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Research paper

Development of an enteric-coated formulation containing freeze-dried, viable recombinant *Lactococcus lactis* for the ileal mucosal delivery of human interleukin-10

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Abstract

Recombinant hIL-10 producing *Lactococcus lactis* (Thy12) looks a promising intestinal mucosal delivery system for treatment of Crohn's disease [L. Steidler, W. Hans, L. Schotte, S. Neirynck, F. Obermeirer, W. Falk, W. Fiers, E. Remaut, Treatment of murine colitis by *L. lactis* secreting interleukin-10, Science 289 (2000) 1352–1355. L. Steidler, S. Neirynck, N. Huyghebaert, V. Snoeck, A. Vermeire, B.M. Goddeeris, E. Cox, J.P. Remon, and E. Remaut, Biological containment of genetically modified *L. lactis* for intestinal delivery of human interleukin-10, Nat. Biotechnol. 21 (7) (2003) 785–789]. As the hIL-10 production is strictly related to Thy12's viability and gastric fluid negatively influences this viability, an enteric-coated formulation had to be developed with maintenance of its viability after production and storage. *L. lactis* MG1363, used for optimization, was grown until stationary phase in milk (glucose/casiton supplemented) and freeze-dried. This resulted in a viability of about 60%. Storage at different conditions showed that viability remained highest at 8 °C/N_2 atmosphere (32.5% of initial remained viable after 6 months). To increase the concentration of bacteria in the freeze-dried powder, they were concentrated by centrifugation. *L. lactis* tolerated this procedure. However, the concentration factor was limited to 10. Freeze-dried Thy12 was filled in ready-to-use enteric-coated capsules. Despite the good enteric properties of the capsules, viability of Thy12 dropped to about 43 and 28% after gastric fluid stage, depending on the enteric polymer used. Freeze-dried Thy12 filled in ready-to-use enteric-coated capsules, packed in Alu sachets (sealed at 20% RH) maintained 6.1 and 44.3% of initial viability after storage for 1 year at 8 and −20 °C, respectively, as well as its hIL-10 producing capacity.

Keywords: Freeze-drying; Recombinant *Lactococcus lactis*; Viability; Enteric-coated capsules

1. Introduction

With the discovery of cytokines and their mechanism of action, there is an increasing interest in their therapeutic use as intestinal mucosal immune modulators. Intestinal mucosal delivery of cytokines would allow a local effect, resulting in fewer side effects than the systemic administration. This encloses the production of an oral formulation of cytokines. 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 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 was not effective [4]. Local delivery of the cytokine by *L. lactis* seemed more promising [2]. Murine IL-10 secreting *L. lactis* (LL-IL-10)

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were used for the in vivo production and mucosal delivery of this cytokine. Daily intragastric inoculation with these bacteria (LL-IL-10) into mice, in which experimental enterocolitis was installed, could cure or prevent the intestinal inflammation [2].

To evaluate the therapeutic potential of this concept in patients with Crohn's disease, a dry formulation containing the bioengineered human IL-10 (hIL-10) producing *L. lactis* (Thy12) [5] had to be developed. Since the hIL-10 production is strictly related to the viability of Thy12, a suitable production technique with maintenance of an acceptable level of viability and shelf life had to be selected. The survival of *L. lactis* has been studied in the human gastrointestinal tract up to the faeces. Klijn et al. [6] showed that the cells recovered in the faeces accounted for approximately 1% of the total number of cells ingested. Moreover, Vesa et al. [7] showed that the survival of *L. lactis* MG1363 was only 1% at the terminal ileum, demonstrating the detrimental effects of gastric acid and bile salts. This implies the enteric coating of the dosage form.

Research to stabilize 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. However, there is no universally applicable protocol for the successful freeze-drying of bacteria. Viability loss of lactic acid bacteria, freeze-dried in the same matrix, can change from 1 log for *Streptococcus thermophilus* to 2 logs for *Lactobacillus bulgaricus* [14]. This indicated that the survival after freeze-drying is strain dependent [13–23] and that for each individual type of bacterium a matrix has to be optimized. Moreover, depending on the cryoprotective agents added, survival after freeze-drying can range from less then 1–67% for *S. thermophilus* [16]. Besides, most of these studies were conducted mainly in dairy or food industry, they lack the precise data on initial viability, stability and bacterial density. None of them report on freeze drying of bioengineered bacteria and/or maintenance of their properties achieved by bioengineering.

In this study, it was the aim to develop an enteric-coated formulation of freeze-dried Thy12. The first requirement was to develop a freeze-dried powder formulation containing viable and hIL-10 producing Thy12 with an acceptable shelf life. In this study, the optimal growth medium, physiological state and freezing rate have been optimized. Next, the stability of *L. lactis* was evaluated in order to select acceptable storage conditions. In order to increase the *L. lactis* concentration in the freeze-dried powder to reach an acceptable dose for oral administration, the culture preparation method was optimized and influence of cell density was evaluated. To increase bacterial viability after freeze-drying and storage, the influence of the incorporated feed components was evaluated. As the second requirement of this study was to protect the bacteria against the detrimental gastric fluid and bile salts, freeze-dried Thy12 was incorporated in enteric-coated capsules. These enteric-coated capsules are especially designed for storage at low temperature and low relative humidity and allowing passage through the stomach with low penetration of gastric fluid [24]. Viability and hIL-10 production in function of storage time and viability after simulated passage through the stomach have been tested.

2. Materials and methods

2.1. Strains used in this study

L. lactis subsp. *cremoris* MG1363 [25].
Thy 12 (human IL-10 producing *L. lactis* subsp. *cremoris* MG1363) [5].

2.2. Preparation of the cell suspensions

The non-bioengineered *L. lactis* subsp. *cremoris* MG1363 culture was prepared by inoculating a stock suspension, stored at −20 °C in glycerol/GM17 (50/50), 1/1000 in growth medium (further specified). To prevent further activity or growth, the culture was kept on ice until use and in between all manipulations.

