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Research paper

Development of an enteric-coated, layered multi-particulate formulation for ileal delivery of viable recombinant *Lactococcus lactis*

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Abstract

Layering of recombinant hIL-10 producing *Lactococcus lactis* (*L. lactis* Thy12) on inert carriers is a promising technique for the preparation of a multi-particulate formulation of viable, hIL-10 producing *L. lactis*. To improve viability after layering and storage, *L. lactis* Thy12 was layered in different matrices (10% skim milk and/or 2.5, 5, 10% inulin). After layering, the highest viability was obtained in the 10% skim milk supplemented with 5% inulin matrix (8.7%). However, upon storage, 10% skim milk alone yielded the highest viability. Thereby, layered *L. lactis* Thy12 showed superior long term stability in comparison with freeze-dried *L. lactis* Thy12. The layering process was performed during 3 h without encountering technical problems, with good layer consistence and constant viability. Enteric properties were obtained with a 30% Eudragit® L30D-55 or 15% Eudragit® FS30D coating and maintained during an initial six months storage period (−20 °C/20% RH). After in vitro simulation of the gastric stage, only 5% of the bacteria remained viable in Eudragit® L30D-55 coated pellets, contrary to 85% in Eudragit® FS30D coated pellets, indicating its superior protective capacity against gastric fluid. After eight months storage (−20 °C), 80% of the initial *L. lactis* Thy12 remained viable in the Eudragit® FS30D coated pellets.

Keywords: Layering; Inulin; Eudragit; Viability; Recombinant *Lactococcus lactis*

1. Introduction

As interleukin-10 (IL-10) plays a central role in down-regulating inflammatory cascades, it is a potential candidate to treat Crohn's disease, a severe, chronic intestinal inflammation [1]. However, IL-10 given through the systemic route is not effective [2]. A local delivery of the cytokine by *Lactococcus lactis* seems a promising alternative [3]. Steidler et al. and Vandenbroucke et al. described the advantageous use of recombinant *L. lactis* strains for the mucosal delivery of immunomodulating cytokines and other therapeutic proteins, respectively [3–5]. Murine IL-10 secreting *L. lactis* were used for the in vivo production and mucosal delivery of this cytokine. Daily intragastric inoculation of these bacteria to mice, in which experimental enterocolitis was installed, could cure or prevent the intestinal inflammation [3].

To evaluate the therapeutic potential of this concept in patients with Crohn's disease, a formulation containing the biologically contained human IL-10 (hIL-10) producing *L. lactis* Thy12 strain had to be developed [6]. Since the hIL-10 production is strictly related to the viability of *L. lactis* Thy12, a suitable production technique with maintenance of an acceptable viability level and shelf life is required. As Klijn et al. showed that the gastric fluid negatively influences the viability of *L. lactis*, the dosage form should be enteric-coated [7]. A multi-particulate formulation has been chosen to ensure rapid gastric emptying [8] while it also offers the advantage of ease of swallowing in case of large doses.

Previous work showed that layering is a promising technique for the production of a multi-particulate formulation of viable and hIL-10 producing *L. lactis* Thy12 [9]. The bacteria culture is atomised and subsequently dried on the surface of fluidising inert carriers. Thereby this is an

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economic and single-step production process for a coated multi-particulate formulation as after layering, coating can be performed in the same apparatus.

The objectives of this study are to evaluate different layering matrices for their potential to preserve viability, to study long term stability, to assess the influence of long time processing in feasibility, layer consistence and viability, to optimise coating and to determine viability of *L. lactis* Thy12 in coated pellets after storage and passage through the gastric fluid.

2. Materials and methods

2.1. Bacterial strain used in this study

L. lactis Thy12 (human IL-10 producing *L. lactis* MG1363 [10] derivative) was obtained from the Department of Molecular Biomedical Research (DMBR) where it was genetically modified in order to produce human IL-10 [6]. The bacteria were stored as a stock suspension in glycerol/GM17 (50/50)/thymidine (−20 °C).

2.2. Layering

2.2.1. Preparation of the layering solutions

An *L. lactis* Thy12 culture was prepared by inoculating the stock suspension 1/1000 in M17 supplemented with 0.5% glucose and 50 μg/ml thymidine (GM17T). The culture was grown for 16 h at 30 °C to reach the stationary phase ($2-3\times10^9$ cfu/ml). The bacteria were collected by centrifugation at 3000 g for 10 min at 4 °C. The cell pellet was resuspended at 10^{10} cfu/ml in different layering solutions i.e. 10% (w/v) skim milk (Difco, Becton Dickinson, MA, USA), 10% (w/v) skim milk + 5% (w/v) inulin (EXL® 608, kindly donated by Sensus, Roosendaal, The Netherlands), 10% (w/v) inulin, 5% (w/v) inulin or 2.5% (w/v) inulin. These solutions were prepared by dissolving inulin in boiling water and skim milk in cold water. To prevent further activity or growth, the cultures were kept on ice in between all handling steps.

