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Research paper

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

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Abstract

Layering of pellets with recombinant *Lactococcus lactis* Thy 12 was optimised for the production of a dosage form with a high load of viable recombinant *L. lactis*. Shear stress induced during the atomisation and the type of carrier used for the layering process did not influence the viability. A 5% lactose matrix resulted in the highest viability of *L. lactis* ($8.9 \pm 1.7\%$) which could be maintained for at least 12 months at −20 °C. A higher bacterial cell load on the pellets was obtained using a longer process time, but the addition of 10% skim milk was essential to maintain the stabilising capacity of the matrix. Increasing the load of viable *L. lactis* was also possible using a higher bacterial cell concentration of the layering suspension and increasing the amount of stabilising matrix to 10% lactose/20% skim milk, yielding a formulation with 1.7×10^9 cfu/100 mg pellets. To protect the bacteria during gastric passage and to obtain ileum targeting, the formulation was enteric coated with 5% Eudragit® FS30D, but after coating and gastric residence for 2 h HCl about 1% of the bacteria remained viable. Application of a subcoating, previous to enteric coating, did not result in a higher viability.

Keywords: Layering; Viability; Recombinant *Lactococcus lactis*; Ileum targeting; Pellets; Eudragit; Lactose; Skim milk

1. Introduction

In the last decade there has been an increasing interest in the use of recombinant lactic acid bacteria (LAB) as intestinal mucosal delivery systems for vaccines and biotherapeutics [1]. Although freeze- and spray-drying can be used for the development of a stable formulation of viable bacteria, both the techniques have several disadvantages. Freeze-drying is an expensive and time-consuming process, while spray-drying can be very detrimental for the bacteria due to the higher process temperature. Moreover, both the techniques result in a powder that must be processed into a pharmaceutical formulation. Layering of multi-particulates (spheres of 1 mm Ø) with bacteria suspended in a stabilising matrix was presented as a valuable alternative for the production of a viable *Lactococcus lactis* dosage form [2], offering several advantages: (a) layering and enteric coating (required due to the sensitivity of *L. lactis* to gastric fluids [3]) can be performed in the same equipment; (b) the small particle size of the pellets assures a fast gastric emptying of the formulation, limiting the contact time with the gastric environment; (c) ease of administration since the small particles can be easily swallowed or can be mixed with food. However, the bacterial cell load of these layered pellets was too low: 36.5 g pellets were required to deliver a single dose of 10^{11} cfu of *L. lactis* Thy 12, a genetically modified strain producing IL-10 which can be used as a therapeutic in the treatment of Crohn's disease [4] and which has already been used in an in vivo study [5]. Hence, the aim of this study was to enhance the viability and bacterial cell load of *L. lactis* Thy 12 on the layered pellets via the optimisation of several parameters. The influence of process parameters (atomising pressure and nozzle diameter) and carrier material (microcrystalline cellulose pellets or

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starch-based pellets) on the viability of *L. lactis* Thy 12 during the layering was evaluated. Different stabilising matrices (skim milk, disaccharides, polysaccharides and polyalcohols) were tested for their stabilising capacities and a 1-year stability study was performed. To further increase the amount of viable *L. lactis* Thy 12 on the pellets, the process time and the bacterial cell load in the layering suspension were increased.

In addition, the gastric resistance of the layered bacteria was improved using an enteric polymer in combination with different subcoatings. As Crohn's disease is mostly occurring in the ileum part of the intestine, the ileum targeting capacity of the used coating polymer was assessed.

2. Materials and methods

2.1. Materials

Growth media M 17 (Difco) and GM17T (i.e. M17 supplemented with 0.5% glucose and 50 μg/ml thymidine) were purchased from Becton Dickinson (MA, USA). Different stabilising matrices were used: skim milk (Difco, Becton Dickinson, MA, USA), lactose (Alpha Pharma, Braine-l' Alleud, Belgium), trehalose (Cerestar, Mechelen, Belgium), sucrose (Federa, Brussels, Belgium), isomalt (Cerestar, Mechelen, Belgium), mannitol (Certa, Braine-l' Alleud, Belgium), calcium caseinate (Miprodan®) (Arla Foods Ingredients, Videbaeck, Denmark), maltodextrin DE 5 (Glucidex 2, Roquette Frères, Lestrem, France), DE 18 and DE 38 (Cerestar, Mechelen, Belgium), and glucose (Bufa, Uitgeest, The Netherlands). Microcrystalline cellulose spheres (Cellets®, Pharmatrans-Sanaq, Basel, Switzerland) and starch pellets were used as inert carriers for the layering experiments. Starch pellets consisted of 7.0% hydroxypropylmethylcellulose (Methocel® E15 Colorcon, Dartford, UK), 10.1% sorbitol (Cerestar, Mechelen, Belgium) and 82.9% starch (Uni-Pure® EX starch, National Starch and Chemical Company, NJ, USA).