To investigate the influence of the freeze-drying matrix, two different growth media were used: M17 broth (Difco, Becton Dickinson, Erembodegem, Belgium) supplemented with 0.5% glucose in order to obtain GM17 or 10% (w/v) skim milk (Difco, Becton Dickinson, Erembodegem, Belgium) supplemented with 0.5% glucose and 0.5% casein hydrolysate (Casiton®, Difco, Becton Dickinson) to obtain GC-milk. Influence of physiological state was investigated by growing the bacteria at 30 °C for 3 h or overnight (ON) to obtain the logarithmic and stationary phase, respectively. The culture in the logarithmic phase had a viable count of about 10^7 cfu/ml while the culture in stationary phase had about 10^9 cfu/ml.

In further experiments, *L. lactis* MG1363 was grown (ON at 30 °C) in GM17 and collected by centrifugation at 3000*g* for 10 min at 4 °C. To investigate the influence of culture preparation, the *L. lactis* MG1363 pellet was suspended in fresh GC-milk, with or without a previous wash step with PBS. As reference, *L. lactis* MG1363 was grown in GC-milk until the stationary phase. To determine the influence of *L. lactis* MG1363 density, the cell pellet was suspended in different volumes of fresh GC-milk, i.e. the initial volume of culturing, 1/5, 1/10, 1/100 and 1/200th of the initial volume in order to obtain different densities of *L. lactis*: initial density (2.3×10^9 cfu/ml), ∼5 times concentrated (9.5×10^9 cfu/ml), ∼10 times concentrated (3.3×10^{10} cfu/ml), ∼100 times concentrated (2×10^{11} cfu/ml) and ∼200 times concentrated (4×10^{11} cfu/ml), respectively.

Thy12 was prepared by inoculating a stock suspension, stored at −20 °C in glycerol/GM17(50/50)/thymidine, 1/1000 in 10% skim milk supplemented with 0.5% glucose,

0.5% Casiton® (Difco, Becton Dickinson) and 50 μg/ml thymidine (GCT-milk) or in GM17 supplemented with 50 μg/ml thymidine (GM17T) and grown as cited above. The cell pellet was resuspended in skim milk at 10^{10} cfu/ml (10 times concentrated). To evaluate the influence of feed components in the freeze-drying matrix on Thy12 viability, the cell pellets were suspended in the initial volume of fresh milk, T-milk (skim milk supplemented with 50 μg/ml thymidine) or GCT-milk.

To determine stability of Thy12 in enteric-coated capsules, the cell pellet was suspended in 1/10th of the initial volume of skim milk (10 times concentrated).

2.3. Freeze-drying of L. lactis cultures

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 or dry nitrogen. To determine influence of freezing rate, vials were instantly frozen, accomplished by immersing the vials into liquid N_2 (−196 °C) prior to freeze-drying. All freeze-drying runs were performed in triplicate ($n=3$), except if mentioned.

2.4. Preparation of enteric-coated capsules containing freeze-dried Thy12

The production of the enteric-coated HPMC capsules is extensively described elsewhere [24]. Briefly, HPMC caps and bodies 00 (Vcaps, Capsugel, Bornem, Belgium) (consisting of hydroxypropyl methyl cellulose, gellan gum as gelling agent and K-ions as gelling promoter) were coated separately in a fluid bed apparatus (GPCG 1, Glatt, Binzen, Germany) with two different enteric coating dispersions, i.e. Eudragit® L30D-55 and FS 30 D coating dispersion. The Eudragit® L30D-55 coating dispersion consisted of 15.3% Eudragit® L30D-55 polymer (an anionic copolymer of methacrylic acid and ethyl acrylate (1:1) dissolving from pH 6.0), 3.1% triethylcitrate (plasticizer), 1.3% glyceryl monostearate (glidant), 0.5% polysorbate 80 (wetting agent) and 43% of water. The Eudragit® FS 30 D coating dispersion consisted of 16.6% Eudragit® FS 30 D polymer (an anionic copolymer of methyl acrylate, methyl methacrylate and methacrylic acid dissolving from pH 7.2), 1.3% glyceryl monostearate (glidant), 0.5% polysorbate 80 (wetting agent) and 41.9% of water. The coated bodies were filled with freeze-dried Thy12 powder or with the freeze-dried Thy12 powder mixed with marker substance (thymidine) at 20% RH to prevent sticking of the hygroscopic powder and closed with coated caps. The enteric-coated capsules were packed in Alu sachets (LPS, Vapor flex barrier bag, New Jersey, US) and sealed at 20% RH. Thymidine has been used as marker substance in this project as it is the essential feed component of Thy12 [5]. Moreover, it is freely water-soluble (5.5 g/100 ml) at all pH values (from pH 2.5 to 7) and has therefore excellent properties to evaluate the enteric properties of an enteric-coated formulation.

2.5. Storage of the freeze-dried powder in vials and in enteric-coated capsules

Table 1 gives an overview of the storage conditions tested. To evaluate the influence of different parameters on stability, vials containing the freeze-dried *L. lactis* MG1363 were stored for 1 week at different conditions: at low temperature (8 °C) and 10% RH (above silica (silica gel for

Table 1
Overview of the parameters evaluated and storage conditions used

Parameter evaluated	Strain used	Storage time	Storage temperature (°C)	Storage atmosphere
(1) Freeze-drying matrix	*L. lactis* MG1363 freeze-dried powder in vials	–	–	–
(2) Stability				
*Short stability test	*L. lactis* MG1363 freeze-dried powder in vials	1 week	8	10% RH, vacuum, N_2
			RT	10%, 60% RH, vacuum, N_2
*Long-term stability test	Thy 12 freeze-dried powder in vials	6 months	8	10%, 20% RH, N_2
(3) Influence of culture preparation	*L. lactis* MG1363 freeze-dried powder in vials	1 week	8	10% RH
(4) Influence of cell density	*L. lactis* MG1363 freeze-dried powder in vials	1 week	8	10% RH
(5) Influence of feed-components	Thy 12 freeze-dried powder in vials	1 week	8	10% RH
(6) Stability in the enteric-coated capsules	Thy 12 freeze-dried powder in capsules	12 months	8, −20	Alu sachets, sealed at 20% RH

desiccation, Sigma, Bornem, Belgium) in a plastic container), vacuum or under N_2 and at room temperature (RT) and 10% RH, 60% RH, vacuum or N_2. The long-term stability of freeze-dried Thy12 in vials was evaluated at low temperature (8 °C) and 10% RH, N_2 and 20% RH (above a saturated potassium acetate solution). Analysis was performed after 1, 3 and 6 months. To evaluate the influence of the culture preparation method, cell density and presence of feed components, freeze-dried *L. lactis* was stored for 1 week at 8 °C and 10% RH. The filled coated capsules, packed in Alu sachets (sealed at 20% RH), were stored at 8 °C and −20 °C. Analysis was performed after 1, 3, 6 and 9 months and 1 year.

