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Research paper

Evaluation of extrusion/spheronisation, layering and compaction for the preparation of an oral, multi-particulate formulation of viable, hIL-10 producing *Lactococcus lactis*

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Abstract

Three formulation techniques were compared in order to develop a multi-particulate formulation of viable, interleukin-10 producing *Lactococcus lactis* Thy12. First, freeze-dried *L. lactis* was compacted into mini-tablets. Next, liquid *L. lactis* culture was used as the granulation fluid for the production of pellets by extrusion/spheronisation. Finally, liquid *L. lactis* culture was layered on inert pellets as an alternative technique for the production of pellets. *L. lactis* viability and interleukin-10 production was evaluated. Viability dropped to 15.7% after compaction of freeze-dried *L. lactis* and to 1.0% after pelletisation of liquid *L. lactis* by extrusion/spheronisation. The viability in the mini-tablets and pellets, stored for 1 week at RT and 10% RH was reduced to 23 and 0.5% of initial viability, respectively. Storage for 1 week at RT and 60% RH resulted in complete loss of viability. Layering of *L. lactis* on inert pellets resulted in low viability (4.86%), but 1 week after storage at RT and 10% RH, 68% of initial viability was maintained. Increasing product temperature and cell density of *L. lactis* in the layering suspension did not significantly change viability after layering and storage. Interleukin-10 production capacity of *L. lactis* Thy12 was maintained after layering.

Keywords: Extrusion/spheronisation; Layering; Compaction; Viability; *L. lactis*; Recombinant

1. Introduction

With the discovery of cytokines and their mechanism of action, there is an increasing interest in their therapeutic use as mucosal immune modulators. Mucosal delivery of cytokines would allow exerting a local effect, resulting in fewer side effects than systemic administration. This encloses the production of an oral formulation for cytokine delivery. Apart from the mere fact that a conventional formulation of recombinant cytokines is extremely expensive, such approaches suffer from many other technical and fundamental obstacles.

Steidler and co-workers [1,2] described the advantageous use of recombinant *Lactococcus lactis* strains for the delivery of cytokines. As interleukin-10 (IL-10) plays a central role in down-regulating inflammatory cascades, it looked a promising candidate to treat Crohn's disease, a severe, chronic intestinal inflammation [3]. However, IL-10 given through the systemic route is not effective [4]. A local delivery of the cytokine by *L. lactis* seems more promising [1]. Murine IL-10 secreting *L. lactis* (LL-IL-10) 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 [1].

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The objective of this study was to develop a dry formulation containing the bioengineered human IL-10 (hIL-10) producing *L. lactis* (Thy12) [5]. Since hIL-10 production is strictly related to the viability of Thy12, a suitable production technique, which maintained an acceptable level of viability had to be selected. Moreover, we wanted this to result in a multi-particulate formulation (~1 mm), as this offers the advantages such as ease of swallowing in case of large doses and fast gastric emptying, decreasing the residence time in the presence of the detrimental gastric fluids [6]. Klijn et al. [7] showed that the gastric fluid negatively influences the viability of *L. lactis*. This implies the enteric coating of the dosage form and requires a spherical shape of the formulation.

Research to stabilise biomaterials by formulating them in dry state is a highly active area. The most commonly used method for preparing solid protein pharmaceuticals [8–10] and dry live (attenuated) bacteria or viruses as vaccines [11,12] or for food application [13] is freeze-drying. Previous experiments showed that freeze-drying is an adequate method to preserve Thy12 (Huyghebaert et al., submitted).

In this study, three formulation techniques were compared. First, freeze-dried *L. lactis* was compacted in order to obtain mini-tablets. Next, liquid *L. lactis* culture was used as granulation fluid for the production of pellets by extrusion/spheronisation. Finally, liquid *L. lactis* culture was layered on inert pellets as an alternative technique for the production of pellets. Moreover, a first screening of storage stability was performed after 1 week to make an appropriate selection of a suitable production technique.

2. Materials and methods

2.1. Strains used in this study

Lactococcus lactis MG1363 [14].
Thy12 (human IL-10 producing *L. lactis* MG1363) [5].