As coating polymer, a 30% (w/w) aqueous Eudragit® FS30D dispersion (Röhm, Darmstadt, Germany) was used in combination with polysorbate 80 (wetting agent) (Tween 80, Alpha Pharma, Nazareth, Belgium) and glycerol monostearate (glidant) (Federa, Braine-l' Alleud, Belgium). HPMC-based (Opadry®) (Colorcon, Kent, UK) or PVA-based coating (Opadry® II and Opadry® AMB) (Colorcon, Kent, UK) were used as subcoatings before enteric coating.

2.2. Bacterial strain

Lactococcus lactis Thy 12 (human IL-10 producing *L. lactis* MG1363 derivate) was obtained from the Department of Molecular Biomedical Research (Ghent University, Belgium). *L. lactis* is a non-pathogenic, non-invasive, gram-positive lactic acid bacteria generally used to produce fermented dairy products. This strain of *L. lactis* was genetically modified in order to produce human IL-10 and was made thymidine dependent to avoid spreading of the bacteria in the environment [6]. The bacteria were stored at −20 °C as a stock suspension in glycerol/GM17T (50/50).

2.3. Preparation of the layering suspensions

Lactococcus lactis Thy 12 cultures were prepared by inoculating the stock suspension 1/1000 in GM17T growth medium. The culture was grown for 16 h at 30 °C to reach the stationary phase (2–3 × 10^9 cfu/ml) (ON culture). The bacteria were collected by centrifugation at 3000*g* for 20 min at 4 °C. The cell pellet was resuspended at 2–3 × 10^{10}, 6–9 × 10^{10} or 1–1.5 × 10^{11} cfu/ml (respectively, 10-, 30- and 50-fold concentrated compared to ON culture) in different layering suspensions (Table 1) To prevent further activity or growth, the cultures were kept on ice in between all the handling steps.

2.4. Evaluation of the influence of atomising pressure and nozzle diameter on viability

A layering suspension consisting of *L. lactis* Thy 12 (2–3 × 10^{10} cfu/ml) in 10% skim milk was atomised at different pressures (0.5, 1.5 and 2 bar) and through different nozzles (Ø 0.8 and 1.2 mm) using a fluid-bed (GPCG 1, Glatt, Binzen, Germany). The atomised suspensions were collected and analysed for viability. Viability of the bacteria was determined by monitoring the growth of the standards and the samples in a Bioscreen (Labsystem, Helsinki, Finland). The viability of the overnight culture was set as 100% value. Different dilutions of the starting culture were

Table 1
Different layering suspensions

Stabilising matrix		Concentration
Influence of stabilising matrix		
Skim milk	Skim milk and components	10%
Lactose		5%
Ca-caseinate		3.8%
Lactose/ Ca-caseinate		5/ 3.8%
Trehalose	Disaccharides	5%
Sucrose		5%
Isomalt		5%
Mannitol	Polyalcohol	5%
Maltodextrin DE 5	Polysaccharides	5%
Maltodextrin DE 18		5%
Maltodextrin DE 38		5%
Glucose		5%
Influence of stabiliser/ bacteria ratio		
Skim milk		5% - 10% - 20%
Lactose		2.5% - 5% - 10%
Trehalose		2.5% - 5% - 10%
Increasing process time		
Lactose		5%
Lactose/ maltodextrin DE 5		5/ 2.5% - 5/ 5%
Lactose/ skim milk		5/ 10%
Increasing bacterial cell concentration in layering suspension		
Lactose/ skim milk		5/ 10% - 10/ 20%

made, inoculated 1/100 in fresh GM17T and loaded in triplicate into microtiter plates for analysis in the Bioscreen. The growth at 30 °C was followed for 21 h. Based on the exponential growth phase, 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. This 50% time was plotted against the natural logarithm of the viability to calculate the equation of the standard curve. The viability of a sample was determined based on the standard curve of the starting culture and expressed as % of the viability of the overnight culture. Three dilutions of each sample were loaded in duplicate into microtiter plates for analysis in the Bioscreen. The viability values were statistically evaluated with a one-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 the variances by means of a Levene test. A multi-comparison among pairs of means was performed using a Scheffé test with $p < 0.05$ as a significance level. All the analyses were performed with SPSS 12.0 for Windows.