2.6. Analysis of the freeze-dried L. lactis cultures

2.6.1. Determination of viability of L. lactis

Viability of the bacteria was determined by following the growth of the standards and the samples in an automated turbidimeter (Bioscreen C, Oy Growth Curves ABLtd, Helsinki, Finland). The viability of the starting culture was set at 100%. In order to prepare standards, different dilutions of the starting culture were made, inoculated 1/100 in fresh GM17 with (Thy12) or without thymidine (*L. lactis* MG1363) and loaded in triplicate onto micro-titer plates. Of all standards, the growth was followed at 30 °C 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 for all standards. 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.

The viability of the samples determined by this method corresponded very well with the results obtained by the plate-count method. Determining viability with the Bioscreen offers the advantage of a broad dynamic range and small error bars. For determining the viability in the freeze-dried powder, 0.1 g powder was dissolved in 1 ml sterile water. When concentrated cultures were used, the powder was diluted further accordingly. For determining the viability in the enteric coated capsules after the gastric fluid stage, the capsules were subjected to 0.1 N HCl (37 °C) (1 capsule/30 ml). After 2 h, the content of the capsule was dissolved in 30 ml GM17T-45 mM CO_3^{2-} (pH 8.7). Three replicas of each sample were analyzed and of each replica, three dilutions were loaded in duplicate. A correction factor was taken into account for the weight loss of the culture during freeze-drying. For calculating the viability in the sample, it was estimated that the weight was reduced by a factor 10 during drying. Viability after storage was expressed as % from viability after freeze-drying (relative viability).

2.6.2. Determination of water content

The water content of the freeze-dried culture was determined using a Mettler DL35 Karl Fisher titrator (Mettler-Toledo, Beersel, Belgium). The samples were stirred in the reaction medium for 60 s. Afterwards the water was titrated with Hydranal® Composite 5 (Riedel-de Haën, Seelze, Germany). The analysis was performed in triplicate.

2.6.3. Determination of hIL-10 production

A sample that had been used for determining the viability was diluted 1/25 in GM17 and incubated for 3 h at 30 °C. Then the cultures were centrifugated for 10 min at 1650*g*, the supernatant was removed and the bacteria were resuspended in BM9. The cultures were incubated for another 3 h at 30 °C. A sample of 1 ml was removed and centrifugated for 3 min at 16,000*g*. The supernatant was removed to determine the hIL-10 concentration 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 1/1000 biotinylated rat anti-human IL10 (Pharmingen) combined with 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.

2.6.4. Determination of the glass transition temperature

T_g was determined using a model 2920 modulated DSC (TA Instruments, Brussels, Belgium). Approximately 7 mg of sample was placed in an aluminum pan that was hermetically sealed. The sample was heated from −40 to 100 °C with an underlying heating rate of 2 °C/min, a modulation period of 60 s and modulation amplitude of 0.5 °C. T_g was reported as the midpoint of the transition. The analysis was performed in duplicate.

2.6.5. Determination of the amorphous properties

The amorphous properties of the freeze-dried powder, stored for 9 months in the enteric-coated HPMC capsules at 8 and −20 °C were determined by X-ray diffraction (diffractometer D5000 Cu Kα, Siemens, Germany) (Counting time 0.8 s, step size 0.020 dg, wave length 1.5406 Å).

2.7. Statistical analysis

Viability values (mean of three values) were statistically evaluated with a one-way ANOVA. The values obtained in the short (1 week at different storage conditions) and long-term (6 months in vials and 1 year in enteric-coated HPMC capsules) stability studies were evaluated with a two-way ANOVA. Both tests were performed 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.

2.8. Dissolution testing

To evaluate the enteric properties of the capsules coated with the different polymers and stored at −20 °C in Alu-sachets, sealed at 20% RH, 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 one capsule per vessel, filled with 250 ml HCl 0.1 N for 2 h. Marker (thymidine) release was measured spectrophotometrically (Perkin–Elmer, Zaventem, Belgium) at 267 nm.

3. Results and discussion

3.1. Development of a freeze-dried powder formulation of viable and hIL-10 producing Thy 12

3.1.1. Influence of freeze-drying matrix, physiological state and rate of freezing on viability of L. lactis MG1363 after freeze-drying