2.2. Preparation of the cell suspensions

The first experiments were carried out with the non-bioengineered *L. lactis* MG1363. For the production of mini-tablets and pellets by extrusion/spheronisation, *L. lactis* culture was prepared by inoculating a stock suspension, stored at −20 °C in 50% glycerol-GM17, 1/1000 in 10% (w/v) skim milk (Difco, Becton Dickinson, MD, USA), supplemented with 0.5% glucose as a C-source and 0.5% casein hydrolysate (Casiton®, Difco, Becton Dickinson) as a N-source. The culture was grown for 16 h at 30 °C to obtain a saturated culture, which had a viable count of $2–3\times10^9$ cfu/ml. To prevent further activity or growth, the culture was kept on ice until use and in between all handling.

For the production of pellets by layering, Thy12 was inoculated in 10% skim milk supplemented with 0.5% glucose, 0.5% Casiton® (Difco, Becton Dickinson) and 50 μg/ml thymidine (GCT-milk) or in M17 supplemented with 0.5% glucose and 50 μg/ml thymidine (GM17T) and prepared as cited above. The bacteria grown in GM17T were collected by centrifugation at $3000\times g$ for 10 min at 4 °C. The cell pellet was resuspended in skim milk at 10^{10} cfu/ml (10 times concentrated).

2.3. Production of a multi-particulate formulation

Fig. 1 shows a schematic overview of the processing steps in order to obtain the three different multi-particulate formulations.

2.3.1. Production of mini-tablets (n = 5)

Approximately 2 g *L. lactis* MG1363 culture was filled in vials (glass type 1, Gaash Packaging, Mollem, Belgium). The vials were covered with a freeze-drying stopper (V9032 FM 257/2 SAF1, bromobutyl with magnesium silicate as filler, kindly donated by Helvoet Pharma, Alken, Belgium). Prior to freeze-drying, the vials were kept on ice.

The vials were loaded on the precooled shelves (−25 °C) of the freeze-dryer (Leybold GT4, Finn-aqua, Sohlberg, Germany). The samples were frozen to −45 °C over 105 min at 1000 mbar. The primary drying (12 h) was performed at −15 °C and 0.8–1 mbar and the secondary drying (9 h) at 10 °C and 0.1–0.2 mbar. After freeze-drying, the vials were closed under vacuum. Samples were taken immediately after freeze-drying and kept on ice until analysis. The freeze-dried cake, containing *L. lactis* in skim milk matrix, was ground to powder by means of pestle and mortar, manually filled as such in the die and subsequently compacted using an eccentric tabletting machine (Korsch EK 0, Frankfurt, Germany) equipped with 2 mm flat punches at a compaction pressure of 52 ± 2 MPa, yielding 1.7 mg mini-tablets.

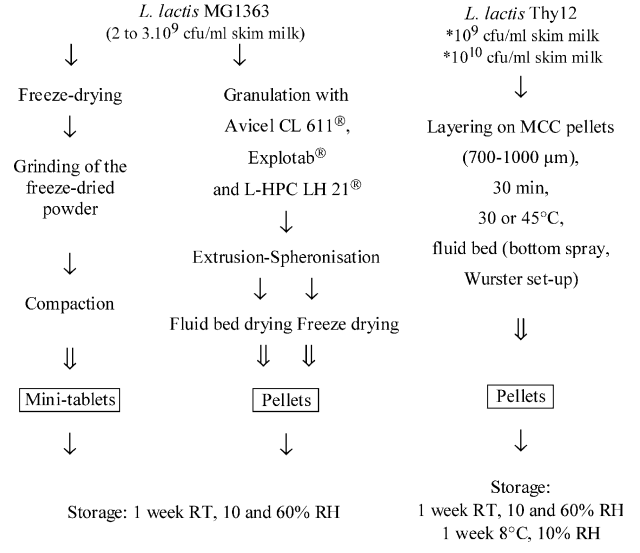

Fig. 1. Schematic overview of the processing steps to obtain the three different multi-particulate formulations.

All handling was performed at 20% RH to prevent sticking of the hygroscopic powder. Samples were taken immediately after production of the mini-tablets and kept on ice until analysis.