2.5. Layering process

Microcrystalline cellulose spheres (Cellets®) and starch pellets were used as inert carriers. Equal amounts of Cellets® 700 (700–1000 μm) and Cellets® 1000 (1000–1250 μm) were mixed to obtain 300 g pellets. Starch pellets (900–1400 μm) were produced by extrusion/spheronisation [7]. 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 3 g/min, product temperature 45 °C, air velocity 7–8 m/s, process time 30 min, atomising pressure 1.5 or 2 bar, inlet air relative humidity 20%). During layering, the layering suspension was kept on ice. After layering, 0.1 g pellets were vortexed for 30 s in 1 ml sterile water and analysed for viability via the Bioscreen turbidity method. A stability study was performed for the 10% skim milk and the 5% trehalose, lactose, sucrose and isomalt matrices. The pellets were packed in Alu sachets (LPS, Vapor flex barrier bag, NJ, USA) and sealed at 20% RH. The sachets were stored for 12 months at 8 and −20 °C, and viability was assessed after 1, 3, 6 and 12 months.

To predict the sensitivity of the bacteria to a longer process time, the pellets layered for 30 min were incubated for 2 h at 45 °C and a relative humidity of 20%. The pellets were evaluated for viability immediately after layering and after a storage time of 30, 60, 90 and 120 min.

2.6. Enteric coating and subcoating of the layered pellets

Layered pellets were enteric coated with Eudragit® FS30D (aqueous dispersion of an anionic copolymer based on methyl acrylate, methyl methacrylate and methacrylic acid) or subcoated with an HPMC-based (Opadry®) or PVA-based coating (Opadry® II and Opadry® AMB) before enteric coating (Fig. 1).

For the preparation of the Eudragit® FS30D coating dispersion polysorbate 80 and glycerol monostearate were added to water and stirred for 10 min with a high-shear mixer until a fine, homogenous dispersion was obtained. This dispersion was gently added to the Eudragit® FS30D dispersion and mixed using magnetic stirrer. The coating dispersion was passed through a 0.3 mm sieve before use. Throughout the coating process, the coating dispersions were stirred. Three hundred grams 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 Ø 0.8 mm). Before coating, the pellets were preheated to the required product temperature. Pellets were enteric coated with Eudragit® FS30D until a polymer weight gain of 5%, 10% or 15% was reached. After coating, the pellets were packed in Alu sachets sealed at 20% RH and stored for minimum 2 days at −20 °C. To prepare the subcoating dispersions, the powders were gently added to water using a magnetic stirrer, until a 10% dispersion was achieved for Opadry® and a 15% dispersion for Opadry® II and Opadry® AMB. Pellets layered with *L. lactis* Thy 12 in 10% lactose/20% skim milk (30 min) were subcoated with 4% Opadry®, Opadry® II and Opadry® AMB prior to enteric coating. The coating and subcoating parameters are shown in Table 2.

2.6.1. Evaluation of the pellets

Viability of the bacteria after layering, subcoating and enteric coating was determined by the Bioscreen turbidity method. For the layered and subcoated pellets, 0.1 g pellets were vortexed for 30 s in 1 ml sterile water. To evaluate the viability after enteric coating, the bacteria were removed from Eudragit® FS30D coated pellets by stirring 1 g pellets for 1 h in 250 ml 0.0125 M phosphate buffer pH 7.4 at 37 °C. A 0.0125 M phosphate buffer was used because a strong phosphate buffer had a detrimental effect on the viability of the bacteria. To evaluate the viability after gastric passage of the enteric coated pellets, a dissolution test was performed using the reciprocating cylinder method (Bio-Dis) with 1 g pellets per vessel of 250 ml during 2 h in 0.1 N HCl, followed by stirring of the pellets in 250 ml 0.025 M phosphate buffer pH 7.4 at 37 °C on a magnetic stirrer until the coating was completely dissolved (visual inspection) and the bacteria released. In this case, a non-toxic 0.0125 M phosphate buffer could not be used as it was not able to dissolve the enteric coating after 2 h contact with 0.1 N HCl. However, the measured viability was corrected for the loss of bacteria due to the toxic effect of a 0.025 M phosphate buffer. Some samples were evaluated for viability using the plating-out method. Via the plate pour method a dilution series of the samples was plated out using GM17T/agar as growth medium and after 48 h incubation at 30 °C, the colony forming units were counted.