L. lactis MG1633 was first freeze-dried in the conventional broth of *Lactococus lactis*: GM17 (1 freeze-drying run ($n=1$)). This resulted in an absolute viability of only 9.3 ± 0.8%. Therefore, the bacteria were grown and freeze-dried in an alternative GC-milk matrix. This resulted in a significantly higher viability (60.0 ± 18.0%, $n=17$). The obtained viability is in accordance with the viability data reported by Carcoba et al. [22] for *L. lactis* subsp. *lactis* CECT5130 (44.3%) and Kilara et al. [16] for *L. cremoris* (63%), both freeze-dried in skim milk. Despite all attempts to standardize the freeze-drying procedure, batch-to-batch variability could not be avoided. Andersen et al. [17] experienced the same problem. The low viability obtained in the GM17 matrix can be explained by the collapsed structure of the freeze-dried product. This problem was overcome by freeze-drying *L. lactis* MG1363 in skim milk, a medium used by many investigators to freeze-dry lactic acid bacteria. It is thought to offer beneficial properties because of its proteins, calcium and lactose [13,16,20–22]. Besides, freeze-drying of skim milk resulted in a glassy matrix (T_g 71.69 ± 5.48 °C). In literature, it has been proposed that for the effective stabilization, macromolecules should be incorporated in an amorphous, glassy matrix, which is characterized by a very high viscosity [26]. 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 stabilizing capacity. When the material is exposed to a temperature below T_g, it occurs in a glassy state. However, T_g is an unsatisfactory indicator for the temperature below which molecular motions and hence chemical and physical degradation reactions are zero [32]. T_0 rather than T_g should be used as a practical guide for selecting the storage temperature, as only below this temperature zero mobility is obtained. T_0 is at least 50 °C below T_g (the T_g-50 °C rule). Next, the amorphous state of a material is unstable and crystallization can occur, leading to loss of stabilizing power [33]. Next, Crowe et al. [28] concluded that direct interaction between sugars (e.g. lactose) and polar groups (e.g. in proteins and phospholipids) is a second stabilization requirement. The proposed mechanism is that sugars replace the water removed during desiccation, which is referred to as the 'water replacement theory'.

From Table 2, it is clear that the influence of freeze-drying stress is dependent on the physiological state of the bacteria: absolute viability after freeze-drying of *L. lactis* MG1363 in the stationary phase was significantly higher than after freeze-drying in the logarithmic phase. These results are in accordance with these published by Bergère for *Lactococci* [29]. However, in literature, there is disagreement about the influence of the physiological state of the bacteria on the viability after freeze-drying. Souzu [18] reported an increasing resistance of *E. coli* as cell growth approached its stationary phase. While Gehrke et al. reported that *E. coli* shows a better resistance against freezing, the most detrimental step of the freeze-drying when the cells are taken from the logarithmic growth phase as compared to the late stationary phase [19].

The rate of freezing is also a critical parameter in the freeze-drying process [18]. In our study, two freezing rates were evaluated: slow freezing by loading the filled vials on the precooled shelves (−25 °C) or instant freezing by immersion of the vials in liquid N_2 (−196 °C) before loading them on the precooled shelves. After freeze-drying, there was no significant difference in absolute viability of *L. lactis*, frozen at the two rates studied (Table 2). Gehrke et al. [19] found that the percentage of living cells decreased

Table 2
Influence of physiological state and rate of freezing on the absolute viability of *L. lactis* MG1363 after freeze-drying (mean ± SD)

Physiological state [a]	Viability (%)	Rate of freezing [b]	Viability (%)
Logarithmic phase	24.1 ± 8.0^{A} ($n=3$)	Slow	67.0 ± 18.2^{C} ($n=8$)
Stationary phase	60.3 ± 20.0^{B} ($n=3$)	Fast (liquid N_2)	67.1 ± 16.5^{C} ($n=8$)

$^{A–C}$Groups with the same superscript are not significantly different from each other ($P>0.05$) (one-way ANOVA).
[a] *L. lactis* grown and freeze-dried in GC-milk.
[b] *L. lactis* grown until stationary phase in GC-milk and freeze-dried in GC-milk.

Table 3
Relative viability of *L. lactis* MG1363 (mean ± SD), grown in GC-milk until the stationary phase and freeze-dried in GC-milk after 1 week in function of storage conditions

Relative viability (%) of *L. lactis* MG1363 stored at different temperatures						
	8 °C	*n*	RT	*n*	Main atmosphere[a]	*n*
10% RH	85.4 ± 24.2	9	13.6 ± 11.9	9	49.5 ± 41.3[A]	18
Vacuum	81.5 ± 31.4	3	26.0 ± 19.6	3	53.7 ± 38.4[A]	6
N_2	74.4 ± 25.0	6	28.2 ± 13.2	6	51.3 ± 30.7[A]	12
Main temperature[b]	81.1 ± 24.5[A]	18	20.6 ± 14.7[B]	18	50.8 ± 36.6	36

[A,B]Groups with the same superscript are not significantly different from each other ($P > 0.05$) (two-way ANOVA, post hoc Scheffé).
[a] Global effect of atmosphere, irrespective of temperature.
[b] Global effect of temperature, irrespective of atmosphere.

dramatically during the freezing process and that the optimal cooling rate depends mainly on cell properties (size, membrane, and structure). An optimum has to be found as rapid freezing of cells leads to formation of intracellular ice-crystals, which results in cell damage. This can be avoided by slow cooling [13,30,31]. However, at slow cooling rates the cells loose water from the cell cytoplasm. This leads to high salt concentrations in the cell, which can cause damage of the cell membrane and protein denaturation. The optimum freezing rate varies from one genus to another [13]. It can be concluded that freeze-drying *L. lactis* MG1363, grown until the stationary phase in GC-milk, without any previous, time-consuming and complicated fast freezing step resulted in the highest survival.

3.1.2. Stability of freeze-dried L. lactis

Next to high viability after production, the product should have an acceptable shelf life. In literature it is mentioned that during storage, contact with oxygen and moisture should be avoided. Moreover, light is also highly detrimental [13]. In a first screening study, the viability of freeze-dried *L. lactis* MG1363 was evaluated after 1 week storage at different conditions (Table 3). Preliminary studies showed that storage at room temperature and 60% RH for 1 week resulted in a complete loss of viability. The increased water content of the freeze-dried powder (from 2.2 after freeze-drying to 10.5% (w/w)) can explain this viability loss. As water acts as a plasticiser, the T_g of the glassy matrix, in which the bacteria are stabilized (71.7 ± 5.6 °C) decreased to −2.8 ± 3.6 °C and resulted in a collapse of the freeze-dried powder. It is known that the stability of biological systems held in rubbery state decreases remarkably [27]. In further studies, this storage condition was excluded.