2.3.2. Production of pellets by extrusion/spheronisation ($n=5$)

Microcrystalline cellulose containing 11.3–18.8% (w/w) sodium carboxymethyl cellulose (Avicel CL611®, FMC Europe, Brussels, Belgium) and low substituted hydroxypropyl cellulose (L-HPC LH 21®, kindly donated by ShinEtsu, Tokyo, Japan) were used as excipients for the production of pellets. Sodium carboxymethyl starch (Explotab®, kindly donated by Penwest Pharmaceuticals, NY, USA) was used as a disintegrant.

L. lactis MG1363 culture was used as the granulation fluid. Avicel CL 611® (60 g), Explotab® (20 g) and L-HPC LH 21® (120 g) were pre-blended for 10 min at 60 rpm using a planetary mixer with a K-shaped mixing arm (Kenwood Major Classic, Hampshire, UK) and subsequently wetted by gradual addition of 375 g *L. lactis* culture and granulated for 15 min. Next the wet mass was extruded in a dome extruder (Type D6-L1, Fuji Paudal Co., Tokyo, Japan) equipped with a 1-mm perforated screen and operating at 45 rpm. The extrudates were spheronized for 3 min on a spheronizer (Caleva model 15, Sturminster Newton, UK) equipped with a cross-hatched friction plate, operating at 1000 rpm. The pellets were dried either by fluid-bed or by freeze-drying. For fluid-bed drying, 160 g wet pellets were dried for 30 min in a fluid-bed dryer (Uniglatt D 7852, Glatt, Binzen, Germany) set at 25 °C inlet air temperature. For freeze-drying, 1 g wet pellets were transferred into glass vials and freeze-dried as described for the culture for the production of mini-tablets. Samples were taken at different stages of the production process (granules, extrudates, wet pellets and dried pellets). To avoid any activity of the bacteria, the samples were kept on ice until analysis.

2.3.3. Production of pellets by layering ($n=3$)

Microcrystalline cellulose spheres were used as inert carriers. To obtain an average diameter of 1000 μm as standard for pellets, 150 g of each Cellets® 700 and 1000, kindly donated by Pharmatrans (Basel, Switzerland) were mixed. The Thy12 culture was layered on 300 g Cellets® 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; spraying rate of 3 g/min for 30 min; atomising pressure 1.5 bar; product temperature 30 or 45 °C). The pellets were preheated for 5 min at an inlet air temperature of 30 or 45 °C before layering. Throughout the layering process Thy12 culture was stirred using a magnetic stirrer. The experiments were performed in a room with low relative humidity (20% RH).

2.4. Storage

The pellets made by extrusion/spheronisation and the mini-tablets were stored for 1 week at room temperature (RT) (23 ± 2 °C) and 10% RH (above silica) and at 60% RH. The pellets produced by layering were stored for 1 week at RT and 8 °C at 10% RH (above silica).

2.5. Evaluation of the multi-particulate formulation

2.5.1. Determination of viability of L. lactis

Viability of the bacteria was determined by following the growth of the standards and the samples in a Bioscreen (Labsystems). 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 or without thymidine and loaded in triplicate into plates. 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 percentage of theoretical.

The viability of the samples determined by this method corresponded very well with the results obtained by plating. Determining viability with the Bioscreen offers the advantage of a broad dynamic range and small error bars. For determining the viability of the freeze-dried cake or the mini-tablets, 0.1 g was dissolved in 1 ml sterile water. Three dilutions of each sample were loaded in duplicate. A correction factor was taken into account for the weight loss of the culture during freeze-drying. For determining viability in the pellets by extrusion/spheronisation, 0.1 g was dissolved in 1 ml M17 and shaked at 1400 rpm for 10 min at 12 °C in a Thermomixer comfort (Eppendorf, Hamburg, Germany). Three dilutions of each sample of pellets by extrusion/spheronisation were loaded in duplicate on a plate for analysis in the Bioscreen. For calculating the viability in the sample, it was estimated that the weight was reduced by a factor 10 during drying. For the pellets by layering, three dilutions of each sample were plated in duplicate. Only the plates with a sufficient and reliable number of bacteria were counted to calculate the viability.