For the determination of the yield, the same layering solutions were used, but the bacteria were replaced by thymidine (Alkemi, Lokeren, Belgium) as a marker substance. Thymidine is easy to quantify by UV-spectrofotometry.

2.2.2. Layering process ($n=3$)

Microcrystalline cellulose spheres were used as inert carriers. Equal amounts of Cellets® 700 (700–1000 μm) and Cellets® 1000 (1000–1250 μm), both kindly donated by Pharmatrans (Basel, Switzerland) were mixed, to obtain 300 g of pellets with an average diameter of 1000 μm as standard for pellets. Layering was performed in a fluid-bed apparatus (GPCG 1, Glatt, Binzen, Germany) used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; spray rate of 3 g/min for 30 min or 3 h; atomising pressure 1.5 bar; product temperature 45 °C). Before layering, the pellets were preheated until a product temperature of 45 °C was reached. Throughout the layering process the layering solution was kept on ice and manually homogenised from time to time. The experiments were performed at 20% relative humidity (RH).

After layering, the pellets were immediately analysed (yield, viability) or packed in Alu sachets (LPS, Vapor flex barrier bag, NJ, US) and sealed at 20% RH. To study the influence of the layering matrix composition on storage stability of *L. lactis* Thy12, the sachets were stored for 12 months at room temperature (RT) (23 ± 2 °C), 8 °C and −20 °C. Before further coating, the sachets were stored at −20 °C.

2.2.3. Evaluation of the layered pellets

2.2.3.1. Determination of viability of L. lactis Thy12. The influence of the layering matrix composition on viability was determined. Therefore, the pellets, layered with five different matrices, were analysed immediately after layering and after 1 month storage at RT, 8 and −20 °C. Next, the long term storage stability was determined by analyzing pellets, layered with 10% skim milk, after storage at −20 °C. Viability of the bacteria was determined by following the growth of the standards and the samples in a Bioscreen (Labsystem, Helsinki, Finland). The viability of the starting culture was set at 100%. Different dilutions of the starting culture were made, inoculated 1/100 in fresh GM17 with thymidine and loaded in triplicate into microtiter plates for analysis in the Bioscreen. The growth at 30 °C was followed for 21 h. The time necessary to reach an optical density at 600 nm (OD_{600}) half way the minimum and maximum OD_{600} (50% time) was calculated based on the exponential growth phase. This 50% time was plotted against the natural logarithm of the viability and the equation of the standard curve was calculated. The viability of a sample was measured based on the standard curve of the starting culture and expressed as % of theoretical. 0.1 g of pellets were manually shaken in 1 ml sterile water. Three dilutions of each sample were loaded in duplicate onto micro-titer plates for analysis in the Bioscreen. The viability values were statistically evaluated with a two-way ANOVA at a significance level of 0.05. The normality of the data was checked by means of a Kolmogorov–Smirnov test and the homogeneity of variances by means of the Levene test. A multi comparison among pairs of means was performed using a Scheffé test with $P<0.05$ as a significance level. All analyses were performed with SPSS 11.0 for Windows.

2.2.3.2. Determination of the amorphous properties of the layered matrix. The 10% inulin matrix, layered on the pellets was focused with a Kaiser Optical Systems Raman Rxn1 Microprobe (microscope objective-10×) coupled to a Raman Rxn1 Analyser (air-cooled CCD detector) via

a proprietary holographic optical module. The RamanRxn1 Analyser is an axial transmissive spectrograph that employs holographic technology. A 785 nm Invictus™ laser with 400 mW of power was employed for excitation. 10 s exposures were used for measurements. The obtained spectrum was compared with that of crystalline and amorphous inulin in order to characterise the molecular arrangement of inulin in the matrix.

2.2.3.3. Determination of the yield of the layering process. Three batches of pellets were layered during 30 min with the marker substance in the 10% skim milk +5% inulin matrix. After sonication of the pellets in demineralised water for 10 min and subsequent filtration (Celtron 30/0.2 CA, Schleicher and Schuell, Dassel, Germany), the concentration of thymidine was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

2.2.3.4. Determination of the influence of process time on yield and viability. Two batches of pellets were layered for 168 and 180 min, respectively with the marker substance in the 10% skim milk +5% inulin matrix. Samples were taken at 30 min interval and yield was determined as described above. Thereby, two batches of pellets were layered for 180 min with *L. lactis* Thy12 incorporated in the 10% skim milk +5% inulin matrix. Samples were taken at 30 min interval and viability was determined as described above.

