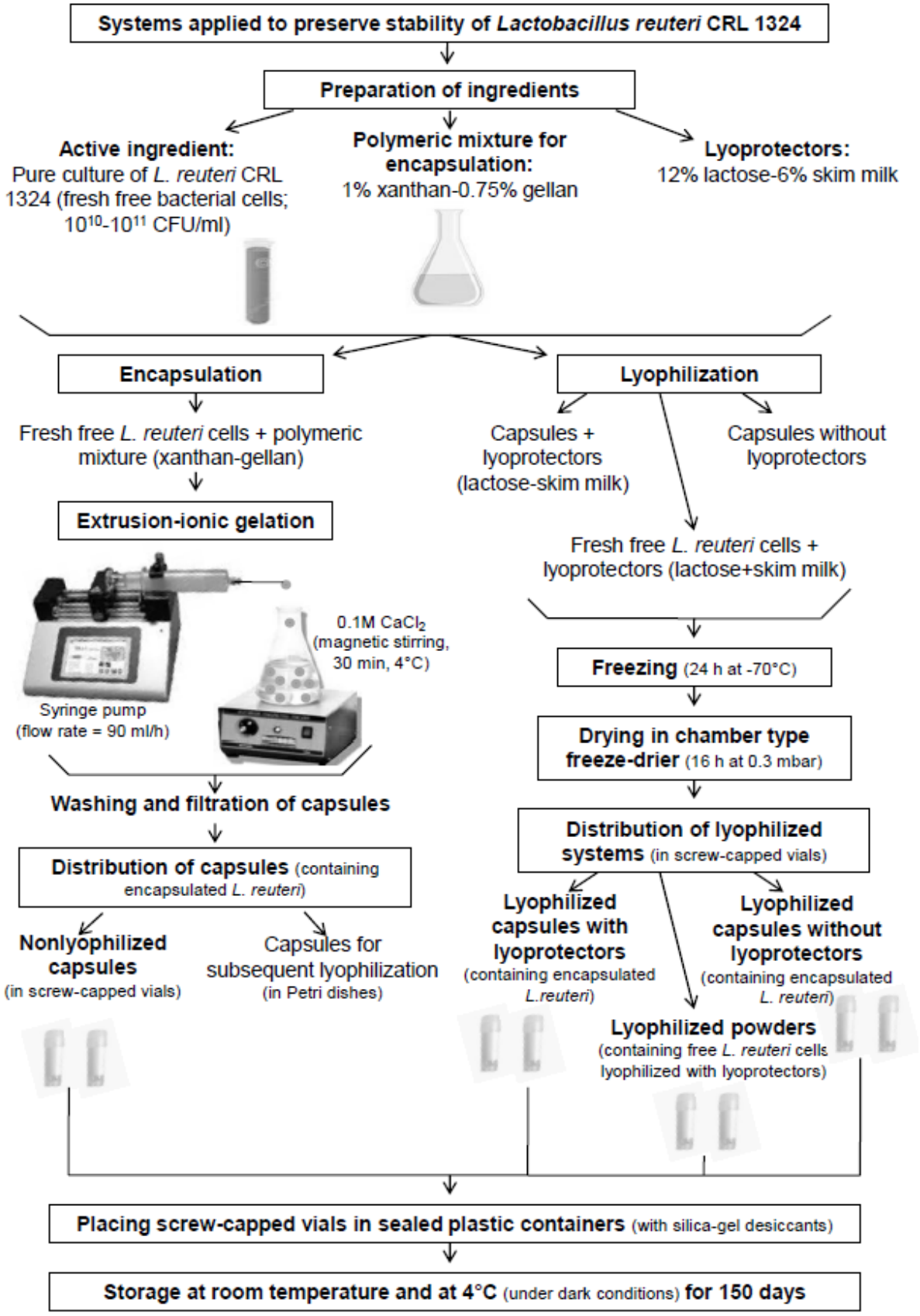

Systems applied to preserve stability of Lactobacillus reuteri CRL 1324
Preparation of ingredients
Active ingredient:
Pure culture of L. reuteri CRL 1324 (fresh free bacterial cells; 10^10-10^11 CFU/ml)
Polymeric mixture for encapsulation:
1% xanthan-0.75% gellan
Lyoprotectors:
12% lactose-6% skim milk
Encapsulation
Lyophilization
Fresh free L. reuteri cells + polymeric mixture (xanthan-gellan)
Capsules + lyoprotectors (lactose-skim milk)
Capsules without lyoprotectors
Extrusion-ionic gelation
Fresh free L. reuteri cells + lyoprotectors (lactose+skim milk)
0.1M CaCl2 (magnetic stirring, 30 min, 4°C)
Syringe pump (flow rate = 90 ml/h)
Freezing (24 h at -70°C)
Drying in chamber type freeze-drier (16 h at 0.3 mbar)
Washing and filtration of capsules
Distribution of lyophilized systems (in screw-capped vials)
Distribution of capsules (containing encapsulated L. reuteri)
Lyophilized capsules with lyoprotectors (containing encapsulated L.reuteri)
Lyophilized capsules without lyoprotectors (containing encapsulated L. reuteri)
Nonlyophilized capsules (in screw-capped vials)
Capsules for subsequent lyophilization (in Petri dishes)
Lyophilized powders (containing free L. reuteri cells lyophilized with lyoprotectors)
Placing screw-capped vials in sealed plastic containers (with silica-gel desiccants)
Storage at room temperature and at 4°C (under dark conditions) for 150 days