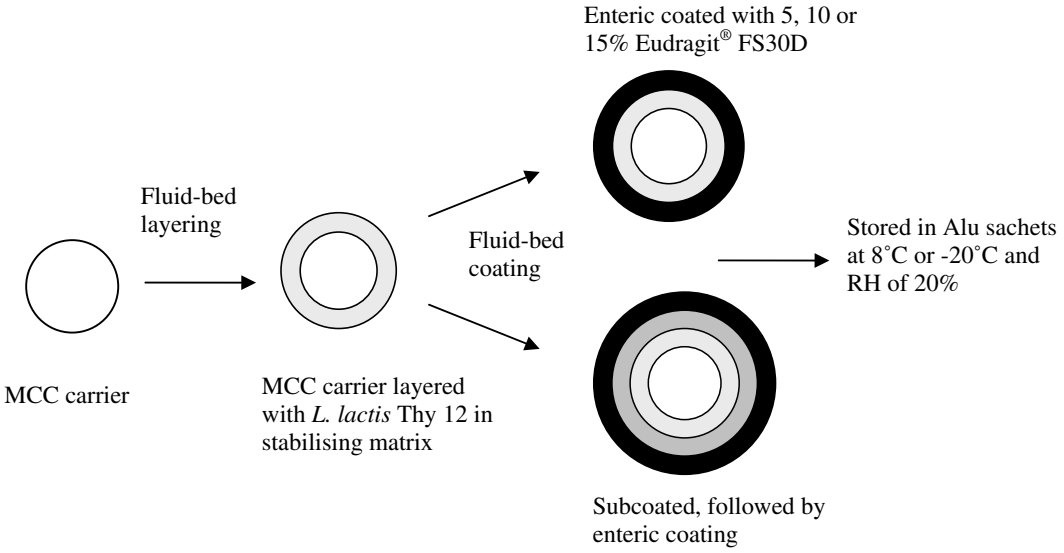

Fig. 1. Flow-chart layering, subcoating, coating and storage of the pellets.

Table 2
Process parameters for enteric- and subcoating

	Eudragit® FS30D	Opadry®	Opadry® II	Opadry® AMB
Spray rate (g/min)	3	3	3	2
Atomising pressure (bar)	1.5	2	2	2
Velocity of air (m/s)	7–8	7–8	7–8	7–8
Product temperature (°C)	25–30	45	45	45

2.7. Ileum targeting

2.7.1. Production of thymidine pellets

To evaluate the impact of coating thickness of Eudragit® FS30D on the release profile from the pellets, thymidine was incorporated in pellets (1%) prepared by extrusion/spheronisation because of its easy quantification in comparison to the quantification of *L. lactis*. Moreover, because of its pH-independent release it has excellent properties for adequate evaluation of enteric properties of the pellets. Thymidine (3.5 g) (Alkemi, Lokeren, Belgium) and microcrystalline cellulose (346.5 g) were preblended and granulated with 350 ml demineralised water in a planetary mixer (Kenwood Major Classic, Hampshire, UK). Extrusion was performed in a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal, Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. The extrudate was spheronized on a spheronizer (Caleva model 15, Sturminster Newton, UK), using a cross-hatched friction plate, operating at 1000 rpm with a residence time of 5 min. The pellets were dried for 12 h in an oven at 40 °C. The 700–1250 μm fraction was separated by sieving.

2.7.2. Enteric coating

Pellets were enteric coated with Eudragit® FS30D until a polymer weight gain of 5% or 15% was reached. After coating, the pellets were packed in Alu sachets (LPS, Vapor flex barrier bag, NJ, USA) sealed at 20% RH and stored for minimum 2 days at −20 °C.

2.7.3. Evaluation of the coated pellets

Dissolution testing was performed using the reciprocating cylinder method (USP apparatus 3) (Bio-Dis, Vankel, NJ, USA) at a dip rate of 21 dpm using 1 g pellets per vessel (250 ml) with two consecutive media: 0.1 N HCl (2 h) and consequently a buffer solution (phosphate buffer 0.05 M) at pH 6.8, 7.0 or 7.4. The concentration of thymidine was measured spectrophotometrically (UV-1650PC, Shimadzu, Antwerp, Belgium) at 267 nm.

3. Results and discussion

3.1. Influence of atomising pressure, nozzle diameter, inert carrier and stabilising matrix

The viability of *L. lactis* Thy 12 after atomisation at different pressures through different nozzles was not significantly different from 100% at every tested condition. It can be concluded that the shear stress caused by atomisation did not influence the viability of *L. lactis* Thy 12. Also, the type of inert carrier had no influence on the viability of *L. lactis* after layering as there was no significant difference in viability between the MCC pellets and the starch pellets using a 5% trehalose or lactose matrix, $6.7 \pm 1.3\%$ and $6.3 \pm 1.1\%$, respectively, for trehalose and $8.9 \pm 1.7\%$ and $7.8 \pm 1.6\%$ for lactose.

Since the effect of stabilisers on the viability depended on the bacterial strain [8], several polyols (disaccharides, polyalcohols, polysaccharides) and a skim milk medium (10% w/v; consisting of 5% lactose, 3.8% proteins (mainly Ca-caseinate), 0.1% fat, minerals and vitamins in water) were evaluated for their stabilising capacity of *L. lactis* Thy 12 during the layering on inert MCC carriers.