2.2.3.5. Scanning electron microscopy. The morphology of the layer surface and the layer thickness of pellets layered for 30, 60, 90, 120, 150 and 180 min were examined by scanning electron microscopy (SEM) (Jeol JSM 5600 LV, Jeol, Tokyo, Japan). Pellets were radially sheared and platina coated using a sputter coater (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan). The layer thickness of five pellets was measured at minimum four sites per pellet.

2.3. Coating

2.3.1. Preparation of the coating dispersions

The composition of the coating dispersions containing Eudragit® FS 30 D and Eudragit® L30D-55 is shown in Table 1. For the preparation of the Eudragit® FS 30 D coating dispersion, a 30% (w/w) aqueous Eudragit® FS 30 D dispersion was used (Röhm, Darmstadt, Germany). Polysorbate 80 (wetting agent) (Tween® 80, Alpha pharma, Nazareth, Belgium) and glyceryl monostearate (glidant) (Federa, Braine-l'Alleud, Belgium) were added to water and stirred for 10 min with a high-speed mixer until a fine, homogenous dispersion was obtained. This dispersion was gently added to the Eudragit® FS 30 D dispersion and mixed by magnetic stirring. For the Eudragit® L30D-55 coating dispersions (A and B), the preparation was identical, except that triethyl citrate (Sigma-Aldrich, Bornem, Belgium) (20% on the dry polymer) was used as plasticiser. For the Eudragit® FS 30 D coating dispersions no plasticiser was needed since Eudragit® FS 30 D exhibits a minimum film-forming temperature (MFT) of 14 °C (contrary to Eudragit® L30D-55 (27 °C)). The difference between the two Eudragit® L30D-55 coating dispersions A and B is the polymer content: 15.3 and 22.8% (w/w), respectively. In order to avoid influence of process time, the polymer content in dispersion B was increased to reach a polymer weight increase on the layered pellets of 30% (w/w) in a process time comparable as to reach a polymer weight increase of 15% (w/w) (dispersion A).

2.3.2. Coating process

The coating dispersions were passed through a 0.3 mm sieve before use. Throughout the coating process the coating dispersions were stirred using a magnetic stirrer. 300 g of pellets were coated in a fluid bed coating apparatus (GPCG 1, Glatt, Binzen, Germany), used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; atomising pressure 1.5 bar). The spray rate was 4 g/min for the Eudragit® FS 30 D and Eudragit® L30D-55 (A) dispersion and 5.3 g/min for the Eudragit® L30D-55 (B) dispersion. For all coating experiments, the product temperature was 23–25 °C. Before coating, the pellets were preheated to the desired product temperature during coating. After coating, the pellets were standard cured in the apparatus for 15 min at the same conditions as the coating process. Then the pellets were packed in Alu sachets (LPS, Vapor flex barrier bag, New Jersey, US) sealed at 20% RH, cured for 2 or 5 days and subsequently stored at room temperature (RT) (23±2 °C), 8 or −20 °C. The pellets, layered for 30 min with the marker substance in the 10% skim milk +5% inulin matrix were coated with 15% (w/w) Eudragit® FS 30 D and 15 and 30% (w/w) Eudragit® L30D-55. The pellets, layered for 180 min with *L. lactis* Thy12 in the 10% skim milk +5% inulin matrix were coated with 15% (w/w) Eudragit® FS 30 D and 30% (w/w) Eudragit® L30D-55.

2.3.3. Evaluation of the coated pellets

2.3.3.1. Evaluation of the enteric properties after coating and storage. Dissolution testing ($n=3$) was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1,3 g pellets per vessel (250 ml) with HCl 0.1 N for 2 h. The concentration of thymidine was measured spectrophotometrically (Perkin Elmer, Zaventem, Belgium) at 267 nm.

2.3.3.2. Determination of viability of L. lactis Thy12 after coating and after passage through the gastric fluid stage. For the determination of the viability after coating, a dissolution test ($n=3$) was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1.3 g pellets per vessel (250 ml) with phosphate buffer (0.0125 M) pH 7.4 for 40 min. For the determination of the viability after passage through the gastric stage, first a dissolution test ($n=3$) was

Table 1
Composition of coating dispersions containing Eudragit® FS 30 D, Eudragit® L30D-55 (A and B)