The viability values obtained after layering 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.

2.5.2. Determination of water content

The water content of the granules, the extrudates and the wet pellets was determined gravimetrically by heating 0.5 g sample for 20 min at 100 °C in an IR oven (Mettler LP 16M, Mettler Toledo, Belgium). The analysis was performed in duplicate. Water content of the freeze-dried and fluid-bed dried pellets was determined using a Mettler DL35 Karl Fisher titrator (Mettler-Toledo, Beersel, Belgium). The samples were stirred in the reaction medium for 80 s. Afterwards the water was titrated with Hydranal® Composite 2 (Riedel-de Haën, Seelze, Germany). The analysis was performed in triplicate.

2.5.3. Determination of hIL-10 production

The hIL-10 concentration was determined in a sandwich ELISA. Maxisorp F96 plates (Nunc, Roskilde, Denmark) were coated with 2 μg/ml rat anti-human IL-10 (Pharmingen). The plates were blocked with a 0.1% casein solution. A 1/2 dilution series of hIL-10, starting from 1 ng/ml and appropriate dilutions of the samples were loaded on the plates. Between each step the plates were washed with PBS +0.05% Tween-20. The bound hIL10 was detected with 1/1000 biotinylated rat anti-human IL10 (Pharmingen) combined with 1/1000 horse radish peroxidase coupled streptavidine. The plates were developed with TMB substrate (Pharmingen). The reaction was stopped after 30 min with 1 M H_2SO_4. The absorbance was measured at 450 nm, with 595 nm as reference wavelength.

3. Results and discussion

The aim of this study was to develop a solid dosage form of viable and hIL-10 producing *L. lactis* for oral administration. To circumvent the detrimental effect of the gastric environment, the formulation should be coated. In order to avoid prolonged gastric residence times and penetration of gastric fluid into the formulation, a multi-particulate formulation is preferred. Mini-tablets and pellets, either produced by extrusion/spheronisation or layering are the two main appropriate formulations.

3.1. Viability in mini-tablets, composed of freeze-dried L. lactis

Previous experiments showed that freeze-drying is an adequate method to dry liquid *L. lactis* MG1363 and Thy12 suspensions (Huyghebaert et al., submitted) with maintenance of an acceptable viability. Viability after freeze-drying was 71.05±18.07% ($n=5$). As a powder containing an acceptable amount of viable *L. lactis* was available and tablets are the most commonly used administration form, the freeze-dried powder was compacted into mini-tablets. In this study, it was evaluated if this production technique allows the production of a multi-particulate formulation containing viable *L. lactis*. A survival of 15.7±4.9% was obtained in the mini-tablets (Fig. 2). This means that 78% of the viability of *L. lactis* in the freeze-dried powder was lost during tabletting indicating the bacteria's sensitivity to pressure. These results are in agreement with Maggi et al. [15,16] who reported that tabletting of freeze-dried *Bifidobacterium adolescentis* and *Lactobacillus gasseri* resulted in a viability loss of 40–99% of initial viability in freeze-dried powder, depending on the strain, the tablet type (single- or double-layered) and the tablet composition (effervescent or slow release layer). The authors did not mention the compaction force. In this study, the viability of *L. lactis* MG1363 in the mini-tablets, stored for 1 week at RT and 10% RH was reduced to 3.7±0.8 or 25.3±6.1% of its initial viability after production (Fig. 2). Storage for 1 week at RT and 60% RH resulted in complete loss of viability. Former experiments (data not shown) indicated that the viability of *L. lactis* stored at RT and 10% RH continues to decrease gradually. Therefore the formulation will definitely not have an acceptable shelf life. Maggi et al. [15] reported a decrease in viability of *B. adolescentis* and *L. gasseri* of 50–20% after storage for 2 months at RT, dependent on tablet type and composition. A dramatic decrease in viability of *B. adolescentis* was reported after storage for 1 year at 4 °C as only 1% remained viable. A mixture of three selected *Lactobacillus* species in one tablet resulted in an unchanged viability after 1 year of storage at 4 °C [15]. It should however be noticed that no specifications were available on the relative humidity conditions during storage. Stadler et al. [17] reported no reduction in viability of freeze-dried *Lactobacillus acidophilus* (3×10^9 cells/tablet) formulated in tablets after storage for 6 months at 10 and 20 °C (2.5×10^9 cells/tablet) while no data are available on the stability of freeze-dried *L. lactis*. These studies indicated that the stability is strain dependent. Moreover, Maggi et al. [15] and Stadler et al. [17] prepared standard sized tablets (10 mm), whereas in this study we used mini-tablets in which the bacteria are less protected from oxygen and moisture as they have a large surface to volume ratio. Furthermore, freeze-dried *L. lactis* formulated in tablets has the tendency to be less sensitive to storage than