The viability of *L. lactis* Thy 12 after layering in a 10% (w/v) skim milk suspension was 3.1 ± 1.2%. To evaluate the stabilising capacity of each individual milk component, *L. lactis* Thy 12 was layered in a 5% lactose and 3.8% Ca-caseinate suspension, resulting in a significantly higher (8.9 ± 1.7%) and lower viability (0.7 ± 0.2%), respectively. A better stabilisation of bacteria using lactose alone instead of skim milk was also found during freeze-drying by Costa et al. [9]. Probably Ca-caseinate, due to its large molecular weight, inhibited direct interaction of lactose with bacterial membranes. Small molecules, like disaccharides, can replace the water molecules surrounding the phospholipids groups of the bacterial membranes during drying, preventing collapse of the membrane [10]. The negative influence of Ca-caseinate on the stabilising capacity of lactose after layering was confirmed by adding 3.8% Ca-caseinate to a 5% lactose solution which resulted in a significant decrease of viability (0.3 ± 0.1%).

Comparison of the viability in matrices containing other disaccharides revealed that the viability of *L. lactis* suspended in a 5% trehalose, isomalt and sucrose matrix was not significantly different from the 5% lactose matrix (Fig. 2). However, the isomalt and sucrose matrices resulted in sticking problems during layering. Trehalose, known as the golden standard for stabilisation during anhydrobiosis [11], did not result in a better stabilisation after layering. Although Efiuvwevwere et al. [12] reported a high viability after drying of *L. lactis* using mannitol as stabilizer, this was not confirmed in our study since layering in a 5% mannitol matrix did not provide protection, probably due to complete crystallization of the mannitol.

Layering of *L. lactis* in a solution of maltodextrins (polysaccharide) with a higher DE (i.e. higher degree of hydrolysis), resulted in a significantly higher viability compared to the lower DE maltodextrins (Fig. 2). However, the viability was much lower compared to disaccharide matrices. It was concluded that the incorporation of *L. lactis* in an amorphous maltodextrin matrix was not sufficient for stabilisation during fluid-bed layering. The higher molecular weight maltodextrins are probably not able to directly interact with the membranes of the bacteria because of steric hindrance [13]. Completely hydrolysed starch (i.e. 5% glucose) resulted in a significantly lower viability of 1.38 ± 0.59%, compared to the MD DE 38 (T_g 67–73 °C), probably because of its low T_g (21–39 °C). The glass transition temperature (T_g) characterizes amorphous materials. At a temperature under the T_g the material is in a glassy state of very high viscosity, inhibiting chemical and biochemical reactions as the mobility in this state is very low. At a storage temperature above the T_g, the material will enter a rubbery state with a lower viscosity and loss of stabilising capacity. Moreover, layering of a glucose solution resulted in sticking problems.

To evaluate the influence of the stabilizer/bacteria ratio on the viability of *L. lactis* the concentrations of skim milk, lactose and trehalose in the layering matrix were varied. Varying the skim milk concentration from 5% to 20% did not result in a significant difference in viability after layering (Fig. 3). For the trehalose and lactose matrix a higher stabilizer/bacteria ratio resulted in a higher viability, but no significant difference was observed between 5% and 10%.

To assure an accurate dosing of these therapeutic IL-10 producing bacteria, a reproducible viability of *L. lactis* is required. Therefore, it was evaluated if the viability could be maintained during storage. The pellets layered with 10% skim milk, 5% lactose, trehalose, isomalt and sucrose were stored at low relative humidity (20%) and a temperature of 8 or −20 °C (Fig. 4). Whereas storage for 1 month at 8 °C already resulted in a significant decrease in viability (except for the 5% sucrose matrix), there was no significant decrease in viability of all matrices after 12 months storage at −20 °C (except for the 5% isomalt matrix).

3.2. Increasing the bacterial cell load on the pellets

From the above shown data, it is clear that the bacterial viability in the layered pellets was low (max. about 10%) independent of the matrix. To increase the load of viable bacteria on the pellets a longer process time and a higher bacterial load in the layering suspension were evaluated

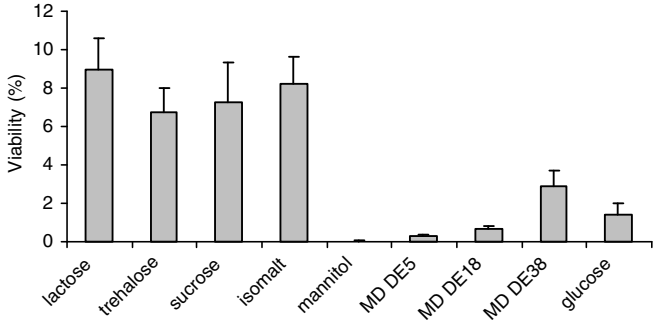

Fig. 2. Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in different matrices (5%) ($n = 3$).