	Eudragit® FS 30 D		Eudragit® L30D-55 (A)		Eudragit® L30D-55 (B)	
	Total (g)	Dry (g)	Total (g)	Dry (g)	Total (g)	Dry (g)
Eudragit® FS 30 D 30% (w/w) aq. disp.	55	16.5	–	–	–	–
Eudragit® L30D-55 30% (w/w) aq. disp.	–	–	51	15.3	51	15.3
Glyceryl monostearate	1.3	1.3	1.3	1.3	1.3	1.3
Tween® 80 (33% aq. sol.)	1.6	0.5	1.6	0.5	1.6	0.5
Triethyl citrate	–	–	3.2	3.2	3.2	3.2
Water	41.7	–	43	–	10	–
Polymer content (%, w/w)	16.4		15.3		22.8	
Solid content (%, w/w)	18.4		20.2		30.3	

performed using 1.3 g pellets per vessel (250 ml) with consequently HCl 0.1 N for 2 h and phosphate buffer (0.05 M) pH 7.4 for 40 min. Next, dissolution testing was performed in phosphate buffer (0.05 M) pH 7.4 for 40 min, without the previous gastric fluid stage. Then viability after gastric fluid stage was calculated as follows: viability after HCl 0.1 N (2 h) + phosphate buffer 0.05 M (40 min)/(viability after phosphate buffer 0.05 M (40 min) × 100.

3. Results and discussion

3.1. Preliminary experiments

In literature, it has been proposed that for the effective stabilisation, macromolecules should be incorporated in an amorphous, glassy matrix, which is characterized by a very high viscosity [11]. The macromolecules are embedded into a 'rocky candy' type of glassy state, inhibiting chemical and biochemical reactions since mobility/diffusion is of the order of micrometers per year. The amorphous structure is characterized by the glass transition temperature (T_g). When the amorphous material is exposed to a temperature above T_g, it will collapse, resulting in a rubbery state and loss of stabilising capacity. When the material is exposed to a temperature below T_g, it occurs in a glassy state. So, in order to ensure optimal stabilization capacity, the material should have a high T_g. Trehalose is mentioned as the golden standard for stabilisation of biomaterials during anhydrobiosis because of its high T_g, compared to other sugars (115 °C). Lactose however, also has a high T_g, (105 °C) but it shows a high tendency to crystallize, leading to loss of stabilizing power [12]. Next, Crowe et al. [11] concluded that direct interaction between trehalose and polar groups in proteins and phospholipids occurs in the dry state. This is referred to as the 'water-replacement' theory since the removed water is replaced by the sugar and is a second requirement for stabilization of macromolecules in dry state. Preliminary freeze-drying experiments confirmed the stabilising capacity of trehalose. Addition of 5% (w/v) trehalose in the 10% (w/v) skim milk matrix resulted in a viability of *L. lactis* MG1363 (10^{10} CFU/ml) after 1 week storage (RT, 10% RH) that was twice as high as obtained in the 10% (w/v) skim milk without additives ($n = 3$) (data not shown). As an alternative of the expensive trehalose, inulins having a high T_g were evaluated for their potential as stabilising matrix. Inulins are fructose oligomers with varying molecular weight. As the T_g of the inulin is related to its chain length, it offers the advantage that the T_g can be adjusted to 154.4 °C (Inulin EXL®). Hinrichs et al. showed that inulins with a DP_n/DP_w (degree of polymerization) above 5.5/6.0 meet the physicochemical characteristics to successfully act as a protectant for proteins [13]. They showed that 50% of the activity of alkaline phosphatase, freeze-dried in inulin EXL® was maintained after storage at 60 °C for 6 days whereas the activity of alkaline phosphatase in trehalose was completely lost. In a preliminary experiment, the stabilising capacity of inulin for *L. lactis* Thy12 was evaluated. *L. lactis* Thy12 (10^{10} cfu/ml) was freeze-dried in 10% skim milk (reference) and in 10% inulin and 10% milk + 5% inulin (alternative matrices) ($n = 3$). After freeze-drying, viability in the different matrices showed no significant difference (data not shown). After subsequent storage for 1 week at 8 °C/10% RH, the 10% skim milk matrix showed a better viability than the 10% inulin matrix, although inulin has the highest T_g (154.4 °C) (Fig. 1). This could be explained by the requirement of milk proteins for successful stabilisation and/or by the better stabilising capacity of lactose (disaccharide) than inulin (polysaccharide). However, addition of 5% inulin to the 10% milk matrix resulted in a significant increase in the stabilising capacity of the 10% milk matrix during storage of freeze-dried *L. lactis* Thy12 at 8 °C/10% RH (Fig. 1). This could be explained by the higher T_g of this matrix (80 °C) compared to that of 10% milk without inulin added (61 °C) and can be supported by the collapse of the freeze-dried 10% skim milk matrix after storage at RT and 60% RH, while the 10% milk + 5% inulin matrix remained an amorphous cake. It could be concluded that addition of 5% inulin to the 10% milk matrix significantly increased the stability of freeze-dried *L. lactis* Thy12 during storage ($P < 0.05$).