Figure 1

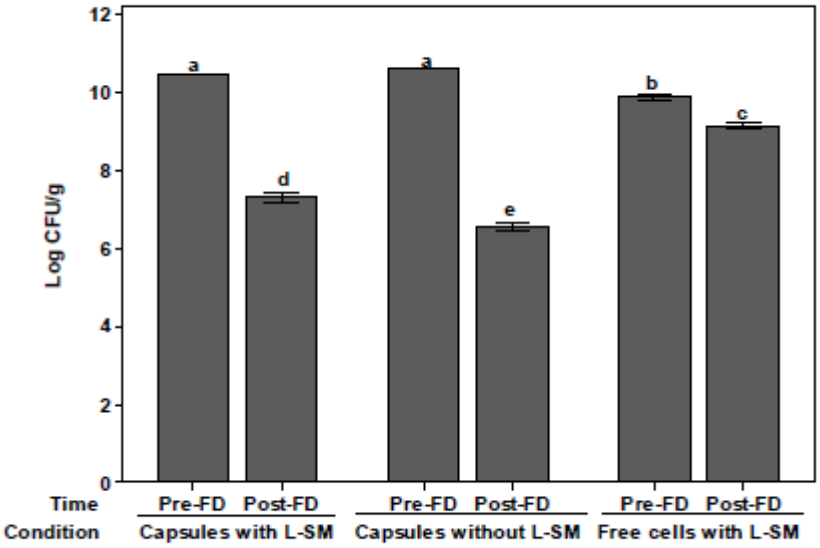


Figure 2

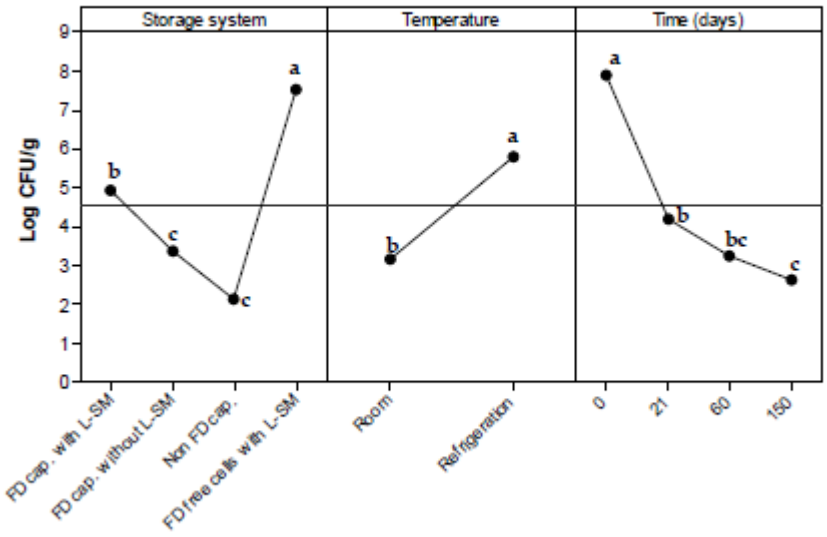