To compare viability data after storage, the relative viability values were used as absolute viability values were batch dependent. Two main storage factors were evaluated for their influence on relative viability as a function of time, temperature and atmosphere. From Table 3 it can be concluded that the storage temperature has a significant influence on the viability, in contrast with the storage atmosphere. Although not statistically significant, viability tended to be higher after 1 week storage at RT and vacuum or N_2 than at 10% RH. This can be explained by the absence of oxygen. Moreover, the water content of the powder increased from 2.2 ± 0.7% (vacuum and N_2) to 3.2 ± 0.2% (10% RH), but this did not cause a significant change in T_g (from 71.7 ± 5.5 to 65.8 ± 7.8 °C). No significant interaction was seen between storage temperature and storage atmosphere (two-way ANOVA).

From these data it could be concluded that long-term stability of *L. lactis* MG1363, freeze-dried in skim milk matrix could not be achieved at room temperature. After 1 week storage at 8 °C, viability has already decreased with approximately 20% of the initial viability after freeze-drying (relative viability of 81.1%, Table 3, main temperature at 8 °C).

A long-term stability test was performed at 8 °C to further investigate the rate of decrease in viability. In contrast to the short-term stability test, the long-term stability test (6 months) was performed with the recombinant strain (Thy12), grown and freeze-dried in GCT-milk ($n = 3$). An additional storage condition was included, i.e. 20% RH as this is an acceptable condition for further processing of the freeze-dried powder into capsules. Fig. 1 shows the relative viability profile as a function of time. A significant effect of storage time was observed (Table 4). Moreover, relative viability decreased remarkably after 1 month, followed by a slower decrease in relative viability during the subsequent months of storage (logarithmic

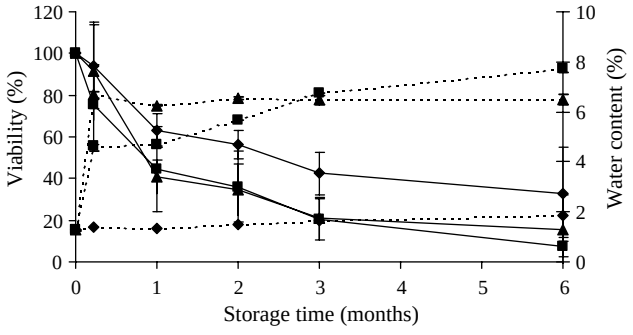

Fig. 1. Long-term stability at 8 °C (6 months) of Thy12, grown in GCT-milk until stationary phase and freeze-dried ($n = 3$): relative viability* (—) and water content (- - -) under N_2 atmosphere (♦), at 20% RH (▲) and 10%RH (■). *Relative viability = (absolute viability after storage/absolute viability after freeze-drying) × 100.

Table 4
Long-term stability at 8 °C (6 months) of Thy12, grown in GCT-milk until stationary phase and freeze-dried: relative viability (%) (mean ± SD) in function of storage time and atmosphere

Relative viability (%) of *L. lactis* Thy12 stored at different atmospheres								
	N_2	*n*	10% RH	*n*	20% RH	*n*	Main time[a]	*n*
1 week	93.9 ± 20.2	3	75.5 ± 19.3	3	91.6 ± 23.3	3	87.0 ± 20.2[A]	9
1 month	63.2 ± 7.8	2	44.5 ± 20.6	2	40.8 ± 7.8	2	49.5 ± 15.0[B]	6
2 months	56.2 ± 6.8	3	36.2 ± 17.1	3	34.7 ± 12.4	3	42.4 ± 15.2[B,C]	9
3 months	42.5 ± 10.2	3	20.5 ± 10.3	3	20.8 ± 9.5	3	27.9 ± 13.9[B,C]	9
6 months	32.5 ± 22.5	3	7.3 ± 4.6	3	15.6 ± 16.4	3	18.5 ± 18.0[C]	9
Main atmosphere[b]	57.2 ± 26.0[A]	14	36.3 ± 27.8[B]	14	40.7 ± 31.2[B]	14	44.7 ± 29.4	42

[A–C]Groups with the same superscript are not significantly different from each other ($P > 0.05$) (two-way ANOVA, post hoc Scheffé).
[a] Global effect of time, irrespective of atmosphere.
[b] Global effect of atmosphere, irrespective of time.

trend). Storage under N_2 atmosphere resulted in a significantly higher relative viability than storage at 10 or 20% RH. The results of the short-term stability test that relative viability tended to be higher after storage under vacuum or N_2 atmosphere than at 10% RH were now confirmed by the results of the long-term storage at 8 °C. The water content of the powder stored at 10 and 20% RH was similar and this was reflected in a similar relative viability. But the water content increased slightly after 6 months when the samples were stored at 10% RH. This could be explained by water penetration through the plastic container in which the vials were stored. This proved again the requirement of protection against moisture, especially in the refrigerator, in which a high relative humidity exists (77%).

Although the water content remained unchanged (2.2 ± 0.4%), T_g (71.7 ± 5.5 °C) remained high and oxygen was absent, a decrease in viability was also observed when stored under an inert N_2 atmosphere. This confirms that T_g is an unsatisfactory indicator for temperatures below which molecular motions are zero [32]. T_0 rather than T_g should be used as a practical guide for selecting the storage temperature, as only below this temperature zero mobility is obtained. T_0 is at least 50 °C below T_g. Although, in this study the difference between the storage temperature (8 °C) and T_g is 63 °C, viability decreased. Deleterious metabolic and/or enzymatic reactions which progress even at low storage temperatures and water content are probably the cause of the decrease in viability [18].