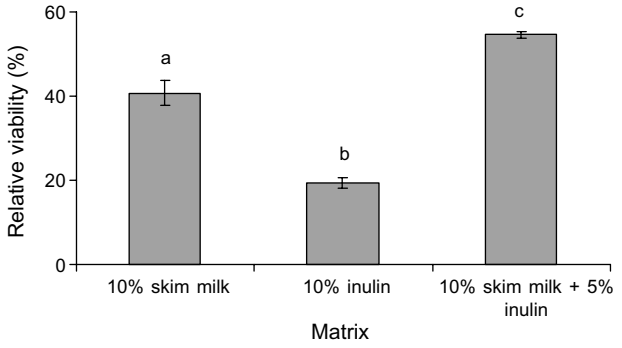

Fig. 1. Viability of *L. lactis* Thy12, freeze-dried in different matrices after 1 week storage (8 °C/10% RH) ($n=3$). (a–c) Groups within the same condition with the same superscript are not significantly different from each other ($P>0.05$) (one-way ANOVA, post hoc Scheffé).

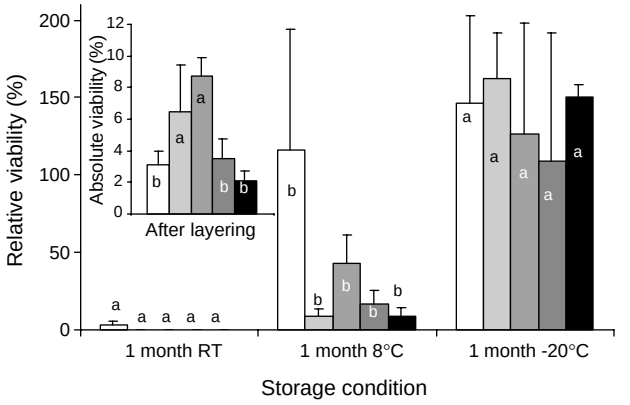

Fig. 2. Relative viability of *L. lactis* Thy12 after layering in different matrices and storage in Alu sachet (sealed at 20% RH) for 1 month at different conditions (RT, 8 and −20 °C). Insert, Absolute viability after layering (□10% skim milk, ▢ 10% inulin, ▢ 10% milk+5% inulin, ▢ 5% inulin, ν 2.5% inulin) ($n=3$) (a,b): Groups within the same condition with the same superscript are not significantly different from each other ($P>0.05$) (one-way ANOVA, post hoc Scheffé).

3.2. Optimisation of the layering matrix to increase viability after layering and storage

Previous experiments showed that, in order to increase the load of *L. lactis* Thy12 on an inert carrier, increasing the product temperature to 45 °C and the bacterial concentration in the layering solution from 10^9 to 10^{10} CFU/ml did not lead to a significant change in viability after layering and storage [9]. Therefore, further experiments were performed at 45 °C with a cell concentration in the layering solution of 10^{10} CFU/ml and a modified layering matrix in an attempt to increase viability after layering and storage. As a reference, 10% skim milk was used. Analogous to the preliminary freeze-drying experiments, 10% skim milk+5% inulin and 10% inulin were evaluated. Besides two other inulin concentrations (5 and 2.5%) were evaluated.

Comparison of the viability values in the different matrices revealed that the viability after layering in the 10% skim milk+5% inulin matrix was significantly higher than in the other matrices (Fig. 2). However, there was no significant difference with the 10% inulin matrix. After storage for 1 month at RT, viability dropped to zero in all matrices, except in the 10% milk matrix (3.3±2.5%) (Fig. 2). After storage for 1 month at 8 °C, no significant differences were observed between the matrices. However, 10% milk matrix tends to be the best performing matrix as viability remained quasi unchanged after 1 month. In the other matrices, the relative viability dropped below 45%. The lowest viability was seen in the pure inulin matrices.

From these data it can be concluded that skim milk is an essential compound for the stabilisation of bacteria during storage and that the addition of inulin results in a destabilising effect. As it is generally known that crystalline structures present in the amorphous matrix lead to destabilisation of the biomaterials included [11], the molecular arrangement of the inulin molecules was determined. However, no crystalline inulin could be identified in the layered matrix using Raman technology.

Independent of the matrix used, no significant differences were seen between the viability values after storage for 1 month at −20 °C. Remarkably, the average relative viability values observed after 1 month storage exceeded those determined immediately after the layering process. This apparent discrepancy could be related to our method of assessing viability (Bioscreen) which in essence measures turbidity of the culture and hence its capacity to multiply. It is conceivable that, although still viable during the layering process, the bacteria have temporarily lost their capacity to resume multiplication. Storage at −20 °C could then lead to restoration of the multiplying capacity.