Figure 3

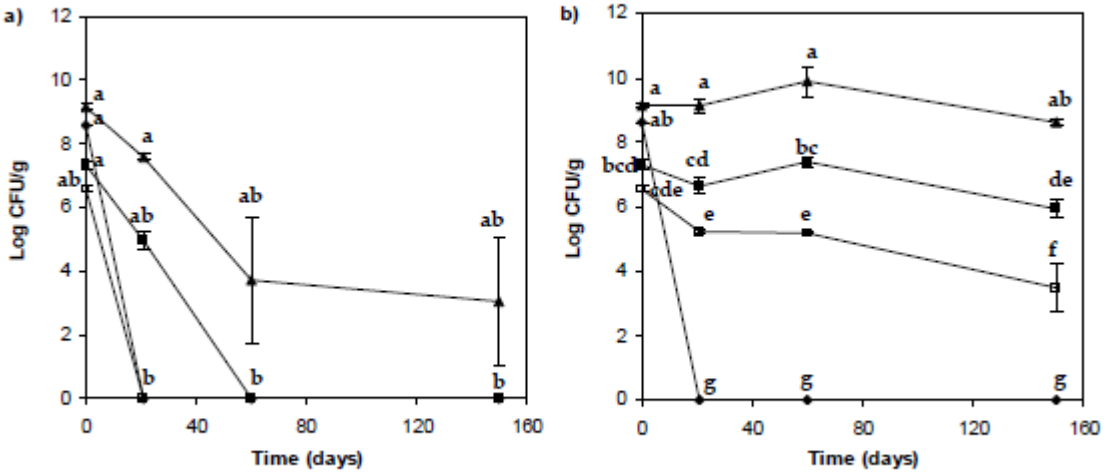


Figure 4