3.1.3. Influence of culture preparation

In order to reach an acceptable dose for oral administration of Thy12 to humans, the bacterial concentration had to be increased in the freeze-dried powder. Therefore, the bacteria could be isolated from the growth medium by centrifugation and further resuspended in fresh medium until the desired bacterial concentration was reached. In this experiment, it was investigated if *L. lactis* tolerates this culture preparation. As a reference, *L. lactis* MG1363 was grown in GC-milk and freeze-dried in this matrix (I). Alternatively, *L. lactis* MG1363 was grown in GM17 and harvested by centrifugation. Next, the cell pellet was resuspended in fresh GC-milk (II). To ensure removal of all possible toxic metabolic products, the cells were subjected to a washing step before resuspension in fresh GC-milk and freeze-drying (III). No significant differences are seen immediately after freeze-drying (data not shown) or after storage for 1 week at 8 °C and 10% RH (Fig. 2). It can be concluded that the time-consuming washing step is not advantageous and can be omitted. Furthermore, although the relative viability of *L. lactis* grown and freeze-dried in GC-milk tends to be higher after 1 week storage at 8 °C and 10% RH, it can be concluded that *L. lactis* tolerated the centrifugation step. This offers the advantage to increase the concentration of the bacteria in the freeze-dried powder. Moreover, the bacteria could be resuspended in a fresh matrix, so avoiding metabolites, which could create GMP problems on large-scale production. Moreover, from these data we can conclude that the growth environment of *L. lactis* has no reflection on the tolerance to freeze-drying stress.

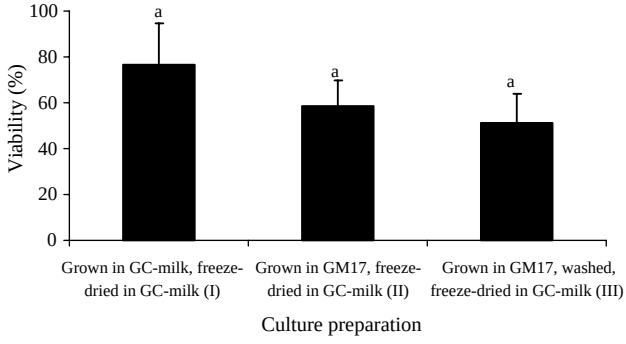

Fig. 2. Influence of the culture preparation on the relative viability* of *L. lactis* MG1363 after storage (1 week, 8 °C and 10% RH) ($n = 3$). [a]Groups with the same superscript are not significantly different from each other ($P > 0.05$) (one-way ANOVA, post hoc Scheffé). *Relative viability = (absolute viability after storage/absolute viability after freeze-drying) × 100.

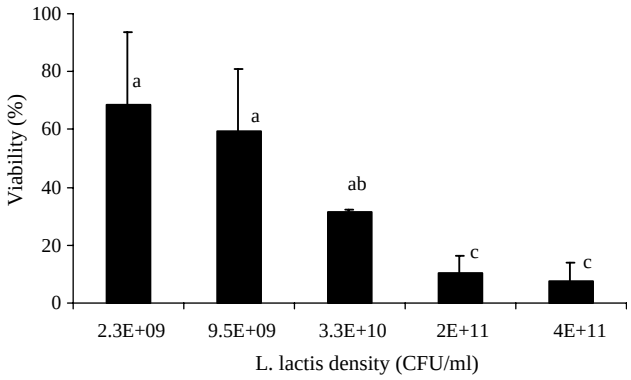

Fig. 3. Influence of density of *L. lactis* MG1363 in the matrix on the relative viability* of *L. lactis* MG1363 after storage (1 week, 8 °C and 10% RH) ($n=3$). [a–c]Groups with the same superscript are not significantly different from each other ($P>0.05$) (one-way ANOVA, post hoc Scheffé). *Relative viability = (absolute viability after storage/absolute viability after freeze-drying) × 100.

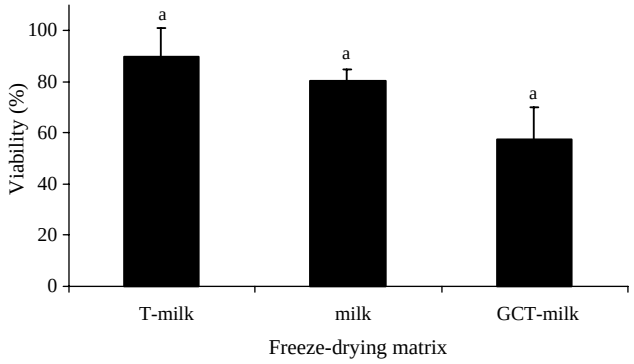

Fig. 4. Influence of feed components incorporated in the freeze-drying matrix on relative viability* of Thy12 after storage (1 week, 8 °C and 10% RH) ($n=3$). [a]Groups with the same superscript are not significantly different from each other ($p>0.05$) (one-way ANOVA, post hoc Scheffé). *Relative viability = (absolute viability after storage/absolute viability after freeze-drying) × 100.

3.1.4. Influence of culture density

In this experiment, it was evaluated if the concentration factor of *L. lactis* in the freeze-drying matrix is subjected to limits. In literature bacterial concentration is reported to affect viability after freeze-drying, especially during freezing [18]. Our data showed no significant influence of cell density on absolute viability immediately after freeze-drying (data not shown). After 1 week storage at 8 °C and 10% RH, relative viability decreased significantly with increasing cell density (Fig. 3). It can be concluded that increasing the cell density of *L. lactis* by a factor 10 seems an acceptable limit (no significant decrease). Bozoglu et al. [23] explained the higher relative viability for a higher cell load by the fact that by increasing the initial bacterial load, the interaction between the microorganisms decreases the exposed area of each cell to the environment and therefore prevents possible damage. However, a very high initial biomass (10^{12} cfu/ml) is harmful because of an unbalanced osmotic pressure.

3.1.5. Influence of feed components in the freeze-drying matrix

Thy 12 lacks the genes for lactose and protein digestion because the essential plasmides are lost by protoplasting. Therefore, it is dependent on glucose and amino acids. Moreover, because Thy12 lacks the genes for thymidilate synthase to ensure its biocontainment [5], it is also dependent on thymidine. To ensure viability and metabolic activity of the bacteria in vivo, the feed components were freeze-dried together with Thy12. In a next experiment, the influence on viability of these feed components in the freeze-drying matrix was investigated after freeze-drying and storage. We observed no significant difference in absolute viability between the three freeze-drying matrices immediately after freeze-drying (data not shown) and after 1 week storage at 8 °C and 10% RH (Fig. 4). However, the incorporation of glucose and casein hydrolysate (amino acids) in the freeze-drying matrix tended to negatively influence the relative viability after 1 week storage at 8 °C and 10% RH ($P=0.091$). It can also be concluded that the thymidine dependent strain (Thy12) was stable in dry state without its essential feed component. Therefore, it was decided to resuspend Thy12 in pure milk before freeze-drying, without any feed component added.