Relying on the previous results, the viability of the three batches of *L. lactis* Thy12 layered in 10% skim milk matrix was evaluated in function of storage time in Alu sachets (filled and closed at 20% RH) at −20 °C (Fig. 3). For batch 1 and 2, viability did not significantly change after 8 and 9 months storage, respectively. For batch 3, viability decreased significantly after 12 months storage (60%). So, the viability profile as a function of time is variable form batch to batch.

However, the viability is adequately maintained in function of storage time. It can be concluded that layering of *L. lactis* Thy12 in the 10% skim milk matrix on inert pellets results in a valuable multi-particulate formulation of *L. lactis* Thy12 since it was designed for in situ production of a therapeutic (hIL-10). Hence, accurate dosing is a necessity and requires the guarantee of a reproducible viability, especially as a function of time. In comparison with the stability of freeze-dried *L. lactis* Thy12 in the same matrix (42% after 9 months storage in Alu sachets at −20° [14], it can be concluded that the layered *L. lactis* Thy12 showed a superior stability.

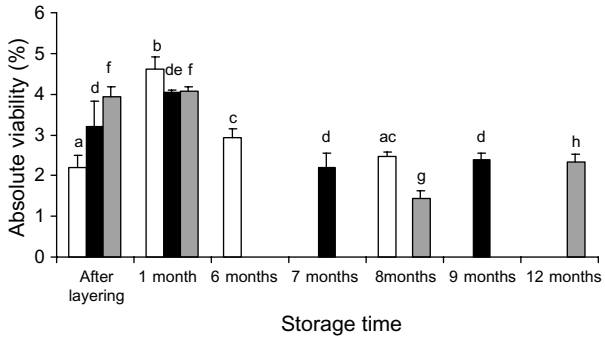

Fig. 3. Absolute viability of *L. lactis* Thy12 after layering in 10% skim milk matrices and storage in Alu sachet (sealed at 20% RH) in function of storage time at −20 °C. (□ Batch 1 (after layering, 1, 6 and 8 months), ■ Batch 2 (after layering, 1, 7 and 9 months), ▣ Batch 3 (after layering, 1, 8 and 12 months)) (a–h): Groups within the same series with the same superscript are not significantly different from each other ($P > 0.05$) (one-way ANOVA, post hoc Scheffé).

3.3. Determination of the yield and reproducibility of the layering process

As the 10% milk +5% inulin matrix resulted in the best viability immediately after layering, the process yield and the reproducibility (inter- and intra-batch variability) was determined using a marker substance (thymidine). Layering of the 10% milk +5% inulin matrix is a reproducible process as the inter- and intra batch variability was below 4.74 and 2.80%, respectively. Moreover, the process yield was high (91.99±4.74%, $n=3$). The loss of yield (∼8%) can be explained by spray drying, as the fluid bed container was not optimally filled.

3.4. Viability as function of process time

It was confirmed that the layering process could be run for at least 3 h without any technical problems e.g. sticking of the pellets and blocking of the nozzle. In order to detect any damage and subsequent pealing off of the matrix layer, the yield was determined after different process times 30, 60, 90, 120, 150 and 180 min for two batches. Fig. 4 indicates that the yield remained constant during the entire layering process.

The layer thickness of the layered pellets was measured in function of process time (SEM) (data not shown). There was a linear increase of the layer thickness as a function of process time (layer thickness = 0.299 × process time + 0.116, $r^2 = 0.998$), indicating a good layer consistence and no pealing off of the matrix layer during the process. The surface appeared smooth and showed very few superficial cracks (Fig. 5). This is in contrast to the surface of pellets layered at 30 °C looking rough and showing deep cracks, probably due to the high T_g of inulin.

The viability of *L. lactis* was also followed as a function of the process time. Fig. 6 shows the viability obtained in two batches after 30 min layering (29 and 27%).

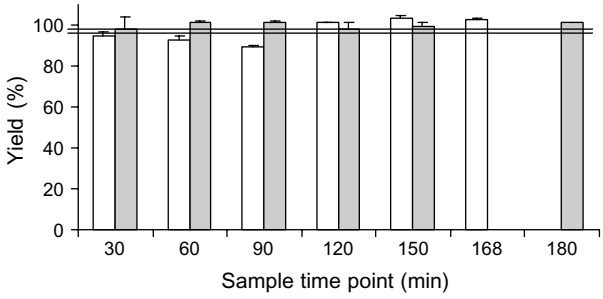

Fig. 4. Layering yield (%) of 2 batches in function of the process time using 10% milk+5% inulin matrix and thymidine as marker substance. The horizontal lines indicate the mean yield for the total process time (□ Batch 1, ▣ Batch 2).