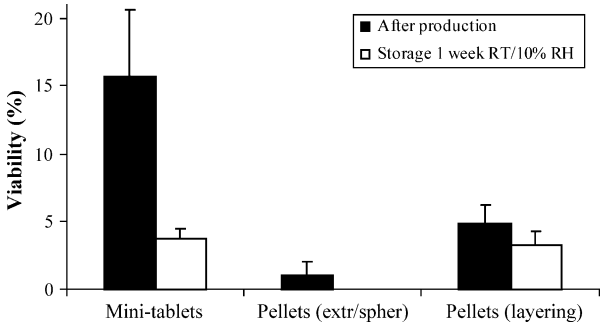

Fig. 2. Absolute viability (% of theoretical, mean±SD) of *L. lactis* MG1363, incorporated in mini-tablets ($n=5$), pellets produced by extrusion/spheronisation ($n=5$) and of Thy12 in pellets produced by layering (30 °C) after production and after 1 week storage at RT and 10% RH.

freeze-dried *L. lactis* powder as such; after 1 week at RT and 10% RH, viability dropped to 25.3±6.1 and 13.6±11.9% of initial viability in the tablets or powder, respectively, but no significant differences were seen.

Next to the problem of viability decrease during storage, the production of mini-tablets is very expensive, especially as the freeze-drying step is an energy-intensive and time-consuming process. In addition, the freeze-dried powder is very hygroscopic and special precautions have to be taken during tabletting. Moreover, it has to be investigated if the hygroscopicity will allow tablets to be efficiently coated and if *L. lactis* will survive this coating process.

3.2. Viability in pellets, produced by extrusion/spheronisation

A cheaper and faster way to produce a multi-particulate formulation containing viable bacteria is the production of pellets by extrusion/spheronisation [18]. It was evaluated whether this technique could be applied to *L. lactis* MG1363. For this, viability of *L. lactis* MG1363 was determined after the different pelletisation steps (Fig. 3). From Fig. 3 it is clear that the viability of *L. lactis* dropped gradually during the production process. A rise in temperature during granulation, extrusion and spheronisation might alter the moisture content of the product due to evaporation of the granulation fluid during processing [19]. This might thereby dramatically decrease the microorganism's viability, but Fig. 3 shows that the loss of viability could not be explained by a decrease of the water content as this remains constant. The shear developed during granulation, and mainly during extrusion/spheronisation with an additional rise in temperature (30–40 °C) might explain the drop in viability during pelletisation. However, the most detrimental step is the drying process; only 5% of the bacteria in the wet pellets survived the fluid-bed drying. Because fluid-bed drying dramatically reduced the viability to 1.0%, freeze-drying was evaluated as an alternative drying technique. However, the viability in the freeze-dried pellets also dropped to 0.81%. From these data, it can be concluded that freeze-drying, the most common method for preservation of microorganisms, offered no advantage for drying the wet pellets in comparison to fluid-bed drying in terms of *L. lactis* viability. After pelletisation, the microorganisms are probably damaged to a certain level that no drying method is appropriate to maintain the *L. lactis* viable in the pellets. Moreover, the 'low-water' stress applied to the bacteria during the two drying techniques brought about cell damage that led to an important loss in viability. The viability of the remaining bacteria was determined after a 1-week storage period (Fig. 2). The viability of *L. lactis* MG1363 formulated in pellets, stored for 1 week at RT and 10% RH was reduced to 0.5% of its viability immediately after production. Viability of *L. lactis* MG1363 in pellets stored at RT and 60% RH dropped to zero. Kim et al. [18] prepared viable and stable dry lactic acid bacteria (LAB)-containing particles for food and agricultural purposes by mixing liquid culture concentrates of three different LAB strains with food grade cellulose. After granulation with a glycerol/water mixture, extrusion and spheronisation, the pellets were fluid-bed dried at 20–25 °C. This production process was not detrimental to the bacteria. Kim et al. [18] reported no loss of viability of *Streptococcus cremoris* and *Pediococcus acidilactici* (both cocci) in pellets after 1 week storage at RT but a loss of viability of more than 1 log of *Lactobacillus plantarum* (rod-shaped) in the pellets stored at the same conditions. So viability after storage at room temperature showed strain dependency. No indication was given about the relative humidity conditions of storage. Viability of *L. plantarum, P. acidilactici and S. cremoris* remained unchanged for 76 days when stored at 4 °C. Again, these results emphasised strain dependency. Thereby it can be concluded that *L. lactis* seemed more sensitive to storage.