3.2. Development of an enteric-coated formulation of Thy 12-incorporation of Thy12 in ready-to-use enteric-coated capsules

3.2.1. Optimisation of enteric-coated capsules

The final goal of this study was to develop an enteric-coated formulation of viable and hIL-10 producing Thy12. From previous experiments, it can be concluded that growing Thy12 until the stationary phase in GM17T, centrifuged and resuspended in 1/10th of the initial volume of fresh skim milk and subsequent freeze-drying resulted in a product with acceptable viability. Viability was best maintained when stored at 8 °C/N_2. Since *L. lactis* is susceptible to gastric fluid and bile salts [6,7], the final formulation had to be enteric-coated. Furthermore, the bacteria should be targeted to the human ileal mucosae as Crohn's disease is mainly situated in the ileum [34]. From previous experiments, in which all commercial available polymers were evaluated for their ileum targeting properties, it could be concluded that none of the polymers or mixtures of polymers are suitable for specific targeting to the ileal mucosae [35]. A possible solution for this problem could be the administration of a combination of doses namely one dose coated with Eudragit® L30D-55 and another coated with Eudragit® FS 30 D, ensuring ileum release in patients with low and high ileum pH, respectively.

Moreover, in other experiments we showed that compaction of freeze dried *L. lactis* powder in order to obtain a formulation that could be enteric coated, resulted in

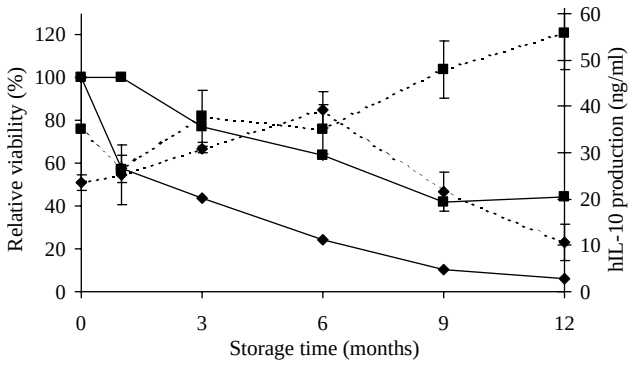

Fig. 5. Relative viability (—) and hIL-10 production (- - -) of freeze-dried Thy12, filled in ready-to-use enteric-coated HPMC capsules (Eudragit® L30D-55), packed in Alu-sachets (sealed at 20% RH) and stored for 1 year at 8 (♦) and −20 °C (■).

a loss of viability [36]. It might also be difficult to obtain an effective coating on these hygroscopic tablets and survival of *L. lactis* after this coating process is questionable. To avoid all handling with the heat and moisture sensitive freeze-dried Thy12, an alternative coating technology was developed resulting in ready-to-use enteric-coated capsules [24]. HPMC caps and bodies, separately coated with Eudragit® L30D-55 or FS 30 D, show excellent enteric properties after manual assembly. Preliminary experiments were performed to optimize the coating composition. First, the appropriate amount of plasticiser was selected allowing storage at low temperature (−20 °C) and low relative humidity (20% RH). Incorporation of 20 and 10% triethyl citrate (calculated on the polymer content) was required for the Eudragit® L30D-55 and Eudragit® FS 30 D polymer, respectively. Dissolution testing showed that the capsules coated with Eudragit® L30D-55 or Eudragit® FS 30 D maintained their enteric properties when stored for 1 year at 8 and −20 °C in Alu sachets (sealed at 20% RH). Both polymers complied with the European Pharmacopoeia requirements for an enteric-coated formulation (release <10% after 2 h in 0.1 N HCl) since release was below 1% at every time point of analysis.

Secondly, to avoid or limit gastric fluid penetration during passage through the stomach, 8% (w/w) glyceryl monostearate (calculated on the polymer content) was incorporated as liphophilic agent. It is often used in coating solutions as glidant, but is not necessary for coating of HPMC capsules [37]. This resulted in significantly lower water content of the powder in the Eudragit® L30D-55 coated capsules after 2 h 0.1 N HCl from 21.7 to 12.9%.

3.2.2. Viability and hIL-10 production after storage for 1 year at 8 and −20 °C

Although capsules were filled in a production room with low relative humidity (20% RH) and the powder was kept on ice, the viability decreased with 33.5±9.5% during manipulation ($n=2$). As previous studies showed the pressure sensitivity of *L. lactis* during compaction [36], grinding the freeze-dried powder in a mortar by a pestle probably caused the cell death. Moreover, the water content of the powder increased from 2.24±0.37 to 4.92±0.26% during this filling operation.

Fig. 5 shows the relative viability of freeze-dried Thy12 in capsules coated with Eudragit® L30D-55 as a function of time and storage temperature ($n=1$). Thy12 in capsules coated with Eudragit® FS 30 D showed a comparable relative viability profile (data not shown). Two main storage factors were evaluated, storage time and temperature. They both had a significant influence on viability as a function of storage (Table 5). Again, a logarithmic trend in the relative viability in function of time could be observed at both temperatures. Relative viability showed a parallel plot at both temperatures, but was higher at −20 °C. From 9 months storage, viability showed no further significant decrease. It can be concluded that after storage for 1 year at 8 and −20 °C, respectively, 6 and 44% of the initial Thy12 count remained viable or about 3.7×10^9, 2.1×10^{10} CFU/capsule, respectively.

The water content of the powder in the capsules did not significantly change during the storage time and, no significant difference in water content was seen between the two storage temperatures, e.g. for capsules coated with Eudragit® FS30D: 5.27±0.21% at 8 °C and −5.11±0.04% at −20 °C. The T_g of the powder in the Eudragit® coated capsules after 1 year storage at 8 °C was 47.71±0.95 °C.