The viability slightly decreased after 60 min, but proceeding the process for another 2 h did not significantly affect the viability. Probably, an equilibrium was reached around 60 min process time: the death rate of newly layered bacteria is constant and once the bacteria are dried on the surface of the inert carriers, they maintain viable.

3.5. Enteric properties of a coated, layered pellet formulation and influence of curing and storage conditions on enteric properties

It is generally accepted (European Pharmacopoeia) that release from an enteric-coated formulation in HCl 0.1N after 2 h may not exceed 10%. In this study, a lower limit of 3.5% was set forward, as a minimum of acid penetration through the coat is prerequisite for maintaining the viability of the acid-sensitive *L. lactis*. The pellets were coated with two different coating polymers i.e. Eudragit® L30D-55 and Eudragit® FS 30 D. Next to protection against the detrimental gastric fluid and bile salts, this coating polymers

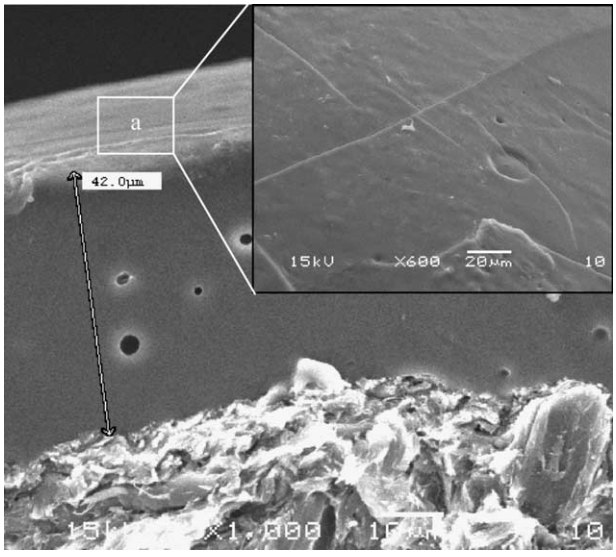

Fig. 5. Cross section of a Cellet® layered for 150 min with a 10% milk+5% inulin matrix: layer thickness. Insert (a): surface of the layer.

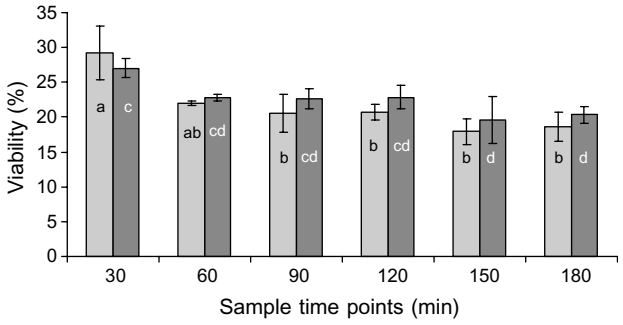

Fig. 6. Viability (%) of *L. lactis* Thy12, layered in 10%milk+5%inulin matrix in function of process time. (a–d): Groups within the same series with the same superscript are not significantly different from each other ($P>0.05$) (one-way ANOVA) (□ Batch 1, ■ Batch 2).

are chosen for their ileum targeting properties in relation to Crohn's disease treatment. A previous study indicated that none of the available coating polymers ensured ileal targeting. This could be circumvented by administration of the combination of one pellet fraction coated with Eudragit® L30D-55 while another was coated with Eudragit® FS30D ensuring ileum release in patients with low and high ileum pH profiles, respectively [15].

Fig. 7 reveals that from pellets, coated with 15% (w/w) Eudragit® L30D-55, release was above the limit of 3.5%, even after curing for longer periods (5 days) and at higher temperature (60 °C). Increasing the amount of polymer applied to the layered pellet (30%, w/w) resulted in a release in HCl 0.1 N after 2 h below 3.5%, already after curing for 2 days at −20 °C. For the Eudragit® FS 30 D polymer, 15% (w/w) weight increase on the pellets was sufficient to result in a release in HCl 0.1 N after 2 h below 2%. Generally, it can be concluded that Eudragit® FS 30 D is a less permeable polymer than Eudragit® L30D-55 as only 15% weight increase is required to obtain the desired enteric properties, contrary to 30% of Eudragit® L30D-55. Fig. 7 also shows