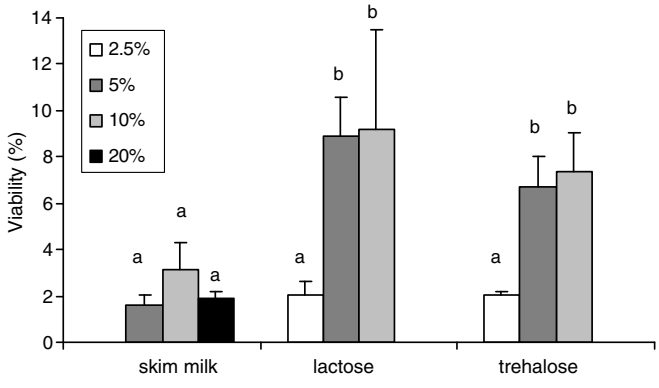

Fig. 3. Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in different matrices at different concentrations ($n = 3$). (a–c) Groups with the same superscript within the same stabilising matrix are not significantly different ($p > 0.05$) (one-way ANOVA, post hoc Scheffé).

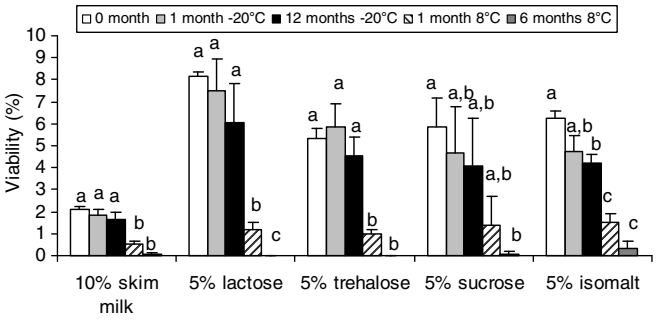

Fig. 4. Viability (mean ± SD) of *L. lactis* Thy 12 after layering and after 1, 3, 6 and 12 months storage in Alu sachet (sealed at 20% RH) at 8 or −20 °C. (a–c) Groups with the same superscript within the same stabilising matrix are not significantly different ($p > 0.05$) (one-way ANOVA, post hoc Scheffé).

for a formulation containing 5% lactose. Increasing the process time from 30 to 120 min reduced viability from 9.6 ± 1.7% after 30 min to 2.9 ± 0.9% after 120 min. Moreover, the stickiness of the pellets occurred after 1 h processing. These observations are probably due to crystallization of lactose during the layering process, damaging the bacterial cell membrane. To inhibit lactose crystallization, different stabilizers were added to the lactose matrix (maltodextrins and skim milk). To evaluate which components could inhibit lactose crystallization, a 2 h process time was simulated by layering the pellets for 30 min followed by a 2 h incubation period at 45 °C (= process temperature). The incubation of layered pellets during 2 h significantly decreased the viability (2.8 ± 0.9% after 120 min) (Fig. 5). However, this confirmed that incubation can be used as predictive tool since a similar viability was determined after 2 h processing in a fluid-bed (2.9 ± 0.9%). Adding 5% MD DE 5 to the 5% lactose solution resulted in a significantly lower viability after 30 min layering (2.5 ± 0.7%), although the viability was maintained during 2 h incubation. Lowering the MD DE 5 concentration to 2.5% increased the initial viability (6.7 ± 1.1%), but this higher value could not be maintained during incubation at 45 °C. A combination of 5% lactose and 10% skim milk resulted in a viability of 7.8 ± 1.2% immediately after layering, which was not significantly different from the viability of the 5% lactose matrix alone, but significantly different compared to a 10% skim milk matrix (3.1 ± 1.2%) as the higher lactose/protein ratio allowed more interaction between lactose and bacterial membranes. Furthermore, there was no significant decrease in viability after 2 h incubation at 45 °C. Jouppila and Roos [14] proposed a hypothesis that the proteins in skim milk protected lactose against crystallization.

To confirm these predictive results, the 5% lactose/10% skim milk matrix was selected for a 2 h layering process resulting in a viability of 10.9 ± 1.6% after 2 h. This relative viability corresponds with an absolute number of 3.6×10^8 cfu/100 mg pellets. No technical problems were encountered during the process.