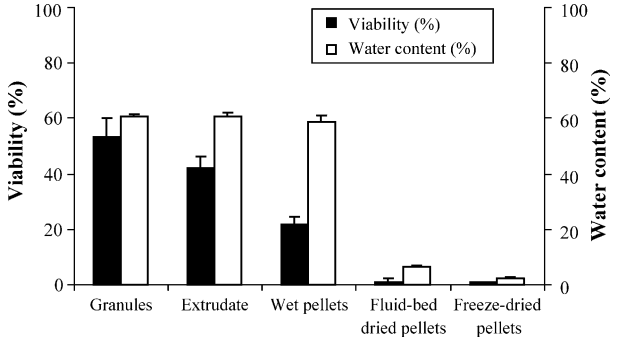

Fig. 3. Absolute viability (% of theoretical, mean±SD, $n=5$) of *L. lactis* MG1363 and the water content during the pellet production process (extrusion/spheronisation) ($n=5$).

3.3. Viability in pellets, produced by layering

As incorporation of *L. lactis* in pellets by means of extrusion/spheronisation resulted in an enormous loss of viability, layering of *L. lactis* on inert carriers was proposed as an alternative production technique for a multi-particulate formulation. While the inert carriers were fluidising, the *L. lactis* culture was atomised and the droplets met the inert carrier and consequently dried on the surface. No literature is available on layering of bacteria. Only spray drying is reported and showed that this technique allows preservation of 62% *L. lactis* [20]. However, this yielded a powder formulation, so subsequent compaction would be required to obtain a multi-particulate formulation [18]. This can be overcome by layering the culture on inert particles. Maa et al. [21,22] showed the possibility to layer lactose powder with recombinant human deoxy-ribonuclease (rhDNAse) by means of a Wurster spray coater at product temperatures ranging from 34 to 43 °C and yielded granules containing rhDNAse with 65% of its initial activity.

Preliminary experiments showed that during layering of non-pareil sugar seeds with the *L. lactis* culture, the sugar

partially dissolved which resulted in sticking. Decreasing the spray rate or increasing the inlet air temperature prevented sticking of the sugar spheres, but resulted in a too long process time and increased risk of viability decrease. Inert microcrystalline cellulose pellets were used as alternative carriers. The layering was conducted in a room with low relative humidity (20%) in order to increase the spray rate and the drying capacity of the inlet air in the fluid bed without increasing the temperature. Thy12 was layered in a 10% (w/w) skim milk solution at a process temperature of 30 °C on Cellets® 700 and 1000 (1/1, w/w). Skim milk was chosen as matrix to layer *L. lactis* on the inert pellets as freeze-drying experiments (Huyghebaert et al., submitted) revealed the stabilising capacity of a skim milk matrix for *L. lactis*. Preliminary layering tests with a reference substance dissolved in skim milk, revealed that the yield of the process was 100%. This indicated the absence of spray drying and a good adherence of the skim milk matrix to the surface of the Cellets®. The viability of Thy12 after layering was 4.9 ± 1.4% (Fig. 2), remarkably lower as compared to freeze-drying and subsequent tabletting. Shear stress generated by atomisation, oxidation and thermal stress may all affect *L. lactis* viability [20]. Moreover, in contrast to spray drying, *L. lactis* experienced a longer exposure to an elevated temperature during layering. This could explain the more dramatic loss of viability than reported by Fu and Etzel [20] during spray drying.