Table 5
Long-term stability (12 months) of freeze-dried Thy12, filled in ready-to-use enteric-coated HPMC capsules (Eudragit® L30D-55), packed in Alu-sachets (sealed at 20% RH): relative viability (mean±SD) in function of storage time and temperature

Relative viability (%) of Thy12 stored at different temperatures						
	8 °C	*n*	−20 °C	*n*	Main time[a]	*n*
1 month	57.4±3.8	3	100.3±1.5	2	74.5±23.7[A]	5
3 months	46.7±0.3	3	77.2±0.5	3	60.4±18.4[B]	6
6 months	24.6±0.3	3	63.7±2.1	3	44.1±21.4[C]	6
9 months	10.4±0.6	3	42.1±3.6	3	26.3±17.5[D]	6
12 months	6.1±0.6	3	44.3±1.9	3	25.2±21.0[D]	6
Main temperature [b]	28.4±20.3[A]	15	63.0±21.1[B]	14	45.1±26.9	29

[A–D]Groups with the same superscript are not significantly different from each other ($P<0.05$) (two-way ANOVA, post hoc Scheffé).
[a] Global effect of time, irrespective of temperature.
[b] Global effect of temperature, irrespective of time.

Taking into account the $T_g - 50$ °C rule, zero mobility can be expected below −2.26 °C (T_0). This can explain why viability decreased during storage at 8 °C. However, during storage at −20 °C, where the storage temperature is far below T_0, viability also decreases. This could be explained by the strong crystallization tendency of the lactose in skim milk matrix as crystallization of the freeze-dried matrix leads to loss of stabilizing power [33]. However, X-ray diffraction patterns showed that the freeze-dried skim milk matrix remained amorphous for at least 9 months at 8 and −20 °C. Deleterious metabolic and/or enzymatic reactions which progress even at low storage temperature and water content are probably the cause of the decrease in viability [18]. Storage at −20 °C assured a better shelf life.

As Thy12 was genetically modified for the production of hIL-10, it was extremely important to evaluate whether this capacity is maintained during production, passage through gastric fluid and storage. In literature, no data are available on the influence of processing on recombinant properties. From Fig. 5, it can be concluded that the hIL-10 producing capacity is maintained after 1 year at both storage temperatures. However, it is hard to correlate the hIL-10 production with the viability of Thy12, since the expression of the protein is dependent on many variables. During storage viability decreases and hIL-10 production remains unchanged. During storage, cell lyses could occur, resulting in the release of the precursor-protein from the cells. As the current ELISA cannot differentiate hIL-10 and the non-active precursor-hIL10, this may lead to an overestimation of the hIL-10 concentration.

3.2.3. Viability and hIL-10 production after passage through gastric fluid

Although the release of marker substance from the enteric-coated capsules was below 1%, even after storage for 1 year at 8 and −20 °C, the viability of Thy12 decreased significantly in the enteric-coated capsules after subjecting them for 2 h to 0.1 N HCl. Fig. 6 shows the fraction of Thy12 which had maintained viability after 2 h 0.1 N HCl. No trend is seen as a function of time, indicating the maintenance of coating performance during storage. After 2 h in 0.1 N HCl, 36.52 ± 8.57% (mean of all the values at different time points) Thy12 in Eudragit® FS 30 D coated capsules remained viable. The increased water content of the powder (10.21 ± 0.69%) could explain this loss of viability. The viability decreased even more when incorporated in Eudragit® L30D-55 coated capsules (26.93 ± 6.37% remained viable after 2 h in 0.1 N HCl) (mean of all the values at different time points). This is also in agreement with the higher water content of the powder (13.11 ± 2.60%). The powder appeared collapsed, in contrast with the powder in the Eudragit® FS 30 D coated capsules. This can be supported by the T_g values: −10.05 ± 6.50 and 11.32 ± 2.18 °C after 2 h 0.1 N HCl in Eudragit® L30D-55 and Eudragit® FS 30 D coated capsules, respectively. Although viability decreased, the formulation provides some protection for Thy12 against the extremely detrimental acidic conditions in the stomach as it is known that no protection would lead to an extremely poor survival.

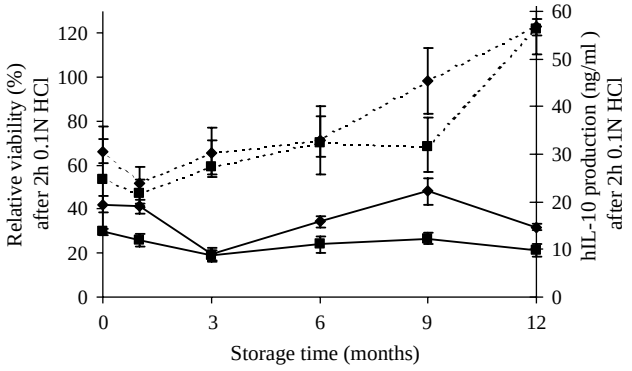

Fig. 6. Relative viability (—) and hIL-10 production (- - -) after 2 h 0.1 N HCl of freeze-dried Thy12, filled in ready-to-use enteric-coated HPMC capsules (Eudragit® L30D-55 (■). and Eudragit® FS 30 D (◆)), packed in Alu-sachets (sealed at 20% RH) and stored for 1 year at −20 °C.

The hIL-10 production capacity was maintained after passage through the gastric fluid stage (Fig. 6).

4. Conclusion

In this study, a pharmaceutical formulation of biological contained recombinant hIL-10 producing *L. lactis* was developed. The incorporation of freeze-dried Thy12 in ready-to-use enteric-coated capsules is a promising formulation since after 1 year storage, the enteric properties, an acceptable viability and hIL-10 producing capacity were maintained. This formulation has been used in a preclinical study in which hIL-10 producing *L. lactis* has been administered to 10 patients suffering from Crohn's disease (Academic Medical Center of Amsterdam). The results suggested a clinical effectiveness of this new therapy.

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