To further increase the bacterial cell load on the pellets a layering suspension with a higher concentration of bacteria was used. 5% lactose/10% skim milk was used as stabilising matrix when the cell concentration in the layering suspension was increased from 2–3 × 10^{10} (10-fold concentration of overnight culture) to 1–1.5× 10^{11} cfu/ml (50-fold concentration of overnight culture). The viability dropped significantly (from 7.8 ± 1.2% to 1.6 ± 0.3%) when the layering suspension was more concentrated (Fig. 6). However, when the concentration of stabilising matrix was increased (10% lactose/20% skim milk) the viability of the concentrated suspension could be maintained during processing: 13.5 ± 3.6% and 11.3 ± 4.1% for a 10- and 50-fold concentrated suspension, respectively. This 10% lactose/20% skim milk matrix was also suitable for a 2 h layering process, as a 30× concentrated suspension yielded a viability of 13.9 ± 1.7%. Via this approach the number of viable *L. lactis* on the pellets could be increased as this

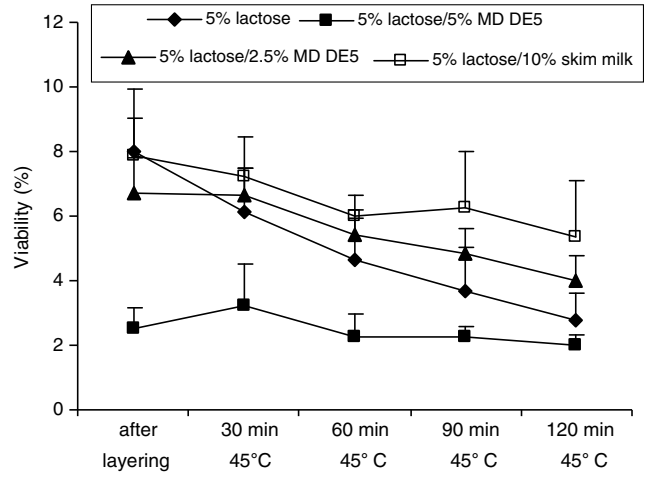

Fig. 5. Viability (mean ± SD) of *L. lactis*Thy 12 after layering and a consequent incubation of 30, 60, 90 and 120 min in Alu sachet (sealed at 20% RH) at 45 °C ($n = 3$).

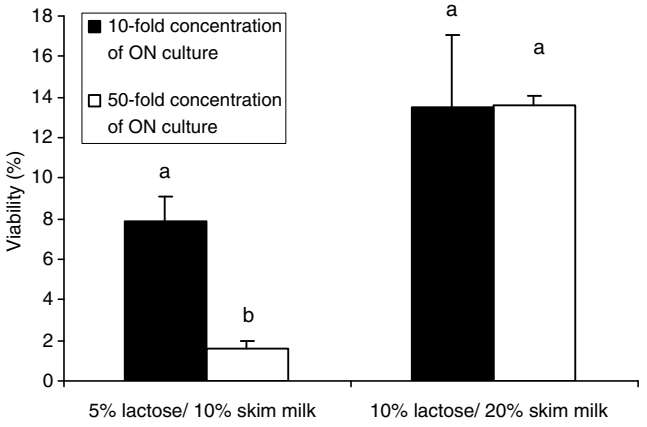

Fig. 6. Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in the function of bacterial concentration and ratio stabilising matrix/bacteria ($n = 3$). (a and b) Groups with the same superscript within the same stabilising matrix are not significantly different ($p > 0.05$) (one-way ANOVA, post hoc Scheffé).

relative viability corresponds with an absolute number of viable bacteria of 1.7×10^9 cfu/100 mg pellets. To administer a dose of 10^{11} cfu, an acceptable amount 6 g pellets should be taken. After 12 months storage of these pellets at −20 °C no significant decrease in viability was observed.

3.3. Enteric coating of the layered pellets

MCC pellets were layered for 30 min with *L. lactis* Thy 12 suspended in a 10% lactose/20% skim milk suspension (using a 10-fold concentration of *L. lactis* compared to the overnight culture) and enteric coated with Eudragit® FS30D. This resulted in a drop in viability (Table 2). Obviously, there is a detrimental effect of the coating process due to the interaction between *L. lactis* Thy 12 and the enteric coating. This drop in viability could not be linked to a longer exposure to a higher temperature as pellets fluidized for the same time at the coating temperature showed no decrease in viability. Moreover, after 2 h in 0.1 N HCl (simulating the gastric residence time) viability further decreased, only about 1% of the bacteria in the coated formulation remained viable independent of coating thicknesses. To avoid direct contact between the bacteria and the toxic enteric coat, subcoatings were applied onto the pellets before enteric coating. For the formulation of drugs like omeprazole, subcoatings have been used to prevent direct contact between acid-labile drug and the acidic functional groups of the enteric coating [15]. In the case of Opadry® and Opadry® AMB, the viability decreased significantly after subcoating. Only Opadry® II offered protection during subcoating. However, when applying an additional enteric coat (5%) this resulted in an even lower viability compared to enteric coating without subcoat (Table 3).