The layered pellets were not stored at room temperature and 60% RH as previous data showed that storage for 1 week at those conditions resulted in a dramatic loss of viability. Comparison of the microorganism's viability in layered pellets and mini-tablets after storage for 1 week at RT and 10% RH, revealed that layered Thy12 showed a better stability as 68 and 25% of its initial viability after production remained, respectively (Fig. 2). Since Thy12 was designed to produce a therapeutic (hIL-10) in situ, accurate dosing is a necessity and requires the guarantee of a reproducible viability, especially as a function of time. With their superior stability, layered pellets were therefore to be preferred over mini-tablets and pellets prepared by extrusion/spheronisation. The results revealed that viability of *L. lactis* could be better guaranteed by the layering technique.

Further experiments were performed in order to increase the load of Thy12 on the Cellets®. In a first approach, the product temperature was increased and so higher drying capacity of the inlet air was obtained resulting in the possibility to increase the spray rate. A second approach consisted in the increase of the Thy12 concentration in the layering suspension. Fig. 4 shows the viability after layering and subsequent storage for 1 week at RT and 10% RH of Thy12 layered at increased product temperature (45 °C) and increased bacterial cell density (10^9–10^{10} cfu/ml). A two-way ANOVA revealed no significant difference ($P > 0.05$) between the viability after layering of *L. lactis* Thy12 at

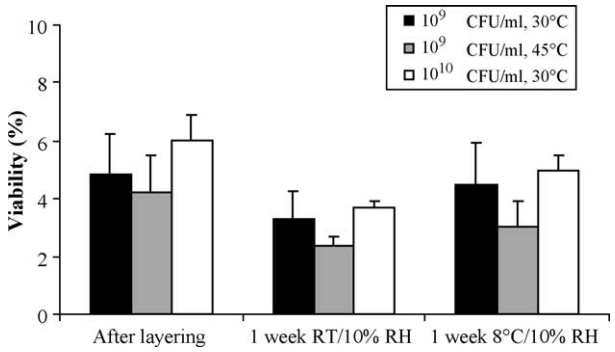

Fig. 4. Absolute viability (% of theoretical, mean ± SD, $n = 3$) of Thy12, 10^9 cfu/ml, layered at 30 and 45 °C product temperature and 10^{10} cfu/ml after layering at 30 °C ($n = 3$) and after 1 week storage at RT and 8 °C (10% RH).

30 °C and 10^9 cfu/ml, at 30 °C and 10^{10} cfu/ml and at 45 °C and 10^9 cfu/ml. From these data, it could be concluded that viability after layering is independent of process temperature (range from 30 to 45 °C) and *L. lactis* concentration in the layering suspension (range from 10^9 to 10^{10} cfu/ml).

It was also evaluated if stability of the viability could be improved by decreasing the storage temperature to 8 °C (Fig. 4). Although viability tends to be higher at 8 °C, a two-way ANOVA revealed no significant difference ($P > 0.05$) between the viability immediately after layering of Thy12, after storage for 1 week at RT and 10% RH or for 1 week at 8 °C and 10% RH. A long term stability test has to confirm this trend.

As Thy12 was bioengineered for in situ production of hIL-10 and hence mucosal delivery in patients with Crohn's disease, the success of the new delivery system depends on the hIL-10 producing capacity. In this study, it was confirmed that the hIL-10 producing capacity was maintained after layering. In vivo experiment have to be performed in order to determine the quantity of Thy12 required to down-regulate inflammation in patients with Crohn's disease.

4. Conclusions

It can be concluded that layering of Thy12 on inert microcrystalline cellulose pellets is a promising technique for the production of a multi-particulate formulation of viable and hIL-10 producing Thy12. It is an economical, single-step production process of a multi-particulate formulation and the layered batch can be subsequently coated in the same apparatus. Further experiments are ongoing to optimise the layering matrix and cell concentration and to evaluate enteric coating of the formulation.

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