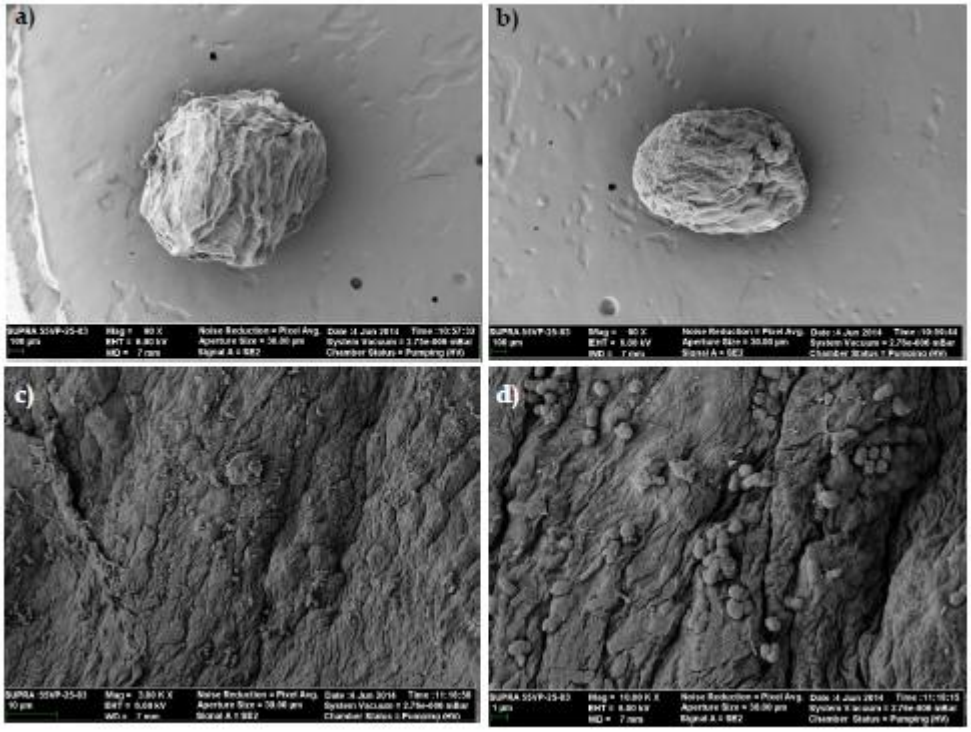


Figure 5

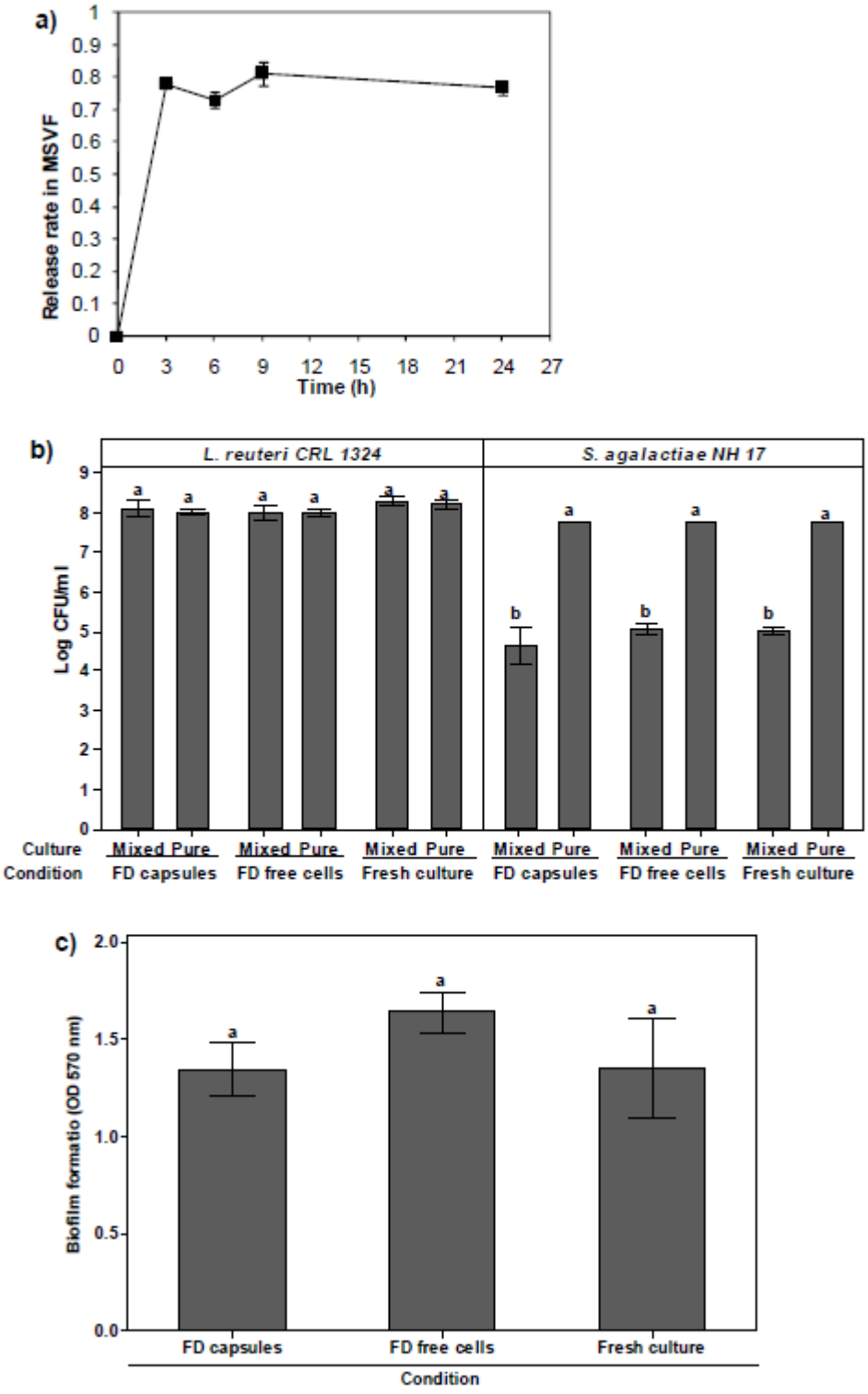