A coating thickness of 5% was selected as a thicker Eudragit® FS30D coat resulted in a decrease of viability. The viability of pellets after layering during 2 h with a concentrated bacteria suspension and after coating with 5% Eudragit® FS30D assessed via the plating-out method was $1.7 \times 10^9 \pm 1.2 \times 10^8$ cfu and $9.9 \times 10^8 \pm 8.8 \times 10^7$ cfu/100 mg pellets, respectively. A dose of 10^{11} cfu corresponds to 10 g pellets. After 2 h in 0.1 N HCl, the viability decreased approximately 1 log U ($1.4 \times 10^8 \pm 6.0 \times 10^7$ cfu/100 mg). However, the drop in viability during in vivo application is probably overestimated as a multiparticulate formulation behaves as a liquid, the pellets are faster emptied from the stomach [16], limiting the degradation of bacteria in acid medium as $3.1 \times 10^8 \pm 1.8 \times 10^6$ cfu/100 mg survived after 30 min gastric residence.

3.4. Ileum targeting

The release of bacteria must be targeted to the ileum, as the ileum is the major site of inflammation in patients with Crohn's disease. Previous results [17] showed that the tested enteric polymers (pH dependent release) did not result in ileum targeting i.e. release at pH 6.8. A 30% Eudragit® L30D55 coating dissolved at a pH lower than 6.8, while a 15% Eudragit® FS30D only dissolved at a pH above the pH of the target site (pH 7.4). An in vivo study showed that HPMC capsules coated with a similar thickness of Eudragit® FS30D resulted in 30% of the cases in a release distal of the ileum target site [18]. The selected 5% coating of Eudragit® FS30D was evaluated for its ileum targeting capacities, compared to a 15% coating thickness. At pH 6.8 there was a very slow release of thymidine, with a slightly faster release for the 5% coated pellets (Fig. 7). At pH 7.0 a complete release of thymidine was obtained after 100 min in phosphate buffer for the 5% coated pellets, whereas for the 15% coated pellets only 50% was released

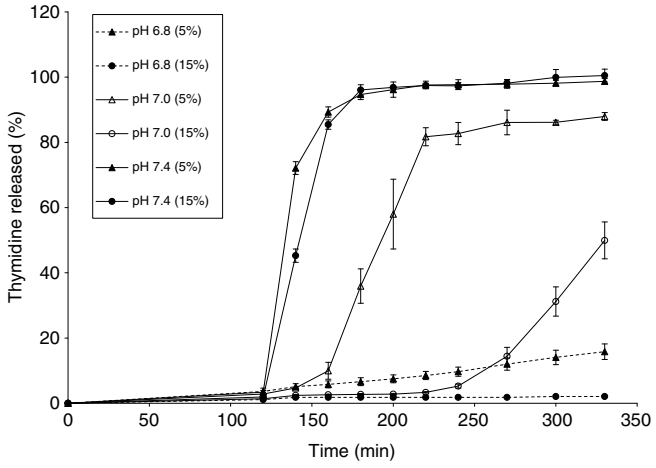

Fig. 7. Release profiles (mean ± SD) of thymidine (mean ± SD, $n = 3$) from pellets coated with 5 (▲) and 15% (●) Eudragit FS30D after 2 h HCl 0.1 N and subsequently buffer solution with pH 6.8 (---, black symbols), 7.0 (—, open symbols) and 7.4 (—, black symbols).

Table 3
Viability (%) (mean ± SD) of *L. lactis* Thy 12 after layering, subcoating, enteric coating and gastric residence

	After layering	Subcoating	Enteric coating	After 2 h 0.1 N HCl
No subcoating	12.4 ± 1.4	/	5%: 8.6 ± 2.8	1.1 ± 0.4
			10%: 4.5 ± 1.8	0.6 ± 0.2
			15%: 2.1 ± 0.5	0.4 ± 0.4
Opadry®		3.7 ± 0.5	/	/
Opadry® AMB		3.2 ± 1.8	/	/
Opadry® II		12.5 ± 2.9	5%: 2.9 ± 1.1	0.9 ± 0.3

after 210 min. The influence of coating thickness on release rate is less pronounced in higher pH range. By reduction of the coating thickness from 15% to 5%, ileum targeting could be improved.

4. Conclusion

By changing the stabilising matrix, process time and bacterial cell concentration in the layering suspension a layered formulation of *L. lactis* Thy 12 with an acceptable load of bacteria on the pellets could be obtained, as a dose of 10 g pellets corresponds to 10^{11} cfu. This formulation with 10% lactose/20% skim milk as stabilising matrix was enteric coated with Eudragit® FS30D, to obtain gastric protection and ileum targeting. The formulation can be stored for at least 12 months at −20 °C without a decrease of viability. This layering technique can also be used to stabilize other types of bacteria or proteins, but every bacterial strain and protein will behave differently during drying and storage. However, a drawback of this technique is the storage requirement at −20 °C as it is essential to maintain the cold chain during the entire life cycle of the product. It was concluded that layering is a valuable technique to develop in a single process step a multi-particulate enteric coated pharmaceutical formulation.

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