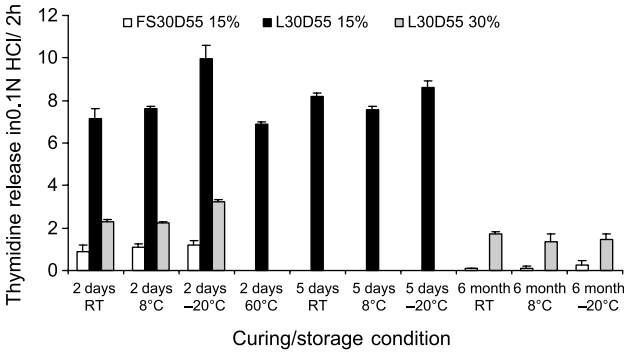

Fig. 7. Release in HCl 0.1 N after 2 h from pellets, layered with 10% milk+5% inulin matrix and coated with 15% (w/w) Eudragit® FS 30 D (□), 15 (■) and 30% (□) (w/w) Eudragit® L30D-55, cured and stored for different times and conditions.

that after storage for 6 months, the release remained low at all conditions for both Eudragit® FS 30 D (15%) and Eudragit® L30D-55 (30%) coated pellets, indicating their maintenance of the enteric properties. It can be concluded that the coating compositions used are convenient for storage at low temperature (−20 °C) and low relative humidity (20% RH), the best storage conditions in terms of *L. lactis'* Thy12 viability.

3.6. Viability of L. lactis Thy12 after coating and after passage through the gastric stage

The two batches, layered for 180 min with *L. lactis* Thy12 (10^{10} cfu/ml) in 10% milk+5% inulin matrix were coated with 30% (w/w) Eudragit® L30D-55 and 15% (w/w) Eudragit® FS 30 D, respectively. Viability in the coated pellets was determined by first performing a dissolution test to ensure release of the bacteria from the coated pellets, followed by a viability test on the dissolution medium in which the bacteria have been released. These data revealed a serious drop in viability in the Eudragit® L30D-55 coated pellets (from 11.4% in the uncoated pellets to 3.8% in the coated pellets), contrary to Eudragit® FS 30 D (17.33±1.13% in the uncoated pellets to 18.36±0.13% in the coated pellets). As the pH of the coating solutions (2.5) and the coating conditions (process time and coating temperature) were similar for both polymers, it can be concluded that the drop in viability after coating with Eudragit® L30D-55 can not be explained by a detrimental effect of coating solution or process. Probably, the coating compounds, released during the dissolution test have a detrimental effect on the *L. lactis'* Thy12 viability. In comparison to the Eudragit® FS 30 D coated pellets, a same quantity of Eudragit® L30D-55 coated pellets contains twice as much polymer and Tween® 80. From previous experiments it is known that Tween® 80 influences the growth of *L. lactis* at a concentration of 50 mg/ml. In this dissolution test, Tween® 80 is only present in a concentration of 0.052 mg/ml. However, a local exposure to higher concentrations immediately after dissolution could have a detrimental effect.

After simulation of the gastric transit (HCl 0.1 N for 2 h) of the Eudragit® FS 30 D coated pellets, about 85% of the bacteria remained intact, indicating the good protective capacity of the polymer against the detrimental gastric fluid. However, after passage through the gastric stage of the Eudragit® L30D-55 coated pellets, only 5% of the bacteria remained viable. It can be concluded that this polymer is much more permeable (cf. above), in spite of the higher coat thickness used (30% weight increase). This polymer is therefore less appropriate for protection of *L. lactis* against the detrimental gastric fluid.

The pellets coated with 15% (w/w) Eudragit® FS 30 D, packed in Alu sachets and sealed at 20% RH, were stored for

8 months at −20 °C. The viability decreased significantly from 18.5 ± 1.1% after layering to 14.8 ± 0.2% after 8 months storage. However, still 80% of *L. lactis* Thy12 remained viable in the enteric-coated pellets during this storage period.

4. Conclusion

In this study, some new aspects of the layering technique, in order to produce an enteric-coated multi-particulate formulation of *L. lactis* Thy12 were shown. First, it can be concluded that the process could be performed for long times without encountering technical problems, with good layer consistence and with maintenance of viability. This broadens the applicability of the technique. Next, the superior stability of layered *L. lactis* Thy12 in comparison with freeze-dried *L. lactis* Thy12 has been shown, based on long term stability data. Besides, coating of the layered multi-particulate formulation with 15% Eudragit® FS30D resulted in good enteric properties: 85% of the bacteria remained viable in Eudragit® FS30D coated pellets after passage through the gastric stage. Moreover, after a 8 month storage period (−20 °C), 80% of *L. lactis* Thy12 remained viable in the Eudragit® FS30D coated pellets. However, the load of *L. lactis* Thy12 on the inert pellets should be further increased in order to reach acceptable dose quantities. Besides, since it has been shown that the Eudragit® L30D-55 polymer, contrary to Eudragit® FS30D, is not acceptable to provide protection against the detrimental fluid, this coating has to be further optimised.

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