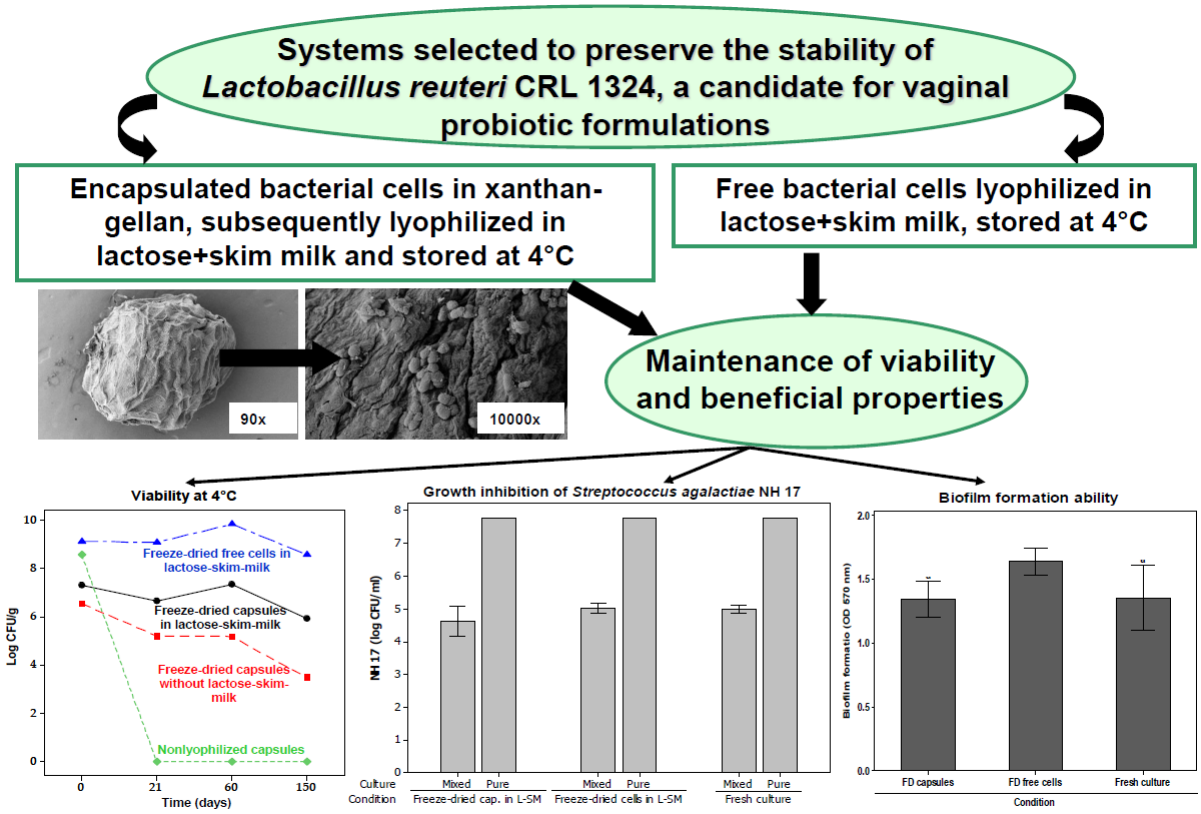


Figure 6



Graphical abstract